Exploring anti-tyrosinase bioactive compounds from the Cape flora

By

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Abstract

Tyrosinase is an enzyme widely distributed in the biosphere and is found in many species of bacteria, fungi, animals, and plants; it is associated with melanin production. Even though it possesses many beneficial properties such as photoprotection, but overproduction causes undesirable effects such melasma, solar lentigines etc. Therefore, tyrosinase enzyme inhibitors are of far-ranging importance in cosmetics, medicinal products, and food industries.

This study is aimed to test anti-tyrosinase activity in 37 plants from 20 families using mushroom tyrosinase inhibition method; each plant was extracted with methanol. The results showed that 17 plant extracts, exerted a considerable level of *in vitro* tyrosinase inhibition comparable to positive controls of kojic acid in the same solvent systems when evaluated spectrophotometrically. Among plant extracts, those that showed an inhibition rate >50 % at 50 µg/ml and >60 % at 200 µg/ml were *A. karroo* (Hayne.), *A. afra* Jacq. Ex Willd, *C. geifolia* (L.), *E. racemosa* (L.), *H. petiolare* Hilliard & B.L.Burt, *M. quercifolia* (L.), *M. communis* (L.), *P. rigida* (Wikstr.), *P. ecklonii* (Benth.), *P. ericoides* (L.), *S. Africana-caerulea* (L.), *S. Africana-lutea* (L.), *S. antarcticus* (Willd.), *S. lucida* (L.) F.A.Barkley, *S. hamilifolius* (L.), *S. furcellata* R.Br and *T riparia* which exhibited great anti-tyrosinase activity.

The active extracts were assayed further for their ability to inhibit melanin biosynthesis and cytotoxicity. Eight of seventeen extracts clearly showed higher values of melanin biosynthesis with the IC_{50} values of *T. riparia* (43.88 µg/ml), *S. furcellata* R.Br (7.13 µg/ml), *C. geifolia* (L.) (36.88 µg/ml), *A. karroo* (Hayne.) (>100 µg/ml), *M. quercifolia* (L.) (<6.26 µg/ml), *P. eroides* (L.) (27.67 µg/ml), *S antarcticus* (Willd.) (20.25 µg/ml) and *M. communis* (L.) (>100 µg/ml). The plant extracts were subjected to cell cytotoxicity 46.94 µg/ml, >100 µg/ml, >100

µg/ml, >100 µg/ml, 97.2 µg/ml, >100 µg/ml, 50.00 µg/ml, >100 µg/ml respectively and did not induce cytotoxicity.

The extract of *M. quercifolia* (L.) showed inhibition zone on the TLC plate and significant tyrosinase and melanin inhibition and was thus selected for purification of bioactive compounds. Fractionation was achieved by using silica column chromatography and sephadex column chromatography. Fraction XII was selected based on TLC bioautography activity; it was then further purified. Three compounds such as quercitin, kaempferol-3-(2,3-diacetoxy-4-trans-*p*-coumaroyl)rhamnoside and kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside were discovered and characterised by semi-prep HPLC. Structure elucidation of the compounds was achieved by analysis of ^{1}H-NMR and ^{13}C-NMR spectrocopic data, information provided by literature, direct comparisons with authentic materials available commercially, and by comparison with data from similar species in the same genus.

A constituent analysis of the aerial parts clarified the structures of 3 compounds. The tyrosinase inhibitory activity of the isolated compounds were measured as indicators of the inhibitory capacity. Compound 1 exhibited potent anti-tyrosinase activity (IC_{50} of <6.25 µg/ml) whereas compound 2 and 3 exhibited weak activity with an IC_{50} of >100 µg/ml. The compounds were further evaluated for cytotoxicity and had an IC_{50} activity of <100 µg/ml for all isolated compounds. The findings therefore indicate that the leaf extract of *M. quercifolia* shows promise as a new source of natural tyrosinase inhibitors and should be explored as a source for depigmentation agents.

Keywords: CFR, *M. quercifolia*, TLC bioautography, tyrosinase inhibition, melanoma B16-F10, melanin biosynthesis inhibition, depigmentation, cosmetics.

DECLARATION

I, **Luveni Sonka,** hereby declare that "**Exploring anti-tyrosinase bioactive compounds from the Cape flora**" 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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And last but certainly not least, I would like to give thanks to my cat, **KAT-lego** and dogs, **Ginger**, **Snowie** and **MJ** for being excellent company and stress relievers.

Dedication

I would like dedicate this dissertation to my parents, especially my dear mother, **Phumza Yvonne Sonka**, for being my pillar and being supportive in all I do; enkosi Tshawekazi. Thanks to the rest of my family for their love and more particularly my lovely brother, **Wandisile Dyosi**, for his unconditional and unwavering support.

Table of contents

Table of Contents

Abstract i
DECLARATION iii
Acknowledgements iv
Dedication v
List of figures ..... **Error! Bookmark not defined.**
List of tables xiii
List of schemes xiv
LIST OF ISOLATED COMPOUNDS xviii
Chapter one 1
General introduction 1
1.1 Why investigate the CFR 2
1.2 Statement of research 3
Aims of this study 3
Objectives of this study 4
Chapter 2 Literature review 5
2.1 The skin 5
2.1.1 Skin anatomy 5
2.1.1.1 Epidermis 5
2.1.1.2 The dermis 7
2.1.1.3 Subcutis 8
2.1.2 Skin function 8
2.1.3 Skin chromophores 8
2.2 Melanin 9
2.2.1 Melanin biosynthesis 9
2.2.2 Melanogenesis regulation 10
2.3 Tyrosinase 10
2.3.1 Distribution and classification of tyrosinase 11
2.3.2 Properties of various tyrosinases 11
2.3.3 Physiological role of tyrosinase 12

2.3.4 Structural mechanism of tyrosinase 13
2.3.5 Lag phase and autoactivation 14
2.3.6 Active centre of tyrosinase 14
2.4 Cosmetics 17
2.4.1 Cosmetic use in medieval Egypt 18
2.4.2 Cosmetics in ancient Europe 19
2.4.3 Cosmetic use in Asian countries 19
2.4.4 Cosmetics in ancient India 20
2.4.5 Cosmeceuticals 20
2.5 Plants used for skin disorders in South Africa 21
2.6 Tyrosinase inhibitors 23
2.6.1 Kojic acid 23
2.6.3 Hydroquinone 24
2.6.4 Arbutin 25
1.6.5 Liquorice 26
2.7 Pigmentation disorders 26
2.7.1 Hyperpigmentary disorders 26
2.7.1.1 Melasma 26
2.7.1.2 Cafe au lait macules 27
2.8 Cape Floristic Region (CFR) 27
2.9 Myricaceae 29
2.9.1 Biological description 29
2.9.2 Genus Myrica 30
2.9.2.1 Distribution and ethnopharmacological uses of Myrica 31
2.9.2.2 Ecological distribution 34
2.10 *Myrica quercifolia* 35
2.10.1 Biological activities and chemical composition of the species from the genus Myrica 36
2.10.2 Anti-cancer properties 36
2.10.3 Anti-tyrosinase and radical scavenging properties 37
2.10.4 Nitrogen fixation 43
Chapter 3 45
Screening of crude extracts for their inhibitory potential against tyrosinase enzyme 45
3.1 Introduction 45

3.2. Materials and methods 46
3.2.1 Plant collection 46
3.2.2 Materials and reagents 48
3.2.3 Equipment and Instruments 48
3.3 Methods 49
3.3.1 Plant extraction 49
3.3.2 Plant sample preparation 49
3.3.3 Tyrosinase enzyme preparation 49
3.3.4 TLC bioautography assay of crude plant extracts 50
3.3.5 Spectrophotometric determination of tyrosinase inhibition of crude extracts using ELISA reader. 50
3.4 Investigation of melanin inhibition and cytotoxicity in melanoma B16-F10 cell culture by crude plant extracts 51
3.4.1 Preparation of test samples 51
3.4.2 Preparation of cells 51
3.4.3 Cell count 52
3.4.4 Cell viability assay 52
3.4.5 Melanin inhibition assay 53
3.5 Results and discussion 56
3.5.1 Ethnopharmacological information 56
3.5.2 Screening of tyrosinase activity of methanolic extracts using TLC bioautography. 57
3.5.3 Screening of tyrosinase inhibitory activities of methanol crude extracts using ELISA reader. 60
3.5.4 Screening of melanin biosynthesis inhibitory effects of methanol crude extracts at 50 µg/ml 62
3.5.5 Screening of melanin biosynthesis inhibitory effects and cytotoxicity of active methanol crude extracts. 64
Chapter 4 66
Extraction, purification, characterisation and anti-tyrosinase activity of compounds from *Myrica quercifolia* (L.). 66
4.1 Introduction 66
4.2 Plant Myrica quercifolia (L.) 68
4.3 Materials and methods 69
4.3.1 Materials and Equipment 69
4.3.2 Reagents and Solvents 69

4.4 Methods 70
4.4.1 Extraction of *M. quercifolia* 70
4.4.2 Thin Layer Chromatography (TLC) 70
4.4.3 Fractionation of crude extract using column chromatography 72
4.4.4 Anti-tyrosinase activity of different fractions 74
4.4.5 Fractionation of fraction XII 75
4.4.6 Purification of compounds from fraction XII using Semi-preparative High Pressure Liquid Chromatography (HPLC) 76
4.4.7 Structure elucidation using Nuclear magnetic resonance (NMR) spectroscopy 77
4.7 Results and discussion 78
4.7.1 Fractionation and purification of *M. quercifolia* constituents. 78
4.7.2 Inhibition of the enzyme tyrosinase by *M. quercifolia* extract and Kojic acid (positive control) using L-tyrosine as a substrate. 88
4.7.3 Melanin biosynthesis inhibition by *M. quercifolia* and kojic acid and Kojic acid using L-tyrosine as a substrate. 89
4.7.4 Effect of bioactive compounds on tyrosinase Inhibition. 90
4.7.5 Effect of bioactive compounds on melanin inhibition 92
Chapter 5 97
5.1 Motivation of the study 97
Conclusion 97
Recommendations 100

List of figures

Figure 2.1: Cross section of the skin 5

Figure 2.11: Four distinct layers of the epidermis under a light microscope.....7

Figure 2.2: Biosynthesis reaction of two types of melanin: Eumelanin which is black/brown and pheomelanin which is red/yellow.....9

Figure 2.3: Active center of tyrosinase.....16

Figure 2.4: Chemical structure of kojic acid.....24

Figure 2.5 Map showing geographical distribution of the Cape Floristic region biodiveristy hotspot.....28

Figure 3.1: TLC bioautography detection of tyrosinase inhibitors. From left to right are plant samples 1 to 39, k is kojic acid which was used as reference standard. Elution was carried out with MeOH/DCM (1:9); (A,C and E) vanillin-sulpuric acid stain used as a detection agent from 1-39; (B, D and F) bioautography for tyrosinase inhibitor detection.....58

Figure 4.1: Leaves of *M. quercifolia* (L.).....68

Figure 4.2 Column chromatography of crude *M. quercifolia* on silica gel.....72

Figure 4.31: Fractions 1-21of crude extract of *M.quercfolia* developed in DCM:MeOH 95:5 viewed under (A) λ 254 nm and (B) λ 366 nm UV wavelength, then inserted in (C) Vanillin-sulphuric acid and heated at ~ 100℃, (D) sprayed with 2mM of tyrosine and 500U/ml of tyrosinase.....74

Figure 4.32: TLC plate of fractions I to X of fraction XII developed in DCM:MeOH and 95:5, the plate was then dipped in vanillin-sulphuric acid and heated at ~ 100 ℃, plates were viewed under (B) 254nm and (C) 366 UV wavelength.....76

Figure 4.5: HPLC Conditions.....77

Figure 4.6: TLC silica gel of isolated compounds, [DCM:MeOH (9:1)] and HPLC chromatogram of XII_5 (Fig. 4.5).....77

Figure 4.71: ^{1}C-NMR of compound 1.....79

Figure 4.72: ^{1}H-NMR spectrum of compound 1.....80

Figure 4.73: HSQC NMR spectrum of compound 1.....80

Figure 4.74: ^{1}C-NMR of compound 2.....81

Figure 4.75: ^{1}H-NMR spectrum of compound 2.....82

Figure 4.76: HMBC NMR of compound 2.....82

Figure 4.77: DEPT NMR spectrum of compound 2.....83

Figure 4.78: ^{1}C-NMR spectrum of compound 3.....84

Figure 4.79: ^{1}H-NMR spectrum of compound 3.....85

Figure 4.8: HSQC NMR spectrum of compound 3.....85

Figure 4.81: HMBC1 NMR spectrum of compound 3.....86

Figure 4.82: DEPT NMR spectrum of compound 3.....86

Figure 4.83: Inhibitory effect on tyrosinase activity by methanolic extract of (A) *M. quercifolia* and (B) kojic acid (positive control).....88

Figure 4.84: The inhibitory effects of (A) *M. quercifolia* (C) Kojic acid (positive control) and cytotoxicity effects of (B) M. *quercifolia and* (D) kojic acid on melanoma B16-F10 cells.....89

Figure 4.85 : Inhibitory effect on tyrosinase activity by Compound 1 (A) Compound 2 (B) Compound 3 (C) and Kojic acid (D).....91

Figure 4.86: The inhibitory effects of (A) Compound 1 (XII-V(1)) on melanoma B16-F10 cells

after a 72 hour treatment (B) cytotoxicity after a 24 hour treatment.....92

Figure 4.87: The inhibitory effects of (A)Compound 3 (XII-V(7)) on melanoma B16-F10 cells after a 72 hour treatment and (B) cytotoxicity after 24 hour treatment.....93

Figure 4.88. Chemical structures of (A) quercetin (B) Kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside, and (C) kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside isolated from the methanol extract of *M. quercifolia*.....99

List of tables

Table 2.1: Distribution and ethnopharmacological uses of *Morella* and *Myrica* species.....32

Table 2.2: Comparison between three genera of the Myricaceae family.....36

Table 2.3: Compounds isolated from the members of the Myrica and Morella genus.....38

Table 3.1: List of plant species used for the present study.....47

Table 3.2: Tyrosinase inhibition by crude plant extracts at two different concentrations.....61

Table 3.3 Melanin inhibition activity of the most active crude extracts evaluated on melanoma B16-F10 cells at 50 µg/ml.....62

Table 3.4: IC_{50} of melanin inhibition and cytotoxicity of crude extracts that were assessed on melanoma cells at 50 ug/ml.....64

Table 4.1 Fractionation of *M. quercifolia* extract.....68

Table 4.2 Solvent system used for fractionation of *M. quercifolia* on column chromatography73

Table 4.3: Tyrosinase **i**nhibitory effects of total fractions of *M. quercifolia* that were evaluated at concentrations of 100 and 50 µg/ml.....73

List of schemes

Scheme 3.1: General schematic representation of screening procedure for 37 crude extracts....59

Scheme 4.1: A flow diagram of experimental procedure for the isolation of pure compounds from *M. quercifolia*.....77

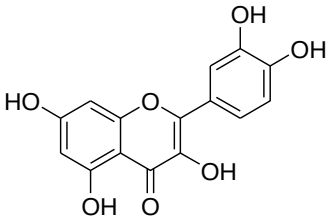
List of abbreviations

AD	Anno domini
AKT	Serine-threonine kinase
BC	Before Christ
cAMP	Cyclic adenoside monophosphate
CREB	cAMP-responsive element
CFR	Cape floristic region
CFNR	Cape flats nature reserve
^{13}C-NMR	Carbon nuclear magnetic resonance Consumer
CO_2	Carbon dioxide
CuA	Copper A
CuA	Copper B
DHI	Dihydroxyindole
DHICA	Dihydroxyindole-carboxylic acid
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPA	l-3,4-dihydroxyphenylalanine
DPPH	1,2-diphenyl-2-picrylhydrazyl
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPR	Electron paramagnetic resonance
EtoAc	Ethyl acetate
EtOH	Ethanol

FCS	Fetal calf serum
FDA	Food and Drug Administration
g	Gram
^{1}H-NMR	Proton nuclear magnetic resonance
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HQ	Hydroquinone
IC_{50}	Half maximal inhibitory concentration
KBG	Kirstenbosch botanical gardens
MAP	Mitogen-activated protein
MC1R	Melanocortin 1 receptor
MeOH	Methanol
MITF	Microphetalmia transcription factor
ml	Millilitres
α-MSH	Melanocyte stimulating hormone
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear magnetic resonance spectroscopy
OH	Hydroxyl radical
O_2	Oxygen
PBS	Phosphate buffer saline
PKB	Protein kinase B
POMC	Proopiomelanocortin
PBS	Phosphate buffer
ROS	Reactive oxygen species
rpm	Rounds per minute

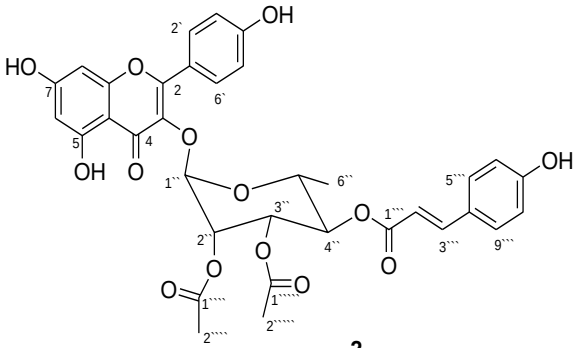
TLC	Thin layer chromatography
TRP-1	Tyrosinase related protein-1
TRP-2	Tyrosinase related protein-2
US	United States
UV	Ultraviolet
UWC	University of the Western Cape
WHO	World health organisation

LIST OF ISOLATED COMPOUNDS



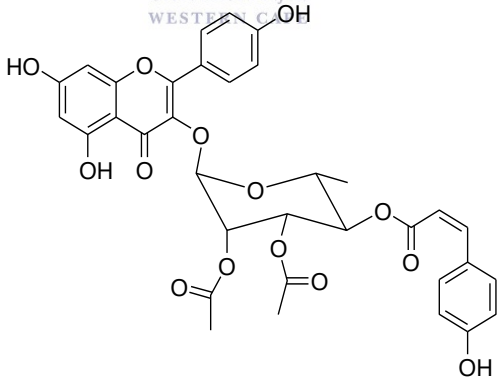
1

Quercitin (Compound 1)



2

Kaempferol-3-(2,3-diacetoxy-4-trans-*p*-coumaroyl)rhamnoside (Compound (2)



3

Kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside (Compound 3)

Chapter one

General introduction

Herbal plants have a significant historical use, population of many countries have relied on herbal plants for healthcare for thousands of years. The World Health Organisation (WHO) states that, 80% of the population throughout the globe are dependent on herbal medicine for primary healthcare needs (Lall and Kishore, 2014).

A large percentage of rural area dwellers in South Africa still depend on herbal plants for skin treatment, this is expected as South Africa inhabits more than 24 000 plant species and various cultural groups have recorded about 3000 plant species as part of their *materia medica* (De Wet *et.al*., 2013).

Apart from being used for healthcare, herbal medicine use in modern cosmetics is increasing quickly due to lack of side effects (Pratim *et.al*., 2005). Herbal extracts comprise a large category in pharmaceutical cosmetics found in the market today due to high consumer demand for natural products. Herbal products exert beneficial biological effects to the skin and provide various proteins, antioxidant, tannins, terpenoids, essential oils and alkaloids (Kapoor, 2005). Maintaining the integrity of the skin is important not only for youthful looks but for many important functions such as thermoregulation, prevention of excess water loss; protection against hostile external/foreign bodies of the environment e.g. bacteria, viruses, fungi, Ultraviolet radiation. The latter is the most common of the environmental elements. UV radiation is the major cause of collagen degeneration, accumulation of elastic fibres which collectively changes the composition of the skin (Moon *et.al*, 2010). Efforts are necessary to associate the modern cosmetology with bioactive ingredients based on traditional system of medicine leading to surfacing of novel cosmeceuticals for skin care.

1.1 Why investigate the CFR

The Cape Floristic Region (CFR), the smallest of the six recognised floral kingdoms of the world, is an area of extremely high and unique diversity and endemism. The CFR houses more than 9 000 vascular plant species, of which 69% are endemic (Odendaal, *et al*., 2008). The ecologically unique CFR contributes approximately 45% of the total trade in traditional medicinal plants that occurs in the Cape Town (Petersen, 2014) for instance *Hypoxis hemerocallidea* "African potato" is sold for use as a traditional tonic, benign prostate hyperplasia skin care, cosmetics; *Salvia africana-caerulea* 'wild sage' is used for respiratory and digestive ailments (Van Wyk, 2011). However, the use of many of these plants haven't been substantiated by scientific studies. Thus, given its remarkable diversity and confined area, CFR provides an excellent model system for scientific studies (Chnitzler, *et al*., 2011).

Also, the criteria for cosmetic scientific research have received very little attention in respect to the CFR, and there is clearly a need for a more rigorous investigation.

1.2 Statement of research

Pigmentation, over production of melanin, is due to a prolong exposure of the human skin to the UV radiation, resulted into over accumulation of free radicals in the body. This condition causes the stimulation of skin degenerative action of tyrosinase enzyme resulting into the formation of undesirable skin pigment. Enzymatic browning of agricultural products after harvesting is another detrimental effect of tyrosinase enzyme, thereby reducing the nutritional quality and downgrading the economic value of such products.

Efforts have been made by scientists towards combating the problem associated with skin pigmentation, however, the market is flooded with products that are inefficient whitening agents which do not suffer from low activity, high cytotoxicity and mutagenicity, poor skin penetration or low stability in formulation. This encouraged us for continuation of research on new anti-pigmentation agents.

The CFR plant species are diverse and are readily available for scientific exploration for their potential application as skin whitening agent. CFR is species dense and lack scientific exploration, thus it may contain species that are a source of depigmenting bioactive ingredients. Therefore, it is of great need to search further for potent natural tyrosinase inhibitors from CFR plant species with the intention of replacing inefficient and toxic products readily available in the market.

Aims of this study

This research work will be identifying anti-tyrosinase bioactive ingredients from plant species indigenous to South Africa. This will be achieved by randomly collecting plant species from great cape floristic region. These species will be extracted and assessed for their ability to inhibit tyrosinase enzymes *in vitro*. Then bioassay-guided isolation of active compounds using

Column chromatography and High performance liquid chromatography (HPLC) will be carried out. The active compounds will then be evaluated for their ability to inhibit tyrosinase

Objectives of this study

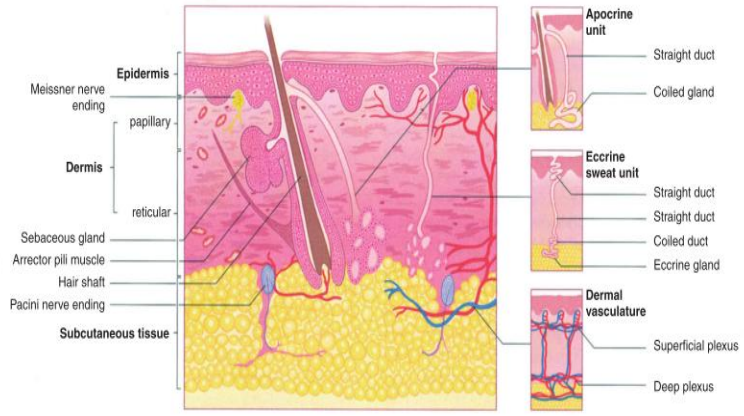
- Randomly collect, document and identify plant species from the CFR.
- Extract all collected plant species with methanol.
- Subject random plant species to wide screening against anti-tyrosinase and anti-melanogenesis related bioassays *in vitro and in vivo* respectively.
- Extract and assess the potential anti-tyrosinase bioactive compounds of the most active species and to evaluate the effects against biological activity.
- Characterise and identify the isolated constituents using spectroscopic techniques

Chapter 2

Literature review

2.1 The skin

The skin extends up to 16 000 cm^2 in surface area, (Igarashi *et al*., 2005) the average adult human skin has a mass value of 5-6 kg in total and constitutes 6% of the total body weight. This makes the skin the largest organ of the body in terms of surface area. At cellular level, the skin constitutes the following primary layers: the epidermis, which is supported and attached firmly to connective tissue, dermis and subcutaneous layer which is made of fatty and connective tissue as illustrated in figure 2.1



(Nicol, 2005)

Figure 2.1: Cross section of the skin

2.1.1 Skin anatomy

2.1.1.1 Epidermis

The epidermis is a stratified, squamous epithelial, nonvascular outer layer of ~ 75-150 µm; the thickness of soles and palms provides for ~ 600 µm. The epidermis terminates at the mucocutaneous junction. Different layers of the epidermis form at embryonic in human beings, so that at birth the functional barrier is already present. This layer harbours several cell

populations, keratinocytes are responsible for the synthesis of long, threadlike protein called keratin, the differentiation process known as keratinisation which starts from the basal layer, and cells migrate outwards to the surface of the skin (D'Orazio *et al*., 2013).

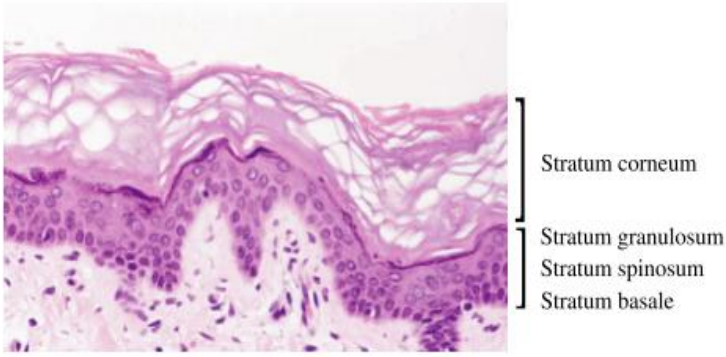
The epidermis consists largely of a constellation of cells, the keratinocytes. Morphologically, the epidermis is divided into 4 well marked layers (illustrated on figure 2.2):

Stratum corneum - consists of biochemically active, but non-viable cells known as corneocytes or horny cells, these cells are arranged in a cornified layer of 15-20 sheets and are (Costin & Hearing, 2007) devoid of cytoplasmic organelles and nuclei. (Tobin, 2012).

Stratum granulosum – This layer consists of 3–5 sheets that are granular, at this layer, keratinocytes are non-divisible that synthesise granules of a protein called keratinohyalin. (Tobin, 2012). These cells form a flattened layer and are pushed to the surface by cells that are progressively dividing. Simultaneously, their cell organelles and nuclei break down and their cell membranes become gradually more impermeable.

Stratum spinosum – also known as the squamous layer, stratum spinosum, is made up of 10-20 layers of keratinocytes and consists of various cell types that differ in subcellular characteristics and structure (Nicol, 2005; Tobin, 2012). Contains cells that are polyhedral in shape, the cells ability to divide is limited, the cellular organelles' dissolution is prepared as the cells are ready for terminal differentiation to form corneocytes the Langerhams' cells of the immune system which are sentinel cells derived from the bone marrow are also found in the stratum spinosa, they play an important in immunological reactions. (Costin & Hearing, 2007)

Stratum basale – also known as stratum germanitivum, the basal layer is made of 1-3 layers of cuboidal cells, whose cytoplasm and nuclei are large (Hendriks, 2005). This layer separates the dermis from the epidermis and thus its cells are attached to the basement membrane. It consists largely of mitotic and differentiating keratinocytes with stem cell-like properties. Other keratinocytes in this layer are Merkel cells, which possesses sensory functions and melanocytes which synthesise the melanin pigment (Tobin, 2012).



(Igarashi, *et.al*., 2005)

Figure 2.12: Four distinct layers of the epidermis under a light microscope.

2.1.1.2 The dermis

The dermis is a thick layer of fibroelastic connective tissues whose thickness ranges from 1-4 mm. The dermis is located between the epidermis and the subcutaneous layer ,they harbour lymphatic, vascular, neural and secretory apparatus (Nicol, 2005). The dermis is comprised of mainly two layers: the upper papillary layer that connects to the epidermis and the lower reticular layer (Tobin, 2012). The dermis is made of fibroblast which produce collagen, proteoglycan, elastin, macrophages and immunocompetent mast cells. The papillary layer is made of loosely packed collagen bundles which is responsible for toughness and strength, elastin is responsible for Collagen makes up approximately 70-80% dry weight of the dermis (Zaidi and Lanigan, 2010).

2.1.1.3 Subcutis

This is made of connective tissue and fatty tissues (Gothelf and Gehl, 2010)

2.1.2 Skin function

The skin is responsible for many important functions such as temperature regulation, prevention of excess water loss; protection against hostile external/foreign bodies of the environment e.g. bacteria, viruses, fungi, Ultraviolet (UV) radiation (Kanitakis, 2002). UV radiation is the major cause of collagen degeneration, accumulation of elastic fibres which collectively changes the composition of the skin (Moon *et.al*., 2010). In developing countries, a large number of population uses herbal preparations for treating a wide variety of skin ailments ranging from pimples, psoriasis, eczema, skin burns, aging etc.

2.1.3 Skin chromophores

Melanin is a polymorphous and multifunctional biopolymer that is represented by eumelanin, pheomelanin, mixed melanins (a combination of the two), and neuromelanin. Mammalian melanocytes produces two chemically distinct types of melanin pigments: black/brownish eumelanin and yellow/reddish pheomelanin. Even though they contain a common arrangement of repeating units linked by carbon-carbon bonds, melanin pigments differ from each other with respect to their structural, chemical, and physical properties (Costin, 2012). Melanin is the main pigment which accounts for skin pigmentation, even though there are four other pigments of the skin such as oxyheamoglobin, haemoglobin, carotene, melanin and melanoid (December & York, 1968). Melanin is the main chromophore in skin colouration; it is widely distributed in nature, varying from the smallest vertebrate to the largest mammal.

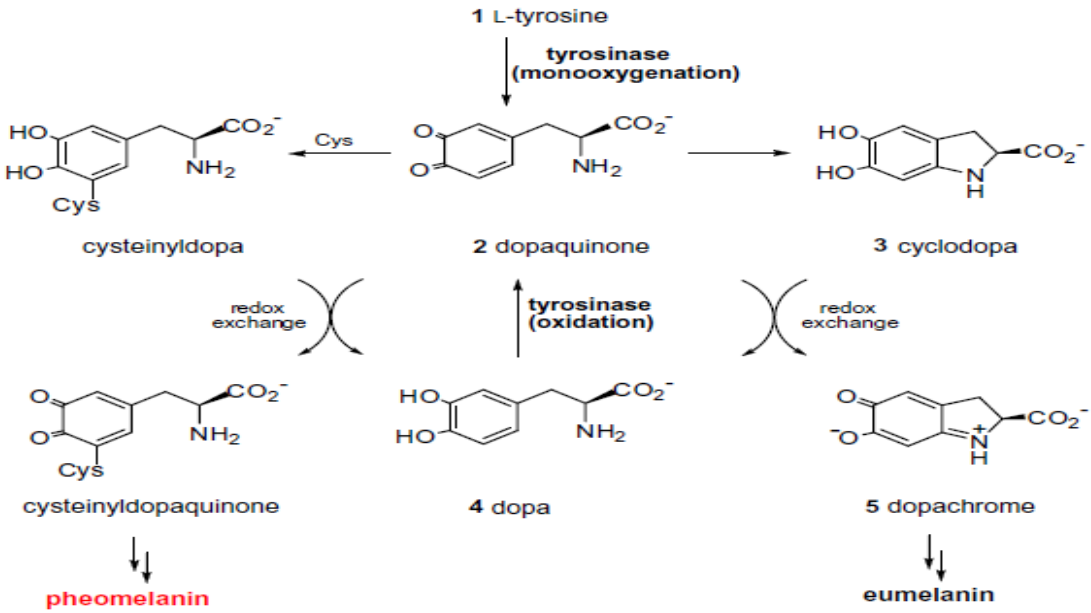
Cells of the epidermal layer known as melanocytes are responsible for the biosynthesis of melanin which is produced in melanosomes, round and membrane-bound organelle via a series of receptor-mediated, enzyme-catalysed, hormone-stimulated reactions through dendrites to keratinocytes (Nicol, 2005).

2.2 Melanin

2.2.1 Melanin biosynthesis

Melanin synthesis takes place within melanocytes, in specialised organelles which are membrane bound organelles known as melanosomes. They are lysosome related organelles and originate from endosomes and multivesular bodies (Amsen, 2009).

The first step during melanin production, L-phenylanine is either hydroxylated to L-tyosinase or L-tyrosine, is directly hydroxylated to L-dihydroxyphenylalanine (L-DOPA), which is a precursor to both catecholamines and melanins on different pathways. Subsequently, L-DOPA, which is also a substrate of tyrosinase, is oxidated to dopaquinone and is a precursor to both pheo and eumelaninogenic pathways. Thereafter, eumelanin is synthesised via a series of oxidoreduction reactions and result to conversion of dopaquinone to leukodopachrome. This reaction produces DHI carbox-ylic acid (DHICA) and dihydroxyindole (DHI) as intermediates. Pheomelanin synthesis also starts with dopaquinone, glutathione or cysteine is added, then dopaquinone is transformed to glutathionyl or cysteinyldopa to form pheomelanin.



Ramsdena and Riley, 2010

Figure 2.2: Biosynthesis reaction of two types of melanin: Eumelanin which is black/brown and pheomelanin which is red/yellow.

2.2.2 Melanogenesis regulation

Transcription of melanogenic enzymes is often controlled by hormonal signals. The predominant POMC-derived ligand produced in skin cells (melanocytes, keratinocytes, fibroblasts), endothelial and inflammatory cells by a peptide hormone, α-melanocyte stimulating hormone (α-MSH) which is essential for melanogenesis and the main intrisic regulator of pigmentation. Stimulation of the melanocortin 1 receptor (MC1R) by α-MSH which are bound on the melanocytes in the basal epidermis leads to intracellular secretion of the GTP-bound Gsα subunit, which then generates adenylate cyclase to produce cAMP (D'Orazio et al. 2013;Amsen 2009). This interaction leads to the activation of protein kinase A (PKA) pathway to phosphorylation of cAMP-responsive element (CREB) transcription factors which acts as a trnscription factor in various genes including the microphthalmia-associated transcription factor (MITF). Then expression of melanogenic enzymes are regulated by MITF in its phosphorylated form which results in the promotion of eumelanogenesis. Phosporylation of MITF is dependent on the mitogen-activated protein (MAP) kinases; its activity is induced by the interaction of keratinocytes produced SCF with c-Kit receptor tyrosinase. (Ebanks et al. 2009;Videira et al. 2013).

2.3 Tyrosinase

The presence of copper is only available in trace amount in living organisms and it functions in electron transfer facilitation. On basis of their spectroscopic properties, there are three different types of copper. Tyrosinase is a metalloenzyme which consists of a binuclear, type 3 copper in the active center. It is responsible for manifestation of two reactions in melanin

biosynthesis including oxidation of monophenols and *o*-diphenols to reactive *o*-quinones that undergoe further oxidation to form melanin via a free radical coupling pathway (Gasparetti, 2012). Tyrosinase catalyses oxidation of phenolic compounds found in fruits, fungi and vegetables shown by browning on the fruits or vegetables that are exposed to air.

2.3.1 Distribution and classification of tyrosinase

Tyrosinase is ubiquitous in biological systems; it is found in animals, plants, bacteria, fungi as well as in human beings. Tyrosinase is the most well studied copper oxygenase. The very first biochemical analysis of tyrosinase was carried out in 1895 on the mushroom *Russula nigricans* whose cut skin initially turns red then black upon exposure to the air, this phenomena was observed by french naturalists, Bertrand and Bourquelot (Gerritsen 2004). The well characterised tyrosinases are isolated from the fungi *Neurospora crassa*, A*garicus bisporus* and the bacteria *Streptomyces glausescen* (Ramsden and Riley., 2014).

2.3.2 Properties of various tyrosinases

Streptomyces glausescen* and *Streptomyces glaucescens - The first bacterial tyrosinase was isolated from *S.glausescen*, they consist of monomeric protein with a low molecular weight of ~ 30 kDa. Tyrosinases found in these bacteria are secreted out of the cell membrane, that is where extracellular melanin synthesis takes place.

Neurospora crassa - Tyrosinase from this fungi is also a monomer with one subunit of 46 kDa. *A. bisporus* tyrosinase is a heterotetramer with two light subunits of ~14 kDa and two heavy subunits of 43 kDa, that make a total molecular mass of 140 kDa.

2.3.3 Physiological role of tyrosinase

The building blocks of melanin are quinones that are synthesised by tyrosinase. Melanin pigments are phylogenetically spread out in nature from bacteria to humans. Melanogenesis fulfils a number of essential physiological functions in different organisms as briefly discussed below.

Melanogenesis in mammals is related to the pigmentation of the eyes, hair and skin, hence, defective tyrosinase leads to hypo or hyperpigmention such as albinism, vitiligo, freckles, lentigines, melasma etc. In mammals, pigmentation is influenced by many factors but the dispersion and degree of melanin is the most important. The distribution of melanin which starts during embryogenesis is under strict genetic control, in animals such as tigers, zebras, leopards etc, it leads to unique yet interesting patterns. Mammalian melanin pigment also play other important roles such as sexual attraction, camouflage and thermoregulation. Human melanin assumes the role of photoprotection by absorbing and radiating UV rays that might cause DNA damage, carcinogenic tumors on the skin and formation of free radicals.

Bacterial tyrosinase activity participates in phenolic compound detoxification, and also, the formed melanin pigments help prevent the cells from dehydrating. Melanin, in plants, also assist in protection by creating a barrier and a toxic environment for the attacker. Tyrosinase and catechol oxidase are responsible for forming catechol melanin which are specifically produced in plants and are formed by derivatives of catechol quinones. Cathechol melanin protects injured plant tissues from microbial infections and insects. In insects, the role of tyrosinase activity is wound healing, sclerotisation of the cuticle and serves as a defense mechanism against parasites.

It has also been suggested that tyrosinase activity in fungi is involved in mechanism of resistance to stressors, it avoids cell lysis by making cell walls resistant to hydrolytic

enzymes, it defends against virulence mechanism. Additionally, melanin pigments in fungi are synthesis during sexual differentiation and spore formation.

2.3.4 Structural mechanism of tyrosinase

Elucidation of monophenolase and diphenolase activities on tyrosinase on a structural level have been reported numerously in literature. Different researchers have proposed various structural mechanisms, however, none of them have any experimental backing. Therefore, even after decades of research, the mechanism of action is still not clear. Below is one of the proposed and accepted mechanism.

The active sites of tyrosinase exists in three different forms during catalytic cycle such as: the two oxidised forms (oxy and met form) and an unstable reduced form (deoxy form) which possess different binuclear structures of the active sites. In the monophenolase cycle, the monophenol only reacts with the oxy form and binds to the axial position of one copper in the oxy form. The atom of the oxygen ($O_{2)}$ molecule that is bound in the oxy form as a peroxide is integrated in the ortho position of the monophenolase substrate (Gasparetti, 2012). The O_2 acts as a catalytic base which subtracts the hydroxyl group of a monophenolase substrate. The monophenol is hydroxylated by the bound peroxide and the resulting product is o-diphenol which is further oxidised to generate o-quinone and subsequently results in the deoxy from. The molecule of O_2 has high affinity to the deoxy form and binds to the active site to produce the oxy form of tyrosinase.

In the diphenolase form, ortho diphenol, both the oxy and met form react with the o-diphenol and oxidises it to form ortho quinone. Then the o-diphenol reacts with the oxy form that is oxidised to o-quinone, generating the met form of the enzyme. Another o-diphenol is

transformed in o-quinone by the met form and is further reduced to the bicuprous deoxy form (Chang, 2009).

2.3.5 Lag phase and autoactivation

The initial monooxidation of phenolic substrates is very slow and oxidation accelerates at a slow rate to its maximum velocity and this period is known as the lag phase. Lag phase characterises the activity of the monophenolase, it is entirely dependent on factors such as the presence of the hydrogen donor, the enzyme and the concentration of the substrate.

2.3.6 Active centre of tyrosinase

Early tyrosinase investigations by Kubowitz., 1938 established the similarity of hemocyanin and tyrosinase which both had copper in their active sites, the copper ions are bound by one carbon monoxide molecule, suggesting the presence of binuclear copper. Furthermore, in 2006, Matoba, *et al.*, studied the crystal structure of tyrosinase from *Streptomyces castaneoglobisporus* which provided an insightful architecture of the enzyme. Tyrosinase and its homologous proteins, hemocyanin and catechol oxidase share very similar active sites. the arrangement of the active sites within these enzymes are characterised as type 3 copper proteins (Ismaya, 2002). The active centre is held by two copper molecules; each of the copper coordinated by three nitrogen donor atoms of histidine residues that are located in α-helices. The copper pair serves as interaction site for tyrosinase with both molecular O_2 and its substrate. Three atoms of ε-nitrogen from His^{38}, His^{54} and His^{63} are bound in the motifs of copper A (CuA), whereas His^{190}, His^{194} and His^{216} are bound in the motif of copper B (CuB) (Nithitanakool, *et al*., 2009).

Type-3 copper exists in different forms depending on the oxidation state of the copper ion. For instance, the two oxygenated forms (oxytyrosinase, E_{oxy};[Cu(II)-O_2^2-Cu(II)]) and mettyrosinase (E_{met};[Cu(II)-OH^--Cu(II)]), the reduced and unstable form deoxytyrosinase (E_{deoxy}; [(Cu(I) Cu(I)]) and the half-mettyrosinase ($E_{h\text{-}met}$;[Cu(I) Cu(II)]) (Zhou *et al*., 2014)

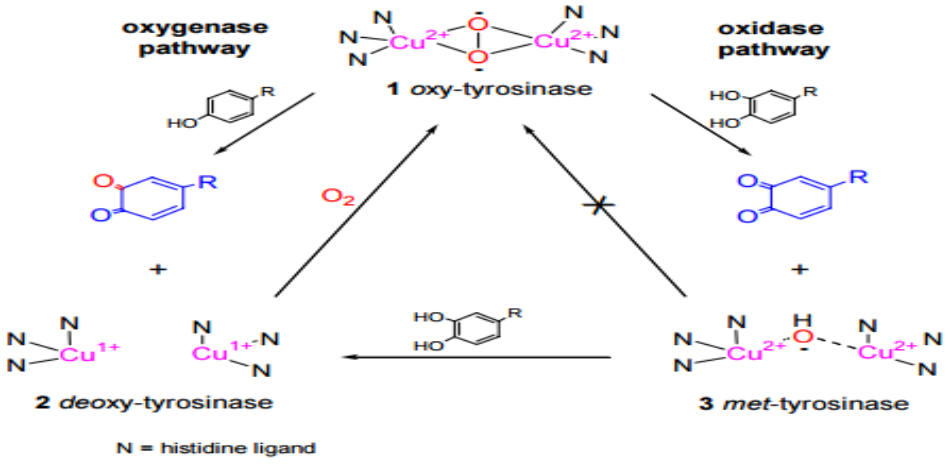
In the E_{met} form, each of the Cu(II) ions are coordinated by 3 N_{His} ligands and the Cu2 bridging atom. Native tyrosinase occurs mainly as met-tyrosinase , approximately 85 to 90 % of tyrosinase is in the E_{met} form (Ramsdena and Riley, 2010). Mettyrosinase, the resting form of the enzyme, consists of two tetragonal Cu(II) ions and is devoid of electron paramagnetic resonance (EPR) signal, this is due to antiferromagnetic coupling between the two copper ions of the E_{met} form thus making EPR silent. Consequently, the antiferromagnetic coupling requires a superexchange pathway associated with endogenous bridging amino acid residues or exogenous bridging ligand (Kim and Uyama, 2005). Mettyrosinase is in the stable Cu(II)2 state, thus it is unable to bind molecular oxygen, therefore monooxygenase activity is not expressed, however, the monooxygenase substrates can bind to the active site (Ramsden and Riley, 2014).

The E_{deoxy} [(Cu(I) Cu(I)], deoxytyrosinase) form, which is also not EPR detectable, contains two copper (I) atoms a co-ordination arrangement similar to that of the met form, but lacks the hydroxide bridge. Each of its copper ions is coordinated by 3 histidine ligands in an approximately trigonal planar geometry (Tepper, 2005). Deoxytyrosinase is formed when 2 electrons are removed from E_{met}, followed by reversible binding of O_2 as a peroxide giving a charge of 2^+ to each of the Cu ions which give rise to the E_{oxy} form.

The E_{oxy} [Cu(II)-O_2^2-Cu(II)], oxytyrosinase) form, similar to the E_{met} form, consists of two tetragonal copper (II) ions, coupled through an endogenous bridge, each coordinated by two

strong N_{His} ligands. The exogenous oxygen molecule is bound as peroxide and bridges the two copper sites (Chang, 2009). E_{oxy} is the primary oxidising form of the tyrosinase and oxidises phenols by a monooxygenase mechanism and oxidises catechols by an oxidase mechanism. As a result, phenols and catechols are oxidised by E_{oxy} to *ortho*-quinones by separate oxidative cycles in the presence of dioxygen. The most important function of tyrosinase is the oxidation of phenols to *ortho*-quinones by E_{oxy} and it is by this process that L-tyrosine is converted to dopaquinone in melanin biosynthesis. Phenolic oxygen binds to CuA, followed by electrophilic monooxygenation of the ring which leads to a complex (substrate binds to both copper atoms) that undergoes homolytic dissociation, yielding ortho-quinone and E_{deoxy}. Then E_{deoxy} is bound to oxygen to give E_{oxy}, this phenol-oxidation cycle continues until the substrates are depleted (Ramsden and Riley, 2014).

In the $E_{half\text{-}met}$ [Cu(I) OH^--Cu(II), half-oxidised tyrosinase] form, only one of the two coppers is oxidised, as a consequence, it is EPR detectable (Tepper, 2005).



(Ramsdena and Riley, 2010)

Figure 2.3: Active center of tyrosinase.

2.4 Cosmetics

Beauty, the quality that gives pleasure to the senses, is perhaps the desire of every human being on earth. Some are born beautiful and some acquire beauty. Aesthetic appearance has always been a matter of primary importance. The word 'beauty' is not only related to women, as is often thought, but men also use cosmetic products. The European Directive 93/35/EEC (European Commission) defines cosmetic products as "any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good conditions (Kole, *et al*., 2005).

The original term for 'cosmetics' is 'kosm tikos' which was derived from the Greek; it means "having the power to arrange, skill in decorating giving kosmein." Kosmein conveys the thought of caring for, adornment, and producing harmony between body and mind. The cosmetics is recorded through history, originally, the concept of cosmetics was associated with fighting, religion and hunting and superstition., then later associated with medicine. In prehistoric era (3000 BC), man used colours to paint their skin for decoration in rituals to for mating and attracting animals they wished to hunt. Additionally, they would paint their body to invoke fear in their enemy, whether animal or man (Sumit, *et al*., 2012). The origin of cosmetics forms a continuous narrative as they develop, throughout the recorded history of mankind. Cosmetics have always been used with the three essential goals, i.e. to decorate the body which then enhances physical appearance, to conceal flaws in the integument and to improve upon nature (Knowlton, 2001). Also, it is said that the term originates from the word 'cosmetae' used to describe Roman slaves whose job was to bathe men and women in perfume. Herbal Cosmetics, referred as Products, are formulated, using various permissible

cosmetic ingredients to form the base in which one or more herbal ingredients are used to provide defined cosmetic benefits only, shall be called as “Herbal Cosmetics”. Herbs do not produce instant cures (Joshi and Pawar, 2015).

2.4.1 Cosmetic use in medieval Egypt

Use of cosmetics is dated back to the first dynasty of Egypt in c 3100-2907 BC (Aboelsoud, 2010) , they also were the first to recognize the health-promoting properties of cosmetics. The Eber papyrus, which is an Egyptian medical papyrus of herbal preparations, written in 1600 BC, made frequent references to several cosmeceutical-based products. Bathing was made a custom in ancient Egypt, their favourite formulation was the use of milk and honey (Wanjari and Waghmare, 2015) this formulation was also used by the queen of Egypt, Cleopatra, for soft and supple skin, milk contains lactic acid and thus acts as a mild exfoliant and honey is a humectant. Some of the herbs for skin disorders that are recorded in the Eber papyrus are *Aloe vera*, *Acacia nilotica*, *Malus sylvetris, Curcumae longoma* (turmeric) and many others (Aboelsoud, 2010).

Throughout the ages, Egyptians were very skilled in the use of face, body and eye paint, ointments and body oils, for example, they used mud and herbs, as far back as 69 BC, for the face to draw out toxin (Oumeish, 2005). They practiced the art of embalming, hair dyeing and waving, , make-up and hair dressing, henna was used to dye paint nails, soles of feet, palm of hands and to dye hair, the principle oil used for mummification was cedar oil and was considered the most sacred of all pure oils. Make-up for the eyes was also common practice, they painted the underside of the eyelid green with malachite ($CuCo_3$-$Cu(OH)_2$), the eyebrows, lids, lashes were made black by painting with kohl whose application was typically in a stick form. Kohl’s dark colour was characterised by PbS, galena or stibnite (Sb_2S_3) and is made of burnt almonds, crushed antimony, ash, ochre, oxidised copper, lead and chrysocolla.

Kohl was also believed to reduce the sun glare, restore poor eye sight and minimise eye infections (Chaudhri and Jain, 2009).

2.4.2 Cosmetics in ancient Europe

Approximately two centuries before Christ, the Greeks used unscented oils for skin treatment after bathing and for massage. They would also dry and grind flowers and use the powder for perspiration. Use of make-up was not restricted to women but men also used it freely. The Greeks seem to be the first to attempt to study dermatology and to consider care of the skin a serious matter. The Romans learned about the aesthetic of personal health care from the Greeks, additionally, they added perfumes to their baths or they would apply oils to their skin after their bath. Nero, a Roman emperor, is said to have sprayed his guests with a mist of perfume from the ceiling of the dining hall. His wife used white lead, ($PbCO_3.(OH)_2$) and chalk ($CaCO_3$), to lighten her skin complexion, kohl for her eyelids and lashes, red paint for her cheeks and lips, flour and butter to cure skin imperfections, and pumice stone to whiten her teeth.

Romans adopted most of their medical knowledge from the Greeks, just like the Geeks, they also applied oil-based perfumes to their skin after bathing, they even applied them to weapons. Also, Roman women would use walnut extracts for darker hair and antimony as eye shadow. For facial masks, they would make a mixture of banana, rose water and oatmeal.

Mediterranean countries and Italy used warm olive oil to treat dry and aged skin, then they would rinse off after bathing.

2.4.3 Cosmetic use in Asian countries

Around 3000 BCE, Chinese used gum arabic, beeswax, gelatin, and egg to paint their fingernails. The colors used were a representation of one's social class, for example, Chou dynasty royals wore gold and silver; later royals wore black or red. The lower classes were permitted to color their nails but prohibited from wearing bright colors.

Beauty “painting” became all the rage in ancient China when legend has it, a plum blossom drifted down onto the forehead of a princess, leaving a floral imprint. Ladies of the court were so impressed they too began to decorate their foreheads with delicate little plum blossom designs and soon it became commonplace. Chinese used the word ‘heang’ to represent perfume, fragrance and incense. Heang was aesthetically divided in six moods, namely, tranquil, beautiful, refined, luxurious, reclusive or noble. During the T’ang dynasty, the upper class Chinese made lavish use of fragrances. This began in the 7th century AD which continued until the end of the Ming dynasty in the 17th century (Chaudhri and Jain, 2009)

In medieval Japan, geisha’s would crush safflower petals to paint their eyebrows, edges of the eyes and lips. They also used sticks of *bintsuke* wax which is a softer version of the sumo wrestlers’ hair wax, as a makeup base. To colour the face and back white, rice powder was used while rouge contoured the eye socket and defined the nose. The geisha also used droppings of birds as the base for lighter colors.

2.4.4 Cosmetics in ancient India

In ancient India, there is evidence of highly advanced ideas of adornment and a range of cosmetic practice both by men and women. The use of cosmetics was not only directed towards enhancing the physical appearance but also towards achieving merit, happiness and longevity with good health. The earliest evidence of cosmetic use dates back to great epic Mahabharata, where the Pandavas were secretly in exile (Patkar, 2008).

2.4.5 Cosmeceuticals

The concept of cosmeceutical was created by the founder of U.S Society of cosmetic chemist, Raymond Reed, it was later popularised in the 1970s by an American dermatologist, Albert Kligman. Even though the Federal, food, drug and cosmetic act (FDA) doesn't recognise this term but it is defined as the cosmetic-pharmaceutical hybrid that is intended to enhance health

and beauty of the skin by exerting specific benefits ranging from acne-control, skin aging, sun protection and many others (Joshi and Pawar, 2015). Cosmeceutical has become the fastest growing segment of the skincare industry. Cosmeceuticals are cosmetic products with active ingredients which exert drug-like benefits, however, the term cosmeceutical has no legal value. These active ingredients promote beneficial actions and provide protection against degenerative skin disorders. Cosmeceuticals improve skin appearance by delivering health promoting nutrients to the skin which help improve skin texture, tone and radiance.

Although natural ingredients have been traditionally used since human civilisation for skin care purposes, they are becoming more prevalent in modern-day formulations. The term "natural" is defined as 'something or an ingredient that is produced by the nature or found in nature and is directly extracted from plants or animal products'. Sources of natural ingredients can be herbs, fruits, flowers, leaves, minerals, water and land. The effect of natural ingredients in skin care products depends on their *in vitro* and *in vivo* efficacy and the form of dermatological base where they are integrated (Ribeiro, *et al*., 2015)

2.5 Plants used for skin disorders in South Africa

Herbal medicines take central stage to African tradition and are also deeply rooted in the treatment of skin ailments and boosting physical appearance. The use of herbs in Africa is as old the first settlers, but many of the African dialects had no written form, which is why there is no written record of the plants that were used at that. Documentation of plants started to take shape when white settlers arrived even though they considered herbal remedies as primitive and inferior to contemporary medicine (Shumba, *et al*., 2009).

Ethnobotanical studies have documented the use of plants by traditional healers for the treatment of various skin ailments. Different plant parts commonly used as cosmetics or face

masks, known as umemezis, are widely used in southern Africa for skin problems like inflammation, wounds, burns, eczema and puberty acne (Lall and Sharma, 2014)

De Wet, *et al*., 2013 conducted a study in Northern Maputaland where 87 individuals were interviewed for plants used for dermatological diseases. The skin diseases included abscesses, acne, burns, boils, incisions, ringworms, rashes, shingles, sores, wounds and warts. The 47 plant species belongs to 35 families, with Fabaceae (*sensu lato*) (eight species) being the most frequently represented family, followed by Asteraceae and Solanaceae (three species each), Anacardiaceae (two species) and the remainder, had one species each. Plant species from the family Fabaceae (*sensu lato*) are well known world-wide for the treatment of wounds. The following nine plant species have been recorded for the first time globally for the use to treat skin disorders; *Acacia burkei*, *Brachylaena discolor*, *Ozoroa engleri*, *Parinari capensis*, subsp. capensis, *Portulacaria afra*, *Sida pseudocordifolia*, *Solanum rigescens*, *Strychnos madagascrariensis* and *Drimia delagoensis*. Although *A. burkei*, B. *discolor*, *O. engleri*, *P. capensis*, *P. afra*, *S. madagascariensis* and *D. delagoensis* have been recorded for other medicinal uses; *S. pseudocordifolia* and *S. rigescens* have no other docu- mented medicinal uses. The indigenous herb *Senecio serratuloides* was by far the most frequently used species by the interviewees to treat skin disorders (17 interviewees). This is a well recorded medicinal plant in South Africa for the treatment of various wounds and dermatological ailments. The second most mentioned species was the indigenous tree *Tabernaemontana elegans*, followed by *Sclerocarya birrea* and *S. madagscariensis*, and *Dialium schlechteri*.

2.6 Tyrosinase inhibitors

The main causative agent for over-expressed melanogenesis is UV radiation of solar light, UV may directly or indirectly act on melanocytes through the release of keratenocytes derived factors named α-MSH (α-Melanocyte stimulating hormones). Melanogenesis can be inhibited, at least partially, through tyrosinase deactivation (Khan, 2007). A number of tyrosinase inhibitors, especially from natural sources have become increasingly important (Chang, 2009). Plants are very rich in bioactive chemicals and consist mostly of no side effects, hence the increased interest in identifying tyrosinase inhibitors (Nithitanakool, 2009).

Below, tyrosinase inhibitors from natural sources are discussed.

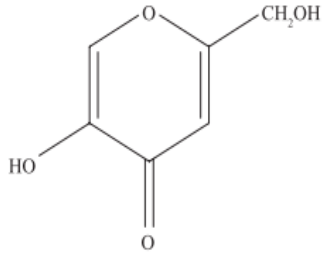
Hydroquinone, kojic acid, arbutin, magnesium ascorbyl phosphate, licorice extract, aloesin, azelaic acid, soybean extract, and niacinamide

2.6.1 Kojic acid

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is a secondary metabolite that is produced in aerobic conditions by various microorganisms, viz. *Aspergillus, Acetobacter* and *Penicillum* (Briganti, *et al*., 2003). Kojic acid was discovered by K. Saito in 1907 in the strain of *Aspergillus ozyrae* that was grown on steamed rice. is a potent and mostly studied inhibitor of tyrosinase and is used widely on a commercial scale as a cosmetic skin whitening agent; it also acts as an antioxidant, bacteriostat, chelates metal ions and prevents photodamage (Ho, *et al*., 2007). Additionally, kojic acid's application is widespread in food, medicine, cosmetics, chemical and agricultural industries. In the medical industry, it is reported to treat antibiotic, treating bacterial, fungal, leukemic and microbial problems. In the food industry, the application of kojic acid include prevention of undesirable browning of crabs, crustaceans, vegetables and fruits during storage. In the chemical field, since kojic acid forms a complex of deep red when

reacted with traces of $Fe3^{+}$, it can be used as an analytical tool for determining cations, also, Kojic acid (Zirak & Eftekhari-Sis, 2015). In the cosmetic field, it is used at concentrations ranging from 0.1% to 2% as a skin-whitening agent; it chelates the copper ion in the tyrosinase through the 4 carbonyl and 5-hydroxyl group

Kojic acid has a molecular mass of 142.11(Burnett, *et al*., 2010)**.**



(Burnett, *et al*., 2010)

Figure 2.4: Chemical structure of kojic acid

2.6.3 Hydroquinone

Hydroquinone-o-β-D-glucopyranoside is a potent skin-whitening agent with high melanocyte-specific cytotoxicity (Gasparetti, 2012). It is regarded as the gold standard in treatment of pigmentation disorders. Hydroquinone is a phenolic compound which is found in coffee, tea, beer, berries, wheat but the liver detoxifies it into an inert compound (Ebanks, *et.al.*, 2009).

Hydroquinone is an effective and widely used skin lightening agent for the treatment of skin pigmentation such as melasma, postinflammatory hyperpigmentation, and other hyperpigmentation disorders. It acts by inhibiting the conversion of tyrosine to melanin.

Hydroquinone is available over the counter in the United States in strengths up to 2% and by prescription in strengths of 3 to 4%. Higher concentrations are available through

compounding pharmacies. Four to 6 weeks of monotherapy with hydroquinone is generally required before whitening effects are visible. Several products are available in the market which contain hydroquinone in combination with vitamin C, tretinoin, retinol, glycolic acid, and fluorinated steroids. The period necessary for these products to take effect varies.

While it is used in depigmenting medication, its use in cosmetic preparations has been discouraged due to numerous side effects, including skin irritation (Gasparetti, 2012)

The most common side effects associated with hydroquinone are skin irritation and contact dermatitis, which can be treated with topical steroids. A rare side effect is the development of exogenous ochronosis, a sooty hyperpigmentation in the treatment area, which can be extremely difficult to reverse. Although this adverse event is uncommon with normal use, it may result from hydroquinone use for extended periods of time. Alternating the use of hydroquinone in 4-month cycles with one of the natural depigmenting agents listed below can prevent or reduce side effects, such as irritation or even exogenous ochronosis (Rendon and Gaviria, 2005).

2.6.4 Arbutin

Arbutin (hydroquinone-O-β-D-glucopyranoside) is a derivative of hydroquinone, a plant derived compound found in several plants including blueberries, cranberries, pears and wheat. Arbutin is used as an effective treatment of hyperpigmentary disorders, and demonstrates less melanocyte cytotoxicity compared to hydroquinone. The compound inhibits melanin synthesis by competitively and reversibly binding tyrosinase without influencing the mRNA transcription of tyrosinase. It also inhibits melanosome maturation, possibly by its influence on DHICA polymerase activity and Pmel-17 protein (Ebanks, 2009).

1.6.5 Liquorice

2.7 Pigmentation disorders

The colour of the skin is principally determined by melanin pigment whose synthesis occurs in melanocytes and is restricted to melanosomes. Melanosomes contain key enzymes, such as tyrosinase and tyrosinase-related proteins (TRPs). Racial and ethnic differences in skin colour are related to the number, size, shape, distribution, and degradation of melanosomes. Changes in skin pigmentation, which induces significant cosmetic problems with effect on quality of life, could result from abnormalities in the formation of melanosomes, melanosome melanisation, melanosomes transfer to keratinocytes with/without degradation in lysosome-like organelles, and the number of melanocytes. Abnormal pigmentation conditions can be divided into two types, that is, hypermelanosis or hypomelanosis, which involve excessive or insufficientmelanin in skin, respectively (Lee and Noh, 2013). Some of the pigmentation disorders are discussed below.

2.7.1 Hyperpigmentary disorders

2.7.1.1 Melasma

Melasma (chloasma) is pigmentation disorder that is acquired due to melanogenesis dysfunction. It is more common in females than in males. The disease is also known as the mask of pregnancy or chloasma. The cause is still unknown, although there are known to be triggering factors, such as pregnancy, menopause and oral contraceptive use. Clinically, brown macules with distinct and irregular margins are seen. These are generally symmetrical and oftеn on the face when exposed to the Sun. There are two subtypes of facial melasma depending on the site of involvement, centrofacial and peripheral. It may also be seen in extrafacial regions such as the arms, forearms or cervical and sternal regions. The course

involves irregular, symmetrical hyperchromic skin discoloration. Involvement in these regions is generally seen in advanced age in patients undergoing the menopause or receiving hormone replacement. Diagnosis is generally clinical. Wood's light and histopathology may be employed on rare occasions (Engin, 2015).

2.7.1.2 Cafe au lait macules.

Cafe au lait macules Cafe au lait macules present as uniformly pigmented macules or patches with sharp margins. Size varies from small confetti macules to large irregular plaques of numerous centimeters. Cafe au lait macules are often present at birth. In normal individuals, only 1 or 2 lesions are usually observed. Light microscopy examination reveals increased epidermal melanin with normal number of melanocytes. Ultrastructural examination shows increased pigment. Giant pigment granules (macromelanosomes) that are a feature of cafe au lait macules of the neurofibromatosis are absent in sporadic cafe au lait macules (Passeron, *et.al*., 2005).

2.8 Cape Floristic Region (CFR)

Extending from 31° and 34°30'S latitude is the biogeographic region located at the South-western tip of South Africa known as the CFR (Manning and Goldblatt, 2012). The CFR boasts one of the richest botanical diversity with approximately 9000 species vascular plants which are native to this area. When compared to other floras of the African region, the Cape flora is unexceptionally species rich especially for its size (it comprises land area of 90 000 km^2). It comprises three of the seven largest plant families in the CFR. For an arid region, the largest families are Asteraceae and Fabaceae which make-up 20% of the total species combined (Manning and Goldblatt, 2000).

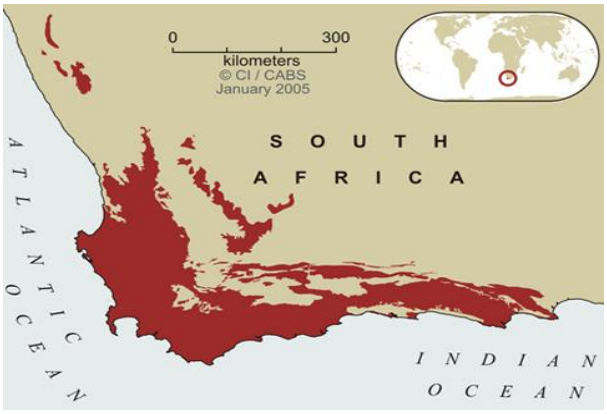


Figure 2.6 Map showing geographical distribution of the Cape Floristic region biodiveristy hotspot.

2.9 Myricaceae

2.9.1 Biological description

The family Myricaceae is a member of the Division Magnoliophyta, Class Magnoliopsida, Subclass Hamamelidae, and is placed in the order Myricales. Several authors placed Myricaceae in the order Myricales alone due to distinctive characteristics including single orthotropous ovule, entire leaves with resinous balloon-like glands and the unilocular ovary (Thorne, 1973). Subclass Hamamelidae is comprised of Myricales and six other orders, Fagales, Juglandales, Leitneriales, Urticales, Hamamelidales and Casuarinales. This subclass is phylogenetically grouped in an order characterised by strongly reduced, typically unisexual flowers which can lack or produce a poorly developed perianth.

Initially, the taxonomic treatment of Myricaceae was monographed by Chevalier in 1901, the family was divided into three genera namely, *Myrica, Gale*, and *Comptonia*. The genera was classified mainly on basis of the nature of the fruit exocarp and different morphological developments of the pistillate flower bracts during fruit maturation. (Lutzow-Felling *et. al*. 1995). Currently, taxonomist recognises four genera of the family including, *Morella* Loureiro, *Myrica* L, *Comptonia* L'Heritier ex Aiton and *Canacomyrica* Guillaumin (Knapp, 2002)

Myricaceae, a small sub-cosmopolitan consisting primarily of shrubs. The flowers of trees are unisex borne in catkins, entire leaves, peltate glands, single orthotropous ovule and a unilocular ovary. With the exception of two monotypic genera, Comptonia and Canacomyrica, the species of Myricaceae have traditionally been referred to the genus Myrica which is of Linnaean origin.

However, Myrica was split into two genera, Myrica *sensu stricto* and Morella, based on phylogenetic analysis of nuclear ITS and chloroplast trnL-F sequence data and morphological differences such as deciduous or evergreen, dry fruits or fleshy fruits; and sunken stoma or not. In Myricaceae, only one genus and four species including *M. adenophora*, *M. rubra*, *M. Nana*, *M. esculenta* and are located in China. These species are easily recognised by their distinct tomentose branchlets and petioles, whereas *M. rubra* and *M. nana* are glabrous or sparsely pubescent. Previous phylogenetic studies strongly supported the monophyly of these four species, but the relationships among *M. rubra*, *M. nana* and *M. adenophora* were not resolved. The genetic diversity and population structure of wild *M. rubra* populations are poorly known (Liu, *et. al*., 2015).

2.9.2 Genus Myrica

When Linnaeus named the genus *Myrica* in 1753,the following five species were known to him: *Myrica gale, M. cerifera, M cordifolia, M quercifolia* and *M. asplenifolia.* There are significant morphological differences between *Myrica gale,* whose fruit is smooth and dry, and *M cerifera, M. cordifolia* or *M. quercifolia,* all of which have papilose waxy fruits. These differences were not recognised formally until the genus was split by Spach in 1984, adopting *Gale dumort* to accommodate *Myrica gale,* retaining *Comptonia,* and placing the remaining species in *Myrica.* In 1901, Chevalier published a monographic study of the family with the division of *Myrica* into three sections (see table 2.1). The majority of literature published since 1901 has recognised only *Myrica* and *Comptonia.*

Ealier taxonomists gave recognition to the differences between *M. gale* and the majority of other species, by recognising the subgenera *Gale* and *Morella* . Some authors (Baird, 1968; Wilbur, 1994) expressed the opinion that *Myrica* (s.l.) should be split into two genera in order

to distinguish between these two groups of species and stated that these should be named *Myrica* and *Morella.* However, this would entail transferring the majority of species to *Morella.* (Verdcourt and Polhill, 1997) made a proposal to conserve the generic names *Myrica* and *Gale,* in order to give these groups generic status whilst minimising the number of name changes that would be required. In line with nomenclatural rules their proposal would involve changing the type specimen of the genus *Myrica* from *M gale* to *M. cerifera. Myrica gale* would then change its name to *Gale belgica* and become the type of the genus *Gale.* However, the proposal was rejected (Brummitt, 1999) and *M. gale* remains the type of the genus *Myrica.* Thus, the generic name *Morella* must be adopted for the majority of those species formerly treated as *Myrica.* Most of the new combinations have now been made. The nomenclature adopted herein follows Wilbur (1994) in recognising *Myrica* s.s., *Morella* and *Comptonia*, with the inclusion of *Canacomyrica.*

2.9.2.1 Distribution and ethnopharmacological uses of Myrica

The genus Morella is the largest of the Myricaceae family, it is described to consist of approximately 50 species with more than half of the species occurring in Africa, the remainder occurs in Europe, North America and Asia (Silva, *et. al*. 2015). The genus Myrica is well represented in South Africa, 15 of these species occur in South Africa in the Cape Peninsula region, 11 in tropical Africa, 6 in the Mascarene Island and the distribution of the remainder occurs throughout the Northern Hemisphere (Lutzow-Felling, *et. al*. 1995).

Seven of which were described from the Cape Peninsula (in the Cape Town region): M. *ovata*, M. *cordifolia, M. burmanni, M. humilis, M. diversifolia, M. dregeana, M. quercifolia, M. elliptica, M. kraussiana, M. zeyheri, M. conifera* and *M. braifolia* Burm. Eleven occur in tropical Africa, six in the Mascarene Islands, and the remainder extensively distributed

throughout the Northern Hemisphere. Distribution and ethnobotanical uses of *Morella* and *Myrica* is further discussed in table 2.1 below.

Table 2.1 Distribution and ethnopharmacological uses of *Morella* and *Myrica* species

Species name	Distribution	Ethnobotanical uses	References
Morella adenophora (Hance) J. Herb.	China and Taiwan	Roots and bark to treat bleeding, diarrhea and stomach pain	Li, 2006
Morella nana (A. Chev.) J. Herb.	China	Roots are used to treat bleeding, diarrhea, stomach pain, burns, and skin diseases.	Wang and Zhang, 2009
Morella serrata (Lam.) Killick	South Africa and Southern African countries extending into tropical Africa	Used to treat asthma, coughing and shortness of breath . The decoction of the root is used to treat painful menstruation, cold, coughs and headaches and to enhance male sexual performance . It is also used in the management of sugar related disorder and as laxative to treat constipation. The stem bark is used to treat headache	Schmidt, 2002; Moffet, 2010; Ashafa, 2013
Morella arborea (Hutch.) Cheek	Cameroon	Bark decoction used to treat fevers and inflammation.	Tene, et al., 2000
Morella cerifera (L.) Small	North America	Herb decoction or tincture used as astringent, diaphoretic, as a circulatory stimulant, to treat irritable bowel syndrome, ulcerative colitis, digestive system disorders, diarrhea, dysentery, leukorrhea, mucous colitis, colds, stomatitis, sore throat,	Hofman and Hofman, 2003

		measles and scarlet fever, convulsions, nasal catarrh and jaundice	
Morella salicifolia (Hochst. ex A.Rich.) Verdc. & Polhill Myrica	Southeast Africa, Ethiopia and Saudi Arabia	Roots infusion is used to treat gastro-intestinal disorders. while roots and bark used in the treatment of headache, pain, inflammation and respiratory diseases.	Kefalew, *et al*., 2015; Teklay, *et al*., 2013; Schlage, *et al*., 2000
	Myrica		
Myrica rubra (Lour.) Siebold & Zucc.	China, Japan, Taiwan and Korea	The various organs are used to treat gastrointestinal diseases, headaches, burns and skin diseases. Leaves are used to treat inflammatory diseases.	Sun, *et al*., 2013;
Myrica esculenta Buch. Ham. ex D. Don	India, South China, Malaysia, Japan, Vietnam and Nepal	Ayuverdic medicine use decoction of bark to treat asthma, bronchitis, fever, lung infection, dysentery, toothache and wounds; leaf, root, bark and fruits juice for worms, jaundice and dysentery; Vietnamese folk medicine uses bark to treat catarrhal fever, cough, sore throat and skin disease	Kirtikar and Basu, 2009; Nadkarni, 2002; Laloo, et al., 2006; Bich, 2004
Myrica gale L.	Europe, Siberia, Canada and Northern USA	Used in the treatment of ulcers, intestinal worms, cardiac disorders and aching muscles.	Small, 2014
Myrica nagi Thunb.	China, Malaya Islands, Pakistan and Nepal	Bark finds its application in reducing inflammations to treat cardiac diseases, bronchitis, gonorrhea, diuresis, dysentery, epilepsy, gargle, heamoptysis, hypothermia, catarrh, headache, menorrhagia, putrid sores, typhoid, face palsy and paralysis and	Panthari, *et al*., 2012; Kumar and Rana, 2012

	wounds. Fruit wax or oil is used for treating ulcers, bleeding piles, body ache, toothache and for regulating the menstrual cycle

2.9.2.2 Ecological distribution

Myricaceae occupy a range of habitats, mostly mesic, claims swampy habitats all eastern U.S. species except for *Comptonia*, mostly which thrives in dry sterile soil (Youngken, 1919). *Morella calfornica* occurs in canyons and moist slopes, whereas *Myrica hartwegii* is found on montane stream banks. The South African species (*Morella*) occur on sandstone or limestone slope (Goldblatt and Manning, 2000), but the sandstone water availability in these localities is not described by some authors.

Most species are commonly found in habitats such as sand-dune, recent volcanism areas, waterlogged areas where nutrients are deficient. They are frequently components of early-successional communities, but also occur in climax vegetation where environmental conditions are not suitable for taller trees. Mostly, the species, if not all, have low tolerance to drought. Several species grow with their roots waterlogged: for example, *Myrica gale* is found in bogs and swamps (Gorham, 1957), *Morella faya* grows in wet forests on the volcanic soils. *M. serrata* occurs in riparian habitats in Cape Town, South Africa. It has been reported that *M. javanica* can grow as an epiphyte on other forest trees in Borneo, this observation is illustrative of the high light requirements that the family has and can strive in nutrient-poor substrates.

Canacomyrica and most species of *Morella* occur in the tropics but they are found in montane habitats at altitudes where the climate is more moderate, the soil often thin and the cloud layer

provides almost constant moisture. Canacomyrica grows on the serpentine soils of southern New Caledonia which are rich in toxic elements, such as iron and nickel, and notoriously poor in essential minerals. Several *Morella* species are also endemic to meandering areas such as those found in Cuba and Malesia. Other species are endemic to the mediterranean regions of South Africa and California, where they are exposed to periods of drought. The requirements of Myricaceae species for abundant water supply cannot, however, be over-emphasised and in these summer-drought regions *Morella* species are mostly restricted to riparian habitats or altitudinal zones where a semi-constant cloud layer provides regular precipitation; two species that may have less stringent requirements for water are *M. cordifolia* and *M. quercifolia*, both South African Cape species. Two notable species are found near sea level; *M. cordifolia* is an important dune stabilising species on coastal sand dunes in the Cape region of South Africa and *M. cerifera* is frequently found in coastal habitats in North America. The latter species is the most wide-ranging *Morella* species, occurring throughout southern North America, Central America and some Caribbean islands. It is possible that this species has a higher tolerance to drought than its congeners, allowing it to populate a wider range of habitats. However, deep root systems are a feature of the family and permit species to tap deep ground-water (Parra-O, 2002).

2.10 Myrica quercifolia

The genus name is *Myrica* from the family Myricaceae and Order Fagales. Dioecious small spreading shrub up to 60 cm, it has obovate leaves, attenuate below, usually pinnatifid, gland dotted. Flowers in axillary spikes. It has wart-textured fruits which are 3-4mm in diameter. It flowers between July and September, grows mostly in coastal sand limestone flats and slopes. NW, SW, AP, SE, LB (Namaqualand to Eastern Cape).

Table 2.2: Comparison between three genera of the Myricaceae family.

	Morella	***Myrica***	***Camptonia***	**References**
Terminal buds	Present	Lacking	Lacking	Wilbur, 1994
Leaves	Thick, usually persistent , entire, toothed or rarely incised.	Thin, deciduous, entire or weakly serrate distally.	Thin, deciduous, roundedly pinnatafid.	Wilbur, 1994
Stipules	Lacking	Lacking	Present	Wilbur, 1994
Aments	Inserted on old wood mainly below the leaves	Inserted at the summit the of the branchletes		

2.10.1 Biological activities and chemical composition of the species from the genus Myrica

Various species from Myrica have many ethnobotanical, the fruits are rich in anti-oxidants and thus bear health promoting properties (Zhang *et.al.* 2015). Few properties are discussed below.

2.10.2 Anti-cancer properties

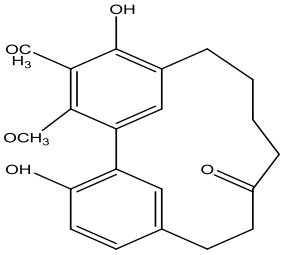
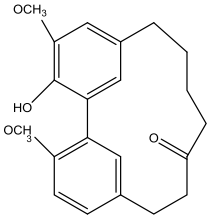
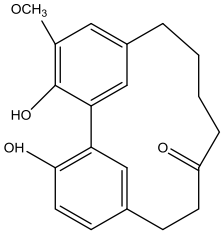
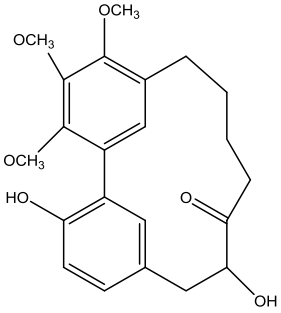
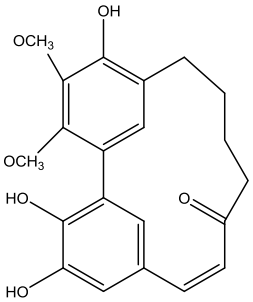
A study was conducted in 2005 by Sylvestre, *et.al*. on *M. gale* to assess its potential against growth of human lung carcinoma cell line A-549 and human colon adenocarcinoma DLD-1, cell line. A total of 53 components were identified, the major compounds isolated were: myrcene (23.18–12.14%), limonene (11.20–6.75%), a-phellandrene (9.90–6.49%) and b-caryophyllene (9.31–10.97%) in the 30 and 60 minutes fractions, respectively, whereas higher caryophyllene oxide content was detected in the 60 min fraction (9.94%) than in the 30 min fraction (3.47%). The 60 min fraction showed higher anticancer activity against both tumor cell lines. The higher cell growth inhibition induced by the 60 minute fraction, as compared to the 30 min fraction, could be due to sesquiterpene enrichment in which anti-cancer properties were detected.

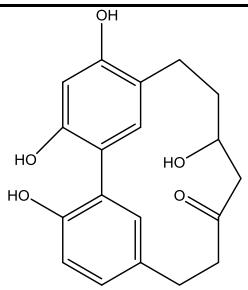
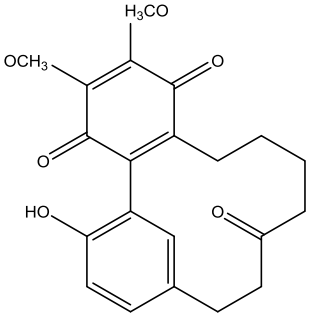
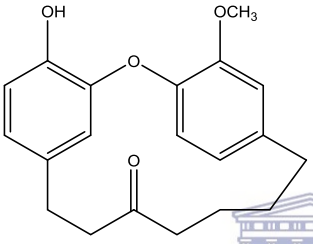
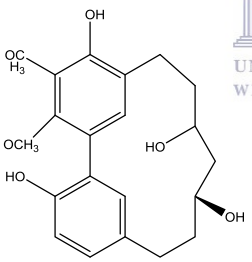
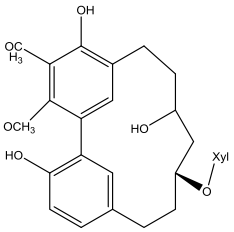
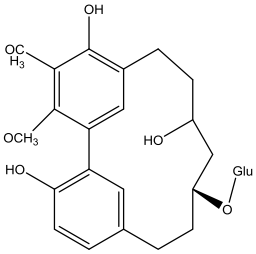
2.10.3 Anti-tyrosinase and radical scavenging properties

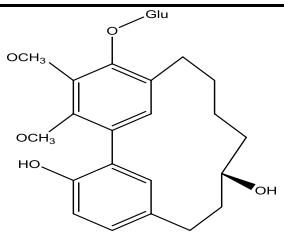
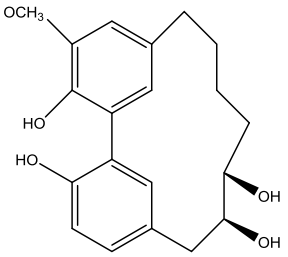
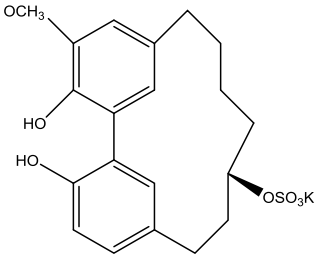
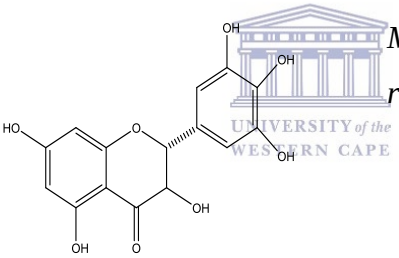
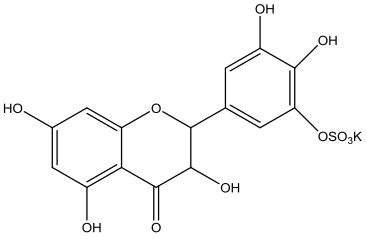
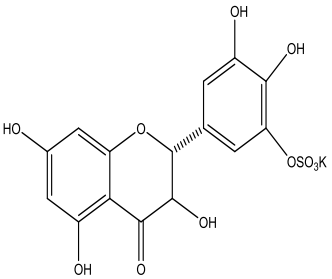
In 1995, Matsuda *et.al*., investigated *in vitro* effects of 50% ethanolic of dried leaves and bark of *M. rubra.* The extracts exhibited tyrosinase inhibition, additionally, production of melanin from dopachrome by autooxidation was inhibited. The extracts also demonstrated superoxide dismutase like (SOD) activity.

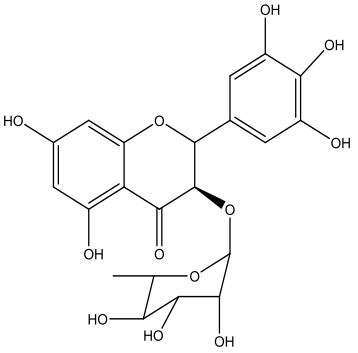
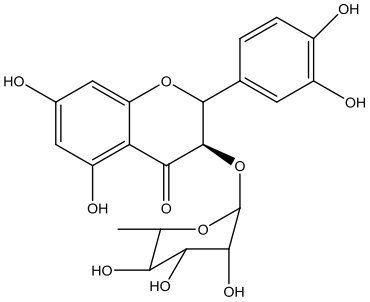
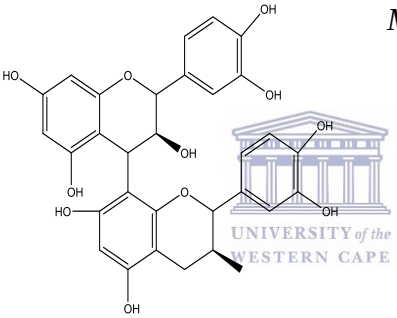
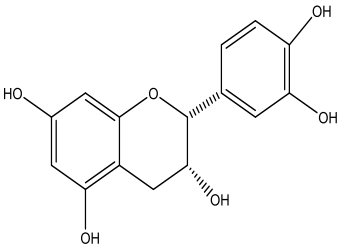
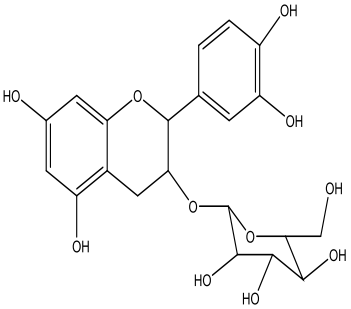
In another study, ten cyclic diarylheptanoids, three new compounds including; myricanone 5-O-a-L- arabinofuranosyl-(1→6)-b-D-glucopyranoside, myricanone 17-O-b-D-(6′-O-galloyl)-glucopyranoside, and 16-methoxy acerogenin B 9-O-b-D-apiofuranosyl-(1Æ6)-b-D-glucopyranoside, along with two flavonoids, were isolated from the extracts of *M. rubra* bark. On evaluation of compounds 1-12 against the melanogenesis in the B16 melanoma cells, six compounds, 3, 5, 7, 8, 10, and 12, exhibited inhibitory effects with 30-56% reduction of melanin content at 25 mg/ml with very weak toxicity to the cells (82-103% of cell viability at 25 mg/mL). In addition, upon evaluation of compounds 1-12 against the scavenging activities of free radicals [against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical], seven compounds, (Akazawa, *et.al*. 2010)

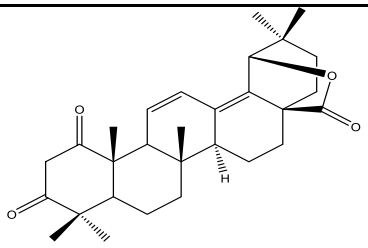
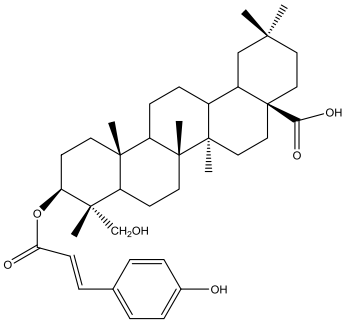
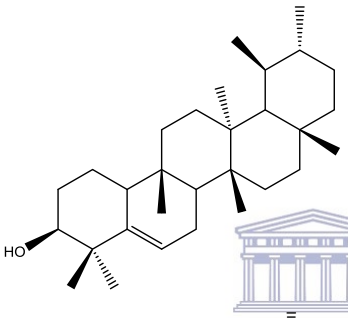
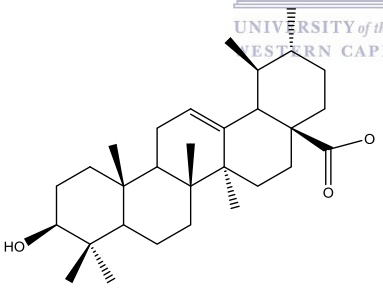
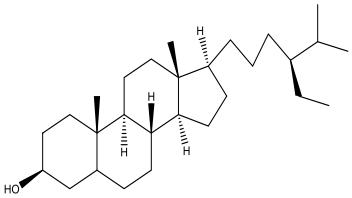
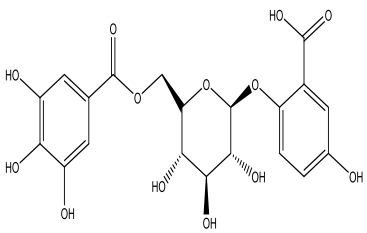
Table 2.3: Compounds isolated from the members of the *Myrica* and *Morella* genus.

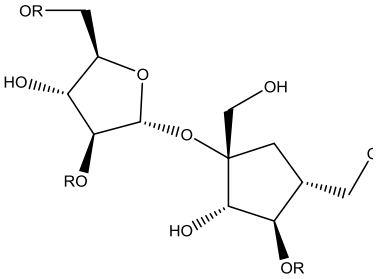
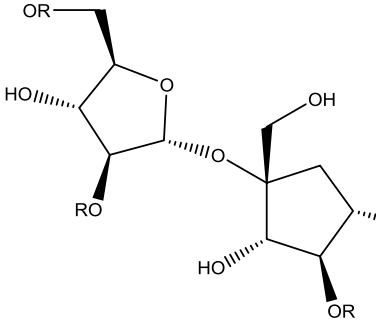
Compound name	Chemical structure	Name of species (plant part(s))	References
	Diarylheptanoids		
Myricanone		*Mo. adenophora*; *Mo. arborea*; *Mo. cerifera*; *My. gale*; *My. rubr*a	Ting, *et al*.,2014
5-Deoxymyricanone		*Mo. adenophora*	Ting, *et al*.,2014
Myricananin C		*Mo. nana*	Wang *et al*., 2008
Porson [b]		*My. Gale*	Nagai *et al*., 1995
Myricananin D		*Mo. adenophora; Mo. nana*	Ting, *et al*.,2014; Wang *et al*., 2008

Alnusonol		*Mo. nana*	Wang *et al*., 2008
Actinidione		*Mo. adenophora; Mo. nana*	Ting, *et al*.,2014; Wang *et al*., 2008
Galeon		*Mo. adenophora; My. gale*	Ting, *et al*.,2014; Morihara, *et al*., 1997
Myricanol		*Mo. adenophora*; *Mo. arborea; Mo. Cerifera*	Ting, *et al*.,2014; Tene, *et al*., 2000; Joshi *et al*., 1996
Myricanol 11-O-β-D-xylopyranoside		*Mo. adenophora; Mo. arborea*	Ting, *et al*.,2014; Tene, *et al*., 2000
Myricanol 11-O-β-D-glucopyranoside		*Mo. adenophora; My. Rubra*	Ting, *et al*.,2014

Myricanol 5-O-β-D-glucopyranoside		*My. rubra*	Akazawa *et al*., 2010
Myricananin A		*Mo. nana*	Wang *et al*., 2008
Juglanin B-11(R)-O-sulphate		*My. rubra*	Kim *et al*., 2014
	Flavonoids		
Myricetin		*Mo. adenophora; My. rubra; My. esculenta.*	Ting, *et al*.,2014; Kim *et al*., 2014; Nhiem, et al., 2013
Myricetin-3′-O-sulfate		*My. rubra*	Kim, *et al*., 2014
Ampelopsin 3′-O-sulfate		*My. rubra*	Kim, *et al*., 2014

Myricitrin		*Mo. adenophora; My. rubra; Mo. cerifera My. esculenta.*	Ting, *et al*.,2014; Kim *et al*., 2014; Nhiem, et al., 2013
Quercitrin		*Mo. adenophora*	Ting, *et al*.,2014
Procyanidin B2		*My. rubra*	Zhang, *et al*., 2013
(−)-Epicathechin		*My. rubra*	Zhang, *et al*., 2013
Cyanidin 3-O-glucopyranoside		*My. rubra*	Sun, *et al*., 2013
Miscellaneous compounds			
Myricalactone		*Mo. adenophora; My. gale*	Ting, *et al*.,2014; Morihara, *et al*., 1997

			
3β-Trans-p-coumaroyloxy-2α,23-dihydroxyolean-12-en-28-oic acid		*Mo. adenophora*	Ting, *et al*.,2014
Rhoiptelenol		*My. rubra*	Tao, *et al*., 2002
Ursolic acid		*My. rubra*	Tao, *et al*., 2002
β-Sitosterol		*Mo. adenophora; My. esculenta*	Ting, *et al*.,2014; Bamola, *et al*., 2009
6′-O-galloyl orbicularin		*Mo. adenophora*	Ting, *et al*.,2014

Myricadenin A		*Mo. adenophora*	Ting, *et al*.,2014
Myricadenin B		*Mo. adenophora*	Ting, *et al*.,2014

2.10.4 Nitrogen fixation

Myricaceae have evolved two important adaptations for survival on nutrient poor soils: an actinorhizal association with a bacterium which provides the ability to fix nitrogen; and cluster roots which improve availability of other essential nutrients, especially phosphorus.

The characteristics of the myricaceae family as a whole is to inhabit moist areas, including habitats where water has been standing for prolonged periods, this results in nitrate deprivation and this phenomena has been repeatedly observed (Carlquist, 2002). Previous study on symbiotic nitrogen fixation have emphasised the role of leguminous plants, particularly in agricultural practice. Such symbioses have been described in some 13,000 species of the family Leguminosae. In contrast, nitrogen fixation by symbiotic bacteria in non leguminous plants has been reported in only 13 genera representing 118 species. The importance of non-leguminous symbiosis have been indicated by the studies in the world's nitrogen cycle and emphasised its significance for plants of pioneer community succession. (Tiffney, 1974)

Myricaceae has evolved two important adaptations to strive on soils with nutrient deficit: an actinorhizal association with a bacterium which has the ability to fix nitrogen and cluster roots which improve availability of other essential nutrients, especially phosphorus.

Myricaceae are one of only nine angiosperm families known to form a symbiotic relationship with Frankia. Frankia are widespread, filamentous, gram-positive, non-endospore forming, mycelial bacteria (Schwintzer, 1979). Traditional classifications based upon morphological characters split actinorhizal plants among four subclasses of dicotyledons

Chapter 3

Screening of crude extracts for their inhibitory potential against tyrosinase enzyme

3.1 Introduction

Hyperpigmentation, overproduction of melanin, which may be caused by chronic exposure to the sun is not desirable on the human skin. Melanin production is facilitated by the enzyme tyrosinase, consequently, a number of tyrosinase inhibitors have been developed for such skin condition (Wang, *et.al*., 2006). The discovery of novel inhibitors of tyrosinase becomes attractive due to their wide potential applications such as improving quality of foods, insect pest control and prevention and treatment of human health problems related to melanin production (García & Furlan, 2015).

The latter is the goal of this project, where a bioactive ingredient from plant species of the Cape Flora that possesses depigmenting properties would be identified. This compound should ideally have the ability to selectively depigment melanoma cells without being toxic to them. A total of 37 plants were screened from different families growing in the Cape region for their anti-tyrosinase potential, These species were extracted and assessed for their ability to possess skin-whitening/lightening properties.

Chapter description

- Detection of tyrosinase inhibitory effects of plant extracts using TLC Bioautography
- Compare tyrosinase inhibitory activity using mushroom tyrosinase inhibition method on various plant extracts using ELISA
- Investigate *in vitro* melanin biosynthesis inhibitory effects of active plant extracts spectrophotometrically (ELISA) on melanoma B16-F10 cells
- Evaluate Cytotoxicity effects of active crude extract on melanoma cells.

3.2. Materials and methods

3.2.1 Plant collection

The plant materials were collected randomly in and around the vicinity of the University of the Western Cape (UWC) campus in Cape Town, Western Cape province in the month of August, 2015. Herbariums of each plant species were created. The leaves of the plants which weighed approximately 100 gram were pulverised using an electric blender.

Table 3.1: List of plant species used for the present study.

Assigned code	Plant species	Family	Sampling location
BC-/31-30	*Acacia karroo* (Hayne.)	Fabacea	CFNR
BC/35-1	*Acokanthera oppositifolia* (Lam.) Codd	Apocynaceae	UWC
BC/29-14	*Antizoma capensis* (L.f.) Diels	Menispermaceae	CFNR
BC/35-3	*Artemisia afra* (Jacq.) Ex Willd	Asteraceae	CFNR
BC/31-23	*Aspalathus hispida* (Thunb.)	Fabaceae	CFNR
BC/27-8	*Chrysanthemoides monilifera* (L.) Norlindh	Asteraceae	CFNR
BC/27-9	*Cineraria geifolia* (L.)	Asteraceae	CFNR
BC/27-5	*Euclea racemosa* (L.)	Ebenaceae	UWC
BC/29-13	*Euphorbia mauritanica* (L.) Var	Euphorbiaceae	CFNR
BC/29-19	*Ficus bengalensis* (L.) 1753	Fabaceae	CFNR
BC/29-22-1	*Helichrysum petiolare* Hilliard & B.L.Burt	Asteraceae	UWC
BC/31-27	*Hyobanche sanguinea* (L.)	Orobanchaceae	CFNR
BC/35-12	*Hypoxis hemerocallidea* Fisch. & C.A. Mey	Hypoxidaceae	Afriplex
BC/37-14	*Laurus nobilis* (L.)	Lauraceae	UWC
BC/31-26	*Leonotis ocymifolia* (Burm. F.)	Lamiaceae	UWC
BC/37-17	*Maytenus bachmanii* (Loes.) Marais	Celastraceae	UWC
BC/29-15-1	*Myrica quercifolia* (L.)	Myricaceae	UWC
BC/37-16	*Myrtus communis* (L.)	Myrtaceae	UWC
BC/29-16	*Nylandtia spinosa* (L.) Dumort	Polygalaceae	CFNR
BC/27-11	*Olea europaea* (L.)	Oleacea	UWC
BC/27-4	*Otholobium fruticans* (L.) C.H.Stirt	*Fabaceae*	CFNR
BC/27-2	*Passerina rigida* Wikstr	Thymelaeaceae	CFNR
BC/27-3	*Phylica ericoides* (L.)	Rhamnaceae	CFNR
BC/37-21	*Plectranthus barbutus* Andrews	Lamiaceae	UWC
BC/37-20	*Plectranthus ecklonii* Benth	Lamiaceae	UWC
BC/37-25	*Salvia africana-caerulea* (L.)	Lamiaceae	CFNR
BC/37-24-1	*Salvia africana-lutea* (L.)	Lamiacea	CFNR
BC/31-25	*Scirpus antarcticus* Willd	Cyperaceae	CFNR
BC/29-20	*Searsia laevigata* (L.)	Anacardicaeae	UWC
BC/29-12	*Searsia lucida* (L.) F.A.Barkley	*Anarcadiaceae*	UWC
BC/27-7	*Senecio halimifolius* (L.)	Asteraceae	CFNR
BC/31-32	*Serruria furcellata* R.Br	Proteaceae	CFNR
BC/39-27	*Sutherlandia frutescens* (L.) R.Br	Fabaceae	Afriplex
BC/39-29-1	*Tetragonia riparia*	Lamiaceae	KBG
BC/31-24	*Trachyandra revoluta* (L.) Kunth	Asphodelaceae	CFNR
BC/27-10	*Viscum capense* (L.f.) Tiegh	Viscacea	CFNR
BC/29-17	*Zygophyllum fulvum* (L.)	Zygophllaceae	CFNR

KBG., Kirstenbosch Botanical Garden, CFN., Cape Flats Nature Reserve, UWC., University of the Western Cape campus.

3.2.2 Materials and reagents

Kojic acid was used a positive control and 5771 U/mg of mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma-Aldrich in Cape Town (South Africa). All organic solvents including methanol (MeOH), ethanol (EtOH), hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and dimethyl sulfoxide (DMSO) and normal-phase silica gel 60 PF254 pre-coated aluminium plates that were used to run thin layer chromatography (TLC) were supplied by Merck from Cape Town (South Africa).

Fetal calf serum (FBS) was purchased from Thermo Scientific , trypsin and EDTA were purchased from Gibco, penicillin/streptomycin and Phosphate saline buffer (PBS) were supplied by Lonza. The Cell Proliferation Kit II 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Roche and 0.4% trypan blue stain was purchased from Invitrogen.

3.2.3 Equipment and Instruments

All spectrophotometric readings were taken using the enzyme-linked immunosorbent assay (ELISA) on AccuReader M 965/965+, Metertech Inc. version 1.11 from Taipei (Taiwan). Cells were grown in a CO_2 incubator from Shel lab: they were counted using Countess^{TM} automated cell couter and Countess^{TM} cell counting chamber. Cells were span down using centrifuge 5417R.

Cells were cultured in Cell culture flasks 25 and 75 cm^2 from SPL Life Science. Both 24 and 96 well plates were supplied by Sigma-Aldrich.

3.3 Methods

3.3.1 Plant extraction

The aerial part of the plant material was used in this study, all the dried material were pulverised using a blender, thereafter, the plant material (~ 100 g) were placed in a conical flask and methanol was added enough to cover the plant material at room temperature for 24 hours, this was repeated three more times. Filtration of the extract was carried out by using Whatman no 1 filter paper and a vacuum pressure.

Combined extracts were concentrated until dryness usingMethanol from the extract was removed by rotary evaporator at 50 ℃. Finally, the residues were collected and used for the experiment.

3.3.2 Plant sample preparation

All crude plant extracts were dissolved in DMSO to a concentration of 20 mg/ml stock, the working solutions were prepared from this DMSO stock into complete DMEM medium to desired concentrations (100, 50, 25, 12.5, 6.25 µg/ml).

3.3.3 Tyrosinase enzyme preparation

A working solution of the mushroom tyrosinase enzyme was prepared from 5771 U/mg of the stock solution to a concentration of 200 U/ml by diluting the enzyme in a phosphate buffer of pH 6.5. The enzyme was then placed in ice, the activity of the enzyme was checked prior to each assay by adding equal amount of enzyme to substrate, colour change would denote that the enzyme is active.

3.3.4 TLC bioautography assay of crude plant extracts

A tyrosinase solution of 250 U/ml was prepared by diluting 1000 U of the enzyme with the phosphate buffer. The plant extracts and the positive control were weighed and dissolved in methanol (1 mg/ml). Then 20 µl of each extract was spotted using a glass capillary. Two copies of TLC plates were spotted; after development, plates were removed from the tank, they were left at room temperature to dry, one plate was sprayed with the 2 mM L-tyrosine then incubated at room temperature for 10 minutes then sprayed with the aforementioned tyrosinase solution. The plate was further incubated at room temperature for 30 minutes; the active compound was indicated by clear white spots or inhibition zones and the background of the plate indicated a purplish colour. The other plate was dipped in vanillin sulphuric acid and heated at a ~110℃. The results are shown in figure 3.1. The Retention factor (R_f) of interesting spots were calculated using the following equation.

Equation 1∴ **Retention factor (R_f)** $= \left(\frac{A}{B}\right)$

A is the distance travelled by the sample from the origin, whereas B is the distance travelled by the solvent from the origin to solvent front

3.3.5 Spectrophotometric determination of tyrosinase inhibition of crude extracts using ELISA reader.

Tyrosinase assay was performed as described previously by Chen *et al*., 2015 with slight modifications. All the plant extracts were weighed and dissolved in DMSO to a final concentration of 20 mg/ml, a working solution was then prepared by diluting a stock solution to 1000 µg/ml in phosphate buffer with a pH of 6.5. The two concentrations of the extracts and the

positive controls, Kojic acid, were 50 and 200 µg/ml, these concentrations were achieved by adding 70 µl of each plant extract together with 30 µl of the tyrosinase enzyme (250 U/ml). The well containing the negative control, 70 µl of the buffer was added instead of the plant extract. Then 110 µl of 2mM L-tyrosine was added to each, the plate was incubated at room temperature for 30 minutes. The wells of the plate were read at an optical density of 490 nm with an ELISA well reader. The tyrosinase inhibition percentage was calculated using the formula below.

Equation 2 ∴ **Tyrosinase inhibition (%) =** $\left(\frac{[(\boldsymbol{A}-\boldsymbol{B})-(\boldsymbol{C}-\boldsymbol{D})]}{(\boldsymbol{A}-\boldsymbol{B})}\right)\boldsymbol{x}\ \mathbf{100}$

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. The results are tabulated in table 3.2.

3.4 Investigation of melanin inhibition and cytotoxicity in melanoma B16-F10 cell culture by crude plant extracts.

3.4.1 Preparation of test samples

Samples were prepared as mentioned in section 3.3.2

3.4.2 Preparation of cells

Melanoma B16-F10 cell lines were grown in a 75 ml T-flask at a density of $1x10^6$ cells/flask. Cells were cultured at a humidified atmosphere of 5% CO_2 at 37℃ Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin. After 24 hours of cultivation, the T-flask and the flow hood was sterilised by spraying it with ethanol, the medium was removed and the cells were rinse two

times with the phosphate buffer saline (PBS) and cells were sub-cultured every 2 days to maintain logarithmic growth.

3.4.3 Cell count

A 1:10 dilution of the cell suspension in trypan blue solution (e.g. 10 µl cells in 90 µl trypan blue) in an eppendorf tube was prepared and well mixed. Ten microlitres of this dilution was transferred to two chambers of the hemacytometer. Cells were counted as per manufacturer's instructions using Countess^{TM} automated cell counter.

3.4.4 Cell viability assay

Cytotoxicity was determined by a modification of the method of Uchida, *et al.,* 2014. Melanoma cells were seeded ($1x10^5$) in a 96 well plate, thereafter, they were incubated for 24 hours for adherence, cells were exposed to different concentrations of plant test samples (preparation described in 3.3.2) and the volume was aspirated; the attached cells were incubated with 100 µl MTT (0.3 mg/ml for 2 hours). A reference plate was prepared by adding 100 µl of the medium, 100 µl of samples and 50 µl of MTT and was also incubated for 2 hours. The absorbance at 570 nm was then measured together with the reference plate which was read at a wavelength of 630 nm using a micro-plate reader. The viability of cells were calculated in percentage using the control as a reference (medium with DMSO). Cell viability was calculated with the following equation:

Equation 3 $\therefore$ **Cell viability (%) =** $\left(\frac{OD\ of\ A}{OD\ of\ B}\right) x\ 100$

where A denotes the OD 570 absorbance of treated cells, and B represents the OD 570 absorbance of control. The dose-dependent inhibition experiments were performed in triplicates to determine the IC_{50} of the test samples (See table 3.5 for results).

3.4.5 Melanin inhibition assay

3.4.5.1 Preparation of plates

This melanin inhibition assay was previously described by Curto, *et al.*, 1999 with slight modification. After cells were (cell preparation described in 3.3.2) confluent, plates were removed from the incubator. The medium was aspirated and cells were rinsed twice with PBS, they were then trypsinised and centrifuged at 970 rpm. Cells were suspended in 2ml DMEM medium and the viability was determined by staining with trypan blue and were counted using an automated cell counter.

The medium with new DMEM medium containing test samples of various concentrations was replaced. After 72 hours of incubation, the adherent cells were washed with phosphate buffered saline (PBS) and detached from the T-flask by trypsinisation. The cells were collected in a test tube and washed twice with PBS.

Melanoma B16-F10 cells with density of 1×10^5 cells/flask were incubated in 60 mm dishes with various concentrations of the test compounds. After treatment, the cells were washed twice with phosphate buffered saline (PBS)

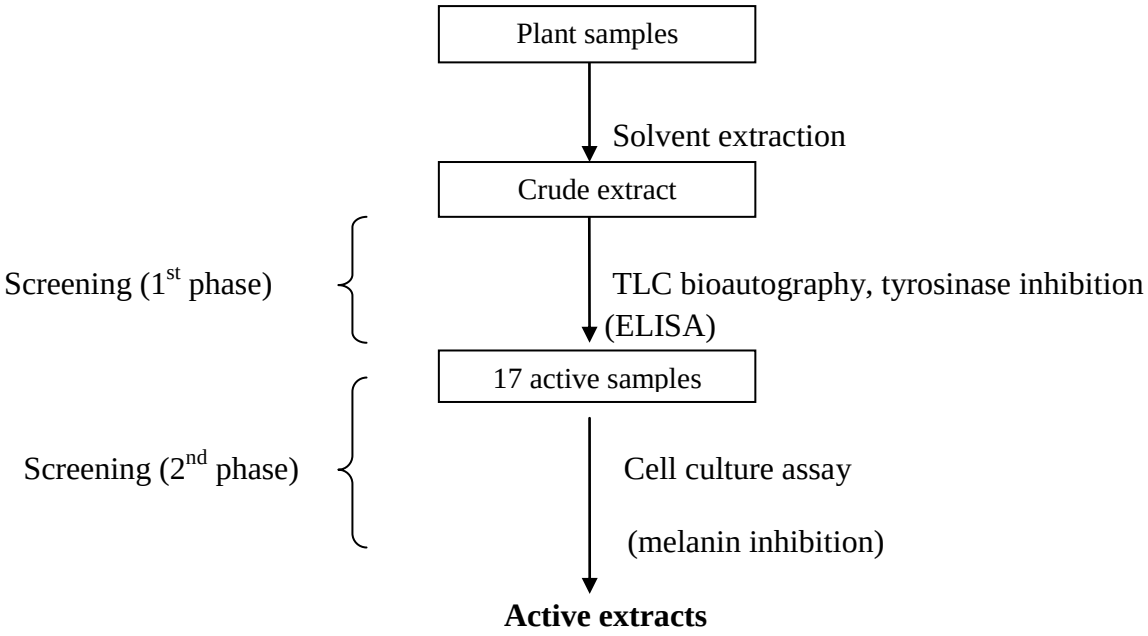
The number of cells were determined by means of staining with tryptan blue and were then counted using a cell counter. In order to determine melanin inhibition, $1x10^6$ B16 -F10 melanoma cells were pelleted in an eppendorf tube and were resuspended in 200 µl of 1 M NaOH and were then solubilised on a heating block at 100℃ for 30 minutes. Solubilised cells were then centrifuged at 16 000 rpm for 20 minutes at room temperature. After centrifugation,

the supernatant was collected and 200 µl was aliquoted in 96 well plates and the melanin was determined by enzyme micro-plate readings at an absorbance of 400 nm on a spectrophotometer. The melanin inhibition was calculated and corrected for the concentrations of proteins, using control cells as 100 %.

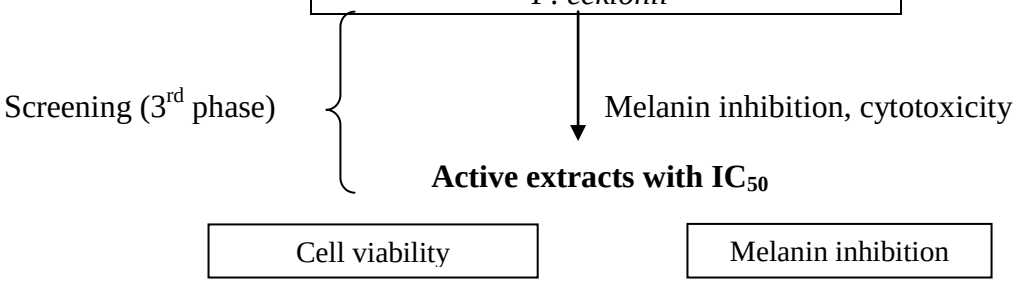
Melanoma B16-F10 cells with density of 1×10^5 cells/flask were incubated in 60 mm dishes with various concentrations of the test compounds. After treatment, the cells were washed twice with phosphate buffered saline (PBS), and lysed in 200 µl of 1 N NaOH for 1 hour at 100˚C.

Equation 4 ∴ **Melanin inhibition %** $= \left(\frac{A-B}{A}\right) x\ 100$

A is the total number of cells who were untreated, whereas B denotes the cells that were treated with test sample or positive control.



E. racemosa	*T. riparia*
M. quercifolia	*C. geifolia*
A. karroo	*S. africana-lutea*
S. hamilifolius	*P. ericoides*
H. petiolare	*S. africana-caerulea*
S. furcellata	*S. antarcticus*
M. communis	*S. lucida*
P. rigida	*A. afra*
P. ecklonii	



50	*C. geifolia*	36.9
>100	*S. antarcticus*	20.3
>100	*S. furcellata*	7.1
97.2	*M. quercifolia*	<6.25
>100	*P. ericoides*	27.7
46.9	*T. riparia*	43.9
>100	*M. communis*	>100
>100	*A. karroo*	>100

Scheme 3.1: General schematic representation of screening procedure for 37 crude extracts.

3.5 Results and discussion

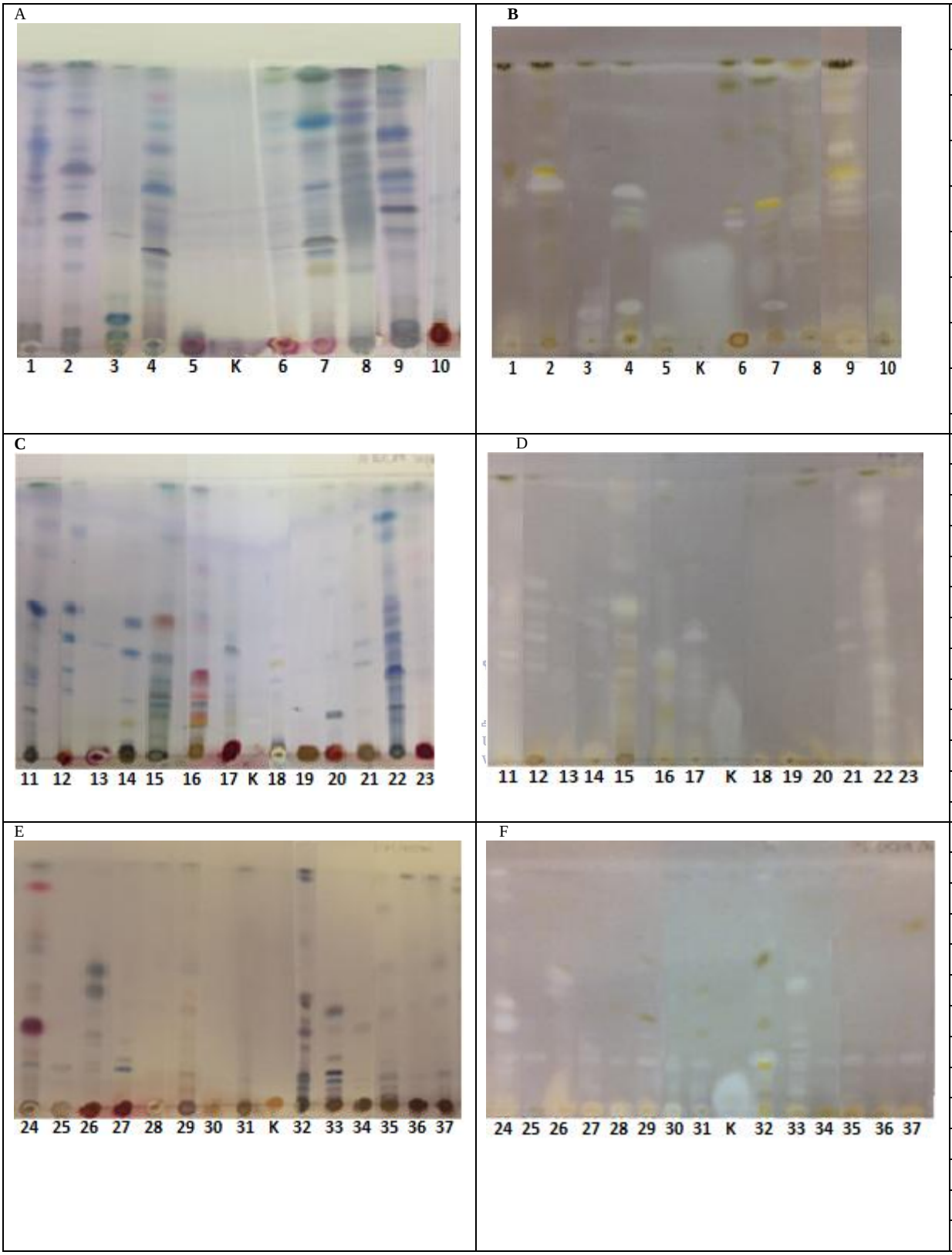
3.5.1 Introduction

Plants are a rich source of bioactive compounds, many of which are free from unpleasant side effects, plant extracts usually occur as a combination of complex bioactive compounds or phytochemicals whose activity as a crude extract might not be sufficiently potent, thus screening and characterisation of bioactive molecules hold industrial importance (Sasidharan, *et al*., 2011). Safe and effective tyrosinase inhibitors have become important for their potential applications in improving the quality of food, depigmentation, and preventing other melanin-related health problems in human beings, in addition to cosmetic applications (Quispe, *et al*., 2017). Thirty nine species to be studied were selected which were collected randomly from the CFR and supported by the fact that there have been no previous studies on their effect on tyrosinase activity. Table 3.1 provides the scientific name, the family name, as well as sampling location of each plant. These 39 species belong to 22 botanical different families. Lamiaceae was the family with the largest species, followed by the Fabaceae, Asteraceae and other families.

For the extraction of these plants, only aerial parts were used in this study, the effect on tyrosinase inhibition of all crude methanolic extracts were investigated. TLC bioautography assay was employed, extracts were spotted at a concentration of 1 mg/ml (results are shown in figure 3.1 in section 3.5.2) and using an ELISA reader, spectrophotometrically at 200 and 50 µg/ml.

3.5.2 Screening of tyrosinase activity of methanolic extracts using TLC bioautography.

The thin layer chromatographic (TLC) plates were developed in a pre-saturated glass tank using MeOH/DCM (1:9), the developed TLC plates were viewed at λ254/λ366 nm UV lamp, they were then sprayed with 250 U/ml of tyrosinase and 2 mM tyrosine (figure 3.1). The white spots, also referred to as inhibition zones on the plates in figure 3.1 (on plates A, C and E) indicate the presence of active compounds in the extract. The white spots appear against a purplish-brownish background and this is a result of formation of coloured quinones from L-tyrosinase.



1. *A. oppositifolia*
2. *T. Riparia*
3. *S. frutescens*
4. *A. Afra*
5. *H. hemerocallidea*
6. *M. bachmanii*
7. *L. Nobilis*
8. *P. barbutus*
9. *S. africana-caerula*
10. *S. furcellata*
11. *S. african-lutea*
12. *E. racemosa*
13. *V. capense*
14. *O. europea*
15. *P. ecklonii*
16. *H. petiolare*
17. *C. geifolia*
18. *S. hamifolius*
19. *H. suinguinea*
20. *A. karroo*
21. *E. mauritanica*
22. *O. fruticans*
23. *M. quercifoila*
24. *S. Lucida*
25. *A. capensis*
26. *P. rigida*
27. *P. ericoides*
28. *C. monilifera*
29. *L. ocymifolia*
30. *Z. fulvum*
31. *N. spinosa*
32. *S. leavigata*
33. *T. revolute*
34 *F. benghalensis*
35. *S. antarcticus*
36. *A. hispida*
37. *M. myrtus*

Figure 3.1: TLC bioautography detection of tyrosinase inhibitors. From left to right are plant samples 1 to 39, k is kojic acid which was used as reference standard. Elution was carried out with MeOH/DCM (1:9); (A,C and E) vanillin-sulpuric acid stain used as a detection agent from 1-39; (B, D and F) bioautography for tyrosinase inhibitor detection.

A series of inhibition zones occurred at a number of points in the TLC chromatograms; *S. furcellata* showed spots at R_f 0.39 and 0.62, the inhibition zones were also observed at R_f 0.21 for *M. quercifolia* (L.) whereas *A. karroo* (Hayne.) did not show any spots of inhibition. *T. riparia* showed the clearest zone of inhibition out of selected active extracts at R_f 0.66 followed by *C. geifolia* (L.) at R_f 0.46 and R_f 0.38. The TLC plate indicated by the letter B, the positive control, kojic acid (K) was spotted too much which led to streaking and slightly covered the next sample (Plate B in figure 3.1).

On the plates that were sprayed with vanillin-sulphuric acid (Chromatograms A,C and E), the various constituents within the samples were clearly distinguishable with *T. riparia* with R_f of 0.29, 0.39, 0.47, 0.55, 0.61, 0.69, 0.75 and 0.88 in TLC plate A, this extract showed clearest and indicating the presence of a number of extra constituents, when compared to the other crude extracts. *A. karroo* (Hayne.) showed the presence of a constituent when detected with vanillin sulphuric acid at R_f 0.38. All the other active extracts showed the bands at the same R_f as on the bioautography plates.

The TLC bioautography assay was further confirmed by conventional spectrophotometric microplate reader, the results are tabulated below in 3.2 in section 3.5.3.

3.5.3 Screening of tyrosinase inhibitory activities of methanol crude extracts using ELISA reader.

The effect on tyrosinase inhibition of all crude methanol extracts were assayed at a concentration of 200 and 50 µg/ml and the results are reported in Table 3.2. Kojic acid was used as the positive control at the same concentrations as the plant samples. Crude extracts were analysed with the help of a spectrophotometer and the readings were taken at 490 nm. The percentage of inhibition were then determined using equation 2 mentioned under subtitle 3.3.5. and the results are reported in Table 3.2.

Table 3.2: Tyrosinase inhibition by crude plant extracts at two different concentrations.

Species	Tyrosinase inhibition (%)	
	200 µg/ml	**50 µg/ml**
A. karroo	77.08	45.07
A. oppositifolia	22.51	31.26
A. capensis	18.53	11.89
A. afra	58.28	69.18
A. hispida	23.58	13.62
C. monilifera	16.98	14.10
C. geifolia	85.06	74.83
E. racemosa	73.79	76.19
E. mauritanica	66.45	40.47
F. bengalensis	28.91	11.04
H. petiolare	59.22	44.28
H. sanguinea	50.51	41.58
H. hemerocallidea	26.78	43.91
L. nobilis	9.08	10.35
L. ocymifolia	17.52	16.38
M. bachmanii	43.57	12.67
M. quercifolia	95.49	80.84
M. communis	71.50	72.56
N. spinosa	11.55	16.11
O. europaea	13.81	5.61
O. fruticans	29.11	18.29
P. rigida	88.25	60.07
P. ericoides	62.85	26.21
P. barbutus	42.49	21.53
P. ecklonii	39.40	46.27
S. africana-caerulea	68.71	70.83
S. africana-lutea	53.98	38.22
S. antarcticus	53.98	35.15
S. laevigata	25.69	9.19
S. lucida	86.61	91.24
S. halimifolius	59.25	59.47
S. furcellata .	96.29	83.22
S. frutescens	3.38	16.04
T. riparia	7.60	0.88
T. revoluta	-3.59	1.09
V. capense	36.93	30.83
Z. fulvum	33.08	10.74

Of the 37 extracts assessed, 17 crude extracts demonstrated significant effects on tyrosinase inhibition with activities of >50 % at 50 µg/ml and >60 % at 200 µg/ml. The 17 plant extracts

which were the most active extracts according to tyrosinase inhibition assay (ELISA) were selected and further screened for their ability to inhibit melanin biosynthesis at 50 µg/ml and the results are demonstrated in table 3.3 in section 3.5.4.

3.5.4 Screening of melanin biosynthesis inhibitory effects of methanol crude extracts at 50 µg/ml.

Table 3.3 Melanin inhibition activity of the most active crude extracts evaluated on melanoma B16-F10 cells at 50 µg/ml.

Species	Inhibition (%)
A. karroo	83.6±7.1
A. afra	37.7±12.5
C. geifolia	73.9±1.9
E. racemosa	59.9±4.3
C. geifolia	73.9±1.9
E. racemosa	59.9±4.3
C. geifolia	73.9±1.9
E. racemosa	59.9±4.3
C. geifolia	73.9±1.9
E. racemosa	59.9±4.3
S. Africana-caerulea	56.0±8.7
S. Africana-lutea	39.5±9.5
S. antarcticus	80.2±4.4
S. lucida	77.2±3.2
S. hamilifolius	48.8±6.9
S. furcellata	94.3±11.4
T. riparia	77.2±5.4

To investigate the effects of the crude extracts on melanin production, B16 melanoma cells were cultured in the presence of crude extracts and Kojic acid (positive control).

Out of 17 plants were evaluated for their ability to inhibit melanin biosynthesis in melanoma cells. The best inhibitory activities were observed in extracts of *M. quercifolia* (L.) which showed inhibitory activity of 80 % ± 7.9, *A. karroo* (Hayne.) with an inhibition of 83.6 % ± 7.1, *S. furcellata* R.Br with inhibition percentage of 94.3 % ± 11.4, *T. riparia* (77.2 % ± 5.4), *C. geifolia* (L.) (73.9 % ± 1.9), *S. antarcticus* (Willd.) (80.2 % ±4.4) *P. eroides* (L.) (65.1±6.1).

The active crude extracts were assayed further and tested for melanin inhibition and cytotoxicity at different concentrations and their IC_{50} were determined respectively. All the results are listed in table 3.4.

3.5.5 Screening of melanin biosynthesis inhibitory effects and cytotoxicity of active methanol crude extracts.

Table 3.4: IC_{50} of melanin inhibition and cytotoxicity of crude extracts that were assessed on melanoma cells at 50 ug/ml.

Species	Inhibition IC_{50} (ug/ml)	
	Melanin inhibition	**Cytotoxicity**
T. riparia	43.88	46.94
S. furcellata	7.13	>100
C. geifolia	36.88	>100
A. karroo	>100	>100
M. quercifolia	< 6.25	97.2
P. eroides	27.67	>100
S. antarcticus	20.25	50.00
M. communis	> 100	>100
Kojic acid (positive control)	>6.25	50.13

Methanolic extract of *M. quercifolia* (L.) showed potent melanin inhibition activity with an IC_{50} of <6.25 µg/ml, subsequently, the cytotoxic effects of the extracts were evaluated which showed an IC_{50} of >100 µg/ml. This extracts also showed significant tyrosinase inhibition with the value of 80.8 % at 50 µg/ml. *S. furcellata* R.Br. also showed significant melanin inhibition at an IC_{50} of 7.13 µg/ml with no cytotoxic effects (>100 µg/ml) followed by *S. antarcticus* (Willd.) (20.25 µg/ml) with moderate cytotoxicity (50 µg/ml)

The plant extracts of *C. geifolia* (L.) and *P. eroides* (L.) showed moderate activity with IC_{50} of 35.88, 27.67 µg/ml respectively followed by *T. riparia* (43.88 µg/ml) and had IC_{50} of >100 µg/ml for cytotoxicity.

There is no information in the literature that has been found regarding the plant extracts in table 3.4, but there is a report on *M. communis* (L.). The inhibitory effects of DCM, EtOAc, EtOH and MeOH extracts of the aerial parts and berries were investigated against cholinesterase and tyrosinase. The highest tyrosinase inhibition was exhibited by EtOAc berry extracts (40.53±0.47%) (Tumen, *et al*., 2012). These results are compare well with the finding of this study, *M. communis* (L.) showed no activity even then it showed significant effects on tyrosinase inhibition of 72.56 %. *M. communis* (L.) exhibited an IC_{50} value of >100 µg/ml with no cytotoxic effects (>100µg/ml).

Conclusion

Findings indicated that the extract of *M. quercifolia* (L.) consistent tyrosinase activity in the assays and a correlation was observed between the aforementioned bioactivities without cytotoxic effects. This plant might have other attributes such as potent antioxidant properties that were not investigated in the present study which could be useful in the treatment of general health promotion. Although the plant extracts of *A. karroo* (Hayne.), *S. furcellata* R.Br, and *S. antarcticus* (Willd.) showed melanin inhibition activity with; these plant materials also exhibited to melanoma cells without cytotoxicity. The plant extracts of *C. geifolia* (L.) and *P. eroides* (L.) also exhibited melanin biosynthesis inhibition activity and had no toxicity to the mouse melanomas; these extracts therefore have potential as anti-tyrosinase agents, and thus should be investigated further.

Chapter 4

Extraction, purification, characterisation and anti-tyrosinase activity of compounds from *Myrica quercifolia* (L.).

4.1 Introduction

Natural products and its derivatives have been the basis of treatment of human illnesses since the beginning of time. The idea that effect of drug in human body are mediated by specific interactions of the drug molecule with biological macromolecules led scientist to the conclusion that pure compounds are the factors required for the biological activity of the drug. This led to the beginning of a completely new era in pharmacology, as pure compounds, instead of extracts, became the standard treatments for diseases. Consequently, bioactive compounds, responsible for the effects of crude extract drugs, and their chemical structure are elucidated in modern science (Lahlou, 2013).

However, natural products are diverse in nature and present distinct physicochemical properties thus, extraction of a pure compound from a crude natural product extract is a formidable task (Cannell, 1998) as a crude material consist of a cocktail of compounds. Therefore it is difficult to apply a single separation technique to isolate an individual compound from a crude extract (Gray *et.al*., 2006).

Bioassay-guided isolation strategies connecting information on the chemical profiles of extracts and fractions with their activity data *in vitro* bioassays performed at micro-scale significantly reduced the time for hit discovery. The extract of *M. quercifolia* exhibited impressive anti-tyrosinase properties and thus it was studied further as well as full chemical studies for the most active compounds.

Chapter description

- Fingerprint analysis of plant sample with the positive control in full panel of anti-tyrosinase-related assays *in vitro.*
- Elucidate the inhibition of melanin biosynthesis of the active plant extract.
- Bioassay-guided isolation of compounds from *M. quercifolia* (L.) using different techniques including HPLC and elucidate their structures.
- Investigation of inhibition of tyrosinase and melanin biosynthesis assays of isolated compound *in vivo.*

4.2 Plant Myrica quercifolia (L.)

Myrica quercifolia (Myricaceae) known as *Morella quercifolia* and commonly known as Bayberry, Oak-leaved Myrica, Waxberry Bush or Waxberry (https://toptropicals.com/catalog/uid/Myrica_quercifolia.htm) is a dioecious small spreading shrub that grows from 60 cm up to 1 meter, it has obovate leaves, attenuate below, usually pinnatifid, with dotten glands. Flowers are arranged in axillary spikes and has wart-textured fruits which are 3-4 mm in diameter. It flowers between July and September, grows mostly in coastal sand limestone flats and slopes (Wilcox and Cowan, 2016; Manning and Goldblatt 2012). This plant species is native to South Africa and is provincially distributed in Eastern Cape, Northern Cape, North West and Western Cape.

https://www.ispotnature.org/node/530909

Figure 4.1: Leaves of *M. quercifolia* (L.)

4.3 Materials and methods

4.3.1 Materials and Equipment

Pre-coated plates of silica gel 60 F254 (Merck, Germany) were used for TLC analysis; visualisation of TLC plates were observed using UV lamp from CAMAG, Switzerland.

Column chromatography was performed using silica gel 60 H (0.040-0.063 mm particle size, Merck, South Africa). Sephadex LH-20 purchased from Sigma-Aldrich, South Africa was used as stationary phase. The purity of isolated compounds was monitored with Agilent Technologies 1200 series, coupled with UV detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), column compartment (G1316A) and reversed phase C18 column SUPELCO (25 x 1.0 cm, 5 μm).

4.3.2 Reagents and Solvents

All organic solvents such as the HPLC graded methanol, ethanol, ethyl acetate, dichloromethane, hexane, deuterated chloroform were supplied by Merck (Darmstadt, Germany).

4.4 Methods

4.4.1 Extraction of *M. quercifolia*

The aerial parts of the fresh plant material (~1 kg) were blended and extracted with methanol (4.5 L) at room temperature (25 ℃) for 24 hours. The methanol extract was filtered and evaporated to dryness under reduced pressure at 40 ℃ to yield 15,1 g (1.51 %). The extracts were kept under cold conditions for further use.

4.4.2 Thin Layer Chromatography (TLC)

Different plant extracts, fractions and compounds were spotted on TLC to detect their constituents and/or purity (See section 3.3.4.) Visualization of TLC plates was done by observing the bands after development under UV at wavelengths λ254 and λ366 nm using UV lamp, followed by phytochemical identification using vanillin/sulphuric acid as a detection agent (See figure 4.3).

M. quercifolia

Silica column chromatography

Main fractions

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV

XII

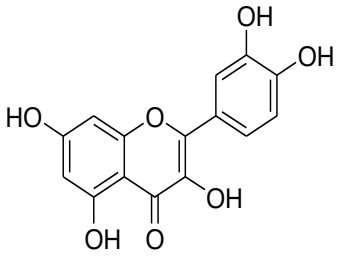
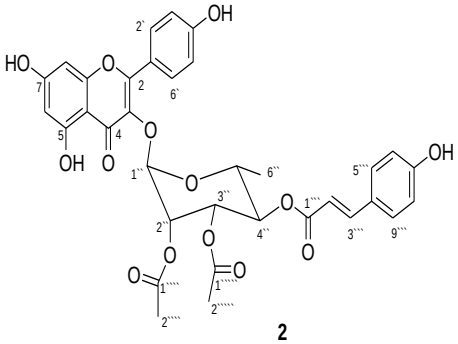
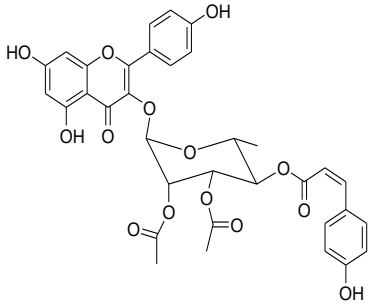
Sephadex column chromatography

1	2	3	4	5	6	7	8	9	10

XII_5

Semi-prep HPLC

Pure compounds

XII_5 (1) Compound 1	XII_5 (2) Compound 2	XII_5 (3) Compound 3
 **1**	 **2**	 **3**

Scheme 4.1: A flow diagram of experimental procedure for the isolation of pure compounds from *M. quercifolia*.

4.4.3 Fractionation of crude extract using column chromatography

The methanolic extract of *M. quercifolia* (L.), which showed the highest anti-tyrosinase activity dried and quantified (15.1 g). This extract was applied to a silica column chromatography and eluted with 100% hexane followed by Hex:EtoAc (9:1), 8:2, 7:3, 6:4, 5:5, 4:6, 2:8 and 100% EtOAc (Table 4.2). The elution process was further carried using EtOAc:MeOH (95:5), 70:30, 50:50. Thirty eight fractions (250 ml each) were collected and spotted on a TLC plate to check profile similarity. The fractions were combined according to their TLC profile to yield 21 main fractions (Table 4.1) and concentrated using a rotary evaporator. The fractions were spotted on TLC plates (Figure 4.3). The obtained fractions were coded by roman numbers (I - XXI) and the results are summarized in table 4.3, fraction XVI-XXI were ignored due their high tannin content. Scheme 4.1 shows the schematic representation of the column purification steps for the isolation of compounds from *M. quercifolia.*

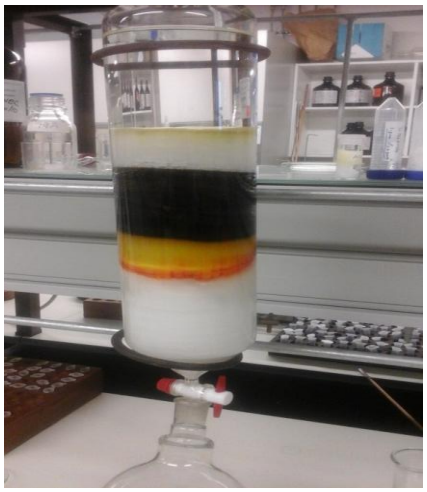


Figure 4.2 Column chromatography of crude *M. quercifolia* on silica gel.

Table 4.2 Solvent system used for fractionation of *M. quercifolia* on column chromatography.

Solvent system	Ratio	Volume (L
Hex:EtoAc	10:0	1
Hex:EtoAc	9:1	1
Hex:EtoAc	8:2	2
Hex:EtoAc	7:3	2
Hex:EtoAc	6:4	2
Hex:EtoAc	5:5	2
Hex:EtoAc	4:6	1
Hex: EtoAc	2:8	1
Hex: EtoAc	0:10	1
EtoAc:MeOH	95:5	2
EtoAc:MeOH	70:30	2
EtoAc:MeOH	50:50	1

Table 4.3 Fractionation of *M. quercifolia* extract.

Assigned Numeric no	Collected fractions	Weight (mg)
I	1-3	1794.3
II	4	1292.4
III	5	1650
IV	6	_
V	7	939.9
VI-VIII	8-12	2813.1
IX-XI	13-18	952.0
XII	19-20	1221.0
XIII	21	263.8
XIV	22-23	604
XV	24-25	931

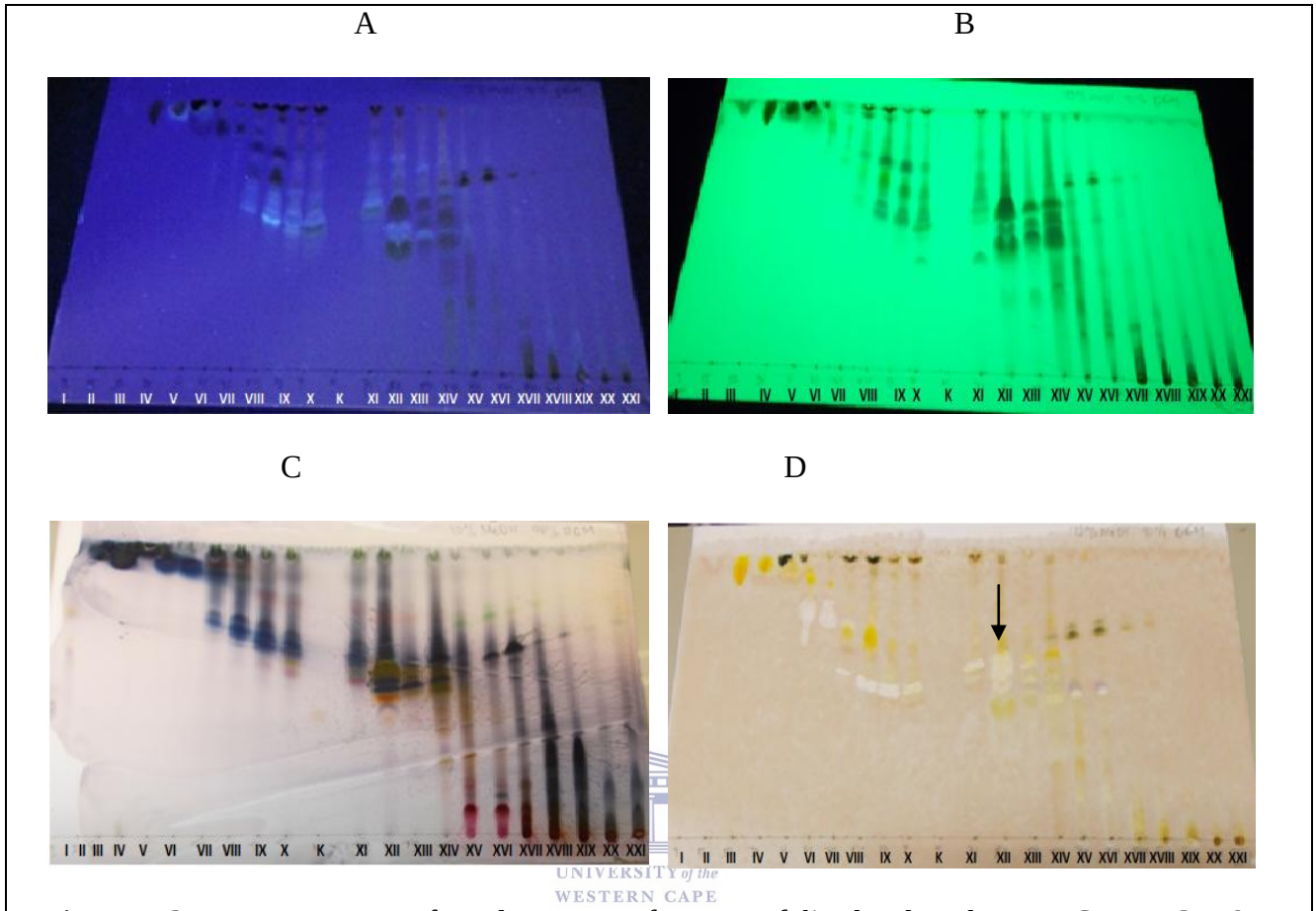


Figure 4.31: Fractions 1-21of crude extract of *M.quercfolia* developed in DCM:MeOH 95:5 viewed under (A) λ 254 nm and (B) λ 366 nm UV wavelength, then inserted in (C) Vanillin-sulphuric acid and heated at ~ 100℃, (D) sprayed with 2mM of tyrosine and 500U/ml of tyrosinase.

4.4.4 Anti-tyrosinase activity of different fractions

All fractions were subjected to biological evaluation against tyrosinase to determine the potent fraction (s). fractions were tested using TLC bioautography (see section 4.4.2), the results are shown in figure 4.3. Also, all fractions were tested for their inhibitory effects against tyrosinase activity at 100 and 50 µg/ml (Table 4.1).

Table 4.2: Tyrosinase **i**nhibitory effects of total fractions of *M. quercifolia* that were evaluated at concentrations of 100 and 50 µg/ml.

Fractions	**Percentage of inhibition (%)**		**TLC bioautography Inhibition zone (Rf) (Relative intensity)**
	100 µg/ml	50 µg/ml	
II	50.56	40.79	
III	82.40	45.07	
IV	46.67	26.49	
V	76.06	30.00	0.80 (+)
VI	99.03	46.69	0.78 (+)
VII	86.11	30.72	0.6 (+)
VIII	67.28	24.36	0.54 (+)
IX	51.36	30.61	0.50 (++)
X	59.24	28.28	0.49 (+)
XI	54.83	29.47	0.56 (+++)
XII	64.09	42.10	0.62 (+)
XIII	62.11	40.61	
XIV	69.44	69.74	
XV	86.28	74.10	

4.4.5 Fractionation of fraction XII

According to table 4.1, fraction XII among the fractions which showed high inhibition as well as clear inhibition zones on the TLC bioautography, therefore, it was selected for further purification.

Fraction XII_5 of *M. quercifolia* was dissolved in methanol it was then applied to a Sephadex column and eluted with ethanol/distilled water mixture (1:1) followed by EtOH:H_2O (9.5:0.5). As the column's elution started, compounds started to form bands and moved down the column at different rates. Ten main fractions were collected and concentrated separately using a rotor vapour (see figure 4.5).

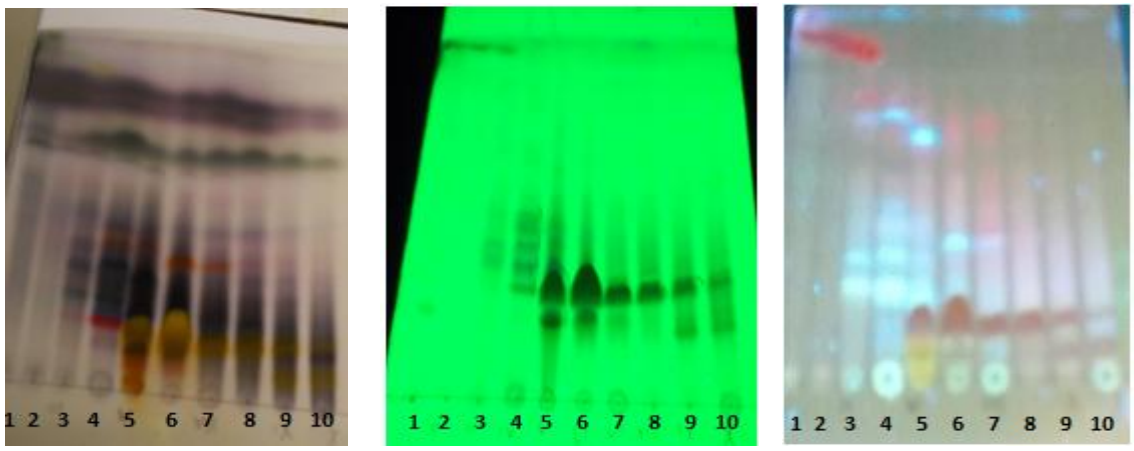


Figure 4.32: TLC plate of fractions I to X of fraction XII developed in DCM:MeOH and 95:5, the plate was then dipped in vanillin-sulphuric acid and heated at ~ 100 ℃, plates were viewed under (B) 254nm and (C) 366 UV wavelength.

4.4.6 Purification of compounds from fraction XII using Semi-preparative High Pressure Liquid Chromatography (HPLC)

Fraction XII was selected because it showed interesting chemical profileThe final purification of the compound from fraction XII_5 (330 mg) was carried using semi-prep HPLC. The flow rate was set at 1.5 ml/min and 50 µl was the sample injection volume. All experiments were done at room temperature (20℃). Briefly, the mobile phase was composed of 65% MeOH to 75% in 20 min, then to 100% in 25 min, keep isocratic 100% to 35 min. The detector monitored the eluent at wavelength λ 254 nm. The peaks were observed at 14.2 min for compound 1, 30.17 min for compound 2 and 30.8 min for compound 3 (figure 4.61)

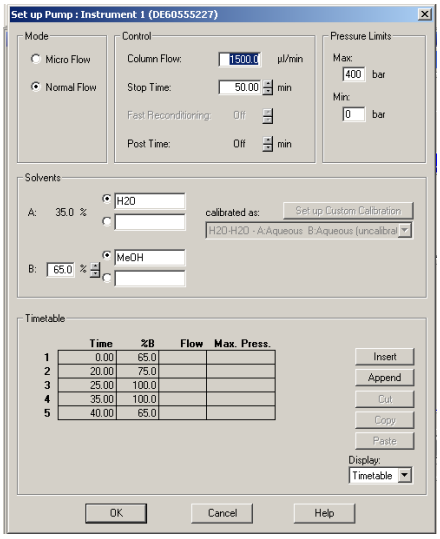


Figure 4.5: HPLC Conditions

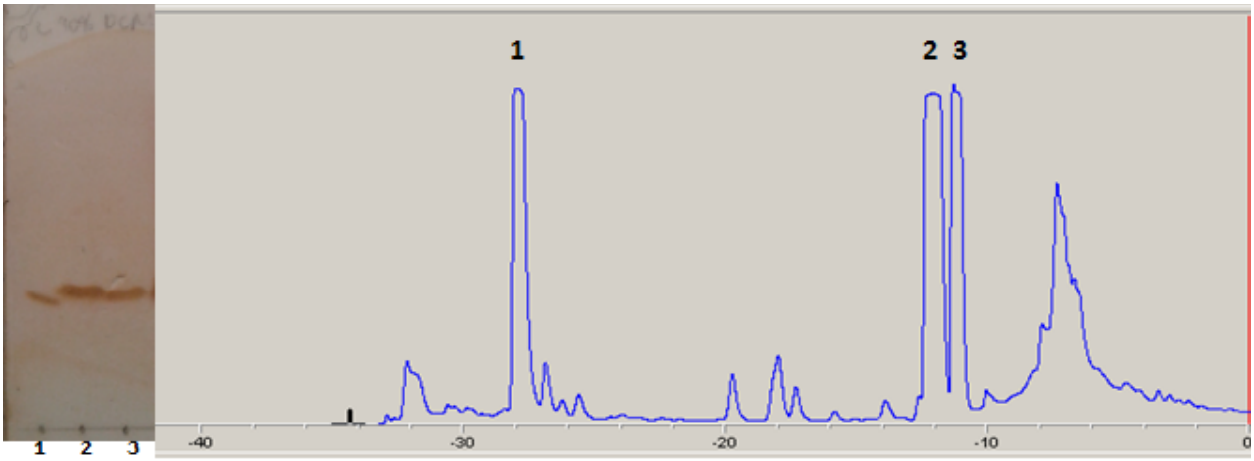


Figure 4.6: TLC silica gel of isolated compounds, [DCM:MeOH (9:1)] and HPLC chromatogram of XII_5 (Fig. 4.5).

4.4.7 Structure elucidation using Nuclear magnetic resonance (NMR) spectroscopy

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

4.7 Results and discussion

4.7.1 Fractionation and purification of *M. quercifolia* constituents.

The preliminary screening of plants extracts indigenous to South Africa using TLC bioautography and ELISA assays indicated that *M. quercifolia* showed the best activity and it was selected for further chromatographic purification to isolate the bioactive compound.

The methanolic extract was subjected to silica gel column chromatography (see table 4.2 for solvent system) and separated into 38 fractions, subsequent to fractionation, all fractions were spotted and combined according to their TLC profile similarity to make 21 main fractions. Fractions 16-21 were not used because they had high tannin content.

A bioactivity-guided process was adopted to fractionate the leaf extract of *M. quercifolia*. The inhibitory activity of each fraction was measured spectrophotometrically (Table 4.1), the most powerful fraction (VI) [99% inhibition (100 µg/ ml)] and second most powerful fraction (VII) [86 % inhibition (100 µg/ml)]. Fraction XII [64 % inhibition (100 µg/ml)] was selected and submitted for further purification because of anti-tyrosinase activity both spectrophotometrically and on the TLC bioaugraphy showing clear inhibtion zone at R_f 0.62.

Figure 4.6 shows the HPLC chromatograms of fraction XII_5. All the major peaks were separated within 40 minutes, with retention factors depending mainly on structural hydrophobicity. After the isolation, three compounds were identified as candidates for tyrosinase inhibition, eluting in different retention times.

Compound **1** appeared at 14.2 min, compound **2** at 30.18 min and compound **3** at 30.8 min.

Compound 1 isolated from fraction XII and identified as quercitin from its NMR data.

^{1}H and ^{13}C NMR data showed typical 6-, and 8-H signals at 6.52/6.25 (1H each/bris) attached to carbons C_6 and C_8.

1,3,4. Trisubstituted rings B was identified from the signals at 7.80 (bis) 7.68 (d) and 6.98 (d) and attached to carbons C_2 ; C_5, and C_6. The remaining of carbon and careful analysis of 2D NMR data (HSQC and HMB) confirmed the structure of quercitin.

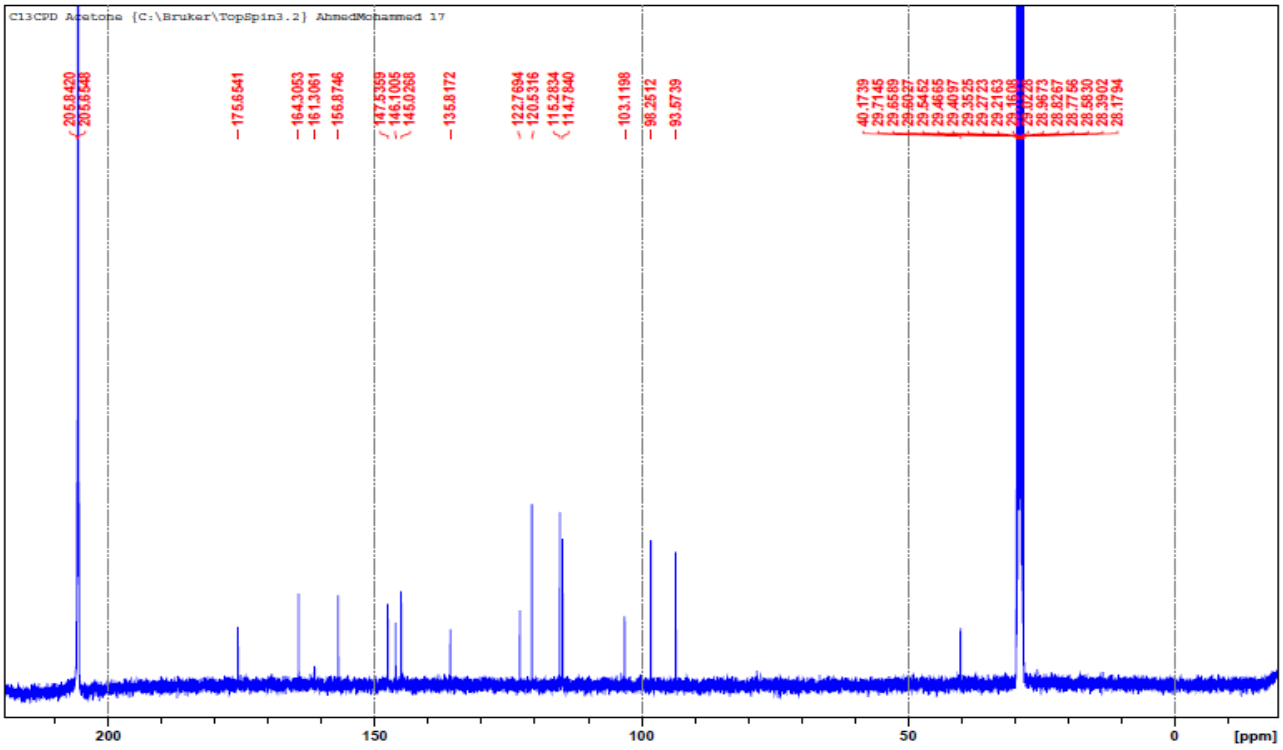


Figure 4.71: ^{1}C-NMR of compound 1

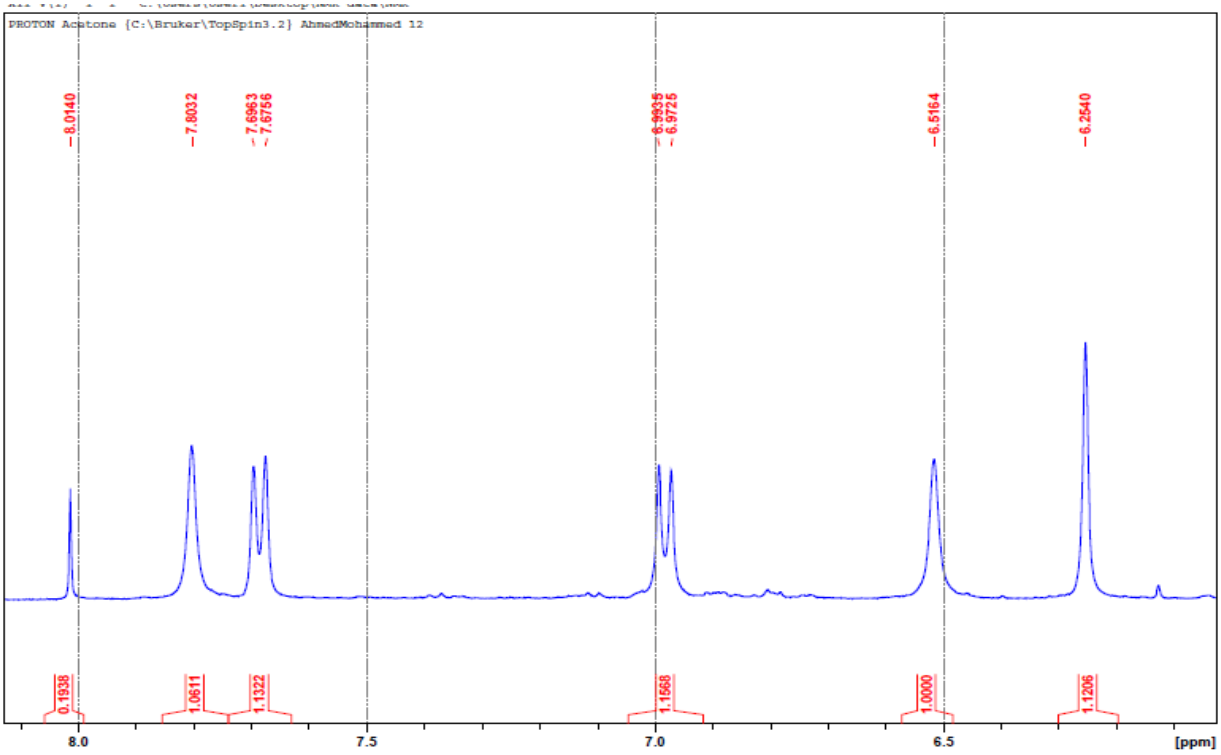


Figure 4.72: ^{1}H-NMR spectrum of compound 1

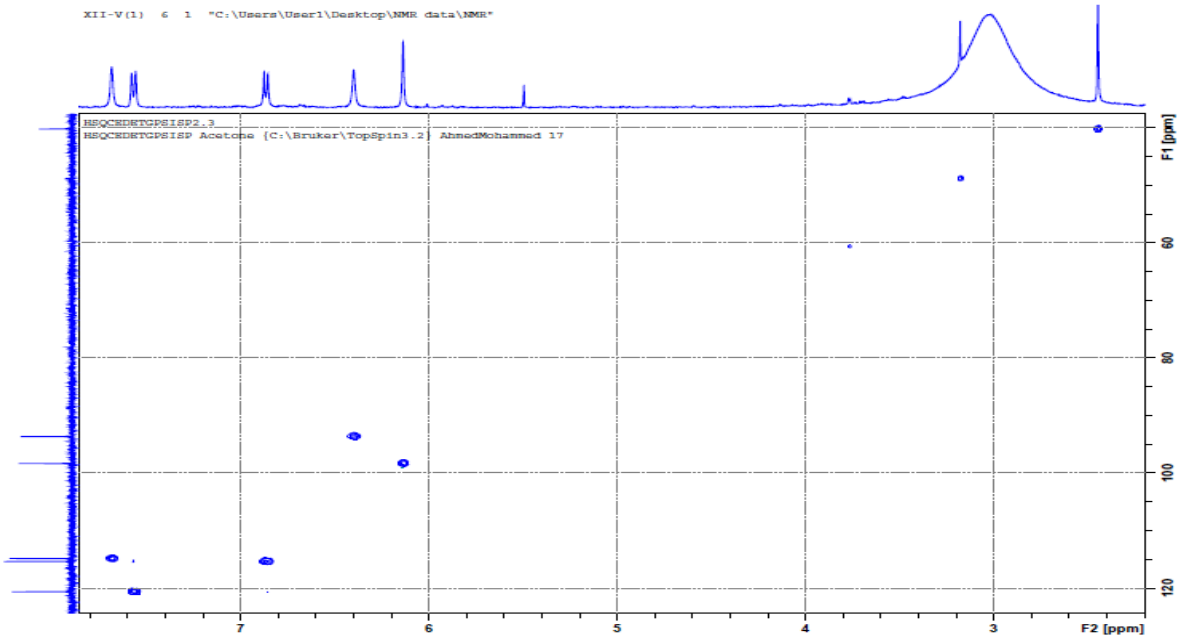


Figure 4.73: HSQC NMR spectrum of compound 1

Compound 2 was isolated as amorphous white-yellowish powder (6 mg). It was isolated using semi-prep HPLC from the polar fraction (XII_5) and gives purple colour under λ 254 nm which indicated unsaturated structure and more specifically, flavonoid. This was confirmed from the UV absorption peak at 265 and 310 nm.

^{1}H- and ^{13}C-NMR showed three different regions, the first region showed a typical kaempferol. The typical 6 and 8-signals protons appearred at 6.28 and 6.48 ppm, and 1,4 disubstituted ring B at 7.89 and 7.11 (2H/each) for 2',6' and 3,5' respectively. ^{13}CNMR supported the kaempferol structure with 15 carbon signals as shown in table 4.4. Therefore, data indicated the presence of kaempferol nucleus attached to Rhamnose which was acylated with 2-acetate groups (C2 and C3) and p-coumaric acid at C-4. Thus compound 2, has the strucure of kaempferol 3-(2,3-diacetoxy-cis-p-coumaroyl)rhamnose.The structure of kaempferol-3-(2,3-diacetoxy-4-*p*-coumaroyl)rhamnoside and was identified previously from *Myrica gale*.

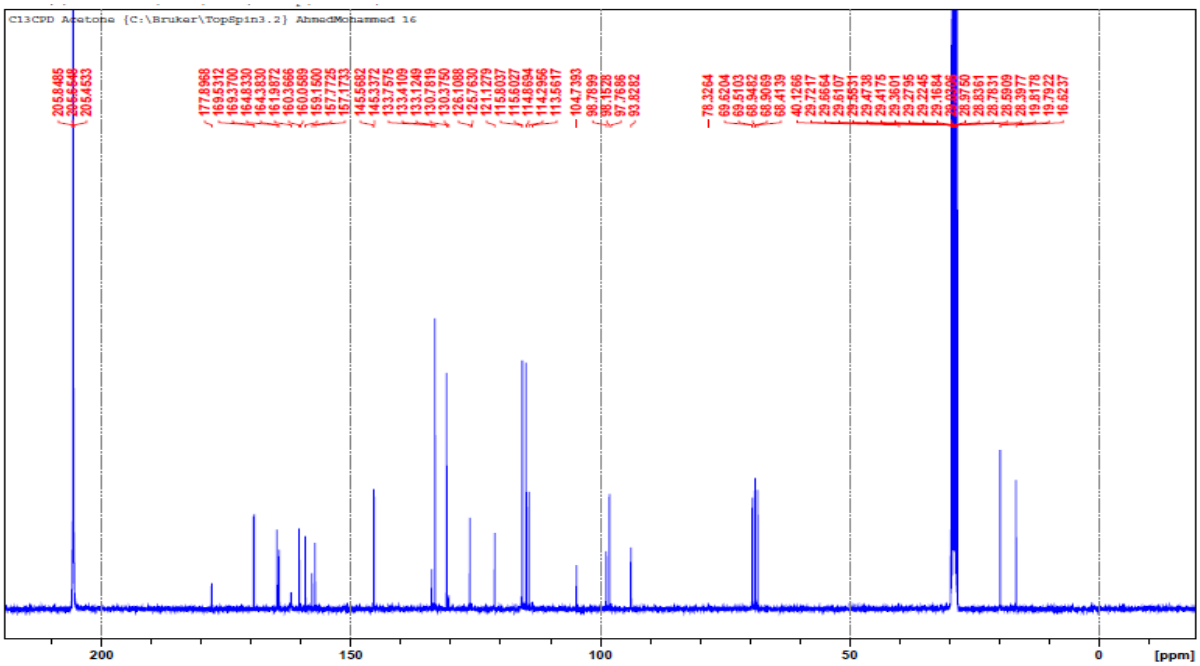


Figure 4.74: [1]C-NMR of compound 2

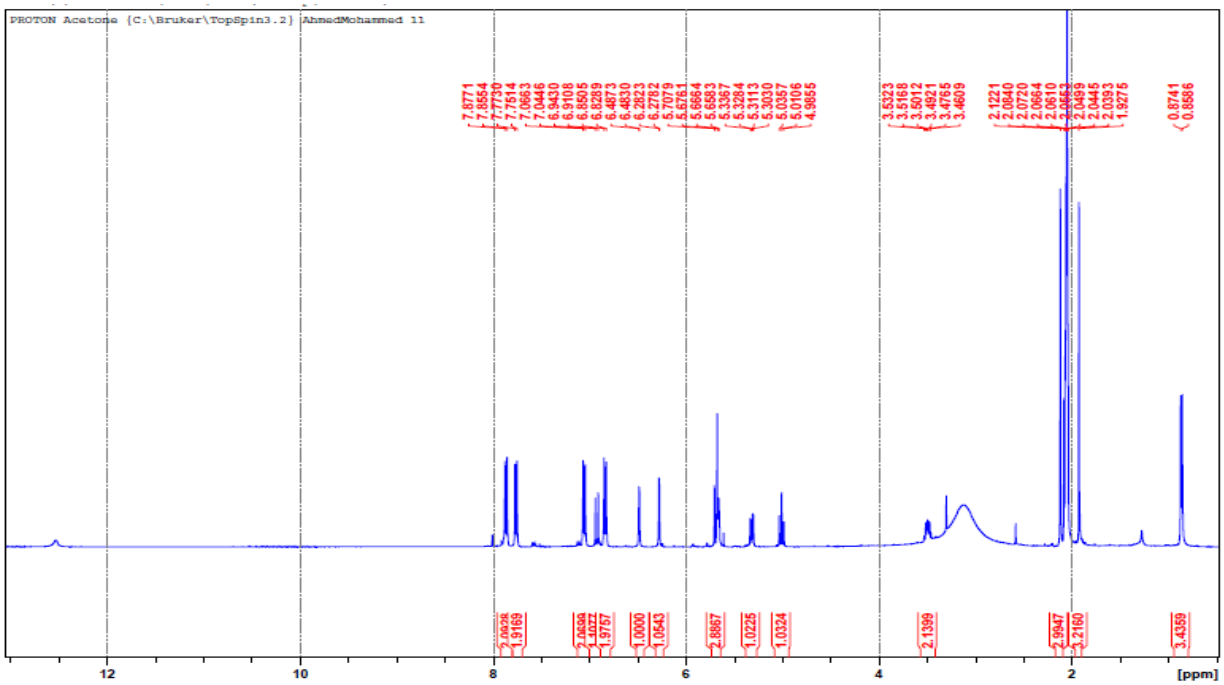


Figure 4.75: [1]H-NMR spectrum of compound 2

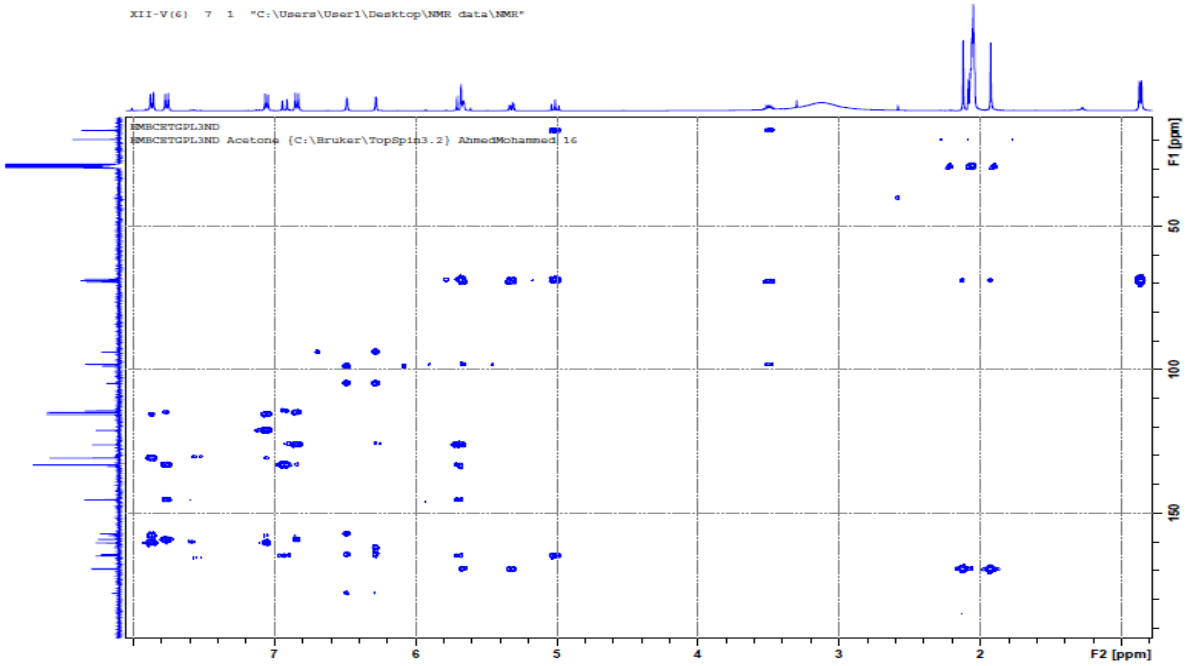


Figure 4.76: HMBC NMR of compound 2

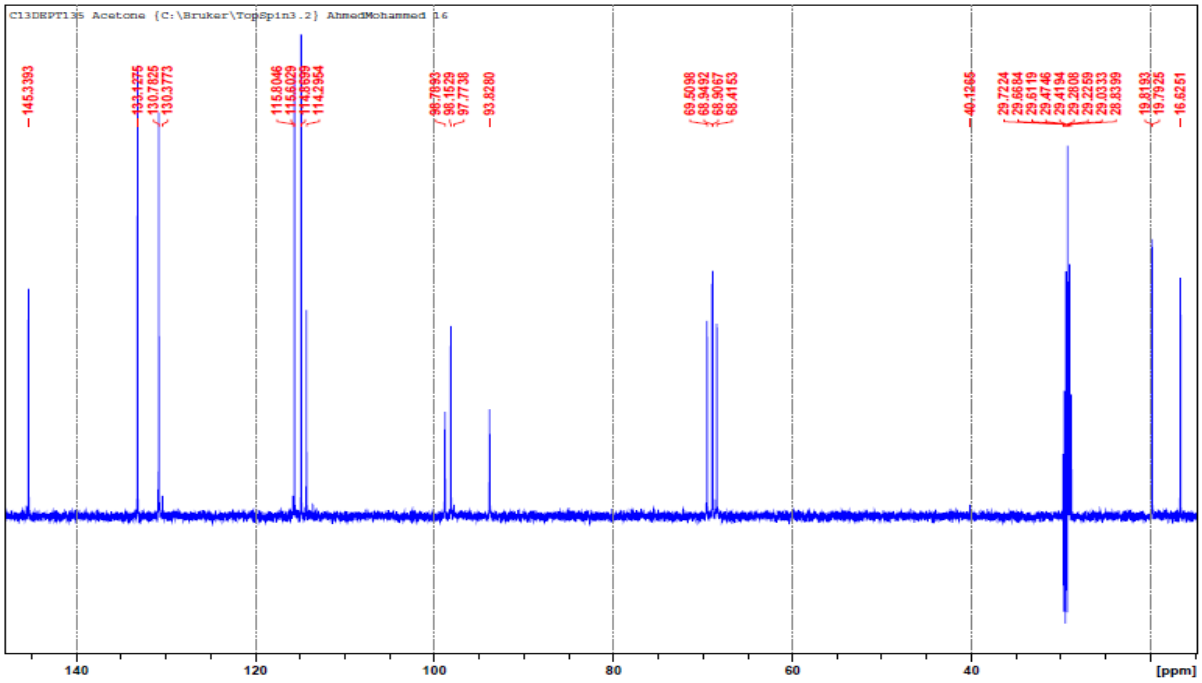


Figure 4.77: DEPT NMR spectrum of compound 2

Region 2 showed a glycoside unit acylated at positions 2,3 and 4. The signal of the methyl group at 0.86 (d, 6.2Hz/ δC16.6) indicated Rhamnose. The HMBC correlation (Fig..) showed a correlation between the two acetyl groups and C-2 and C-3 respectively, the C-4 was acylated with phenolic acid.

Region 3; showed a typical signal of *p*-coumaric acid (Table 4.4), it showed signals of trans coupled two protons at 7.57 and 6.26 (d, J=16.0 Hz), 1,4-disubstituted benzene ring at 7.58/130.4 and 6.90/115.8 (2H each; d, 8.5 Hz), in addition to carbonyl group at 165.6.

Compound 3 was isolated from the same fraction (XII_5), and showed a typical flavonoidal skeleton on TLC and UV analysis.

The ^{1}H- and ^{13}C-NMR data were very similar to compound 2 (Table 4.4) except for the region of *p*-coumaric acid and especially C-1-C-4, and proton signals of the double bond

which showed clear shift from compound 2.

Careful analysis of the data including 2D (HMBC) indicated that the compound has a typical structure of compound 2 except the double bond geometry which showed different chemical shift and coupling identical with cis *p*-coumaric acid. This was further confirmed on typical and this confirmed by the coupling constant between (H-2 and H-3).

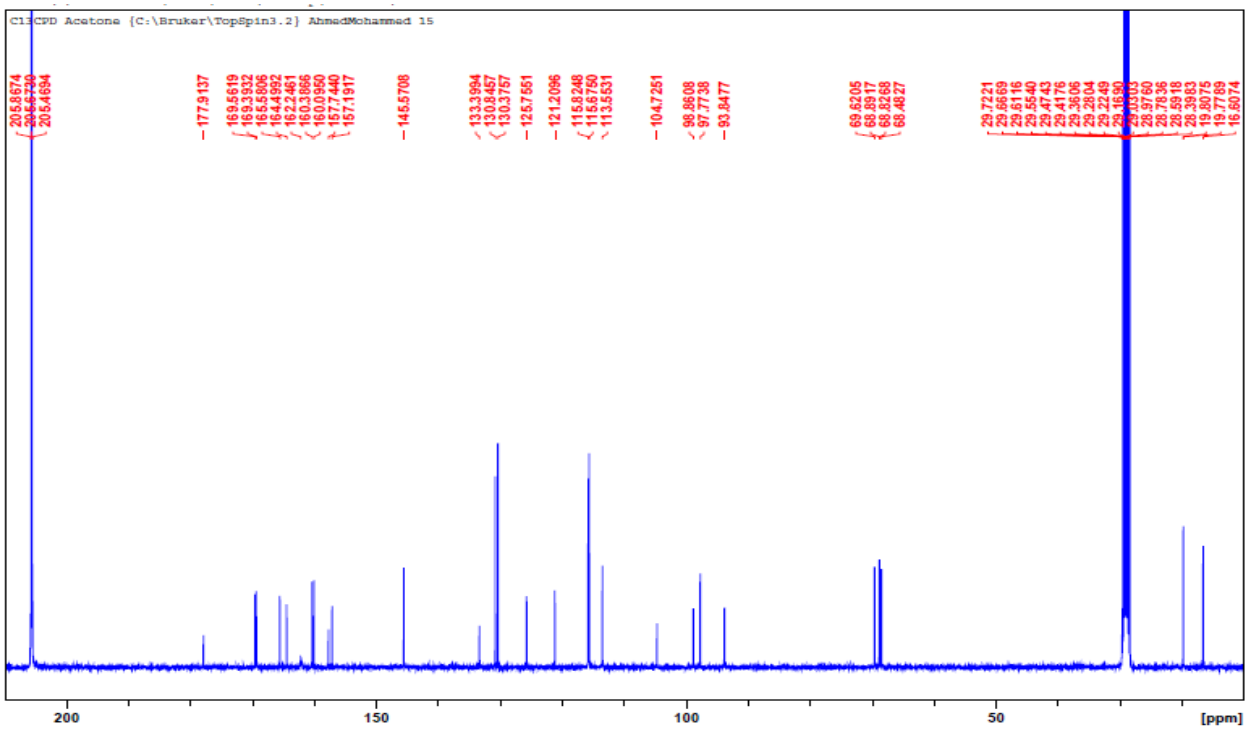


Figure 4.78: ^{1}C-NMR spectrum of compound 3

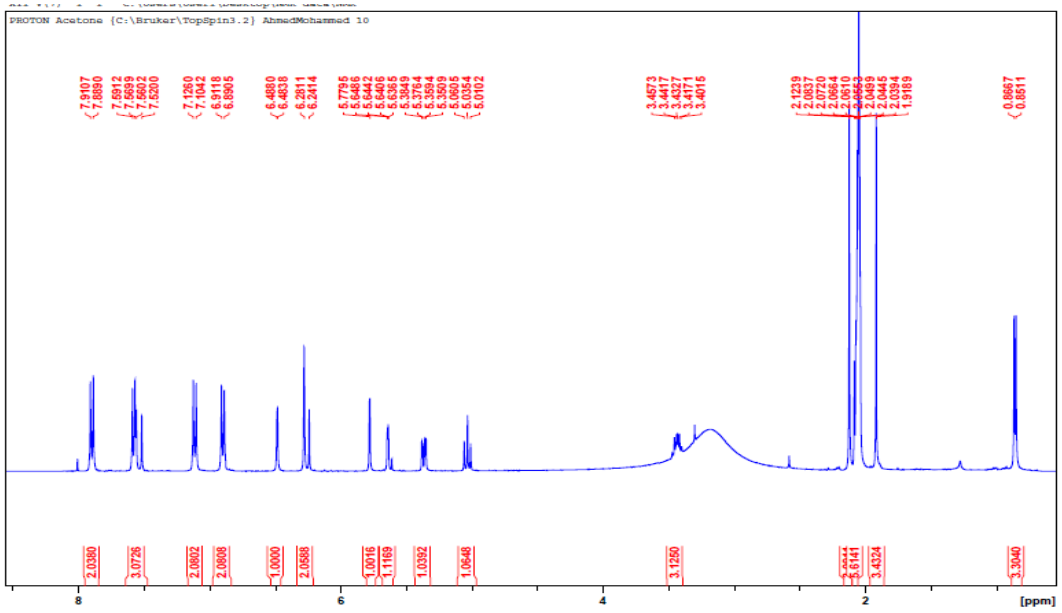


Figure 4.79: ^{1}H-NMR spectrum of compound 3

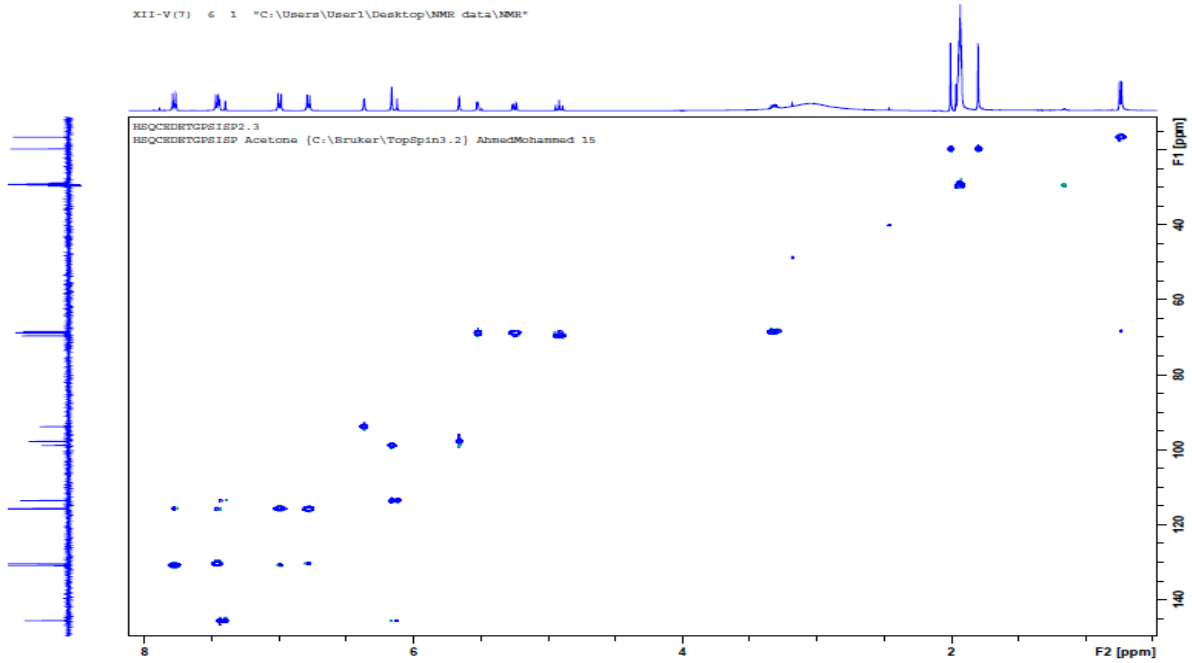


Figure 4.8: HSQC NMR spectrum of compound 3

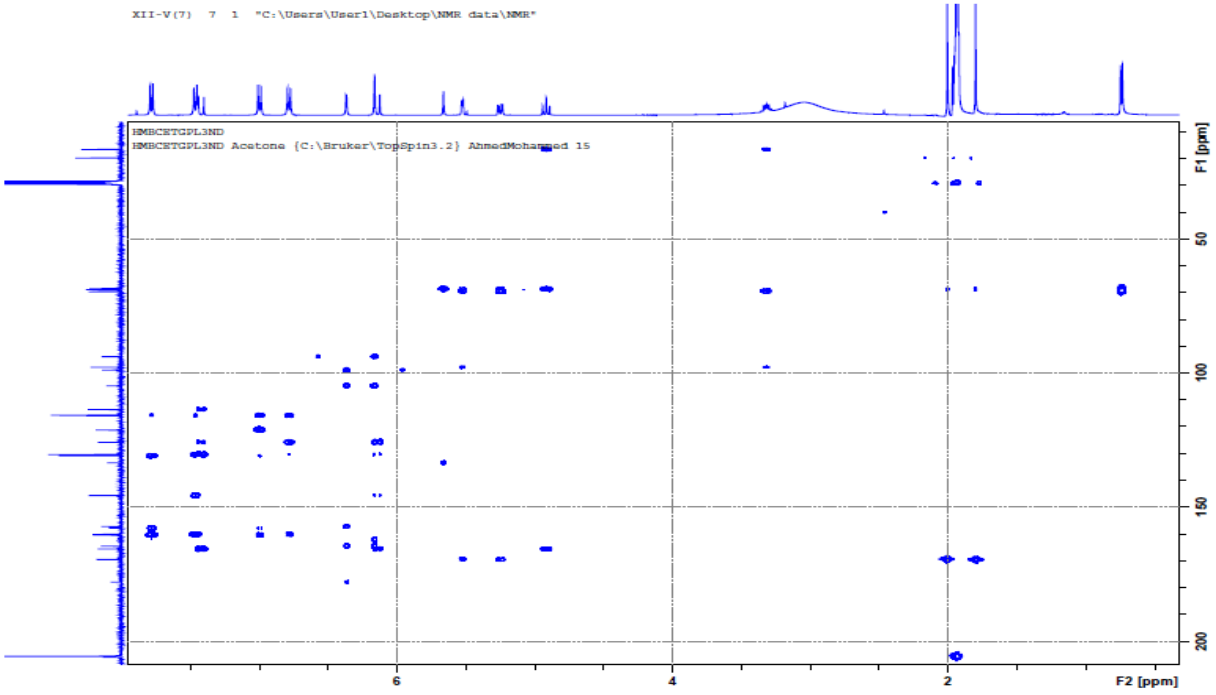


Figure 4.81: HMBC1 NMR spectrum of compound 3

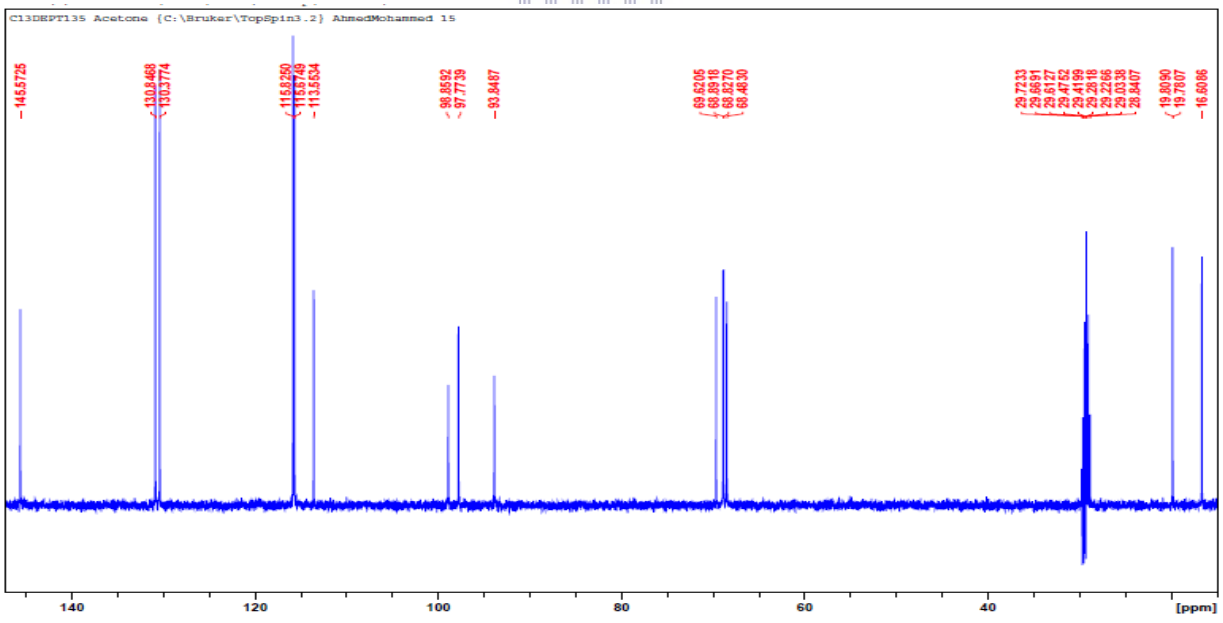


Figure 4.82: DEPT NMR spectrum of compound 3

Table 4.4

No	XII-V(7)					
	C	H	C	H	C	H
2	157.2 s		157.8 s			
3	133.4 s		133.8 s			
4	177.9 s		177.9 s			
5	157.7 s		157.2 s			
6	98.9 d	6.28 d, 1.7	98.8 d	6.28 d, 1.7		
7	164.5 s		164.8 s			
8	93.9 d	6.48 d, 1.7	93.8 d	6.48 d, 1.7		
9	162.3 s		162.0 s			
10	104.7 s		104.7 s			
1`	121.2 s		121.1 s			
2`, 6`	130.8 d	7.89 d, 8.7	130.8 d	7.89 d, 8.7		
3`, 5`	115.79 d	7.11 d, 8.7	115.6 d	7.11 d, 8.7		
4`	160.4 s		160.4 s			
1``	97.8 d	5.77 s	98.2 d	5.77 s		
2``	68.6 d	5.64 t, 1.7	68.9 d	5.64 t, 1.7		
3``	68.9 d	5.36 d, 3.4, 10.1	69.0 d	5.36 d, 3.4, 10.1		
4``	69.9 d	5.04 d, 10.1	69.5 d	5.04 d, 10.1		
5``	68.5 d	3.43 m	68.4 d	3.43 m		
6``	16.6 q	0.86 d, 6.2	16.6 q	0.86 d, 6.2		
1```	165.6 s		162.0 s			
2```	145.6 d	7.57 d, 16.0	145.3 d	7.57 d, 16.0		
3```	113.6 d	6.26 d, 16.0	114.9 d	6.26 d, 16.0		
4```	125.6 s		126.1 s			
5```, 9```	130.4 d	7.58 d, 8.5	133.1 d	7.58 d, 8.5		
6```, 8```	115.8 d	6.90 d, 8.5	114.8 d	6.90 d, 8.5		
7```	160.1 s		160.1 s			
1````	169.4 s		169.4 s			
2````	19.78‡ q	2.12 s	19.78‡ q	2.12 s		
1`````	169.6 s		169.6 s			
2`````	19.81‡ q	1.92 s	19.81‡ q	1.92 s		

4.7.2 Inhibition of the enzyme tyrosinase by *M. quercifolia* extract and Kojic acid (positive control) using L-tyrosine as a substrate.

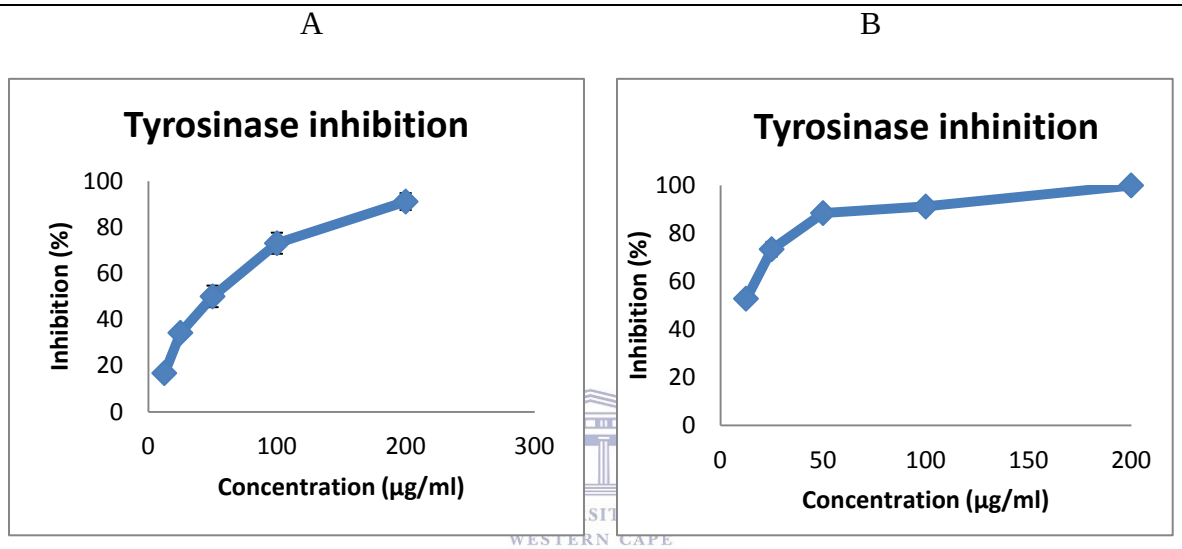


Figure 4.83 Inhibitory effect on tyrosinase activity by methanolic extract of (A) *M. quercifolia* and (B) kojic acid (positive control).

According the results obtained from the present study, tyrosinase inhibitory effect of methanolic leaves of *M. quercifolia* were evaluated on ELISA. As indicated in figure 4.3A, MeOH extract of *M. quercifolia* showed inhibitory effects with an IC_{50} value of 66.9 µg/ml comparable with kojic acid with an IC_{50} of <12.5 µg/ml. All experiments were repeated 3 times in triplicates and the extract of *M. quercifolia* exhibited the same consistency throughout all the experiments.

4.7.3 Melanin biosynthesis inhibition by *M. quercifolia* and kojic acid and Kojic acid using L-tyrosine as a substrate.

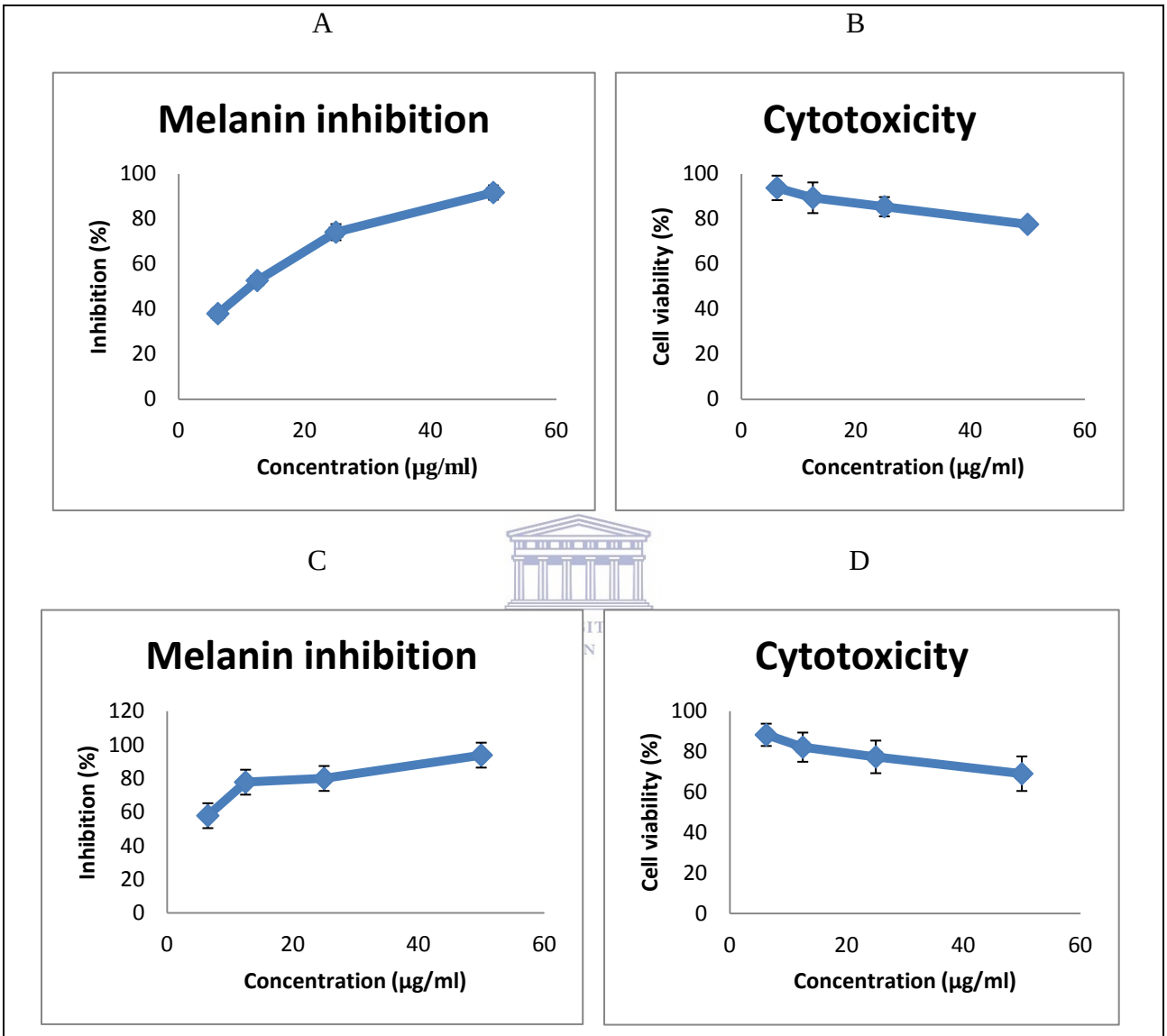


Figure 4.84: The inhibitory effects of (A) *M. quercifolia* (C) Kojic acid (positive control) and cytotoxicity effects of (B) M. *quercifolia and* (D) kojic acid on melanoma B16-F10 cells.

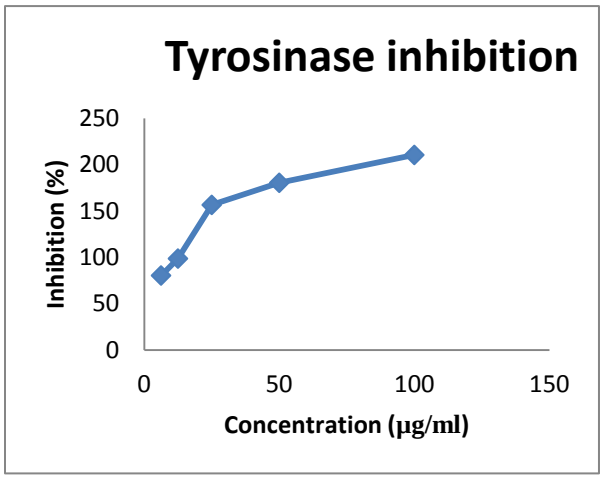
In the current study, melanoma B16-F10 cells were employed in assaying melanin inhibition as it contains moderate amount of the pigment and are suitable for observing the the melanin

pigment. Melanoma cells were treated with different concentrations of plant extracts ranging from 6.25 to 50 µg/ml; the activity was show in a concentration dependant manner (see figure 4.9). *M. quercifolia* exhibited potent inhibtory effects on melanoma cells (<6.25 µg/ml) without cytotoxic effects (>100 µg/ml) kojic acid showed the same inhibitory and cytotoxic results as *M. quercifolia*.

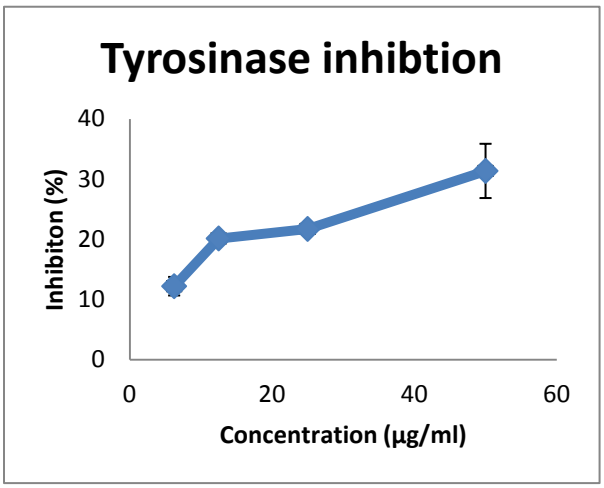
4.7.4 Effect of bioactive compounds on tyrosinase Inhibition.

Methanolic extract of *M. quercifolia* was chosen for further examinination as they displayed the highest and consistent tyrosinase inhibition activity among 37 plant species that were screened.

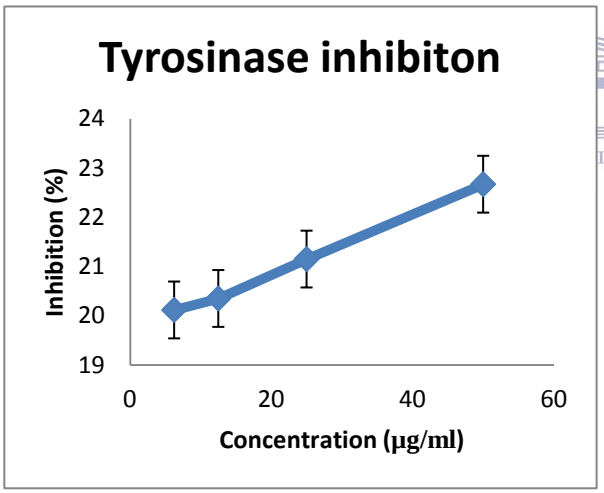
A



B



C



D

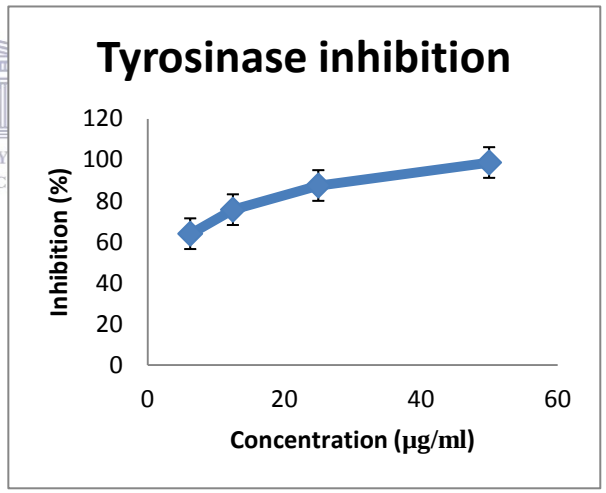


Figure 4.85: Inhibitory effect on tyrosinase activity by Compound 1 (A) Compound 2 (B) Compound 3 (C) and Kojic acid (D).

The tyrosinase inhibitory activities of the bioactive components of *M. quercifolia* (L.) were confirmed spectrophotometrically using a tyrosinase assay. As shown in Figure 4.6, all isolated compounds did not show any anti-tyrosinase activity except for compound 1 which showed potent inhibitiory effects with an IC_{50} of <6.25 µg/ml; compounds 2 and 3 had IC_{50} values of > 100 µg/ml. Kojic acid had a significant inhibitory effect with an IC50 of < 6.25 µg/ml.

4.7.5 Effect of bioactive compounds on melanin inhibition

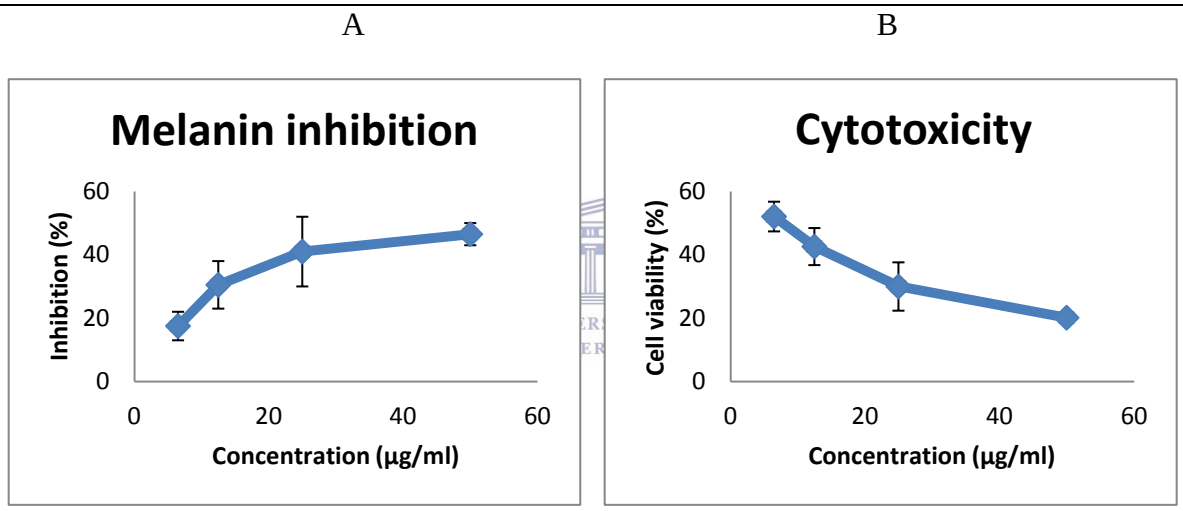


Figure 4.86: The inhibitory effects of (A) Compound 1 (XII-V(1)) on melanoma B16-F10 cells after a 72 hour treatment (B) cytotoxicity after a 24 hour treatment.

A B

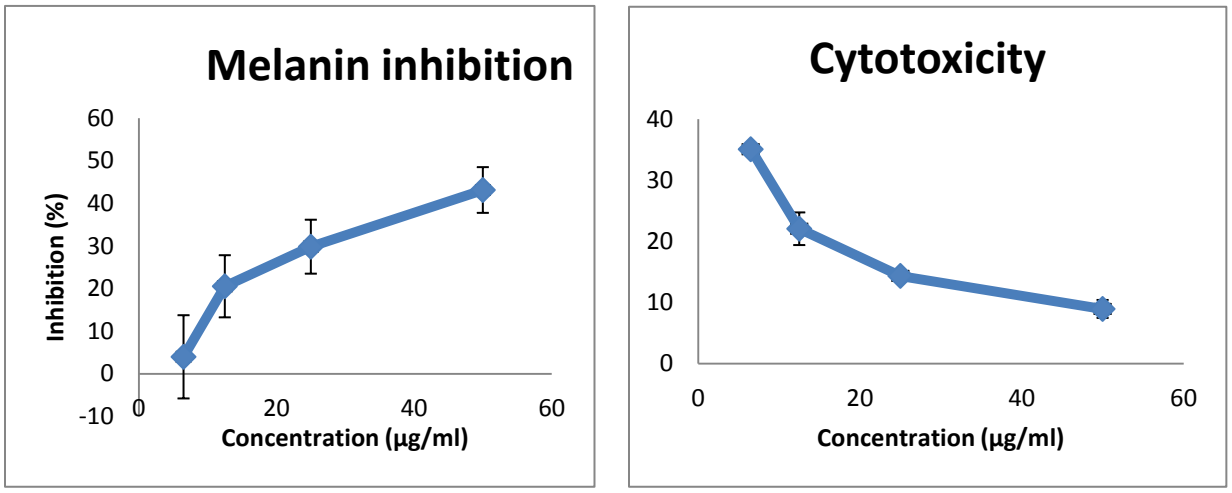


Figure 4.87: The inhibitory effects of (A)Compound 3 (XII-V(7)) on melanoma B16-F10 cells after a 72 hour treatment and (B) cytotoxicity after 24 hour treatment.

Table 4.5. IC_{50} values of pure compounds isolated from *M. quercifolia* (L.)

Sample	**IC_{50} (µg/ml)**		
	Inhibition (tyrosinase)	Inhibition (melanin)	Cytotoxicity
Myrica quercifolia	66.9	< 6.25	97.2
Compound 1	< 6.25	> 100	< 100
Compound 2	> 100	> 100	< 100
Compound 3	> 100	> 100	< 100
Kojic acid	< 6.25	< 6.25	50.3

Three compounds were isolated from the MeOH extracts, then evaluated for their melanogenesis inhibitory activity. By addition of these compounds at the concentration of 6.25, 12.5, 25 and 50 µg/ml, to an incubation medium of melanoma B16-F10 cells, all of the compounds showed weak inhibition of melanogenesis (melanin inhibition of 3.99-46.5%). However, all three compounds did not possess the same degree of activity when compared to the crude plant extract at comparable concentrations; this could be because the compounds in the plant matrix have a multi-factorial effect or they form a synergistic interaction to inhibit melanin or the compounds that are responsible for inhibition were not isolated.

Cell viability was assessed by the MTT reduction assay. Mouse melanoma cells were only resistant to compound 3 at a low concentraions (6.25 µg/ml) showing viability of 52.06%. All three of the isolated compounds, showed toxicity to the cells (cell viability 8.91-52.06%) compared to the toxicity of the crude plant extract that they were isolated from whose viability

was 77% at 50 µg/ml. In 2010 Akazawa and colleagues carried a melanogenesis inhibitory study where *Myrica rubra* was evaluated against mushroom tyrosinase; myricanol, myricanone, myricetin among other compounds were isolated and exhibited very high toxic effects to the melanoma B16 cells and thus induced cell death adversely with 1.9-17.7% cell viability at 25 µg/ml.

There is little literature on *M. quercifolia* but according to Plantzafrica it has been traditionally used for its wax to make candles and for stomach aches. Up to date, *M. quercifolia* has not been reported for tyrosinase inhibition, melanin inhibition or for any other scientific bioassays. However, there are reports of *Myrica* genera of which *M. rubra* is the most researched and is also popular for its rich phyto-nutritional content. The leaf consists of, proanthocyanidins, triterpenes, diarylheptanoids and flavonoids (Sun *et al.*, 2013) and flavonol glycosides (myricetin-3-O-β-D-glucoside, myricetin-3-O-α-L-rhamnoside, quercetin-3-O-α-L-rhamnoside, and quercetin-3-O-β-D-glucoside) has been isolated from the leaves and kernels of *M. rubra* (Wang *et al*., 2010).

In 2010, Matsudi *et al*., assessed methanolic extracts from leaves of *M. rubra* against tyrosinase activity. The extract showed potent tyrosinase activity; the IC_{50} of the tyrosinase inhibitory effect was 0.23 mg/ml.

In 1995 Matsudi and colleagues, conducted a tyrosinase inhibitory study; ethanolic extract of the leaf and bark of *Myrica rubra* were evaluated. The bark which was tested at a concentration of 500 µg/ml exhibited the highest inhibition of 73% and the leaves showed activity of 70% at the same concentration. The inhibitory activity of all other plant species mentioned above are not reported. Figure 3.3 illustrates crude extracts which showed activity

higher (these extracts were selected from the 37 extracts that were screened for tyrosinase inhibition, see table 3.2). Crude extracts were further selected and re-evaluated for melanin inhibition, cytotoxicity and IC_{50} determination.

One study by Ohguchi and Ozaki, 2017 , reported the stem bark of *Myrica esculenta* which was investigated for its ability to inhibit α-MSH induced melanogenesis. When melanoma B16 cells were treated with α-MSH in the presence of *M. esculenta* bark extracts, α-MSH induced melanin production decreased significantly, compared to the cells that were not treated with *M. esculenta* bark extracts. The amount of melanin content decreased in a dose-dependent manner by *M. esculenta* bark extracts, with the maximal level at 10 µg/ml.

Chapter 5

5.1 Motivation of the study

As a contribution to the on-going search for novel, effective, stable, safe and affordable treatments to be used for depigmentation in southern Africa, it was necessary to advocate scientific research on plants from the CFR.

The analysis and identification of natural antioxidants with practical applications in food, medicine, and cosmetics, and the determination of anti-tyrosinase compounds become more attractive and of great interest scientific research on plants used for skin diseases.

Conclusion

With its unsurpassed botanical diversity, the CFR holds unexplored potential natural resources of global significance. For that reason, 37 plants from the CFR were evaluated for tyrosinase inhibition using TLC bioautography in order to develop new potential skin whitening agents; kojic acid was used as a positive control. The activity was confirmed with tyrosinase assay spectrophtometrically; seventeen methanolic extracts including *A. karroo* (Hayne.), *A. afra* Jacq. Ex Willd, *C. geifolia* (L.), *E. racemosa* (L.), *H. petiolare* Hilliard & B.L.Burt, *M. quercifolia* (L.), *M. communis* (L.), *P. rigida* (Wikstr.), *P. ecklonii* (Benth.), *P. ericoides* (L.), *S. Africana-caerulea* (L.), *S. Africana-lutea* (L.), *S. antarcticus* (Willd.), *S. lucida* (L.) F.A.Barkley, *S. hamilifolius* (L.), *S. furcellata* R.Br and *T riparia* showed significant inhibition for mushroom tyrosiase. Melanogenesis inhibitory activity for the 17 active extracts was employed *in vivo* at 50 µg/ml. Of the 17 extracts, eight showed the best activity [*T. riparia*, *S. furcellata* R.Br, *C. geifolia* (L.), *A. karroo* (Hayne.), *M. quercifolia* (L.), *P. eroides* (L.), *S antarcticus* (Willd.) and *M. communis* (L.)], consequently the active extracts were selected for

further *in vivo* studies; melanogenesis inhibitory and cytotoxic studies were employed. *M. quercifolia* exhibited the best inhibitory effects compared to other plant extracts and was selected for purification and characterisation of bioactive compounds. There is no information in literature to describe the inhibitory effects of *M. quercifolia*, however, it is known that the genus *Myrica* has a variety of molecules with different biological activities such as antioxidant, melanogenesis, activities from various species including as *M. rubra* and *M. esculenta*. The compounds that were isolated from *M. rubra* that have been attributed to melanin inhibition are myricanol 11-O-b-D-glucopyranoside, myricanol 5-O-b-D-(6′-O-galloyl)-glucopyranoside, Myricanone 5-O-a-L-arabinofuranosyl-(1→6)-b-D-glucopyranoside, myricitrin (myricetin 3-O-a-L-rhamnopyranoside and 16-Methoxy acerogenin B 9-O-b-D-apiofuranosyl- (1 →6)-b-D-glucopyranoside.

In this study, the bioactive compounds of *M. quercifolia* were isolated following bioactivity-guided isolation using chromatographic techniques and subsequently identified using spectropic techniques. Three compounds, such as quercitin, kaempferol-3-(2,3-diacetoxy-4-trans-*p*-coumaroyl)rhamnoside and kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside were isolated from the methanolic fraction (XII_5) as the active principles.

Quercitin (compound 1) strongly inhibited tyrosinase activity but showed weak activity on B16 melanoma cells in comparison with a known melanogenesis inhibitor, kojic acid, the cells were only resistant to toxicity at the lowest concentration tested. Kaempferol and compound 3 showed weak activity both in tyrosinase and melanin inhibition assays and were toxic to cells at all tested concentrations.

This study gives some validation towards the use of some medicinal plants for the treatment of skin disorders. Moreover, selective plants from the CFR could be targeted for future study on melanin inhibition which plays an important curative role in the overall health of the skin. This study indicated that *M. quercifolia* is a suitable plant extract for isolation of suitable bioactive candidates for skin-whitening.

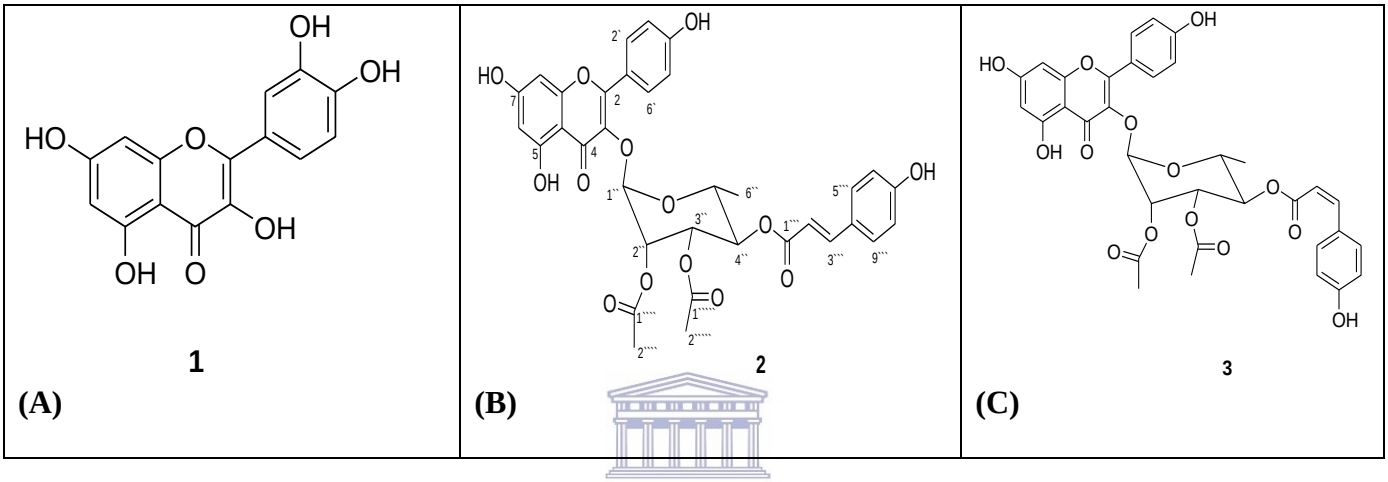


Figure 4.88. Chemical structures of (A) quercetin (B) Kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside, and (C) kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside isolated from the methanol extract of *M. quercifolia.*

Recommendations

This study is a starting point for further investigations on tyrosinase inhibitors with respect to CFR plant extracts. Simultaneously, more CFR plants should be explored in order to identify useful chemical compounds that exhibiting broad-spectrum activities against the tyrosinase activity. The present data would certainly help to determine the potency of the tested plant materials as potential source of tyrosinase inhibitors to be used for cosmetics, pharmaceutical, medical and functional food applications.

Further research is needed to identify individual components from *M. quercifolia* that have noteworthy inhibition against tyrosinase. It may be beneficial to test compounds together to address the synergistic effects on skin lightening, particularly when the active components influence distinct steps of melanogenesis. Further study including the evaluation of tyrosinase inhibitory activity is required to clarify the mechanisms of the active extracts and compounds.

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