DETECTION OF SELECTIVE TYROSINASE INHIBITORS FROM SOME SOUTH AFRICAN PLANT EXTRACTS OF LAMIACEAE FAMILY

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By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Various dermatological disorders, such as formation of black pigmented patches on the surface of the skin arise from the over-activity of tyrosinase enzyme's degenerative action. This enzyme is further implicated in the involvement of melanin in malignant melanoma, the most life-threatening skin tumors. Although, synthetic products were found effective to combat this menace, nevertheless, overtime detrimental effect on human skin is a challenge. Investigation of natural tyrosinase inhibitors from methanol extracts of medicinal plants of Lamiaceae family using L-tyrosine as substrate on three different complementary assays (TLC bio-autography, spectrophotometry and cyclic voltammetry) was carried out accordingly.

The result indicated *Salvia chamelaeagnea*, *Salvia dolomitica*, *Plectranthus ecklonii*, *Plectranthus namaensis,* and *Plectranthus zuluensis*, with significant zone of inhibition against tyrosinase on TLC bio-autography, spectrophotometry result showed that extracts of *Plectranthus ecklonii* (IC_{50} = 21.58 μg/mL), *Plectranthus zuluensis* (IC_{50} = 23.99 μg/mL), *Plectranthus madagascariensis* (IC_{50} = 23.99 μg/mL) and *Salvia lanceolata* (IC_{50} = 28.83) demonstrated good anti-tyrosinase activity when compared with kojic acid (IC_{50} = 3.607 µg/mL). On the other hand, cyclic voltammetry are in consonant with above results thereby supported the nomination of some of the extracts as strong anti-tyrosinase agents.

Salvia chamelaeagnea showed strong activity in cyclic voltammetry and clear zone of inhibition on TLC bioautography, these reasons gave us justification for further chemical study to isolate the bioactive constituents.

Phytochemical investigation of the bioactive extract of *Salvia chamelaeagnea* using different chromatographic methods including column chromatographic and semi preparative HPLC

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afforded six (6) known compounds *viz* carsonol (**C1**), carnosic acid (**C2**), 7- ethoxylrosmanol (**C3**), ursolic acid (**C4**), rosmanol (**C5**) and ladanein (**C6**). Their chemical structures were elucidated by analyses of spectroscopic (^{1}H and ^{13}C NMR) data as well as correlations with existing literature. The methanolic extract of *S. chamelaeagnea* (SC) showed moderate anti-tyrosinase (IC_{50} = 267.4 µg/mL) activity, total antioxidant capacities measured as: Oxygen radicals absorbance capacity (ORAC; 14970 ± 5.16 µM TE/g), ferric-ion reducing antioxidant power (FRAP; 9869.43 ± 7.87 µM AAE/g) and trolox equivalent absorbance capacity (TEAC; 13706.5 ± 0.95 µM TE/g). Excellent total antioxidant capacities were demonstrated by **C1** and **C5** respectively as FRAP (9338.92 ± 1.72; 8622.73 ± 1.92) µM AAE/g; TEAC (16505 ± 0.86; 10641.5 ± 0.52) µM TE/g; ORAC (14550.5 ± 3.65; 14633.90 ± 3.84) µM TE/g and including the inhibition of Fe^{2+} -induced lipid peroxidation (IC_{50} = 32.5; 30.25) µg/mL. All the compounds except **C4** are electro-active with well-defined oxidation-reduction peaks while **C1** demonstrated the highest tyrosinase inhibitory activity by strongly decreased the inhibition current with time using cyclic voltammetry method.

The isolated compounds especially **C1**, **C2** and **C5** are well known to combat with ageing problems and documented for their powerful activity against oxidative stress and alzheimer's diseases, which are ageing related symptoms. The isolation of such bioactive compounds indicated the synergetic effect of the results of the three methods used in this thesis.

This is the first report on the evaluation of both anti-tyrosinase and total antioxidant capacities of the isolated compounds from *S. chamelaeagnea*. The findings therefore can be used as background information for exploitation of skin depigmentation and antioxidant agents from natural source.

Keywords: Lamiaceae; tyrosinase inhibitor; cyclic voltammetry; TLC bioautography; medicinal plants; cosmetics; antioxidant; *S. chamelaeagnea*.

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DECLARATION

I, **Ninon Geornest Eudes Ronauld ETSASSALA** hereby declare that "Detection of Selective Tyrosinase Inhibitors from Some South African Plant Extracts of Lamiaceae Family" is my original work and to the best of my knowledge, it has not been submitted before for any degree or assessment in any other University, and all the sources that I used or quoted have been indicated and acknowledged by means of complete references.

Date…… Signed……..

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Special thanks to my colleagues **Luveni Sonka, Abdulrahman Elbagory**, **Abobaker Ibrakaw**, **Masande Yalo**, **Mthandazo Rondo**, **Mkhuseli Koki**, **Ndikho Nako**. Above all, I give all the glory to **God** for the provision of strength, wisdom towards successful completion of this work.

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DEDICATION

I would like to dedicate this dissertation to my family, especially my father **Georges Etsassala** and my mother **Ernestine Gayaba**, who gave me their unwavering support throughout my life. Also to my wife **Grace Milounguidi**, my sisters **Georgea** and **Horcia Etsassala**, friends and to the Congolese communities.

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LIST OF ABBREVATIONS

NMR	Nuclear magnetic resonance
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
1D-NMR	One-dimensional nuclear magnetic resonance
2D NMR	Two-dimensional nuclear magnetic resonance
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
COSY	Correlation spectroscopy
$CDCl_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
H_2SO_4	Sulphuric acid
MeOH	Methanol
KCl	Potassium chloride
ABTS	2, 2- Azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) diammoniun salt
AAPH	2,2- Azibis (2-methylpropionamidine) dihydrochloride, perchloric acid
FRAP	Ferric-ion reducing antioxidant power

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LIST OF ABBREVATIONS

ORAC Oxygen radicals absorbance capacity

TLC Thin layer chromatography

TEAC Trolox equivalent absorbance capacity

TE/g Trolox equilalent per gram

TCA Trichloroacetic acid

Trolox 6-Hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid

TPTZ (2,4,6-tri[2-pyridyl]-s-triazine, Iron (III) chloride hexahydrate

UV Ultraviolet

BHT Butylated hydroxytoluene

AAE/g Ascorbic acid per gram

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DIW De-ionized water

DNA Deoxyribonucleic acid

ECM Extra cellular matrix

EDTA Ethylenediaminetetraacetic acid

EGCG Epigallocatechingallate

Fig Figure

g Gram

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LIST OF ABBREVATIONS

L	Liter
mg	Milligram
min	Minute
HPLC	High performance liquid chromatography
ROS	Reactive oxygen specie
SANBI	South African National Biodiversity Institute
SD	Standard deviation
Spp	Species
IC_{50}	Half maximal inhibitory concentration

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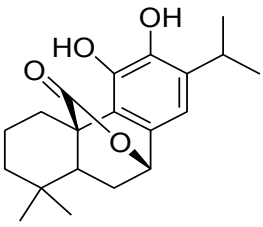
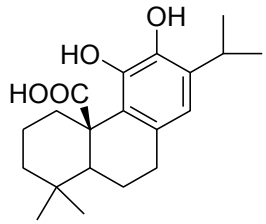
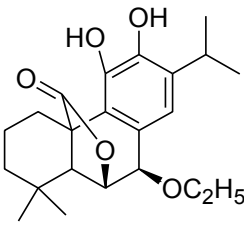
LIST OF ISOLATED COMPOUNDS



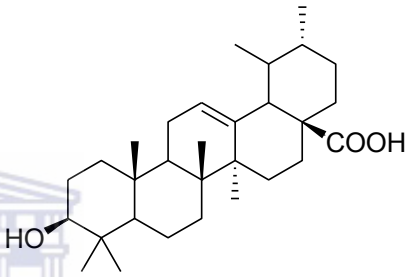
carnosol (C1)



carnosic acid (C2)



7- ethoxy-rosmanol (C3)



ursolic acid (C4)

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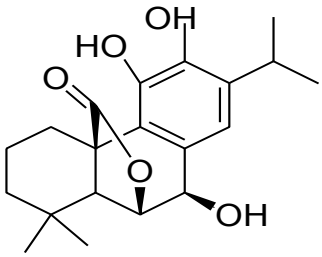
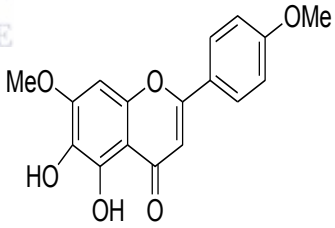


rosmanol (C5)



ladanein (C6)

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LIST OF PUBLICATIONS ARISING FROM THIS RESEARCH

1. Ninon G.E.R. Etsassala, Tesfaye Waryo, Christopher N. Cupido, Olugbenga K. Popoola, Emmanuel I. Iwuoha, and Ahmed A. Hussein. Screening of Selected South African Lamiaceae Plant Species against Tyrosinase using Three Complementary Assays.

Manuscript submitted

2. Ninon G.E.R. Etsassala, Tesfaye Waryo, Christopher N. Cupido, Olugbenga K. Popoola, Emmanuel I. Iwuoha, and Ahmed A. Hussein. Antityrosinase and total antioxidant capacities of *Salvia chamelaeaegnea* constituents.

   Manuscript in preparation

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CHAPTER 1: INTRODUCTION

1.1 General overview of the use of plants as source of medicines

Plants have been used as a source of medicine since time immemorial for the purpose of curing numerous human afflictions (Fernnell, et al., 2004). The practice of using herbal medicine at early stage was primitively considered either an option for the less privileged or low-income earners (Rates, 2001). The use of folk medicine in developing countries has been a common practice and people have been able to treat ailments using plants within their respective locality in the mode of decoction and infusion (Rojas, et al., 2006). It has been estimated that up to 80 % of the population living in Africa uses traditional medicine as part of their primary healthcare system (Ekor, 2014). The use of traditional herbal remedies plays a vital role in South Africa, and forms the backbone of rural communities (Coopoosamy & Naidoo, 2013). Up to 60 % of the populations of the rural area in South Africa consult an approximation of 200,000 traditional healers. This huge turnout was ascribed to affordability, accessibility and most importantly freedom of expression of personal health status unlike orthodox medicines (Taylor, et al., 2001). Report shown that out of 30,000 species of higher plants present in South Africa, 10 % of these species have been found useful in traditional medicine around the country to treat different kind of disease such as cough, diarrhea, headaches, heart related problems, inflammation and prevention of abortion (Thring & Weitz, 2006). From the aforementioned, the beneficial usage of traditional medicine is receiving global icon of recognition compared to the synthetic drugs due to environmental friendly nature of the former (Iniaghe, et al., 2010).

Medicinal plants contain a wide range of substances that can promote health and alleviate disease burden (Prajapati, et al., 2011). They have curative properties due to the presence of numerous bioactive constituents termed secondary metabolites (Karthikeyan & Balasubramanian, 2014).

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These secondary metabolites range from terpenoids, phenolics, flavonoids, tannins and alkaloids which are of more beneficial effect to human than the plant itself (Shaukat, et al., 2013).

1.2 Natural products as source of drugs

Drugs derived from plants represent one of the most important sources of new and safe therapeutic agents. Many medicinal plants contain bioactive compounds that serve as precursors to synthesized drugs (Karthikeyan & Balasubramanian, 2014). Drugs derived from natural products have assumed greater value in recent days, due to the tremendous potential they offer in the formulation of new drugs, which may protect humankind against many diseases (Balunas & Kinghorn, 2005; Khalid, et al., 2013). It is estimated that 25-50 % of modern drugs are derived from plant origin with an approximation of 50 % of new drugs introduced in this time being has a natural product origin (Kingston, 2011). In Germany, 50 % of phytomedicinal products were sold based on the medical prescription (Rates, 2001; Bussmann, et al., 2010). Further evidence of the importance of natural products is provided by the fact that almost half of the world's 25 best-selling pharmaceuticals in 1991 were either natural products or their analogues (Ashour, et al., 2013). Nowadays, plant derived drugs are predominantly important to the world population because of their safety profile (Vinha, et al., 2012; Nag, et al., 2013).

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1.3 Cosmeceutical exploration of African medicinal plants

Traditional herbal medicines provide a very interesting source of potential new drugs and skin-care products (Kiken & Cohen, 2002). There are innumerable South African plants that have wide application in cosmetic industry as additives to shampoo, shaving, skin care creams, skin disorders, treatment of burns and skin depigmenting agents (Lall & Kishore, 2014).

Egyptians were the first since the ancient time to use herbal medicine such as the whipped ostrich eggs, olive oil, and resin mixed with milk for treating various skin ailments. Another

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notable trend in the history of African medicinal plants is the usage of *Eclipta prostrate* commonly known as false daisy in Egypt as the fresh juice of the plant and applied directly to the scalp, and it is people's believed to blacken the hair and beard (Lall & Kishore, 2014).

In Rwanda, the purpose of lightening the skin using plants is a common practice, especially during the major ceremonies such as marriage. The leaves of numerous herbal plants have been used by Rwandese to treat skin disease, hyperpigmentation, leprosy, eczema and skin mycosis (Kamagaju, et al., 2013).

The leaves of the plant species of Combretaceae (*Anogeissus leiocarpus, Anogeissus schimperi* and *Conocarpus leiocarpus)* are used in Nigeria in the treatment of skin diseases. *Plumbago zeylani*ca (Plumbaginaceae), *Artemisia absinthium* (Asteraceae), *Artemisia herba-alba* (Asteraceae) are extremely popular throughout Africa and Asia as a remedy for parasitic skin diseases, especially leprosy, scabies, acne vulgaris, sores and leg ulcers (Dweck, 1996). Other medicinal plants with significant recognition as anti hyperpigmenting agents are *Aloe* species (Xanthorrhoeaceae), *Cassipourea malosana* (Rhizophoraceae), *Sideroxylon inerme* (Sapotaceae) (Van Wyk & Gericke, 2000; Momtaz, et al., 2008).

There are numerous popular South African plants such as *Aloe ferox Mill* (Bitter Aloe: Xanthorrhoeaceae), *Aspalathus linearis* (Rooibos: Fabaceae), *Calodendrum capense L.f. Thunb.* (Cape Chestnut: Rutaceae), *Cyclopia intermedia E. Mey* (Honeybush: Fabaceae), *Sideroxylon inerme L.* (White milkwood: Sapotaceae) and *Salvia stenophylla Burch. Ex Benth* (Blue mountain sage: Lamiaceae) which are frequently used in various skin creams for slowing down aging process and other skin related ailments (Lall & Kishore, 2014).

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1.4 Historical perspective of skin pigmentation

Skin is the largest organ of the human body, accounting for about 15 % of the total adult body weight (Kanitakis, 2002). It serves as an important environmental interface that provides a protective envelope which is crucial for homeostasis (Chompo, et al., 2012). The structure of the skin is mainly made of two layers (Fig 1.1). The outer layer also called the epidermis, characterizing mainly of keratinocytes responsible for protecting the body against environmental damage, while the inner part, known as the dermis constitutes connective tissue and structural components such as collagen (responsible for skin firmness), elastic fibers (responsible for skin elasticity), and extracellular matrix (ECM) also known as structural components (Sparavigna, et al., 2013). However, human skin is exposed to the ultra violet (UV) radiation which on prolong exposure cause excessive accumulation of free radicals and other form of reactive oxygen species (ROS) including hydroxyl and peroxyl radicals, and hydrogen peroxide, which are primarily generated in the body as a result of physiological and biochemical processes (Aiyegoro & Okoh, 2010). Other notable ways of accumulating ROS in the body, is through continuous contact with the series of environmental cues such as UV radiation, human activities and lifestyles (Fearon & Faux, 2009; Chompo, et al., 2012). The free radical accumulation, when in excess in the body, can cause oxidative damage to some important macromolecules such as proteins, lipids and deoxyribonucleic acid (DNA), which eventually lead to many chronic diseases such as cancer, early aging, atherosclerosis, neurodegenerative disorders (Fig 1.2) (Niki, et al., 2005; Makrantonaki, et al., 2007), and other degenerative diseases in humans (Aiyegoro & Okoh, 2010). Other significant pathological implications of ROS in the body is the activation of skin enzyme's degenerative actions, resulting in early or premature skin aging processes such as skin pigmentation (Corstjens, et al., 2007; Ndlovu, et al., 2013), sagging and wrinkle formation (Porcheron, et al., 2014). UV- exposed human skin may be an accessible model system in which

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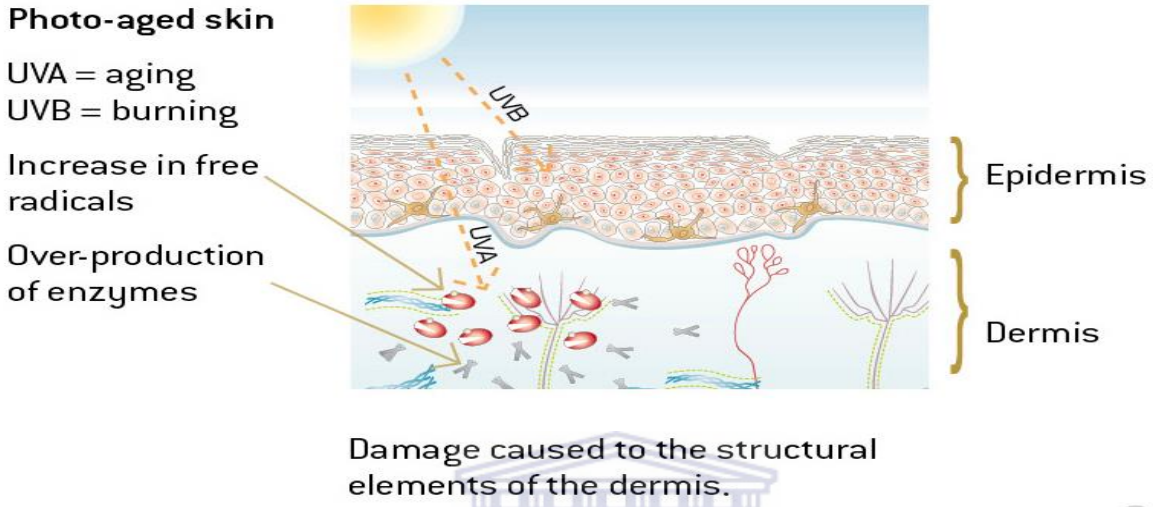
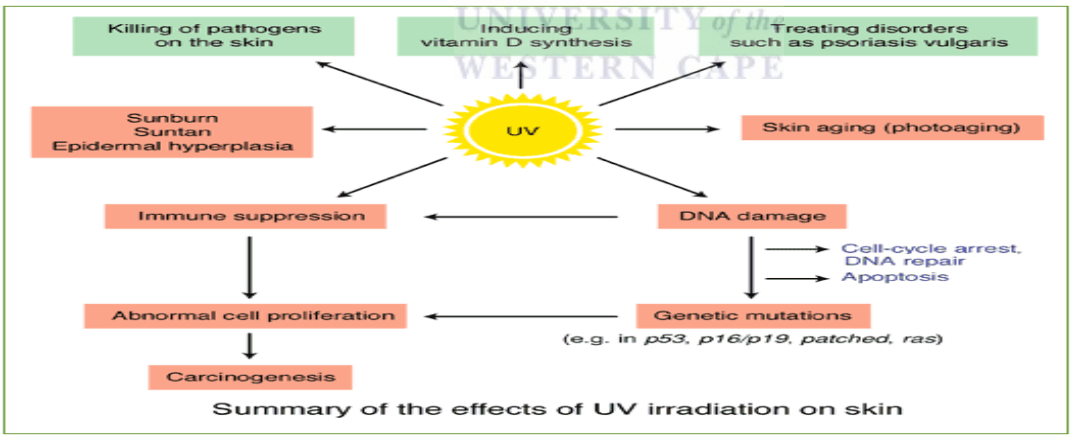
to characterize the role of oxidative damage in both internal and external tissue, given the compelling evidence for the role of ROS as mediators of photoaging (Watson, et al., 2014).



* Source: www.leadinginstanteyelift.com

Figure 1.1: Damage caused by UV to the structural elements of the skin



*Source: www.healthyfellow.com

Figure 1.2: Effects of UV radiation on human skin

The occurrence of pigmented patches as shown in Figure 1.3 resulting from the accumulation of abnormal amount of melanin in different specific parts of the skin is referred to as skin pigmentation (Chompo, et al., 2012). This undesirable situation is caused by prolong exposure of human skin to the UV radiation, which resulted into over accumulation of free radicals in the

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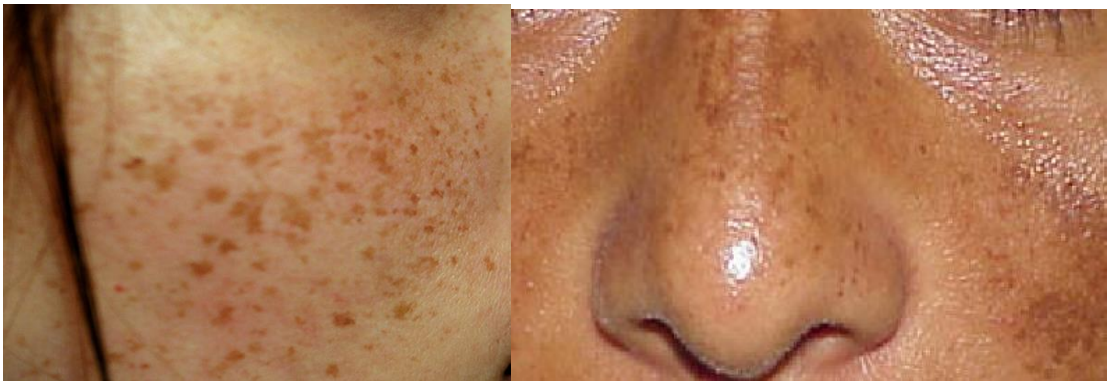
body (Wangthong, et al., 2007). The presence of excess of free radicals in the human body stimulated the activation of tyrosinase enzyme, which caused over-activity of the enzyme, thereby resulting into skin hyperpigmentation (Mapunya & Nikolova, 2012).



Source: http://monsterblog.com.my

Figure 1.3: Effect of skin pigmentation

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1.5 Oxidative Stress

The formation of free radicals in human body is a continuous process which occurred through evolution after prolong exposure to UV radiations. Free radicals play vital roles in both inter and intracellular signaling (Munné-Bosch & Alegre, 2003), controlling vascular tone, defense against microorganisms, cell generation-degeneration, and basal regulation of homeostasis (Basu, 2010). However, when in excess, they are involved in the pathogenesis of diseases by damaging DNA and proteins causing gene modifications by altering the protein structures and functions, while glycoxidative damage and oxidative degeneration of lipids in cell membranes can also be a result (Carocho & Ferreira, 2013). The body defense mechanism generally declines with age, and can be compromised by various forms of oxidative stress from environmental factors to cancer, diabetes, atherosclerosis, and neurodegenerative disorders. All these conditions, as well as the aging process, are associated with oxidative stress due to the elevation of ROS or insufficient ROS detoxification (Limon-Pacheco & Gonsebatt, 2009; Igwe & Echeme, 2014). Oxidative stress therefore occurs when the formation of bioactive oxidative products such as oxidizing

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agents, free radicals and reactive oxygen species, greatly overwhelms the capacity of the endogenous cellular antioxidant defense system, thus leading to potential damage of the cells and organs and to the progression of degenerative diseases in humans (Schrader & Fahimi, 2006; Basu, 2010).

1.6 Tyrosinase

Tyrosinase is a copper-containing enzyme present in animal, plant and human tissues. It catalyzes two distinct reactions using molecular oxygen (O_2): hydroxylation of tyrosine to 3,4 – dihydroxyphenylalamine (DOPA) by monophenolase action and oxidation of DOPA to DOPA-quinone by diphenolase action (Figure 1.4), which is considered as rate-limiting enzyme for controlling the production of melanin pigment that plays a vital role as a photo-protective agent against the harmful effects of UV radiation thereby absorbing UV light and removing reactive oxygen species (Summers, 2006; Mapunya & Nikolova, 2012). Melanin is produced naturally during a physiological process called melanogenesis by specific cells shown as melanocytes, which are distributed in the basal layer of the dermis (Baurin, et al., 2002).

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In food industry, tyrosinase is a very important enzyme in controlling the quality of foods, fruits and vegetables. It catalyzes the oxidation of phenolic compounds to the corresponding quinones responsible for the browning of fruits and vegetables during post harvesting (Huang, et al., 2006).

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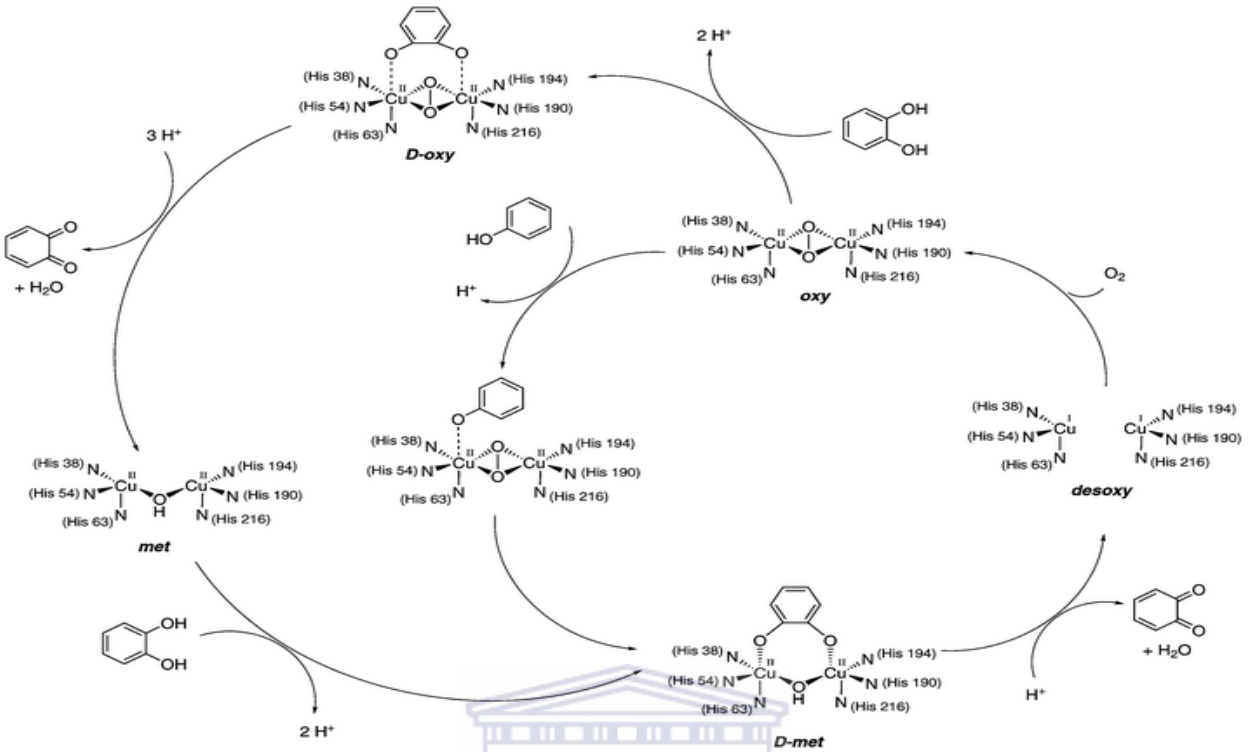

Source: www.pub.rcs.org

Figure 1.4: Monophenolase and diphenolase cycle of tyrosinase

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1.7 Natural product as source of tyrosinase inhibitors

Discovering of tyrosinase inhibitors from natural source is of great importance in cosmetic products formulation for the prevention of skin hyperpigmentation (Chen, et al., 2015). Enzymatic browning of agricultural products during post harvesting is a common phenomena resulting into a decrease in nutritional quality and economic depreciation of such products (Mayer, 1987; Whittaker, 1995; Friedman, 1996). Hyperpigmentation in human skin and enzymatic browning in fruits are undesirable, so the occurrence of these unpleasant changes have motivated researchers to seek for new potent tyrosinase inhibitors for use as anti-browning and skin depigmenting agents (Kim & Uyama, 2005). There are numerous tyrosinase inhibitors from natural source such as hydroquinone, aloesin, arbutin, kojic acid and glabridin. However, the effectiveness of these products is of reasonable doubt owing to overtime adverse effects. An

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example for this claim is carcinogenic effect demonstrated by kojic acid (Fuyuno, 2004), skin irritation, mutagenic effects to mammalian cells and cytotoxic to melanocytes demonstrated by hydroquinone (Curto, et al., 1999; Parvez, et al., 2006), and poor skin penetrations and stability demonstrated by aloesin and glabridin (Hermanns, et al., 2000). Thus it is in great need to seek for new potent tyrosinase inhibitors from natural source with reasonable human safety limits.

1.8 Why investigate the family Lamiaceae

The family Lamiaceae comprises approximately 980 species in Southern Africa which are mainly found in the Mediterranean, Central Asia and Sub-Sahara Africa regions, and display enormous morphological diversity (Klopper, et al., 2006). There are widely distributed in South Africa and comprises about 255 species assigned to 35 genera (Szentmihályi, et al., 2004; Strelitzia, 2013). Most of these plants possess aromatic sweet smell and widely used as culinary herbs and skin-care products (Lee, et al., 2011). The South African plants species of this family (e.g. *Salvia*) are used locally in the treatment of colds, coughs and bronchial infections. Industrially, these plants are widely employed as food additives, cosmetic formulations, aromatherapy and insecticides (Kamatou, et al., 2008). *Tetradinia riparia* has also been used traditionally in the treatment of coughs, dropsy, diarrhea, fever, headaches, malaria and toothaches (Gazim, et al., 2010), while *Plectranthus ecklonii* is used in the treatment of skin disorders, nausea, vomiting and cancer (Burmistrova, et al., 2015). Other notable economic value of plant species of this family is highlighted in the literature review presented in the next Chapter.

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1.9 Statement of research

The process of pigmentation caused by over accumulation of melanin in specific parts of the skin is due to a prolong exposure of the human skin to the UV radiation, resulted into over

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accumulation of free radicals in the body. This condition caused the stimulation of skin degenerative action of tyrosinase enzyme resulting into the formation of unpleasant skin pigmented patches as one of the factor of premature skin aging. Enzymatic browning of perishable agricultural products after post harvesting is another detrimental effect of tyrosinase enzyme, thereby reducing the nutritional quality and depreciation market value of such products.

It is noteworthy to state that tremendous effort has been achieved by scientists towards combating the problem associated with skin pigmentation. One of such landmark achievement is the invention of sun screen for absorbing UV radiation. Regrettably, apart from the recent shortcomings of the usage of sunscreen, it is not affordable for the people of the continent. Hence there is urgent need to search for more potent skin depigmenting agents that can replace already listed agents as illustrated in section 1.6 due to their various bottlenecks.

Lamiaceae plant species are widely distributed in South Africa and are readily available for human exploration for their potential application as anti-pigmenting agents. It is an established fact that this family contains phenolic compounds such as flavonoids, phenolic acid and diterpenes which are expected to play an important role in controlling undesirable skin conditions. Numerous plant species of this family such as *Lavandula* species, *Origanum majorana*, *Ballota nigra*, *Eremostachys laevigota Burge* have been reported to display potent tyrosinase inhibitory activity and also as antioxidant (Lee, et al., 2011; Hashemi & Zarei, 2014). Therefore, it is of great need in searching further for potent natural tyrosinase inhibitors from Lamiaceae plant species with proposition of replacing unsafe products readily available in the market. Further hypothesis was made to investigate the degree of antioxidant capacities in an *in vitro* system in order to complement the natural antioxidant already existing in the body through dietary intakes.

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1.10 Aims of this study

The main aim of this project is directed towards the preliminary screening of South African plants species belonging to Lamiaceae for their potency of tyrosinase inhibitory activity using different complementary assays such as thin layer chromatography (TLC) bioautography, spectrophotometry and cyclic voltammetry methods. The outcome of this screening will be used as prerequisite to nominate the bioactive plant materials for chromatographic isolation. Compounds emanated from such chromatographic isolation will be fully characterized chemically and biologically, and finally established their various structure-activity relationship between the compound (s) and their biological specificity demonstrated therefrom.

1.11 Objectives of this study

The main objectives of the study are:

- Collection, documentation and identification of Lamiaceae plants.
- Extraction of each plant materials with methanol.
- Conduct the preliminary phytochemical screening on the methanolic extracts to identify various classes of compounds.
- Carry out the preliminary biological screening using TLC bio-autographic and colorimetric assays on the methanolic extracts.
- Investigate the tyrosinase inhibition current of the methanol extracts using cyclic voltammetry screening.
- Perform chromatographic (TLC-guided column) isolation of bioactive constituents present in the most biologically and phytochemically active plant extract.
- Perform final purification of chromatographic fractions using semi-prep HPLC to obtain compounds in pure state.

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- Carry out structural elucidation of isolated compounds using different spectroscopic techniques.
- Carry out biological investigations on the isolated compounds for their *in vitro* total antioxidant capacities.
- Determine other biological characterization of the isolated compounds on tyrosinase inhibitory activity using spectrophotometric and cyclic voltammetry methods.

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CHAPTER 2: A REVIEW OF SOUTH AFRICAN LAMIACEAE: TRADITIONAL, PHYTOCHEMISTRY AND BIOLOGICAL IMPORTANCE

2.1 Introduction

The aim of this chapter is to review the South African plant species of Lamiaceae from their ethno-medicinal perspective to the existing scientific data as depicted from Scifinder. The comprehensive review includes highlights on the phytochemistry of the family and their respective biological and pharmacological importance as applicable to this study.

2.2 South African Lamiaceae family

Lamiaceae is a family of flowering plants with 233 to 263 genera and 6900 to 7200 species (Kaurinovic, et al., 2010), which are classified into seven (7) subfamilies such as Ajugoideae kostel, Lamioideae Harley, Nepetoideae (Dumort) Luerss, Prostantheroideae Luerss, Scutillarioidea (Dumort) Caruel, Symphorematoideae Briq and Viticodeae Briq (Theodoridis, et al., 2012). They are mainly found in Mediterranean, Central Asia and Sub-Saharan Africa region. An approximation of 980 southern Africa species is widespread and display enormous morphological diversity. South African floral contains about 255 species assigned to 35 genera (Klopper, et al., 2006). Most of these plants are aromatic with fragrance smells and are widely used as culinary herbs and skin-care cosmetics traditionally (Lee, et al., 2011).

2.2.1 Plant Morphology: Description and distribution

The leaves of the plants belonging to Lamiaceae are opposite or whorled, decussate and gland-dotted, while the flowers are usually bisexual arranged in compact auxiliary cymes. Zygomorphic flowers are characteristic of this family with calyxes usually persistent with 5 lobes. However, actinomorphic (regular) with 4 or 5 lobed flowers occur in some genera (Raja, 2012). The corolla is tubular and typically 2-lipped with 2 lobed upper lips and 3 lobed lip,

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variously colored and are hairy most of the time. The species are predominantly abundant in summer compared to the winter in rainforest areas. The often habitats for the plants are rocky, woodland or grassland, but also occur along forest margins and in fynbos (Strelitzia, 2013).

2.3 Traditional and biological uses of Lamiaceae plant species

The use of traditional medicine plays a vital role in South Africa and constitutes the backbone of rural areas where 70 % of the population relies on traditional medicine for their primary health care needs (Scott & Springfield, 2004). Numerous plant of Lamiaceae such as *Salvia* species have been used in South Africa to treat different kind of diseases including fever, digestive disorders, rheumatism, sexual debility, loss of memory, inflammation, microbial infections, and cancer (Kamatou, et al., 2005). *Salvia* species have been reported to have anti-inflammatory and antioxidant properties and their traditional usage include in the treatment of wounds (Kamatou, et al., 2008). *Plectranthus* species are plant of economic and medicinal value, and numerous species are used in South Africa as traditional medicines for the treatment of various diseases such as cough, wounds, gastrointestinal disorders, and skin infections (Maree, et al., 2014). *Tetradenia riparia* have been assessed to be very potent in the treatment of common skin ailments in many rural communities of South Africa, and the herbal plant has been found to be the most frequently used by traditional healers for the treatment of cough and chest related diseases (Ndamane, et al., 2013). More details about the traditional uses of Lamiaceae plants species are highlighted on the Table 2.1 as follows.

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Table 2.1: Medicinal uses and biological activities reported on South African plant species of Lamiaceae

Name of the plant	Traditional/Biological uses	Reference
Ballota africana	Fever, cough, asthma, influenza, insomnia, stress, sores	(Scott & Springfield, 2004; Lall & Kishore, 2014)
Leonotus leonurus	Skin infections, skin rashes, eczema, boils, bronchitis, cough, influenza, menstrual disorders, wound healing, asthma, cold, antioxidant, anti-inflammatory, dermatological disorders	(Scott & Springfield, 2004; Mazimba, 2015)
L. ocymifolia	Skin irritation, eczema, diabetes, hypertension, anemia, purgative	(Scott & Springfield, 2004)
Mentha longifolia	Ulcerative colitis, liver complaints, anti-inflammatory, analgesic, antiplasmodic, anti-mutagenic, sores, wound, urinary tract antioxidant	(Scott & Springfield, 2004; Lall & Kishore, 2014)
Plectranthus ambiguus	Anti-oxidant	(Liu & Ruedi, 1996)
P. barbatus	Respiratory ailments, liver disturbance, heart diseases, nervous problems	(Reis, et al., 2015)
P. ecklonii	Skin disorders, stomachaches, nausea, vomiting, meningitis, cancer	(Burmistrova, et al., 2015)
Salvia africana-caerulea	Stomachache, headache, bronchial congestion, antiseptic wash to ulcer	(Scott & Springfield, 2004)
S. africana- lutea	Cough, sexual debility, throat inflammation, chronic bronchitis, tuberculosis, influenza, diarrhea, anticancer, anti- inflammatory, antioxidant	(Scott & Springfield, 2004; Nkomo, et al., 2014)

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S. aurita	Antioxidant, anti-inflammatory	(Fisher, 2005)
S. chamelaeagnea	Cough, whooping cough, bronchitis, cold, flu, diarrhea, stomachache, headache, fever, burn wounds, microbial infections, antioxidant	(Huang & Van Staden, 2002; Scott & Springfield, 2004; Kamatou, et al., 2007)
S. disermas	Sores, rheumatism, hypertension, antioxidant, antimicrobial, antituberculosis, anti-inflammatory, antiplasmodial	(Fisher, 2005; Hawas, et al., 2009)
S. dolomitica	Anti-inflammatory	(Kamatou, et al., 2008)
S. namaensis	Antioxidant	(Kamatou, et al., 2008)
S. radula	Antioxidant, anti-inflammatory	(Kamatou, et al., 2008)
S. repens	Sores on the body, stomach problem, diarrhea, anti-inflammatory, anti-oxidant	(Kamatou, et al., 2005)
S. runcinata	Antioxidant, fever, anti-inflammatory, purgative, headache	(Kamatou, et al., 2005)
S. stenophylla	Antioxidant, anti-inflammatory, disinfectant, purgative, cold, cough, chest congestion	(Kamatou, et al., 2005; Lall & Kishore, 2014)
Tetradenia riparia	Cough, whooping, bronchitis, cold, diarrhea, headache, dropsy, fever, stomach ache, burn wounds, antiseptic, toothaches, skin diseases, and antioxidant.	(Scott & Springfield, 2004; Gairola, et al., 2009; Ndamane, et al., 2013)

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2.4 Phytochemistry of Lamiaceae

The chemistry of Lamiaceae family is still scanty with little chemical characterisation, but a large number of diterpenoids including kauranes, abietanes, clerodanes, phyllocladenes, neoclerodane, labdanes, pimiranes, isopimaranes, beyeranes, sesquiterpenes, and flavonoids are

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widely found in different species of *Plectranthus* genus (Abdel-Mogib & Albar, 2002). *Salvia* species have been reported to be rich source of phenolic and polyphenolic compounds such as carnosic acid (**8**), rosmarinic acid (**21**), caffeic acid (**20**) (Table 2.3.3 and 2.3.5) (Hamidpour, et al., 2014). It has been reported that the genus *Ballota* is rich in diterpenoids especially clerodane and tetracyclic diterpenes, the aromatic fragrance smell is an indication that characterized the plant of this genus (Ahmad, et al., 2004). Widespread occurrence of flavonoids, labdane diterpenoids, monoterpenoids and sesquiterpenoids in *Tetradenia* genus is an indication that characterized the plant from this genus (Mazimba, 2015). Lamiaceae family is a source of flavonoids and phenolic acid compounds such as rosmarinic acid (**21)**, *p*-coumaric acid, caffeic acid (**20**), ferulic acid, chlorogenic acid, luteolin (**22**), apigenin (**23**) (Table 2.3.5 and 2.4.1), genkwanin, quercitrin, rutin, epicatechin and catechin (Lee, et al., 2011). Caffeic acid is very common in *Salvia* species and the majority of phenolic acids isolated from *Salvia* are derivatives of caffeic acid which is the building block of a variety of plant metabolites (Kamatou, et al., 2009). Caffeic acid plays a vital role in the biochemistry of Lamiaceae and occurs predominantly in a dimer form as rosmarinic acid (Table 2.3.5) (Hamidpour, et al., 2014). The chemistry and the biological importance of the isolated compounds from Lamiaceae with skin anti-aging properties such as anti-tyrosinase, anti-melanogenesis, antioxidant, anti-inflammatory and antibacterial are highlighted on the tables (Table 2.2, 2.3, 2.4, … etc.) below.

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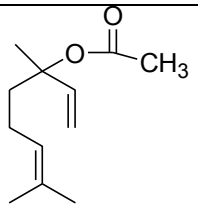
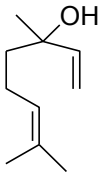
Table 2.2: Selected secondary metabolites with anti-ageing properties

Table 2.2.1: Monoterpenes

S/N	Compound	Plant source	Biological activity	Reference
1	 Linalyl acetate	*S. dolomitica*	Anti-inflammatory	(Kamatou, et al., 2008)
2	 Linolol	*S. dolomitica*	Anti-inflammatory	(Kamatou, et al., 2008)

S: Salvia

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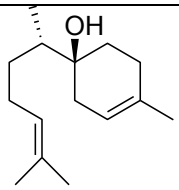
Table 2.2.2: Sesquiterpene

S/N	Compound	Plant source	Biological activity	Reference
3	 $\propto -$ bisabolol	*S. runcinata*	Antioxidant Anti-inflammation	(Kamatou, et al., 2008)

S: Salvia

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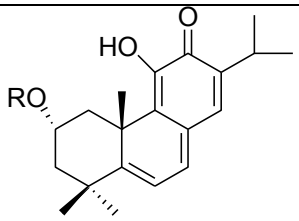
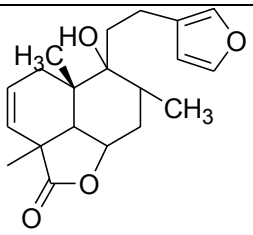
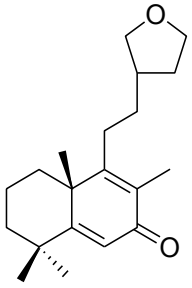
Table 2.2.3: Abiatene diterpenoid

S/N	Compound	Plant source	Biological activity	Reference
4	 Parvifloron D	*P.ecklonii*	Antioxidant	(Rosa, et al., 2015)

P: Plectranthus

Table 2.2.4: Labdane diterpenoids

S/N	Compound	Plant source	Biological activity	Reference
5	 Marrubin	*L. leonurus*	Antioxidant	(Mazimba, 2015)
6	 Leoleorin C	*L. leonurus*	Antioxidant	(Mazimba, 2015)

L: Leonotus

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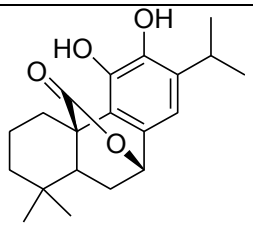
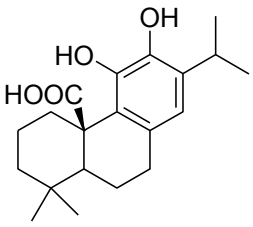
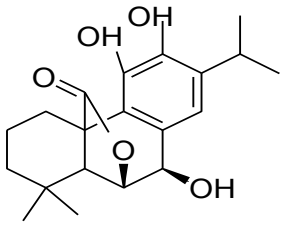
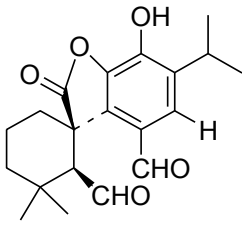
Table 2.2.5: Phenolic diterpenes

S/N	Compound	Plant source	Biological activity	Reference
7	 Carnosol	*S. chamelaeagnea*	Antioxidant Anti-melanogenic Antibacterial	(Kamatou, et al., 2009)[a] (Shirasugi, et al., 2010)[b] (Kamatou, et al., 2007)[d]
8	 Carnosic acid	*R.officinalis*	Antioxidant Anti-inflammatory	(Oliveira, et al., 2016)[a] (Bai, et al., 2010)[b]
9	 Rosmanol	*R. officinalis*	Antioxidant	(Inatani, et al., 1982)
10	 Rosmadial	*S. officinalis*	Antioxidant	(Brewer, 2011)

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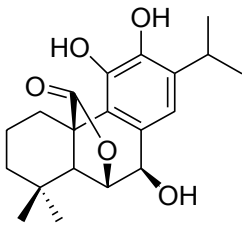
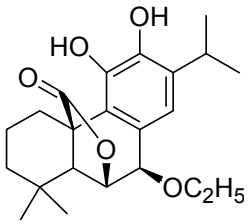
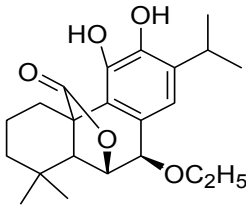
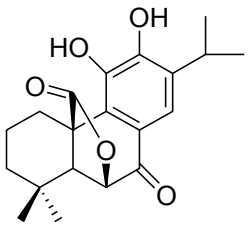
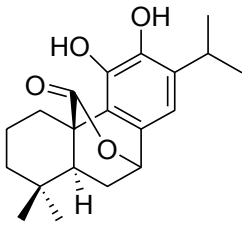
11	 epirosmanol	*S. officinalis*	Antioxidant	(Miura, et al., 2002)
12	 7- ethoxy-rosmanol	*S. officinalis*	Antioxidant	(Miura, et al., 2002)
13	 7-O-methylepirosmanol	*S. namaensis*	Antioxiant	(Kamatou, et al., 2009)
14	 galdosol	*S. officinalis*	Antioxidant	(Miura, et al., 2002)
15	 isorosmanol	*S. officinalis*	Antioxidant	(Miura, et al., 2002)
16			Antioxidant	(Gazim, et al., 2014)

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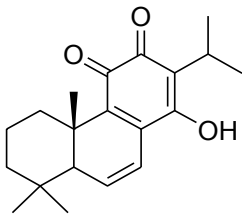

6,7- dehydroroyleanone

T. riparia

a: antioxidant; b: anti-melanogenesis; d: antimicrobial*; S:Salvia; R:Rosmarinus; T:Tetradenia*

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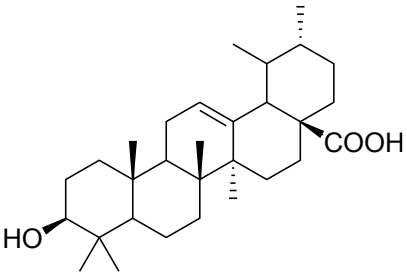
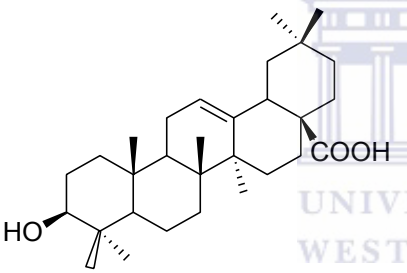
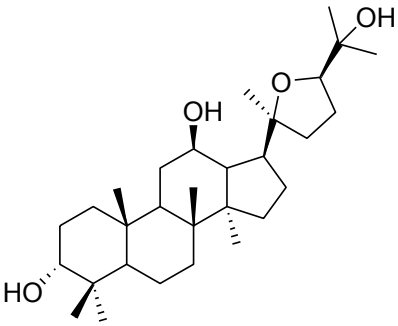
Table 2.2.6: Triterpenes

S/N	Compound	Plant source	Biological activity	Reference
17	 Ursolic acid	*S. officinalis*	Anti-inflammatory Antibacterial	(Baricevic, et al., 2001)[c] (Horiuchi, et al., 2007)[d]
18	 Oleanolic acid	*S. chamelaeagnea*	Antioxidant Anti-inflammatory Antimicrobial	(Kamatou, et al., 2007)[a] (Hwang, et al., 2014)[c] (Horiuchi, et al., 2007)[d]
19	 betulafolientriol oxide	*S. radula*	Antioxidant	(Kamatou, et al., 2009)

a: antioxidant; c: anti-inflammatory; d: antimicrobial;***S: Salvia***

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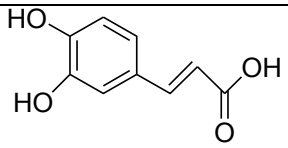
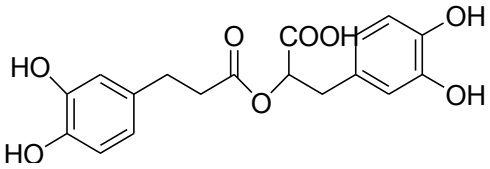
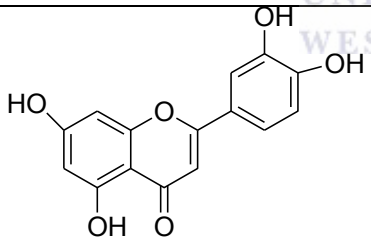
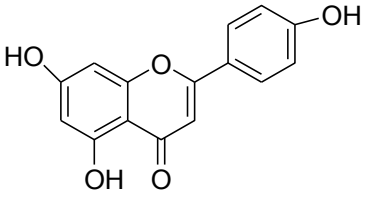
Table 2.2.7: Phenolic acid

S/N	Compound	Plant source	Biological activity	Reference
20	 Caffeic acid	*S. muirii*	Antioxidant Anti-melanogenic	(Kamatou, et al., 2009)[a] (Thangboonjit, et al., 2014)[b]
21	 Rosmarinic acid	*R.officinalis*	Anti-inflammatory Antioxidant Anti-melanogenic	(Petersen & Simmonds, 2003)[a,c] (Oliveira, et al., 2013)[b]

a:antioxidant; b:anti-melanogenesis; c: anti-inflammatory; S: Salvia;R: Rosmarinus

Table 2.2.8: Flavones

S/N	Compound	Plant source	Biological activity	Reference
22	 Luteolin	*Teucrium .chamaedrys*	Antioxidant Anti-inflammatory	(Panovska, et al., 2005)[a] (Lopez-Lazaro, 2009)[c]
23	 Apigenin	*T. polium*	Antioxidant	(Panovska, et al., 2005)

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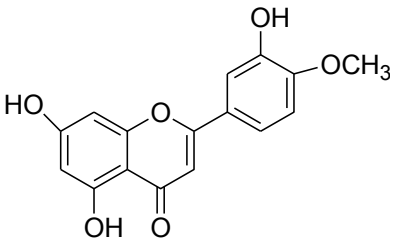
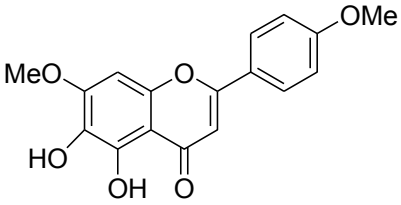
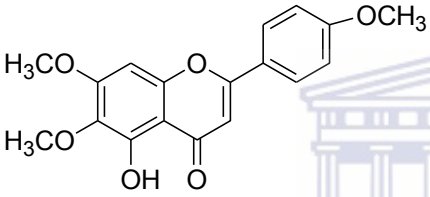
24	 Diosmetin	*T. polium*	Antioxidant	(Panovska, et al., 2005)
25	 Ladanein	*P. ambiguus*	Antioxidant	(Liu & Ruedi, 1996)
26	 Salvigenin	*S. radula*	Antioxidant	(Kamatou, et al., 2009)

a: antioxidant; c: anti-inflammatory; T: Teucriun; P: Plectranthus; S: Salvia

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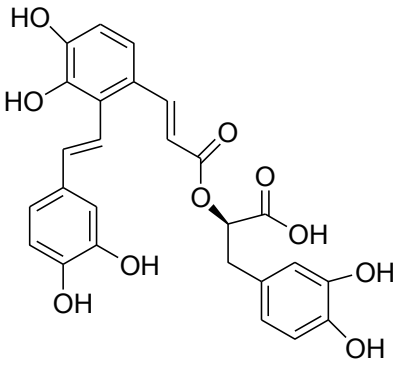
Table 2.2.9: Phenylpropenoid

S/N	Compound	Plant source	Biological activity	Reference
27	 salvianolic acid	*S. officinalis*	Antioxidant	(Lu & Foo, 2001)

S: Salvia

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2.5 Biological activities of Lamiaceae

2.5.1 Anti-tyrosinase activity

Based on the available information on the SciFinder database, there is no report on South African Lamiaceae plant species with significant activity against tyrosinase enzyme. However, it has been documented that the methanol extract of the aerial part of *S. africana lutea* demonstrated marginal inhibition of tyrosinase with inhibition of 48 % and 36 % at 500 µg/mL using tyrosine and L-DOPA as substrate (Momtaz, et al., 2008). The unpublished result conducted on the ethyl acetate fraction of *P. ecklonii* demonstrated moderate anti-tyrosinase inhibition with IC_{50} = 61.73 ± 2.69 µg/mL (Nyilan & Lall, 2010).

Nevertheless, there are numerous plants species of this family from other geographical locations reported to have significant tyrosinase inhibitory activity. Such example with significant inhibition are indicated in Table 2.6

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Table 2.3: Plant species with tyrosinase inhibitory activities

Name of the plant	Location	IC_{50} (µg/mL)	Reference
Origanum majorana	Taiwan	107.00	(Lee, et al., 2011)
Lavendula stoechas	Taiwan	127.00	(Lee, et al., 2011)
Lavendula latifolia	Taiwan	145.00	(Lee, et al., 2011)
Lavendula allardu	Taiwan	113.00	(Lee, et al., 2011)
Ballota nigra	Iran	3.67	(Hashemi & Zarei, 2014)
Eremostachys laevigota	Iran	9.51	(Hashemi & Zarei, 2014)

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2.5.2 Antioxidant activities

The family Lamiaceae is considered to be a promising source of natural antioxidants by the fact that polyphenolic sincluding carnosol (**7**), rosmanol (**10**) (Table 2.3.3) are the predominant components of this family and it has been reported to possess good antioxidant activities (Kamatou, et al., 2009). It has been established that there is a relationship between the chemical structure and antioxidant activity demonstrated by each constituents. The increase in number of hydroxyl groups in the aromatic ring increased with the free radical-scavenging and antioxidant activities (Lee, et al., 2011). The South African *Salvia* species were found to display good antioxidant activity with DPPH (IC_{50} 1.66-74.50 µg/mL), while $ABTS^{\cdot+}$ showed IC_{50} values between the range of 11.88 to 69.26 (Kamatou, et al., 2008). The antioxidant values displayed by the South African *Salvia* are mainly attributed to the presence of phenolics including phenolic acid such as rosmarinic acid (**21**), carnosic acid (**8**) and carnosol (**7**) as indicated in Table 2.3.3 and 2.3.5 (Huang & Van Staden, 2002; Petersen & Simmonds, 2003). Other compounds that may contribute to the antioxidant activities of *Salvia* include the presence of polyphenolic compounds such as flavonoids (Exarchou, et al., 2002; Kouřimská, et al., 2013). *Teucrium* species have been reported to possess radical scavenging activity as well as antioxidant activity in an *in vitro* system (Panovska, et al., 2005).

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CHAPTER 3: PRELIMINARY PHYTOCHEMICAL AND BIOLOGICAL SCREENING OF SELECTED SOUTH AFRICAN LAMIACEAE

3.1 Introduction

This chapter describes the preliminary phytochemical and biological investigation of South African Lamiaceae collected in the Cape floristic region.

The preliminary phytochemical screening was carried out with TLC using different solvent system and detection the spot physically (UV, short and long wavelengths) and chemically using vanillin/H_2SO_4 spray.

The preliminary biological (anti-tyrosinase activity) screening of the methanolic extracts of 25 South African Lamiaceae involved the following methods:

- TLC bioautography, a quick and simple screening method for detection of tyrosinase inhibitors. It is commonly used for bio-guided fractionation of tyrosinase inhibitors from mixtures or extracts (Wangthong, et al., 2007).
- Spectrophotometric measurement as the most widely used and reliable methodfor detection of tyrosinase inhibitors due to its best sensitivity, selectivity and versatility (Xia, et al., 2013).
- Cyclic voltammetry measurement, an electrochemical technique in which the current is measuring between a working and counter electrode as a function of the potential (Aristov & Habekost, 2015). CV is commonly used for detection of phenolic compounds, antioxidant agents (Dobes, et al., 2013; Magarelli, et al., 2013). However, this method has not been reported previously to measure or predict tyrosinase activity of plant extracts. So this is the first report on screening of tyrosinase inhibitors from natural source using cyclic voltammetry as a tool for detection of tyrosinase inhibitors.

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3.2 Materials and methods

3.2.1 Plant materials

Some of the plant materials used in this study were sourced from Kirstenbosch Garden Centre and the Nursery at the Cape Flats Nature Reserve, Bellville, while others were collected in the Cape Flats Nature Reserve and Hantam National Botanical Garden, Nieuwoudtville (Table 3.1). The wild collected plants were identified at the Compton Herbarium, Kirstenbosch by Dr. Christopher Cupido (SANBI, Kirstenbosch); a voucher specimen of each plant was deposited at the Compton Herbarium at Kirstenbosch.

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Table 3.1: List of the collected plant species

Assigned Code	Name of the plant	Place of collection
S1	*Ballota africana*	Hantam National Botanical Gardens
S2	*Leonotis leonurus*	Cape Flats Nature Reserve
S3	*L. nepetifolia*	Cape Flats Nature Reserve
S4	*Mentha longifolia*	Kirstenbosch Garden Centre
S5	*Plectranthus ambiguus*	Kirstenbosch Garden Centre
S6	*P. ciliates*	Cape Flats Nature Reserve
S7	*P. ecklonii*	Kirstenbosch Garden Centre
S8	*P. fruticosus*	Kirstenbosch Garden Centre
S9	*P. grandidentatus*	Kirstenbosch Garden Centre
S10	*P. hadiensis*	Kirstenbosch Garden Centre
S11	*P. madagascariensis*	Cape Flats Nature Reserve
S12	*P. saccatus*	Cape Flats Nature Reserve
S13	*P. strigosus*	Kirstenbosch Garden Centre
S14	*P. verticillatus*	Kirstenbosch Garden Centre
S15	*P. zuluensis*	Kirstenbosch Garden Centre
S16	*Salvia africana-caerulea*	Cape Flats Nature Reserve
S17	*S. chamelaeagnea*	Kirstenbosch Garden Centre
S18	*S. disermas*	Hantam National Botanical Gardens
S19	*S. dolomitica*	Kirstenbosch Garden Centre
S20	*S. lanceolate*	Kirstenbosch Garden Centre
S21	*S. muirii*	Kirstenbosch Garden Centre
S22	*S. namaensis*	Kirstenbosch Garden Centre
S23	*Stachys rugose*	Hantam National Botanical Gardens
S24	*Tetradenia riparia*	Kirstenbosch Garden Centre
S25	*Thorncroftia succulenta*	Kirstenbosch Garden Centre

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3.2.2 Preparation of plant extracts

The fresh aerial parts of the fresh plant materials were blended and extracted with methanol for 24 hours at room temperature (25 ^{o}C). The methanol extracts were filtered and evaporated to dryness under reduced pressure at 40 ^{o}C. The extracts were kept under cold conditions for further use.

3.2.3 Chemical and Reagents

The mushroom tyrosinase (EC 1.14.18.1) 5771 Unit/mg, L-Tyrosine and Kojic acid were purchased from Sigma Aldrich (Cape Town, South Africa). Organic solvents such as methanol (MeOH), ethanol (EtOH), hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and dimethyl sulfoxide (DMSO) were supplied by Merck (Cape Town, South Africa). Thin layer chromatography (TLC) was conducted on normal-phase silica gel 60 PF_{254} pre-coated aluminium plates (Merck, Cape Town, South Africa).

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3.2.4 Apparatus

All spectrophotometry experiments were performed using the enzyme-linked immunosorbent assay (ELISA) on AccuReader M 965/965+, Metertech Inc. version 1.11, Taipei, Taiwan. The cyclic voltammetry was performed using BASI EPSILON system as potentiostat. Three electrodes were used such as glass carbon electrode as a working electrode, a platinum (Pt) wire electrode as a counter electrode and an auxiliary electrode Ag/AgCl as reference electrode. The cyclic voltammetry experiments were recorded at 50 mV/s or 25 mV/s in two different potential ranges comprised between -1300 and 1300 mV; -200 and 200 mV. All experiments were performed at room temperature.

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3.2.5 TLC bioautographic assay for tyrosinase inhibition

The TLC bioautographic assay of tyrosinase inhibition was performed as described previously (Wangthong, et al., 2007) with slight modifications. Phosphate buffer (PBS) was prepared by mixing two stock solutions of monosodium phosphate 50 mM and disodium phosphate 50 mM to the pH 6.5.Tyrosinase solution was prepared by dissolving 1 mL of 1000 U enzyme in 1 mL of phosphate buffer. The substrate was prepared by dissolving 0.0036 g of L-tyrosine in 10 mL phosphate buffer. Extracts and kojic acid were dissolved in methanol to a stock solution of 1 mg/mL, 15 µL of the extracts and kojic acid (as positive control) were spotted onto the TLC plate and the plate was developed using two different solvent systems Hex:EtOAc (9:1) and DCM:MeOH (97:3). The developed plate was sprayed with tyrosinase and incubated at room temperature for 5 min, followed by L-tyrosine (20 mM) and left at room temperature for 30 min, then photographed.

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3.2.6 Tyrosinase enzyme Assay

This assay was performed using spectrophotometric method as previously described (Popoola, et al., 2015). Samples were dissolved in DMSO to a stock solution of 1 mg/mL, and further dilutions were done with 50 mM sodium phosphate buffer (pH 6.5) for all working solutions. Kojic acid was used as positive control. In the wells of a 96-well plate, 70 µL of each sample working solution was combined with 30 μL of tyrosinase (500 Units/mL in sodium phosphate buffer) in triplicate. After incubation at room temperature for 5 min, 110 μL of substrate (2 mM L-Tyrosine) was added to each well. Incubation commenced for 30 min at room temperature and the enzyme activity was determined by measuring the absorbance at 490 nm. The percentage of tyrosinase inhibition was calculated as follows:

$$(\%) = [(A - B) - (C - D)]/ (A - B) \times 100 \quad (1)$$

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Where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the test sample with the enzyme and D is the absorbance of the test sample without the enzyme.

3.2.7 Cyclic voltammetry measurement

Cyclic voltammetry was used for monitoring the behavior (increase or decrease) of the inhibition current with time in the presence of kojic acid or extract(s).

It was performed using the BAS100B electro-chemical analyzer, a glassy carbon electrode (GCE) as working electrode, Ag/AgCl as a reference electrode and platinum (Pt) as a counter electrode. Cyclic voltammograms (CVs) were recorded at two different scan rates 50 mV/s and 25 mV/s within the potential window ranging from -1300 to 1300 mV and from -200 to 200 mV. Both extracts and kojic acid were made to a stock solution of 10 mg/mL with DMSO. Tyrosinase and L-tyrosine were diluted in phosphate buffer to a working concentration and the experiments were carried out at room temperature.

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In CV experiments, glassy carbon electrode was polished with emery paper and Alumina powder (1.0, 0.3, 0.05 μm) to a minor finish, and then sonicated in doubly purified using ethanol and distilled water for 15 min each. After each CV scan, the electrode was re-polished using the same procedure to maintain the consistency of the surface area of the electrode.

To a cell containing 10 mL of 50 mM PBS at pH 6.5, a fixed volume of extracts and tyrosinase enzyme were added (700 μL) and (300 μL) respectively, to which a specific volume of the L-tyrosine (1100 μL) was also added. To understand the activity of the control either positive or negative, the extract was replaced with kojic acid and DMSO. Cyclic voltammetry of each addition was recorded.

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3.3 Results and Discussion

In the search of discovering new natural tyrosinase inhibitors, three complementary methods such as TLC bioautography, spectrophotometric and cyclic voltammetry were used for screening the selected plant extracts against tyrosinase. However, ELISA is predominantly used, the TLC bioautography has been reported for the same purpose while the use of cyclic voltammetry for the detection of tyrosinase inhibitors was limited to detect phenolic compounds through electrochemical measurement.

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Table 3.2: Percentage yield of the plant extracts

Name of the plant	Weight of the plant (g)	Weight of the extract (g)	% Yield
Ballota africana	797.5	74.2	9.3
Leonotis leonorus	134	10.1	1.32
L. nepetifolia	89.8	10.6	11.8
Mentha longifolia	10.1	1.9	18.8
Plectranthus ambiguus	88.8	2.1	2.36
P. ciliates	202.5	3.9	1.92
P. ecklonii	130.2	8.5	6.51
P. fruticosus	113.7	4	3.52
P. grandidentatus	197.4	5	2.5
P. hadiensis	138.7	3.8	2.74
P. madagascariensis	294.5	4.5	1.51
P. saccatus	163.4	7.1	4.34
P. strigosus	72.6	12.6	17.35
P. verticillatus	494.8	9.4	1.89
P. zuluensis	101.2	3.5	3.45
Salvia africana-caerulea	55	16	29.1
S. chameleagnea	1088.7	29.55	2.71
S. disermas	541.1	40.3	7.44
S. dolomitica	93.3	6.5	6.97
S. lanceolate	63.8	16.4	25.7
S. muirii	56.5	4.6	8.14
S. namaensis	130.8	10.3	7.87
Stachys rugosa	229.6	43	18.72
Tetradenia riparia	170.2	8.1	4.79
Thorncroftia succulent	167.4	5.3	3.16

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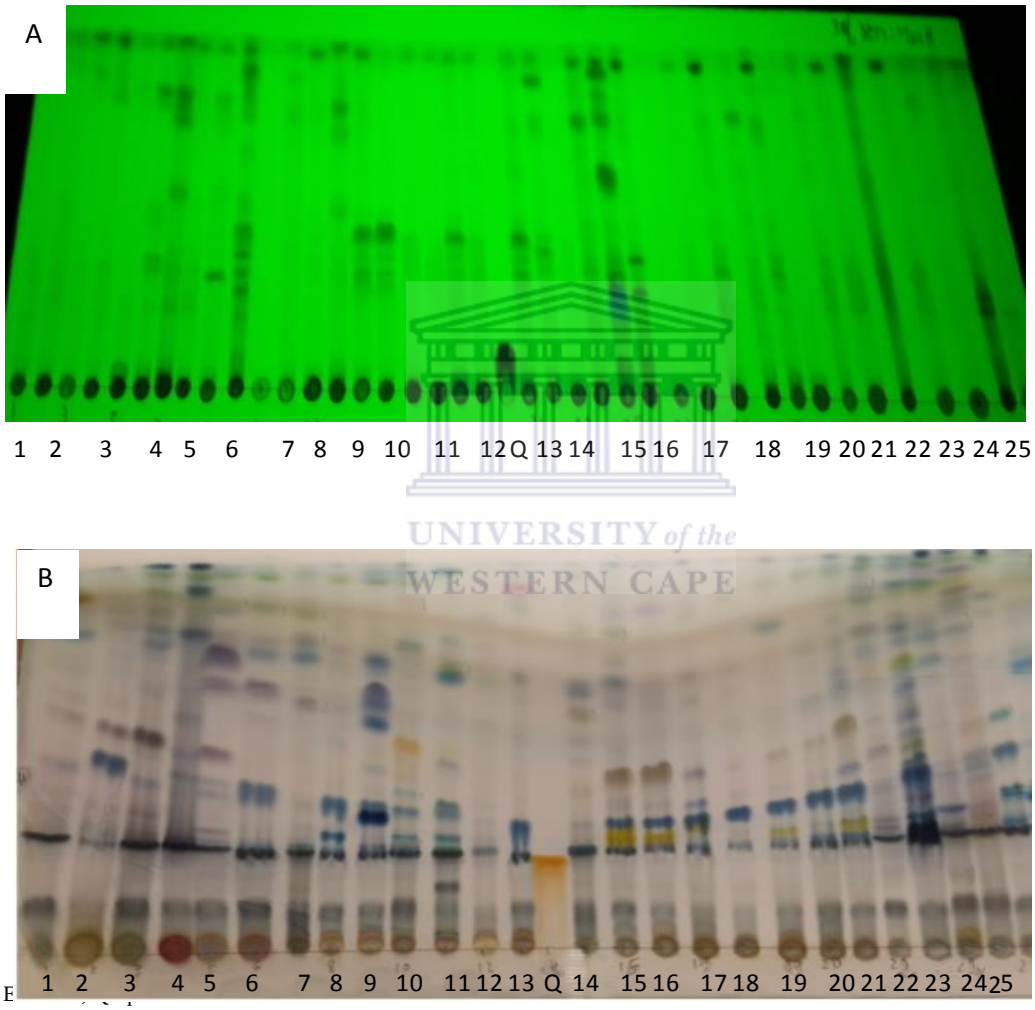
3.3.1 Preliminary phytochemical screening

The phytochemical screening of the selected plant species of Lamiaceae revealed the presence of many classes of compounds as shown on the TLC plate (Fig. 3.1). Such compounds include terpenoids (blue), flavonoids (yellow), and other phenolic compounds (red, orange).



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1-25: E

1-25: Extracts (S1-S25); Q: quercetin

Figure 3.1: TLC silica gel of the phytochemical screening of the plant species, solvent system DCM:MeOH (9.5:05); under UV (254nm; A), and spraying with H_2SO_4/ vanillin then heated (B).

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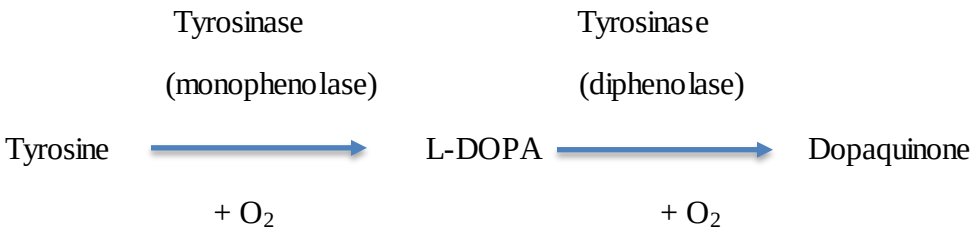
3.3.2 Cyclic voltammetry measurement

It is well known that tyrosinase catalyzes the oxidation process of L-tyrosine to dihydroxy-phenylalamine (L-DOPA) and from L-DOPA to dopaquinone (Ozer, et al., 2007).



CVs recorded for monitoring the enzymatic oxidation of L-tyrosine catalyzed by tyrosinase with time in the presence of molecular oxygen as shown in Figure 3.2. The broad-scan range set of CVs (from -1300 to 1300 mV at 50 mV/s) showed interesting electro-oxidation peak between -200 to 200 mV (Fig. 3.2 A), which evolved only gradually, i.e. its peak current increasing with reaction time. This was attributed to the electron transfer reaction involving a product of the enzymatic reaction, in this case, dopaquinone, which accumulated in the reaction medium as time went by (Fauziyah, et al., 2012). This peak was confirmed to exist at about 131 mV independent of the other peaks by running another set of CVs in a narrower scan range (-200 and 200 mV at 25 mV/s) as shown in Figure 3.2 B. Henceforth, this peak will be referred to as the inhibition peak and its peak current as the inhibition current (i_{inh}) because it is expected to be affected by the presence of tyrosinase inhibiting agent.

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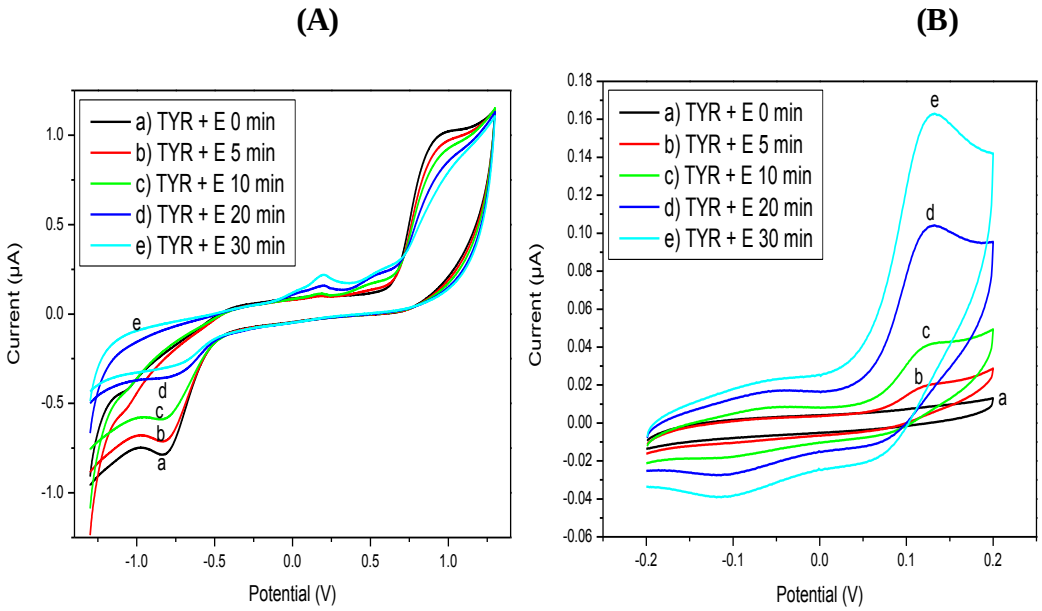

Figure 3.2: CVs recorded at 50 mV/ s in wide scan range (A), and at 25 mV/ s at the narrow scan range (B) for the reaction mixture of tyrosine and tyrosinase in 50 mM PBS (pH 6.5) at different times. Curve (a) 0 min, curve (b) 5 min, curve (c) 10 min, curve (d) 20 min, and then curve (e) 30 min.

The inhibition peak for the same reaction mixture was next studied in the presence of kojic acid, a standard tyrosinase inhibitor (Chang, 2009) as a positive control, and DMSO as a negative control (blank). As shown in Figure 3.3, an oxidation peak was observed and the oxidation current increased in time from 8.99 X 10^{-3}µA to 96 X 10^{-3}µA at 131 mV, indicating that the oxidation of tyrosine catalyzes by tyrosinase have been demonstrated. Figure 3.3 B showed no redox peak and the inhibition current decreased and almost dropped to the background level in time, indicating that the activity of tyrosinase was inhibited by kojic acid. These results are in agreement with the previous work described (Xia, et al., 2013), and the proposed method will be applicable for screening tyrosinase inhibitors from natural sources.

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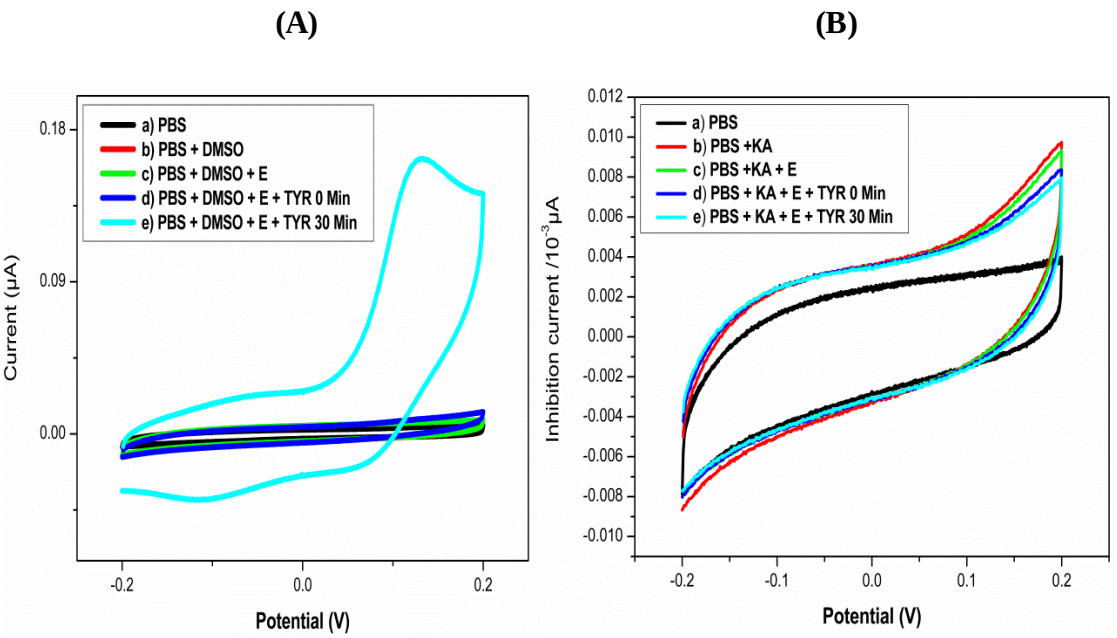

Figure 3.3: CVs recorded at 25 mV/ s for the reaction mixtures during negative control (A) and positive control (B) experiments, first for 50 mM PBS (pH 6.5) (curve a) and then after consecutive addition of DMSO (left, curve b) or kojic acid (right, curve b), tyrosinase (curve c), and tyrosine (0 min – curve d, 30 min- curve e).

Based on the CVs, the inhibitory behavior of each extracts toward the enzyme were classified as either positive or negative depending on the similarity to CVs recorded by replacing the extract with kojic acid as positive control or DMSO as negative control. An inhibitory behavior was "positive" when the inhibition current decreased with time but "negative" when it increased with time. Such decisions were made based on the differences between CVs recorded at 0 min and 30 min after addition of the substrate to a mixture of the extract and the enzyme in the buffer. Ultimately, the extracts were classified into two groups: active extracts and inactive extracts.

Group 1: Extracts that behave as positive control by decreasing the inhibition current with time, indicating that the activity of tyrosinase was inhibited and were therefore refers to this group as << active >>.

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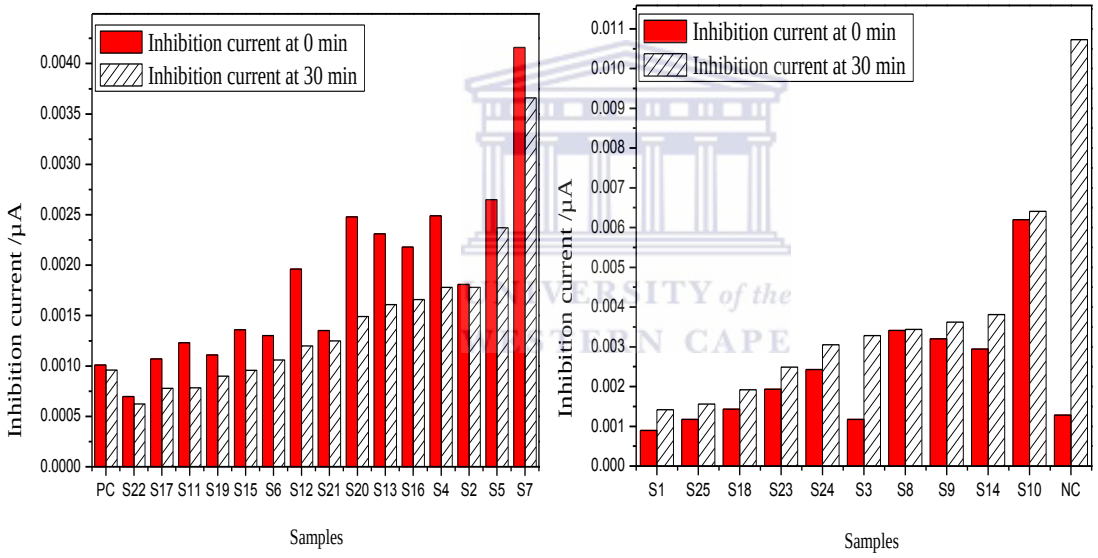
Group 2: Extracts that behave as negative control by increasing the inhibition current with time, indicating that the inhibition of tyrosinase doesn't take place and were therefore tagged this group as << inactive >>.

The histograms of each group were obtained by integrating the anodic area at the narrow potential ranging from 0.05 V to 200 V at 0 and 30 min after adding tyrosinase and tyrosine to the extract(s) or control(s) as shown in Figure 3.4.



Group 1: Active extracts : Inactive extracts

PC>S22>S17>S11>S19>S15>S6>S12>S21>S20>S13>S16>S4>S2>S5>S7 S1>S25>S18>S23>S24>S3>S8>S9>S14>S10>NC

Figure 3.4: Illustration of the effect of each group on inhibition of tyrosinase activity

The results demonstrated that the tyrosinase inhibitory activity of the tested extracts and kojic acid obtained by measuring the inhibition current with time are in agreement with the previous work described (Xia, et al., 2013). It has been reported that during the screening of tyrosinase inhibitors using cyclic voltammetry, the current increase with the increase of tyrosinase

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concentration and time in the absence of inhibitor, while the inhibition current decrease and almost dropped to the background level with the increase of kojic acid concentration and time, indicating that the activity of tyrosinase was inhibited by kojic acid (Xia, et al., 2013).

All the active extracts including kojic acid decreased the inhibition current with time (from 0 to 30min) as shown in Figure 3.4A, an indication that tyrosinase activity was inhibited by the extracts. The preliminary phytochemical investigation (Fig. 3.1) on the 25 plants (methanol extracts) revealed the presence of polyphenolic compounds as well as terpenoids when sprayed with vanillin sulfuric acid. The behavior of the inhibition current is due to the fact that the extracts contain phenolic constituents that have the properties to chelate or bind with the copper ions in the active site of tyrosinase, which result in its inhibition (Huang, et al., 2006). All the inactive extracts including DMSO increased of the inhibition current with time as shown in Figure 3.4B, an indication that there is no inhibition of tyrosinase by the tested extracts. This might be presumably related to their phytochemical constituents. Structural differences in phenolic compounds found in species of Lamiaceae family may also play a critical role in occurrence of tyrosinase inhibition (Suntar, et al., 2011).

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To evaluate the amenability of the method adopted, the tyrosinase inhibitory activity was determined by measuring the inhibition based on charge of each extracts and controls. The percentage of inhibition was calculated as follows.

$$[(Q30' - Q0')/ Q0'] X 100 \quad (2)$$

Where Q30' is the inhibition based on charge at 30 min, Q0' is the inhibition based charge at 0 min,

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Based on the result obtained in Table 3.3, 60 % of the plant extracts exhibited the percentage of inhibition more than 100, and were therefore refers as <<active>>, while 40 % of the extracts demonstrated the percentage of inhibition less than 100, and were therefore tagged as <<inactive>>

Table 3.3: Inhibition based on charge of the extracts, positive and negative controls at 0 and 30 min in the presence of tyrosinase and tyrosine obtained by integrating the anodic area from the corresponding CVs

	Abg	A0'	A30'	Qbg	Q0'	Q30'	Q30'-Q0'	
	Area before rnx/10^{-4}	**Area at 0 min/10^{-4}**	**Area at 30 min/10^{-4}**	Qbg/ mC	Q0min/ mC	Q30min/ mC	DQ/ mC	%DQref
Ballota africana	7.39	8.97	14.2	29.56	35.88	56.8	20.92	95.7
Leonotus leonurus	16.9	18.1	17.8	67.6	72.4	71.2	-1.2	100.2
L. nepetifolia	8.24	11.7	32.8	32.96	46.8	131.2	84.4	82.8
Mentha longifolia	12.9	24.9	17.8	51.6	99.6	71.2	-28.4	105.8
Plectranthus ambiguus	17.2	26.5	23.7	68.8	106	94.8	-11.2	102.3
P. ciliates	6.36	13	10.6	25.44	52	42.4	-9.6	102.0
P. ecklonii	16.2	41.61	36.6	64.8	166.44	146.4	-20.04	104.1
P. fruticosus	25.8	34.1	34.4	103.2	136.4	137.6	1.2	99.8
P. grandidentatus	12.2	32	36.2	48.8	128	144.8	16.8	96.6
P. hadiensis	31.6	62	64.1	126.4	248	256.4	8.4	98.3
P. madagascariensis	2.91	12.3	7.83	11.64	49.2	31.32	-17.88	103.6
P. saccatus	9.76	19.6	12	39.04	78.4	48	-30.4	106.2
P. strigosus	14.3	23.1	16.1	57.2	92.4	64.4	-28	105.7
P. verticillatus	16	29.4	38.1	64	117.6	152.4	34.8	92.9
P. zuluensis	4.87	13.6	9.56	19.48	54.4	38.24	-16.16	103.3
S. africana-caerulea	21.4	21.8	16.6	85.6	87.2	66.4	-20.8	104.2
S.chamelaeagnea	6.06	10.7	7.78	24.24	42.8	31.12	-11.68	102.4
S. disermas	8.88	14.3	19.2	35.52	57.2	76.8	19.6	96.0

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S. dolomitica	8.91	11.1	9.01	35.64	44.4	36.04	-8.36	101.7
S.lanceolata	9.81	24.8	14.9	39.24	99.2	59.6	-39.6	108.1
S. muirii	10.4	13.5	12.5	41.6	54	50	-4	100.8
S. namaensis	5.24	6.96	6.24	20.96	27.84	24.96	-2.88	100.6
Stachys rugosa	16.1	19.3	24.9	64.4	77.2	99.6	22.4	95.4
Tetradenia riparia	21.5	24.3	30.5	86	97.2	122	24.8	94.9
Thorncroftia succulenta	9.96	11.7	15.6	39.84	46.8	62.4	15.6	96.8
Positive control	12.6	10.1	9.59	50.4	40.4	38.36	-2.04	100.4
Negative control	13.8	12.8	107.3	55.2	51.2	429.2	378	0.0

3.3.3 TLC bioautographic assay for tyrosinase inhibition

The anti-tyrosinase bioautography assay was carried out on TLC plate developed with solvent systems Hex:EtOAc (9:1) and DCM:MeOH (97:3) respectively as indicated in Figure 3.5 (A & B). The plate was sprayed with both enzyme and the substrate and then incubated for 30 minutes. Clear white zones against the purple background, which were visually detected, are indication of zone of inhibition as demonstrated by respective extracts (Misra & Dey, 2012). The size of the white zones of inhibition is related to the degree of tyrosinase inhibition. Kojic acid demonstrated the highest zone of inhibition followed by *Salvia chamelaeagnea*, *S. dolomitica*, *Plectranthus ecklonii*, *P. namaensis,* and *P. zuluensis.*

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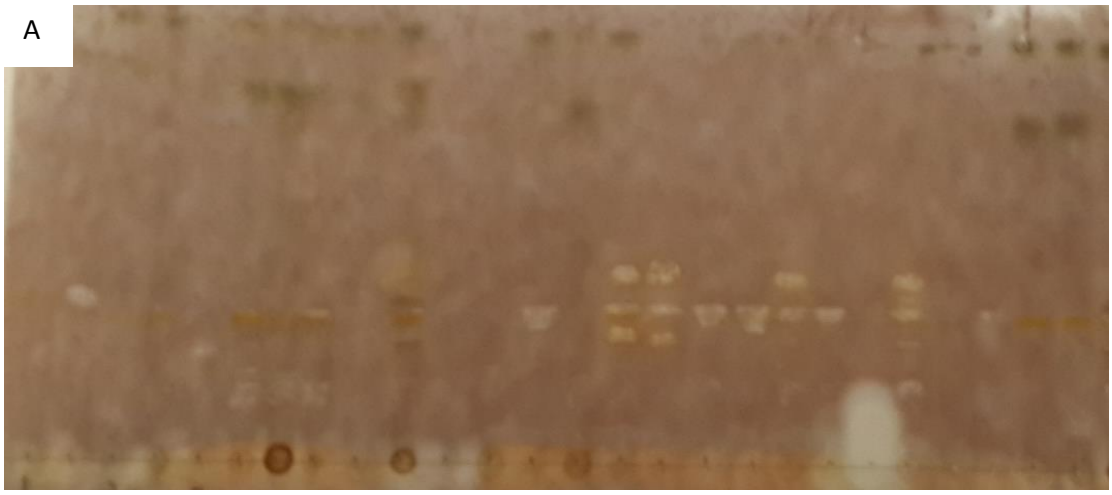
A



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 KA 22 23 24 25

1-25 Extracts (S1-S25); KA: Kojic acid

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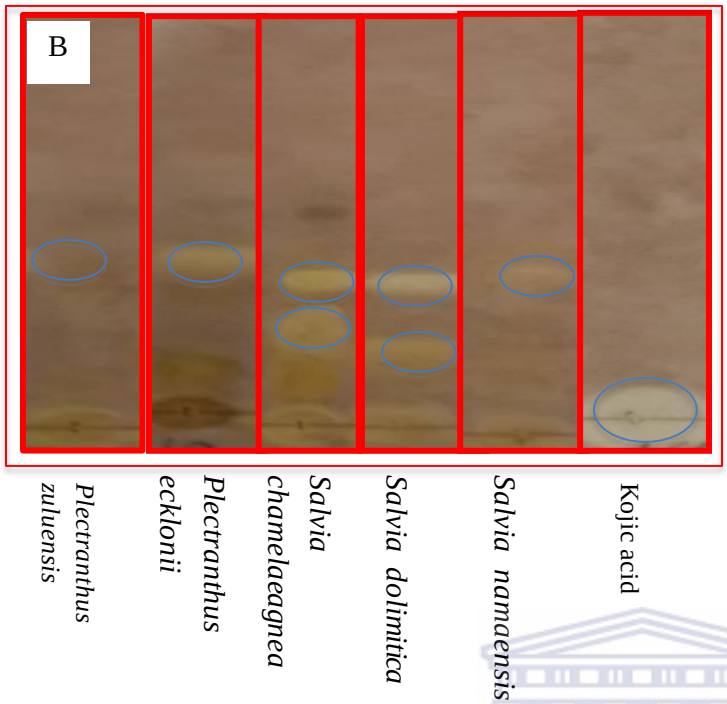

Figure 3.5*:* TLC silica gel bioautography of all the extracts and kojic acid (A), the active extracts and kojic acid (B), solvent system Hex:EtOAc (9:1) andDCM:MeOH (97:3) respectively.

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3.3.4 Tyrosinase enzyme Assay

The screening of 25 plant extracts was studied for quantitative measurements by spectrophotometry method. Table 3.4 shows, the concentration at which 50 % of tyrosinase enzyme is inhibited (IC_{50}) by the methanolic extracts. This study revealed that four plant extracts (16 %) such as *Plectranthus ecklonii*, *P. madagascariensis*, *P. zuluensis* and *Salvia lanceolata* inhibited tyrosinase strongly with IC_{50} values of < 30 μg/mL, six plant extracts (24 %) inhibited tyrosinase moderately with IC_{50} values comprising between 30 and 100 μg/mL while fifteen five plant species (60 %) inhibited tyrosinase poorly with IC_{50} values of >100 μg/mL compared to the positive control (kojic acid IC_{50} = 3.42 μg/mL).

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Table 3.4: Screening of plant extracts for tyrosinase inhibitory activities

Sample	IC_{50} (µg/mL)	TLC- Bioautography ($R_f$value)
Ballota africana	> 500	-
Leonotis leonurus	> 500	-
L. nepetifolia	>500	-
Mentha longifolia	212.46	-
Plectranthus ambiguus	115.81	-
P.ciliates	36.08	-
P. ecklonii	21.58	0.43
P. fruticosus	229.38	-
P. grandidentatus	280.12	-
P. hadiensis	36.08	-
P. madagascariensis	23.99	-
P. saccatus	38.49	-
P. strigosus	45.74	-
P. verticillatus	287.37	-
P. zuluensis	23.99	0.42
Salvia africana caerulea	81.98	-
S. chamelaeagnea	267.4	0.38 ; 0.26
S. disermas	226.96	-
S. dolomitica	243.87	0.37 ; 0.2
S. lanceolata	28.83	-
S. muirii	103.73	-
S. namaensis	301.86	0.4
Stachys rugosa	> 500	-
Tetradenia riparia	171.39	-
Thorncroftia succulenta	36.08	-
Kojic acid	3.42	0.06

*IC_{50} means the concentration of samples required for 50 % inhibition of tyrosinase activity.- : Not active

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To summarize the outcome of the findings from the screening of plant materials for tyrosinase inhibitors using three complementary methods, cyclic voltammetry commonly used as suitable tool for detection of phenolic and antioxidant compounds through electrochemical measurement (Dobes, et al., 2013), was used for the first time for detection of tyrosinase inhibitors based on the behavior of each extracts toward tyrosinase and tyrosine by increasing or decreasing the inhibition current with time as controls. CVs developed nominated *Leonotis leonurus, Plectranthus madagascariensis, P. saccatus, P. zuluensis P. saccatus, P. ecklonii, S. lanceolata, S. dolomitica* and *Salvia chamelaeagnea,* as strong tyrosinase inhibitors due to the fact that they strongly decreased the inhibition current with time in the comparative manner with kojic acid. TLC bioautography, known as a quick and simple technique for screening of tyrosinase inhibitors (Wangthong, et al., 2007) was developed to detect extracts which have the potency to inhibit tyrosinase activity. Five extracts such as *P. ecklonii, P. zuluensis, S. chamelaeagnea, S. dolimitica,* and *S. namaensis,* demonstrated very clear white zone of inhibition on TLC plate. The appeared of white spots against a browning-purple background onto the TLC plate is an indication of the inhibition of the conversion of tyrosinase catalyzed by tyrosine to melanin (Misra & Dey, 2012). Spectrophotometric method nominated *P. ecklonii, P. madagascariensis, P. zuluensis* and *S. lanceolata* as strong tyrosinase inhibitors.

These three complementary methods showed significant level of agreement as indicated, suggesting how reliable of the methods adopted by the investigation. For instance, *P. zuluensis* and *P. ecklonii* were identified by the three methods carried out, while two out of the three assays nominated *P. madagascariensis, S. chamelaeaegnea, S. namaensis, S. lanceolata,*and *S. dolomitica* as natural plants for anti-tyrosinase activities.

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CHAPTER 4: ANTI-TYROSINASE AND TOTAL ANTIOXIDANT ACTIVITIES OF ISOLATED CONSTITUENTS OF *SALVIA CHAMELAEAGNEA*

4.1 Introduction

The preliminary phytochemical, bioautography, cyclic voltammetry and spectrophotometry screening of 25 plant species of Lamiaceae family carried out in Chapter 3 nominated *S. chamelaeagnea* as one of the most biologically active extract. However, the clear zone of inhibition in TLC-bioautography determined the final selection of this species. Thus, chromatographic isolation of compounds responsible for these biological profiles was carried out to elucidate their chemical structures and establish various biological specificity demonstrated by each compounds.

4.2 Background information on *Salvia chamelaeagnea*

Salvia chamelaeagnea (Lamiaceae; Salvia) is a flowering plant, commonly known as "sage", a slow growing evergreen shrub growing up to 2 m with a spread of about 1 m originating from the South Western area of the Cape of Good Hope (Fig 4.1 B). In its natural habitat, *S. chamelaeagnea* develops into attractive foliage and flowering landscape plant, with small mid-green egg shaped leaves and masses of bright blue or white flowers (Fig 4.1 A) (Kamatou, et al., 2007). This plant has been used for medicinal purposes such as treating coughs and colds, colic and heartburn. History also documented that extract of this plant exhibit pharmaceutical effect against various microbial and bacterial infections (Kamatou, et al., 2008; Strelitzia, 2013)

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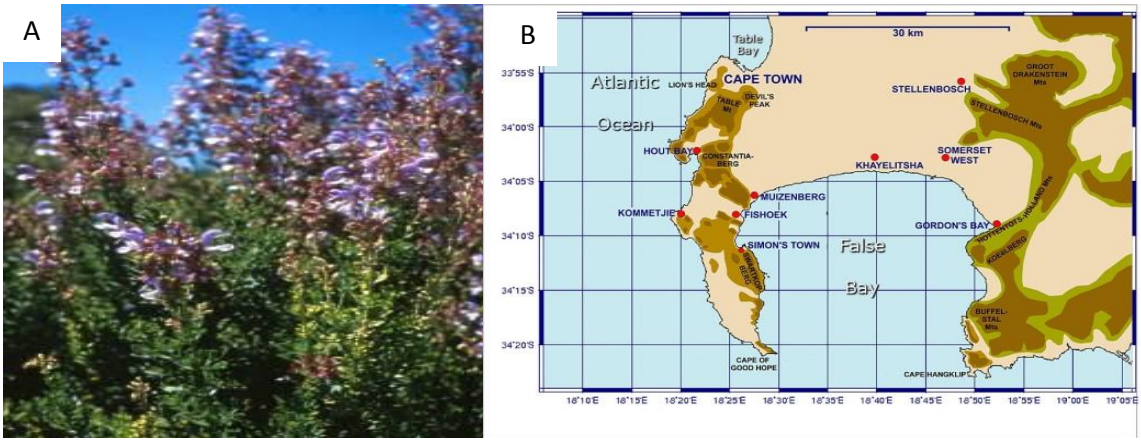

Figure 4.1: *Salvia chamelaeagnea* description (A), and distribution along South Western area of the Cape of Good Hope. www.plantzafrica.com

This chapter describes:

- Isolation of the chemical constituents present in methanol extract of *S. chamelaeagnea*, using different chromatographic methods.
- Elucidation of the chemical structure of each compound using different spectroscopic techniques.
- Determination of tyrosinase inhibitory activity of the isolated compounds.
- Evaluation of the total antioxidant capacities of isolated compounds.
- Establishment of the relationship between the chemical structures of each constituents with the various biological behaviors through mechanism of reactions

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4.3 CHEMICAL CHARACTERIZATION OF *SALVIA CHAMELAEAGNEA* CONSTITUTUENTS

4.3.1 Reagents and Solvents

Organic solvents of methanol (HPLC graded), ethanol, ethyl acetate, dichloromethane, hexane (redistilled), deuterated chloroform was supplied by Merck (Darmstadt, Germany).

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4.3.2 Chromatography

4.3.2.1 Thin layer chromatography (TLC)

Pre-coated plates of silica gel 60 F_{254} (Merck, Germany) was used for TLC analysis. Visualization of TLC plates was done by observing the bands "spots" after development under UV at wavelengths 254 and 366 nm using UV lamp (CAMAG, Switzerland), followed by vallinin/sulphuric acid spray reagent and then heated for phytochemical identification. Unless otherwise stated, the solvent systems generally used for the TLC development of *S. chamelaeagnea* fractions are indicated in Table 4.1

Table 4.1: TLC solvent systems

Solvent system	Ratio	Assigned code
Hex - EtOAc	9:1	A
Hex - EtOAc	7:3	B
DCM - MeOH	9.5:0.5	C
DCM - MeOH	9:1	D
DCM - MeOH	7:3	E

Hex: hexane; EtOAc: ethylacetate; DCM: dichloromethane; MeOH: methanol

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4.3.2.2 Column chromatography

The column chromatography (different diameters) was performed using silica gel 60 H (0.040-0.063 mm particle size, Merck, South Africa) and sephadex LH-20 (Sigma-Aldrich, South Africa) as stationary phases.

4.3.2.3 Semi-preparative High Pressure Liquid Chromatography (HPLC)

Final purification was carried out using Agilent Technologies 1200 series, equipped with UV

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detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), column compartment (G1316A) and reversed phase C18 column SUPELCO (25 x 1.0 cm, 5 μm). The flow rate was set at 1.5 mL/min. All experiments were done at room temperature (20 ^{o} C).

4.3.3. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded at 20^{o}C, using deuterated chloroform on a Bruker Avance 400 MHz NMR spectrometer (Germany), Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to tetramethylsilane (TMS) as internal reference.

4.4 Collection and identification of plant material

The plant material used in this study was collected from the Cape Flats Nature Reserve as mentioned in chapter 3.

4.5 Extraction and Fractionation of total extract

The aerial parts of the fresh plant material (1.0887 Kg) were blended and extracted with methanol (4.5 L) at room temperature (25 ^{o}C) for 24 hours. The methanol extract was filtered and evaporated to dryness under reduced pressure at 40 ^{o}C to yield 29.55 g (2.71 %). The extracts were kept under cold conditions for further use. The total extract of *S. chamelaeagnea* (29 g) was applied to a silica gel column (30 x 18 cm) and eluted using gradient of hexane and ethyl acetate in order of increasing polarity as indicated in Table 4.2. Sixty four (64) fractions (500 mL each) were collected during the process and numbered 1-64.

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Table 4.2: Fractionation of the extract of *S. chamelaeagnea* (SC)

Solvent system	Solvent volume	Fraction collected
Hexane	1L	1-2
Hexane – ethyl acetate (95:5)	2L	3-6
Hexane – ethyl acetate (90:10)	2L	7-10
Hexane – ethyl acetate (85:15)	3L	11-16
Hexane – ethyl acetate (80:20)	3L	17-22
Hexane – ethyl acetate (75:25)	2L	23-26
Hexane – ethyl acetate (70:30)	2L	27-30
Hexane – ethyl acetate (60:40)	1L	31-32
Hexane – ethyl acetate (50:50)	1L	33-34
Hexane – ethyl acetate (40:60)	2L	34-37
Hexane – ethyl acetate (30:70)	2L	38-41
Hexane – ethyl acetate (20:80)	2L	42-45
Hexane – ethyl acetate (10:90)	3L	46-51
Hexane – ethyl acetate (5:95)	3L	52-57
Ethyl acetate	3L	58-64

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The collected fractions (1-64) were concentrated and combined according to their TLC profiles (Fig 4.2) using solvent system (B &D) to yield 15 main fractions (Fig 4.3). The obtained fractions were coded by roman numbers (I - XV) and the results are summarized in table 4.3

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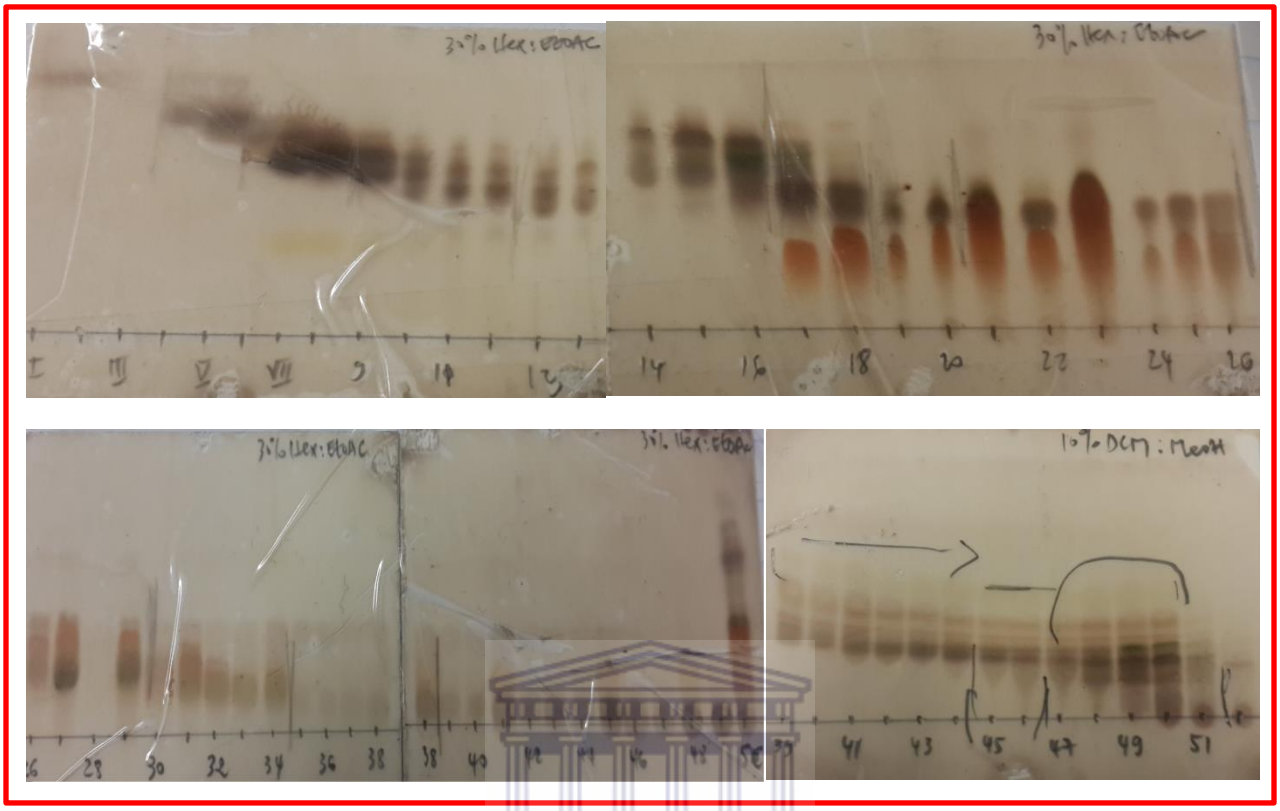

Figure 4.2: TLC silica gel of the collected fractions of SC, solvent system Hex:EtOAc (7:3) and DCM:MeOH (9:1).

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Table 4.3: Fractions obtained upon fractionation of total extract of *S. chamelaeagnea*

Combined fraction	Designated number	Code	Weight of fractions (mg)
1-3	I	SC-I	470.0
4-5	II	SC-II	366.0
6-8	III	SC-III	10.5
9-12	IV	SC-IV	305.0
13-16	V	SC-V	697.2
17-18	VI	SC-VI	620.1
19-20	VII	SC-VII	550.0
21-29	VIII	SC-VIII	1046.9
30-32	IX	SC-IX	1457.8
33-34	X	SC-X	465.2
35-38	XI	SC-XI	562.0
39-44	XII	SC-XII	1097.6
45-46	XIII	SC-XIII	116.0
47-50	XIV	SC-XIV	560.9
51-64	XV	SC-XV	503.8

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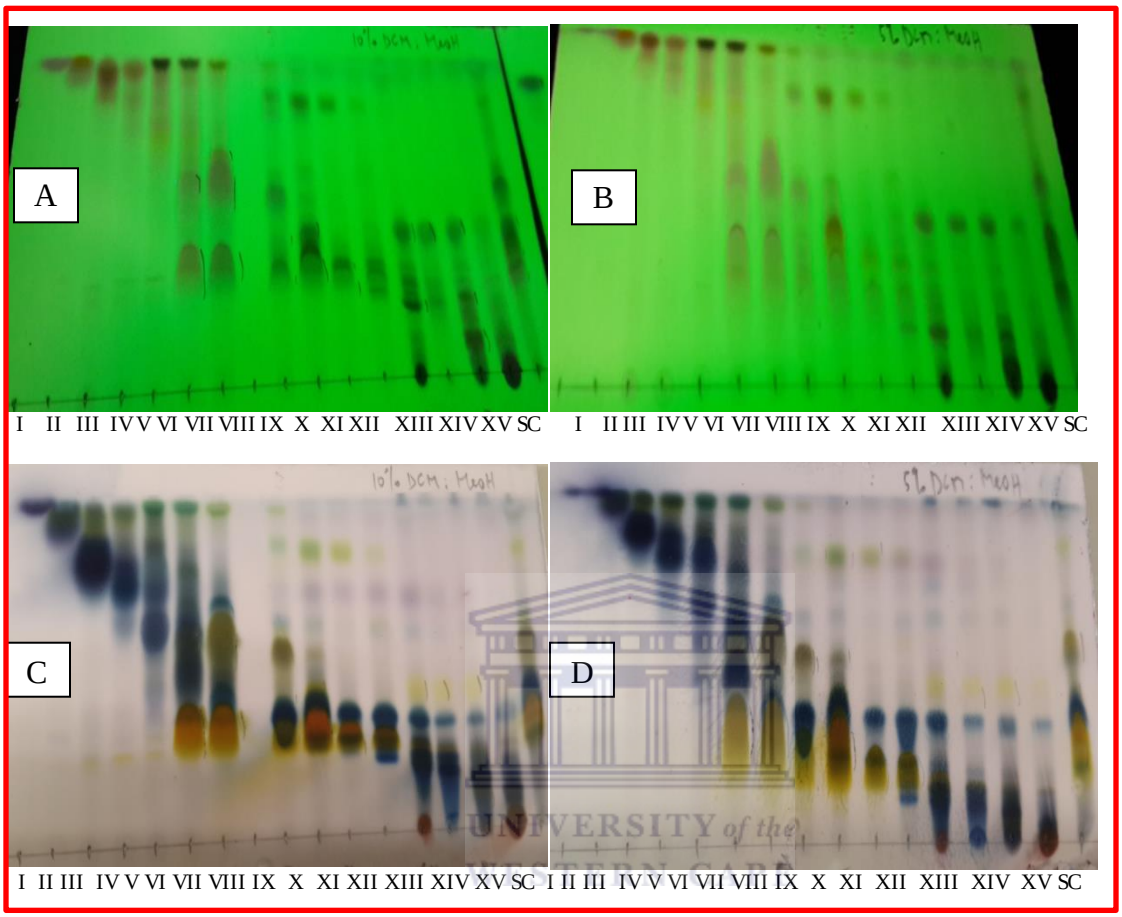

Figure 4.3: TLC silica gel profile of combined fractions under UV (254 nm; A & B), and After spraying with H_2SO_4/ vanillin and then heated (C & D).

TLC plate (A & C) of combined fractions (I-XV) was developed using solvent system D.

TLC plate (B & D) of combined fractions (I-XV) was developed using solvent system C.

4.6 Isolation of pure compounds

4.6.1 Isolation of compound C1 – Column chromatography of main fraction SC-VII

Main fraction SC-VII (550 mg) was chromatographed on sephadex column using isocratic elution of 5 % aqueous ethanol. Fractions of 5 mL each were collected and evaporated using

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rotary evaporator. Fractions obtained were developed on TLC using solvent system C and the fractions that displayed same profiles on the TLC plate were combined as indicated in Table 4.4.

Table 4.4: Fractions grouped from the column

Fraction	Weight (mg)	Assigned code
5-9	33.2	SC-VII-1
10-15	56.1	SC-VII-2
16-20	69.2	SC-VII-3
21-23	155.8	SC-VII-4
24-31	195.2	SC-VII-5

Figure 4.4: TLC silica gel of combined fractions of SC-VII, solvent system DCM:MeOH (9.5:0.5).

Fractions 24-31 were suspected pure due to its single spot after development on TLC using solvent system C (Fig. 4.5). These fractions (24-31) were pooled together, evaporated and re-spotted using solvent system C to further confirm the purity of the combined fractions. The TLC showed only single spot after development and confirmed the purity (Fig. 4.4) and labeled the compound as (**C1**, 195.2 mg, 0.67%).

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Figure 4.5 TLC silica gel of SC-VII-5 (**C1**), solvent system silica gel plate; solvent system DCM:MeOH (9.5:05).

4.6.2 Isolation of compound C2 – Column chromatography of main fraction SC-VIII

Main fraction SC-VIII (554 mg) was chromatographed on sephadex column using isocratic elution 5 % aqueous ethanol. Fractions of 5 mL each were collected and evaporated using rotary evaporator. Fractions obtained were developed on TLC using solvent system C and the fractions of same TLC profiles were combined as indicated in Table 4.5

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Table 4.5: Fractions grouped from the column

Fraction	Weight (mg)	Assigned code
1-7	10.7	SC-VIII-1
8-13	29.1	SC-VIII-2
14-21	179.6	SC-VIII-3
22-23	84.8	SC-VIII-4
24-32	57.4	SC-VIII-5
33-45	50.1	SC-VIII-6

After spotting and developing the fractions on TLC plate using solvent system C, single spot was observed and suspected to be a pure compound as shown on the Figure 4.6. The fractions that

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gave the single spot (22-23) were combined together and labeled SC-VIII-4 (**C2**; 84.8 mg; 0.29 %).

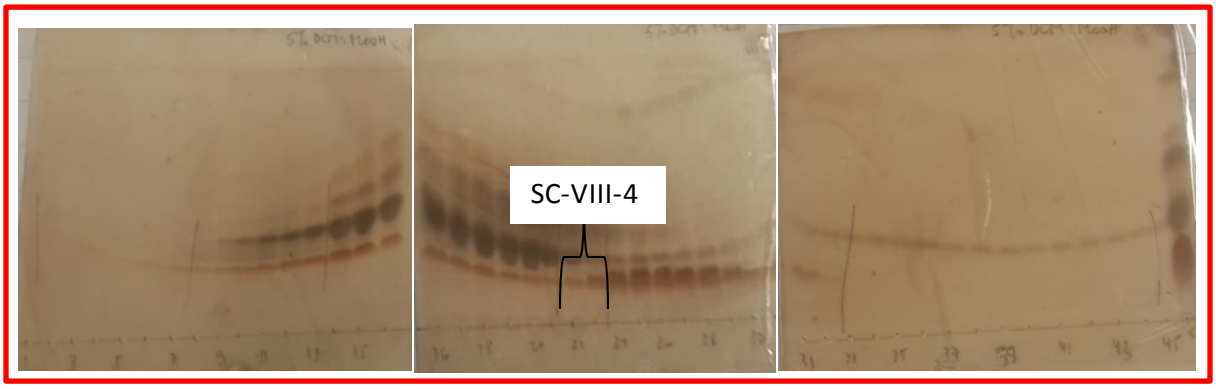
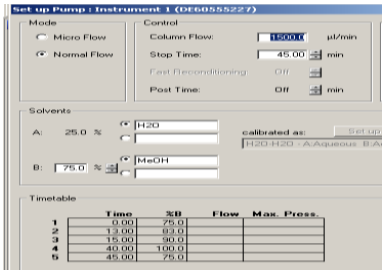


Figure 4.6: TLC silica gel of combined fractions of SC-VIII, solvent system DCM:MeOH (9.5:05).

4.6.3 Isolation of compound C3 Column chromatography of main fraction SC-VIII-6

Injection of sub fraction SC-VIII-6 (50 mg) to the HPLC using gradient solvent system of MeOH and de-ionized water (75:25 to 100% MeOH in 40 min) afforded five (5) peaks (Figure 4.7). The prominent peak collected was labeled as SC-VIII-6-5 (**C3**, 28 min, 3.7 mg; 0.01 %).



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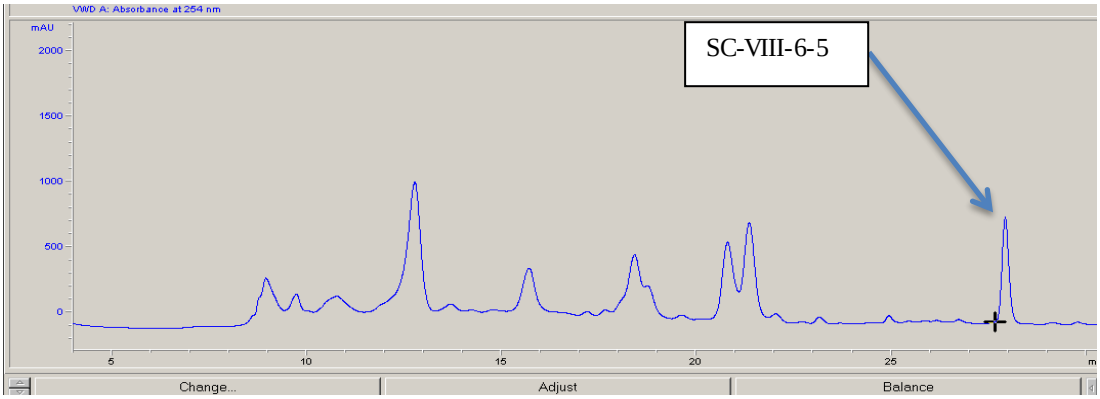


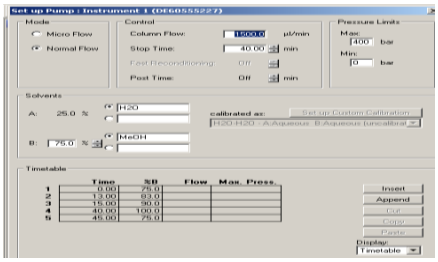
Figure 4.7: HPLC chromatogram of **C3**.

***Conditions**

Solvent	MeOH:DIW 75:25 to 100% in 40 min
Column	SUPELCO, RP C-18 (25 x 1 cm)
Flow rate	1.5 mL/min
Detection	UV at 254 nm

4.6.4 Isolation of compound C4 Column chromatography of main fraction SC-VIII-2

Injection of sub fraction SC-VIII-2 (629 mg) to the HPLC using gradient solvent system of MeOH and de-ionized water (75:25 to 100% MeOH in 40 min) afforded eighteen (18) peaks (Figure 4.8), the prominent peak collected and labeled as SC-VIII-2-17-A. After spotting and developing the fractions on TLC plate using solvent system C, a single spot suspected to be a pure compound was observed (Fig 4.11). The fraction that afforded this single spot were pooled together and labeled as SC-VIII-2-17-A (**C4**, 32.1 min, 13.79 mg; 0.04 %).



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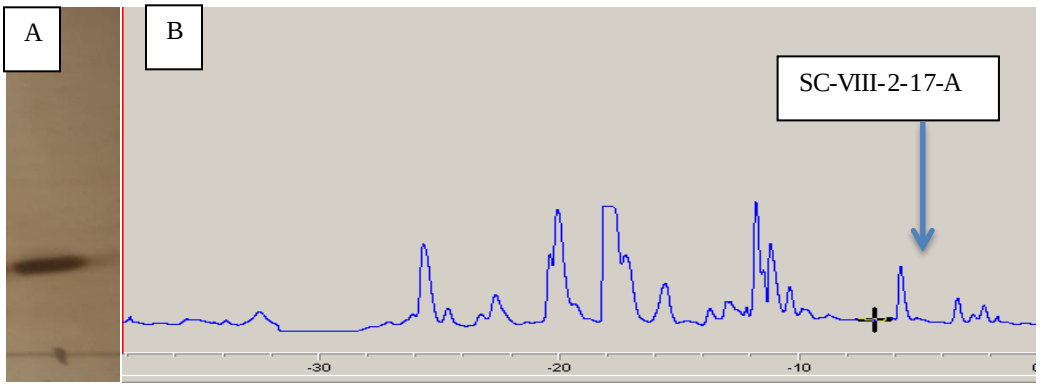


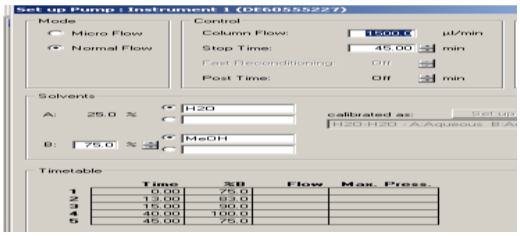
Figure 4.8: TLC silica gelof SC-VIII-2-17-A (Fig. 4.8 A), solvent system DCM:MeOH (9.5:0.5) and HPLC chromatogram of **C4** (Fig. 4.8B).

***Conditions**

Solvent	MeOH:DIW 75:25 to 100% in 40 min
Column	SUPELCO, RP-18 (25 x 1 cm)
Flow rate	1.5 mL/min
Detection	UV at 254 nm

4.6.5 Isolation of compound C5 Column chromatography of main fraction SC-VIII-5

Injection of sub fraction SC-VIII-5 (57.4 mg) to the HPLC and eluted using gradient solvent system of MeOH and de-ionized water (75:25 to 100% MeOH in 45 min) afforded eighteen (6) peaks (Figure 4.9), the prominent peak was collected and labeled as SC-VIII-5-1 (**C5**, 32.1 min, 7.1 mg; 0.02 %).



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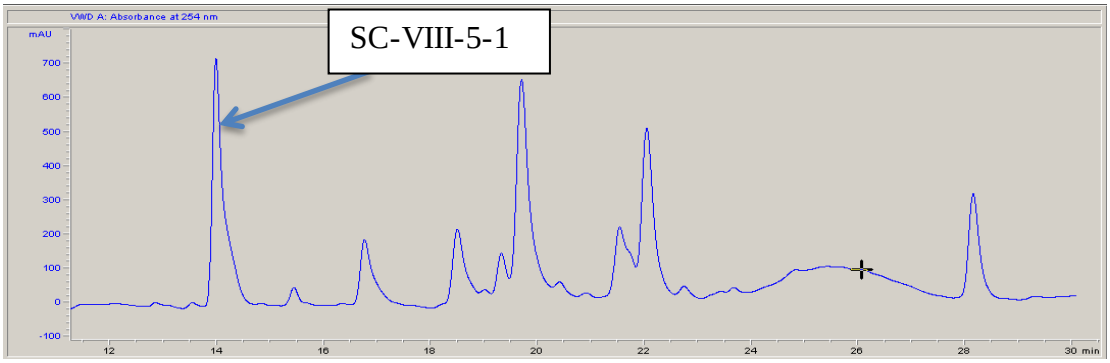

Figure 4.9: HPLC chromatogram of **C5**.

***Conditions**

Solvent	MeOH:DIW 75:25 to 100% in 45 min
Column	SUPELCO, RP-18 (25 x 1 cm)
Flow rate	1.5 mL/min
Detection	UV at 254 nm

4.6.6 Isolation of compound C6 Column chromatography of main fraction SC-XIV

Fraction XIV (500 mg) was chromatographed on sephadex column (5 % aqueous ethanol). 5 mL each of the fraction were collected, and evaporated using rotary evaporator. Fractions obtained were developed on TLC using solvent system D and fraction displayed same profiles on TLC were combined as indicated in Table 4.6

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Table 4.6: Fractions grouped from the column

Fraction	Weight (mg)	Assigned code
1-9	24.7	SC-XIV-1
10-13	13.4	SC-XIV-2
14-20	37.5	SC-XIV-3
21-26	73.2	SC-XIV-4
27-32	24.5	SC-XIV-5
33-39	37.4	SC-XIV-6
40-51	17.8	SC-XIV-7
52-64	24.9	SC-XIV-8

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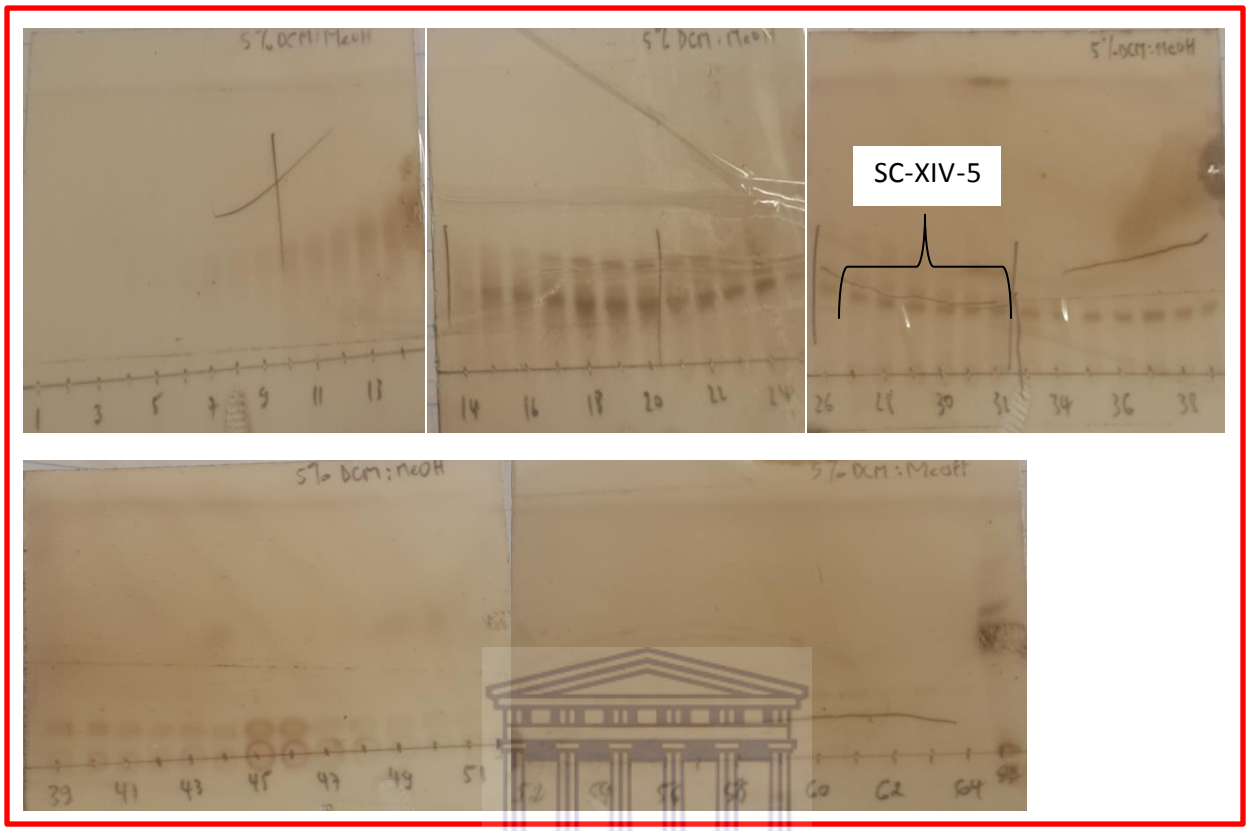

Figure 4.10: TLC silica gel of combined fractions of SC- XIV, solvent system DCM:MeOH (9:1).

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Sub fraction**XIV-5** (24.5 mg) was injected to the HPLC and eluted using gradient solvent system of MeOH and de-ionized water (75:25 to 100% MeOH in 45min) which afforded a prominent peak in Figure 4.11, collected and labeled as **SC-XIV-5-2**.

After spotting and developing the fractions on TLC plate using solvent system C, a single spot suspected to be a pure compound was observed (Fig 4.11). The fractions that afforded this single spot were pooled together and labeled as **SC-XIV-5-2** (**C6**, 12.7 min, 13.6 mg, 0.04 %).

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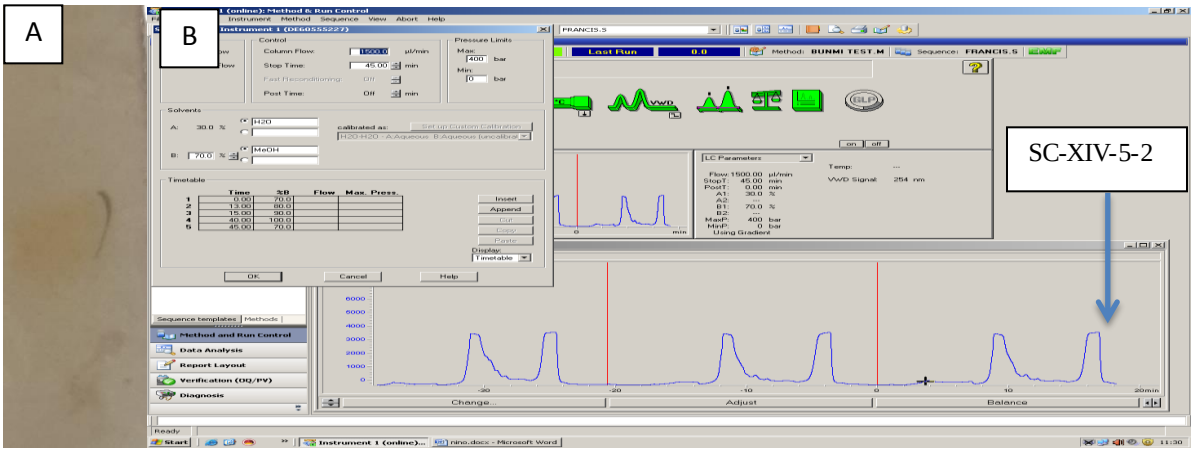

Figure 4.11: TLC silica gel of SC-XIV-5-2 (Fig. 4.11 A, solvent system DCM:MeOH (9.5:05) and HPLC chromatogram of C6 (Fig. 11.B).

***Conditions**

Solvent	MeOH:DIW 75:25 to 100% in 45 min
Column	SUPELCO, RP-18 (25 x 1 cm)
Flow rate	1.5 mL/min
Detection	UV at 254 nm

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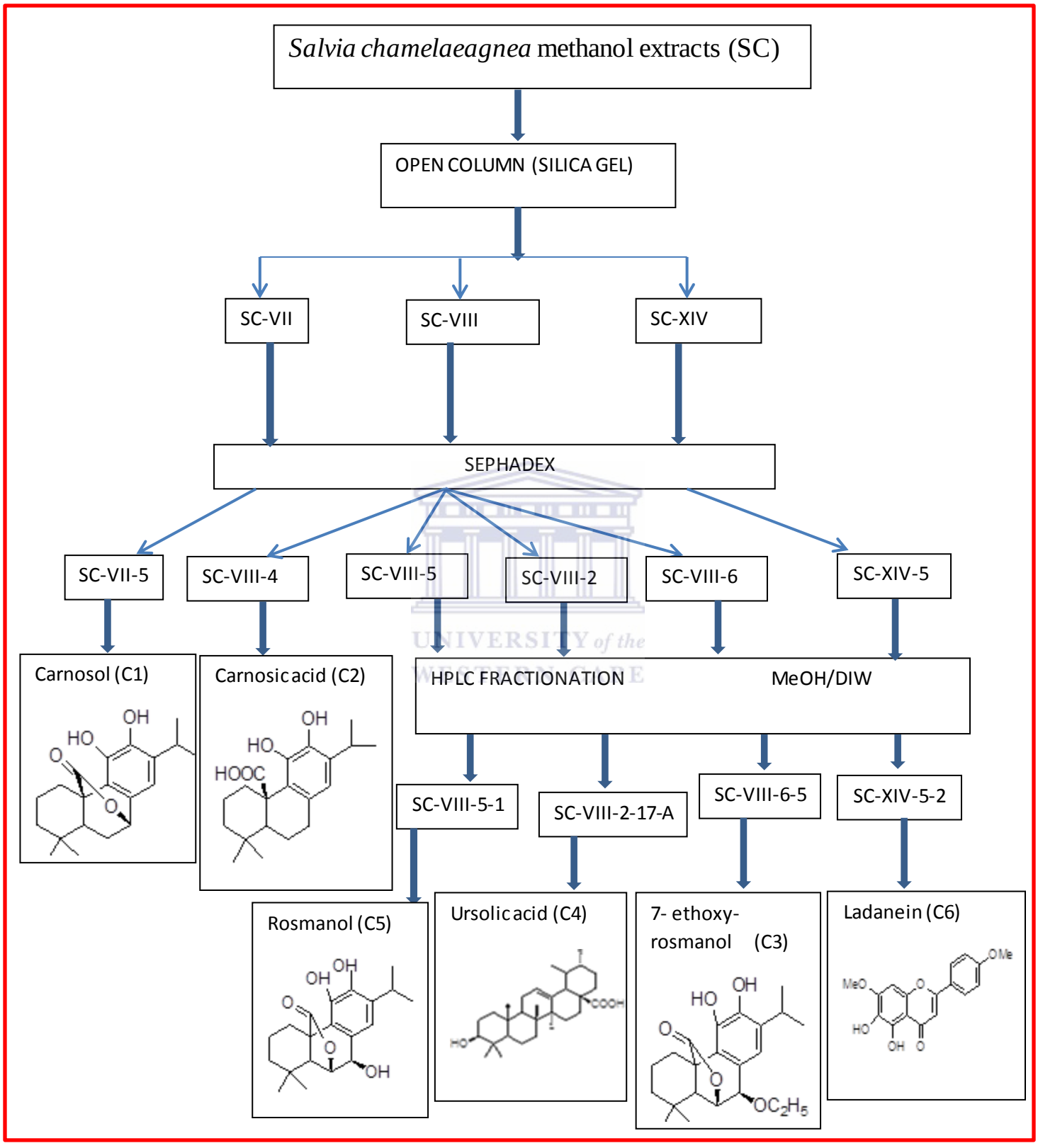

Scheme 4.1: A flow diagram of experimental procedure for the isolation of compounds from *S. chamelaeagnea*

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CHAPTER 4: ANTI-TYROSINASE AND TOTAL ANTIOXIDANT ACTIVITIES OF ISOLATED COMPOUNDS

4.7 BIOLOGICAL CHARACTERIZATION OF ISOLATED COMPOUNDS

4.7.1 General experimental procedure for biological assays

4.7.1.1 Reagents

Standards (purity > 99.0%) for the total antioxidant capacities (ORAC, FRAP & TEAC), inhibition of Fe^{2+}-induced lipid peroxidation, and anti-tyrosinase assays such as kojic acid, EGCG (epigallocatechingallate), trolox (6-hydroxyl-2, 5, 7, 8- tetramethylchroman-2-carboxylic acid), and other reagents including ABTS (2,2- azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt), potassium peroxodisulphate, fluorescein sodium salt, AAPH (2,2-Azobis (2-methylpropionamidine) dihydrochloride, perchloric acid, TPTZ (2,4,6-tri[2-pyridyl]-s-triazine, iron (III) chloride hexahydrate, copper sulphate, hydrogen peroxide, were secured from Sigma-Aldrich, Inc. (Cape Town, South Africa). All antioxidant assays including FRAP, TEAC, lipid peroxidation, and tyrosinase inhibition were measured by Multiskan spectrum plate reader, while automated ORAC assay was determined using Floroskan spectrum plate reader.

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4.7.2 Tyrosinase enzyme assay

This assay was performed using the method previously described (Chompo, et al., 2012) with slight modifications. Samples were dissolved in DMSO (dimethyl sulphoxide) to a stock solution of 1 mg/mL (w/v). Further dilutions were done with phosphate buffer (pH 6.5) for all working solutions to the concentrations of 1000, 500, 100, 50, 10 µg/mL. Kojic acid was used as a positive control. In each well of a 96-well plate, 70 µL of each sample working solution (extract/pure compounds) was combined with 30 µL of tyrosinase [from mushroom, 500 Units/mL in phosphate buffer (pH 6.5)] in triplicate. After incubation at room temperature for 5 minutes, 110 µL of substrate (2 mM L-tyrosine) was added to each well. The sample control was

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made up of each sample with phosphate buffer in the absence of tyrosinase enzyme. The reacting mixture was then incubated for 30 minutes at room temperature. The enzyme activity was determined by measuring the absorbance at 490 nm using plate reader. The percentage of tyrosinase inhibition was calculated as follows.

$$[(A - B) - (C - D)] / (A - B) \text{ X } 100 \quad (1)$$

Where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the test sample with the enzyme and D is the absorbance of the test sample without the enzyme.

4.7.3 Cyclic voltammetry measurement

Cyclic voltammetry was used for monitoring the oxidation-reduction potentials of the isolated compounds, and the behavior (increase or decrease) of the inhibition current with time in the presence of kojic acid or compound(s).

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It was performed using the BAS100B electro-chemical analyzer, a glassy carbon electrode (GCE) as working electrode, Ag/AgCl as a reference electrode and platinum (Pt) as a counter electrode. Cyclic voltammograms (CVs) were recorded at two different scan rates 50 mV/s and 25 mV/s within the potential window ranging from -1300 to 1300 mV and from -200 to 200 mV. Both compounds and kojic acid were made to a stock solution of 1 mg/mL with DMSO. Tyrosinase and L-tyrosine were diluted in phosphate buffer to a working concentration and the experiments were carried out at room temperature. To a cell containing 10 mL of 50 mM PBS at pH 6.5, a fixed volume of compound and tyrosinase enzyme were added (700 μL) and (300 μL) respectively, to which a specific volume of the L-tyrosine (1100 μL) was also added. To

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understand the activity of the control either positive or negative, the compound was replaced with kojic acid and DMSO. Cyclic voltammetry of each addition was recorded.

4.7.4 Total Antioxidant capacities assays

4.7.4.1 Ferric-ion reducing antioxidant power (FRAP) assay

The FRAP assay was carried out in accordance to the method described previously (Benzie & Strain, 1996). In a 96-well plate, 10 µL of the stock solution (1 mg/mL w/v) of the isolated compounds (**C1 – C6**) and a methanol extract (**SC**) were mixed with 300 µL FRAP reagent. The FRAP reagent was prepared by mixing (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20mM), Incubation commenced at room temperature for 30 min, and the plate was read at a wavelength of 593 nm in a Multiskan spectrum plate reader (Thermo Fisher Scientific). L-Ascorbic (Sigma Aldrich, South Africa) was used as a standard with concentrations varying between 0 and 1000 µM. Further dilutions were done to the samples that were highly concentrated and such dilution factors were recorded and used for calculations of the affected samples. The results were expressed as µM ascorbic acid equivalents per milligram dry weight (µM AAE/g) of the test samples.

4.7.4.2 Automated oxygen radicals absorbance capacity (ORAC) assay

ORAC assay was done according to the previous method (Prior, et al., 2003) with slight modifications (Cao & Prior, 1998). The method measures the antioxidant scavenging capacity of thermal decomposition generated by peroxyl radical of 2,2-azobis (2-amino-propane) dihydrochloride (AAPH) as peroxyradical (ORAC $_{ROO^{\cdot}}$) generator. The loss of fluorescence of fluorescein (probe) was an indication of the extent of its oxidation through reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the fluorescence area under the curve (AUC) plot relative to that of blank in which no antioxidant

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was present. The analyzer was programmed to record the fluorescence of fluorescein every 2 minutes after AAPH was added. The fluorescein solution and sample were added in the wells of an illuminated 96 well plate, 12 µL of each of sample (in stock solution of 1 mg/mL was combined with 138 µL of a fluorescein working solution followed by addition of 50 µL of 150 mg of AAPH prepared in-situ in 6 mL phosphate buffer. Absorbance was measured with Fluoroskan spectrum plate reader with the excitation wavelength set as 485 nm and the emission wavelength at 530 nm. A calibration curve was used, using a trolox stock solution of concentration in the range of 83 - 417 µM (R^2=0.9514). The ORAC values were calculated using a regression equation ($Y = a + bX + Cx^2$) between Trolox concentration (Y in µM) and the net area under the fluorescence decay curve (X). ORAC values were expressed as micromoles of trolox equivalents (TE) per milligram of test sample. Samples without perfect curve were further diluted and the dilution factors were used for the calculation of such samples.

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4.7.4.3 Trolox equivalent absorbance capacity (TEAC) assay

The total antioxidant activity of the test sample was measured using previously described methods (Pellegrini, et al., 1999). The stock solutions which contain 7 mM ABTS and 140 mM $K_2S_2O_8$ was prepared and kept at -2 ^oC. The working solution was then prepared by adding 88 µL $K_2S_2O_8$ solution to 5 mL ABTS solution. The two solutions were mixed and allowed to react for 24 hours at room temperature in the dark. Trolox was used as the standard with concentrations ranging between 0 and 500 µM. After 24 hours, the ABTS mix solution was diluted with ethanol to read a start-up absorbance (control) of approximately 2.0 (±0.1). The stock solution (1 mg/mL) of a methanol extract (SC) and purified compounds (25 µL) were allowed to react with 300 µL ABTS in the dark at room temperature for 30 min. The absorbance was read at 734 nm at

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25 ^{o}C in the plate reader. The results were expressed as μM trolox equivalents per milligram dry weight (μM/TE/g) of the test samples.

4.7.4.4 Inhibition of Fe (II) - Induced Microsomal Lipid Peroxidation assay

A method described by (Snijman, et al., 2009)with a few modifications was adopted. The reaction mixture contained microsomes (1mg of protein/mL in 0.01 M potassium phosphate buffer; pH 7.4, supplemented with 1.15 % KCl). The positive control includes microsomes, buffer and ferrous sulphate, in the absence of the samples to be tested.

The sample stock solutions (SC and **C1-C6**) were prepared in DMSO (1mg/mL, w/v). The working sample solutions were prepared in 0.01 M potassium phosphate buffer pH 7.4, supplemented with 1.15 % KCl diluted to100, 50 and 10 μg/mL concentrations. 100 μL of each sample (working solutions) were dissolved in potassium phosphate buffer and pre-incubated with 500 μL microsomes a 37 ^{o}C for 30 minutes in a shaking water bath. 200 μL of KCl-buffer were added to the mixture, followed by 200 μL of a 2.5 mM ferrous sulfate solution and incubated at 37 ^{o}C for 1 hour in a shaking water bath. The reaction was terminated with 10 % trichloroacetic acid (TCA) solution (1 mL) containing 125 μL butylated hydroxytoluene (BHT, 0.01 %) and 1 mM ethylene diamine tetra acetic acid (EDTA). Samples were centrifuged at 2000 rpm for 15 minutes, and 1 mL of each supernatant was mixed with 1 mL of 0.67 % thiobarbituric acid (TBA) solution. The reaction mixture was then incubated in a water bath at 90 ^{o}C for 20 minutes and the absorbance were measured at 532 nm using plate reader. The percentage inhibition of TBARS formation relative to the positive control was calculated by

$$[(A_{control} - A_{sample)/} A_{control} \text{ X } 100)] \quad (2)$$

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4.8 Chemical characterization: Results and discussion

4.8.1 Structure elucidation of carnosol (C1)

Compound **1** (195.2 mg) (Fig. 4.12) was isolated as described in Scheme 4.1 and obtained as a white crystal. It was identified as carnosol based on its NMR data. ^{1}H-NMR showed singlets of two methyls at δ_H 0.75, and 0.72 (Me-18, 19) and two doublets counted for six protons at 1.06 and 1.05 (Me-16, and 17); septet at 3.36, (H-15), a low field shift proton at 5.26 dd (H-7$_\alpha$), a singlet at 6.51 for the aromatic proton H-14. The ^{13}C-NMR showed 20 carbons, which confirmed the diterpene skeleton. DEPT-135 and HSQC split those carbons into 4 methyls (22.4 X 2/C16, 17; 31.4/18 and 19.3/19); four methylene (28.6/C1; 18.6/C2; 40.7/C3; 29.5/C6); four methines; one of them aromatic (111.6/C14); one hydroxylated (78.1/C7); in addition the five signals of 5 aromatic carbons (131.6/C8; 121.5/C9; 142.4/C11; 142.3/C12; 134.2/C13) and a carbonyl group (177.5). The HMBC showed cross peaks (among others) of H14 with C15; C7; C9; C11 and/or C12, H7/C5; C6; C14; C9; C8; C20; C12, H15/C16,17; C14; C13; C11 and C12. The above data with 2D NMR experiments (HSQC, HMC, and COSY) established the structure of carnosol (**1**) and finally confirmed by comparing the experimental data with literature (Inatani et al., 1982). Carnosol was first isolated from *Salvia carnosa* in 1942 and its chemical structure was first established in 1964 (Brieskorn, et al., 1964) and widely distributed within the family members of lamiaceae most especially *Salvia* and *Rosemary*.

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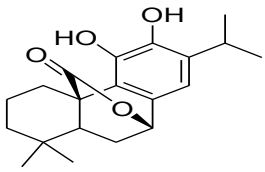

Figure 4.12: Chemical structure of carnosol

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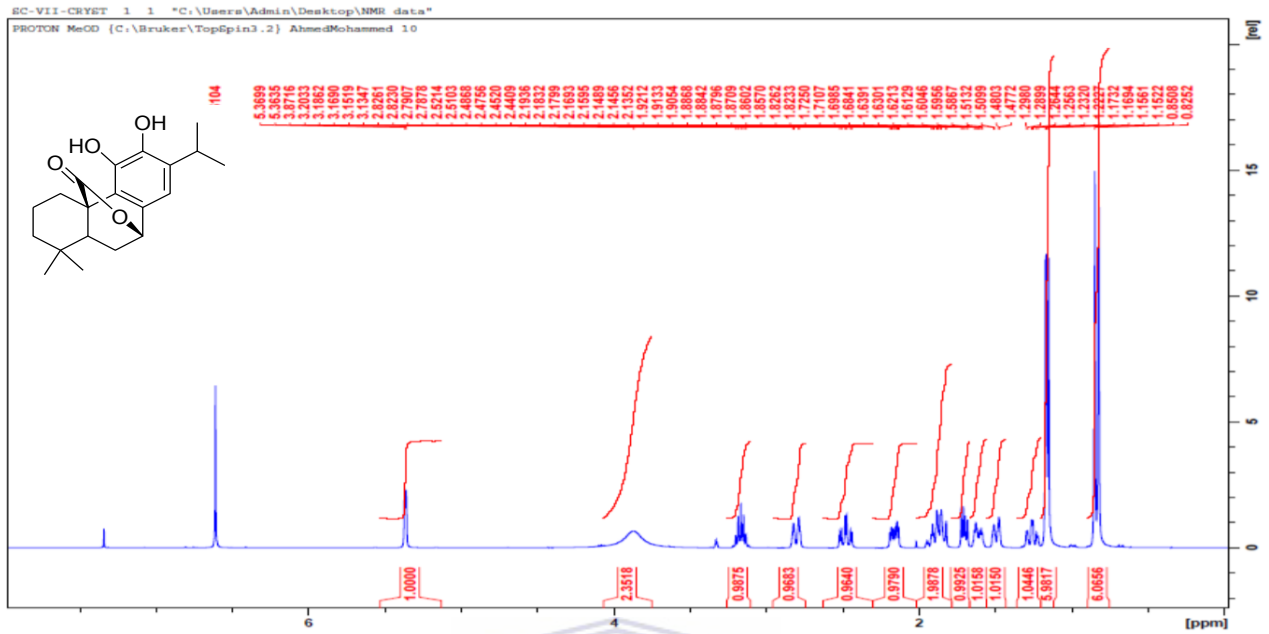

Figure: 4.13: ^{1}H NMR spectrum of compound **1**

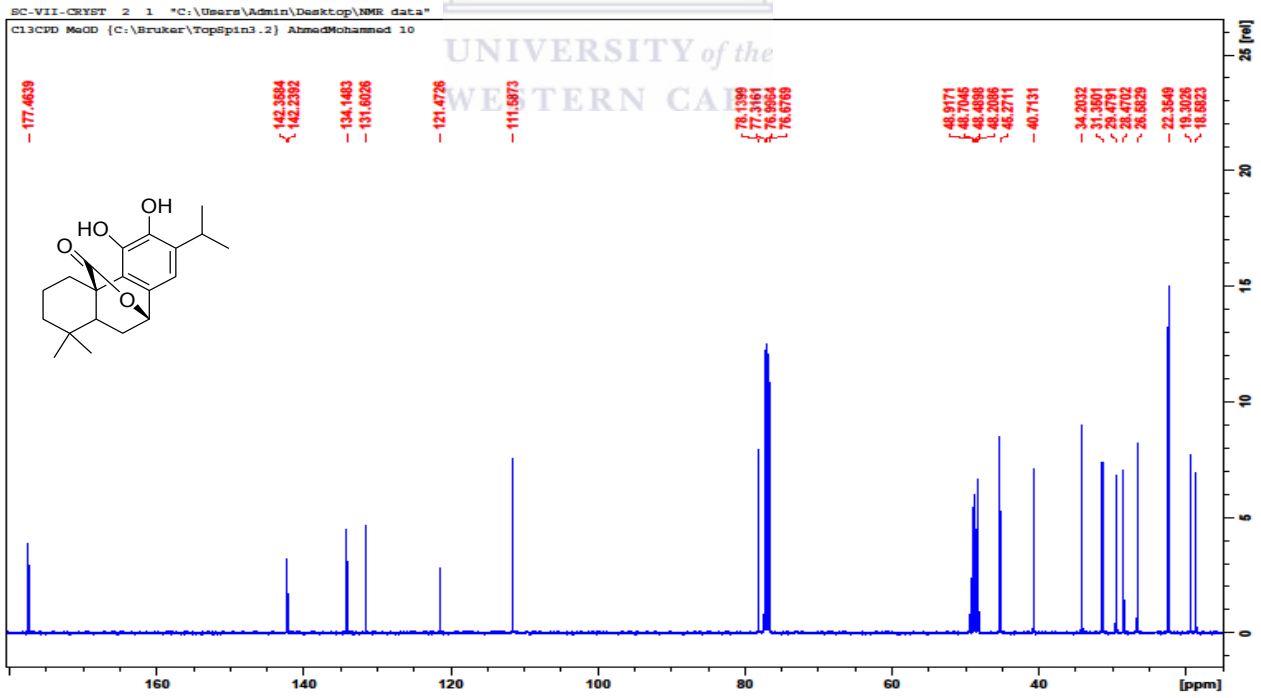

Figure: 4.14: ^{13}C NMR spectrum of compound **1**

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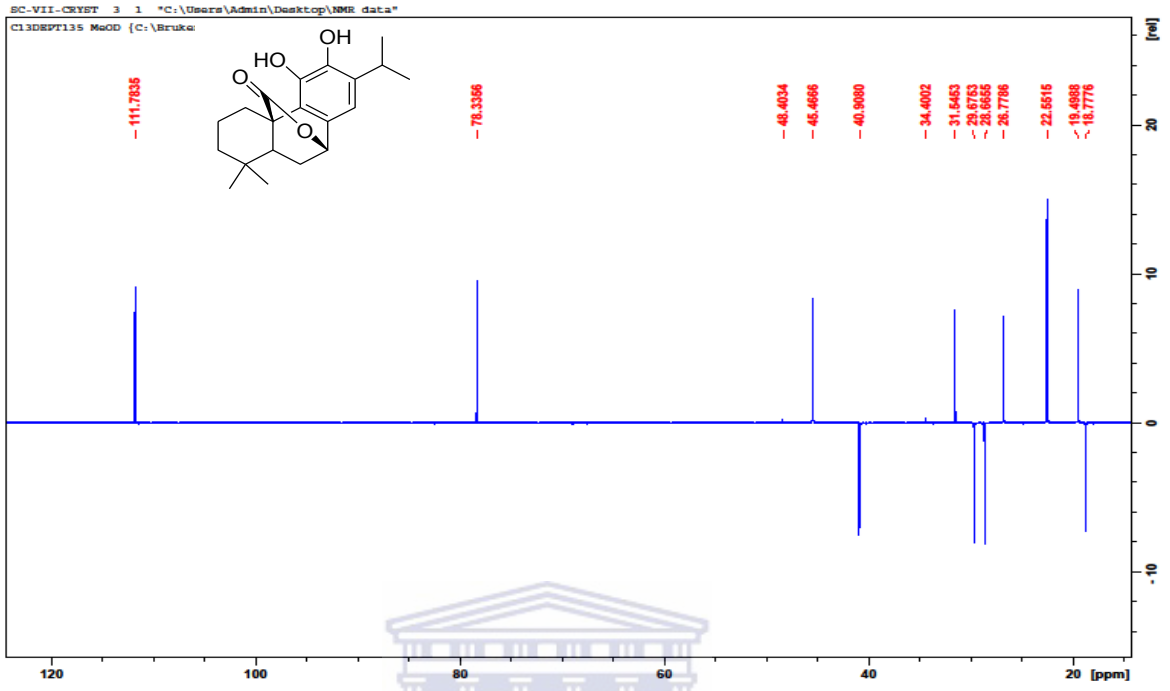

Figure 4.15: DEPT NMR spectrum of compound **1**

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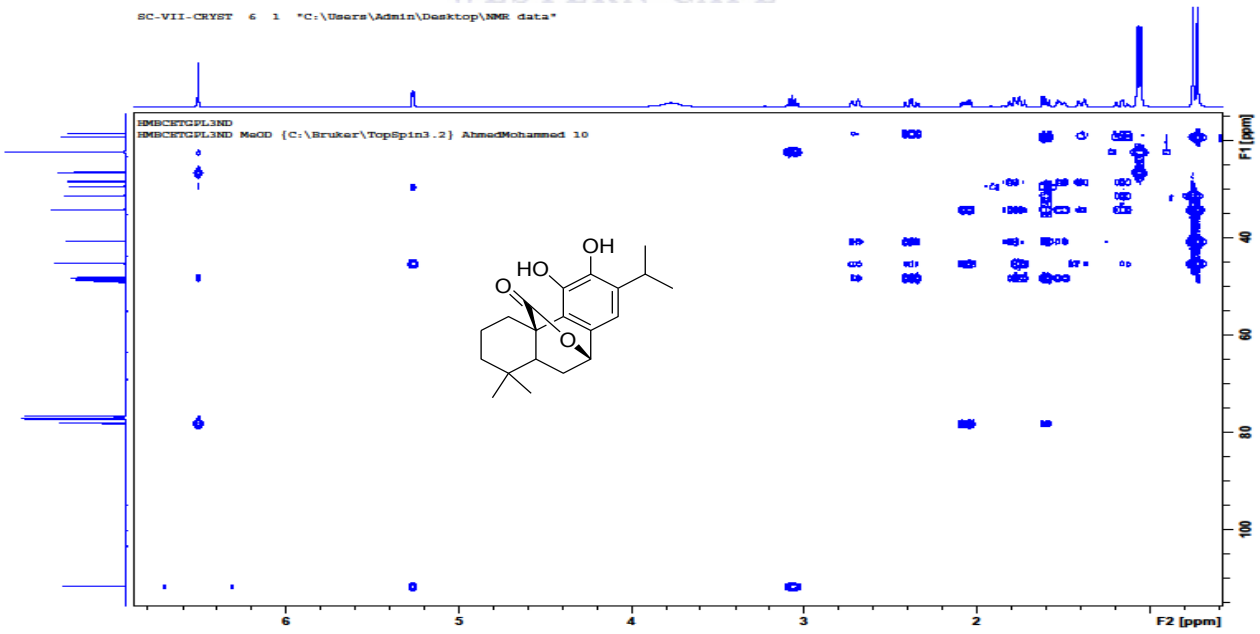

Figure 4.16: HMBC NMR spectrum of compound **1**

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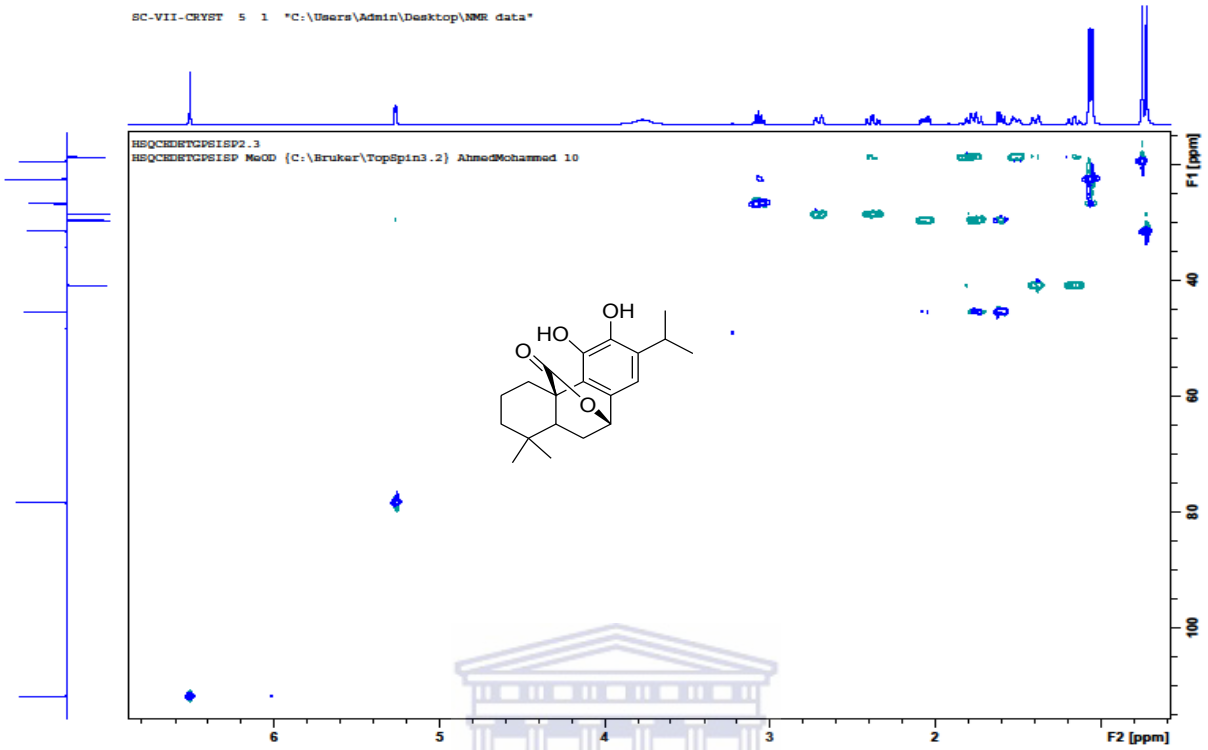

Figure 4.17: HMQC NMR spectrum of compound **1**

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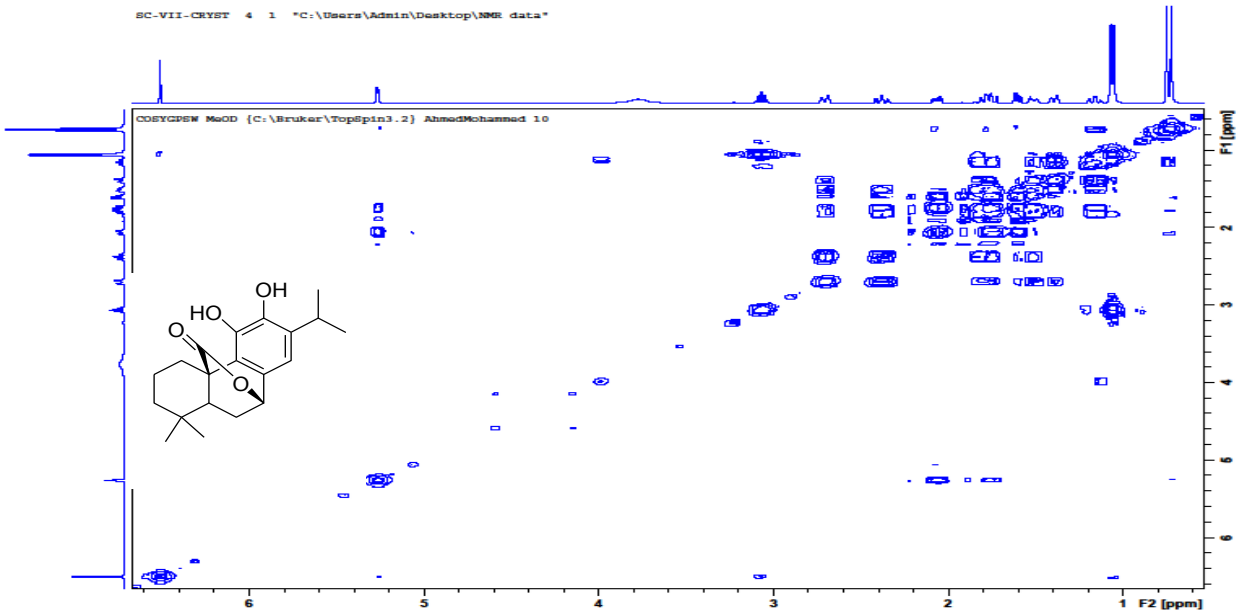

Figure 4.18: COSY NMR spectrum of compound **1**

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4.8.2 Structure elucidation of carnosic acid (C2)

Compound **2** (84.8 mg) (Fig. 4.13) was isolated as described in Scheme 4.1 and obtained as amorphous powder. It was identified as carnosic acid from its NMR data. NMR data was similar to that of compound **1** except the absence of the signal at 5.58. The carbon signals showed five methylene (extra one than **1**) and three methines (less one than **1**). The ^{13}C signal of the carbonyl carbon was shifted to a lower field (182.1) which indicate free carboxyl group. The above data with comparison of the obtained data with literature confirmed the structure of compound **2** as carnosic acid. Many literatures (Topcu, et al., 2013) described the isolation of carnosic acid as its methyl ester derivative due to the fact that, the free carboxyl group makes the chromatographic separation very difficult and also contribute to the instability of the compound during the separation process.

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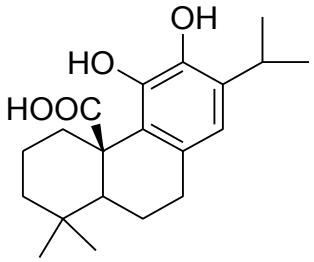

Figure 4.19: Chemical structure of carnosic acid

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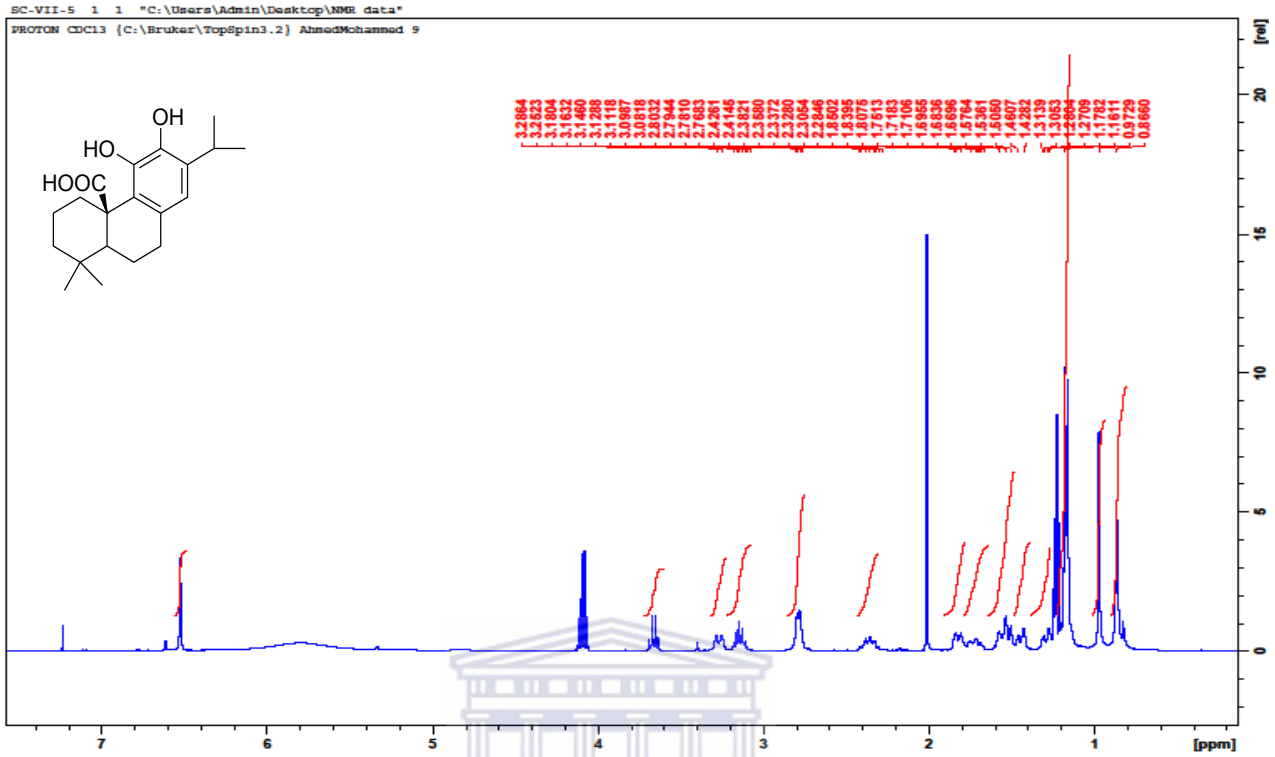

Figure 4.20: ^{1}H NMR spectrum of compound **2**

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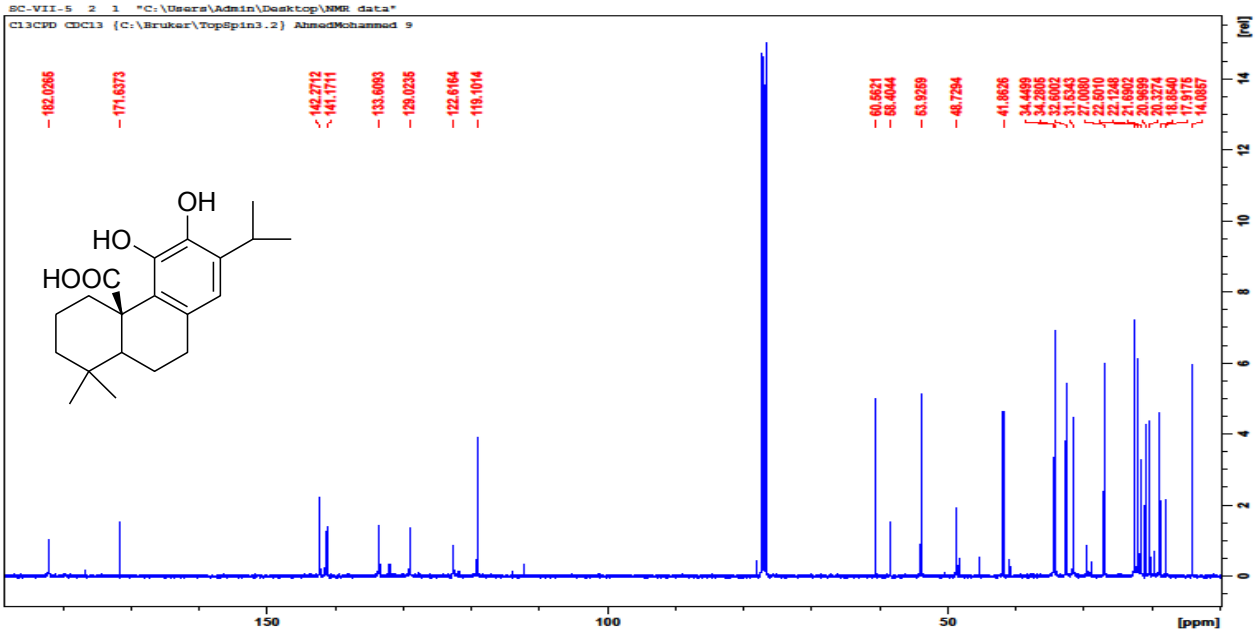

Figure 4.21: ^{13}C NMR spectrum of compound **2**

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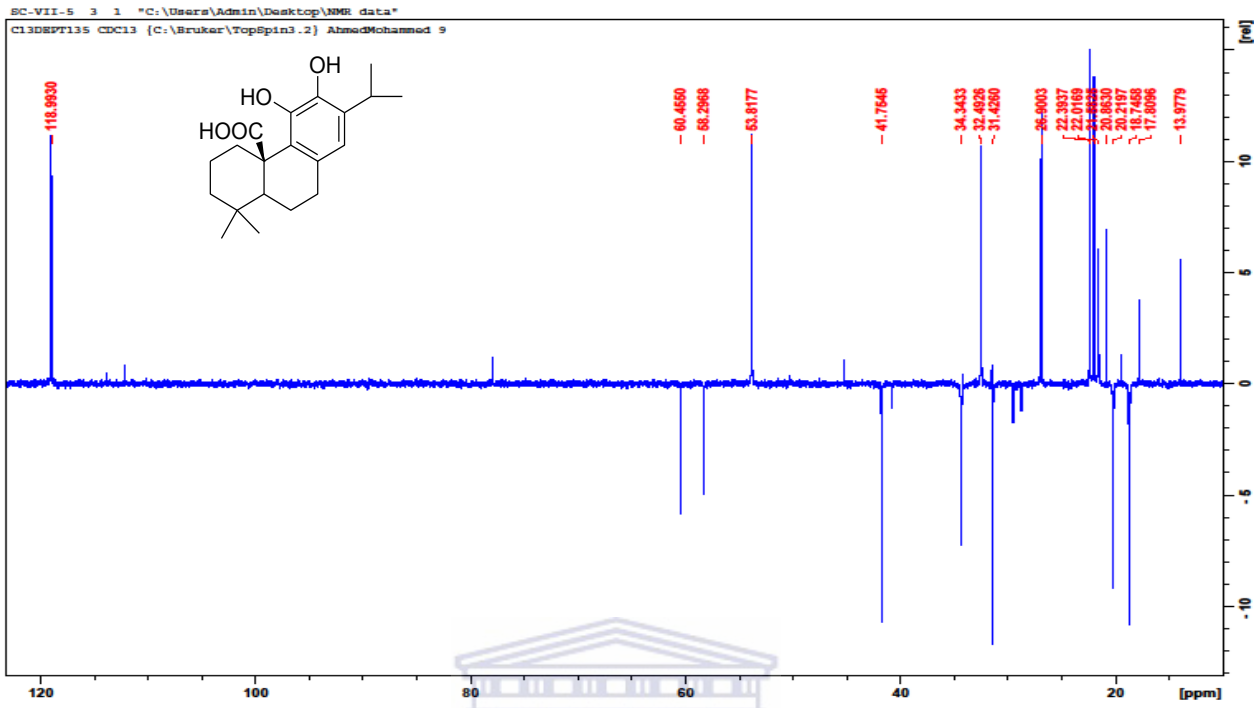

Figure 4.22: DEPT NMR spectrum of compound **2**

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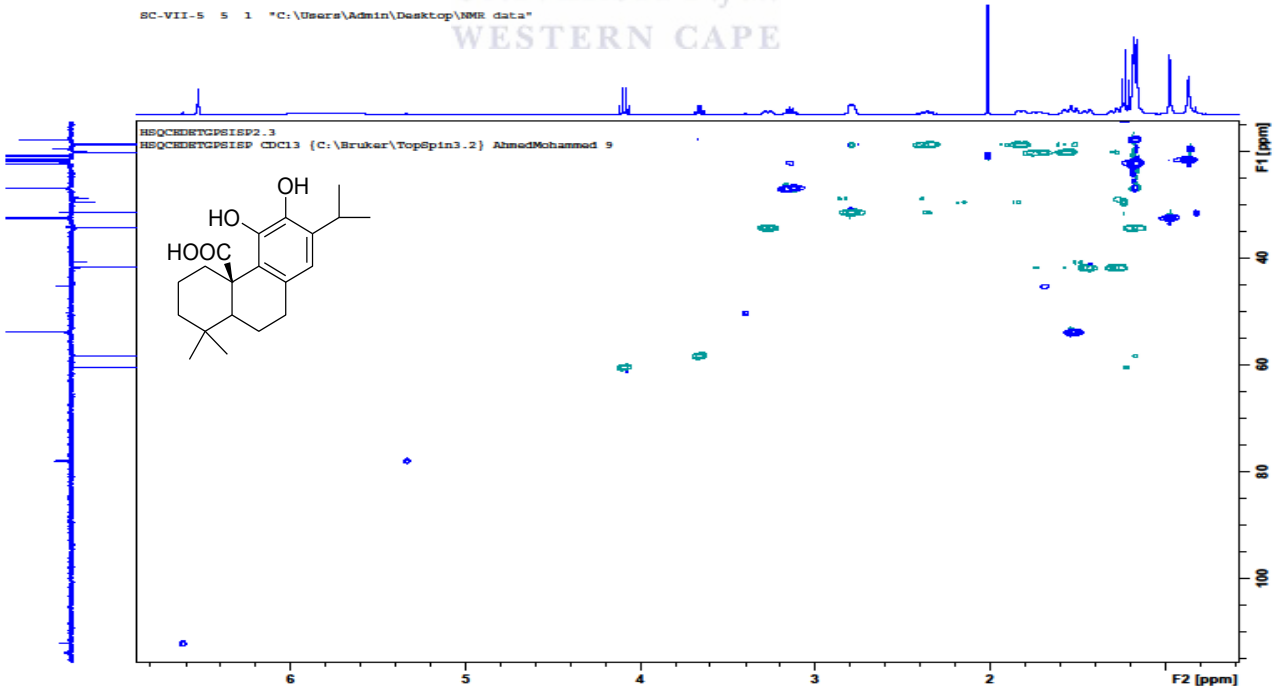

Figure 4.23: HMQC spectrum of compound **2**

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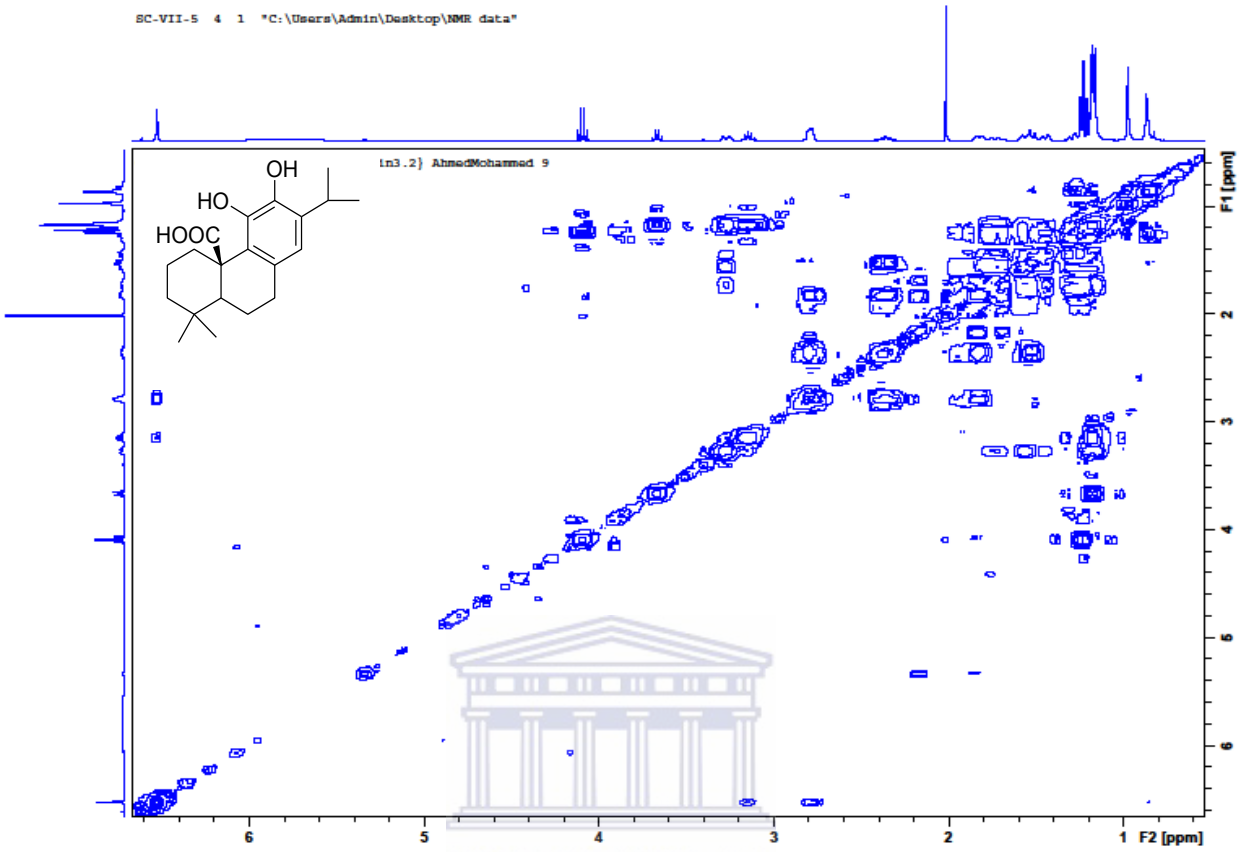

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Figure 4.24: COSY spectrum of compound **2**

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4.8.5 Structure elucidation of rosmanol (C5)

Compound **5** (7.1 mg) (Fig. 4.16) was isolated as described in Scheme 4.1 and obtained as brown amorphous solid. The NMR data of **C5** showed a typical abietane diterpene skeleton and similar to compound **1** and **2**. ^{1}H NMR showed two doublet signals at 4.49 (d, $J_{6.7}$ = 2.3; H-6); 4.66 d ($J_{6.7}$ = 2.3 H-7) 6.80 s (H-14); 3.07 Septet ($J_{15.6/7}$ = 6.8; H-15); 3.11 (m, H-1α) in add to four methyl signals, two of them appeared as doublets at 1.19; 1.18 (Me-16; 17; J = 6.8 Hz); and the other two as singlets at 0.88 (Me-18) and 0.87 (Me-19). The ^{13}C-NMR and DEPT-135 showed 20 carbons, four of them are methyls at 22.1/C16 (or C17); 22.6/C17 (or C16); 32.0/C18 and 20.0/C19, three methylene signals at 27.3; 19.1 and 38.2 (C1-C3 respectively); five methines, two of them are oxygenated at 78.2/C6 and 68.5/C-7 and one aromatic at 120.3/C-14, the other two methines attributed to C5 (50.8) and C15/27.3. In addition to seven quaternary carbons, five of them belong to the aromatic ring (128.1, 124.5, 142.9, 141.8, 135.1 (C8, C9, C11-C13 respectively) and a carbonyl group at 178.9 (C-20); the other two belong to C-10 and C-4 (at 47.2 and 31.3). The chemical shift of C-20 appeared at higher field than compound **2** which indicated the formation of lactone ring also, the H-6 only coupled with H-7 but not with H-5, which indicate the 90^{o} coplanar of H-5 and H-6 and confirm the lactonization at C-6. Finally, the NMR data of rosmanol was identical with those published in literature (Inatani et al., 1982) and established the structure of compound **5** as rosmanol.

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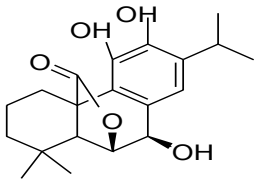

Figure 4.25: Chemical structure of rosmanol

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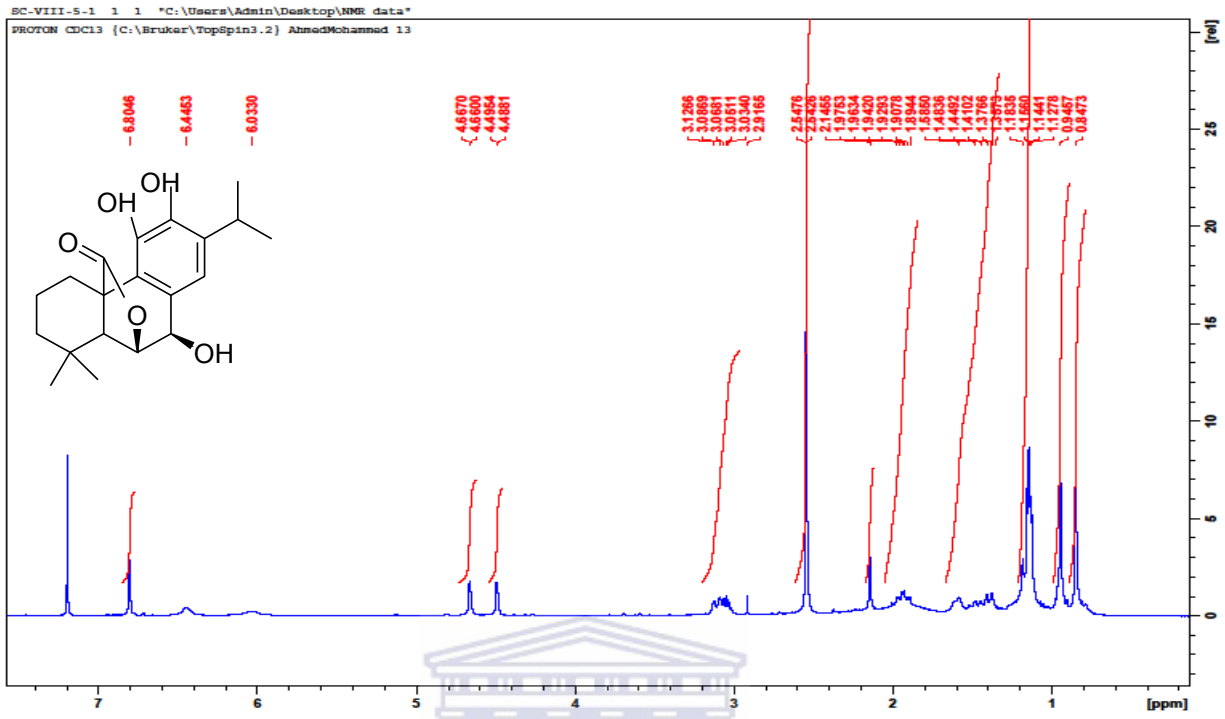

Figure 4.26: ^{1}H NMR spectrum of compound **5**

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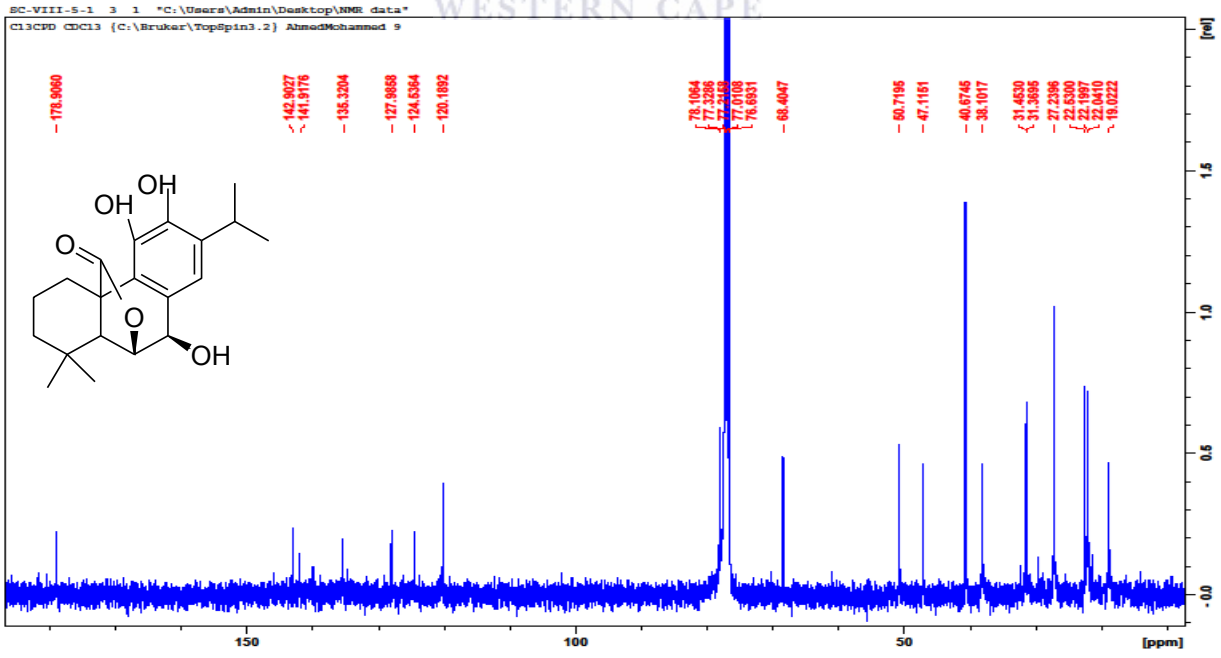

Figure 4.27: ^{13}C NMR spectrum of compound **5**

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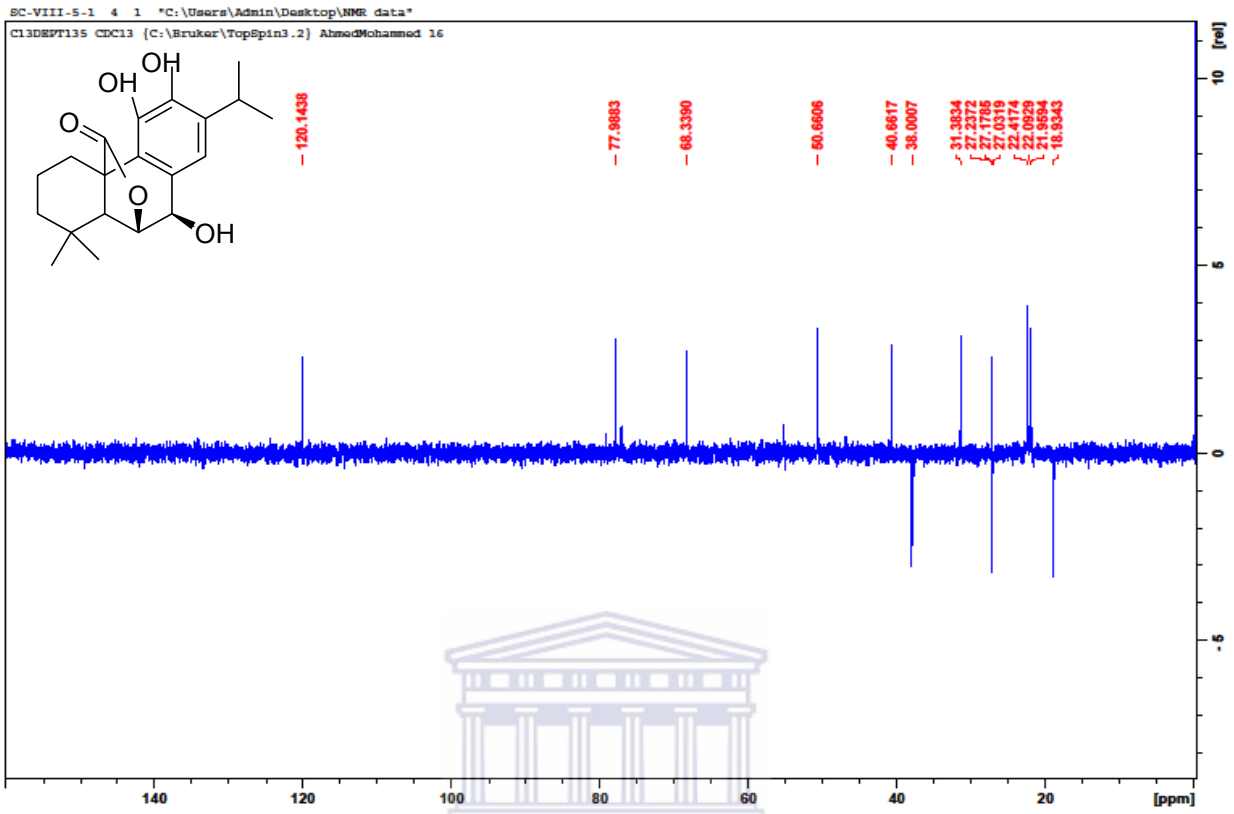

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Figure 4.28: DEPT spectrum of compound **5**

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4.8.3 Structure elucidation of 7-ethoxyrosmanol (C3)

Compound **3** (3.7 mg) (Fig. 4.14) was isolated as described in Scheme 4.1 and obtained as a yellow amorphous powder. It was identified as 7-ethoxyrosmanol based on its NMR data. It showed identical signal with that of compound **3** except the chemical shift of C-7 and the presence of ethoxy group in compound **3** (^{13}C, 66.4 t; ^{1}H, 16.2 q) indicated that 7-ethoxy derivative of **3**. This was confirmed by comparison of the obtained NMR data with those published in literature (Arisawa, et al., 1987).

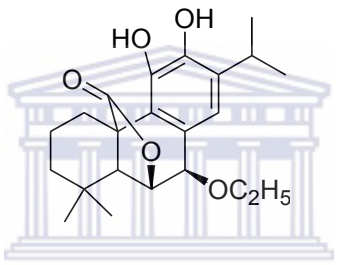

Figure 4.29: Chemical structure of 7-ethoxyrosmanol

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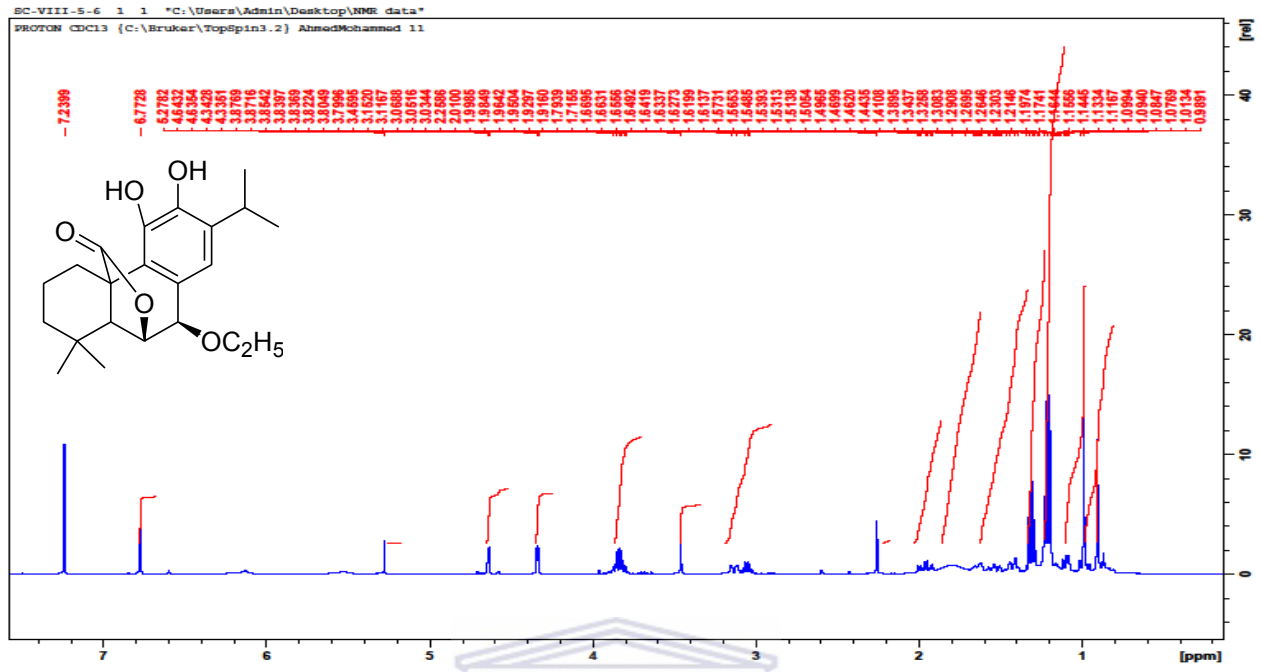

Figure 4.30: ^{1}H NMR spectrum of compound **3**

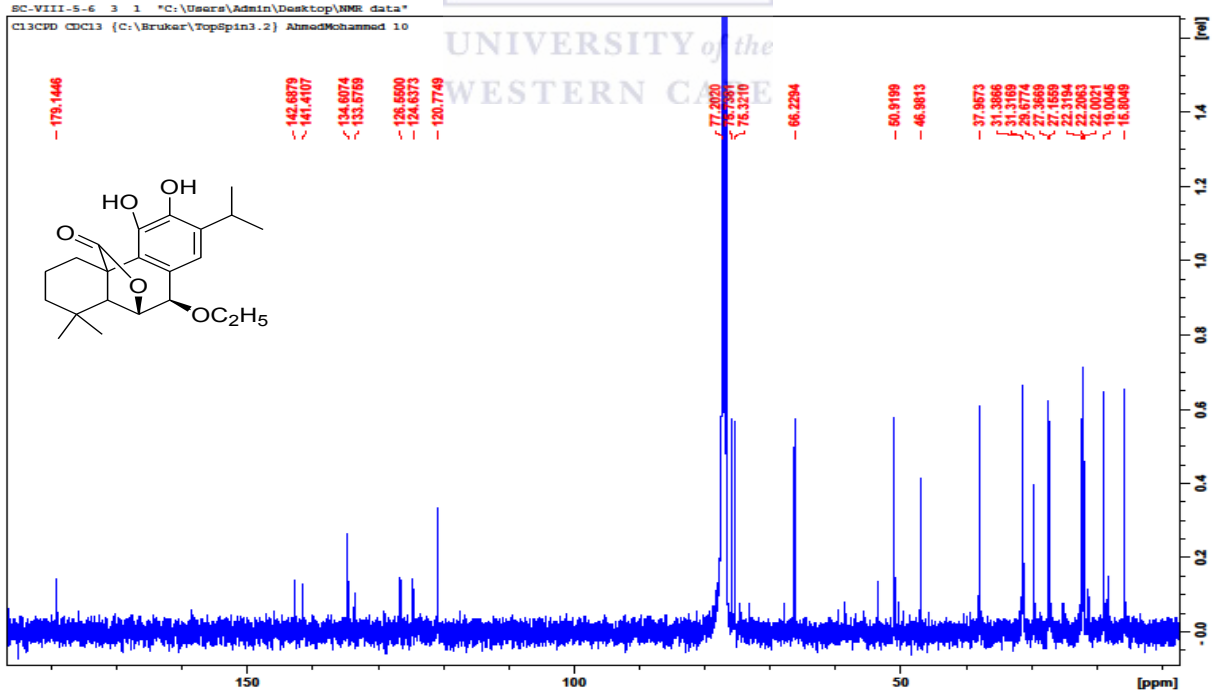

Figure 4.31: ^{13}C NMR spectrum of compound **3**

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4.8.4 Structure elucidation of ursolic acid (C4)

Compound **4** (13.79 mg) (Fig. 4.15) was isolated as described in Scheme 4.1 and obtained as white powder. It was identified as ursolic acid based on its NMR data, which showed typical triterpene signals with 3 β-OH; carboxylic group (C-28) and $C_{12\text{-}13}$ double bond. The identity of the compound confirmed by comparing the experimental data with literature (Guvenalp, et al., 2006). Ursolic acid is a well-known triterpene and widely distributed in plants, the same compound was identified previously from *Leonurus cardiac* (Ali, et al., 2007).

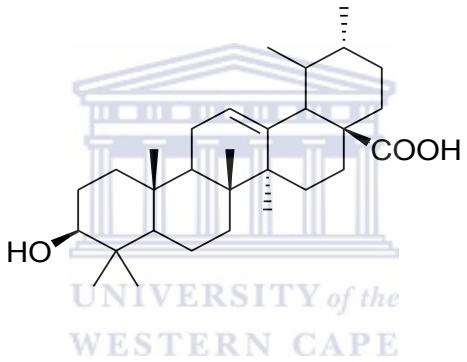

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Figure 4.32: Chemical structure of ursolic acid

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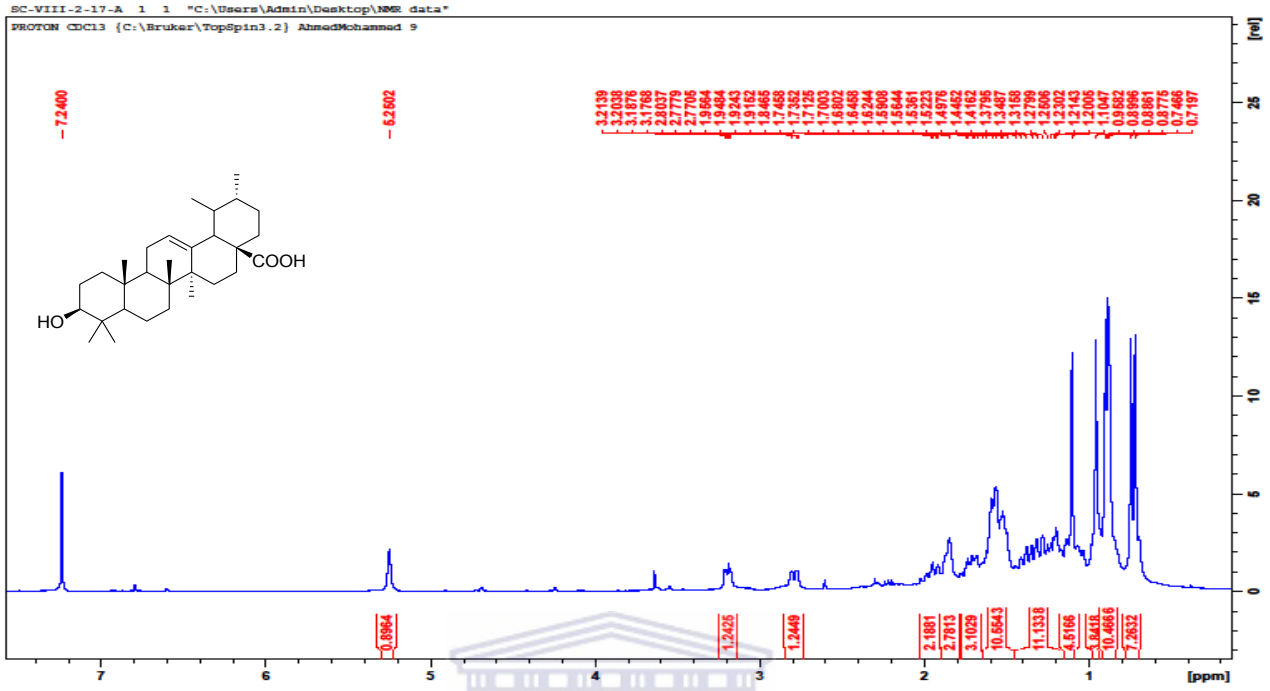

Figure 4.33: ^{1}H NMR spectrum of compound **4**

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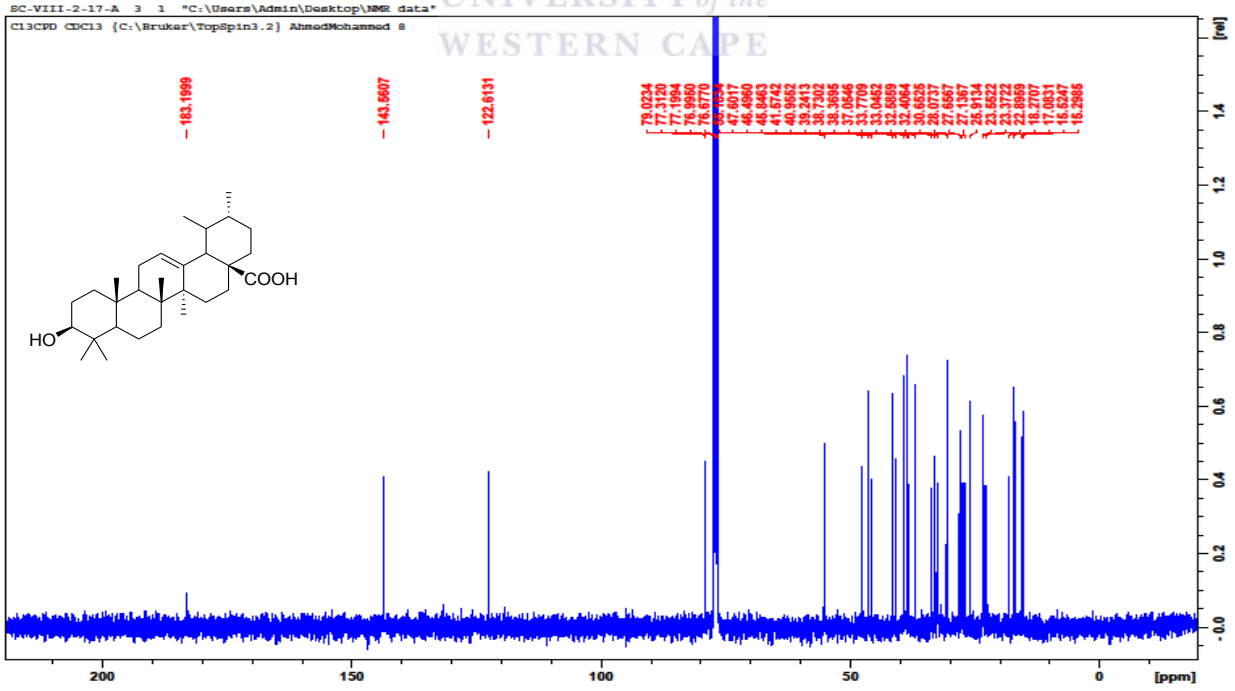

Figure 4.34: ^{13}C NMR spectrum of compound **4**

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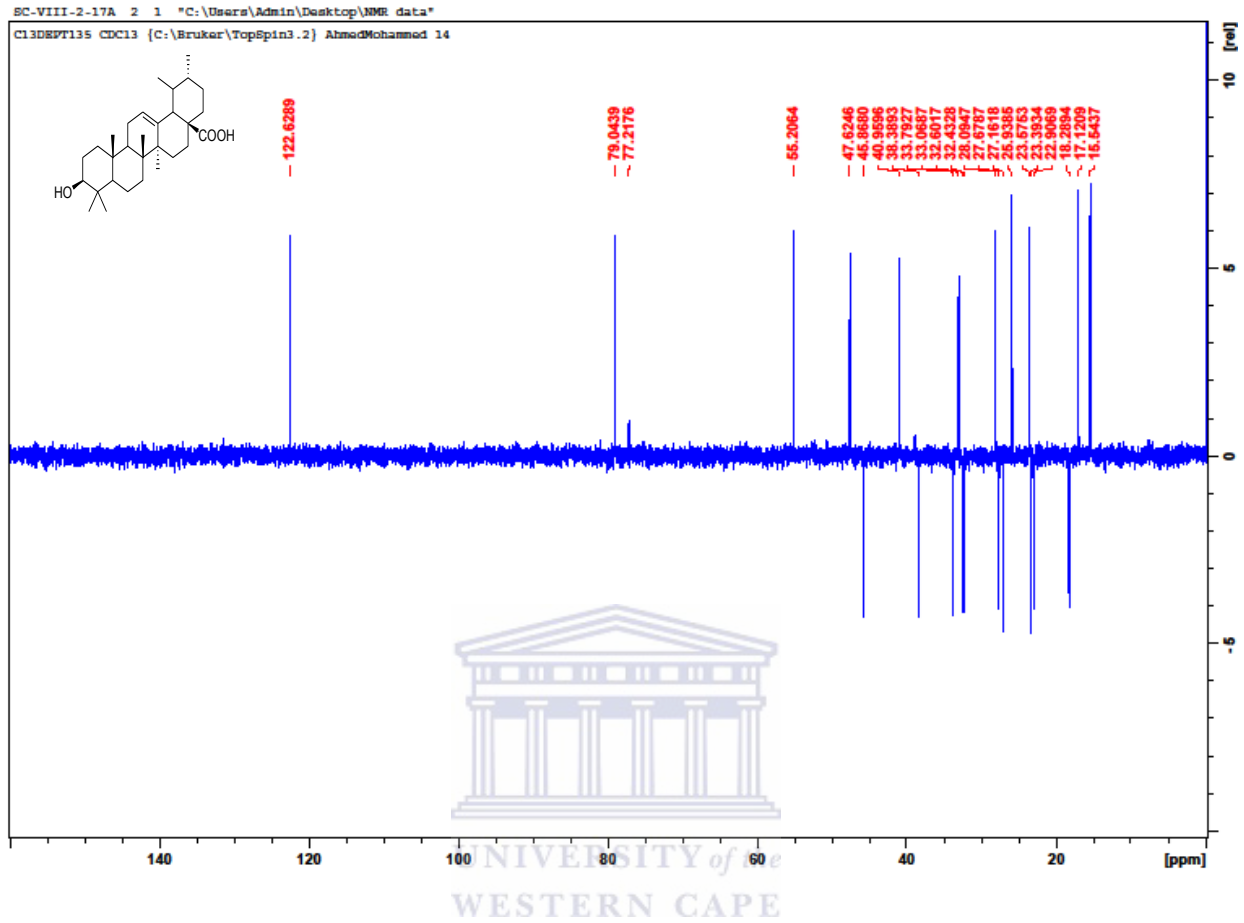

Figure 4.35: ^{1}H NMR spectrum of compound **4**

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4.8.6 Structure elucidation of ladanein (C6)

Compound **6** (13.6 mg) (Fig. 4.15) was isolated as described in Scheme 4.1 and obtained as yellow amorphous solid. The compound showed 15 carbons in addition to two O-Me signals at (δ_H/ δ_C); 3.87/55.6 and 3.98/56.76. The 15 carbons of the main skeleton indicated Flavonoid type of Apigenin nucleus. The compound showed in NMR signals at 12.57 attributed to 5-OH, and showed cross-peaks in HMBC with C_{10} (105.9); C_5 (145.9) and C_6 (129.5). The singlet signal at 6.56 (counted 2H) attributed to H-3 and H-8; it showed HMBC correlations with C_9, C_{10}, C_7, C_6, [H8] and C2; C4; C10; C1 [H-3]. The positions of the two OMe were established by HMBC. The NMR data of ladanein was identical with those published in literature (Farjam, et al., 2013) and other 2-D NMR correlations confirm the structure of compound **6**as 6–hydroxy-7,4`-dimethoxyapigenin (ladanein).

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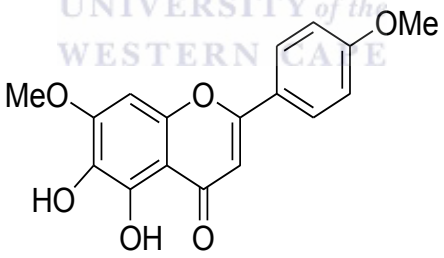

Figure 4.36: Chemical structure of Ladanein

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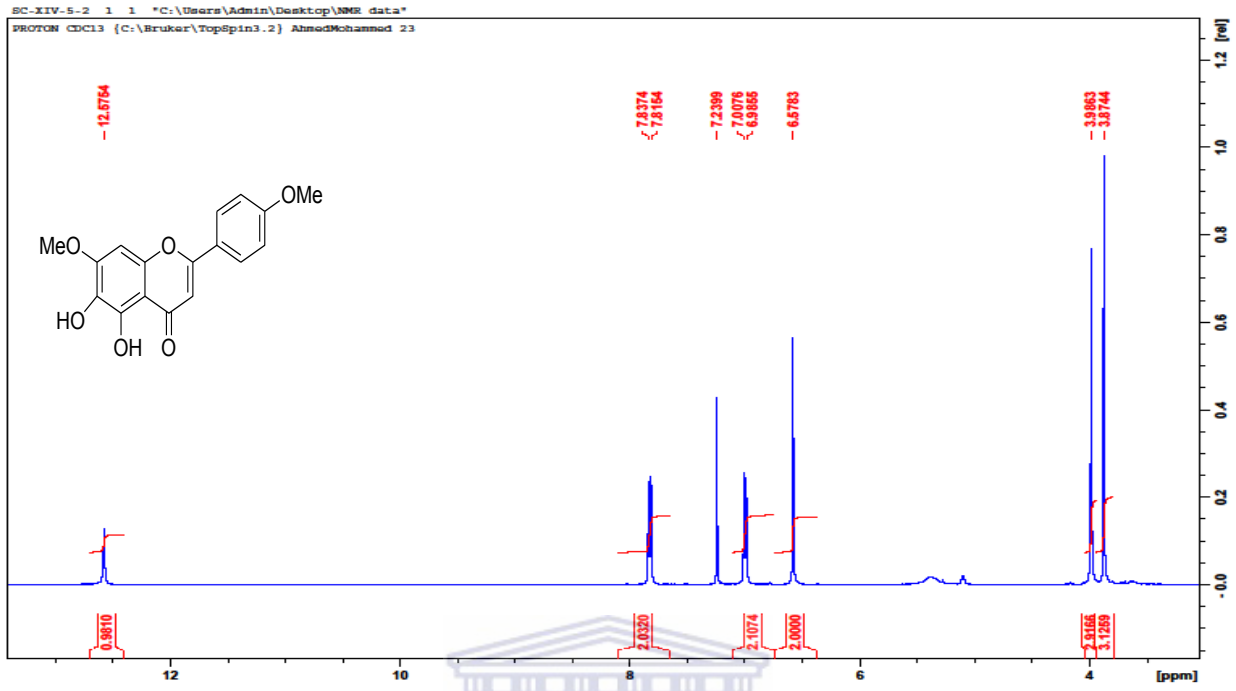

Figure 4.37: ^{1}H NMR spectrum of compound **6**

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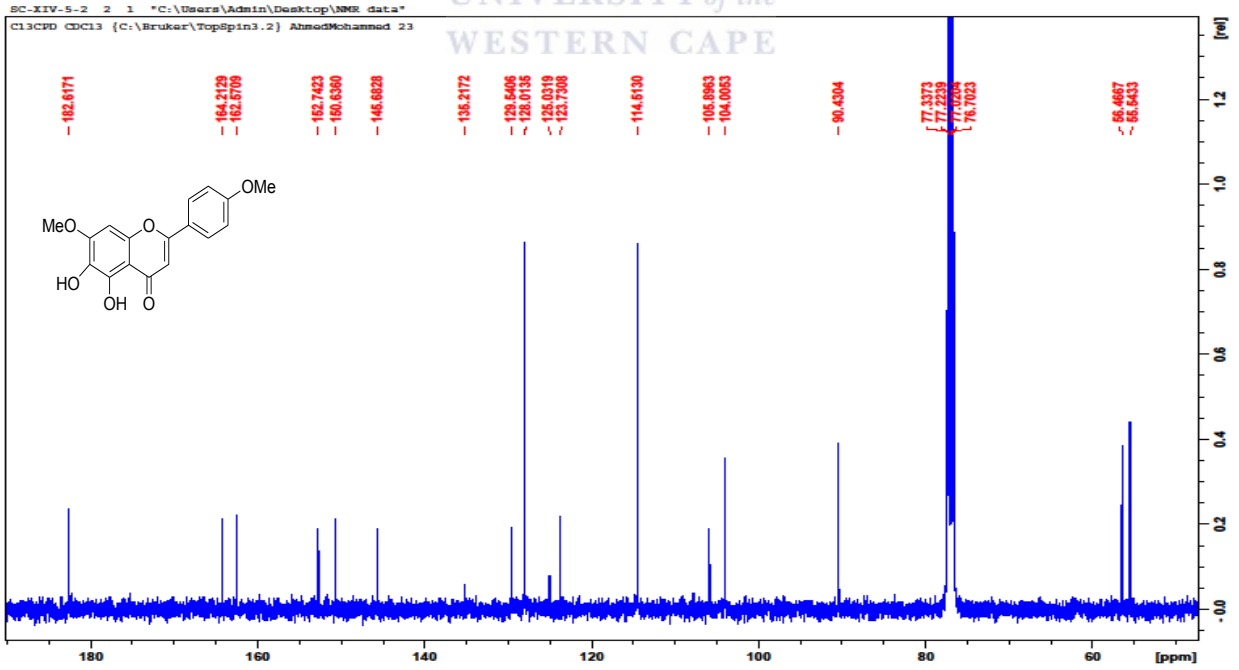

Figure 4.38: ^{13}C NMR spectrum of compound **6**

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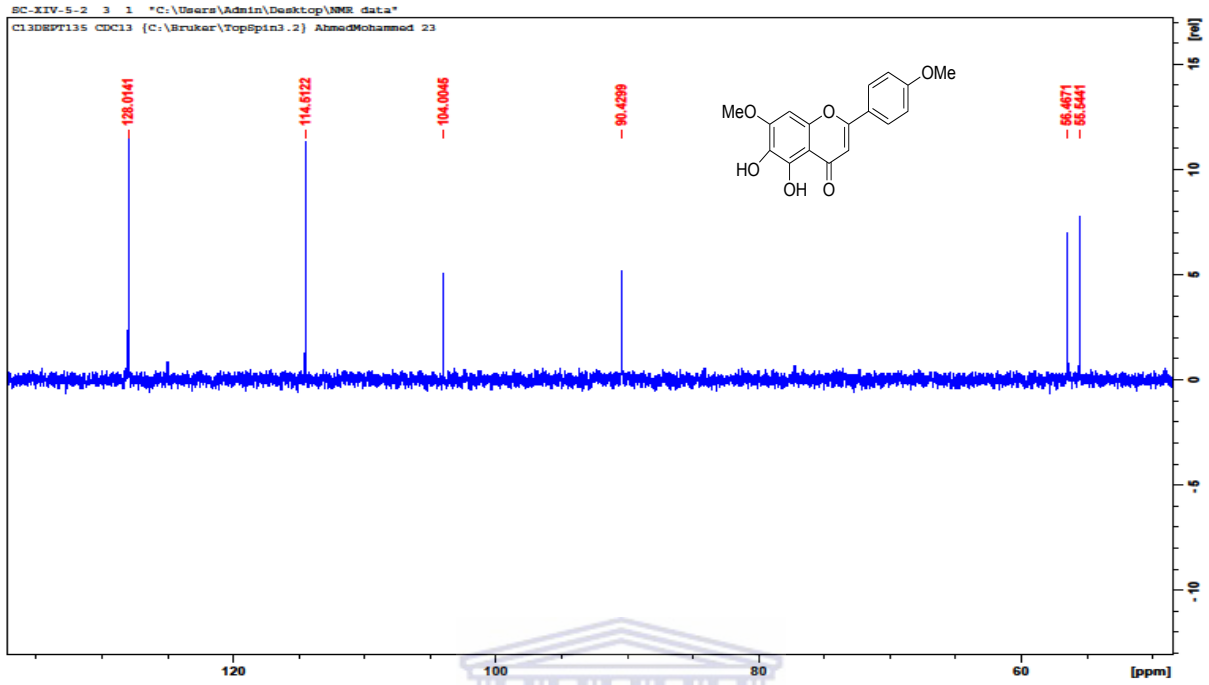

Figure 4.39: DEPT spectrum of compound **6**

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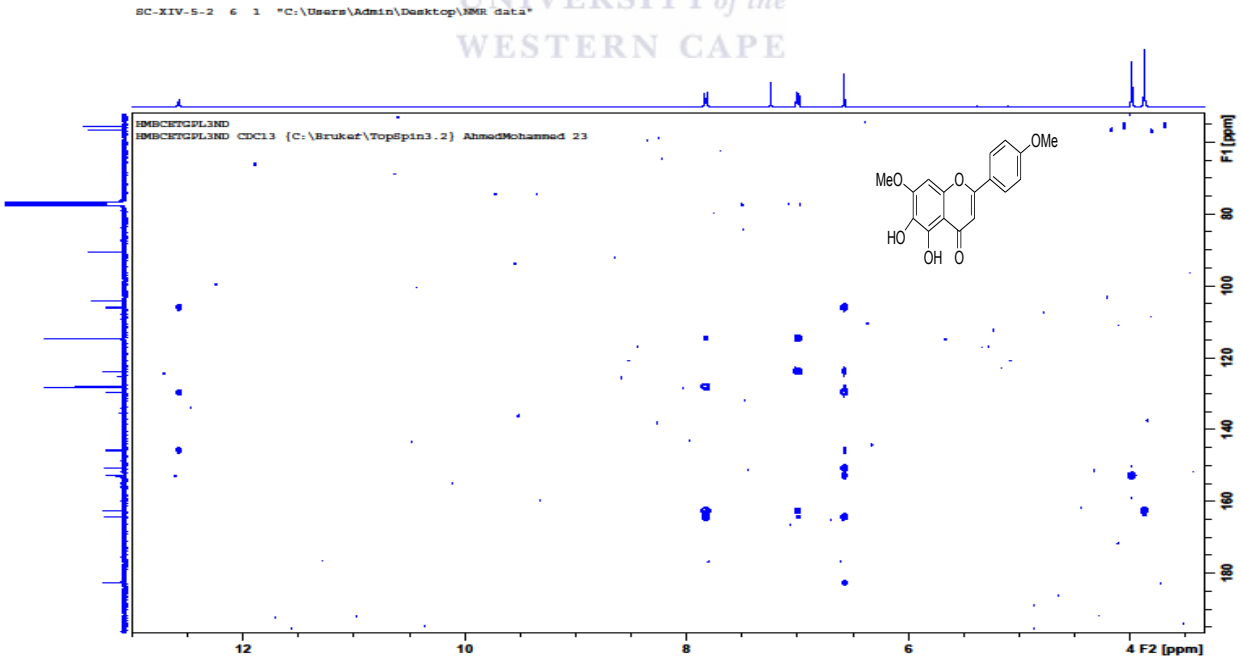

Figure 4.40: HMBC spectrum of compound **6**

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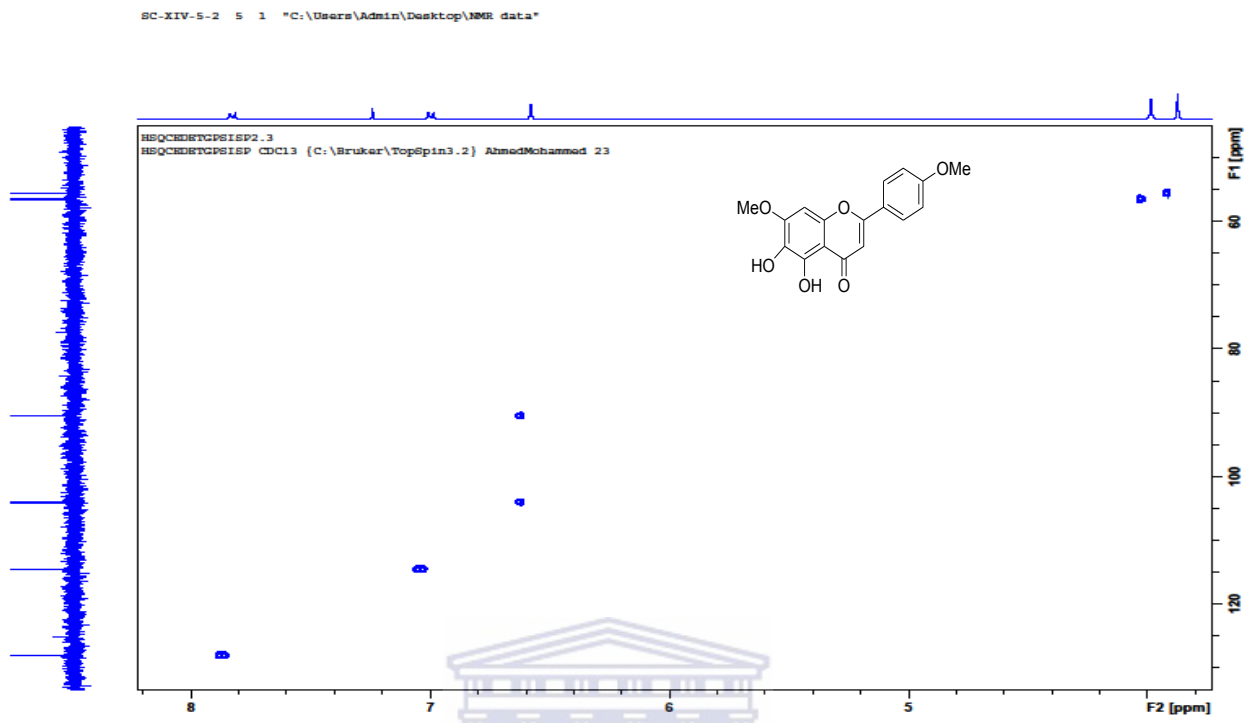

Figure 4.41: HMQC spectrum of compound **6**

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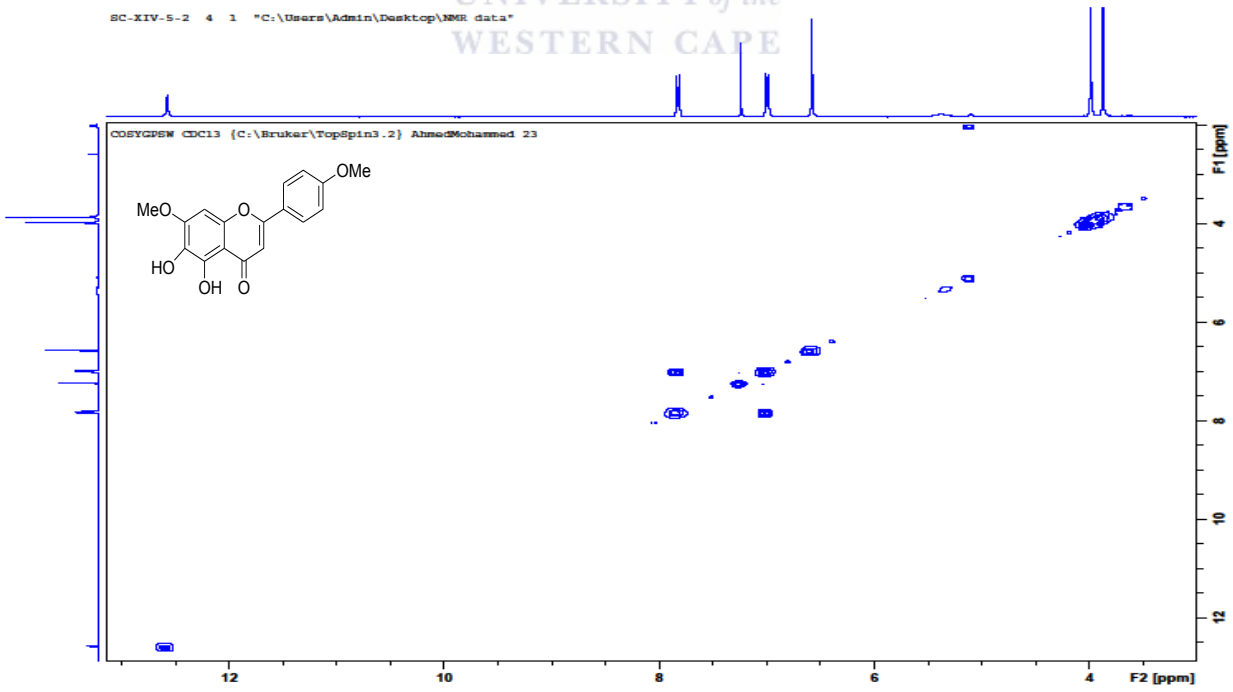

Figure 4.42: COSY spectrum of compound **6**

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Table 4.7: ^{13}C NMR data of **C1**, **C2**, **C3** and **C5** compared to the NMR data from the literature

	Exp.*	Reported**	Exp.*	Exp.*	Reported**	Exp.*	Reported**
	C	C	C	C	C	C	C
	28.6	29.8 t	34.4	27.3 t	28.4 t	27.4 t	28.3 t
	18.6	19.7 t	20.3	19.1 t	19.9 t	19.0 t	19.8 t
	40.7	41.9 t	41.6	38.2 t	39.1 t	38.0 t	38.9 t
	34.2	35.1 s	34.5 s	31.3 s	32.0 s	31.4 s	32.0 s
	45.3	46.4 d	53.9 d	50.8 d	51.0 d	50.9 d	51.5 d
	29.5	30.6 t	18.9	78.2 d	78.8 d	75.3 d	75.4 d
	78.1	78.2 d	31.5	68.5 d	69.1 d	75.7 d	76.8 d
	131.6	133.6 s	129.0	128.1 s	130.4 s	126.6 s	128.4 s
	121.5	123.1 s	122.6	124.5 s	124.9 s	124.6 s	124.1 s
	48.2	49.2 s	48.7 s	47.2 s	47.7 s	47.0 s	47.6 s
	142.3	143.8 s	142.3	142.9 s	144.7 s	142.7 s	144.6 s
	142.4	143.4 s	141.2	141.8 s	142.5 s	141.4 s	142.6 s
	134.2	135.1 s	133.6	135.1	136.5 s	134.6 s	136.3 s
	111.6	112.4 d	119.1 d	120.3	120.2 d	120.8 d	120.6 d
	26.6	27.6 d	27.0 d	27.3	27.5 d	27.2 d	27.4 d
	22.4	23.0 q	22.5 q	22.1 q	22.9 q	22.2 q	22.9 q
	22.4	23.1 q	22.1 q	22.6 q	23.1 q	22.3 q	23.1 q
	31.4	32.0 q	32.6 q	31.4 q	31.8 q	31.3 q	31.8 q
	19.3	20.0 q	21.5 q	22.3 q	22.4 q	22.0 q	22.3 q
	177.5	175.9 s	182.1	178.9 s	178.3 s	179.1 s	178.4 s
						66.2 t	66.4 t
						15.8 q	16.2 q

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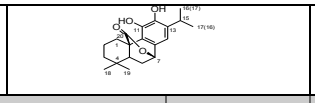
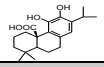
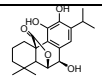
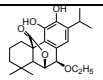
Table 4.8: ^{1}HNMR data of **C1**, **C2**, **C3** and **C5** compared to the NMR data from the literature

								
	Exp.*		Reported**	Exp.*	Exp.*	Reported**	Exp.*	Reported**
α	2.36 ddd, 13.8, 4.4, 4.4		2.55 ddd	1.17#	1.94 ddd, 13.5, 5.0, 5.0	1.98 ddd	1.96 ddd, 14.0, 5.6, 5.6	1.96 m
β	2.71 dddd, 13.8, 13.8, 4.4, 3.3		2.82 dddd	3.26 br. d. 14.4	3.11 brd, 16.0	3.29 m	3.31 brd, 14.0	3.30 m
α	1.51 ddddd, 13.8, 3.9, 3.9, 3.9, 3.9		1.57 ddddd	1.73 br.d. 14.3	1.46 m	1.59 dddd	1.64 m	
β	1.81 ddddd, 13.8, 13.8, 13.8, 3.9, 3.9		1.93 dddd	1.56 m	1.39 m	1.47 dddd	1.40 m	
α	1.39 ddd, 13.9, 3.9, 3.9		1.31 ddd	1.29 ddd 13.7, 3.4, 3.4	1.10 brd, 13.5	1.27 ddd	1.09 m	
β	1.13 ddd, 13.9, 13.9, 3.9		1.50 dddd	1.44 br.d 13.1	1.37 m	1.44 ddd	1.53 m	
	1.60 dd, 10.6, 5.4		1.70 dd	1.53 br.d 13.1	2.15 s	2.29 s	2.26 s	2.22 s
α	2.06 ddd, 13.4, 5.4, 4.0		2.19 ddd	2.37 m	4.49 d, 2.3	4.52 d	4.34 d 3.1	4.35 d
β	1.75 dddd, 13.4, 10.6, 4.0, 1.4		1.85 ddd	1.82 m	----	--		
α	5.26 dd, 2.0, 5.0		5.43 dd	2.79 m	4.66 d, 2.3	4.64 d	4.63 d 3.1	4.75 d
β				2.79 m				
	6.51 s		6.77 s	6.53 s	6.80 s	6.89 s	6.77 s	6.84 s
	3.07 sept, 7.0		3.36 sept	3.14 sept 6.0	3.07 sept, 6.8	3.27 sept	3.05 sept, 6.8	3.27 m
	1.06 d, 1.5		1.18 d	1.17 d 6.0	1.16 d, 6.8	1.17 d	1.20 d, 6.8	1.17 d
	1.05 d, 1.5		1.19 d	1.17 d 6.0	1.13 d, 6.8	1.18 d	1.20 d, 6.8	1.20 d
	0.75 s		0.88 s	0.97 s	0.95 s	1.02 s	0.99 s	1.02 s
	0.72 s		0.87 s	0.87 s	0.85 s	0.90	0.91 s	0.91 s
							3.83 m	
							1.31, 7.0	

* in $CDCl_3$

**in Acetone – d_6 (see Inatani et. al. 1982 and Arisawa et. al. 1987)

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4.9 Biological evaluations: Results and discussion

In this study, an *in vitro* investigation were carried out to determine the anti-tyrosinase activity and the total antioxidant capacities of a methanol extract of *S. chamelaeagnea* and its 6 isolated constituents (**C1-C6**). Spectrophotometry and cyclic voltammetry assays were carried out for the evaluation of tyrosinase inhibitory activities with kojic acid as a positive control, while the FRAP, TEAC, ORAC and Fe^{2+}- induced lipid peroxidation assays were used to evaluate the antioxidant and oxidative damage modulatory capacities of the extract and isolated compounds while, trolox, ascorbic acid and EGCG were used as reference.

4.9.1 Evaluating the anti-tyrosinase activity of the isolated compounds

The methanolic extract (**SC**) and the isolated compounds (**C1-C6**) were evaluated for their tyrosinase inhibitory activity at different concentration as illustrated in Table 4.9. The result revealed that **C1** demonstrated the highest tyrosinase inhibitory activity (IC_{50} = 455.5 µg/mL**)** among the isolated compounds tested. Recently, tyrosinase inhibitors have received great attention, due to its alleviating properties to deliver skin lightening and anti-aging benefits, caused by undesirable skin hyperpigmentation (Narayanaswamy, et al., 2011). The results showed anti-tyrosinase activity in order of **C1>C3>C4>C2>C6>C5** as illustrated in Table 4.9**.**

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Table 4.9: Percentage of tyrosinase inhibition and IC_{50} of *S. chamelaeagnea* constituents

	% inhibition (µg/mL)					
Sample	**1000**	**500**	**100**	**50**	**10**	**IC_{50}**
C1	99.30	67.44	9.71	4.89	4.19	455.5
C2	91.69	55.4	10.49	4.19	3.88	514.9
C3	/	53.72	11.22	2.94	-5.1	465.7
C4	97.12	54.39	11.96	10.25	2.09	485.2
C5	/	42.28	1.7	-4.48	-1.31	>500
C6	99.77	29.33	9.52	4.87	4.50	554.5
SC	94.79	58.73	51.54	44.56	20.25	267.4
KA	98.88	98.50	98.42	98.12	67.74	3.4

KA: kojic acid;SC: methanol extract of *S. chamelaeagnea;* **C1-C6**: Isolated compounds (1-6)

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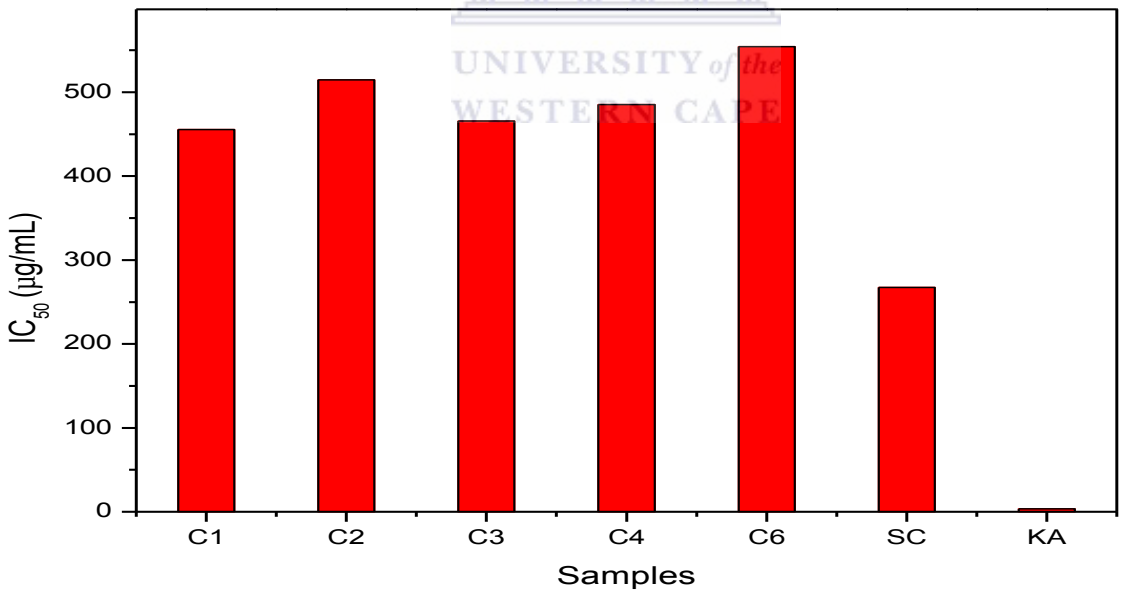

KA>SC>C1>C3>C4>C2>C6

Figure 4.43: Effects of *S. chamelaeagnea* constituents on inhibition of tyrosinase enzyme

*Data are expressed as IC_{50} with all samples screened at 1000µg/mL

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4.9.2 Cyclic voltammetry (CV) measurement

4.9.2.1 Cyclic voltammetry measurement of redox peaks of isolated compounds

CV is the most common reliable technique used for the characterization of redox system. It gives information about the number of redox states as well as qualitative information about the stability of these oxidation states and the electron transfer kinetics (Sochor, et al., 2013).

CV on glassy carbon electrode recorded for monitoring the oxidation-reduction potentials of isolated compounds (**C1-C6**) and extract at the broad scan range from -1300 to 1300 mV at 50 mV/s indicated all the compounds except **C4** are electro-active with well-defined oxidation-reduction peaks**.** The electro-activity of these compounds are illustrated by the fact that redox peaks are shown on Figure 4.35, an indication that the compounds possessed hydroxyl groups which are susceptible to be oxidized to their corresponding quinone, and reduced back to the initial compound. This electro-oxidation can be attributed to the electron transfer reaction involving to the oxidized product. Therefore, CV is a suitable method for the detection of phenolic compounds (Magarelli, et al., 2013). It has been reported that compound with the ability to be oxidized more easily appear to be in higher amount as antioxidant capacity is more extensive (Dobes, et al., 2013). There is a relationship between electrochemical behavior of compounds with antioxidant activity and their resulting antioxidant power capacity, and it is known that low oxidation potential corresponds to the high antioxidant power (Sochor, et al., 2013). **C1** demonstrated the highest electron transfer capacity with the highest anodic peak current value (0.7 µA), which is in agreement with TEAC (16505.0 ± 0.86 µM TE/g) and FRAP (9338.92 ± 1.72 µM AAE/g). The mechanism of these two antioxidant assays (FRAP & TEAC) are measure of electron transfer as well as that of CV. The results obtained therefore are in agreement with **C1** demonstrated highest values for the 3 assays that measure electron transfer

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mechanism. This electron transfer property may be attributed to the presence of an ortho-dihydroxy group on the C-ring of **C1** (Miura, et al., 2002). Table 4.10 showed the anodic peak current and potential of the tested samples.

Table 4.10: Anodic peak current of *S. chamelaeagnea* constituents

Sample	i_{pa}/µA	E_{pa}/V
C1	0.70	0.16
C2	0.33	0.15
C3	0.18	0.33
C4	/	/
C5	0.260	0.11
C6	0.60	0.16
SC	0.14	0.157

SC: methanol extract of *S. chamelaeagnea;* **C1-C6**: Isolated compounds (1-6)

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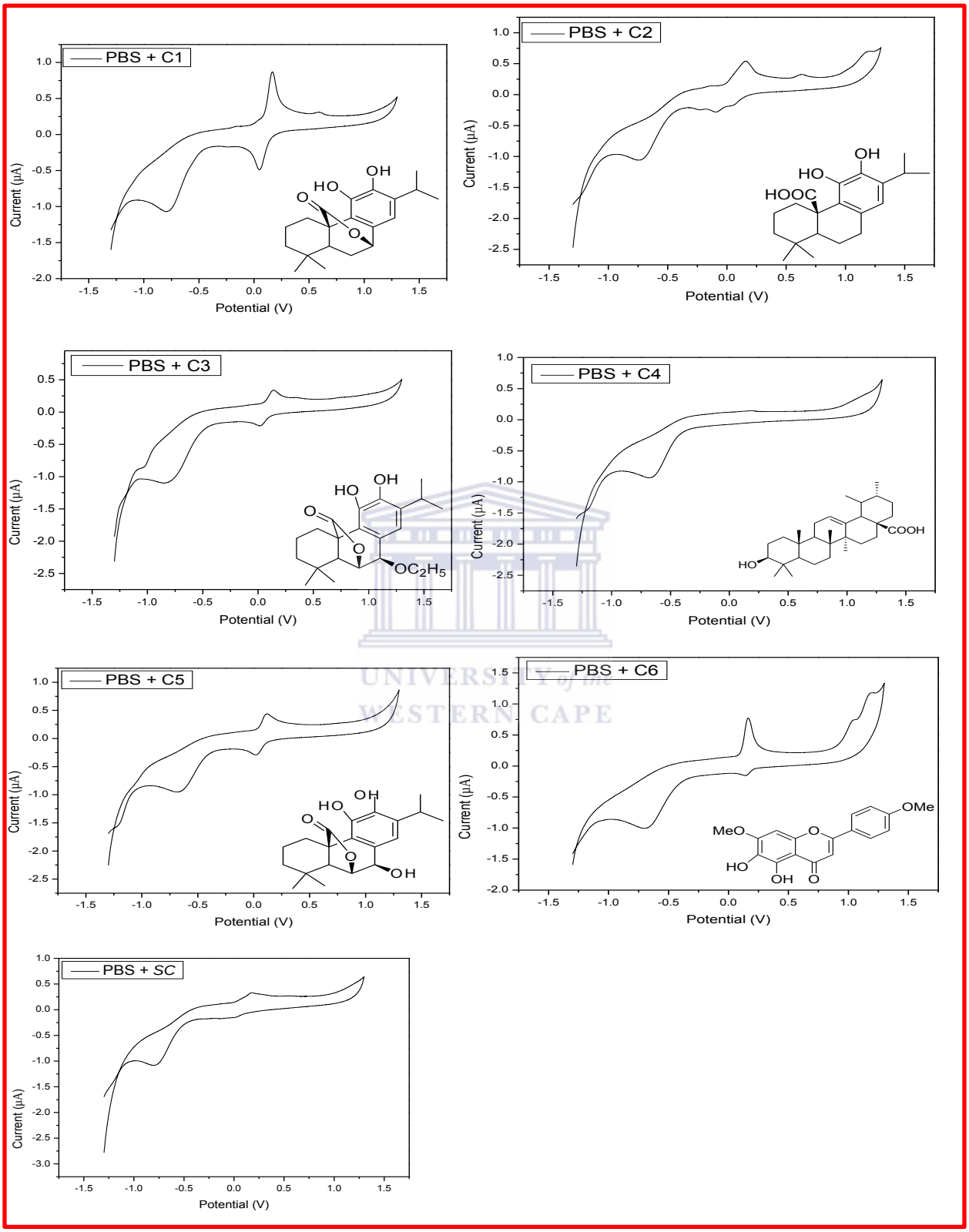

Figure 4.44: CVs recorded at 50 mV/ s in broad scan range from -1300 to 1300 mV for 1 mg/L of each compounds in phosphate buffer (pH 6.5).

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4.9.2.2 Cyclic voltammetry measurement of tyrosinase inhibitory activity

The tyrosinase inhibitory activity of the isolated compounds (**C1-C6**) was investigated using CV by direct measurement of the inhibition current with time at the scan range (from -200 to 200 mV). The result indicated **C1** demonstrated the strongest tyrosinase inhibitory activity by decreasing the inhibition current with time in comparison to kojic acid, indicating that the inhibitory activity demonstrated by kojic acid is 1.5 times more active than **C1**. Among the tested compounds, only **C4** increased the inhibition current with time as shown in Table 4.11 and Figure 4.45, 4.46. According to the result, **C1** demonstrated the highest anti-tyrosinase activity among the tested compound by strongly decreased the inhibition current with time as kojic acid.

Table 4.11: Inhibition current in the presence of the compounds, positive and negative controls 0 and 30 min after addition of tyrosinase and tyrosine based on the anodic process at 131 mV from the corresponding CVs

Sample	i_{inh}/ 10^{-2}µA at 0 min	i_{inh}/ 10^{-2}µA at 30 min
C1	11.5	2.7
C2	16.8	8.1
C3	10.3	5.4
C4	3.1	6.2
C5	12.2	6.9
C6	14.5	11.1
SC	3.4	2.5
PC	2.1	1.8
NC	2.08	80.9

PC: positive control; NC: negative control; SC: methanol extract of *S. chamelaeagnea;* **C1-C6**: Isolated compounds (1-6)

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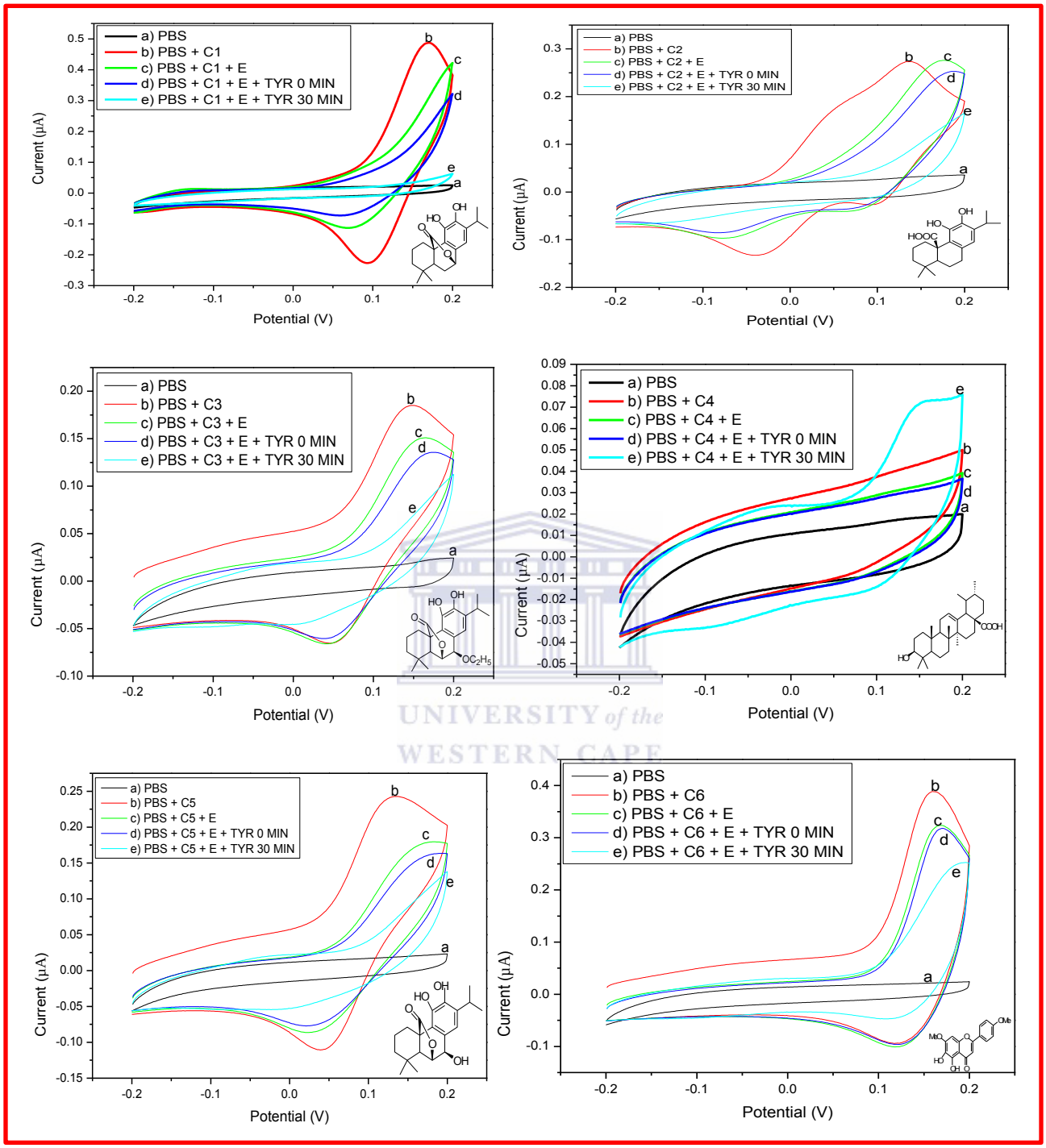

Figure 4.45: CVs recorded at 25 mV/ s in wide scan range from -200 to 200 mV for each isolated compounds in PBS for the reaction mixture of tyrosine and tyrosinase in 50 mM PBS (pH 6.5) at different times.

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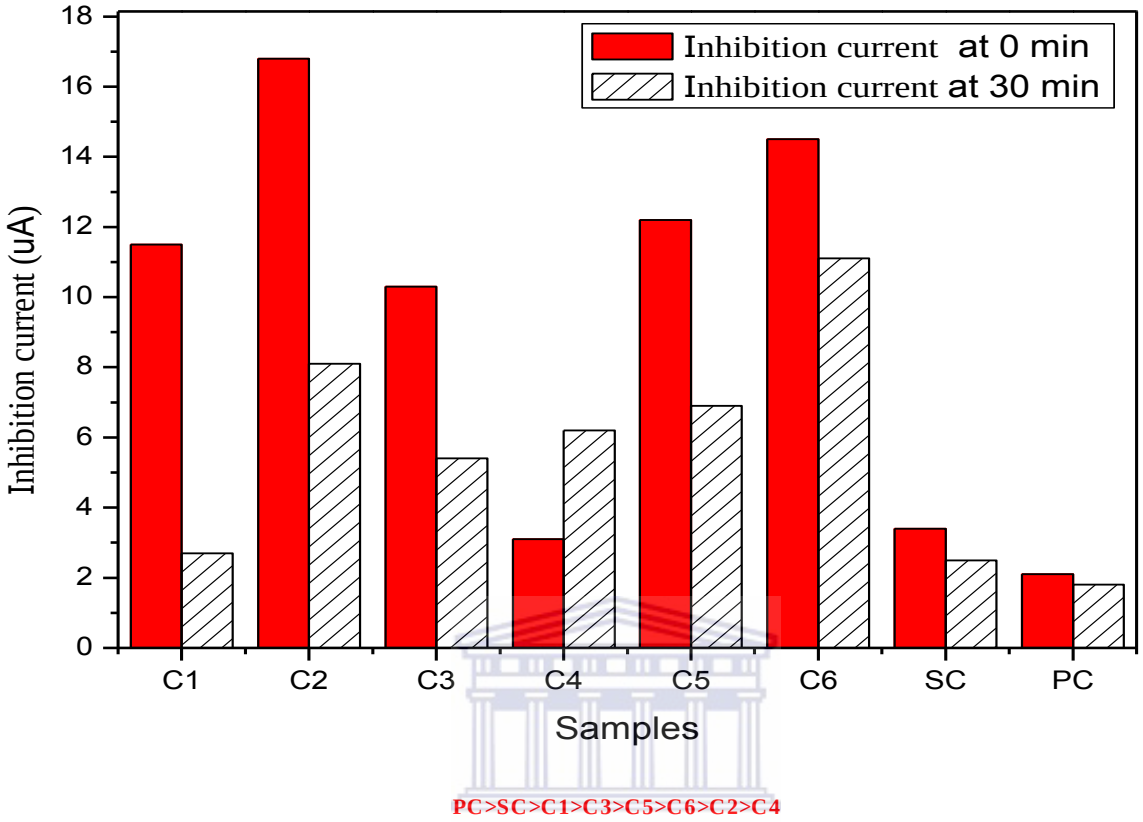

Figure 4.46: Effect of *S. chamelaeagnea* constituents on inhibition of tyrosinase activity

To evaluate the amenability of the method adopted, the enzyme activity was determined by measuring the inhibition based on charge of each isolated compounds and controls. The percentage of inhibition was calculated as follows.

$$[(Q30' - Q0')/ Q0'] X 100 \quad (2)$$

Where Q30' is the inhibition based on charge at 30 min, Q0' is the inhibition based on charge at 0 min.

The result presented in Table 4.12 is an indication about the percentage of inhibition of each compound. According to the obtained result, all the tested compounds except **C4** demonstrated

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some activity by decreasing the inhibition based on charge with time and **C1** showed the highest percentage of inhibition amongst the tested compounds by strongly decreased the inhibition current based on charge with time.

Table 4.12: Inhibition based on charge of the compounds, positive and negative controls at 0 and 30 min in the presence of tyrosinase and tyrosine obtained by integrating the anodic area from the corresponding CVs

	Abg	A0'	A30'	Qbg	Q0'	Q30'	Q30'-Q0'	
	Area before 0 min/10^{-4}	**Area at 0 min/10^{-4}**	**Area at 30 min/10^{-4}**	Qbg/ mC	Q0min/ mC	Q30min/ mC	DQ/ mC	%DQref
C1	30.07	21.19	4.71	120.28	84.76	18.84	-65.92	113.4
C2	30.35	25.51	12.8	121.4	102.04	51.2	-50.84	110.4
C3	18.9	15.6	8.99	75.6	62.4	35.96	-26.44	105.4
C4	7.4	7	10.95	29.6	28	43.8	15.8	96.8
C5	19.5	17.1	11.56	78	68.4	46.24	-22.16	104.5
C6	27.7	25.5	22.6	110.8	102	90.4	-11.6	102.4
SC	8.03	4.18	1.65	32.12	16.72	6.6	-10.12	102.1
PC	4.8	4.9	4.24	19.2	19.6	16.96	-2.64	100.5
NC	3	4.2	12.42	12	16.8	49.68	32.88	0.0

PC: positive control; NC: negative control; SC: methanol extract of *S. chamelaeagnea;* **C1-C6**: Isolated compounds (1-6)

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4.9.3 Evaluating the FRAP and TEAC activity of the isolated compounds

The TEAC is based on single electron transfer (SET) mechanism in which the antioxidant (test samples) donates an electron to the corresponding cationic radical to neutralize it (Pérez-Fons, et al., 2010). The FRAP assay measures the sample's capacity to reduce the intense color of blue ferric TPTZ complex to its ferrous form in acidic medium (Fe^{3+} to Fe^{2+}), thereby changing its absorbance (Firuzi, et al., 2005). The results showed that **C1** and **C5 (**9338.92 ± 1.72; 8622.73 ± 1.92) µM AAE/g respectively demonstrated excellent activity on FRAP when compared to the

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commercial antioxidant EGCG (4722.51 ± 2.22) µM AAE/gas illustrated in Table 4.13. On the other hand, **C1** and **C5 (**16505.5 ± 0.86; 10641.5 ± 0.52) µM TE/g respectively also demonstrated the highest TEAC value when compared to EGCG (10455 ± 0.81) µM TE/g.

The excellent activity demonstrated by **C1** and **C5** is due to the presence of an ortho-dihydroxy group on the C-ring which has the radical scavenging property by transferring electron to the corresponding free radicals (Miura, et al., 2002). In general, phenolic compounds can easily transfer or donate electrons to the reactive radicals because of the resonance stability of phenoxy radical and this induced radical chain reactions delay. The obtained phenoxy radical is less active than the initial one (Özgen, et al., 2011).

Table 4.13: FRAP and TEAC antioxidant capacities of *S. chamelaeagnea* constituents

Sample	FRAP (µM AAE/g)	TEAC (µM TE/g)
C1	9338.92 ± 1.72	16505.5 ± 0.86
C2	4695.98 ± 2.59	5897.5 ± 1.03
C3	1113.05 ± 5.6	4618.2 ± 1.11
C4	117.26 ± 2.6	-57 ± 1.62
C5	8622.73 ± 1.92	10641.5 ± 0.52
C6	5027.55 ± 4.62	8296.2 ± 1.18
SC	9869.43 ± 7.87	13706.5 ± 0.95
EGCG	4722.51 ± 2.22	10455.1 ± 0.81

EGCG: Epigallocacatechingallate; SC: methanol extract of *S. chamelaeagnea;* **C1-C6**: Isolated compounds (1-6)

4.9.4 Evaluating the ORAC activities of the isolated compounds

Results obtained from the investigation of the oxygen radical antioxidant capacity revealed that **C1** and **C5** demonstrated significant ORAC value (14550.5 ± 3.65; 14633.90 ± 3.84) µMTE/g in a competitive manner to that of EGCG (14970 ± 5.53 µMTE/g) as shown in Table 4.14.The findings showed activity in order of **C5>C1>C2>C6>C3>C4.** Therefore, the activity of **C1** and

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C2 is related to the fact that they have a single aromatic ring, hydroxylated by 2 OH groups, which serve as hydrogen donating agent to peroxyl radicals thereby stabilizing them and giving rise to a relatively stable radical (Özgen, et al., 2011).

Table 4.14: Oxygen radical's antioxidant capacity of *S. chamelaeagnea* constituents

Sample	Automated oxygen radical Absorbance capacity (ORAC µMTE/g)
C1	14550.50 ± 3.65
C2	10398 ± 1.81
C3	8247.35 ± 6.83
C4	2080.19 ± 8.52
C5	14633.90 ± 3.84
C6	8380.08 ± 4.52
SC	14338.49 ± 5.16
EGCG	14970 ± 5.53

EGCG: Epigallocacatechingallate; SC: methanol extract of *S. chamelaeagnea;* C1-C6: Isolated compounds (1-6)

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4.9.5 Evaluating the Fe^{2+}-induced anti-lipid peroxidation activity of the isolated compounds

Lipid peroxidation is a process whereby polyunsaturated lipids of the membranes are susceptible to oxidative damage via the reaction attack of free radical. It is one of the consequences of oxidative stress (Ayala, et al., 2014).

The results showed that **C5** and **C1** in comparison with (EGCG) display excellent inhibitory activity against Fe^{2+}-induced anti-lipid peroxidation (IC_{50} = 30.25; 32.5; 41.50) µg/mL respectively, followed by **C2** (IC_{50} = 38.5 µg/mL) as shown in Table 4.15. Their significant activity recorded were due to the presence of vicinal –OH groups in **C1** and **C2** that can chelate with pro-oxidative metalsor scavenge peroxyl radical thereby preventing oxidation. However, the

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unreactivity of **C4** in lipid peroxidation may be explained by the lack of a phenolic moiety (Özgen, et al., 2011).

Table 4.15: Lipid peroxidation of *S. chamelaeagnea* constituents

Sample	100 µg/mL	50 µg/mL	10 µg/mL	IC_{50}
C1	82.2	81.05	24.66	32.5
C2	81.28	78.31	20.11	38.35
C3	19.86	20.54	11.54	>100
C4	17.77	14.58	11.12	>100
C5	82.01	79.71	27.94	30.25
C6	36.93	51.94	20.41	>100
SC	43.98	22.11	10.26	>100
EGCG	91.31	43.19	37.48	41.50

Data are given as IC_{50} with tested sample screened at 100 µg/mL; **SC** methanolic extract

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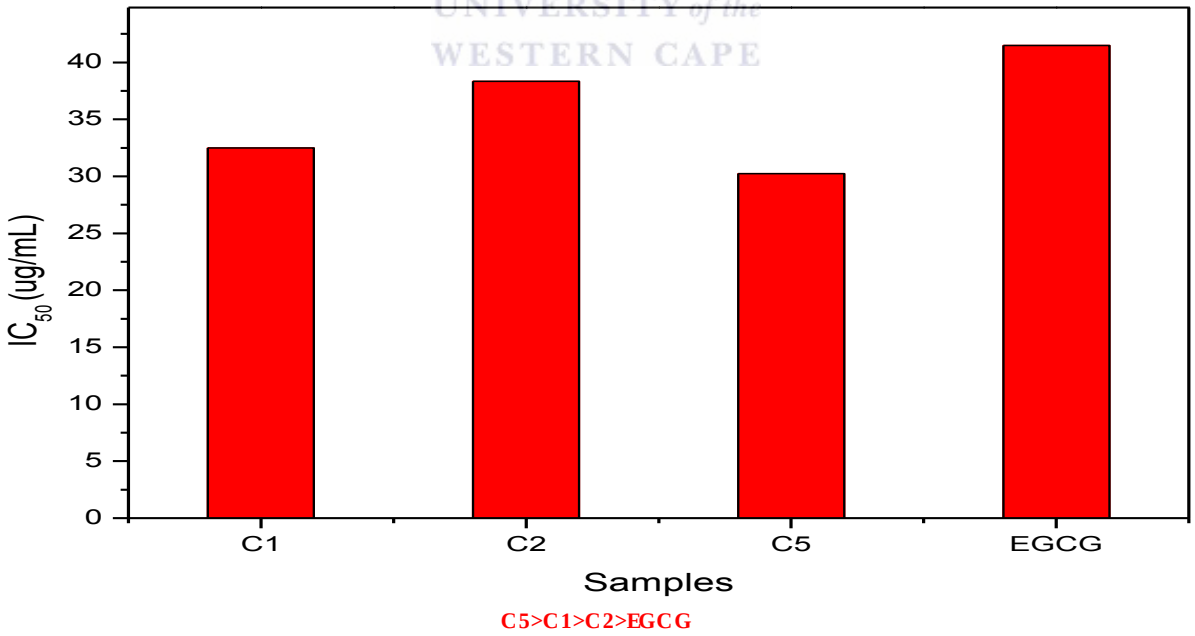

Figure 4.47: Effects of *S. chamelaeagnea* constituents on inhibition of Fe^{2+}-induced microsomal lipid peroxidation.

*Data are expressed as IC_{50} with isolated compounds screened at 100 µg/mL

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CHAPTER 4: ANTI-TYROSINASE AND TOTAL ANTIOXIDANT ACTIVITIES OF ISOLATED COMPOUNDS

Six known compounds were tested for total antioxidant activity, most of them have been shown to possess antioxidant activity and have been implicated as inhibitors of lipid peroxidation. Excellent total antioxidant activities were demonstrated by **C1**, **C5** and **C2** respectively as FRAP (9338.92 ± 1.72; 8622.73 ± 1.92; 4695.98 ± 2.59) µM AAE/g; TEAC (16505 ± 0.86; 10641.5 ± 0.52; 5897.5 ± 1.03) µM TE/g; ORAC (14550.5 ± 3.65; 14633.90 ± 3.84; 10398 ± 1.81) µM TE/g and including the inhibition of Fe^{2+} -induced lipid peroxidation (IC_{50} = 32.5; 30.25; 38.35) µg/mL, and dedicated them as potent antioxidant agents. It has been reported that the antioxidant activity of *Salvia* and Rosemary is related to the presence of carnosic acid and carnosol, which account for approximatively 90 % of the total antioxidant activity of the plant (Kamatou, et al., 2009). **C1, C2** and **C5** have been reported to exhibit remarkably strong antioxidant activity (Miura, et al., 2002), which corroborate with our findings. **C2** has been reported to have strong antioxidant activity with TEAC value of (5600.0 ± 1.0 µM TE/g) (Erkan, et al., 2008) and ORAC (6489 µM TE/g)(Ibarra, et al., 2010), which is due to the presence of the ortho-dihydroxy group in the C ring (Miura, et al., 2002). These findings are in agreement with our result with TEAC (5897.5 ± 1.03 µM TE/g) and ORAC (10398 ± 1.81) µM TE/g). **C1**has been reported to be the most potent antioxidant phenolic diterpenoid of Lamiaceae such sage (Masuda, et al., 2005). It can be confirmed by the result obtained during our research where **C1** demonstrated the highest antioxidant activity on TEAC and FRAP due to its higher capacity of transferring electron to the corresponding cationic radical, while **C5** demonstrated the potent antioxidant activity on ORAC and LPO. This is the first report on the total antioxidant activity of the isolated constituents of *S. chamelaeagnea* and the obtained result is a good indication for the establishment of the relationship between the chemical structure and the antioxidant activity of the compounds in term of electron transfer or hydrogen transfer agents.

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CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The aim of this study was directed towards the preliminary screening of twenty five (25) South African Lamiaceae for their potency of tyrosinase inhibitory activity using different three complementary assays. The outcome of this screening nominated *S. chamelaeagnea*, the bioactive plant materials for chromatographic isolation of such metabolites responsible for the biological activity demonstrated at the preliminary stage. The extraction procedure of the 25 plant materials were successfully carried out using methanol, after which the methanol extracts were filtered and concentrated under vacuum.

The preliminary phytochemical screening using solvent system DCM: MeOH (97:3) on TLC showed *S. chameleagnea* to possess variety of class of compounds varying from terpenoids, flavonoids, and other phenolic compounds (Kamatou, et al., 2007).

The preliminary biological screening of the plant extracts against tyrosinase indicated *Salvia chamelaeagnea*, *Salvia dolomitica*, *Plectranthus ecklonii, Plectranthus namaensis,* and *Plectranthus zuluensis*, with significant zone of inhibition against tyrosinase on TLC bio-autography, spectrophotometry result showed that extracts of *Plectranthus zuluensis* (IC_{50} = 23.99 μg/mL), *P. ecklonii* (IC_{50} = 21.58 μg/mL), *Thorncroftia succulent* (IC_{50} = 36.08 μg/mL), and *P. madagascariensis* (IC_{50} = 23.99 μg/mL) demonstrated good anti-tyrosinase activity when compared with kojic acid (IC_{50} = 3.607 µg/mL). On the other hand, cyclic voltammetry indicated kojic acid as the strongest tyrosinase inhibitory activity by decreasing the inhibition current with time, followed by the extracts of *P. madagascariensis, P. zuluensis, S. chamelaeagnea* and *S. dolomitica*. Some level of agreement between these assays was therefore observed and considered significant.

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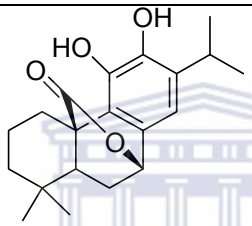
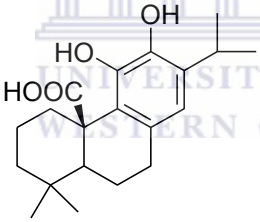
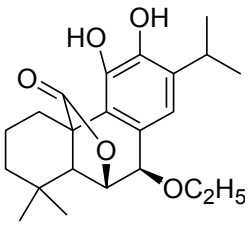
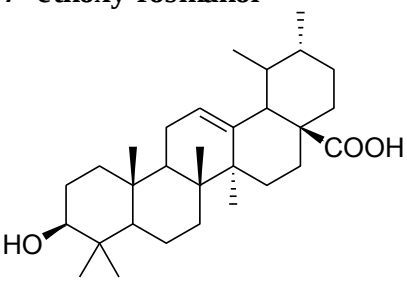
CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

Phytochemical investigation carried out on the bioactive extract of *Salvia chamelaeagnea* using different chromatographic methods including column chromatographic and semi preparative HPLC afforded six (6) known compounds identified as carsonol (**C1**), carnosic acid (**C2**), 7-ethoxylrosmanol (**C3**), ursolic acid (**C4**), rosmanol (**C5**) and ladanein (**C6**). Their chemical structures were fully elucidated by analyses of spectroscopic (^{1}H and ^{13}C NMR) data as well as correlations with existing literature. These compounds are listed as follows (Table 5.1):

Table 5.1: List of the isolated compounds from *S. chamelaeagnea*

Code	Name and chemical structure
C1	 **Carnosol**
C2	 **Carnosic acid**
C3	 **7- ethoxy-rosmanol**
C4	 **Ursolic acid**

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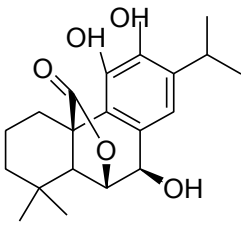
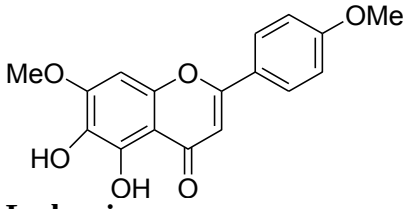
C5



Rosmanol

C6



Ladanein

The methanolic extract of *S. chamelaeagnea* (SC) showed moderate anti-tyrosinase (IC_{50} = 267.4 µg/mL) activity, while the total antioxidant activities measured as: Oxygen radicals absorbance capacity (ORAC; 14970 ± 5.16 µM TE/g), ferric-ion reducing antioxidant power (FRAP; 9869.43 ± 7.87 µM AAE/g) and trolox equivalent absorbance capacity (TEAC; 13706.5 ± 0.95 µM TE/g). Excellent total antioxidant capacities were demonstrated by **C1** and **C5** respectively as FRAP (9338.92 ± 1.72; 8622.73 ± 1.92) µM AAE/g; TEAC (16505 ± 0.86; 10641.5 ± 0.52) µM TE/g; ORAC (14550.5 ± 3.65; 14633.90 ± 3.84) µM TE/g and inhibition of Fe^{2+}-induced lipid peroxidation (IC_{50} = 32.5; 30.25) µg/mL. All the compounds except **C4** are electro-active with well-defined oxidation-reduction peaks while **C1** demonstrated the highest tyrosinase inhibitory activity by strongly decreased the inhibition current with time using cyclic voltammetry method.

Based on the findings of this study, the prerequisite for strong antioxidant activity assessed in these assays was the presence of an ortho dihydroxy group on the C-ring, which may be valuable as a potential antioxidant and lipid peroxidation preventer (Miura, et al., 2002). The total antioxidant capacities demonstrated can be considered as significant information which can be used as a guide for further studies.

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From the above, it is recommended that

- Further studies on this plant should be carried out to investigate the compound(s) responsible for the tyrosinase inhibitory activity demonstrated by the extract *S. chamelaeagnea*.
- Further improvement is required in the electrochemical method as a new method for detection of tyrosinase inhibitors.
- Applications of these chemical constituents in cosmetic, pharmaceutical and food industry will require proof of its antioxidant and skin-enzyme inhibitory effects in an *in vivo* model. Further analyses such as cytotoxicity and effects of the compounds on melanoma cells, carcinogenic and mutagenic effect, as well as clinical trials are recommended in the purpose of translation the current findings into final cosmetic, pharmaceutical and food product formulations.

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References

Abdel-Mogib, M., Albar, H.A. and Batterjee, S.M. 2002. Chemistry of the Genus *Plectranthus*. *Molecules,* 7(2), pp. 271-301.

Ahmad, V.U., Farooq, U., Abbaskhan, A., Hussain, J., Abbasi, M.A., Nawaz, S.A. and Choudhary, M.I. 2004. Four new diterpenoids from *Ballota limbata*. *Chemical & Pharmaceutical Bulletin*, 52(4), pp. 441-3.

Ali, S.M., Ibrahim, S.A., Jalil, S. and Choudhary, M.I. 2007. Ursolic Acid: A potent inhibitor of superoxides produced in the cellular system *Phytotherapy Research,* 21, pp. 558–561.

Aiyegoro, A.O. and Okoh, I.A. 2010. Preliminary phytochemical screening and in vitro antioxidant activities of the aqueous extract of *Helichrysum longifoium*. *BMC Complementary & Alternative Medicine,* 10(21), pp. 21-28.

Al-Jaber, N.A., Awaad, A.S. and Moses, J.E. 2011. Review on some antioxidant plants growing in Arab world. *Journal of Saudi Chemical Society*, 15(4), pp. 293–307.

Arisawa, M., Hayashi, T., Ohmura, K., Nagayama, K., Shimizu, M. and Morita, M. 1987. Chemical and pharmaceutical studies on medicinal plants in Paraguay: Studies on "romero," part 2. *Journal of Natural Products,* 50(6), pp. 1164-1166.

Aristov, A. and Habekost, A. 2015. Cyclic voltammetry - A versatile electrochemical method investigating electron transfer process. *World Journal of Chemical Education*, 3(5), pp. 115-119.

Ashour, A., El-Sharkawy, S., Amer, M., Bar, A.F., Kondo, R. and Shimizu, K. 2013. Melanin biosynthesis inhibitory activity of compounds isolated from unused parts of Ammi visinaga. *Journal of Cosmetics, Dermatological Sciences & Applications,* 3(3), pp. 40-43.

Ayala, A., Muñoz, M. and Argüelles, S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine & Cellular Longevity*, 8, pp. 1-32.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

REFERENCES

Badria, FA., and El Gayyar, M.A. 2001. A new type of tyrosinase inhibitors from natural products as potential treatments for hyperpigmentation. *Bollottino Chimico Farmaceutico,*140(4), pp. 267-271.

Bai, N., He, K., Roller, M., Lai, C.S., Shao, X., Pan, N.H. and Ho, C.T. 2010. Flavonoids and phenolic compounds from *Rosmarinus officinalis*. *Agricultural & Food Chemistry,* 58(9), pp. 5363–5367.

Balunas, M.J. and Kinghorn, A.D. 2005. Drug discovery from medicinal plants. *Life sciences,* 78(5), pp. 431-441.

Baricevic, D., Sosa, S., Loggia, R.D., Tubaro, A., Simonovska, B., Krasna, A. and Zupancic, A. 2001. Topical anti-inflammatory activity of *Salvia officinalis L*. leaves: The relevance of ursolic acid. *Journal of Ethnopharmacology,* 75(2-3), pp. 125– 132.

Bartosz, M., Kedziora, J. and Bartosz, G. 1996. The copper complex of captopril is not a superoxide dismutase mimic. Artefacts in DMPO spin trapping. *Free Radical Research,*24(5), pp. 391-396.

Basu, S. 2010. Fatty acid oxidaton and isoprostanes: Oxidative strain and oxidative stress. *Prostaglandins, Leukotrienes & Essential Fatty Acids*. 82(4-6), pp. 219-225.

Baurin, N., Arnoult, E., Scior, T., Do, Q.T. and Bernard, P. 2002. Preliminary screening of some tropical plants for anti-tyrosinase activity. *Journal of Ethnopharmacology,* 82(2-3), pp. 155-158.

Benzie, I. and Strain, J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP Assay. *Analytical Biochemistry,* 238(1), pp. 70-76.

Brewer, M. 2011. Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science & Food Safety,* 10(4), pp. 221-246.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

REFERENCES

Brieskorn, C.H., Fuchs, A., Brendenberg, J.B., McChesney, J.D. and Wenkert, E. 1964. The Structure of Carnosol, *Journal of Organic Chemistry* 29, pp. 2293-2298.

Burmistrova, O., Perdomoa, J., Simões, M.F., Rijo, P., Quintana, J. and Estevez, F. 2015. The abietane diterpenoid parvifloron D from *Plectranthus ecklonii* is a potent apoptotic inducer in human leukemia cells. *phytomedicine*, 22(11), pp. 1009-1016.

Bussmann, R.W., Glenn, A., Meyer, K., Kuhlman, A. and Townesmith, A. 2010. Herbal mixtures in traditional medicine in Northern Peru. *Journal of Ethnobiology & Ethnomedicine*, 6(1), pp. 10.

Cao, G. and Prior, R. 1998. Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology*, 22(5), pp. 749-760.

Carocho, M. and Ferreira, I. 2013. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food & Chemical Toxicology*, 51, pp. 15-25.

Checker, R., Sandur, S.K., Sharma, D., Patwardhan, R.S., Jayakumar, S., Kohli, V., Sethi, G., Aggarwal, B.B. and Sainis, K.B. 2012. Potent anti-inflammatory activity of ursolic acid, a triterpenoid antioxidant, is mediated through suppression of NF-κB, AP-1 and NF-AT. *PLoS ONE*, 7(2), pp. e31318.

Chen, W.C., Tseng, T.S., Hisiao, N.W., Lin, Y.L., Wen, Z.H., Tsai, C.C., Lee, Y.C., Lin, H.H. and Tsai, K.C. 2015. Discovering of highly potent tyrosinase inhibitor, T1, with significant anti-melanogenesis ability by zebrafish in vivo assay and computational molecular modeling. *Scientific Report*, 10, pp. 1-8.

Chompo, J., Upadhyay, A., Fukuta, M. and Tawata, S. 2012. Effect of Alpinia Zerumbet components on antioxidant and skin disease-related enzymes. *BMCComplementary & Alternative Medicine*, 12, pp. 106-114.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

REFERENCES

Coopoosamy, R.M. and Naidoo, K.K. 2013. A comparative study of three aloe species used to treat skin diseases in South African rural communities. *Journal of Alternative & complementary Medicine,* 19(5), pp. 425-428.

Corstjens, H., Declercq, L., Hellemans, L., Sente, I. and Maes, D. 2007. Prevention of oxidative damage that contributes to the loss of bioenergetics capacity in ageing skin. *Experimental Gerontology,* 42(9), pp. 924-9.

Costin, G.E. and Hearing, V.J. 2007. Human skin pigmentation: melanocytes modulate skin color in response to stress. *Faseb,* 21(4), pp. 976-994.

Curto, E. V., Kwong, C., Hermersdorfer, H., Glatt, H., Santis, C., Virador, V., Hearing, V.J.J. and Dooley, T.P. 1999. Inhibitors of mammalian melanocyte tyrosinase: In vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. *Biochemical Pharmacology,* 57(6), pp. 663–672.

Dobes, J., Zitka, O., Sochor, J., Ruttkay-Nedecky, B., Babula, P., Beklova, M., Kynicky, J., Hubalek, J., Klejdus, B., Kizek, R. and Adam, V. 2013. Electrochemical tools for determination of phenolic compounds in plants. A review. *International Journal of Electrochemistry Sciences,* 8, pp. 4520 - 4542.

D'Orazio, J., Jarrett, S., Amaro-Ortiz, A. and Scott, T. 2013. UV radiation and the skin. *International Journal of Molecular Sciences,* 14(6), pp. 1222–1248.

Dweck, A.C. 1996. Ethnobotanical plants from Africa. *Cosmetics & Toiletries Magazine*

Ekor, M. 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology,* 4, pp. 177-187.

Erkan, N., Ayranci, G., Ayranci, E. 2008. Antioxidant activities of rosemary (*Rosmarinus Officinalis* L.) extract, blackseed (Nigella sativa L.) essential oil, carnosic acid, rosmarinic acid and sesamol.*Food Chemistry*, 110, pp. 76-82

http://etd.uwc.ac.za/

REFERENCES

Espín, J.C. and Wichers, H.J. 2001. Effect of captopril on mushroom tyrosinase activity *in vitro*. *Biochimica Biophysica Acta,* 1544(1-2), pp. 289-300.

Exarchou, V., Nenadis, N., Tsimidou, M., Gerothanassis, I.P., Troganis, A. and Boskou, D. 2002. Antioxidant activities and phenolic composition of extracts from Greek Oregano, Greek Sage, and summer savory. *Journal of Agricultural & Food Chemistry,* 50, pp. 5294-5299.

Farjam, M., Rustaiyan, A., Rustaiyan, E. and Rustaiyan, A. 2013. Labdane-type diterpene and two flavones from *Salvia sharifii Rech. f*. *Iranian Journal of Pharmaceutical Research,* 12(2), pp. 395-399.

Fauziyah, R.S., Gobikrishnan, S., Indrawan, N., Park, S.H., Park, J.H., Min, K., Yoo, Y.J. and Park, D.H. 2012. A Study on the electrochemical synthesis of L-DOPA using oxidoreductase enzymes: Optimization of an electrochemical process. *Journal of Microbiology &Biotechnology,* 22(10), pp. 1446-1451.

Fearon, M.I. and Faux, P.S. 2009. Oxidative stress and cardiovascular disease: Novel tools give (free) radical insight. *Journal of Molecular & Cellular Cardiology,* 47(3), pp. 372-381.

Fernnell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M. and Van stand, F. 2004. Assessing African medicinal plants for efficacy and safety: Pharmacology screening and toxicology. *Journal of Ethnopharmacology,* 94(2-3), pp. 205-17.

Fisher, V. 2005. Indigenous Salvia species - An investigation of the antimicrobial activity, antioxidant activity and chemical composition of leaf extracts. *Msc dissertation University of the Witwatersrand, johannesburg, South Africa*.

Friedman, M. 1996. Food browning and its prevention: An overview. *Journal of Agriculture & Food Chemistry,* 44(3), pp. 631–653.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

REFERENCES

Fuyuno, I. 2004. Spotlight turns on cosmetics for Asian skin. *Nature,* 432(10), pp. 938-1038.

Gad, A.S. and Sayd, A.F. 2015. Antioxidant properties of Rosemary and its potential uses as natural antioxidant in dairy products: A review. *Food & Nutrition Sciences,* 6(1), pp. 179-193.

Gairola, S., Naidoo, Y., Bhatt, A. and Nicholas, A. 2009. An investigation of the foliar trichomes of *Tetradenia riparia* (Hochst.) Codd [Lamiaceae]: An important medicinal plant in Southern Africa. *Flora,* 204(4), pp. 325-330.

Gazim, Z.C., Amorim, A.C.L., Hovell, A.M.C., Rezende, C.M., Nascimento, I.A., Ferreira, G.A. and Cortez, D.A.G. 2010. Seasonal variation, chemical composition, and analgesic and antimicrobial activities of the essential oil from leaves of *Tetradenia riparia* (Hochst.) Codd in Southern Brazil. *Molecules,* 15(8), pp. 5509-5524.

Georges, A., Chinnappan, S., Chintamaneni, M., Kotak C.V., Choudhary, Y., Kueper, T. and Radhakrishnan, A.K. 2014. Anti-inflammatory effects of *Polygonum minus* (Huds) extract (Lineminus™) in *in-vitro* enzyme assays and carrageenan induced paw edema. *BMCComplementary & Alternative Medicine,* 14, pp. 355-369.

Guvenalp, Z., Kilic, N., Kazaz, C., Kaya, Y. and Demirezer, O. 2006. Chemical constituents of Galium tortumense. *Turkey Journal of Chemisty,* 30, pp. 515-523.

Hamidpour, M., Hamidpour, R., Hamidpour, S. and Shahlari, M. 2014. Chemistry, pharmacology, and medicinal property of Sage (Salvia) to prevent and cure illnesses such as obesity, diabetes, depression, dementia, lupus, autism, heart disease, and cancer. *Journal of Traditional & Complementary Medicine,* 4(2), pp. 82–88.

Hashemi, F. and Zarei, M.A. 2014. Tyrosinase inhibitory activity within hexane extract of ten screened plants from Kurdistan province of Iran. *International Journal of Advanced Biological & Biomedical Research,* 2(11), pp. 2795-2799.

Hawas, U.W., Gamal-Eldeen, A.M., El-Toumy, S.A.A., Meyer, J.J.M. and Hussein, A.A. 2009. Inhibition of the initiation stage of carcinogenesis by *Salvia disermas* constituents. *Journal of Nature Research,* 64(11-12), pp. 831-839.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Horiuchi, K., Shiota, S., Hatano, T., Yoshida, T., Kuroda, T. and Tsuchiya, T. 2007. Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant enterococci (VRE). *Biological & Pharmaceutical Bulletin*, 30(6), pp. 1147-1149.

Hermanns, J. F., Pierard-Franchimont, C. and Pierard, G. E. 2000. Skin colour assessment in safety testing of cosmetics. An overview. *International Journal of Cosmetic*, 22(1), pp. 67-71.

Huang, L. and Staden, J. 2002. *Salvia chamelaeagnea* can be micropropated and its callus induced to produce rosmarinic acid. *South African Journal of Botany*, 68(2), pp. 177-180.

Huang, X.H., Chen, Q.X., Wang, Q., Song, K.K., Wang, J., Sha, L. and Guan, X. 2006. Inhibition of the activity of mushroom tyrosinase by alkylbenzoic acids. *Food Chemistry*, 94(1), pp. 1-6.

Hwang, Y., Song, J., Kim, H. & Hwang, K. 2014. Oleanolic acid regulates NF-κB signaling by suppressing MafK expression in raw 264.7 cells. *Biochemistry & Molecular Biology Reports*, 47(9), pp. 524–529.

UNIVERSITY of the
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Igwe, O.M. and Echeme, J.O. 2014. Isolation, structural elucidation and anti-lipid peroxidation activity of 3-(5-Hydroxyphenyl)-4-Methyl-Chromen-3-YI) propanoic acid from the stem bark of *Brachstegia eurycoma Harms*. *International Research Journal of Pure & Applied Chemistry*, 4(4), pp. 447-455.

Inatani, R., N, Nakatani, N. F. H. and Seto, H. 1982. Structure of a new antioxidative phenolic diterpene isolated from Rosemary (*Rosmarinus officinalis L*.). *Journal ofAgricultural & Biological Chemistry*, 46(6), pp. 1661-1666.

Iniaghe, O.M., Malomo, S.O. and Adebayo, J.O. 2010. Proximate composition and phytochemical constitution of leaves of some Acalypha species. *Pakistan Journal of Nutrution*, 8(3), pp. 256-8.

http://etd.uwc.ac.za/

Kamagaju, L., Morandini, R., Bizuru, E., Nyetera, P., Nduwayezu, J.B., Stevigny, C., Ghanem, G. and Duez, P. 2013. Tyrosinase modulation by five Rwandese herbal medicines traditionally used for skin treatment. *Journal of Ethnopharmacology,* 146(3), pp. 824-834.

Kamatou, G.P.P., Makunga, N.P., Ramogola, W.P.N. and Viljoen, A.M. 2008. South African Salvia species: A review of biological activities and phytochemistry. *Journal of Ethnopharmacology,* 119(3), pp. 664-672.

Kamatou, G.P.P., Van Vuuren, S.F., Van Heerden, F.R., Seaman, T. and Viljoen, A.M. 2007. Antibacterial and antimycobacterial activities of South African Salvia species and isolated compounds from *S. chamelaeagnea*. *South African Journal of Botany,* 73(4), pp. 552-557.

Kamatou, G.P., Viljoen, A.M., Gono-Bwalya, A.B., van Zyl, R.L., van Vuuren, S.F., Lourens, A.C., Başer, K.H., Demirci, B., Lindsey, K.L., Van Staden, J. and Steenkamp, P. 2005. The in vitro pharmacological activities and a chemical investigation of three South African Salvia species. *Journal of Ethnopharmacology,* 102(3), pp. 382-90.

Kamatou, G., Viljoen, A. and Steenkamp, P. 2009. Antioxidant, antiinflammatory activities and HPLC analysis of South African Salvia species. *Food Chemistry,* 119(2), pp. 684-688.

Kanitakis, J. 2002. Anatomy, histology and immunohistochemistry of normal human skin.*European Journal of Dermatology*, 12(4), pp. 390-399.

Kaurinovic, B., Vlaisavljevic, S., Popovic, M., Vastag, D. and Brenesel, M.D. 2010. Antioxidant properties of *Marrubium peregrinum L.* (Lamiaceae) essential oil. *Molecules,* 15(9), pp. 5943-5955.

Karthikeyan, M. and Balasubramanian, T. 2014. Phytochemical analysis of *Cynanchum callialatum* through GCMS and LCMS.. *Homeopathy & Ayurvedic Medicine,* 3(1), pp. 1-6.

Kermasha, S., Bao, H. and Bisakowski, B. 2001. Biocatalysis of tyrosinase using catechin as substrate in selected organic solvent media. *Journal of Molecular Catalysis B: Enzymatic,*11(4-6), pp. 929-938.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

REFERENCES

Khalid, S., Rizwan, G.H., Yasin, H., Perveen, R., Abrar, H., Shareef, H., Fatima, K. and Ahmed, M. 2013. Medicinal importance of Holoptela (Roxb). Planch Its biological and pharmacological activities. *Natural Products Chemistry & Research* , 2(1), pp. 1-4.

Kiken, D.A. and Cohen, D.E. 2002. Contact dermatitis to botanical extracts. *American Journal of Contact Dermatitis*, 13(3), pp. 148-152.

Kim, Y.J. and Uyama, H. 2005. Tyrosinase inhibitors from natural and synthtic sources: Structure, inhibition mechanism and perspective for the future. *Cellular & Molecular Life Sciences*, 62(15), pp. 1707-1723.

Kingston, D.G.I. 2011. Modern natural products drug discovery and its relevance to biodiversity conservation. *Journal of Natural Products*, 74(3), pp. 496–511.

Klopper, R.R., Chatelain, C., Banninger, V., Habashi, C., Steyn, H.M., De Wet, B.C., Arnold, T.H., Gautier, L., Smith, G.F. and Spichiger, R. 2006. Checklist of the flowering plants of Sub-Saharan Africa. An index of accepted names and synonyms. Southern African Botanical Diversity Network (ed), Pretoria: SANBI.

Kouřimská, L., Chrpová, D., Nový, P. and Pánek, J. 2013. The effect of concentration on the antioxidant activity of selected culinary herbs. *Journal of Medicinal Plants Research*, 7(13), pp. 766-771.

Kubo, I., Kinst-Hori, I., Ishiguro, K., Chaudhuri, S.K., Sanchez, Y. and Oruga T. 1994. Tyrosinase inhibitory flavonoids from Heterotheca inuloides and their structural functions. *Bioorganic & Medicinal Chemistry Letters*, 4(12), pp. 1443-1446.

Lall, N. and Kishore, N. 2014. Are plants used for skin care in South Africa fully explored. *Journal of Ethnopharmacology*, 153(1), pp. 61-84.

Lazaro, M. 2009. Distribution and biological activities of the flavonoid luteolin. *Mini-Reviews in Medicinal Chemistry*, 9(1), pp. 31-59.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Lee, C.J., Chen, L.G., Chang, T.L., Ke, W.M., Lo, Y.F. and Wang, C.C. 2011. The correlation between skin-care effects and phytochemical contents in Lamiaceae plants. *Food Chemistry,* 124(3), pp. 833-841.

Limon-Pacheco, J. and Gonsebatt, M.E. 2009. The role of antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutation Research,* 674(1-2), pp. 137-147.

Liu, G. and Ruedi, P. 1996. Phyllocladanes (13B-kauranes) from *Plectranthus ambiguus*. *Phytochemistry,* 41(6), pp. 1563-1568.

Lu, Y. and Foo, L. 2001. Salvianolic acid L, a potent phenolic antioxidant from *Salvia officinalis*. *Tetrahedron Letters,* 42(46), pp. 8223-8225.

Magarelli, G., Gomes da Silva, J., Filho, I.A.S., Lopes, I.S.D., SouzaDe, J.R., Hoffmann, L.V. and Pires de Castro, C.S. 2013. Development and validation of a voltammetric method for determination of total phenolic acids in cotton cultivars. *Microchemical Journal,* 109, pp. 23–28.

Makrantonaki, E., Zouboulis, C.C. and German National Genome Research Network 2. 2007. The skin as a mirror of the aging process in the human organism-state of the art and results of the aging research in the German National Genome Research Network 2 (NGFN-2). *Experimental Gerontology,* 42(9), pp. 879-86.

Mapunya, M.B., Nikolova, R.V. and Lall, N. 2012. Melanogenesis and antityrosinase activity of selected south african plants. *Evidence Based Complementary & Alternative Medicine,* 24, pp. 1-6.

Maree, J.E., Khondkar, P., Kwapong, A.A., Oyedemi, B.L., Aljarba, T.M., Stapleton, P., Viljoen, A.M. and Gibbons, S. 2014. Bioactive acetophenones from *Plectranthus venteri*. *Phytochemistry Letters,* 10, p. cxli-cxliv.

Masuda, T., Kirikihira, T., Takeda, Y. 2005. Recovery of antioxidant activity from carnosol quinone: Antioxidants obtained from a water-promoted conversion of carnosol quinone. *Journal of Agriculture & Food Chemistry*. 53, pp. 6831-6834.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

REFERENCES

Mayer, A.M. 1987. Polyphenol oxidases in plants: Recent progress. *Phytochemistry*, 26, pp. 11-20.

Mazimba, O. 2015. *Leonotis leonurus*: A herbal medicine review. *Journal of pharmacognosy & phytochemistry*, 3(6), pp. 74-82.

Misra, B.B. and Dey, S. 2012. TLC-Bioautographic evaluation of In vitro anti-tyrosinase and anti-cholinesterase potentials of Sandalwood oil. *Natural Product Communication*, 8(2), pp. 253-256.

Miura, K., Kikuzaki, H. and Nakatani, N. 2002. Antioxidant activity of chemical components from Sage (*Salvia officinalis L*.) and Thyme (*Thymus vulgaris L*.) measured by the officinalis L. and Thyme (*Thymus vulgaris L*.). *Journal of Agricultural & Food Chemistry*, 50(7), pp. 1845-1851.

Momtaz, S., Lall, N. and Basson, A. 2008. Inhibitory activities of mushroom tyrosine and DOPA oxidation by plant extracts. *South African Journal of Botany*, 74(4), pp. 577-582.

Munné-Bosch, S. and Alegre, L. 2003. Drought-induced changes in the redox state of α-tocopherol, ascorbate, and the diterpene carnosic acid in chloroplasts of Labiatae species differing in carnosic acid contents. *Plant Physiology*, 131(4), p. 1816–1825.

Nag, S., Paul, A. and Dutta, R. 2013. Photochemical analysis of methanol extracts of leaves of some medicinal plants. *International Journal of Science Research Publication*, 3(1), pp. 1-5.

Narayanaswamy, N., Duraisamy, A. and Balakrishnan, K.P. 2011. Screening of some medicinal plants for their antityrosinase and antioxidant activities. *International Journal of PharmTech Research*,3(2), pp. 1107-1112.

Ndamane, Y., Kambizi, L., Songca, S. and Oluwafemi, O. 2013. Antibacteria effectiveness of *Tetradenia riparia* extract, a plant traditionally used in the Eastern Cape province to treat diseases of the respiratory system. *Journal of Medicinal Plants Research*, 37(7), pp. 2755-2760.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Ndlovu, G., Fouche, G., Tselanyane, M., Cordier, W. and Steenkamp, V. 2013. In vitro determination of the anti-aging potential of four African medicinal plants. *BMC Complementary and Alternative Medicine,* 13(304), pp. 1-7.

Niki, S., Paul, A. and Dutta, R. 2005. Lipid peroxidation: Mechanisms, inhibition, and biological effects. *Biochemical & Biophysical Research Communications,* 338(1), pp. 668-676.

Nkomo, M., Katerere, D.R., Vismer, H.F., Cruz, T., Balayssac, S., Malet-Martino, M. and Makunga, N.P. 2014. Fusarium inhibition by wild populations of the medicinal plant *Salvia africana-lutea* L. linked to metabolomic profiling. *BMC Complementary & Alternative Medicine,* pp. 14-99.

Nyilan, M. and Lall, M. 2010. Antilisterial bioactivity and /orbiofilm-formation by compounds from *Plectranthus ecklonii Benth*. and Acacia karroo Hayne. *Doctor of Philosophiae: Plant Science University of Pretoria,* pp. 1-150.

Oliveira, G., Oliveira, A., Conceição, E. and Leles, M. 2016. Multiresponse optimization of an extraction procedure of carnosol and rosmarinic and carnosic acids from *rosemary*. *Food Chemistry,* pp. 465–473.

Oliveira, K., Palú, E., Weffort-Santos, A. and Oliveira, B. 2013. Influence of rosmarinic acid and *Salvia officinalis* extracts on melanogenesis of B16F10 cells. *Revista Brasileira de Farmacognosia,* 23(2), pp. 249-258.

Ozer, O., Mutlu, B. and Kivcak, B. 2007. Antityrosinase activity of some plant extracts and formulations containing ellagic acid. *Pharmaceutical Biology,* 45(6), pp. 519-524.

Özgen, U., Mavi, A., Terzi, Z., Kazaz, C., Aşçı, A., Kaya, Y. and Seçen, H. 2011. Relationship between chemical structure and antioxidant activity of luteolin and its glycosides isolated from *thymus sipyleus subsp. sipyleus var. sipyleus*. *Records of Natural Products,* 5(1), pp. 12-21 .

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Panovska, T., Kulevanova, S. and Stefova, M., 2005. In vitro antioxidant activity of some *Teucrium species* (Lamiaceae). *Acta Pharmaceutica*, 55, pp. 207–214.

Parvez, S., Kang, M., Chung, H.S., Cho, C., Hong, M.C., Shin, M.K. and Bae, H. 2006. Survey and mechanism of skin depigmenting and lightening agents. *Phytotherapy Research*, 20(11), pp. 921-934.

Pellegrini, N., Re, R., Yang, M. and Rice-Evans, C. 1999. Screening of dietary carotenoid rich fruit extracts for antioxidant activities applying ABTS radical cation decolorisation assay. *Methods in Enzymology*, 299, pp. 379-389.

Pérez-Fons, L., Garzón, M. and Micol, V. 2010. Relationship between the antioxidant capacity and effect of rosemary (*Rosmarinus officinalis L*.) polyphenols on membrane phospholipid order.*Journal of Agricultural &Food Chemistry*, 58(1), pp. 161-71.

Petersen, M. and Simmonds, M. 2003. Rosmarinic acid. *Phytochemistry*, 62(2), pp. 121-125.

Popoola, O., Marnewich, J.L., Rautenbach, F., Ameer, F., Iwuoha, E.I. and Hussein AA. 2015. Inhibition of oxidative stress and skin aging-related enzymes by prenylated chalcones and other flavonoids from *Helichrysum teretifolium*. *Molecules*, 20(4), pp. 7143-7155.

Porcheron, A., Latreille, J., Jdid, R., Tschachler, E. and Morizot, F. 2014. Influence of skin ageing features on Chinese women's perception of facial age and attractiveness. *International Journal of Cosmetic Science*, 36(4), pp. 312-320.

Prajapati, R., Kalariya, M., Umbarkar, R., Parmar, S. and Sheth, N. 2011. *Colocasia esculenta*: A potent indigeous plant. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, 1(2), pp. 90-96.

Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampschwoodill, M., Huang, D., Ou, B. and Jacob, R. 2003. Assays for hydrophilic antioxidant capacity ($ORAC_{FL}$). *Journal of Agricultural & Food Chemistry*, 51(11), pp. 3273-3279.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Raja, R. 2012. Medicinally potential plants of Lamiaceae family: An overview. *Research Journal of Medicinal Plants,* 6, pp. 203-213.

Rates, S.M.K. 2001. Plants as source of drugs. *Toxicon,* 39(5), pp. 603-613.

Reis, A., Viccini, L. and Marçal de Sousa, S. 2015. Contributions to cytogenetics of *Plectranthus barbatus Andr*. (Lamiaceae): A medicinal plant. *Comparative Cytogenetics,* 9(3), pp. 451–463.

Rice, L., Brits, G., Potgieter, C. and Van Staden, J. 2011. Plectranthus: A plant of future. *South African Journal of Botany ,* 77, pp. 947-959

Rojas, J.J., Ochoa, V.J., Ocampo, A.S. and Muñoz, J.F. 2006. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections. *BMCComplementary & Alternative Medicine,* 6(2), pp. 1-6.

Rosa, S., Correia, V., Ribeiro, I., Rijo, P., Simões, F., Saraiva, N. and Fernandes, A. 2015. In vitro antioxidant properties of the diterpenes parvifloron D and 7α-acetoxy-6β-hydroxyroyleanone. *Biomedical & Biopharmaceutical Research* , 12(1), pp. 59-67.

Schrader, M. and Fahimi, H.D. 2006. Peroxisomes and oxidative stress. *Biochimica & Biophysica Acta,* 1763(12), pp. 1755-1766.

Scott, G., Springfield, E.P. and Coldrey, N. 2004. A pharmacognostical study of 26 South African plant species used as traditional medicines. *Pharmaceutical Biology,* 42(3), pp. 186-213.

Shaukat, K., Ghazala, H.R., Hina, Y., Rehana, P., Hina, A., Huma, S., Kaneez, F. and Maryam, A. 2013. Medicinal importance of holoptela integrifolia (Roxb.). Planch its biological and pharmacological activities. *Natural Products chemistry & Research,* 2(1), pp. 1-4.

Shirasugi, I., Sakakibara, Y., Yamasaki, M., Nishiyama, K., Matsui, T., Liu, M.C. and Suiko, M. 2010. Novel screening method for potential skin-whitening compounds by a luciferase reporter assay. *Bioscience, Biotechnology, & Biochemistry,* 74(11), p. 2253–2258.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Sima, V.H., Patris, S., Aydogmus, Z., Sarakbi, A., Sandulescu, R and Kauffmann, J.M. 2011. Tyrosinase immobilized magnetic nanobeads for the amperometric assay of enzyme inhibitors: Application to the skin whitening agents. *Talanta*, 83(3), pp. 980-987.

Snijman, P.W., Joubert, E., Ferreira, D., Li, X., Ding, Y., Green, I.R. and Gelderblom, W.C.A.2009. Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (Aspalathus linearis) flavonoids, epigallocatechin gallate, and trolox.*Journal of Agricultural & Chemistry*, 57(15), pp. 6678-6684.

Sochor, J., Dobes, J., Krystofova, O., Ruttkay-Nedecky, B., Babula, P., Pohanka, M., Jurikova, T., Zitka, O., Adam, V., Klejdus, B. and Kizek, R. 2013. Electrochemistry as a tool for studying antioxidant properties. *International Journal of Electrochemistry Sciences*, 8, pp. 8464-8489.

Summers B. 2006. A lightening tour of skin-brightening options. *Pharmaceutical & Cosmetic Review*, pp. 29–33.

Suntar, I., Akkol, EK., Senol, F.S., Keles, H. and Orhan, I.E. 2011. Investigating wound healing, tyrosinase inhibitory and antioxidant activities of the ethanol extracts of *Salvia cryptantha* and *Salvia cyanescens* using *in vivo* and *in vitro* experimental models. *Journal of Ethnopharmacology*, 135(1), pp. 71-77.

Sparavigna, A., Tenconi, B., Deponti, I., Scarci, F. and Caserini, M. 2013. An innovative concept gel to prevent skin aging. *Journal of Cosmetics, Dermatological Sciences & Applications*, 3(4), pp. 271-280.

Strelitzia. 2013. Plants of the greater Cape floristic Region. Snijman, D.A (ed). Pretoria: SANBI.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Szentmihályi, K., Then, M. and Csedő, C. 2004. Comparative study on tannins, flavonoids, terpenes and mineral elements of some Salvia species. *Acta Hortorticulturae*, 629, pp. 463-470.

Tavares da Mota, G.S., Arantes, A.B, Sacchetti, G., Spannoletti, A., Ziosi, P., Scalambra, E., Vertuani, S. and Manfredini, S. 2014. Antioxidant activity of cosmetic formulations based on novel extracts from seeds of Brazilian Araucaria angustifolia (Bertoll) Kuntze. *Journal of Cosmetics, Dermatological Sciences & Applications,* 4, pp. 190-202.

Taylor, J.L.S., Rabe, T., Mc Gaw, L.J., Jager, A.K. and Van staden, J. 2001. Towards the science validation of trditional medicinal plants. *Plant Growth Regulation,* 34(1), pp. 23-37.

Thangboonjit, W., Limsaeng-u-rai, S., Pluemsamran, T. and Panich, U. 2014. Comparative evaluation of antityrosinase and antioxidant activities of dietary phenolics and their activities in melanoma cells exposed to UVA. *Siriraj Medical Journal,* 66(1), pp. 1-6.

Theodoridis, S., Stefanaki, A., Tezcan, M., Aki, C., Kokkini, S. and Vlachonasios, K.E. 2012. DNA barcoding in native plants of the Labiatae (Lamiaceae) family from Chios Island (Greece) and the adjacent Çeşme-Karaburun Peninsula (Turkey). *Molecular Ecology Resources,* 12(4), pp. 620-33.

Thring, T.S. and Weitz, F.M. 2006. Medicinal plant use in the Bredasdorp/Elim region of the Southern Overberg in the Western Cape Province of South Africa. *Journal of Ethnopharmacology* , 103(2), pp. 261-75.

Topcu, G., Ozturk, M., Kusman, T., Demirkoz, A.A.B., Kolak, U. and Ulubelen, A. 2013. Terpenoids, essential oil composition, fatty acid profile, and biological activities of Anatolian *Salvia fruticosa* Mill.*Turkish Journal of Chemistry,* 37, pp. 619 – 632.

Van Wyk, B.E. and Gericke, N. 2000. People's Plants. A guide to useful plants of Southern Africa. *Briza Publications, Pretoria.* pp. 352.

http://etd.uwc.ac.za/

REFERENCES

Vinha, A.F., Soares, M.O., Castro, A., Santos, A., Oliveira, M.B. and Machado, M. 2012. Phytochemical characterizationand radical scavenging activity of aqueous extracts of medicinal plants from portugal. *European Journal Medicinal Plants,* 2(4), pp. 335-347.

Xia, N., Zhang, L., Feng, Q., Deng, D., Sun, X. and Liu, L. 2013. Amplified voltammetric detection of tyrosinase and its activity with dopamine-gold nanoparticles as redox probes. *International Journal of Electrochemistry,* 8, pp. 5487-5495.

Wangthong, S., Tonsiripakdee, I., Monhaphol, T., Nonthabenjawan, R. and Wanichwecharungruang, P.S. 2007. TLC developing technique for tyrosinase inhibitor detection. *Biomedical Chromatography,* 21(1), pp. 94-100.

Watson, R.E.B., Gibbs, N.K., Griffiths, C.E.M. and Sheraft, M.J. 2014. Damage to skin extracellular matrix induced by UV exposure. *Antioxidant & Redox Signaling,*21(7), pp. 1063-1077.

Whittaker, J. 1995. In food enzymes, structure and mechanism. New York, pp. 1-6

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