

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



**UNIVERSITY of the
WESTERN CAPE**

By

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

Master of Science
**UNIVERSITY of the
WESTERN CAPE**

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ABSTRACT

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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* ($IC_{50} = 21.58 \mu\text{g/mL}$), *Plectranthus zuluensis* ($IC_{50} = 23.99 \mu\text{g/mL}$), *Plectranthus madagascariensis* ($IC_{50} = 23.99 \mu\text{g/mL}$) and *Salvia lanceolata* ($IC_{50} = 28.83$) demonstrated good anti-tyrosinase activity when compared with kojic acid ($IC_{50} = 3.607 \mu\text{g/mL}$). 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

ABSTRACT

afforded six (6) known compounds *viz* carsonol (**C1**), carnosic acid (**C2**), 7- ethoxyrosmanol (**C3**), ursolic acid (**C4**), rosmanol (**C5**) and ladanein (**C6**). Their chemical structures were elucidated by analyses of spectroscopic (^1H and ^{13}C NMR) data as well as correlations with existing literature. The methanolic extract of *S. chamelaeagnea* (SC) showed moderate anti-tyrosinase ($\text{IC}_{50} = 267.4 \mu\text{g/mL}$) activity, total antioxidant capacities measured as: Oxygen radicals absorbance capacity (ORAC; $14970 \pm 5.16 \mu\text{M TE/g}$), ferric-ion reducing antioxidant power (FRAP; $9869.43 \pm 7.87 \mu\text{M AAE/g}$) and trolox equivalent absorbance capacity (TEAC; $13706.5 \pm 0.95 \mu\text{M TE/g}$). Excellent total antioxidant capacities were demonstrated by **C1** and **C5** respectively as FRAP (9338.92 ± 1.72 ; 8622.73 ± 1.92) $\mu\text{M AAE/g}$; TEAC (16505 ± 0.86 ; 10641.5 ± 0.52) $\mu\text{M TE/g}$; ORAC (14550.5 ± 3.65 ; 14633.90 ± 3.84) $\mu\text{M TE/g}$ and including the inhibition of Fe^{2+} -induced lipid peroxidation ($\text{IC}_{50} = 32.5$; 30.25) $\mu\text{g/mL}$. 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*.

DECLARATION

DECLARATION

I, **Ninon Geornest Eudes Ronald 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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ACKNOWLEDGMENTS

ACKNOWLEDGEMENTS

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DEDICATION

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 ABBREVIATIONS

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NMR	Nuclear magnetic resonance
^{13}C -NMR	Carbon-13 nuclear magnetic resonance
^1H -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) diammonium salt
AAPH	2,2- Azibis (2-methylpropionamide) dihydrochloride, perchloric acid
FRAP	Ferric-ion reducing antioxidant power

LIST OF ABBREVIATIONS

ORAC	Oxygen radicals absorbance capacity
TLC	Thin layer chromatography
TEAC	Trolox equivalent absorbance capacity
TE/g	Trolox equivalent 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
DIW	De-ionized water
DNA	Deoxyribonucleic acid
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechingallate
Fig	Figure
g	Gram

LIST OF ABBREVIATIONS

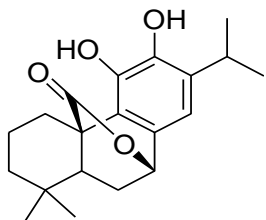
L	Liter
mg	Milligram
min	Minute
HPLC	High performance liquid chromatography
ROS	Reactive oxygen specie
SANBI	South African National Biodiversity Institute
SD	Standard deviation
Spp	Species
IC ₅₀	Half maximal inhibitory concentration



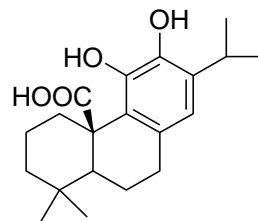
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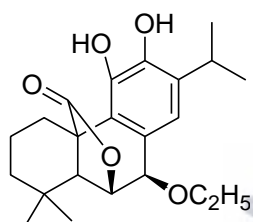
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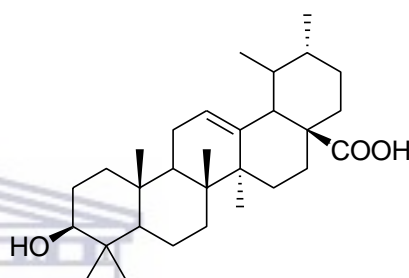
carnosol (C1)



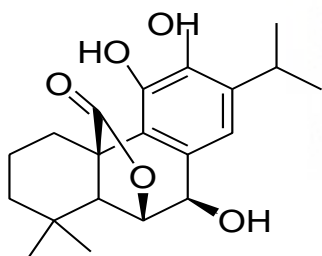
carnosic acid (C2)



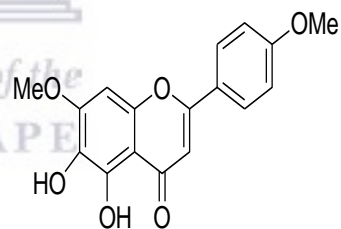
7-ethoxy-rosmanol (C3)



ursolic acid (C4)



rosmanol (C5)



ladanein (C6)

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.

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Manuscript in preparation



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CHAPTER 1: INTRODUCTION

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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 (Fernell, 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 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).

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 zeylanica* (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).

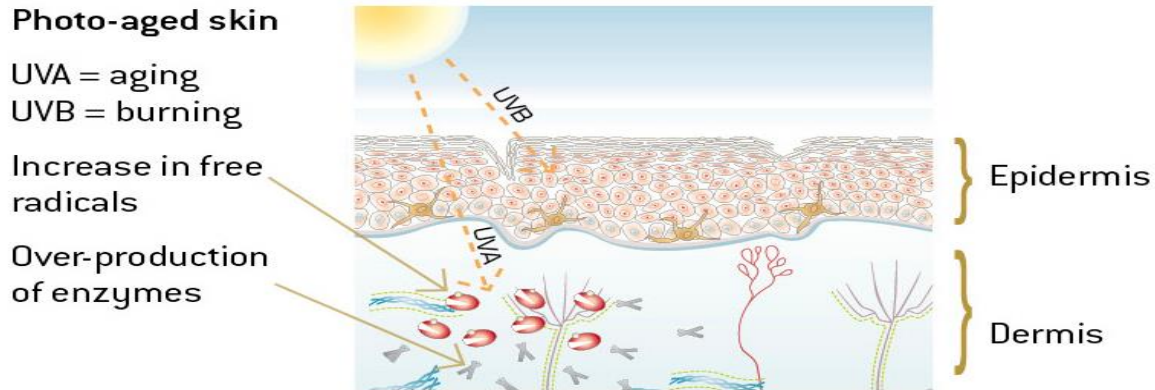
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1.4 Historical perspective of skin pigmentation

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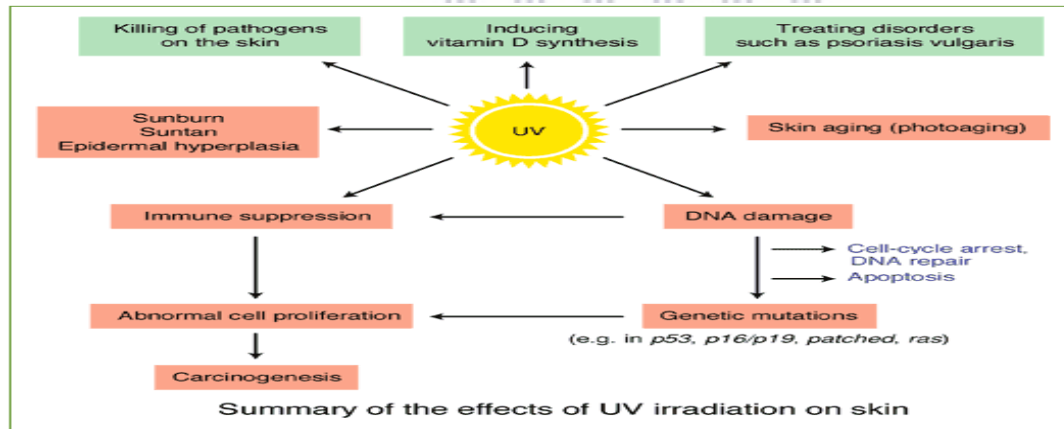
to characterize the role of oxidative damage in both internal and external tissue, given the compelling evidence for the role of ROS as mediators of photoaging (Watson, et al., 2014).



Damage caused to the structural elements of the dermis.

* Source: www.leadinginstanteyelift.com

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



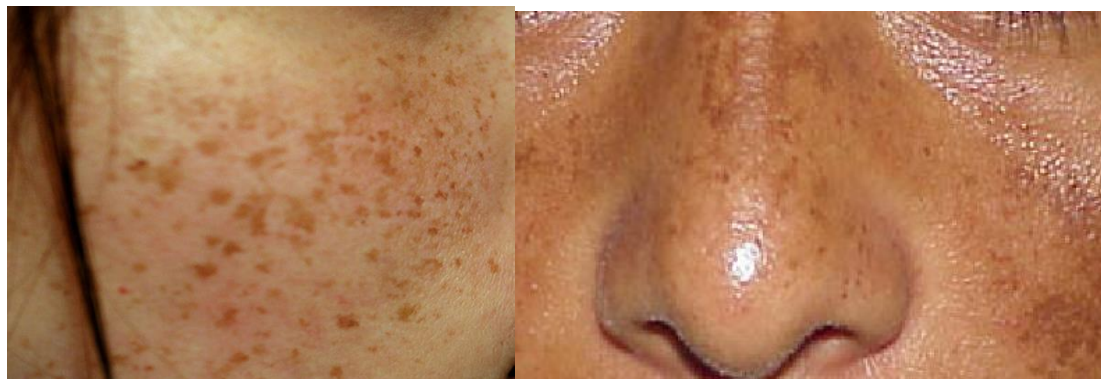
*Source: www.healthyfellow.com

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

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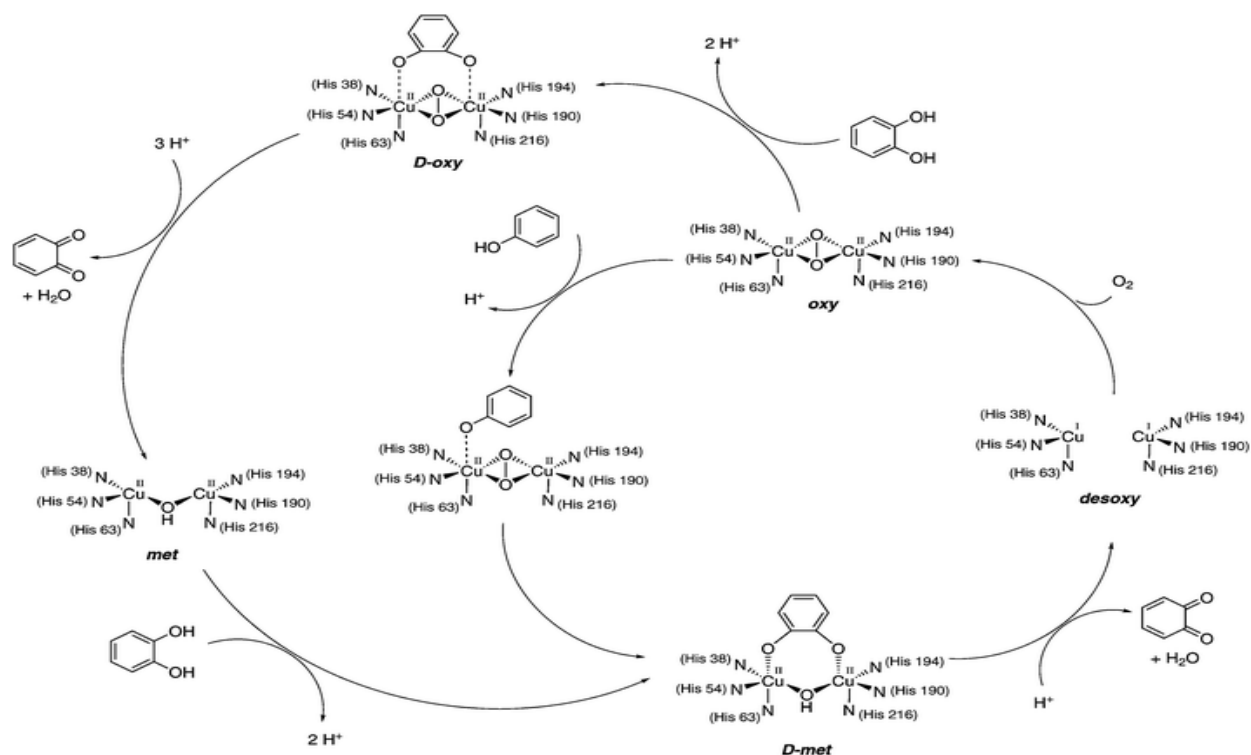
agents, free radicals and reactive oxygen species, greatly overwhelms the capacity of the endogenous cellular antioxidant defense system, thus leading to potential damage of the cells and organs and to the progression of degenerative diseases in humans (Schrader & Fahimi, 2006; Basu, 2010).

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 – dihydroxyphenylalanine (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).

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

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 (Burnistrova, et al., 2015). Other notable economic value of plant species of this family is highlighted in the literature review presented in the next Chapter.

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.

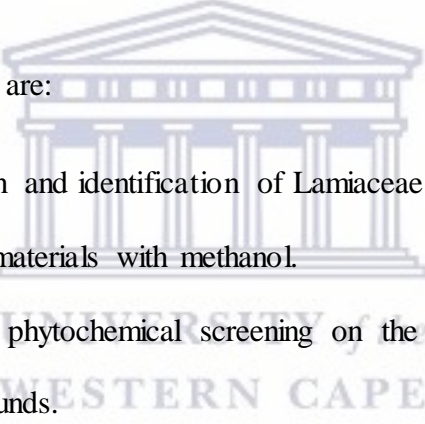
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1.10 Aims of this study

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) Luer, Prostantheroideae Luer, Scutillarioideae (Dumort) Caruel, Symphorematoideae Briq and Viticoideae 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 flora 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
<i>Ballota africana</i>	Fever, cough, asthma, influenza, insomnia, stress, sores	(Scott & Springfield, 2004; Lall & Kishore, 2014)
<i>Leonotus leonurus</i>	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)
<i>L. ocymifolia</i>	Skin irritation, eczema, diabetes, hypertension, anemia, purgative	(Scott & Springfield, 2004)
<i>Mentha longifolia</i>	Ulcerative colitis, liver complaints, anti-inflammatory, analgesic, antiplasmodic, anti-mutagenic, sores, wound, urinary tract antioxidant	(Scott & Springfield, 2004; Lall & Kishore, 2014)
<i>Plectranthus ambiguus</i>	Anti-oxidant	(Liu & Ruedi, 1996)
<i>P. barbatus</i>	Respiratory ailments, liver disturbance, heart diseases, nervous problems	(Reis, et al., 2015)
<i>P. ecklonii</i>	Skin disorders, stomachaches, nausea, vomiting, meningitis, cancer	(Burmistrova, et al., 2015)
<i>Salvia africana-caerulea</i>	Stomachache, headache, bronchial congestion, antiseptic wash to ulcer	(Scott & Springfield, 2004)
<i>S. africana-lutea</i>	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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<i>S. aurita</i>	Antioxidant, anti-inflammatory	(Fisher, 2005)
<i>S. chamelaeagnea</i>	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)
<i>S. disermas</i>	Sores, rheumatism, hypertension, antioxidant, antimicrobial, antituberculosis, anti-inflammatory, antiplasmodial	(Fisher, 2005; Hawas, et al., 2009)
<i>S. dolomitica</i>	Anti-inflammatory	(Kamatou, et al., 2008)
<i>S. namaensis</i>	Antioxidant	(Kamatou, et al., 2008)
<i>S. radula</i>	Antioxidant, anti-inflammatory	(Kamatou, et al., 2008)
<i>S. repens</i>	Sores on the body, stomach problem, diarrhea, anti-inflammatory, antioxidant	(Kamatou, et al., 2005)
<i>S. runcinata</i>	Antioxidant, fever, anti-inflammatory, purgative, headache	(Kamatou, et al., 2005)
<i>S. stenophylla</i>	Antioxidant, anti-inflammatory, disinfectant, purgative, cold, cough, chest congestion	(Kamatou, et al., 2005; Lall & Kishore, 2014)
<i>Tetradenia riparia</i>	Cough, whooping, bronchitis, cold, diarrhea, headache, drowsy, fever, stomach ache, burn wounds, antiseptic, toothaches, skin diseases, and antioxidant.	(Scott & Springfield, 2004; Gairola, et al., 2009; Ndamane, et al., 2013)

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, phyllocladanes, neoclerodane, labdanes, pimiranes, isopimaranes, beyeranes, sesquiterpenes, and flavonoids are

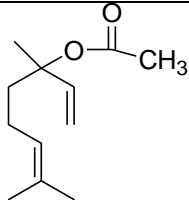
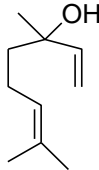
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widely found in different species of *Plectranthus* genus (Abdel-Mogib & Albar, 2002). *Salvia* species have been reported to be rich source of phenolic and polyphenolic compounds such as carnosic acid (**8**), rosmarinic acid (**21**), caffeic acid (**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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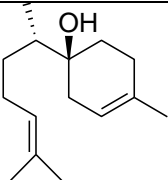
Table 2.2: Selected secondary metabolites with anti-ageing properties

Table 2.2.1: Monoterpenes

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

S: Salvia

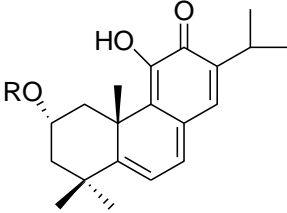
Table 2.2.2: Sesquiterpene

S/N	Compound	Plant source	Biological activity	Reference
3	 α - bisabolol	<i>S. runcinata</i>	Antioxidant Anti-inflammation	(Kamatou, et al., 2008)

S: Salvia

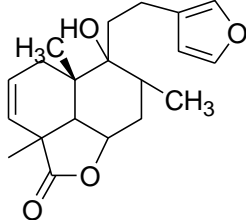
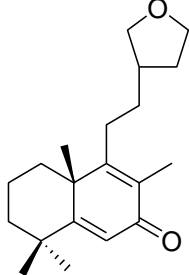
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Table 2.2.3: Abiatene diterpenoid

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

P: Plectranthus

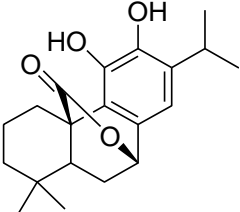
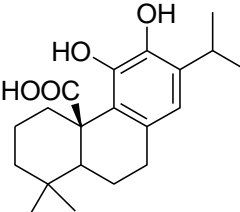
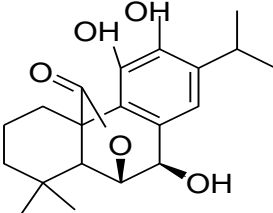
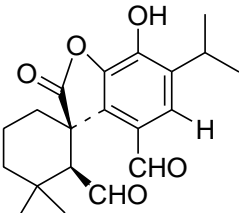
Table 2.2.4: Labdane diterpenoids

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

L: Leonotus

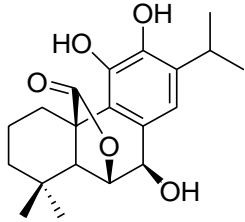
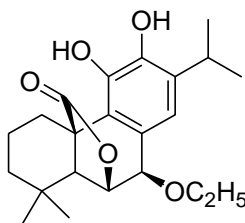
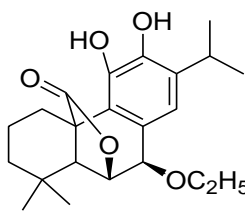
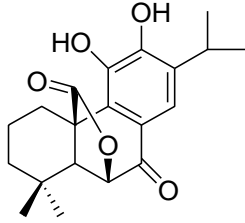
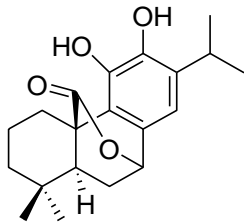
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Table 2.2.5: Phenolic diterpenes

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

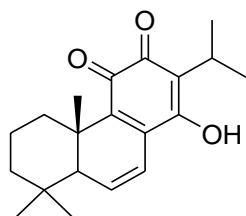


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11	 <p style="margin-top: 5px;">epirosmanol</p>	<i>S. officinalis</i>	Antioxidant	(Miura, et al., 2002)
12	 <p style="margin-top: 5px;">7-ethoxy-rosmanol</p>	<i>S. officinalis</i>	Antioxidant	(Miura, et al., 2002)
13	 <p style="margin-top: 5px;">7-O-methylepirosmanol</p>	<i>S. namaensis</i>	Antioxidant	(Kamatou, et al., 2009)
14	 <p style="margin-top: 5px;">galdosol</p>	<i>S. officinalis</i>	Antioxidant	(Miura, et al., 2002)
15	 <p style="margin-top: 5px;">isorosmanol</p>	<i>S. officinalis</i>	Antioxidant	(Miura, et al., 2002)
16			Antioxidant	(Gazim, et al., 2014)



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T. riparia

6,7- dehydroroyleanone

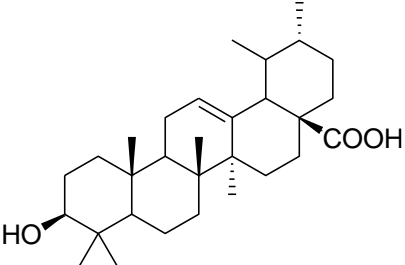
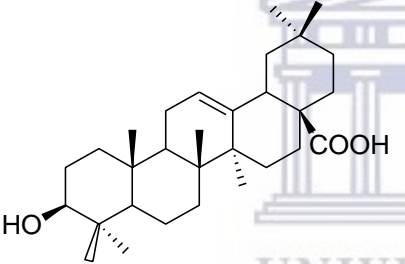
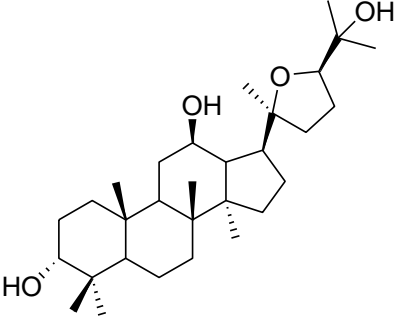
a: antioxidant; b: anti-melanogenesis; d: antimicrobial; *S:Salvia*; *R:Rosmarinus*; *T:Tetradenia*



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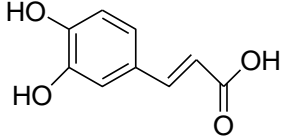
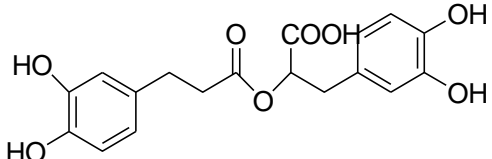
Table 2.2.6: Triterpenes

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

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

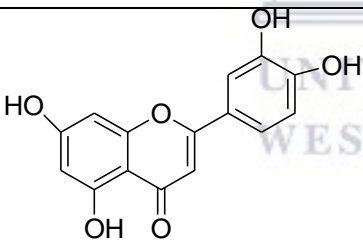
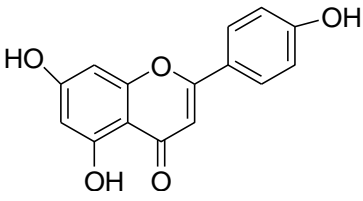
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Table 2.2.7: Phenolic acid

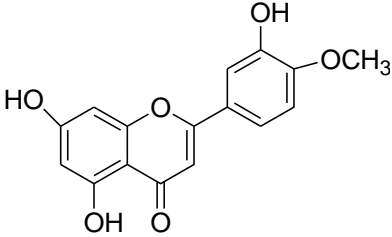
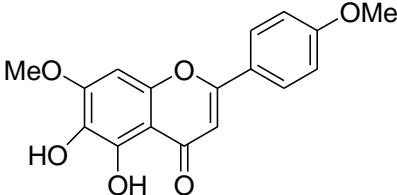
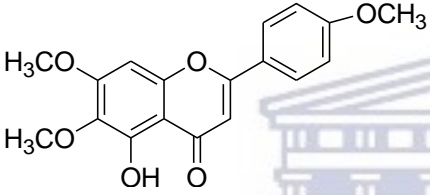
S/N	Compound	Plant source	Biological activity	Reference
20	 Caffeic acid	<i>S. muiirii</i>	Antioxidant Anti-melanogenic	(Kamatou, et al., 2009) ^a (Thangboonjit, et al., 2014) ^b
21	 Rosmarinic acid	<i>R.officinalis</i>	Anti-inflammatory Antioxidant Anti-melanogenic	(Petersen & Simmonds, 2003) ^{a,c} (Oliveira, et al., 2013) ^b

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

Table 2.2.8: Flavones

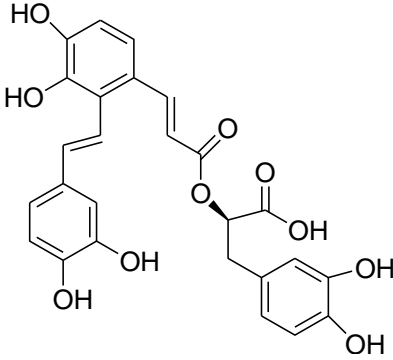
S/N	Compound	Plant source	Biological activity	Reference
22	 Luteolin	<i>Teucrium chamaedrys</i>	Antioxidant Anti-inflammatory	(Panovska, et al., 2005) ^a (Lopez-Lazaro, 2009) ^c
23	 Apigenin	<i>T. polium</i>	Antioxidant	(Panovska, et al., 2005)

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

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

Table 2.2.9: Phenylpropenoid

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

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 $IC_{50} = 61.73 \pm 2.69$ 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

Table 2.3: Plant species with tyrosinase inhibitory activities

Name of the plant	Location	IC ₅₀ (µg/mL)	Reference
<i>Origanum majorana</i>	Taiwan	107.00	(Lee, et al., 2011)
<i>Lavendula stoechas</i>	Taiwan	127.00	(Lee, et al., 2011)
<i>Lavendula latifolia</i>	Taiwan	145.00	(Lee, et al., 2011)
<i>Lavendula allardu</i>	Taiwan	113.00	(Lee, et al., 2011)
<i>Ballota nigra</i>	Iran	3.67	(Hashemi & Zarei, 2014)
<i>Eremostachys laevigota</i>	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 (IC₅₀ 1.66-74.50 µg/mL), while ABTS^{•+} showed IC₅₀ 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).

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/H₂SO₄ 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 method for 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	<i>Ballota africana</i>	Hantam National Botanical Gardens
S2	<i>Leonotis leonurus</i>	Cape Flats Nature Reserve
S3	<i>L. nepetifolia</i>	Cape Flats Nature Reserve
S4	<i>Mentha longifolia</i>	Kirstenbosch Garden Centre
S5	<i>Plectranthus ambiguus</i>	Kirstenbosch Garden Centre
S6	<i>P. ciliates</i>	Cape Flats Nature Reserve
S7	<i>P. ecklonii</i>	Kirstenbosch Garden Centre
S8	<i>P. fruticosus</i>	Kirstenbosch Garden Centre
S9	<i>P. grandidentatus</i>	Kirstenbosch Garden Centre
S10	<i>P. hadiensis</i>	Kirstenbosch Garden Centre
S11	<i>P. madagascariensis</i>	Cape Flats Nature Reserve
S12	<i>P. saccatus</i>	Cape Flats Nature Reserve
S13	<i>P. strigosus</i>	Kirstenbosch Garden Centre
S14	<i>P. verticillatus</i>	Kirstenbosch Garden Centre
S15	<i>P. zuluensis</i>	Kirstenbosch Garden Centre
S16	<i>Salvia africana-caerulea</i>	Cape Flats Nature Reserve
S17	<i>S. chamelaeagnea</i>	Kirstenbosch Garden Centre
S18	<i>S. disermas</i>	Hantam National Botanical Gardens
S19	<i>S. dolomitica</i>	Kirstenbosch Garden Centre
S20	<i>S. lanceolate</i>	Kirstenbosch Garden Centre
S21	<i>S. muirii</i>	Kirstenbosch Garden Centre
S22	<i>S. namaensis</i>	Kirstenbosch Garden Centre
S23	<i>Stachys rugose</i>	Hantam National Botanical Gardens
S24	<i>Tetradenia riparia</i>	Kirstenbosch Garden Centre
S25	<i>Thorncroftia succulenta</i>	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 °C). The methanol extracts were filtered and evaporated to dryness under reduced pressure at 40 °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 PF₂₅₄ pre-coated aluminium plates (Merck, Cape Town, South Africa).

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.

CHAPTER 3: PHYTOCHEMICAL AND BIOLOGICAL SCREENING

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 μ L 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.

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 μ L of each sample working solution was combined with 30 μ L of tyrosinase (500 Units/mL in sodium phosphate buffer) in triplicate. After incubation at room temperature for 5 min, 110 μ L 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.

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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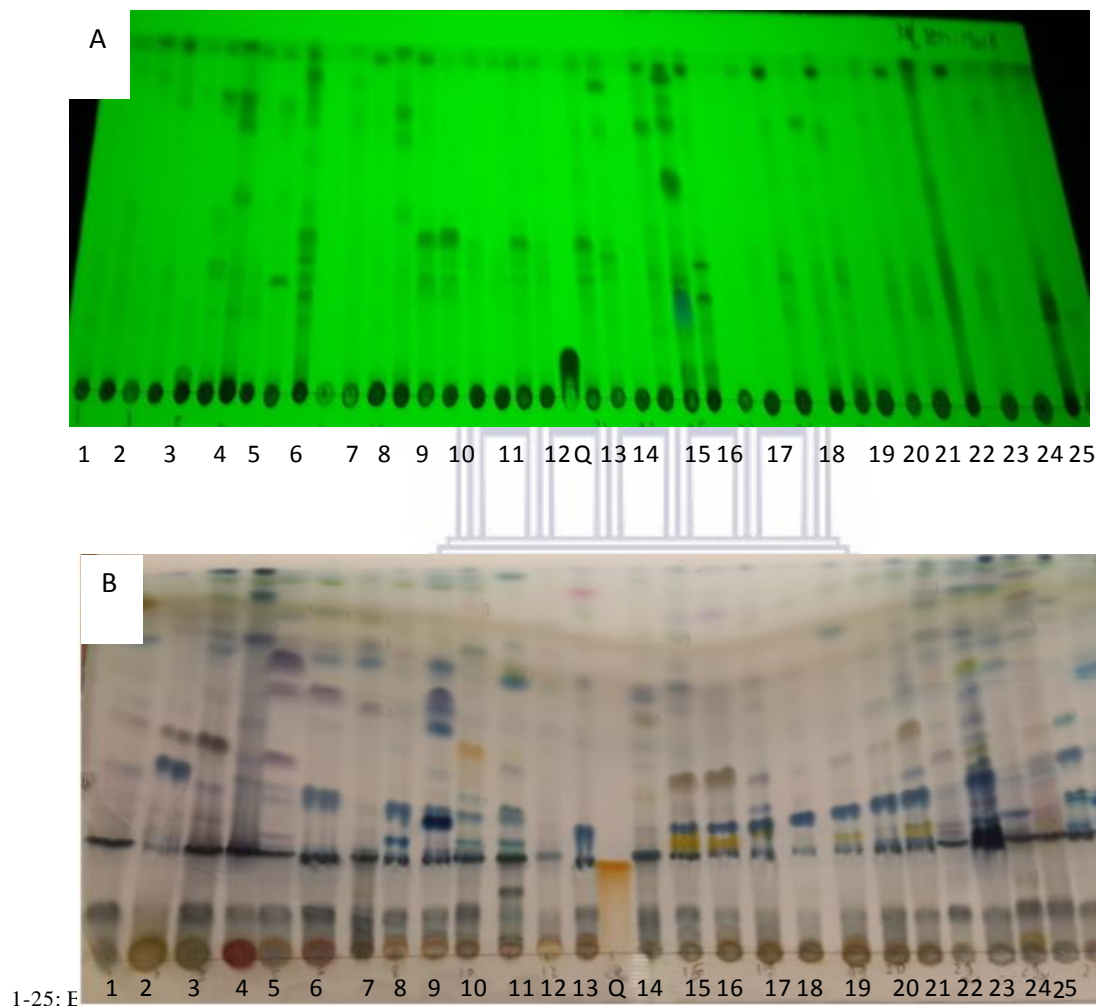
Table 3.2: Percentage yield of the plant extracts

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

CHAPTER 3: PHYTOCHEMICAL AND BIOLOGICAL SCREENING

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).



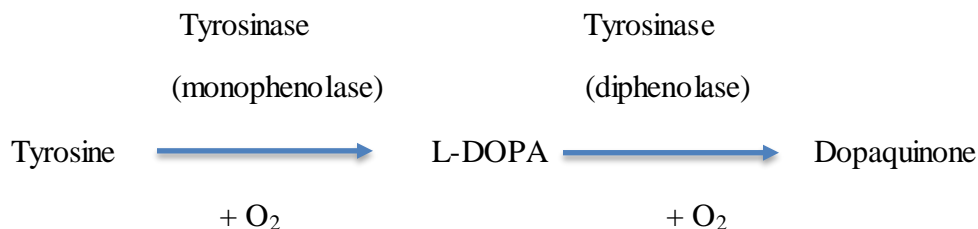
1-25: Extracts (S1-S25); Q: quercetin

Figure 3.1: TLC silica gel of the phytochemical screening of the plant species, solvent system DCM:MeOH (9.5:0.5); under UV (254nm; A), and spraying with H₂SO₄/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 dihydroxyphenylamine (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.

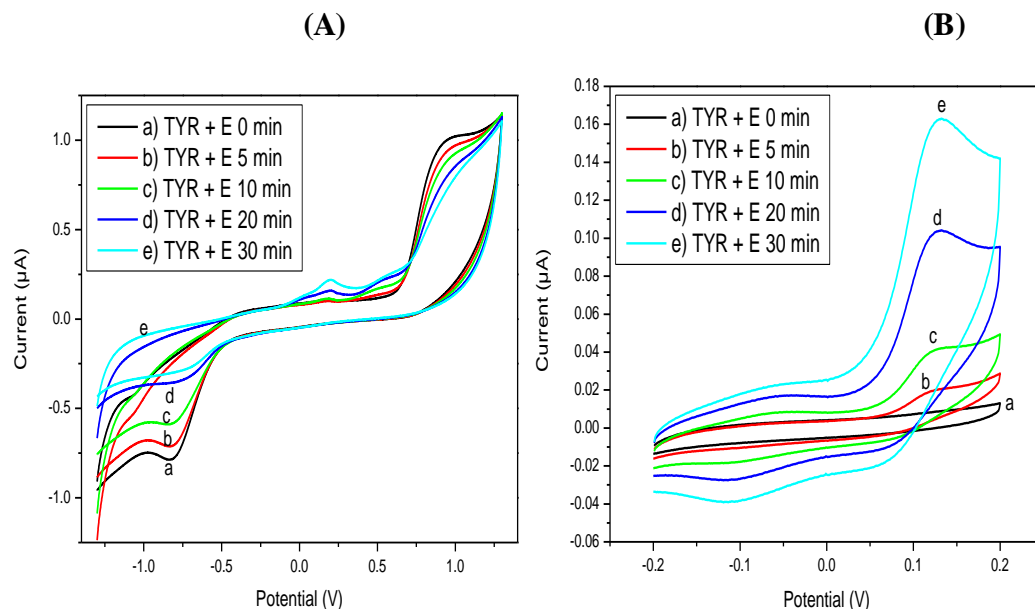


Figure 3.2: CVs recorded at 50 mV/ s in wide scan range (A), and at 25 mV/ s at the narrow scan range (B) for the reaction mixture of tyrosine and tyrosinase in 50 mM PBS (pH 6.5) at different times. Curve (a) 0 min, curve (b) 5 min, curve (c) 10 min, curve (d) 20 min, and then curve (e) 30 min.

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 $8.99 \times 10^{-3} \mu\text{A}$ to $96 \times 10^{-3} \mu\text{A}$ at 131 mV, indicating that the oxidation of tyrosine catalyzed by tyrosinase has 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.

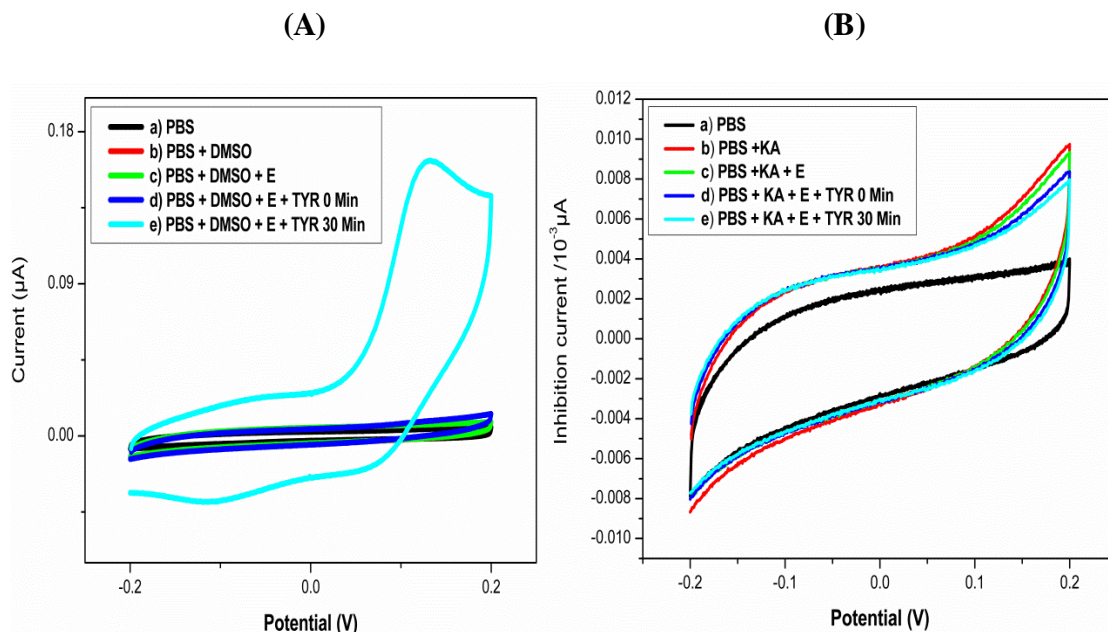


Figure 3.3: CVs recorded at 25 mV/ s for the reaction mixtures during negative control (A) and positive control (B) experiments, first for 50 mM PBS (pH 6.5) (curve a) and then after consecutive addition of DMSO (left, curve b) or kojic acid (right, curve b), tyrosinase (curve c), and tyrosine (0 min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

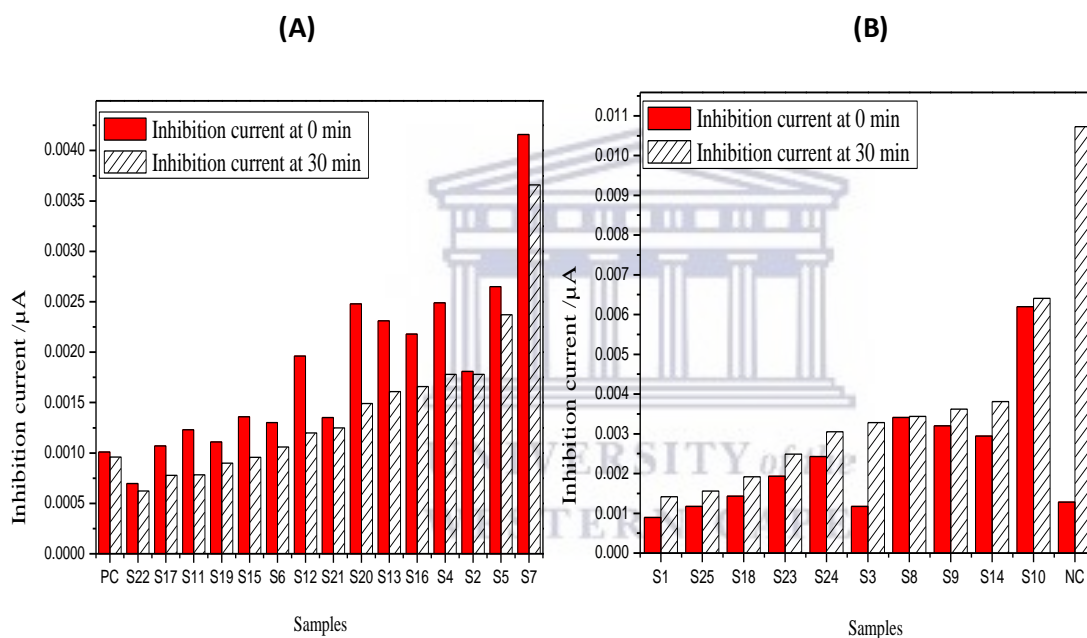
Based on the CVs, the inhibitory behavior of each extracts toward the enzyme were classified as either positive or negative depending on the similarity to CVs recorded by replacing the extract with kojic acid as positive control or DMSO as negative control. An inhibitory behavior was “positive” when 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

Group 2: 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).

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.

$$[(Q_{30'} - Q_0') / Q_0'] \times 100 \quad (2)$$

Where $Q_{30'}$ is the inhibition based on charge at 30 min, Q_0' 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 rxn/10 ⁻⁴	Area at 0 min/10 ⁻⁴	Area at 30 min/10 ⁻⁴	Qbg/ mC	Q0min/ mC	Q30min/ mC	DQ/ mC	%DQref
<i>Ballota africana</i>	7.39	8.97	14.2	29.56	35.88	56.8	20.92	95.7
<i>Leonotus leonurus</i>	16.9	18.1	17.8	67.6	72.4	71.2	-1.2	100.2
<i>L. nepetifolia</i>	8.24	11.7	32.8	32.96	46.8	131.2	84.4	82.8
<i>Mentha longifolia</i>	12.9	24.9	17.8	51.6	99.6	71.2	-28.4	105.8
<i>Plectranthus ambiguus</i>	17.2	26.5	23.7	68.8	106	94.8	-11.2	102.3
<i>P. ciliates</i>	6.36	13	10.6	25.44	52	42.4	-9.6	102.0
<i>P. ecklonii</i>	16.2	41.61	36.6	64.8	166.44	146.4	-20.04	104.1
<i>P. fruticosus</i>	25.8	34.1	34.4	103.2	136.4	137.6	1.2	99.8
<i>P. grandidentatus</i>	12.2	32	36.2	48.8	128	144.8	16.8	96.6
<i>P. hadiensis</i>	31.6	62	64.1	126.4	248	256.4	8.4	98.3
<i>P. madagascariensis</i>	2.91	12.3	7.83	11.64	49.2	31.32	-17.88	103.6
<i>P. saccatus</i>	9.76	19.6	12	39.04	78.4	48	-30.4	106.2
<i>P. strigosus</i>	14.3	23.1	16.1	57.2	92.4	64.4	-28	105.7
<i>P. verticillatus</i>	16	29.4	38.1	64	117.6	152.4	34.8	92.9
<i>P. zuluensis</i>	4.87	13.6	9.56	19.48	54.4	38.24	-16.16	103.3
<i>S. africana-caerulea</i>	21.4	21.8	16.6	85.6	87.2	66.4	-20.8	104.2
<i>S. chamelaeagnea</i>	6.06	10.7	7.78	24.24	42.8	31.12	-11.68	102.4
<i>S. disermas</i>	8.88	14.3	19.2	35.52	57.2	76.8	19.6	96.0

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<i>S. dolomitica</i>	8.91	11.1	9.01	35.64	44.4	36.04	-8.36	101.7
<i>S.lanceolata</i>	9.81	24.8	14.9	39.24	99.2	59.6	-39.6	108.1
<i>S. muiirii</i>	10.4	13.5	12.5	41.6	54	50	-4	100.8
<i>S. namaensis</i>	5.24	6.96	6.24	20.96	27.84	24.96	-2.88	100.6
<i>Stachys rugosa</i>	16.1	19.3	24.9	64.4	77.2	99.6	22.4	95.4
<i>Tetradenia riparia</i>	21.5	24.3	30.5	86	97.2	122	24.8	94.9
<i>Thorncroftia succulenta</i>	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*.

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

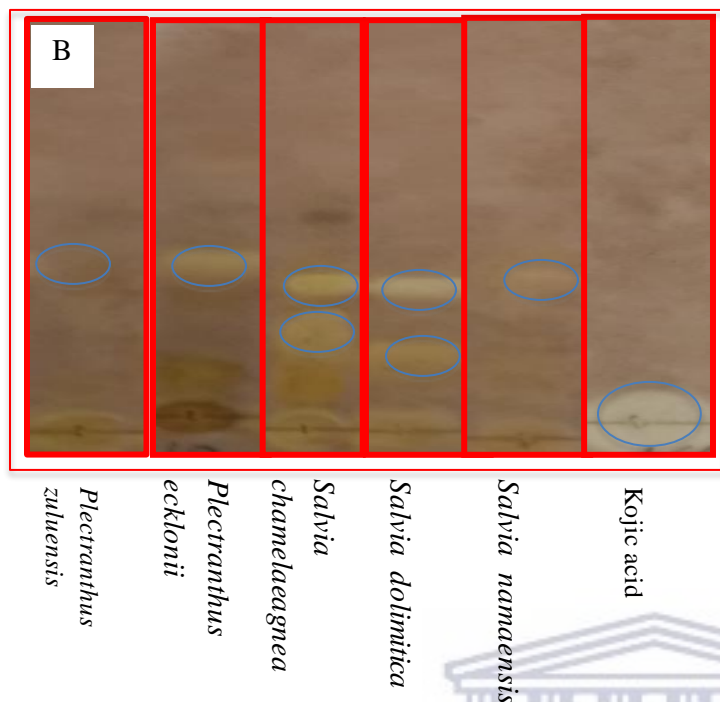


Figure 3.5: TLC silica gel bioautography of all the extracts and kojic acid (A), the active extracts and kojic acid (B), solvent system Hex:EtOAc (9:1) and DCM:MeOH (97:3) respectively.

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 $< 30 \mu\text{g/mL}$, six plant extracts (24 %) inhibited tyrosinase moderately with IC_{50} values comprising between 30 and $100 \mu\text{g/mL}$ while fifteen five plant species (60 %) inhibited tyrosinase poorly with IC_{50} values of $>100 \mu\text{g/mL}$ compared to the positive control (kojic acid $IC_{50} = 3.42 \mu\text{g/mL}$).

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Table 3.4: Screening of plant extracts for tyrosinase inhibitory activities

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

*IC₅₀ 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. dolomitica*, 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. chamelaeagnea*, *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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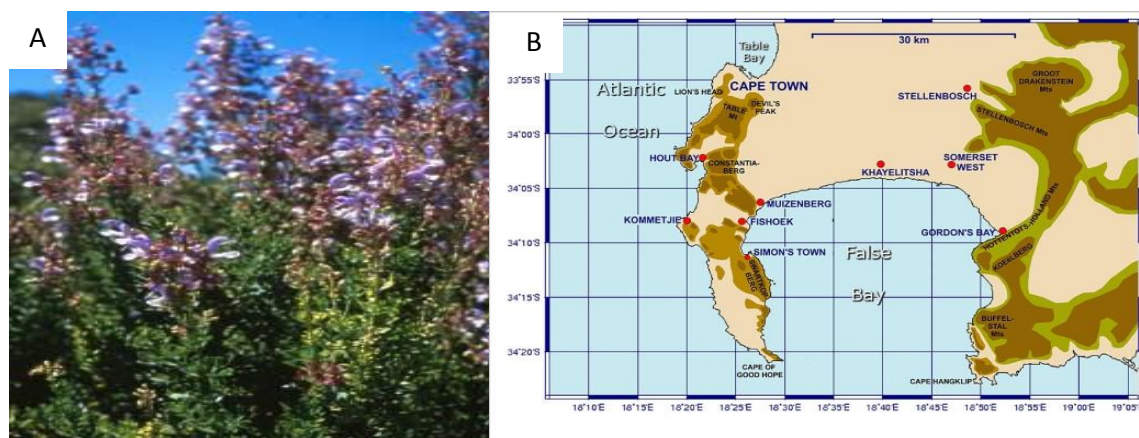


Figure 4.1: *Salvia chamelaeagnea* description (A), and distribution along South Western area of the Cape of Good Hope. www.plantzafrica.com

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

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 F₂₅₄ (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 vanillin/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

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 μ m). The flow rate was set at 1.5 mL/min. All experiments were done at room temperature (20 °C).

4.3.3. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded at 20°C, using deuterated chloroform on a Bruker Avance 400 MHz NMR spectrometer (Germany), Chemical shifts of ^1H (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 °C) for 24 hours. The methanol extract was filtered and evaporated to dryness under reduced pressure at 40 °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

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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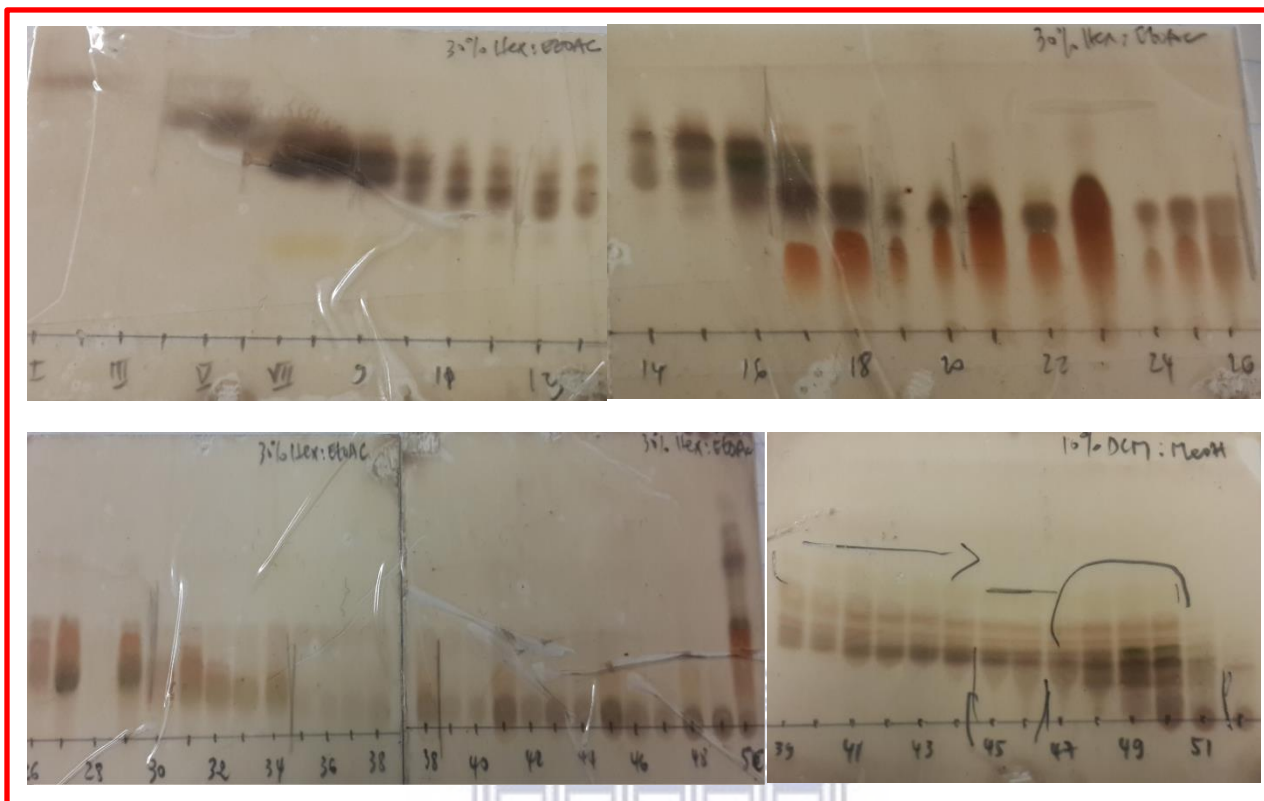


Figure 4.2: TLC silica gel of the collected fractions of SC, solvent system Hex:EtOAc (7:3) and DCM:MeOH (9:1).

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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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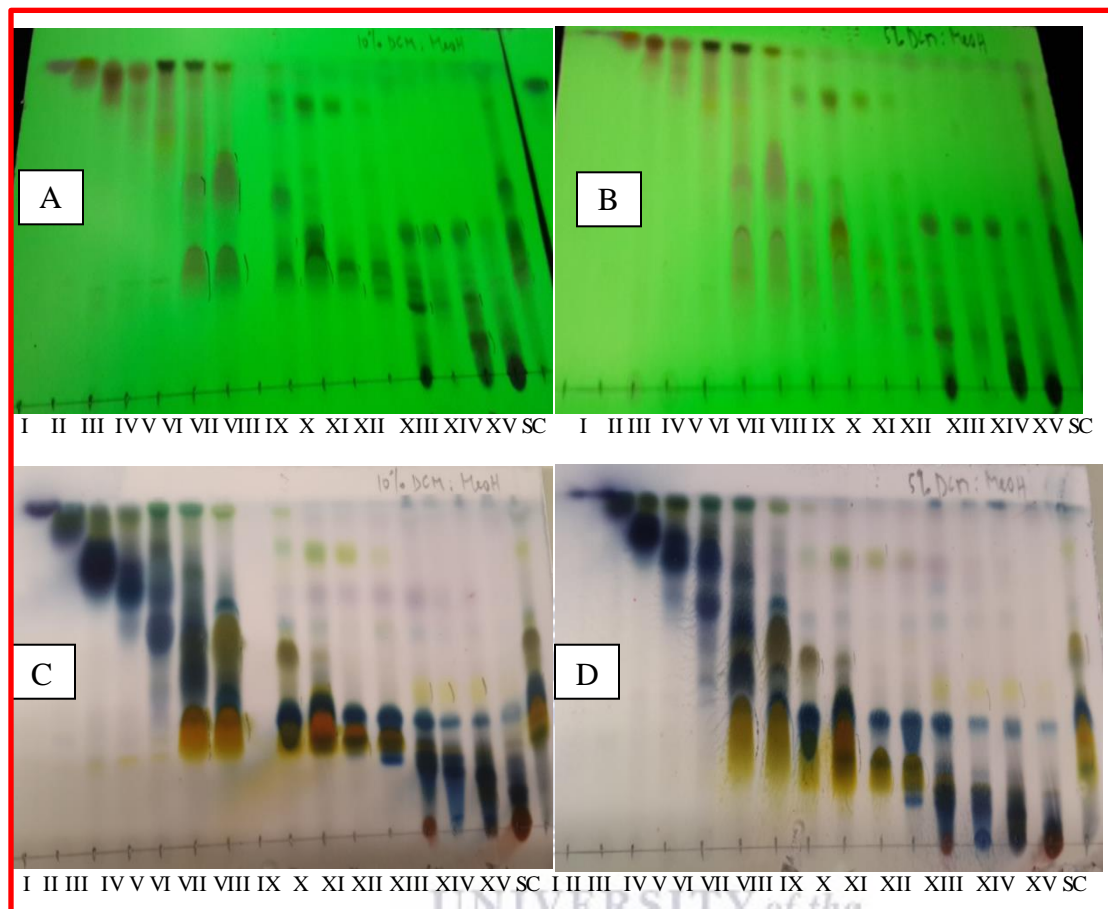


Figure 4.3: TLC silica gel profile of combined fractions under UV (254 nm; A & B), and after spraying with H₂SO₄/vanillin and then heated (C & D).

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

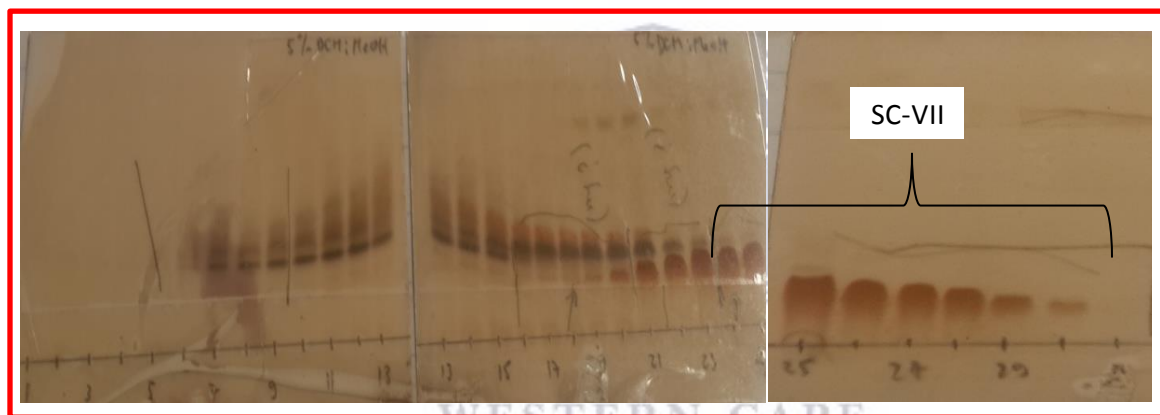


Figure 4.4: TLC silica gel of combined fractions of SC-VII, solvent system DCM:MeOH (9.5:0.5).

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

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 %).

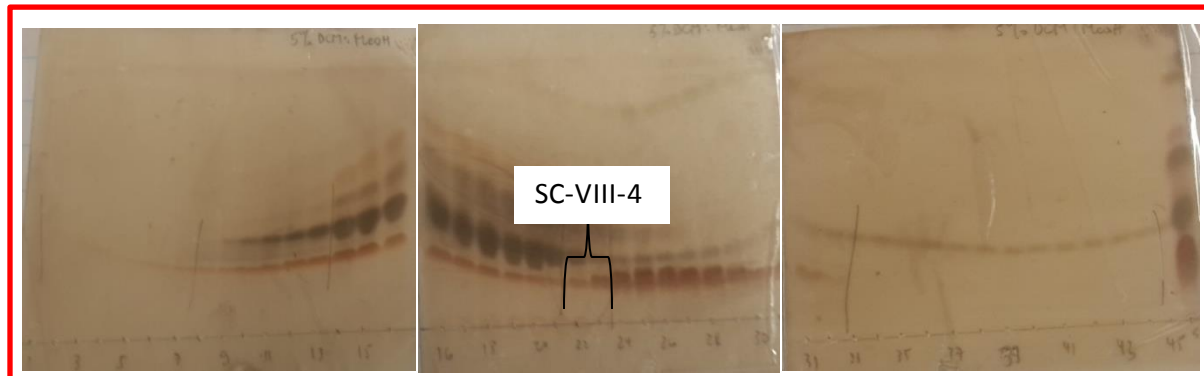
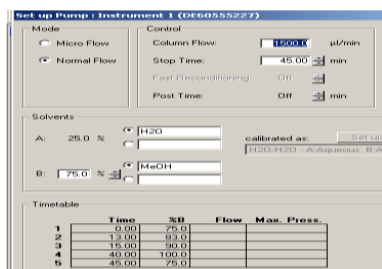


Figure 4.6: TLC silica gel of combined fractions of SC-VIII, solvent system DCM:MeOH (9.5:05).

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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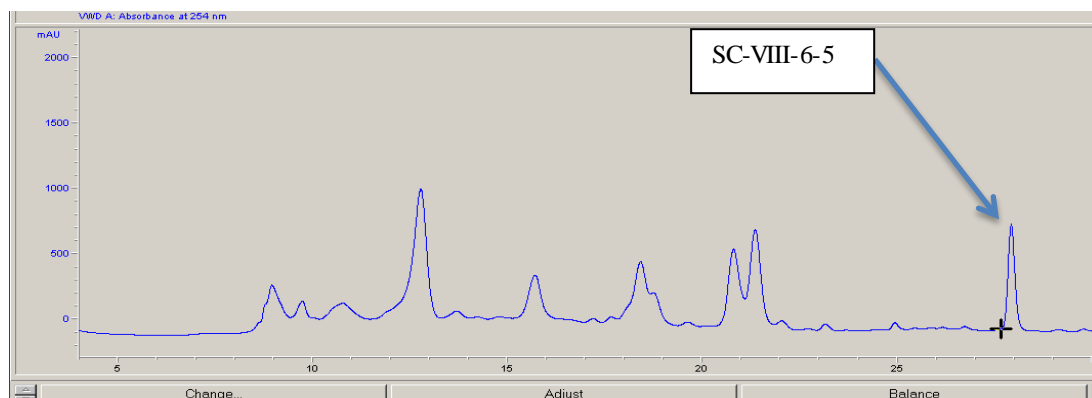


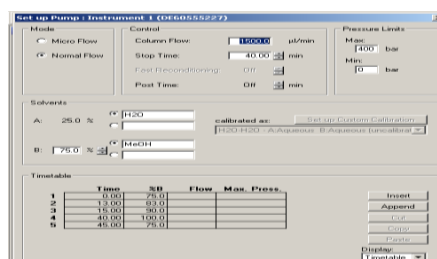
Figure 4.7: HPLC chromatogram of C3.

*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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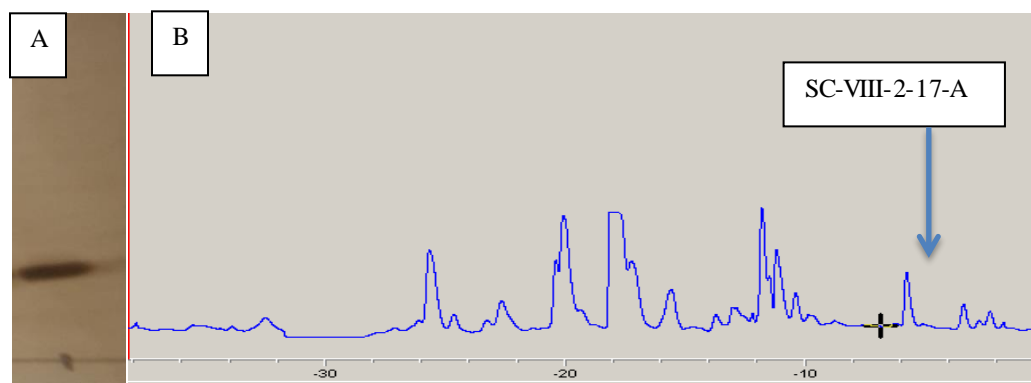


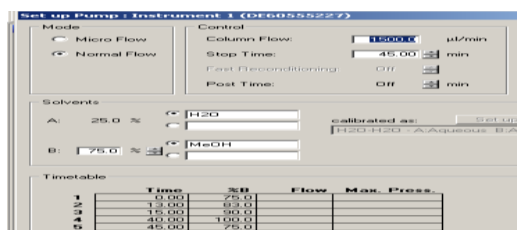
Figure 4.8: TLC silica gel of SC-VIII-2-17-A (Fig. 4.8 A), solvent system DCM:MeOH (9.5:0.5) and HPLC chromatogram of **C4** (Fig. 4.8B).

*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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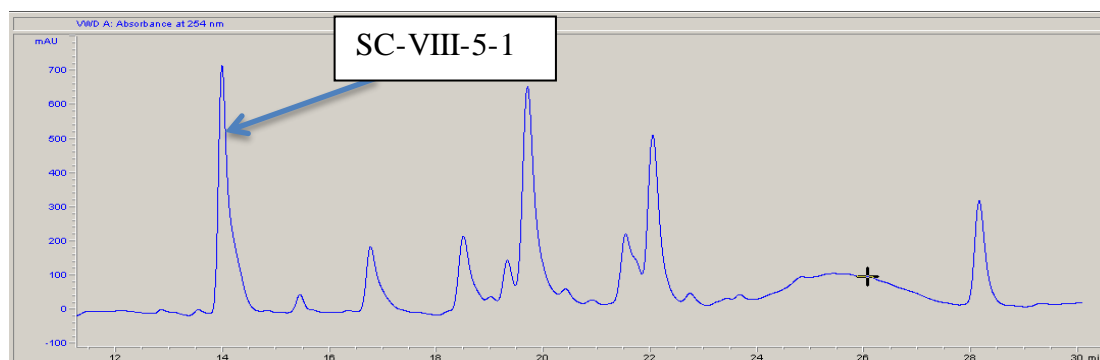


Figure 4.9: HPLC chromatogram of C5.

*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

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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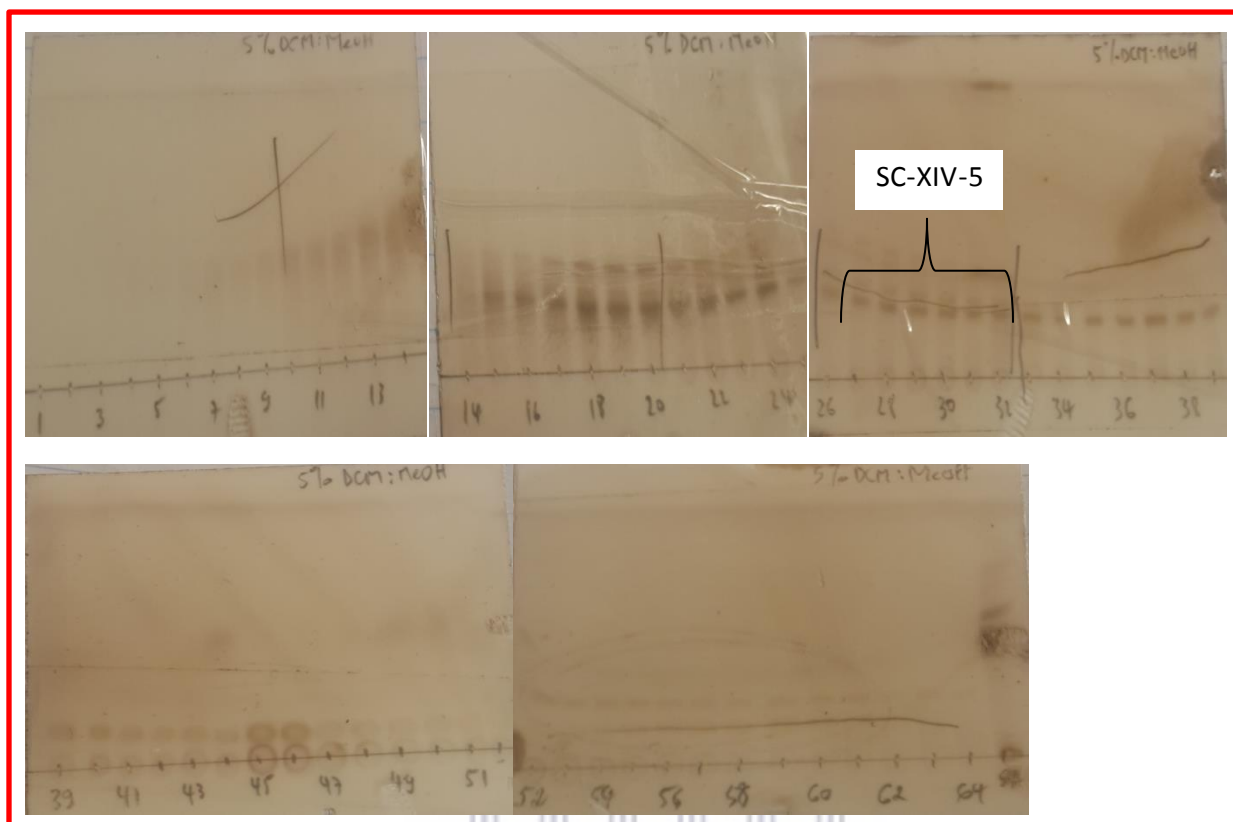


Figure 4.10: TLC silica gel of combined fractions of SC- XIV, solvent system DCM:MeOH (9:1).

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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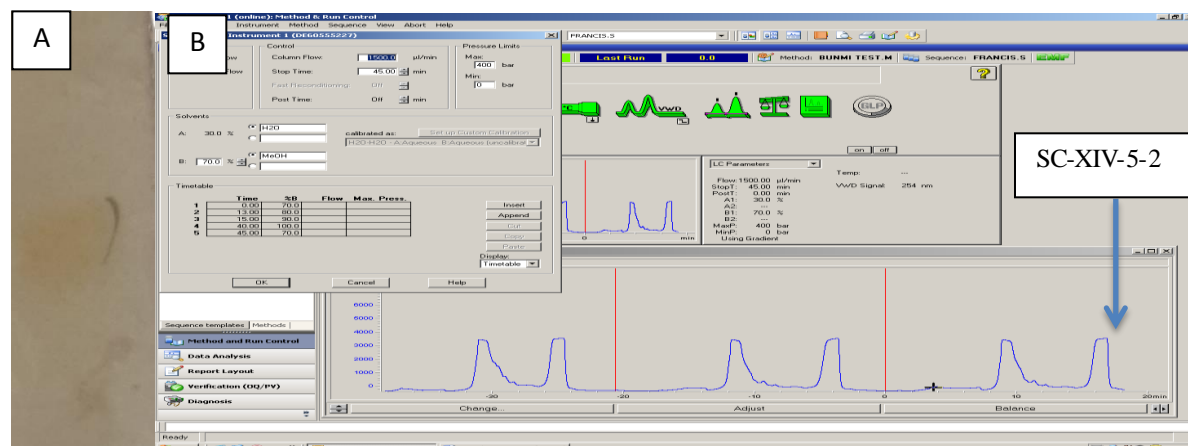
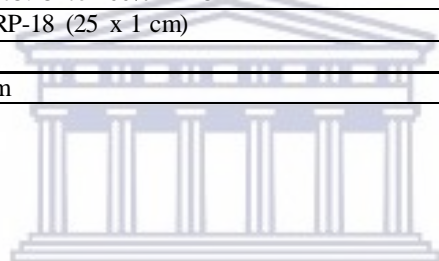


Figure 4.11: TLC silica gel of SC-XIV-5-2 (Fig. 4.11 A, solvent system DCM:MeOH (9.5:05) and HPLC chromatogram of C6 (Fig. 11.B).

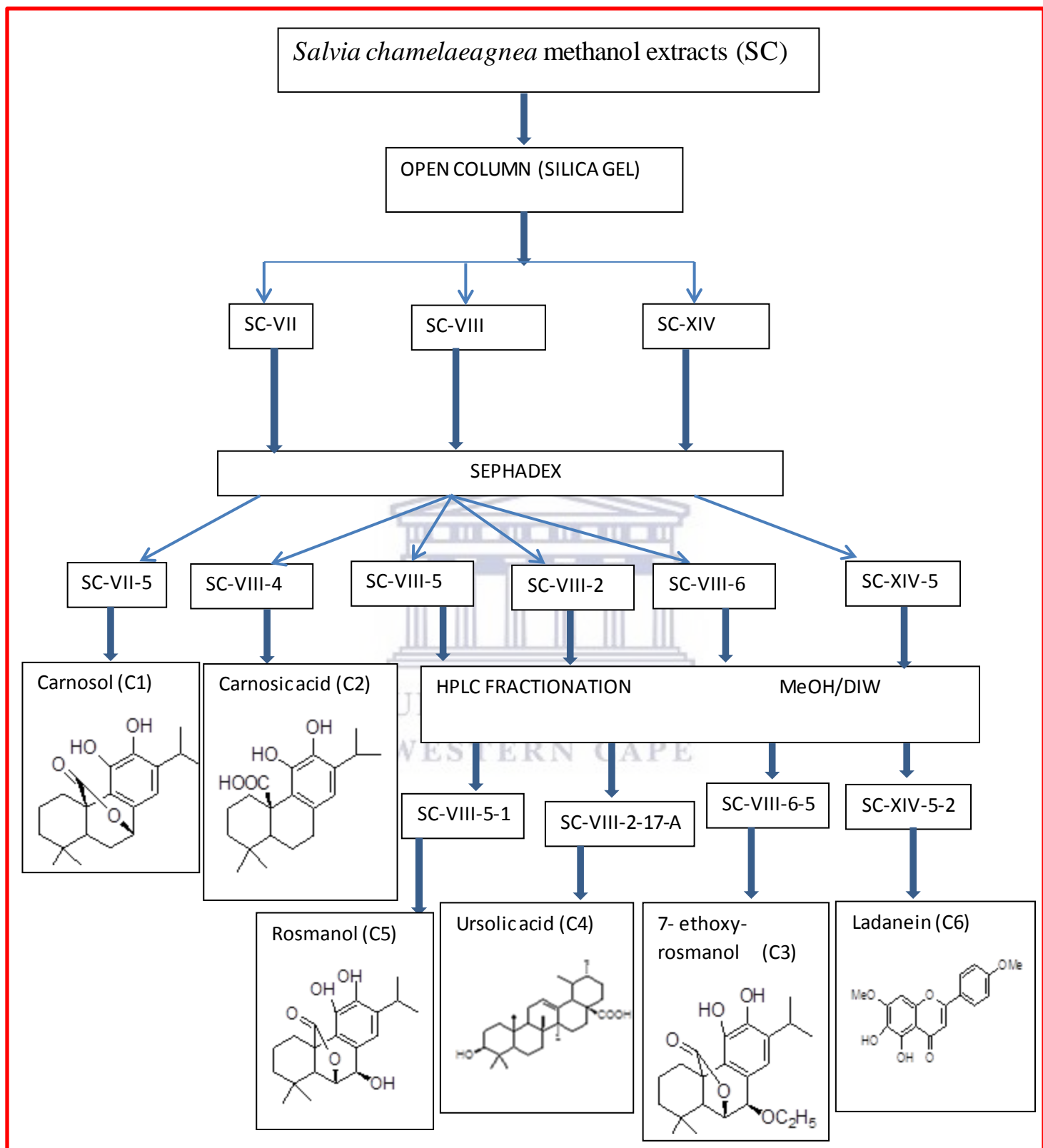
*Conditions

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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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 Fe²⁺-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-methylpropionamide) 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.

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.

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

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).

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 μ L) and (300 μ L) respectively, to which a specific volume of the L-tyrosine (1100 μ L) 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 μ L of the stock solution (1 mg/mL w/v) of the isolated compounds (C1 – C6) and a methanol extract (SC) were mixed with 300 μ L 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 FeCl₃.6H₂O (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 μ M. 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 μ M ascorbic acid equivalents per milligram dry weight (μ M 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 peroxy 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 peroxy 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.

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 $\text{K}_2\text{S}_2\text{O}_8$ was prepared and kept at -2°C . The working solution was then prepared by adding 88 μL $\text{K}_2\text{S}_2\text{O}_8$ 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 °C in the plate reader. The results were expressed as μM trolox equivalents per milligram dry weight ($\mu\text{M}/\text{TE}/\text{g}$) 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 to 100, 50 and 10 $\mu\text{g}/\text{mL}$ concentrations. 100 μL of each sample (working solutions) were dissolved in potassium phosphate buffer and pre-incubated with 500 μL microsomes at 37 °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 °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 °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

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

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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. $^1\text{H-NMR}$ 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 $^{13}\text{C-NMR}$ 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 carnososa* 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*.

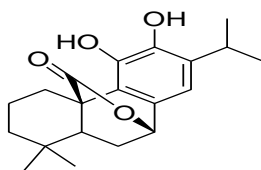


Figure 4.12: Chemical structure of carnosol

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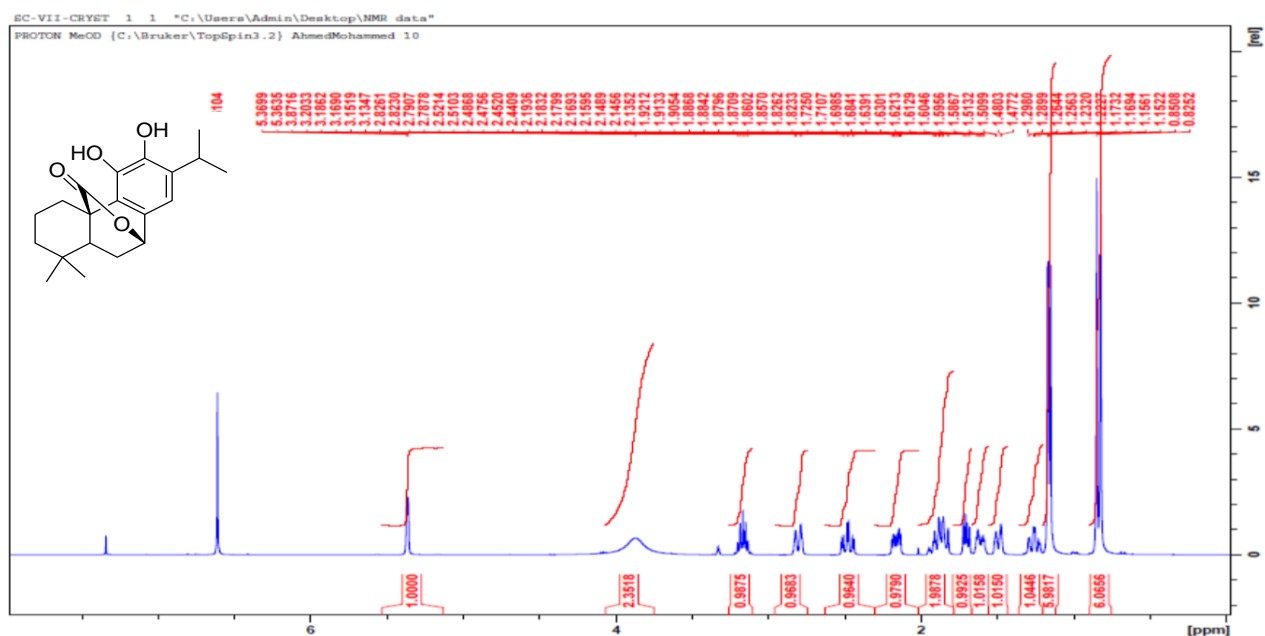


Figure 4.13: ¹H NMR spectrum of compound 1

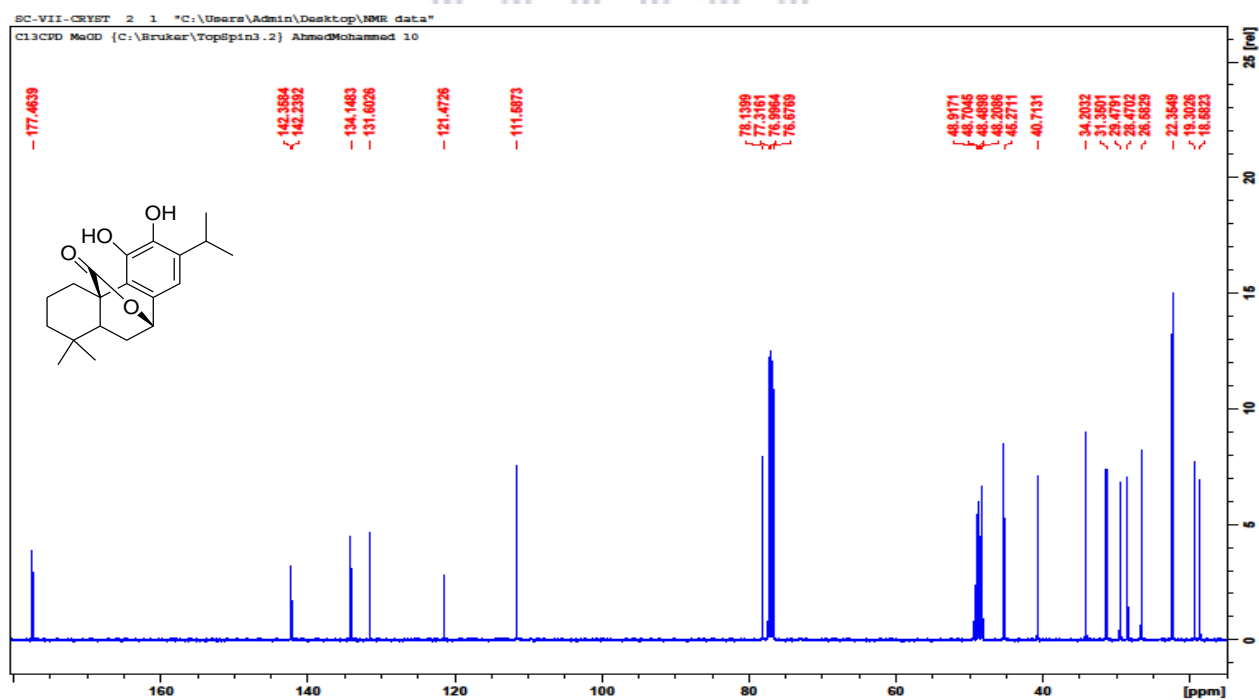


Figure 4.14: ¹³C NMR spectrum of compound 1

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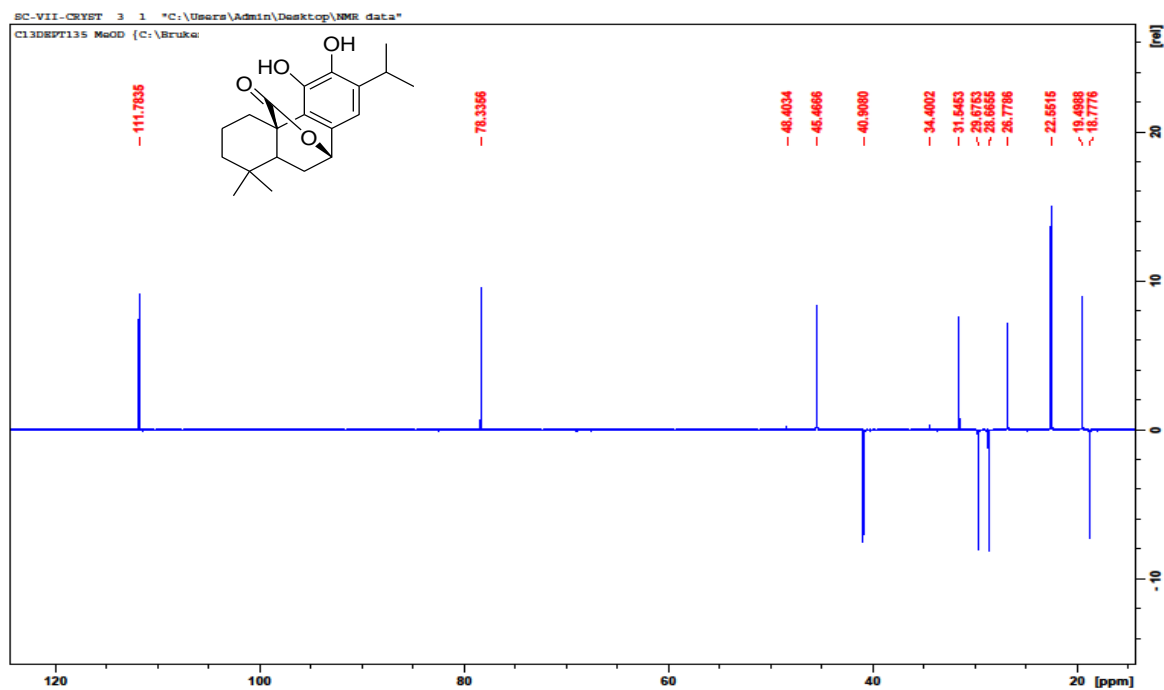


Figure 4.15: DEPT NMR spectrum of compound 1

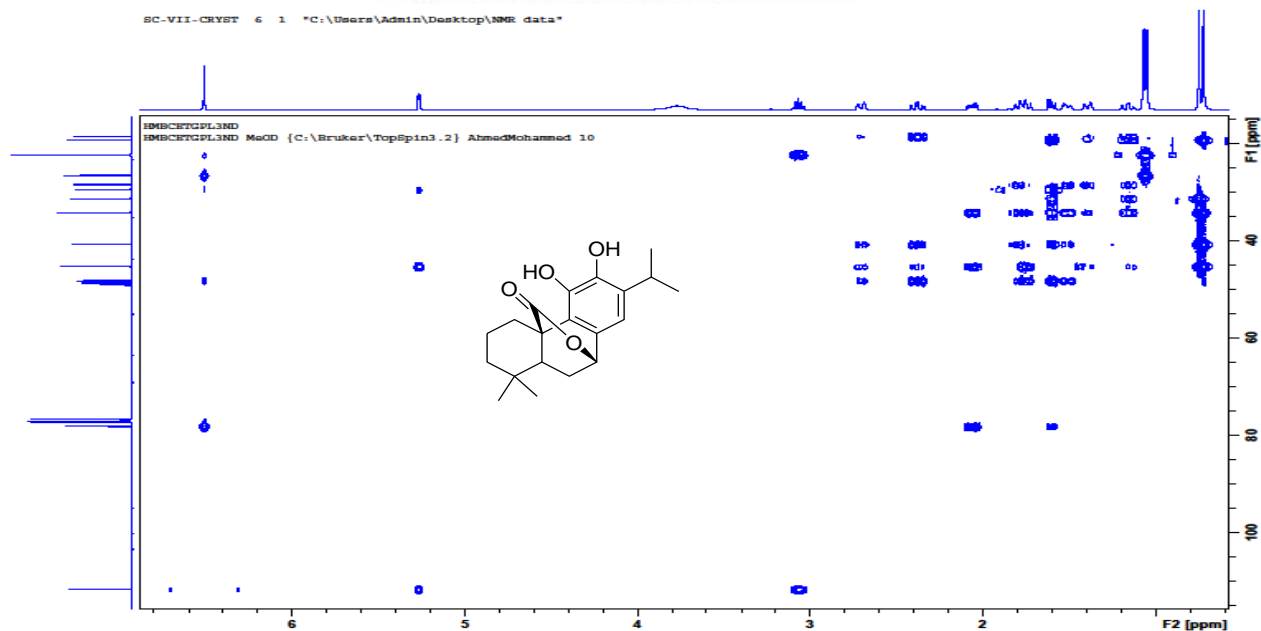


Figure 4.16: HMBC NMR spectrum of compound 1

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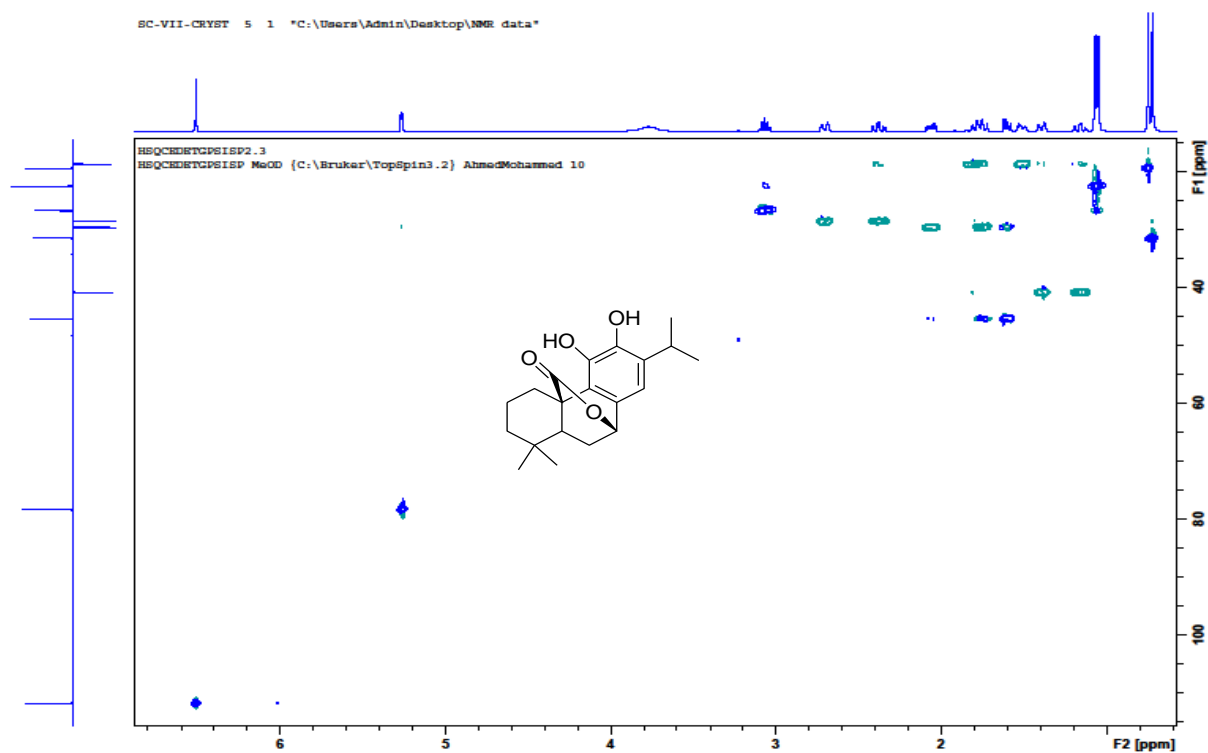


Figure 4.17: HMQC NMR spectrum of compound 1

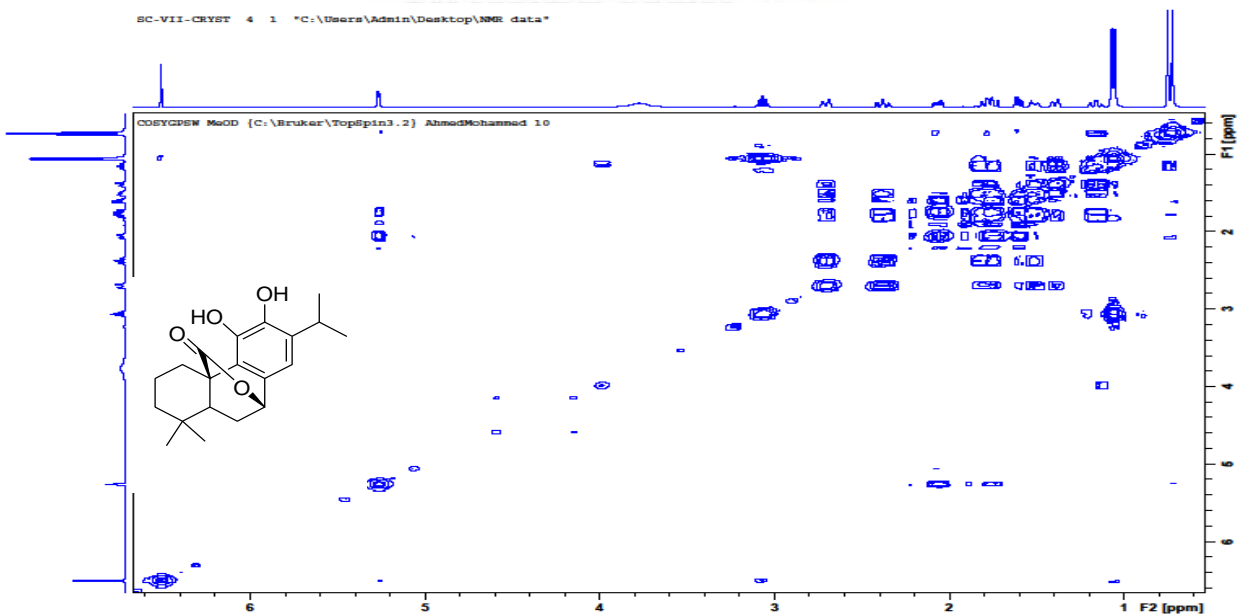


Figure 4.18: COSY NMR spectrum of compound 1

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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.

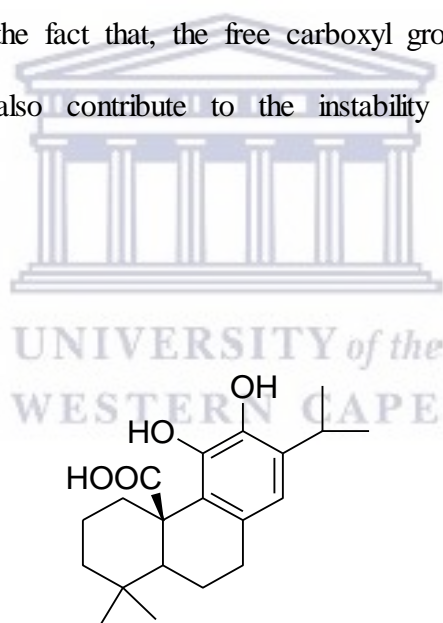


Figure 4.19: Chemical structure of carnosic acid

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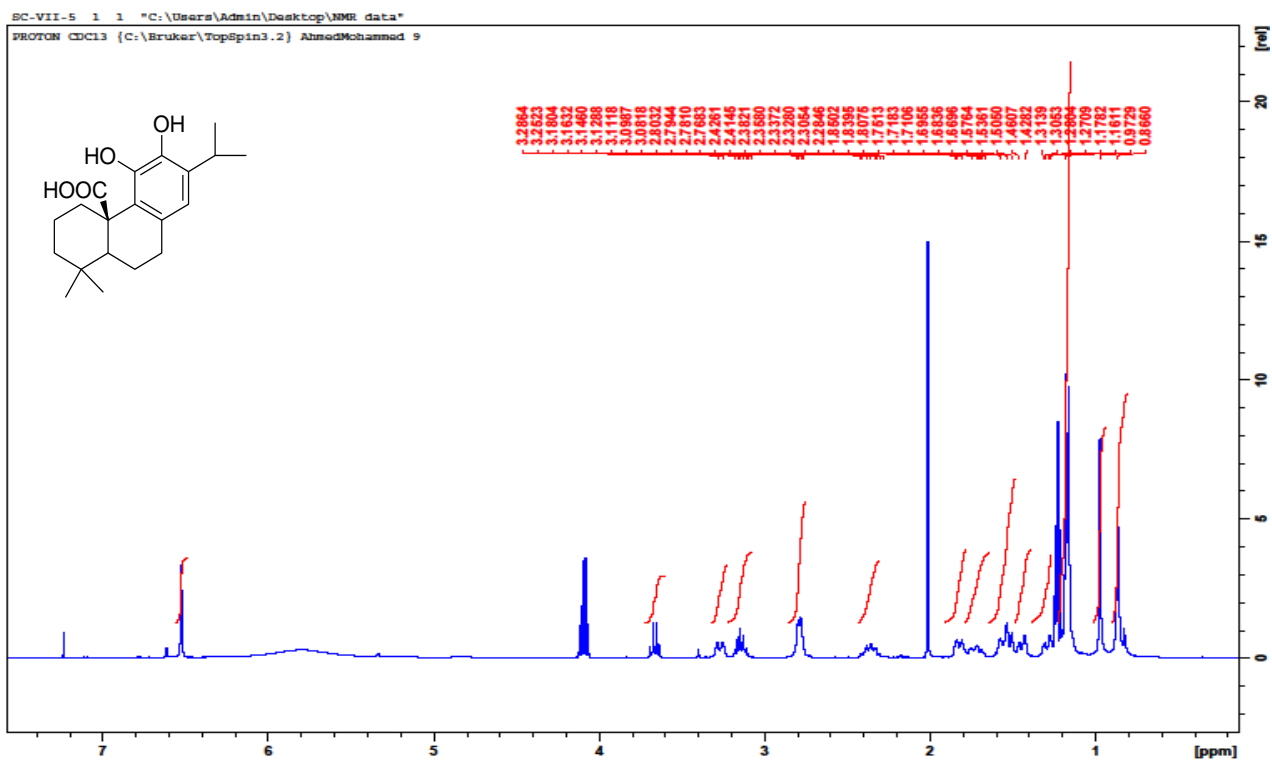


Figure 4.20: ¹H NMR spectrum of compound 2

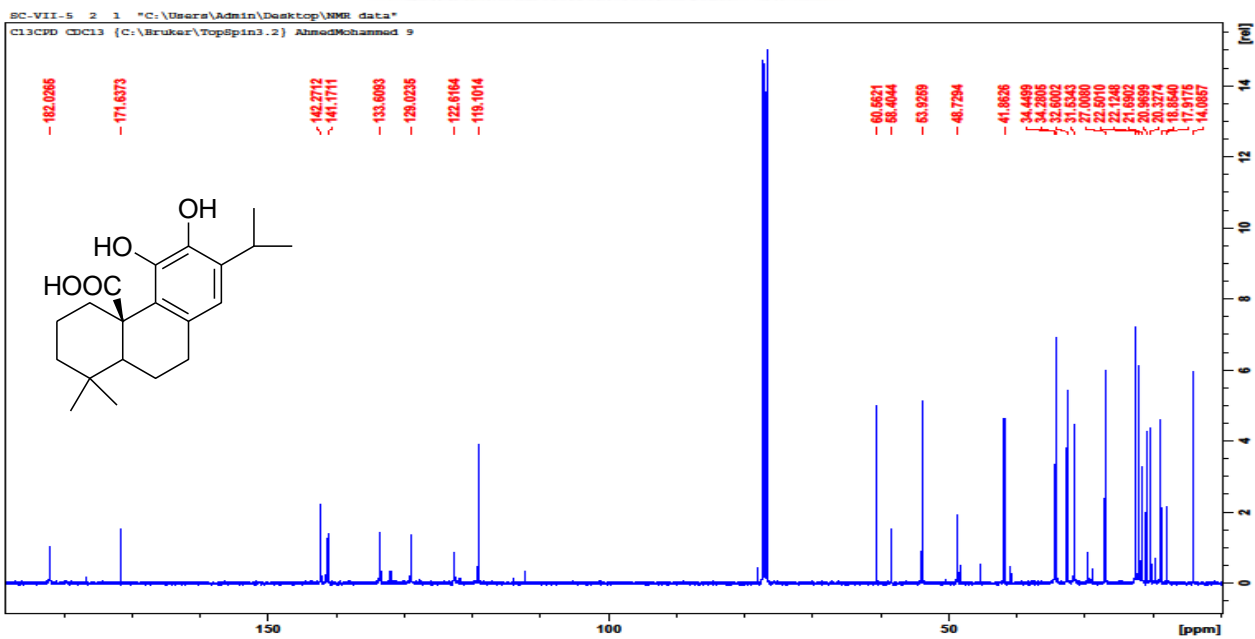


Figure 4.21: ¹³C NMR spectrum of compound 2

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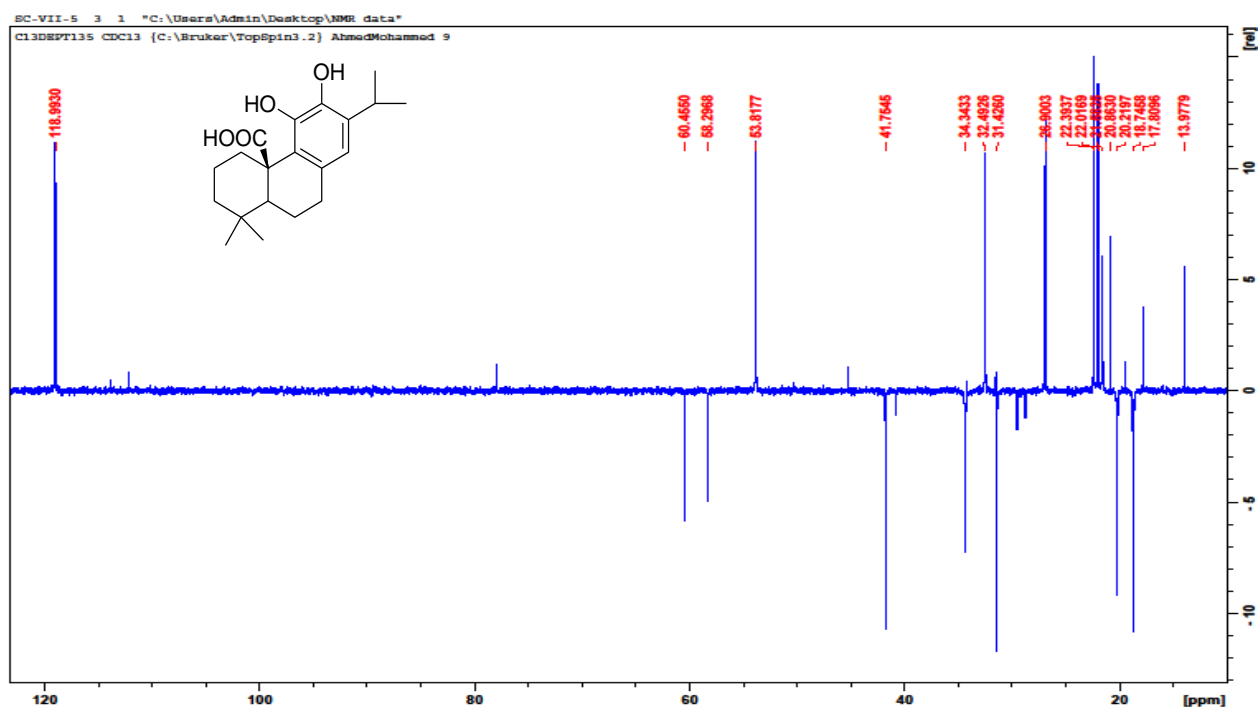


Figure 4.22: DEPT NMR spectrum of compound 2

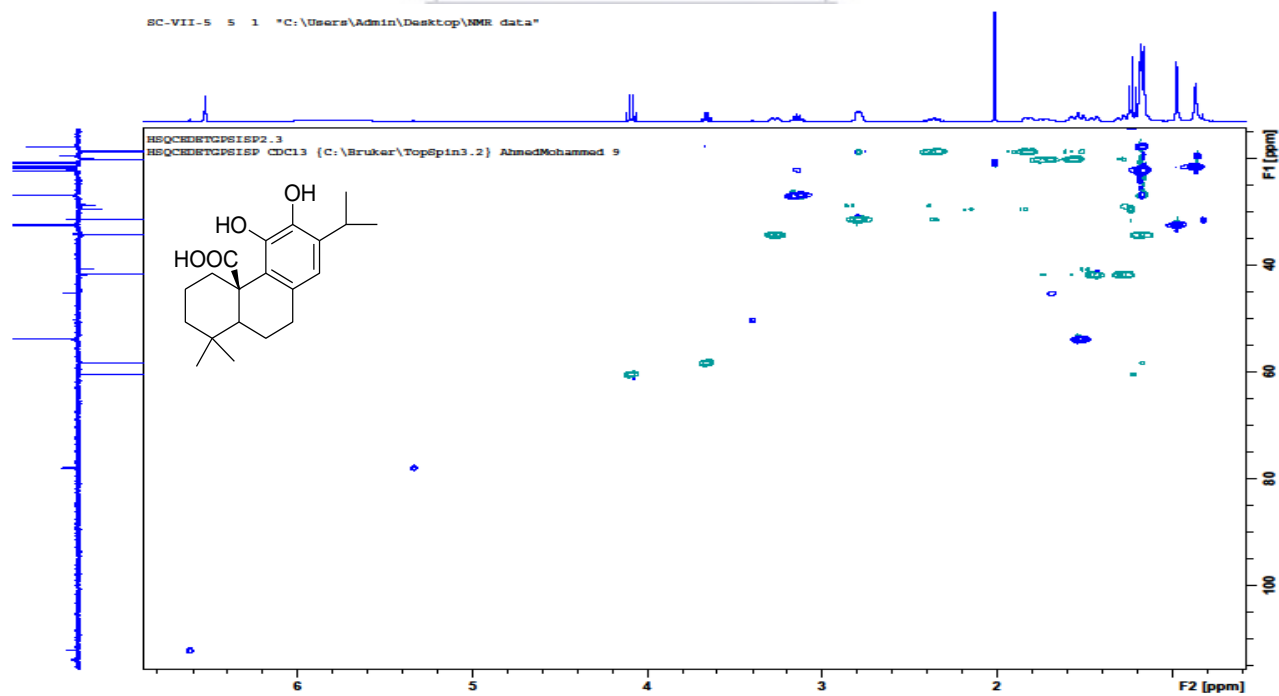


Figure 4.23: HMQC spectrum of compound 2

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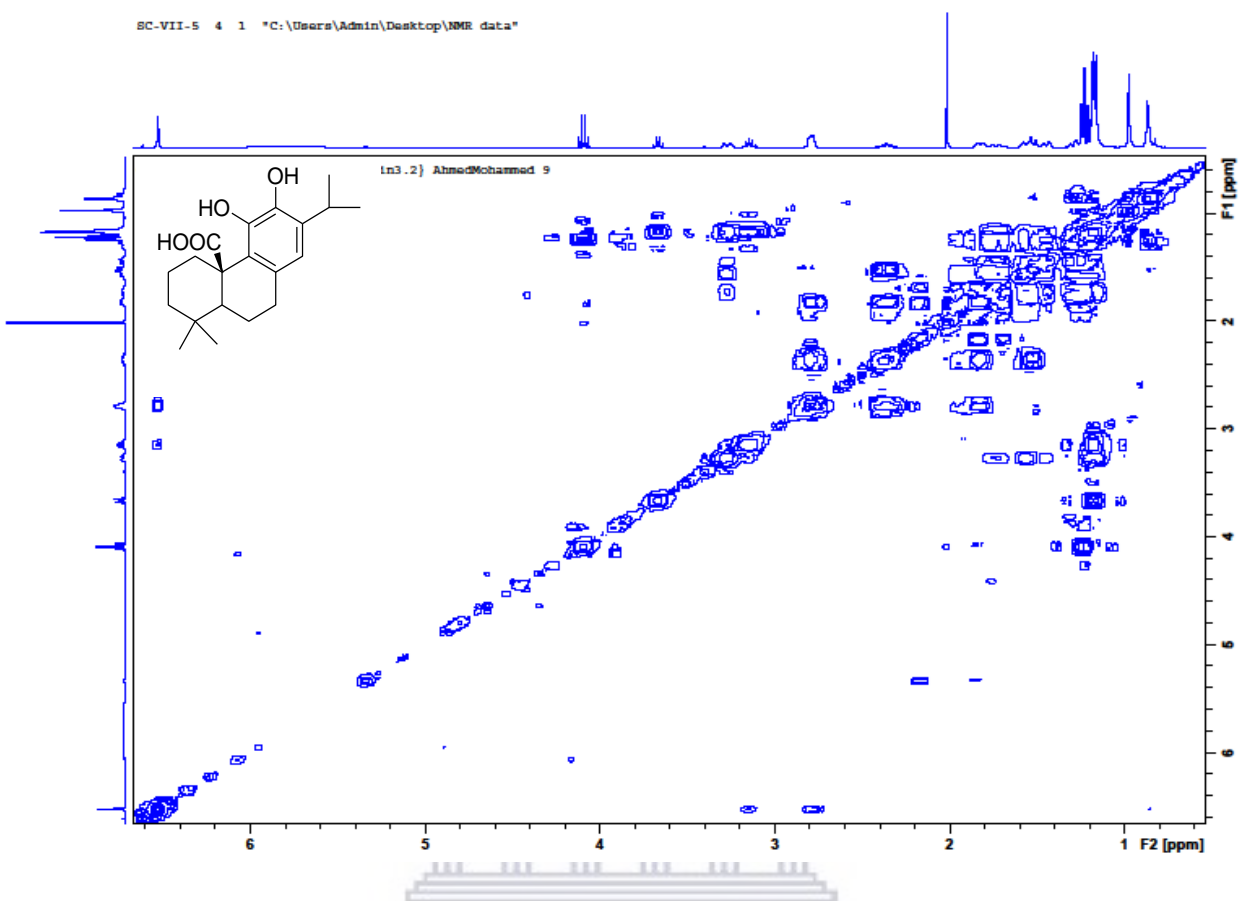


Figure 4.24: COSY spectrum of compound 2

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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**. ^1H 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-1 α) 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° 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.

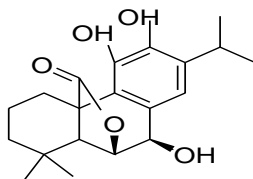


Figure 4.25: Chemical structure of rosmanol

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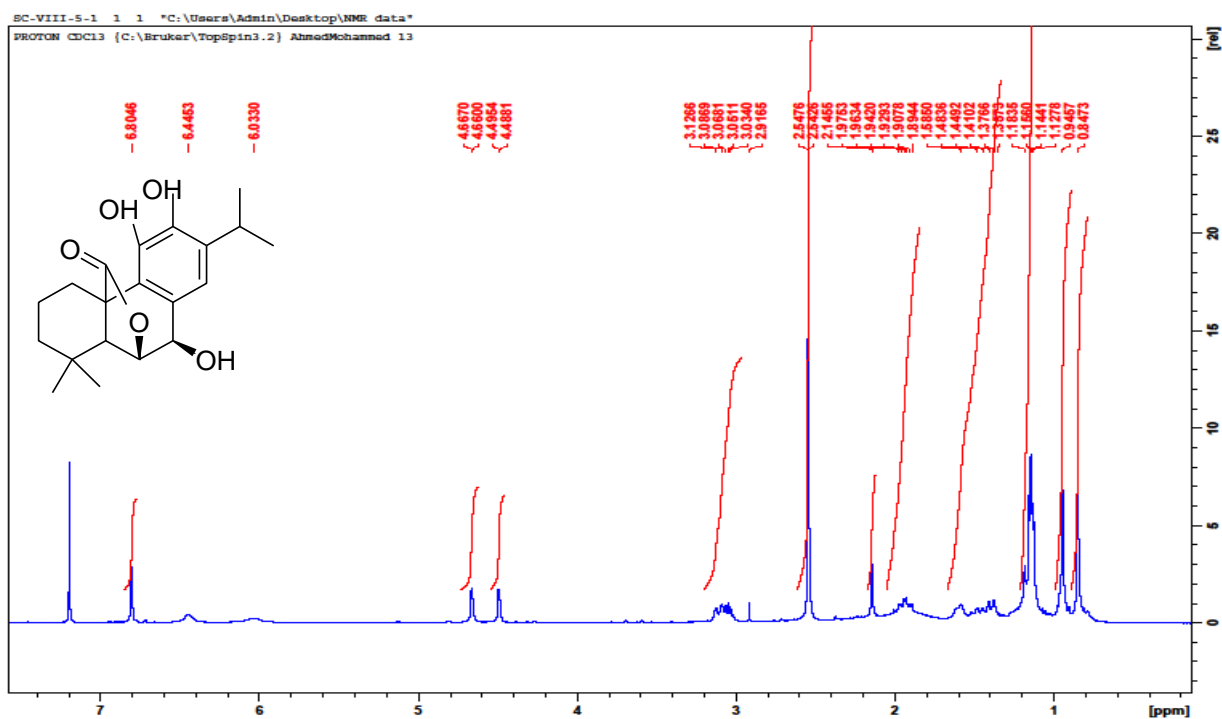


Figure 4.26: ¹H NMR spectrum of compound 5

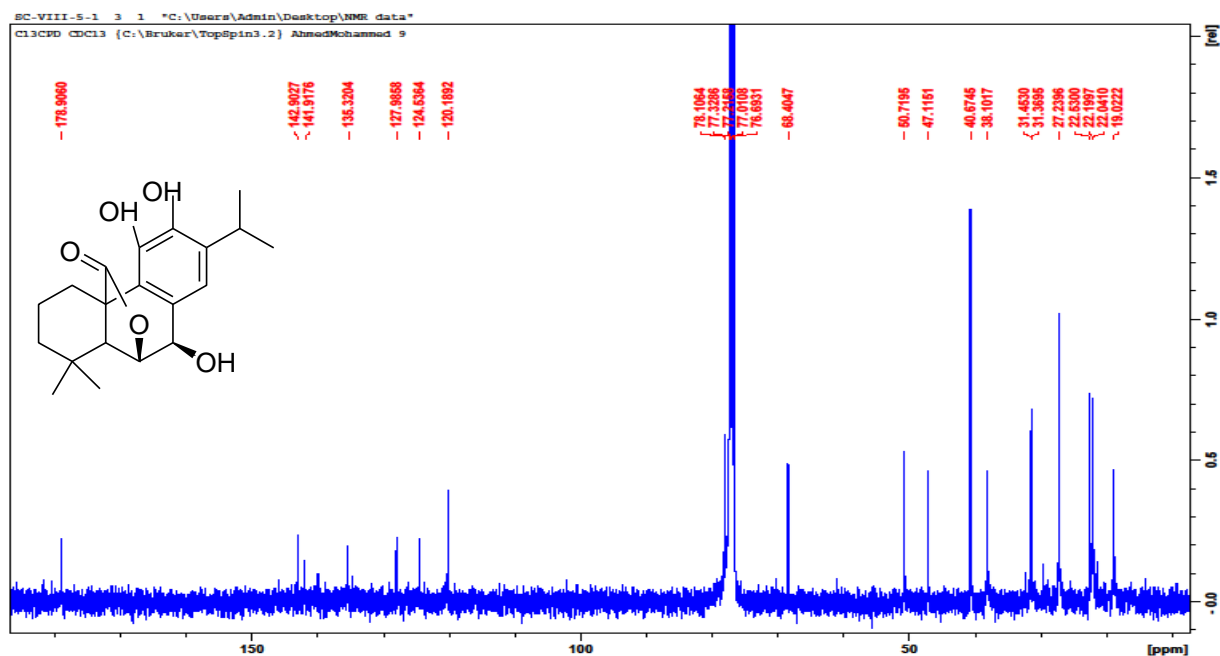


Figure 4.27: ¹³C NMR spectrum of compound 5

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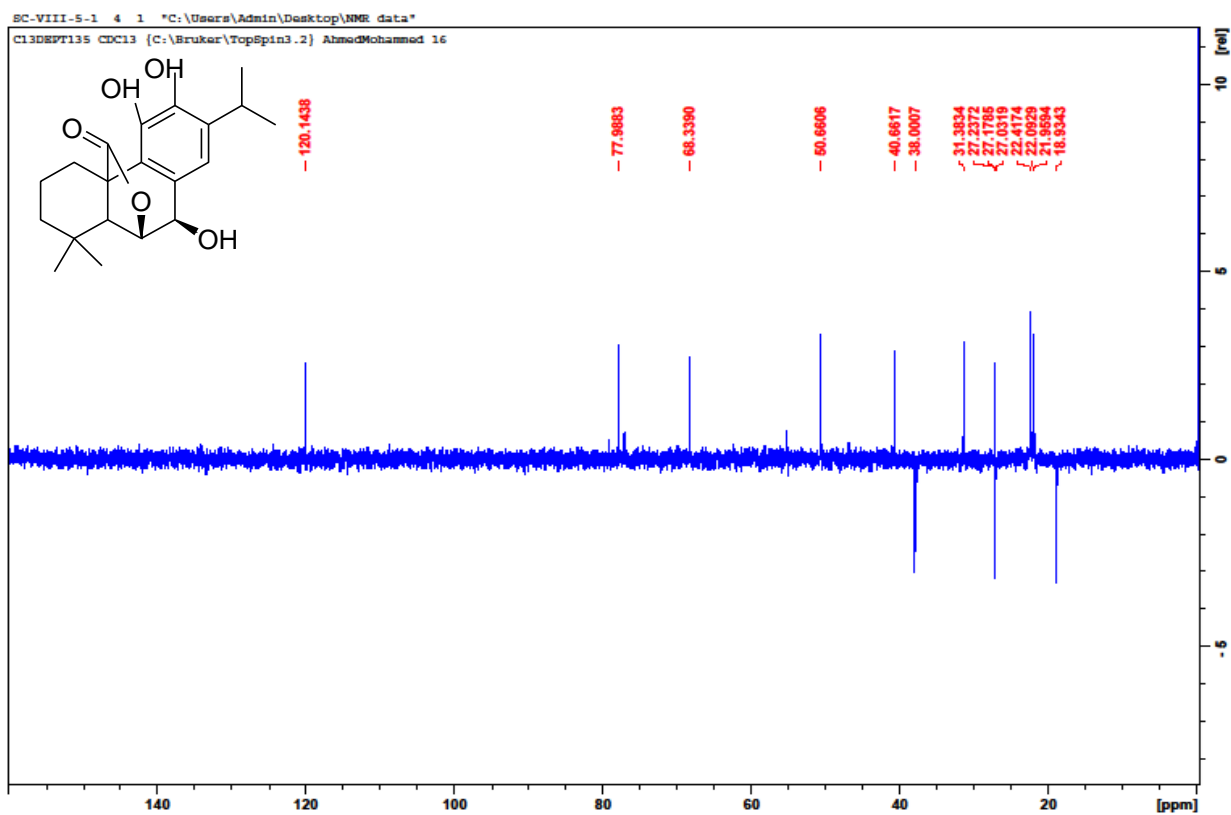


Figure 4.28: DEPT spectrum of compound 5

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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; ^1H , 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).

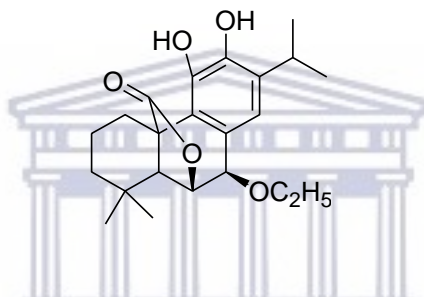


Figure 4.29: Chemical structure of 7-ethoxyrosmanol

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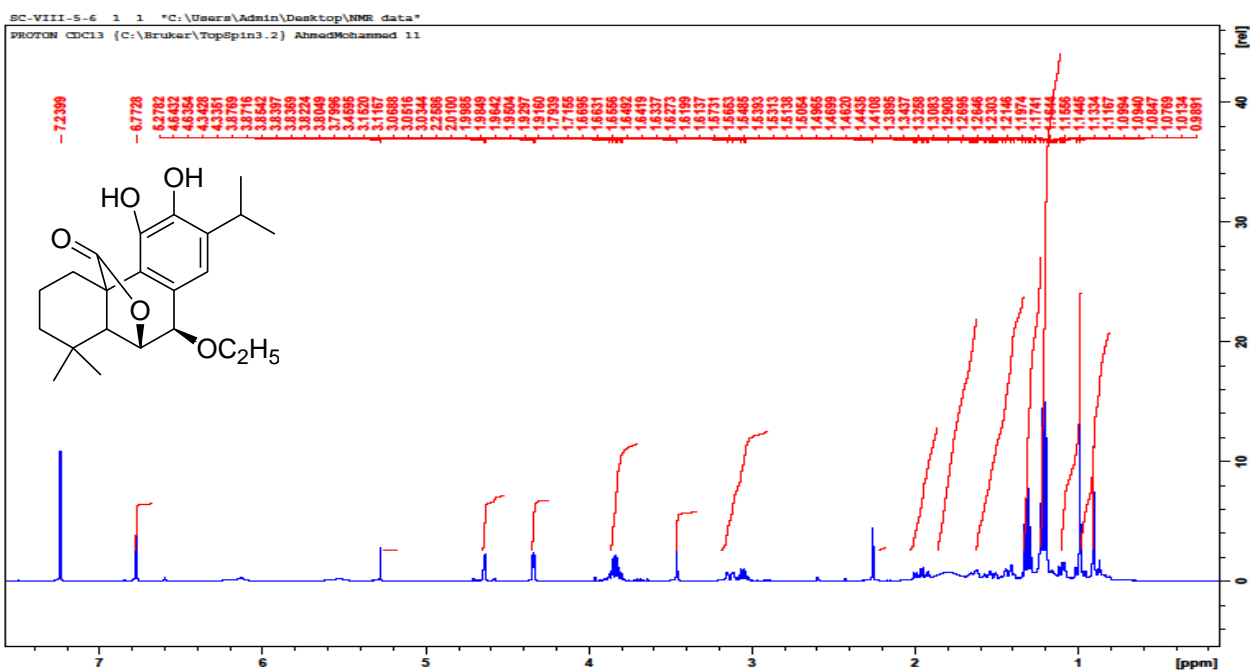


Figure 4.30: ¹H NMR spectrum of compound 3

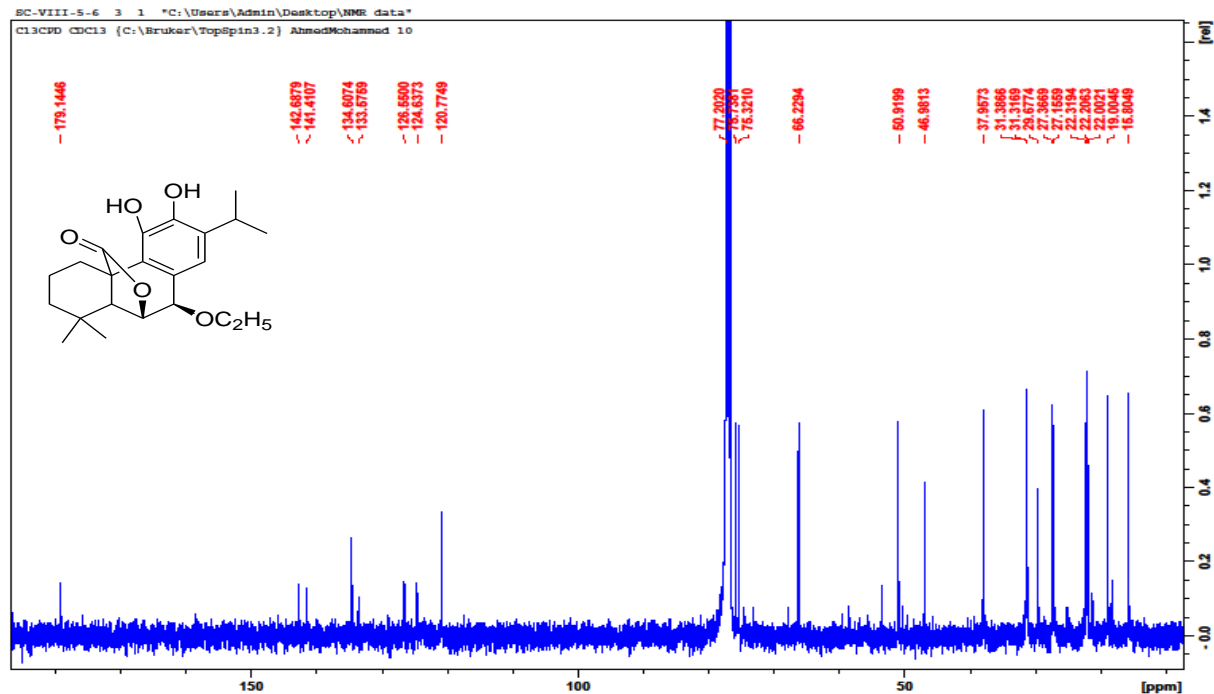


Figure 4.31: ¹³C NMR spectrum of compound 3

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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 C₁₂₋₁₃ 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).

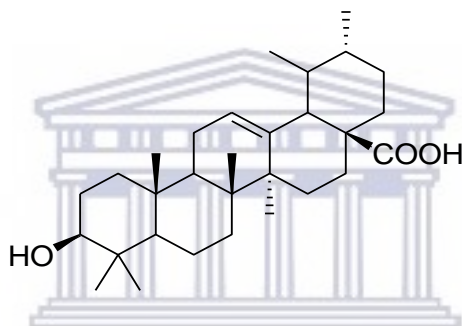


Figure 4.32: Chemical structure of ursolic acid

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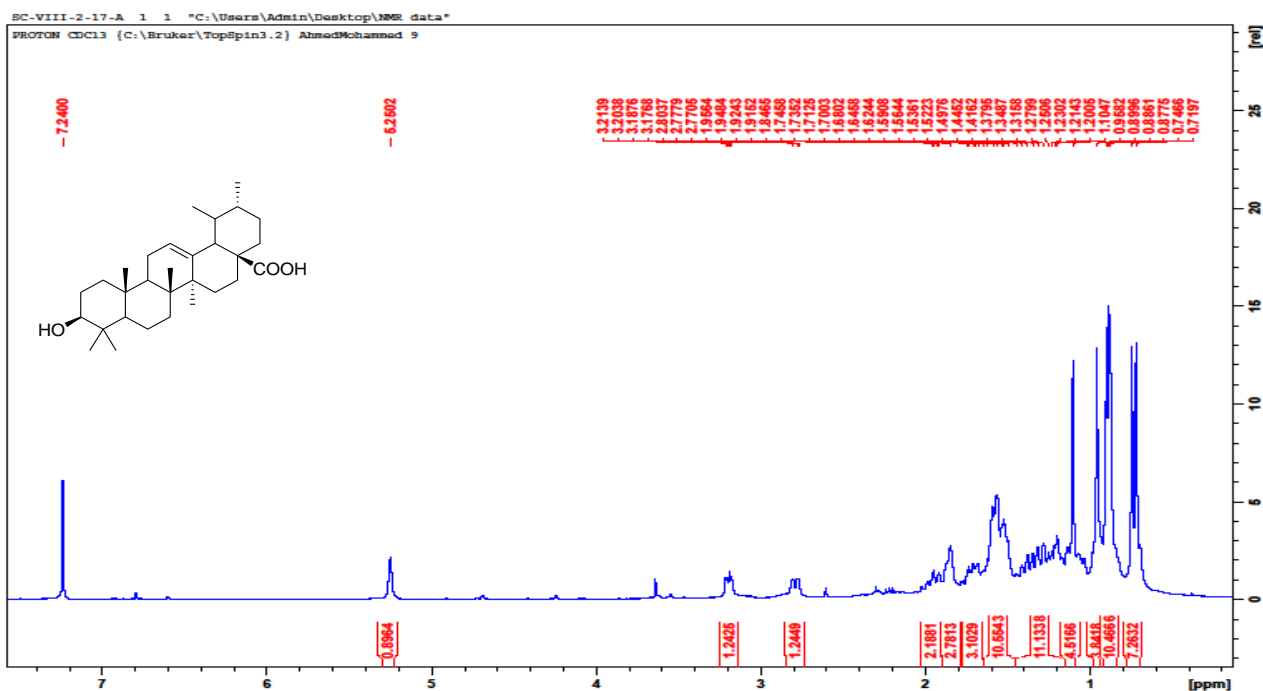


Figure 4.33: ^1H NMR spectrum of compound 4

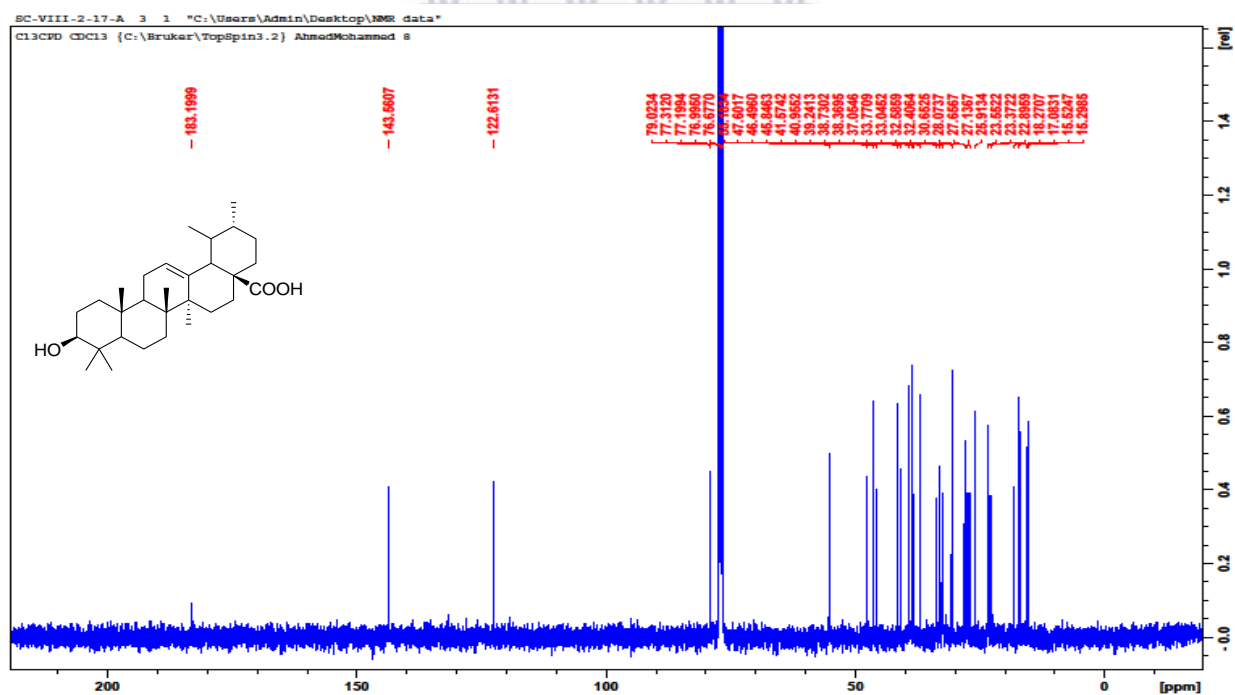


Figure 4.34: ^{13}C NMR spectrum of compound 4

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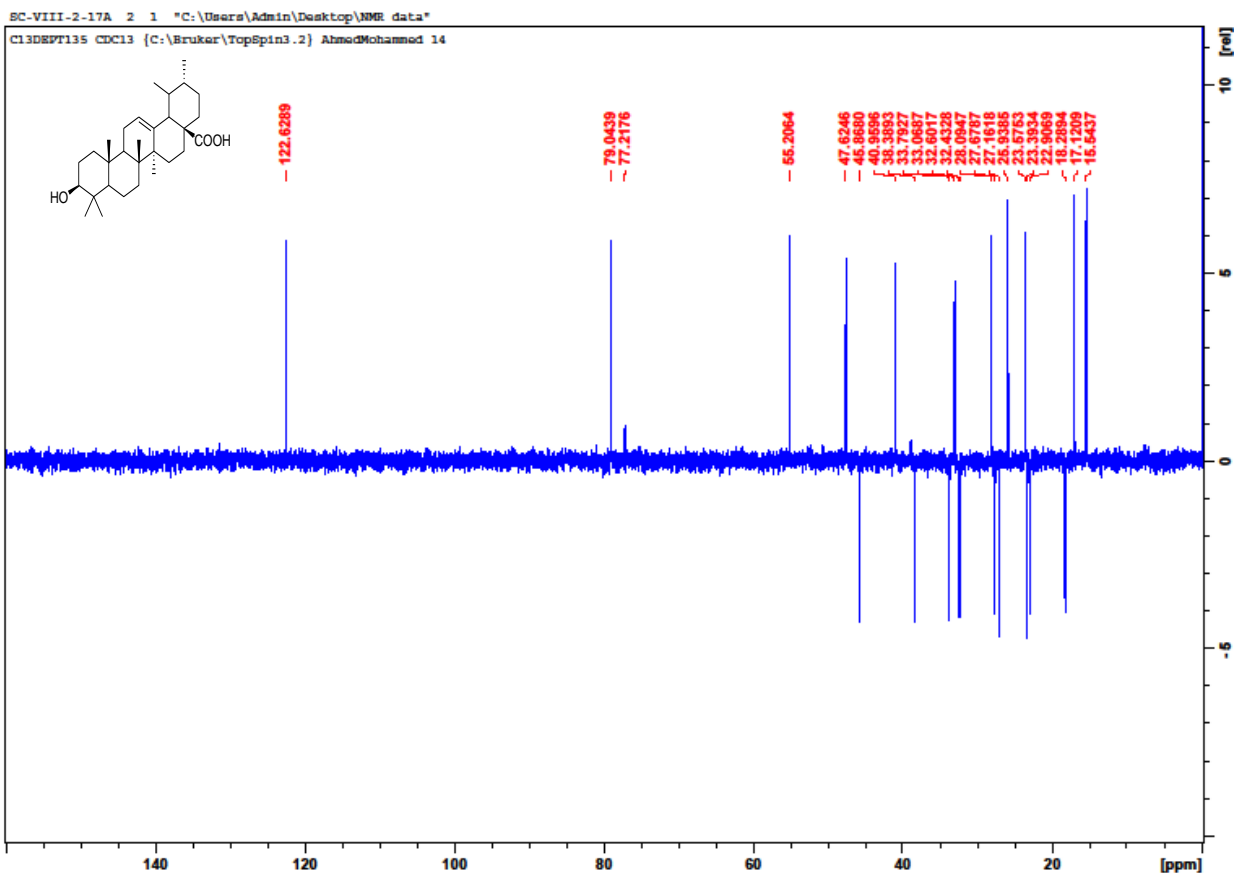


Figure 4.35: ^1H NMR spectrum of compound 4

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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 C₁₀ (105.9); C₅ (145.9) and C₆ (129.5). The singlet signal at 6.56 (counted 2H) attributed to H-3 and H-8; it showed HMBC correlations with C₉, C₁₀, C₇, C₆, [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).

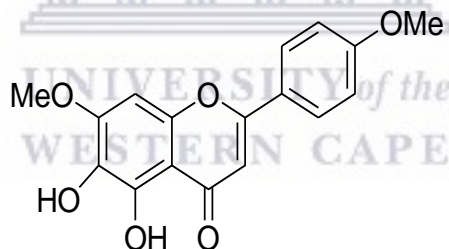


Figure 4.36: Chemical structure of Ladanein

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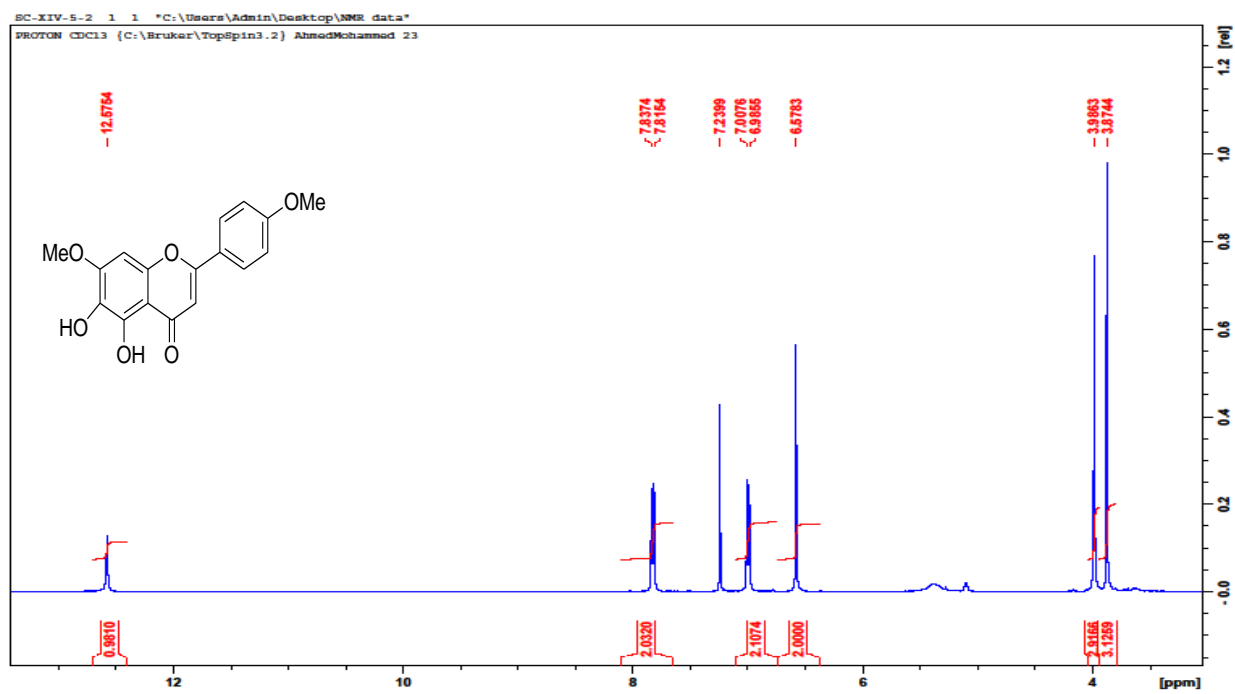


Figure 4.37: ^1H NMR spectrum of compound 6

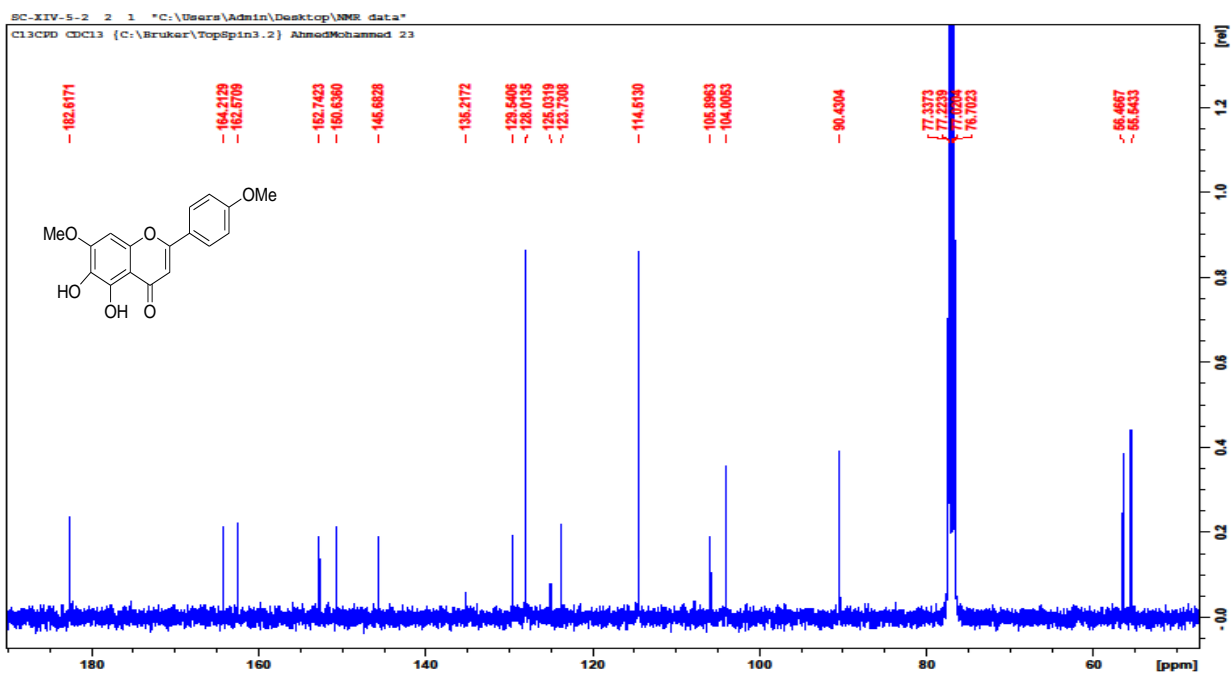


Figure 4.38: ^{13}C NMR spectrum of compound 6

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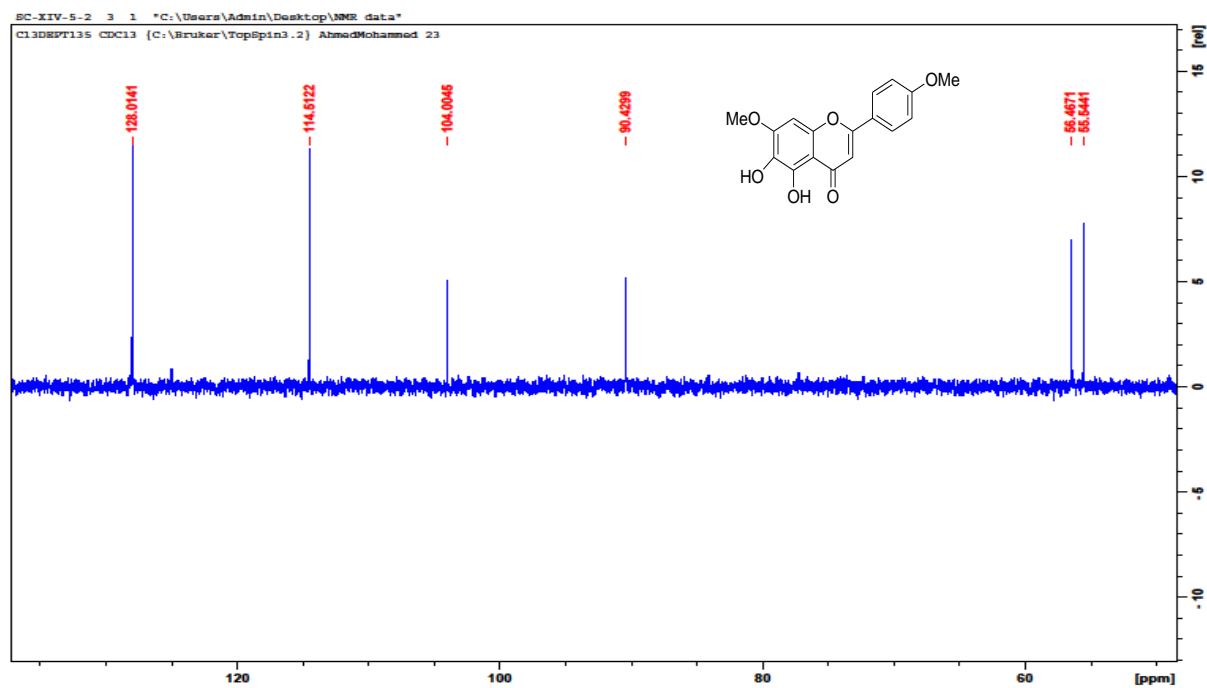


Figure 4.39: DEPT spectrum of compound 6

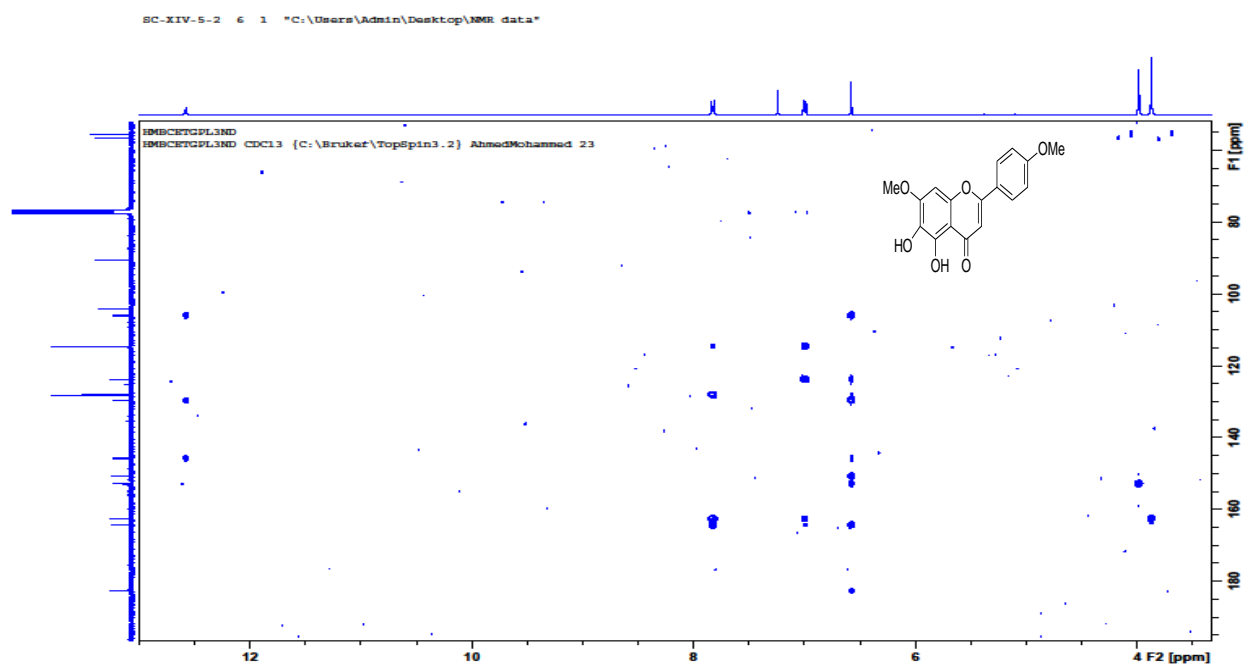


Figure 4.40: HMBC spectrum of compound 6

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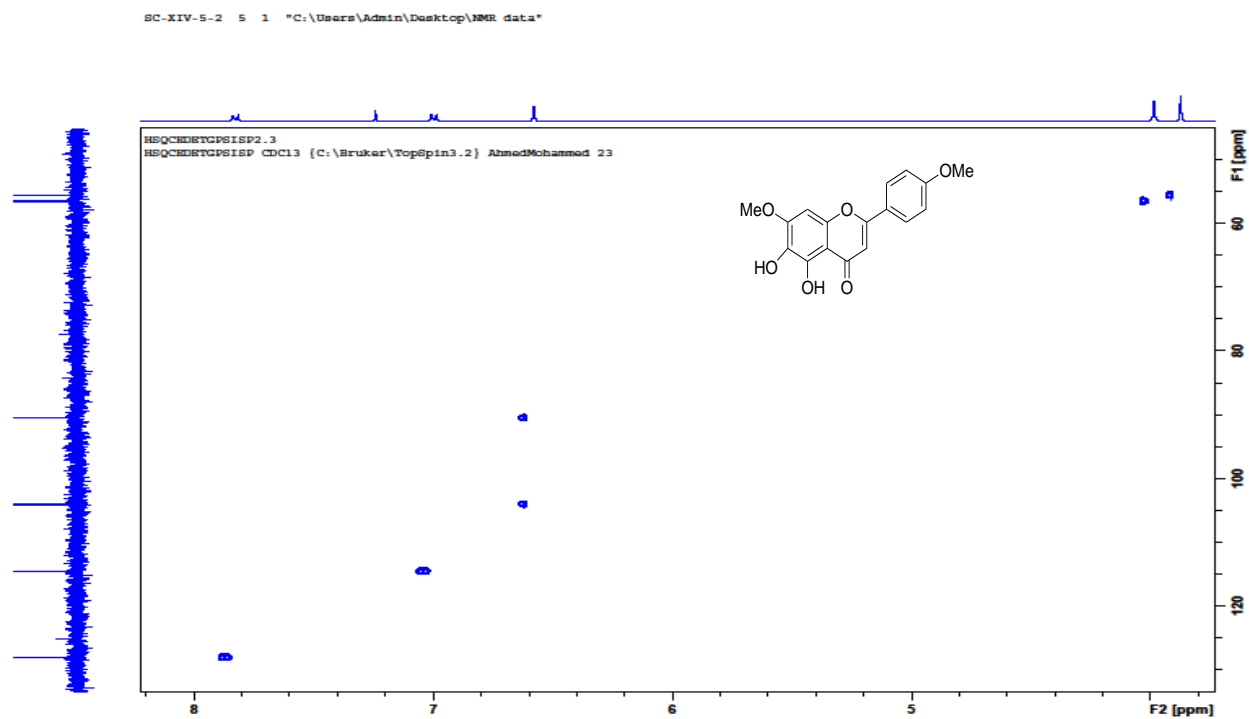


Figure 4.41: HMQC spectrum of compound 6

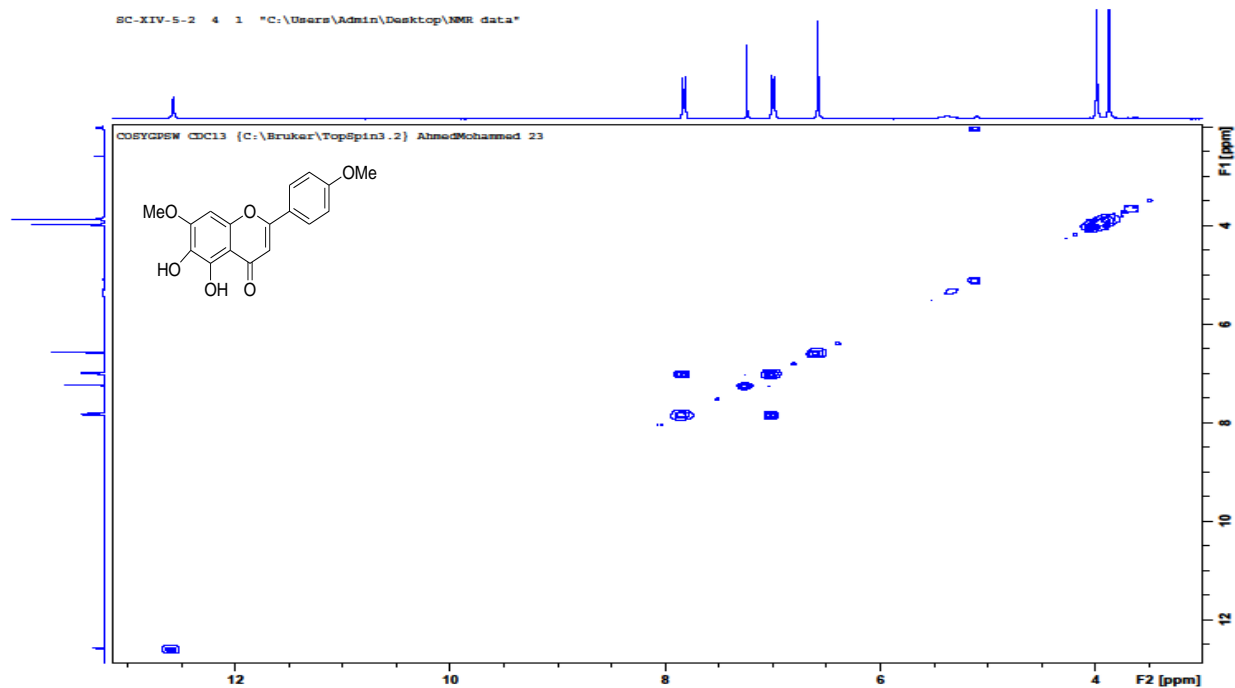


Figure 4.42: COSY spectrum of compound 6

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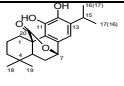
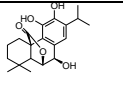
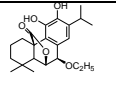
Table 4.7: ^{13}C NMR data of **C1**, **C2**, **C3** and **C5** compared to the NMR data from the literature

	Carnosol		Carnosic acid		rosmanol		7-ethoxy rosmanol	
	Exp.*	Reported**	Exp.*	Exp.*	Reported**	Exp.*	Reported**	
	C	C	C	C	C	C	C	
1.	28.6	29.8 t	34.4	27.3 t	28.4 t	27.4 t	28.3 t	
2.	18.6	19.7 t	20.3	19.1 t	19.9 t	19.0 t	19.8 t	
3.	40.7	41.9 t	41.6	38.2 t	39.1 t	38.0 t	38.9 t	
4.	34.2	35.1 s	34.5 s	31.3 s	32.0 s	31.4 s	32.0 s	
5.	45.3	46.4 d	53.9 d	50.8 d	51.0 d	50.9 d	51.5 d	
6.	29.5	30.6 t	18.9	78.2 d	78.8 d	75.3 d	75.4 d	
7.	78.1	78.2 d	31.5	68.5 d	69.1 d	75.7 d	76.8 d	
8.	131.6	133.6 s	129.0	128.1 s	130.4 s	126.6 s	128.4 s	
9.	121.5	123.1 s	122.6	124.5 s	124.9 s	124.6 s	124.1 s	
10.	48.2	49.2 s	48.7 s	47.2 s	47.7 s	47.0 s	47.6 s	
11.	142.3	143.8 s	142.3	142.9 s	144.7 s	142.7 s	144.6 s	
12.	142.4	143.4 s	141.2	141.8 s	142.5 s	141.4 s	142.6 s	
13.	134.2	135.1 s	133.6	135.1	136.5 s	134.6 s	136.3 s	
14.	111.6	112.4 d	119.1 d	120.3	120.2 d	120.8 d	120.6 d	
15.	26.6	27.6 d	27.0 d	27.3	27.5 d	27.2 d	27.4 d	
16.	22.4	23.0 q	22.5 q	22.1 q	22.9 q	22.2 q	22.9 q	
17.	22.4	23.1 q	22.1 q	22.6 q	23.1 q	22.3 q	23.1 q	
18.	31.4	32.0 q	32.6 q	31.4 q	31.8 q	31.3 q	31.8 q	
19.	19.3	20.0 q	21.5 q	22.3 q	22.4 q	22.0 q	22.3 q	
20.	177.5	175.9 s	182.1	178.9 s	178.3 s	179.1 s	178.4 s	
-CH₂ CH₃						66.2 t	66.4 t	
CH₂ CH₃						15.8 q	16.2 q	

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

	Carnosol		Carnosic acid	Rosmanol		7-ethoxyrosmanol	
	Exp.*	Reported* *	Exp.*	Exp.*	Reported**	Exp.*	Reported**
H-1α	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
H-1β	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
H-2α	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	
H-2β	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	
H-3α	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	
H-3β	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	
H-5	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
H-6α	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
H-6β	1.75 dddd, 13.4, 10.6, 4.0, 1.4	1.85 ddd	1.82 m	---	--		
H-7α	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
H-7β			2.79 m				
H-14	6.51 s	6.77 s	6.53 s	6.80 s	6.89 s	6.77 s	6.84 s
H-15	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
H-16(Me)	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
H-17(Me)	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
H-18(Me)	0.75 s	0.88 s	0.97 s	0.95 s	1.02 s	0.99 s	1.02 s
H-19(Me)	0.72 s	0.87 s	0.87 s	0.85 s	0.90	0.91 s	0.91 s
-CH₂ CH₃						3.83 m	
CH₂ CH₃						1.31, 7.0	

* in CDCl₃

**in Acetone – d₆ (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 Fe²⁺- 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

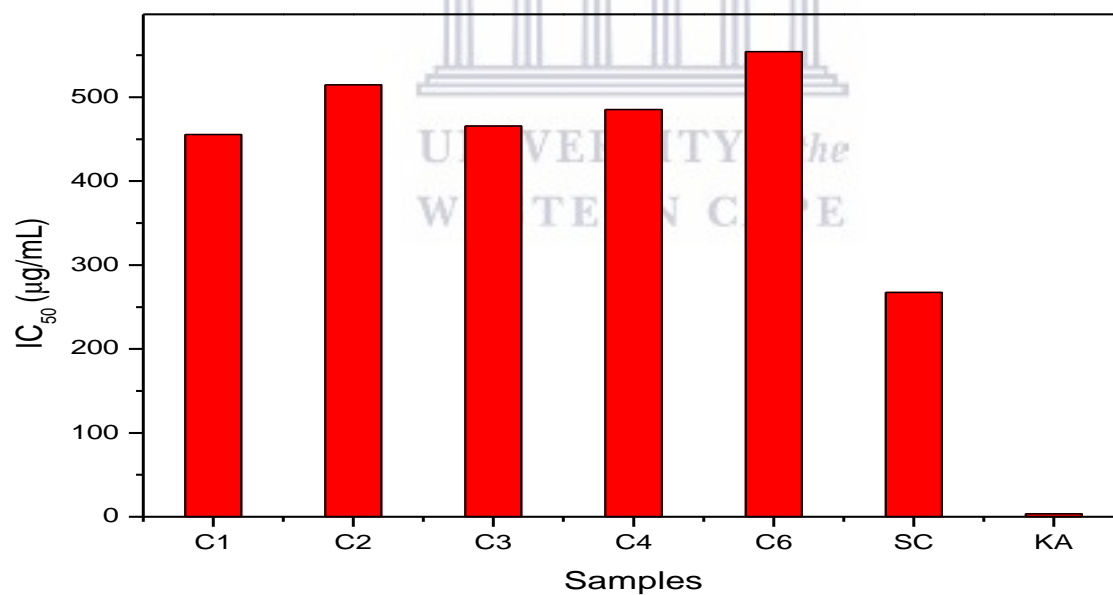
The methanolic extract (SC) and the isolated compounds (C1-C6) were evaluated for their tyrosinase inhibitory activity at different concentration as illustrated in Table 4.9. The result revealed that C1 demonstrated the highest tyrosinase inhibitory activity (IC₅₀ = 455.5 µg/mL) among the isolated compounds 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 IC₅₀ of *S. chamelaeagnea* constituents

Sample	% inhibition (µg/mL)					IC ₅₀
	1000	500	100	50	10	
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)



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

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

*Data are expressed as IC₅₀ 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 μ A), which is in agreement with TEAC ($16505.0 \pm 0.86 \mu$ M TE/g) and FRAP ($9338.92 \pm 1.72 \mu$ M 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

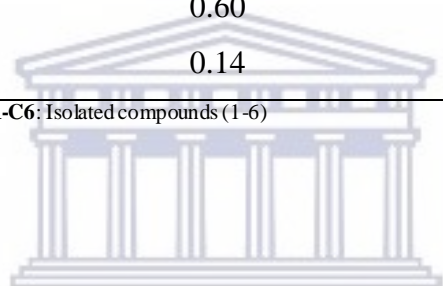
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mechanism. This electron transfer property may be attributed to the presence of an ortho-dihydroxy group on the C-ring of **C1** (Miura, et al., 2002). Table 4.10 showed the anodic peak current and potential of the tested samples.

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

Sample	$i_{pa}/\mu A$	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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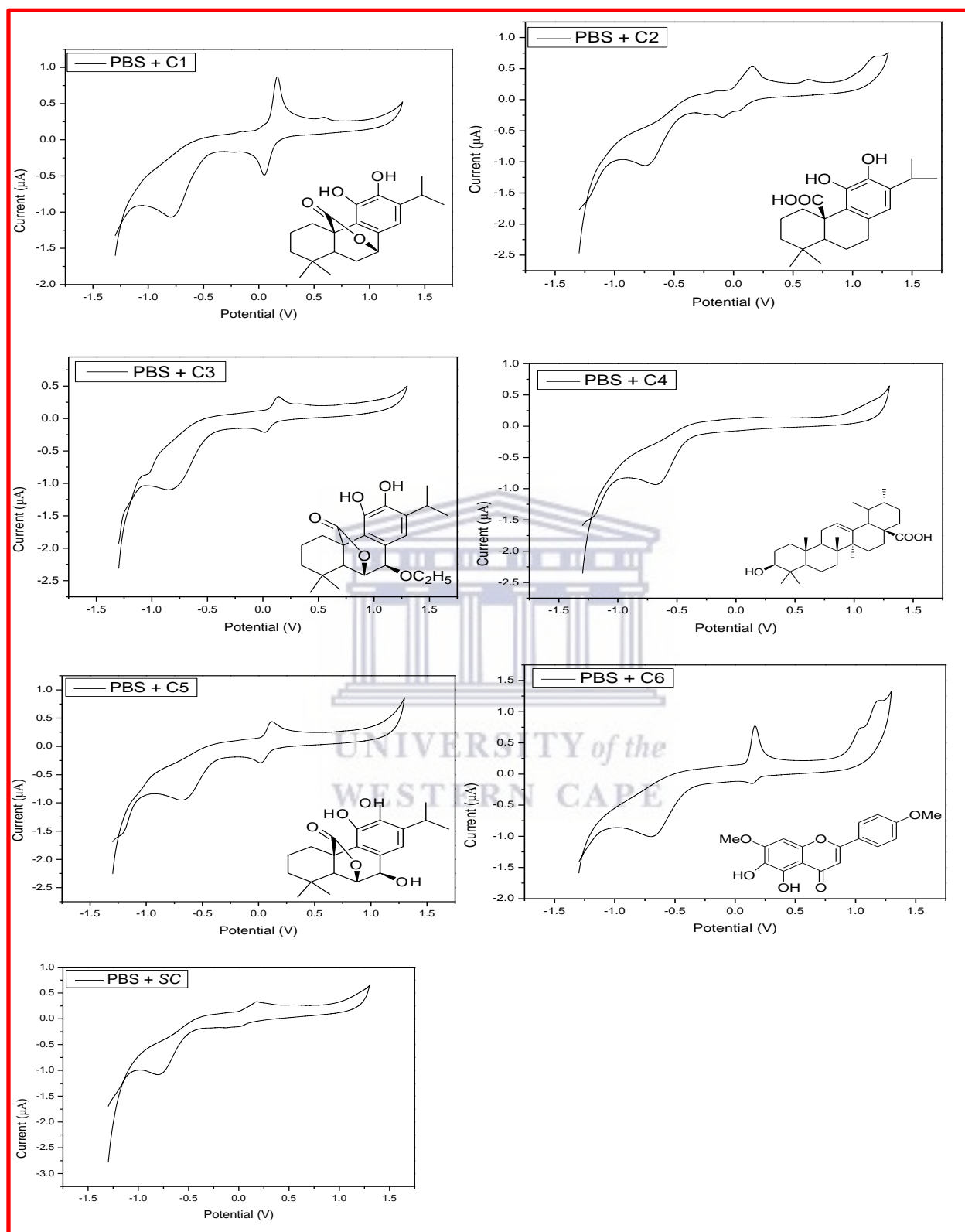


Figure 4.44: CVs recorded at 50 mV/s in broad scan range from -1300 to 1300 mV for 1 mg/L of each compound in phosphate buffer (pH 6.5).

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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	$i_{inh}/10^{-2}\mu\text{A}$ at 0 min	$i_{inh}/10^{-2}\mu\text{A}$ 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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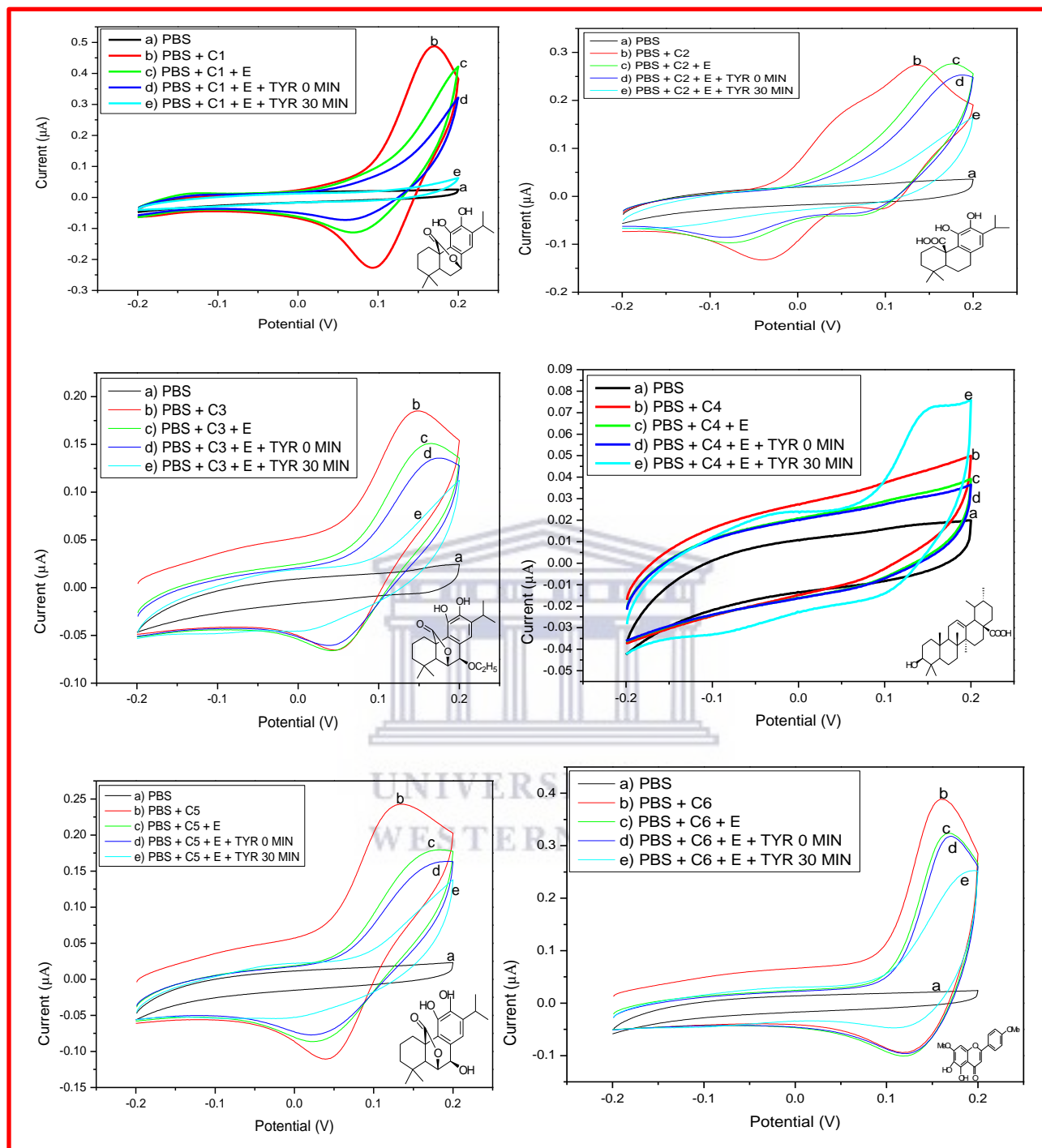
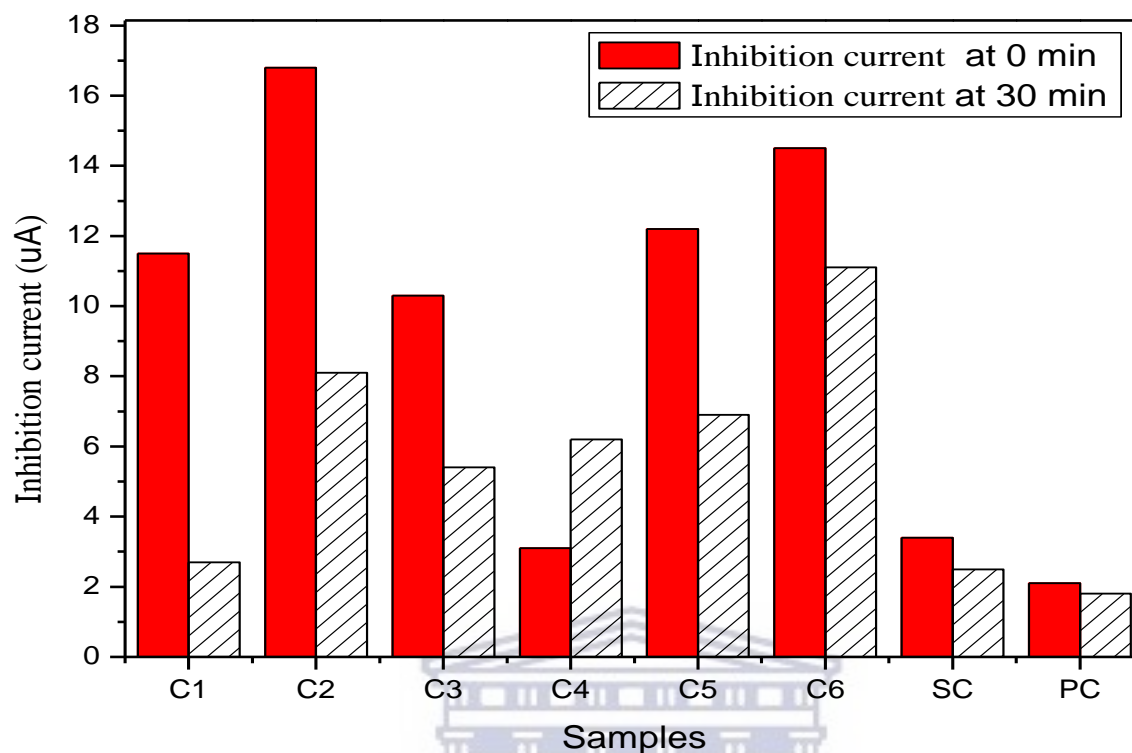


Figure 4.45: CVs recorded at 25 mV/ s in wide scan range from -200 to 200 mV for each isolated compounds in PBS for the reaction mixture of tyrosine and tyrosinase in 50 mM PBS (pH 6.5) at different times.

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PC>SC>C1>C3>C5>C6>C2>C4

Figure 4.46: Effect of *S. chamelaeagne* 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.

$$[(Q_{30'} - Q_{0'}) / Q_{0'}] \times 100 \quad (2)$$

Where $Q_{30'}$ is the inhibition based on charge at 30 min, $Q_{0'}$ 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)

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) μ M 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: Epigallocatechingallate; 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) $\mu\text{MTE/g}$ in a competitive manner to that of EGCG (14970 ± 5.53 $\mu\text{MTE/g}$) 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 peroxy 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 μ MTE/g)
C1	14550.50 \pm 3.65
C2	10398 \pm 1.81
C3	8247.35 \pm 6.83
C4	2080.19 \pm 8.52
C5	14633.90 \pm 3.84
C6	8380.08 \pm 4.52
SC	14338.49 \pm 5.16
EGCG	14970 \pm 5.53

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

4.9.5 Evaluating the Fe²⁺-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 Fe²⁺-induced anti-lipid peroxidation (IC₅₀ = 30.25; 32.5; 41.50) μ g/mL respectively, followed by **C2** (IC₅₀ = 38.5 μ g/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 metals or scavenge peroxy 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 IC₅₀ with tested sample screened at 100 µg/mL; SC methanolic extract

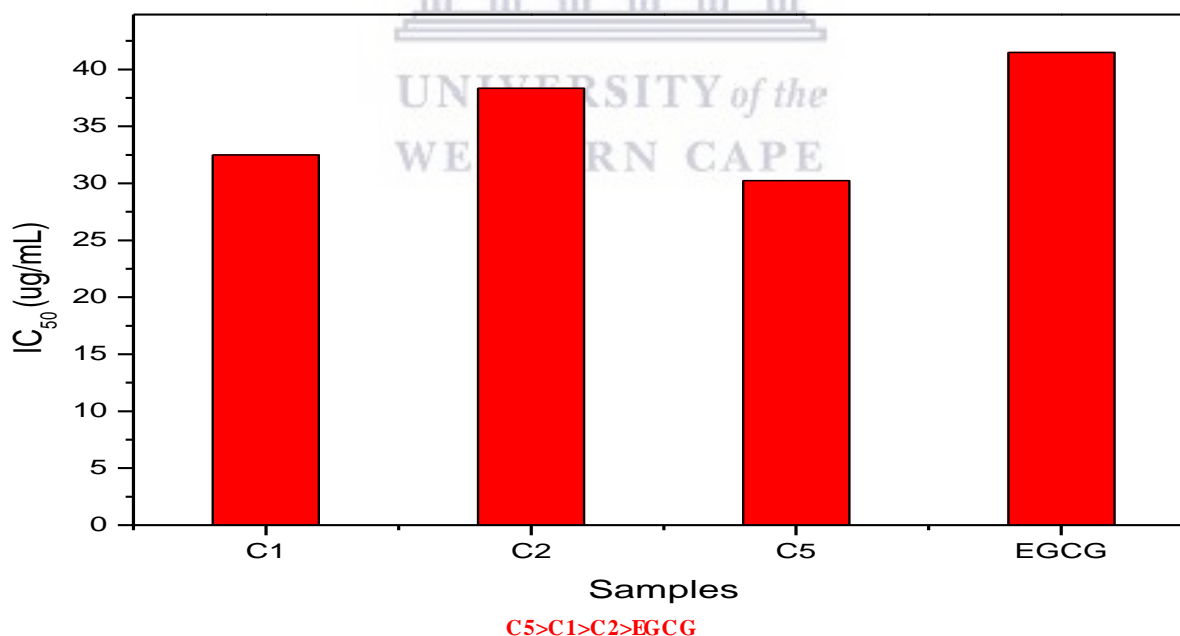


Figure 4.47: Effects of *S. chamelaeagnea* constituents on inhibition of Fe²⁺-induced microsomal lipid peroxidation.

*Data are expressed as IC₅₀ with isolated compounds screened at 100 µg/mL

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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) $\mu\text{M AAE/g}$; TEAC (16505 ± 0.86 ; 10641.5 ± 0.52 ; 5897.5 ± 1.03) $\mu\text{M TE/g}$; ORAC (14550.5 ± 3.65 ; 14633.90 ± 3.84 ; 10398 ± 1.81) $\mu\text{M TE/g}$ and including the inhibition of Fe^{2+} -induced lipid peroxidation ($\text{IC}_{50} = 32.5$; 30.25 ; 38.35) $\mu\text{g/mL}$, 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 approximately 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 ($5600.0 \pm 1.0 \mu\text{M TE/g}$) (Erkan, et al., 2008) and ORAC ($6489 \mu\text{M TE/g}$)(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 ($5897.5 \pm 1.03 \mu\text{M TE/g}$) and ORAC ($10398 \pm 1.81 \mu\text{M TE/g}$). **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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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.

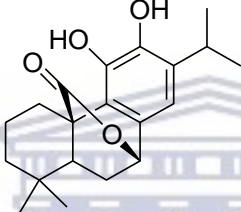
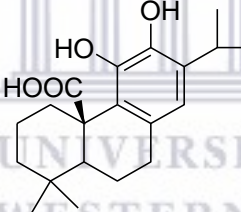
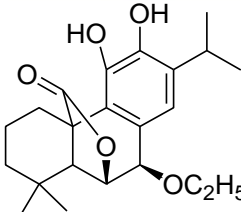
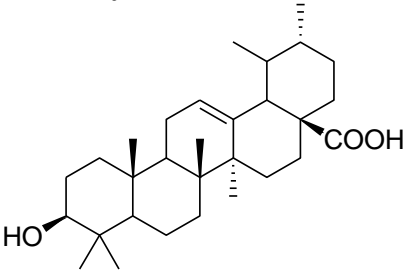
The preliminary phytochemical screening using solvent system DCM: MeOH (97:3) on TLC showed *S. chameleagnea* to possess variety of class of compounds varying from terpenoids, flavonoids, and other phenolic compounds (Kamatou, et al., 2007).

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 bioautography, spectrophotometry result showed that extracts of *Plectranthus zuluensis* ($IC_{50} = 23.99 \mu\text{g/mL}$), *P. ecklonii* ($IC_{50} = 21.58 \mu\text{g/mL}$), *Thorncroftia succulent* ($IC_{50} = 36.08 \mu\text{g/mL}$), and *P. madagascariensis* ($IC_{50} = 23.99 \mu\text{g/mL}$) demonstrated good anti-tyrosinase activity when compared with kojic acid ($IC_{50} = 3.607 \mu\text{g/mL}$). 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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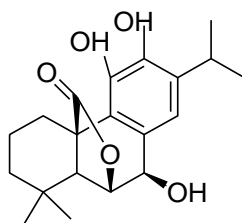
Phytochemical investigation carried out on the bioactive extract of *Salvia chamelaeagnea* using different chromatographic methods including column chromatographic and semi preparative HPLC afforded six (6) known compounds identified as carsonol (C1), carnosic acid (C2), 7-ethoxyrosmanol (C3), ursolic acid (C4), rosmanol (C5) and ladanein (C6). Their chemical structures were fully elucidated by analyses of spectroscopic (^1H 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

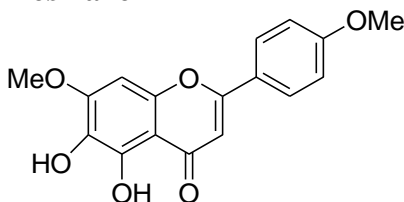
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C5



Rosmanol

C6



Ladanein

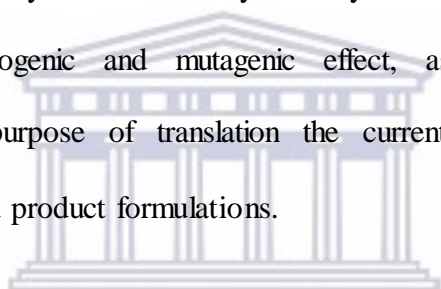
The methanolic extract of *S. chamelaeagnea* (SC) showed moderate anti-tyrosinase ($IC_{50} = 267.4$ $\mu\text{g/mL}$) activity, while the total antioxidant activities measured as: Oxygen radicals absorbance capacity (ORAC; 14970 ± 5.16 $\mu\text{M TE/g}$), ferric-ion reducing antioxidant power (FRAP; 9869.43 ± 7.87 $\mu\text{M AAE/g}$) and trolox equivalent absorbance capacity (TEAC; 13706.5 ± 0.95 $\mu\text{M TE/g}$). Excellent total antioxidant capacities were demonstrated by **C1** and **C5** respectively as FRAP (9338.92 ± 1.72 ; 8622.73 ± 1.92) $\mu\text{M AAE/g}$; TEAC (16505 ± 0.86 ; 10641.5 ± 0.52) $\mu\text{M TE/g}$; ORAC (14550.5 ± 3.65 ; 14633.90 ± 3.84) $\mu\text{M TE/g}$ and inhibition of Fe^{2+} -induced lipid peroxidation ($IC_{50} = 32.5$; 30.25) $\mu\text{g/mL}$. 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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