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POTENTIAL ANTI-MELANOGENIC EFFECTS OF SELECTED SOUTH AFRICAN PLANTS ON B16 MELANOMA CELLS

by

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DECLARATION

I, Oyekunle Olubunmi Simeon, hereby declare that the dissertation “Potential anti-melanogenic effects of selected South African plants on B16 melanoma cells” hereby submitted by me for the PhD degree in Medical BioSciences at the University of the Western Cape has not been submitted previously at this or any other university, and that it is my own work in design and in execution, and that all reference materials contained herein have been duly acknowledged.

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Date Signed: : 12 August, 2019

DEDICATION

This dissertation is dedicated to the memory of my late first born, Israel Oluwasetemipe (16/01/2007 – 01/08/2009), rest in peace dear son. To my wife, Olufunke Bukola (Queen), for her support and steadfastness towards my academic success and for keeping my home for 5 years while outside the country in pursuance of my doctoral degree. To my children: Oluwasemilogo, Oluwasaanumi, and twins: Toluwanimi and Toluwalase, for their endurance and cooperation during my absence at home.

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ABSTRACT

Dyspigmentation is one of the commonest dermatological presenting complaints from patients, particularly hyperpigmentation. Hyperpigmentation can cause dangerous psychological and emotional impact on self-perception and health quality of lives of people affected. However, of all the diseases encountered globally, epidemiological data has shown that skin diseases account for almost 34% of all the diseases and these dermatological disorders have gotten worse over time. The gold standard for treatment of hyperpigmentation is hydroquinone. Despite its efficacy, hydroquinone and other current modalities of treatments are associated with some side effects. There are a number of natural products derived from medicinal plants that have proven to be an abundant source of biologically active compounds and a lot of these have served as the basis for the development of new lead chemicals for pharmaceutical. The present study focused on screening of selected South African plants (*Maclura pomifera, Otholobium fruticans, Phyylica ericoides, Psoralea aphylla, Rhynchosia villosa,* and *Serruria furcellata*) for their antimelanogenic potentials.

Methanol and ethyl acetate were used for the extraction of plant materials. Standard methods were employed for evaluation of cytotoxicity of the methanolic leaf extracts (MLE), ethyl acetate leaf extracts (ELE) and melanin synthesis potentials on B16 melanoma cells. To elucidate mechanisms of melanin reduction action, intracellular tyrosinase activity was determined by measuring the rate of L-DOPA oxidation. Tyrosinase activity was assessed further with dihydroxyphenylalanine (DOPA) staining. The mode of action was further determined by evaluating reactive oxygen species (ROS) and expressions of melanogenesis gene using qPCR.

The results showed that *O. fruticans* and *S. furcellata* reduced melanin synthesis without cytotoxicity.

O. fruticans inhibited tyrosinase activity, increased ROS and suppressed the expression of TYR, TRP-1, TRP-2/Dopachrome tautomerase, MITF, MC1R but upregulated β-Catenin. *S. furcellata* stimulated tyrosinase activity and did not increase ROS. It upregulated the expression of TYR, TRP-1, TRP-2, and MC1R while MITF and β-Catenin were suppressed. The results showed that *O. fruticans* reduced melanin synthesis via cAMP pathway while *S. furcellata* reduced the synthesis via possibly degradation of melanin pigment. The present study on *O. fruticans* and *S. furcellata* has shown that leaves of these plants are candidate anti-melanogenic agents. However, more work still needs to be done to elucidate other possible mechanisms that are relevant to antimelanogenic effects of these two plants.

Key words: melanin**,** hydroquinone, *Maclura pomifera, Otholobium fruticans, Phyylica ericoides, Psoralea aphylla, Rhynchosia villosa, Serruria furcellata*, flow cytometry, plants and ROS, hyperpigmentation

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
B-MSH	Beta- melanocyte- stimulating hormone
cAMP	Cyclic Adonosine Monophsphate
cDNA	Complementary deoxyribonucleic acid
CK1	Casein kinase 1
CM H2DCFDA	5- (and -6)- Chloromethyl -2' 7- dichlorodihydrofluorescein diacetate
CREB	cAMP Response Element Binding Protein
DCF	Dichlorofluorescein
DCT	Dopachrome tautomerase
DHICA	Dihydroxyindole-2-carboxylic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoside
DNA	Deoxyribonucleic acid

DOPA	5, 6-Dtrhydroxypheny lalanne
EDTA	Ethylenediaminetetraacetic acid
ELE	Ethylacetate leaf extract
ERK	Extracellular Signal-regulated Kinase
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GSK3β	Gycogen synthase-3 β
HPA	Hypothalamo-pituitary-adrenal axis
JNK	cJun N-terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
MC1R	Factor melanocortin .1. Receptor
MITF	Microphthalmia-Associated Transcription
MLE	Methanolic Leaf Extract
MP	*Maclura pomifera*
MPs	Medicinal plants
mRNA	Messenger ribonucleic acid
MSH	Alpha- melanocyte- stimulating hormone
MTT	3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NADPH	Nicotineamide adenine dinucleotide phosphate
PA	*Psoralea aphylla*
PBS	Phosphate Buffered Saline
PE	*Phylica ericoides*
PI3K	Phosphatidylinositol-3 Kinase
PIH	Postinflainmatory hyperpigmentation
PMSF	Phenylmethanesulfonylfluoride
qPCR	Quantitative polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactiveoxygen species
RV	*Rhynchosia villosa*
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel
SF	*Serruria furcellata*
TRP1	Tyrosinase-related protein 1
TRP2	Tyrosinase-related protein 2
TYR	Tyrosinase
UV	Ultraviolet

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CHAPTER ONE

INTRODUCTION

1.1.1 Introduction and Background to the Study

Skin pigmentation is an important variable phenotype in humans, and thus symptoms of cutaneous pigmentation disorders frequently present in different forms. Dyspigmentation is defined as an abnormality in the formation or distribution of pigment in the skin and it is one of the most common dermatological presenting complaints from patients with skin of colour, particularly hyperpigmentation (Vashi et al., 2017). Hyperpigmentation is the darkening of the skin mostly caused by an abnormally high content of the pigment, melanin (Vashi et al., 2017, Mehregan, 1975). The prevalence of skin pigmentation disorders varies with regions of the world (Davis and Callender, 2010), however, epidemiological data has shown that skin diseases account for almost 34% of all the diseases encountered globally and the prevalence of these dermatological disorders have gotten worse over time (Abbasi et al., 2010).

There are many disorders of skin pigmentation; some develop in areas of the skin exposed to sunlight such as melasma (Kwon and Park, 2014), post-inflammatory hyperpigmentation (Callender et al., 2011), and lentigines (Bastiaens et al., 2004). Post-inflammatory hyperpigmentation is very common among skin diseases with a high prevalence of 70% in both women and men before age of 35 years amidst Indian population (Hourblin et al., 2014). Moreover, widespread hyperpigmentation can be caused by medications or systemic diseases as seen in Addison's disease (Bensing et al., 2016) and hyperthyroidism (Stulberg et al., 2003). Neck and facial pigmentations

(cervicofacial pigmentations) are the most important cosmetically because of their visibility. These are more common in young women and are related to exogenous and endogenous factors, including the use of cosmetics and perfumes, and exposure to sun radiation (Serrano and Fernandez, 1996). Severe hyperpigmentation can also result from some photodynamic substances that induce phototoxic contact dermatitis as exemplified in berloque dermatitis occurring after the application of eau de cologne or perfumes containing bergamot oil, an ultraviolet sensitizer. In addition, furocoumarines-containing plants may induce prairie dermatitis (phytophotodermatitis) when patients are exposed to sunlight after contact with the particular plant. Typically, hyperpigmentation is not harmful but it can cause significant psychological and emotional impact on self-perception and health quality of lives of the affected people (Ikino et al., 2015, Pawaskar et al., 2007).

Various modalities have been adopted in the management of hyperpigmentation including topical and oral medications, as well as noninvasive procedures. The gold standard for the topical management, is hydroquinone, a hydroxyphenolic chemical (Woolery-Lloyd and Kammer, 2011) often associated with such side effects as exogenous ochronosis, skin irritation, and contact dermatitis in people of darker complexion (Zhu and Gao, 2008). For pigment spot lightening, a number of other topical agents like azelaic acid (Grimes, 2008), kojic acid (Ortonne and Passeron, 2005), retinoic acid (vitamin A) (Gupta et al., 2006), ascorbic acid (vitamin C) (Jutley et al., 2014), arbutin (Sarkar et al., 2013a), licorice, niacinamide, and N-acetyl glucosamine have also been used.

Cosmeceuticals are increasingly popular alternatives to standard depigmenting agents (Levin and Momin, 2010) mostly because of affordability and over-the-counter

availability (Fisk et al., 2014). Plant-derived compounds (phytochemicals) have been documented to exhibit various degrees of cellular actions for various skin pigment diseases (Chahal et al., 2013, Fisk et al., 2014, Leo and Sivamani, 2014), and notable among them are aloesin (Jones et al., 2002), arbutin which is obtained from bearberry plant (Hori et al., 2004) alpha-bisabolol (Kim et al., 2008), and many others.

Medicinal plants have been used in many parts of the world as traditional treatments for a plethora of human diseases for thousands of years. In some rural areas of many developing countries, medicinal plants are still being used as the primary source of medicine (Chitme et al., 2004). Many natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds most of which have served as the molecular templates for the development of new lead chemicals for pharmaceuticals. However, the huge resource of medicinal and aromatic plants in most rural areas of developing countries is still not fully explored.

In this study, six selected South African plants were used: *Otholobium fruticans (OF), Rhynchosia villosa (RV), Phylica erricoides (PE), Psoralea aphylla (PA), Serruria furcellata (SF),* and*Maclura pomifera (MP). OF, RV* and *PA* belong to Fabaceae or Leguminosae family; *SF*, a member of Proteaceae family, *MP*, a member of Moraceae family, and PE, a member of Rhamnaceae family. According to literature, there is no report of any biological study done on any of the plants; however, some family members of these plants have been reported to have folklore medicinal uses.

1.2 Aims of the Study

The aims of this study were:

1. To screen selected South African medicinal plants for melanogenesis inhibition potentials.

2. To evaluate the molecular mechanisms involved in the inhibition of melanogenesis by the plants with melanogenesis inhibition potentials.

The Objectives were:

1. To perform solvent extraction of the plant materials.

2. To evaluate cytotoxicity of the extracts on Murine B16 melanoma cells and normal human keratinocytes (HaCaT).

To achieve these goals, the following steps were taken:

- Collection of plant materials.
- Evaluation of the antimelanogenic activity of the crude extract of the plants.
- Cytotoxicity profiling using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) reduction assay.
- Determination of cellular melanin content.
- Evaluation of tyrosinase enzyme kinetics by performing intracellular tyrosinase activity assay.

- Further evaluation of tyrosinase activity by performing Dihydroxyphenylalanine (DOPA) Staining Assay (zymography).

- Estimation of ROS induction using fluorescence microscopy in the presence of 5-(and-6)-chloromethyl-2', 7' dichlorodihydrofluorescein diacetate (CM-H2DCFDA).

- Melanogenesis gene expression profiling using quantitative polymerase chain reactions.

1.3 Overview of Chapters

The layout of this thesis is as follows:

Chapter One outlines the background of the study, providing brief overview of burden of skin diseases and the prevalence of hyperpigmentation; research aims and objectives, as well as steps taken to achieve the goals.

Chapter Two provides a literature review focusing on important concepts for the purpose of this study. The review of literature presents descriptions of skin and skin pigmentation disorders, melanocytes, melanin structure and synthesis, role of melanin in skin. Current management of hyperpigmentation and its drawbacks are discussed. The chapter ends with review of reactive oxygen species, traditional medicinal plants, and mechanisms of actions on inhibition of melanin synthesis.

Chapter Three describes the research methodology used in this study. The quantitative approach is used in this study and the data collection and analyses are presented. Detailed information on the materials and various methods used in this study is also provided.

Chapter Four describes results of melanin inhibition of all the 6 selected South African medicinal plants used in this study.

Chapter Five describes the results of evaluation of molecular mechanism of action of methanolic extract of *Serruria furcellata* leaves, the discussion, and chapter references.

Chapter Six describes the results of evaluation of molecular mechanism of action of methanolic extract of *Otholobium fruticans* leaves, the discussion, and chapter references.

Chapter Seven provides synthesis of findings of this study as well as the future perspectives and recommendations.

1.4 Chapter One Conclusion

This chapter contextualizes the topic under investigation. It provides background literature to support the research inquiry. The problems observed are presented as part of the background. This chapter also describes the aims and objectives of the study. Chapter two provides a review of literature on the topics relevant to the aims and objectives of this study.

CHAPTER TWO

LITERATURE REVIEW

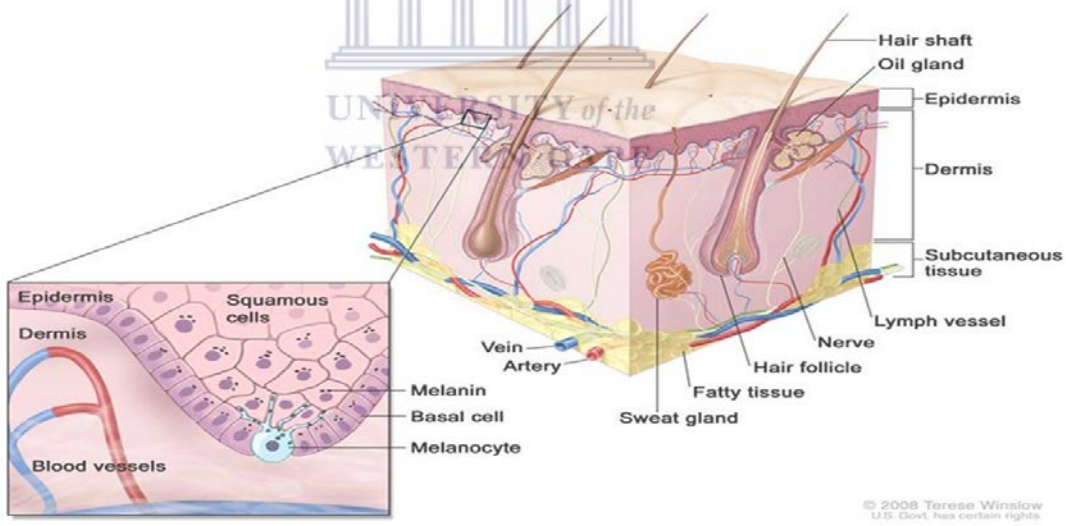
2.1 Skin

The skin represents about 10-15% of the body weight, and is the largest organ of the body with an average surface area of about 2 m^2(Bolognia et al., 2008). Its location on the body underscores its function to act as a barrier with the environment and in the preservation of body's homeostasis (Slominski et al., 2012).

The skin is formed in the first week of pregnancy in humans and is made up of three different compartments namely, the epidermis, dermis and subcutaneous tissue. The main components of the epidermis are the keratinocytes, which differentiate as they move towards the upper layer of the surface of the skin, forming spinous, granular, and lipid-rich cornified layers, Merkel cells, melanocytes and Langerhans cells (Elias, 2007, Elias et al., 2010). The cell populations of the basal layer are the melanocytes which produce melanin pigment that is transferred to surrounding keratinocytes (Slominski et al., 2004).

The skin surface is covered by the stratified squamous epithelium of the epidermis which develops to form a highly impermeable stratum corneum (Fuchs, 1990, Suter et al., 1997). There are no blood vessels in the epidermis and cells present in the deepest layers are nourished almost exclusively by diffused oxygen from the surrounding air (Stücker et al., 2002) (Fig 2.1). Also present in the epidermis are the Merkel cells, which are round-shaped, slow-adapting, type I mechanoreceptors located in areas of

high tactile sensitivity and are attached to basal keratinocytes by desmosomal junctions. They are located in the digits, lips, regions of the oral cavity, and outer root sheath of the hair follicle and are occasionally assembled into specialized structures known as tactile discs or touch domes (Moll, 1994). Cells of the immune system in epidermis are the Langerhans cells. They constitute about 2-8% of the total epidermal cell population and maintain almost constant numbers and distributions in a particular area of the body. The cells are distributed mainly among the squamous and granular layers in the epidermis, with fewer cells in the basal layer. They are involved in a variety of T-cell mediated immune responses. Formed in the bone marrow, these cells migrate to a suprabasal position in the epidermis early in embryonic stage and continue to circulate and repopulate the epidermis throughout life. Because the cells are dendritic, they do not form cellular junctions with neighboring cells (Chu, 2008).



Retrieved from National Cancer Institute websiteon September 10, 2018: https://www.cancer.gov/images/cdr/live/CDR579033-750.jpg

Figure 2.1: Anatomy of the skin, showing the epidermis, dermis, and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis.

2.2 Skin Pigmentation Disorders

Skin pigmentation is an important variable phenotype in humans, and cutaneous pigmentation disorders are frequent symptoms, which present in different forms. People with lighter skin colour are most commonly affected by the dermatological condition called dyspigmentation, particularly its extreme form known as hyperpigmentation (Vashi et al., 2017). Hyperpigmentation, which can either be localized or generalized, is the darkening of the skin mostly caused by abnormally high concentration of the pigment, melanin (Mehregan, 1975). The prevalence of skin pigmentation disorders varies with regions of the world (Davis and Callender, 2010). However, global epidemiological data has shown that skin diseases account for almost 34% of all diseases and the skin disorders have worsened over time (Abbasi et al., 2010).

There are various types of skin pigmentation disorders which include melasma (Kwon and Park, 2014), post-inflammatory hyperpigmentation (Callender et al., 2011) and lentigines (Bastiaens et al., 2004) that usually develop in areas of the skin exposed to sunlight. However, generalized hyperpigmentation can result from medications or systemic diseases as seen in Addison's disease (Bensing et al., 2016) and hyperthyroidism (Stulberg et al., 2003). In most cases, hyperpigmentation is not fatal but it can engender serious psychological and emotional impact on self-perception and quality of lives of people affected (Pawaskar et al., 2007, Ikino et al., 2015). At the other extreme is hypopigmentation that is mostly caused by a reduction in melanin content, leading to lighter skin colour, vitiligo. It is usually due to destruction of the melanocytes (Ezzedine et al., 2012) in the epidermal layer of the skin. As seen in albinotic phenotype, generalized hypopigmentation exemplified by occulocutaneous

type of albinism results from mutations of melanin biosynthesis tyrosinase (TYR) and tyrosinase-related protein 1(TRP-1) genes (Rooryck et al., 2006).

2.2.1 Melasma

Melasma is a pigmentation disorder that presents with light-to-dark brown-coloured macules which are irregular in areas of the skin that are exposed to the sun at such parts as the face (Kwon and Park, 2014). It is commoner in females aged 30's and 40's and a higher percentage of cases have facial patterns: centrofacial, malar, and mandibular (Guinot et al., 2010, Tamega et al., 2013). Recently, extra-facial melasma has been found to occur at such non-facial body parts as the neck, sternum, forearms, and upper extremeties (Ritter et al., 2013). Research has shown that prolonged ultraviolet (UV) rays exposure, sex hormones, and genetics are etiological factors involved in the pathogenesis of melasma (Ortonne et al., 2009).

At the molecular level, melasma occurs due to increased melanogenesis. Studies have revealed that various mechanisms are involved in its pathogenesis which include increased production of alpha-melanocyte-stimulating hormone, overexpression of dermal stem cell factor and its c-kit receptor (Kang et al., 2006), and paracrine melanogenic factors from keratinocytes, mast cells, or dermal fibroblasts (Lee, 2015). Moreover, inflammatory processes have also been implicated in the development of melasma (Noh et al., 2014).

Different epidemiological studies have reported the prevalence of melasma in the general population to be 1%, while a prevalence range of 9-50% has been reported in higher-risk populations (Taylor, 2003, Moin et al., 2006, Werlinger et al., 2007, Rathore et al., 2011)

2.2.2 Lentigines

Lentigines, also known as solar lentigines or lentigo senilis, are reticulated patches or spots, which are light brown to dark brown or even-coloured occurring mostly in sun-exposed areas of the human bodies. About one-third of 50 years-old women were affected in a study carried out in an Indian population (Hourblin et al., 2014). Lentigines are commonly seen on the extensor forearms, upper trunk, dorsum of the hands and the face and may appear solitary or in multiple forms. The rate of occurrence increases with age, thus, lentigines are clinical sign of photoaging (Rahman and Bhawan, 1996, Bastiaens et al., 2004).

In lentigines patient, the basal layer of the skin is hyperpigmented due to increased levels of melanin content of the epidermis (Mehregan, 1975) with an increase in the number of melanocytes (Yamada et al., 2014), or not (Mehregan, 1975).

2.2.3 Postinflammatory Hyperpigmentation

Postinflammatory hyperpigmentation (PIH) is an acquired disorder of skin pigmentation (Callender et al., 2011) with a prevalence of about 70% among both women and men before the age of 35 years for the Indian population (Hourblin et al., 2014). PIH occurs sequel to inflammatory reaction by many skin diseases such as impetigo, acne vulgaris, lichen planus, psoriasis, atopic dermatitis, pityriasis rosea, as well as complications resulting from laser therapy. For more than a decade, PIH has been observed as one of the common hyperpigmentation disorders together with melasma and lentigines (Cayce et al., 2004). Stimulation of epidermal melanocytes leading to significant increase in synthesis of total melanin in PIH is caused by inflammation of the epidermis which results in the production and release of several

cytokines, leukotrienes, and prostaglandins (Lacz et al., 2004, Davis and Callender, 2010). PIH can arise in all skin types especially skin-of-colour patients (Fitzpatrick skin types IV through VI), including Native Americans, African Americans, Hispanics/Latinos, Pacific Islanders, Asians, and those of Middle Eastern descent (Davis and Callender, 2010).

2.2.4 Vitiligo

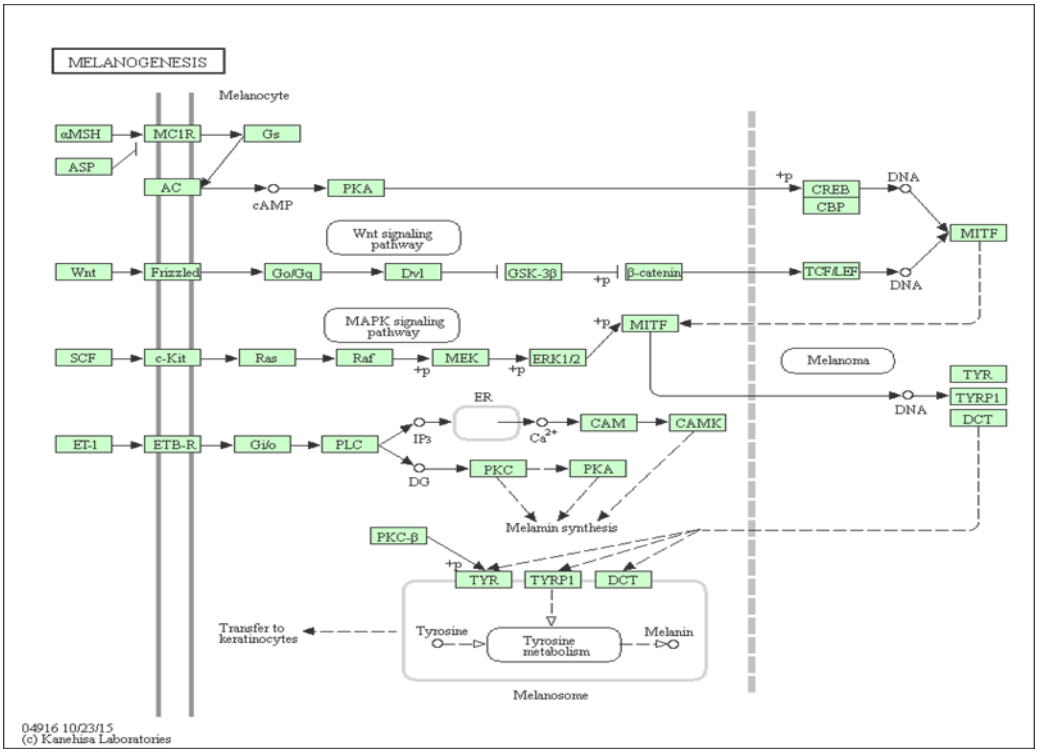
Vitiligo is an acquired condition causing depigmentation of the skin, and occasionally of the mucosa, leading to the selective loss of melanocytes (Ezzedine et al., 2016). About 0.5% to 1% of the population is affected, with no difference in the rate of occurrence with respect to skin type or race (Allam and Riad, 2014). Studies have shown that chronic stress can cause a reduction in cutaneous melanogensis and the amount of expression of the elements of the hypothalamo-pituitary-adrenal (HPA) axis (Pang et al., 2014). Thus, stress may suppress the activation of the cutaneous HPA axis via glucocorticoids and consequently reduce melanogenesis. Stimulation of melanogenesis by systemic administration of alpha-melanocyte stimulating hormone(α-MSH), beta-melanocyte stimulating hormone (β-MSH), and adrenocorticotropic hormone (ACTH) has led to the recognition of the involvement of ACTH and α-MSH in the pigmentation of human skin (Levine et al., 1991). Clinical observations have provided additional evidence suggesting the role of these neuropeptides in skin pigmentation, for instance, protracted therapeutic administration of α-MSH and ACTH, or chronically increased levels of α-MSH and ACTH, as occurs in Addison's disease, could lead to hyperpigmentation in humans (Slominski et al., 2004). Moreover, α-MSH and ACTH act via melanocortin receptor 1(MC1) for their epidermal melanogenesis in humans (Tsatmalia et al., 1999, Tsatmali et al., 2002).

2.3 Melanocytes

Melanocytes are specialized dendritic-shaped pigment-producing cells of the skin epidermis that produce melanosome (Dell'Angelica et al., 2000). These cells are located in the stratum basale of the epidermis and produce the pigment granules, eumelanin and pheomelanin, which are responsible for the colour of the skin and hair (Holbrook et al., 1989) (Fig 2.1). In this basal layer, these cells make epidermal melanin units as a result of the interaction between one melanocyte and 30-40 keratinocytes (Fitzpatrick and Breathnach, 1963, Haass and Herlyn, 2005). However, melanocytes are also present in other areas of the body including the inner ear, heart, and nervous system (Brito and Kos, 2008). At the molecular level, melanocytes are identified by the presence of such proteins as tyrosinase (TYR), tyrosinase-related protein 1 and tyrosinase-related protein 2/dopachrome tautomerase (TYRP1, TYRP2/DCT), microphthalmia transcription factor (MITF), as well as melanosomal matrix proteins (Pmel17, MART-1) (Brenner and Hearing, 2009).

2.4 Melanin synthesis

Melanogenesis is a physiological process that results from exposure to UV radiation (Fig 2.2). There are two major types of melanin, the brown/black eumelanin and red/yellow pheomelanin, which differ in colour, granule packaging, shape, and size (Videira et al., 2013). Melanin synthesis in mammals is catalysed by such enzymes as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 1 (TRP-2) which are specific to melanocytes (Tsang et al., 2012, An et al., 2008).



KEGG Melanogenesis pathways (Kanehisa et al., 2016)

Figure 2.2: Melanin pigments are synthesized by melanogenesis within melanosome

In melanogenesis, tyrosinase is the primary enzyme that catalyses the first two different chemical reactions which are the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone (Nishio et al., 2016). Oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to indole-5,6-quinone-2-carboxylic acid by TRP-1 occurs in mice and not in humans. However, the rearrangement of DOPA to DHICA is catalysed by TRP-2 (Kim et al., 2015). Furthermore, microphthalmia-associated transcription factor (MITF) has been documented to be the master regulator of melanocyte differentiation, proliferation, pigmentation, and survival (Levy et al., 2006). As a major transcription factor, it regulates TYR, TRP-1, and TRP-2 expression (Baek and Lee, 2015). Decrease in MITF expression leads to the down-regulation of differentiation markers and inhibits melanogenesis (Jiménez-Cervantes et al., 2001).

The Wnt/β-catenin pathway is one of the other signalling pathways involved in melanin synthesis. β-catenin is phosphorylated by a complex of many proteins comprising casein kinase 1 (CK1), axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β). When this pathway is not activated, it is then affected by ubiquitin and degraded via ubiquitin proteasomes (Wang et al., 2017). However, on activation of the Wnt pathway, interactions of Wnt 1, Wnt 3a, and Wnt 8 with frizzled receptors and low density lipoprotein receptor-related (Lrp) 5/6 co-receptors leads to GSK3β being negatively regulated (Giles et al., 2003). Then, cytoplasmic β-catenin will translocate into the nucleus and bind to the MITF promoter, causing transcriptional activation of MITF (Latres et al., 1999, Bellei et al., 2008, Shin et al., 2015). An additional pathway involved in the regulation of synthesis of melanin is phosphatidylinositol-3-kinase (PI3K)/Akt signalling. Its activation induces melanogenesis through the upregulation of MITF, tyrosinase and TRPs (Shen et al., 2012).

The intracellular cyclic adenosine monophosphate (cAMP)-mediated pathway is an important pathway that induces melanogenesis in melanocytes. The binding of α-Melanocyte-stimulating hormone (α-MSH) to the melanocortin 1 receptor (MC1R), activates cAMP (Suzuki et al., 1996, Bertolotto et al., 1998) resulting in an increase in the expression of MITF via the activation of the cAMP-response element binding protein (CREB) transcription factor (Jung et al., 2009). Extracellular stimuli also activate members of the mitogen-activated protein kinase (MAPK) family that include extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK) (Kim et al., 2003, Pang et al., 2014). Furthermore, p38 and JNK are stimulated by various factors, which include heat, DNA damage, and UV irradiation. MITF can be activated by phosphorylated JNK and p38 however, the phosphorylation of ERK

can cause a reduction of melanin synthesis by suppressing the expression of MITF (Ng et al., 2014, Kang et al., 2015).

The synthesis of melanin pigments occurs within melanosome through the process known as melanogenesis and the arrangement of melanosomes in compartments serves to protect the cell from such substances as hydrogen peroxide and quinines which are by-products of the melanogenesis process (Cichorek et al., 2013). Formed melanosomes are transported to the keratinocytes (Cichorek et al., 2013) and the transfer of synthesized melanin into keratinocytes, which determines skin and provides photo-protection, requires that there is contact between the dendritic processes of differentiated melanocytes and keratinocytes. Melanin pigment granules are deposited superiorly to keratinocyte nucleus and are removed with shed epidermal cells (Brenner and Hearing, 2009).

2.5 Structure of Melanin

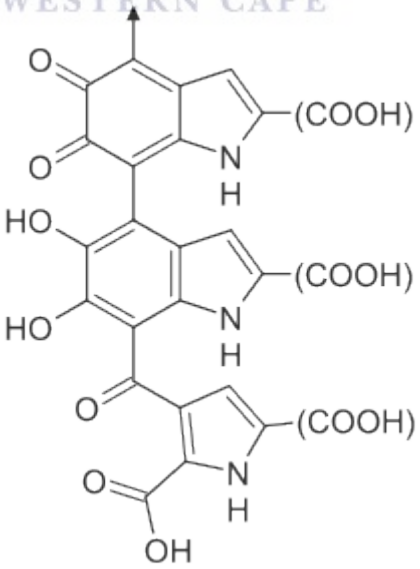


Figure 2.3: Structure of Melanin

Retrieved from http://www.chm.bris.ac.uk/motm/melanin/melaninh.htm on July 3rd, 2019

Melanin is a polymer with a heterogeneous structure that is synthesized within melanosomes by hydroxylation of tyrosine or oxidation of 3,4-dihydroxylphenylalanine (DOPA) by tyrosinase (Fig 2.3). It has different oxidative states consisting of 5,6-dihydroxylindole-2-carboxylic acid (DHICA) units and pyrole units which are derived from their peroxidative cleavage (Simon et al., 2009). The size of the epidermal melanin units is similar in all humans regardless of ethnicity or race, but varies between body areas (Miyamura et al., 2007).

2.6 Functions of Melanin

Melanin primarily protects cells from the damage caused by ultraviolet (UV) radiation by absorbing part of the radiation and through reactive oxygen species (ROS) scavenging (Wang et al., 2008). Melanin can also act as a photosensitizer, producing ROS after UV irradiation (Korytowski et al., 1987), as well as after exposure to visible light (Chiarelli-Neto et al., 2014). In addition, melanin promotes cell cycle arrest and the intermediates of their synthesis, DHICA; it is involved in calcium metabolism, promotion of DNA breakage and the repair of enzymes which is prevented by its interaction with DNA, and it interferes in the ADP/ATP ratio (Hoogduijn et al., 2004, Cunha et al., 2012, Suzukawa et al., 2012, Pellosi et al., 2014).

2.7 Managements of hyperpigmentation

Hyperpigmentation, especially post-inflammatory hyperpigmentation, is very common among skin diseases with a high prevalence of 70% among both women and men under 35 years of age in India (Hourblin et al., 2014). It is one of the most common dermatological presenting complaint among dark-skinned people (Davis and Callender, 2010). Various modalities have been adopted in the management of hyperpigmentation including topical and oral medications, as well as non-invasive

procedures. The gold standard for the topical management of this condition is the hydroxyphenolic compound known as hydroquinone, (Woolery-Lloyd and Kammer, 2011). Despite its efficacy, hydroquinone is associated with some side effects including exogenous ochronosis, skin irritation, and contact dermatitis in people of darker skin complexion (Zhu and Gao, 2008). For the removal of pigment spots on the skin, a number of other topical agents like azelaic acid (Grimes, 2009), kojic acid (Ortonne and Passeron, 2005), retionoic acid (vitamin A) (Gupta et al., 2006), ascorbic acid (vitamin C) (Jutley et al., 2014), arbutin (Sarkar et al., 2013a), licorice, niacinamide, and N-acetyl glucosamine have also been used.

Cosmeceuticals are increasingly popular alternatives to standard depigmenting agents (Levin and Momin, 2010) and are preferred alternatives because they are often much cheaper and easily available over the counter (Fisk et al., 2014). Phytochemicals used as cosmeceuticals, notably aloesin (Jones et al., 2002), arbutin obtained from bearberry plant (Hori et al., 2004), alpha-bisabolol (Kim et al., 2008), etc. have been documented to exhibit various degrees of cellular actions for various skin pigment diseases (Chahal et al., 2013, Fisk et al., 2014, Leo and Sivamani, 2014). However, phytochemicals are not completely devoid of adverse effects as allergic reactions may sometimes occur (Reuter et al., 2010a). Studies have shown that plant extracts may cause photosensitization and phytodermatitis but such side effects as toxic epidermal necrolysis (Kokcam, 2009) are seldomly reported, except with high concentration and oral formulations (Fisk et al., 2014). Furthermore, evidence from clinical trials on the safety and efficacy of many botanicals has shown that there are fewer side effects compared with patients managed with the gold standard therapies (Costa et al., 2010).

Another modality of management of hyperpigmentation is oral medication. Low-dose oral tranexamic acid (a synthetic derivative of the amino acid lysine) has been shown to be an effective adjunct in the treatment of refractory melasma (Tan et al., 2017). Tranexamic acid was found to produce ameliorative effects in the management of hyperpigmentation of melasma among Chinese women (Wu et al., 2012).

Chemical peeling (chemexfoliation) is a non-invasive modality which has been used as an alternative treatment for hyperpigmentation (Grimes, 2008, Moubasher et al., 2014) and other studies have shown laser/light therapy is one of the treatment modalities of skin hyperpigmentation (Chan et al., 2016, Levin et al., 2016, Ohshiro et al., 2016). Associated complications of the above treatment methods include oedema, dyspigmentation, erythema, pain, crust formation, and permanent dyspigmentation (Levin et al., 2016).

2.8 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are together known as free radicals (Valko et al., 2006a). Reactive oxygen species describes broadly the O_2-derived free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and alkoxyl ($RO^{\cdot}$) radicals, as well as hydrogen peroxide (H_2O_2) which is an O_2-derived non-radical species (Halliwell and Cross, 1994, Dickinson and Chang, 2011).

ROS are continuously being produced by many normal cellular events, and majorly from aerobic respiration. The ROS generated during these events are constantly counteracted by several antioxidant proteins (Valko et al., 2007, Chang and Chuang, 2010). Depending on the levels produced, ROS are essential to cell signalling and

regulation or can also be injurious to cell survival as by-products of metabolic processes. At elevated levels, ROS can engender impaired physiological functions via cellular damage of proteins, phospholipids, DNA, and other macromolecules which can lead to certain human diseases (Shen et al., 1996, Stoner et al., 2008, Ray et al., 2012). Despite the imminent danger of increased levels of ROS, many studies have demonstrated that moderate levels of ROS are critical mediators of intracellular signalling pathways in different cell types (Byun et al., 2008, Bulua et al., 2011, Tormos et al., 2011).

ROS generation could either be endogenous or exogenous (Valko et al., 2006a); endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, peroxisome and inflammatory cell activation (Inoue et al., 2003, Bigarella et al., 2014). Other sources of endogenous ROS are macrophages, neutrophils and eosinophils, NADPH oxidase and xanthine oxidase (Santos et al., 2016). Increase in oxygen uptake caused by activated macrophages gives rise to ROS which includes nitric oxide, superoxide anion, and hydrogen peroxide (Conner and Grisham, 1996). Endogenous ROS production is majorly from the mitochondrion and of the total oxygen consumed, 1-2% forms ROS essentially at the level of complex I and complex III of the respiratory chain. The diversion is however believed to be tissue and species dependent (Turrens, 2003).Exogenous sources of ROS-mediated pathogenesis include fatty acids in foods, transition metals, tobacco smoke, and ethanol, which produce lipid peroxides, organic radicals, and hydroxyl radicals (Dreher and Junod, 1996).

It is reported that each cell in the body is attacked by about 10,000 to 20,000 free radicals each day (Valko et al., 2006b). The excessive production of ROS/RNS either through an endogenous or exogenous source beyond the innate threshold of the cell

antioxidant defence system is known as oxidative stress (Valko et al., 2006a, Keane et al., 2015). Oxidative stress causes oxidative degeneration of biomolecules and the building up of oxidative damage has been discovered to disrupt cellular physiologic functions as exemplified in signal transduction and gene expression to mitogenesis, transformation, mutagenesis and cell death (Araki, 2010). Some of the pathologies that have been attributed to oxidative lesion include atherosclerosis, rheumatoid arthritis, myocardial infarction, cancer, and neurodegenerative disorders (Sies et al., 2017).

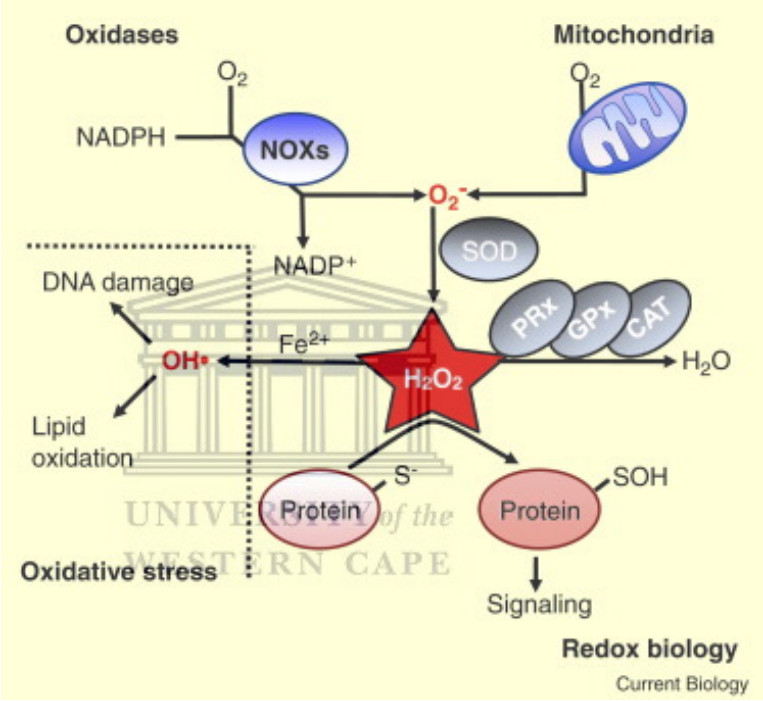


Figure 2.3: ROS signaling. Basics of ROS (Schieber and Chandel, 2014)

2.8.1 Reactive Oxygen Species and Melanogenesis

Reports have implicated reactive oxygen species in melanogenesis (Liu et al., 2012) which is considered to involve oxidation reactions and superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) generation which expose melanocytes to oxidative stress (Koga et al., 1992, Simon et al., 2009). Homeostasis of melanocytes can be disrupted by oxidative stress causing either their death or malignant transformation (Fried and

Arbiser, 2008, Guan et al., 2008). The catalytic activity of tyrosinase, which is the rate-limiting enzyme for melanin synthesis, results in the generation of O_2^-(Tomita et al., 1984, Koga et al., 1992).

ROS generation has been proposed as a cause for natural depigmentation processes such as the hair becoming grey and many other pathological conditions including vitiligo (Jiménez-Cervantes et al., 2001). Previous studies have shown that MITF is phosphorylated and thereafter degraded in response to ROS stimulation (Liu et al., 2009, Kim et al., 2014), consequently leading to depigmentation. Moreover, non-cytotoxic levels of ROS could lead to hypopigmentation through negative regulation of MITF and other melanogenic enzymes (Liu et al., 2009, Kim et al., 2014). In addition, a short pulse of H_2O_2 applied to B16 murine melanoma and human melanoma cells was found to induce a transient repression of melanogenesis by mechanisms that include MITF-dependent down-regulation of melanogenic enzymes (Jiménez-Cervantes et al., 2001).

2.9 Traditional Medicinal Plants

Ethnomedicine is a multi-disciplinary complex system that entails the use of spirituality, natural environment and plants, and has been the source of treatment for millennia (Jamshidi-Kia et al., 2018). Any plant which provides health-promoting characteristics and is used to maintain health or administered for a specific condition, whether in allopathic or traditional medicine is considered a medicinal plant (Smith-Hall et al., 2012). Medicinal plants (MPs) have been used in many parts of the world, including Africa, as traditional treatments for a plethora of human diseases for thousands of years such as essential hypertension and type-2 diabetes mellitus (Davids et al., 2016). MPs are continuously being used as the primary source of medicine in

rural areas of most developing countries (Chitme et al., 2004), and have proven to be an abundant source of biologically active compounds most of which serve as lead chemicals for the development of new pharmaceuticals. Africa has a large number of medicinal and aromatic plant resources and a large percentage of its population relies on these plants for healthcare (Cunningham, 1993) often based on traditional knowledge. However, not much of the huge botanical resources have been explored in most of these developing countries especially for economic benefits.

2.9.1 Medicinal Plants and Skin Diseases

Dermatological diseases are an abundant and frequently occurring health challenge in the population, affecting all ages from the neonates to the elderly (Marks and Miller, 2017). About 70-90% of the world's population in developing countries depends solely on MPs for their primary health care, sometimes as the only system available in many rural areas. The treatment of skin infections using MPs is quite common in the rural areas (Naidoo and Coopoosamy, 2011) possibly due to their ability to arrest bleeding, speed up wound healing, treat burns and mitigate other skin conditions (Naidoo and Coopoosamy, 2011). MP-based treatment regimens are cheap, readily accessible and their use is based on extensive knowledge and skill amongst the local communities (Shai et al., 2008).

The aetiology of dermatological infections could be either viruses, parasites, bacteria or fungi. The infections could range from mild skin rashes, dermatitis, psoriasis, acute erythema, vitiligo to burns and deep wounds (Weckesser et al., 2007). The traditional use of plants and herbal components for the treatment of skin diseases and the management of dyspigmentation, as well in cosmetology as skin-whitening agents, is a common practice in many cultures (Pieroni et al., 2004). Plant-derived medicines are

gaining wider acceptance due to such advantages as having fewer side effects, better tolerance by patients, and affordability due to a long history of use. Research reports within a 17-year period (1995-2012), have shown that about 31 different plants could be effective for the treatment of various skin diseases (Tabassum and Hamdani, 2014).

2.9.1.1 Medicinal Plants used for Hyperpigmentation

Increasing evidence has established that a plethora of medicinal plants have demonstrated effectiveness in the treatment of dermatophytes, sunburn, actinic keratosis, vitiligo, and alopecia (Reuter et al., 2010b). Extracts of soybean (*Glycine maxima*) have been demonstrated to be effective against signs of photoaging such as pigmentation, lentigines and reduced skin tone (Wallo et al., 2007) and Licorice (*Glycyrrhiza glabra*) extracts were found to effectively inhibit melanogenesis in animal models (Yokota et al., 1998).

Proanthocyanidins which is the active compound in extract of grape seeds improved chloasma in a short period of administration to Japanese women (Yamakoshi et al., 2004). Also, the inflorescences of hawthorn (*Crataegus monogyna* Jacq.) are used as herbal medicine in North Africa, Europe, Asia, and America. Creams and lotions with glycolic extract of the flowers produce lightening action on the skin tissue (da Cunha, 2004). *Arctostaphyllos uva* ursi (Bearberry) is used traditionally in herbal therapy to treat urinary tract infections, but it is often used in the cosmetics industry for formulating skin-lightening products. The leaves of this plants contain 17% arbutin (Lukas et al., 2010). In China, *Phyla nodiflora* herbal teas used to treat inflammatory skin conditions equally has depigmenting action which is due to the tyrosinase-inhibitory property of eupafolin, its active molecule (Ko et al., 2014).

2.10 The Fabaceae Family

The Fabaceae or Leguminosae, very often known as the legume, pea, or bean family, are a large and economically useful family of flowering plants. Included in this family are shrubs, trees, herbaceous plants, perennials or annuals, which are easily identified by their fruits (legume) and their compound, stipulated leaves. The group is widely distributed, and in terms of number of species, it is the third largest land plant family after Orchidaceae and Asteraceae, with 730 genera and over 19,400 species (Judd et al., 2002, Wojciechowski, 2006). From chemical and pharmacological point of view, Fabaceae is one of the most evaluated groups (Wink, 2013, Neves et al., 2017). Valuable chemical components such as flavonoids, alkaloids, coumarins etc. used for treatment of various diseases are derived from this family (Wink, 2013).

This botanical group has been recognized as the most diversified family in the most of studies involving medicinal plants in the North Eastern Cerrado area of Brazil (Silva et al., 2015, Vieira et al., 2015, Macêdo et al., 2016). Leaves of *Dalbergia sisso* are used as antipyretic, anti-inflammatory, and analgesic, while leaves of *Cassia fistula* and *Pissum sativum* are used in skin diseases, as purgatives and blood purifier (Ramrakhiyani et al., 2016). In addition, Eland's bean, found across most of the South African grassland (Van Wyk et al., 1997) belongs to the Fabaceae family. It has a number of unbranched, annual stems of about 1 m growing from an underground red rhizome with clusters of small, cream-coloured flowers arranged halfway up the aerial stem. Its characteristic feature is its pod which is about 200 mm long. Roots and rhizome are boiled in water for topical treatment of acne. In combination with *Pentanisia prunelloides* Walp, it is used to treat acne and eczema (Van Wyk and Gericke, 2000, Mabona et al., 2013). Moreover, the root is pulverized and immersed

in water for a whole day and thereafter strained and used topically. It is reported to form protective layer on the mucosa and the skin; this property has been linked to the improvement of tissue regeneration (Van Wyk et al., 1997). Phytochemical screening of this plant showed the presence of active ingredients like tannins and catechins in rhizomes (Maphosa et al., 2010). The red colour of the rhizome reflected the presence of phenolic compounds (Van Wyk et al., 1997, Maphosa et al., 2010). Oral administration of the plant was reported to cause a decrease in respiratory rate (Maphosa et al., 2010).

The Fabaceae family is the most represented in the ethnobotanical survey of medicinal plants used in the management of hypertension in the maritime region of Togo (Gbekley et al., 2018) and it is also reported that plants of this family are used traditionally for the treatment of eye and earache at Dahod District of India (Maru et al., 2018).

Three of the plants evaluated in this study, *Otholobium fruticans, Rhynchosia villosa*, and *Psoralea aphylla* belong to Fabaceae family.

2.10.1 The Fabaceae Family and Depigmentation

Many plants of different genera belonging to the Fabaceae family have been used in folk medicine for treating different ailments in the human body. For depigmentation, an ethnobotanical survey of medicinal plants used in Rwanda for voluntary depigmentation showed 5 different species which are being used for voluntary bleaching including *Erythrina abyssinica, Indigofera arrecta, Kotschya aeschynomenoides, Leptoderris harmsiana,*and *Tephrosia vogelii*(Kamagaju et al., 2013). The methanolic extract and bioactive compounds isolated from the flowers of

Vicia faba L (broad bean) were recently demonstrated to exhibit melanin synthesis inhibition potentials (Allam et al., 2018). This is an important winter crop in Mediterranean areas and spring crop in other regions of Europe and South America, and a major plant food item for the Nile River populations (Amarowicz and Pegg, 2008). Also, *Sophora flavescens*, otherwise known as Kushen, belongs to the Sophora genus in Fabaceae family. The inhibitory effects of bioactive compounds isolated from this plant on the catalytic action of tyrosinase was recently determined by Kim et al. and they have demonstrated that the bioactive compounds isolated exhibited high inhibitory effects on catalytic actions of tyrosinase and consequently, on the synthesis of melanin (Kim et al., 2018). In addition, methanolic extracts of *Onobrychis argyrea* Boiss. Subsp. *Isaurica* (Fabaceae), endemic to the eastern Mediterranean region, have been reported to exhibit potent inhibitory action against tyrosinase enzyme (Guler et al., 2018).

The inhibition of melanin synthesis by plants in the family Fabaceae has been documented in clinical trials done on *Glycyrrhiza glabra* (Liquorice).Results showed that 2.5% of *Glycyrrhiza glabra* applied to 100 females for four weeks produced significantly improved symptoms of melasma compared to the group without any side effects (Badria, 2015). On B16 murine melanoma cells, it was observed that Glabridin, bioactive compound from Liquorice, inhibited tyrosinase activity at 1.0 µg/mL without affecting DNA synthesis (Yokota et al., 1998).

2.10.2 Genus Otholobium

Otholobium C. H. Stirt is one of the genera of flowering plants in the family of legumes, Fabaceae (Psoraleeae) and there are 51 species of this genus that were thought to be endemic to Africa. They all have different morphology such as prostate herbs,

less than 50 cm tall rhizomatous suffrutices, and shrubs to small trees to 3 m tall, and occur mostly at Southern and Eastern Africa, and possibly South America. However, eight South American species that were treated as belonging to Psoralea s.l. were transferred to the genus by Grimes (Grimes, 1990). DNA phylogenetic studies have shown that the American species are different from the African Otholobium genetically (Egan and Crandall, 2008, Dludlu et al., 2013). The African Otholobium occur mainly in the Mediterranean area at the south western tip of Africa (Stirton, 2005). They are centred in the Fynbos biome in Fynbos and Renosterveld vegetation units (Mucina and Rutherford, 2006). The genus is described by a number of features which include recurved-mucronate leaf apex, bracteates flowers that are borne in triplets and which are subtended by a single bract and indehiscent fruits containing one seed (Stirton, 1986).

2.10.2.1 Otholobium fruticans (L.) Strirt

2.10.2.1.1 Taxonomy

Otholobium fruticans is a poorly studied medicinal plant. It is a trailing semi-shrub that grows up to the height of about 400 mm, with a spread of 500 mm to 1 m. It is a resprouter with semi-prostate branches that appear to spread out in all directions from the centre of the bush. The leaves of *Otholobium fruticans* are trifoliate with smooth top and hairy undersides, with stipules at the base of the leaf stalk. The florescence is bracteates with triplet flowers, and each triplet subtended by a single differently shaped bract. The flowers of *Otholobium fruticans* are pea-like, purple to violet colour, calyx is glandular and thinly silky, and the lowest sepal is larger and prominent. Flowering occurs from spring to early summer, which is September to December in South Africa. Usually after flowering, the fruits are stored in pods.

2.10.2.1.2 Distribution and Habitat

Otholobium fruticans grows in the Cape Peninsula only, in mountain fynbos at 180-600 m altitude on steep, rocky slopes. It is simply confused with *O. bracteolatum* but they are differentiated by the colour of the flowers and growth habitat. *O. bracteolatum* has a lager distribution and a different habitat and grows in coastal sandveld and on limestone hills below 170 m that extend from Saldanha to Grahamstown. In addition, it is a sprawling shrub about 1.5 m tall having white, violet or blue flowers in summer. Moreover, calyx bract below the keel in Otholobium fruticans is larger and often blackish or purple.

2.10.2.1.3 Traditional Medicinal Uses

Otholobium fruticans is a poorly studied medicinal plant with no scientific work reported yet on the medicinal use of this plant to the best of our knowledge. However Bakuchiol, a bioactive compound isolated from *Otholobiumpubescens*, another species of Otholobium, has been reported to have hypoglycaemic effects in mice (Krenisky et al., 1999).

Otholobium fruticans (Fig 2.4) has great potential for horticultural use; its growth habitat makes it a beautiful hanging basket plant, filler and a groundcover. It is suited to spill over retaining walls and to rockeries and terraces and is used in landscaping also because of its showy sprays of blue and purple colour in spring. It is equally suitable for fynbos gardens or water-wise, winter rainfall (Manning and Goldblatt, 2012).

Figure 2.4: Shows photomicrograph of *Otholobium fruticans* (L.) Stirt.

2.10.2.2 Genus Rhynchosia

There are about 230 species in the genus Rhynchosia which are distributed mostly in Africa and Madagascar but extends to northern Australia, warm temperate and tropical Asia, and tropical and subtropical America (Schrire, 2005). This genus is grouped into two sections and six series, which are Copisma, comprising of four series, and Arcyphyllum, made up of two (Grear, 1978). Baker divided the South African species of Rhynchosia into five sections in 1923 and these include Eurhynchosia Baker f. (59 ssp.), Polytropia Presl. (two spp.), Chrysoscias E. Mey. (four spp.), Cyanospermum (Wight and Arnott) Benth. (one spp.), and Arcyphyllum Torrey and Gray (two spp.) (Baker, 1923).

2.10.2.2.1 *Rhynchosia villosa*

Rhynchosia villosa (Fig 2.5) is endemic to South Africa and is commonly found in the Eastern Cape, KwaZulu-Natal, and Mpumalanga provinces.

Figure 2.5: Shows photomicrograph of *Rhynchosia villosa* (L.) Stirt.

2.10.2.3 Genus Psoralea

There are many genera of plants that have contributed to traditional as well as orthodox medicine. Prominent among this is the genus Psoralea of the legume (Fabaceae) family which was first established by Linnaeus in 1742. *Psoralea Americana* which is native to America was the only species that was recognized and described by Linnaeus in 1953. Psoralea, according to The Plant List (2013), is a widespread genus consisting of 105 accepted species. About 23 species of this genus have been reported in Australia and 14 species in Northern Territory. There are two very common species in Dominican Republic. ‘Psoralea’, derived from the Greek term ‘Psoraleos’, mean “affected with itch or with leprosy” (Chopra et al., 2013).

Genus Psoralea is a source of a number of bioactive compounds belonging to the chemical class of flavonoids, coumarins, furanocoumarins, chalcones, terpenoids, and meroterpenes. Some of the Medicinally useful compounds sourced from Psoralea

species include ‘psoralen’, ‘isopsoralen’ (angelicin), ‘bakuchiol’, ‘corylifol’, ‘psoralidin’, ‘bavachinin’, ‘corylifolinin’, ‘caryophyllene’, ‘β-farnesene’, ‘α-pinene’, ‘camphene’ and ‘germacrene D’ (Bertoli et al., 2004, Tava et al., 2007, Li et al., 2016). Currently, pharmacological research reports are available on only few species viz. *P. canescens* Michx. [synonym: *Pediomelum canescens* (Michx.) Rydb.], *P. bituminosa* (accepted name: *Bituminaria bituminosa*), *P. corylifolia* L. [accepted name: *Cullen corylifolium* (L.) Medik.], *P. esculenta* Pursh, *P. plicata* Delile [accepted name: *Cullen plicatum* (Delile) C.H.Stirt.] and *P. glandulosa* L. *P. corylifolia*, a member of this group, is commonly known as ‘babachi’, ‘bakuchi’, ‘bavachi’, ‘Indian bread root’, ‘fountain-bush’ or ‘scurf pea’ (Shilandra et al., 2010, Zhang et al., 2016, Alam et al., 2018). It is a very important but endangered medicinal herb that is grown in subtropical and tropical areas of the world (China, Japan, Burma and India) due to its folkloric medicinal properties (Sehrawat et al., 2013).

Psoralea species have scientifically investigated and proven pharmacological activities (Li et al., 2016, Alam et al., 2018). They have been used in the treatment of psoriasis, leucoderma, leprosy and many other pathologies (Newton et al., 2002, Ali et al., 2008, Khushboo et al., 2010, Wong et al., 2013, Zhang et al., 2016).

2.10.2.3.1 *Psorallea aphylla*

Psoralea aphylla is a reseeding, tall shrub or small tree that could be 5 m tall (Fig 2.6). The stem is greenish-tan with storied white lenticels, seasonal shoots that are floppy during flowering, and the flowers are mauve to deep blue (Dludlu et al., 2015)

Figure 2.6: Shows photomicrograph of *Psoralea aphylla*

2.10.2.3.2 Distribution and Habitat

In South Africa, *Psoralea aphylla* is scattered across the Cape Peninsula and surrounding low foothills of mountains. It was commonly found on the Cape Flats, but it is now quite scarcethere. It has an altitude that ranges from10 to 300 m above sea level and grows on Peninsula Sandstone Fynbos i.e. FFs 9 and Cape Flats Sand Fynbos i.e. FFd 5 (Rebelo et al., 2006); open areas, stream banks, gulleys and seepages. Flowering occurs between September to April (Dludlu et al., 2015).

2.11 The Proteaceae Family

The Proteaceae family of flowering plants is predominantly distributed in the Southern Hemisphere with 83 genera and about 1,660 species (Christenhusz and Byng, 2016) with Australia and South Africa as centres of its diversity. Other areas where it is found include Central Africa, South and Central America, India, Eastern and South Eastern Asia, and Oceania (Flora and Study, 1981). The genera of Proteaceae are very

diversified, however, the genus, *Banksia*, provides a striking example of adaptive radiation in plants (Mast and Givnish, 2002). Proteaceae are commonly trees about 40 m in height, and are usually of medium height or low or perennial shrubs, except for some species that are herbs.

Crude extracts of leaves of *Dilobeia thouarsii*, a member of Proteaceae family, were reported to have antimicrobial effects *in vitro*. The high antimicrobial activity of *D. thouarsii* leaf extracts against *S. aureus* supports its traditional use to treat skin infections in Madagascar (Razafintsalama et al., 2013). Masevhe et al. reported that *Faurea Saligna* Harv (Proteaceae) is used traditionally for treating epilepsy in Venda area of South Africa (Masevhe et al., 2015). Decoction of the leaves of *Grevillea robusta*. A. Cunn have been used as antifungal, antibacterial and anti-inflammatory by the irula tribes in India (Dhivya and Kalaichelvi, 2016), while roasted leaves powder of *Gravillea robusta* (Silver Oak) has also been reported to be used for the treatment of scalds and burns by some Indian tribes (Tiwari et al., 2017).

2.11.1 The Proteaceae Family and Depigmentation

A number of plants in the Proteaceae family have been reported to possess melanin inhibition potentials. Polar extracts of root of *Protea madiensis* Oliv. inhibited melanogenesis when used traditionally as herbal medicine for treatment of skin diseases in Rwanda (Kamagaju et al., 2013). A compound, 2,4-dihydroxyphenyl-(6′-*O*-benzoyl)-*O*-*β*-D-glucoside, isolated from *Protea neriifolia* (Proteaceae) possesses structure that is very similar to arbutin which has been used as a whitening agent in cosmetics (Maeda and Fukuda, 1996, Sarkar et al., 2013a). Chemical synthesis of this natural product showed tyrosinase inhibition even though the IC_{50} could not be determined in concentrations lower than 1000 μM (Matsumoto et al., 2018).

2.11.2 *Serruria furcellata* R.BR.

Serruria furcellata is a resprouting, multi-stemmed, erect shrub reaching a height of 0.5 m and spanning 1 m across (Fig 2.7). It grows in acid sandy soils on the Cape Flats with a weather which is cold and wet during winter and hot, dry and windy during summer.

Figure 2.7: Shows photomicrograph of *Serruria furcellata*

2.12 The Moraceae Family

Many plants in the family Moraceae have been used in traditional medicinal practices. In India, oil prepared from the rook bark of *Ficus religiosa*, Linn. is being used topically to manage rheumatism, leprosy and eczema (Lekha and Menakashree, 2018). *Ficus polita* Vahl.is a tropical evergreen shrub of Moraceae family. While the leaves are occasionally harvested for food, the fruits are sometimes eaten as aphrodisiac and stimulant. The fruits and young leaves are chewed for the treatment of dyspepsia (Kuete et al., 2011), infusions prepared from the roots and bark for infectious disease,

diarrhoea, dyspepsia and abdominal management (Etkin and Ross, 1982, Kamga et al., 2010, Kuete et al., 2011). Other plants in the Moraceae family used as folklore medicine in Bangladesh include *Ficus religiosa L.*, Ficus *benghalensis L.*, *Ficus hispida* L.f. and *Artocarpus heterophyllus* Lamk. They are used for diseases which include inflammation, asthma, rheumatic pains, lumbago, diabetes mellitus, and diarrhoea (Khatun and Rahma, 2018).

2.12.1 The Moraceae Family and Depigmentation

Melanin synthesis inhibition potentials of some plants in few genera of Moraceae family have been reported. Stilbenes and flavonoids which have remarkable tyrosinase inhibition potentials have been isolated from the wood and twigs of *Artocarpus heterophyllus*(Ilkay Erdogan and Mahmud Tareq Hassan, 2014). The flavanones steppogenin and artocarpone, and the flavones isocarpesin, norartocapetin and artocarpesin from the wood of *Artocarpus heterophyllus*, have been found to inhibit the monophenolase activity of mushroom tyrosinase in vitro. In a similar work, artocarpanone inhibited the diphenolase activity of mushroom tyrosinase and melanin synthesis in B16 melanoma cells (Arung et al., 2006). In addition, Mulberroside F isolated from the leaves of *Morus alba*, inhibited melanin biosynthesis in cultured Melan-a cells (Lee et al., 2002), while tyrosinase and melanogenesis inhibitory activities of ether extract of *Artocarpus altilis* heartwood containing about 45% w/w of artocarpin have also been reported (Donsing et al., 2008, Buranajaree et al., 2011).

2.12.2 Maclura pomifera

2.12.2.1 Taxonomy

Maclura pomifera is a member of the Moraceae family (Fig 2.8). It is also known as Osage orange, horse apple, mock orange or hedge apple (Wolfrom and Bhat, 1965) and is indigenous to Oklahoma and northern Texas, and cultivated widely in the United States (Mahmoud, 1981).

Figure 2.8: Shows photomicrograph of *Maclura pomifera*

2.12.2.2 Distribution and Habitat

Maclura pomifera, commonly found in open sunny areas, can survive in drought environment and thrives in a wide range of soil types and soil moisture conditions. It grows well in well-drained soils and has a lower soil pH limit of 4.5. As it is found in hedgerows or pastures, it also occurs in disturbed and floodplain forests (Sternberg, 1989).

2.12.2.3 Traditional Medicinal Uses of *Maclura pomifera*

Different parts of *Maclura pomifera*have been used in folklore medicine. The Comanche Indians in North America used decoctions made from roots for the treatment of eye sores (Carlson and Volney, 1940), while the plant sap is used for tooth pain, and the bark and leaves for uterine haemorrhage in Bolivia (Bourdy et al., 2004). In addition, the plant is used for the treatment of cancer by the Native Americans (Mahmoud, 1981). Many biological activities antimalarial, antitumour, oestrogenic, antifungal, antitumour, cytotoxic and antibacterial activities of *Maclura pomifera* and its components have been reported (Peterson and Brockemeyer, 1953, Jones and Soderberg, 1979, Mahmoud, 1981, Voynova et al., 1991, Maier et al., 1995, Bunyapraphatsara et al., 2000, Hay et al., 2004).

2.13 Rhamnaceae Family

Rhamnaceae are a family of trees, shrubs, and climbers which consist of about 50 genera and 900 species (Richardson et al., 2000). Because of their tendency towards xeromorphism, they have ability to survive in drier habitats. It is difficult to truly trace the history of this family due to their representation both in temperate and tropical regions of the world (Raven and Axelrod, 1974).

Species in the family Rhamnaceae are known for their striking morphological diversity and high genetic variation, possibly as evolutionary consequences associated with its cosmopolitan distribution and many different habitat (Hardig et al., 2000, Hauenschild et al., 2016). Little chemistry is documented from this family despite identification of some family-specific metabolites such as triterpenoids (Kang et al., 2016) and cyclopeptide alkaloids (Tuenter et al., 2017).

2.13.1 Traditional Medicinal Uses of Rhamnaceae Family

Species of plants of family Rhamnaceae are being used as folklore medicine in Bangladesh. Hossan et al., in their study of traditional use of medicinal plants in Bangladesh to treat urinary tract infections and sexually transmitted diseases, reported that the Tripura tribeuse the root of *Zizyphus oenoplia* (L.) Mill., locally known as Boroi, to treat burning sensations in urinary tract, less urination, and frequent urination (Hossan et al., 2010). In Pakistan, the fruits and bark of *Zizyphus jujube* (ZJD7), traditionally named - Bera, is used as a tonic expectorant and as antiasthma agent. While the fruits are used directly, the bark are slightly ground and applied to wounds. Another species of the same genus is used in Pakistan. *Zizyphus nummularia* (ZND14), locally called Karkanrah, is used as folklore medicine and its fruits are eaten directly while the leaves are chewed (Ullah et al., 2010).

2.13.2 Genus Phylica

Phylica is a genus of plants that belong to the family Rhamnaceae. There are about 150 species in the Genus (Richardson et al., 2003) and the majority are South Africa where they are members of the fynbos. A few species are found in other areas including other parts of southern Africa, islands of Madagascar, Mascarene, and Gough. It is also present in Amsterdam, Saint Helena, and Tristan da Cunha (Richardson et al., 2003).

2.13.3 *Phylica ericoides* L

Phylica ericoides is a highly-branched and compact shrub (Fig 2.9). It is bushy, evergreen and grows to height of about 0.6 m with equal spread and could attain 1 m allowed. This species has slender branches with sparse covering of short, fine, grey hairs. The leaves of this plant are either linear or lance-shaped(Ns Pillans, 1942).

Figure 2.9: Shows photomicrograph of *Phylica ericoides*

2.13.4 Distribution and Habitat of *Phylica ericoides*

Phylica ericoides has cosmopolitan distribution. It grows on coastal slopes, dunes and deep sands from Saldanha to Paarl and the Cape Peninsula, to Port Elizabeth and into southern tropical Africa.

The taxonomy of plants reviewed above is shown in Table 2.1 below.

Table 2.1: Taxonomy of Plants Evaluated in This Study

	Otholobium fruticans	*Rhynchosia villosa*	*Psoralea aphylla*	*Serruria furcellata*	*Maclura pomifera*	*Phylica ericoides*
Kingdom	Plantae	Plantae	Plantae	Plantae	Plantae	Plantae
Division	Magnoliophyta	Tracheophyta	Tracheophyta	Tracheophyta	Tracheophyta	Tracheophyta
Class	Magnoliopsida	Magnoliopsida	Magnoliopsida	Magnoliopsida	Magnoliopsida	-
Order	Fabales	Fabales	Fabales	Proteales	Rosales	Rosales
Family	Fabaceae	Fabaceae	Fabaceae	Proteaceae	Moraceae	Rhamnaceae
Genus	*Otholobium*	*Rhynchosia*	*Psoralea*	*Serruria*	*Maclura*	*Phylica*
Species	*fruticans*	*Villosa*	*Aphylla*	*furcellata*	*Pomifera*	*ericoides*

2.14 Traditional Medicinal Plants and Mechanisms of Action on Inhibition of Melanin Synthesis

Melanogenesis regulation, which leads to melanin synthesis or its inhibition, is brought about by several signal transduction pathways (Busca and Ballotti, 2000). Many approaches used to elucidate the specific mechanism controlling biosynthesis of melanin via tyrosinase regulation have been documented. Because medicinal plants are a good source of bioactive chemicals and potentially safe and effective skin lightening agents, there has been a surge in interest to identify tyrosinase inhibitors from plants which finds its application in skin care products (Coe and Anderson, 1996, Movahhedin et al., 2016).

Tyrosinase inhibitors from natural sources are present in several chemical classes like flavonols, phenolics, flavanols, isoflavanoids, flavonones, terpenes, steroids, alkaloids, chalcones, flavonoids, coumarins, stilbenes, long chain fatty acids, biscoumarins, bipiperidines, oxadiazole, tetraketones, etc. (Chang, 2009). Various natural products inhibit melanogenesis via different mechanisms of action. The phenol structural group present in flavonoids may be a structural analogue of L-tyrosine substrate (Chang, 2009) which may compete with the substrate for the active site of tyrosinase causing inhibition of its catalytic activity. *Nardostachys chinensis*, an aromatic herb, is used traditionally as a sedative to mitigate psychiatric symptoms such as hysteria, insomnia, and depression (Dhingra and Goyal, 2008). However, ethyl acetate extract of this medicinal plant inhibits melanin production and tyrosinase activity in B16F10 melanoma cells by suppressing the expression of MITF, tyrosinase, and TRPs through MEK/ERK or PI3/Akt phosphorylation (Jang et al., 2011). Other reports have also

demonstrated inhibition of melanin synthesis via ERK-dependent MITF pathway in B16F10 melanoma cells (Saba et al., 2018, Zhao et al., 2018).

According to Lin et al., melanogenesis inhibition by raspberry ketone derived from *Rheum Officinale* in murine B16 cells is through post-transcriptional regulation of tyrosinase gene expression leading to down-regulation of cellular tyrosinase activity and tyrosinase protein content without affecting the level of tyrosinase mRNA transcription (Lin et al., 2011). Other melanin synthesis inhibition experiments have shown that antimelanogenesis occurred by tyrosinase inhibition, tyrosinase expression inhibition and melanin pigment degradation. However, the most potent compound's inhibitory effect was by degradation of melanin pigment in B16 melanoma cells without affecting the tyrosinase activity or tyrosinase expression (Hosoya et al., 2012).

Intracellular production of ROS has been proposed as an aetiological factor for natural depigmentation processes which include greying of hair and many other pathological conditions such as vitiligo (Jiménez-Cervantes et al., 2001). It has been reported in previous studies that MITF is phosophorylated and then degraded in response to ROS stimulation (Liu et al., 2009, Kim et al., 2014). In addition, inhibition of hyperpigmentation has been demonstrated to occur by degradation of MITF through ROS-induced ERK phosphorylation (Ko and Cho, 2018). Impairment of the scavenging system for ROS eventually leads to the destruction of melanocytes by oxidative stress which in turn leads to reduction of melanin synthesis, and by extension, vitiligo, which is a pathological condition characterized by skin depigmentation (Kroll et al., 2005, Gong et al., 2015). However, a few authors have reported a contradictory role of oxidative stress on melanin homeostasis (Yokozawa and Kim, 2007, Kim, 2013). Masaki, in his work on the role of antioxidants in the skin, reported that

oxidative stress caused up-regulation of melanin synthesis and consequently increased skin pigmentation (Masaki, 2010).

2.15 Evaluation of Cytotoxicity

Cells grown in culture for experimental purposes are evaluated for their viability after treatment with extracts, compounds, nanoparticles, etc. using various cytotoxicity assay kits. Arung et al. used 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to evaluate the viability of B16 melanoma cells in their work "Artocarpin, A Promising Compound as Whitening Agent and Anti-skin Cancer" (Arung et al., 2018). Several previous works on B16 melanoma cells used MTT to evaluate the viability of cells after various treatment protocols (Bae et al., 2018, Cheimonidi et al., 2018, Nishina et al., 2018). Other kits used to determine viability of cells by some researchers include WST-1 (Kanda et al., 2013), XTT (Stapelberg et al., 2018) and CCK-8 (Bian et al., 2013).In the present study, the MTT assay was used to evaluate the cytotoxicity of extracts of plants reviewed above on murine B16 melanoma cells.

Chapter Conclusion

This chapter provided a general review of literature on this study. It highlighted pigmentation disorders and management; melanin structure, its role and its synthesis pathway, as well as the role of reactive oxygen species. Six different plant materials used in this study were profiled, and the mechanisms by which medicinal plants inhibit melanin synthesis were briefly discussed.

The next chapter describes the materials and methods used in this study and gives detailed processes of each experiment performed.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

This chapter explains the design, setting, and methodology used in this study, and these include the extracts, handling of cancer cells, assay kits, chemicals and different experiments done at various stages of the study.

3.2 Plants Collection

The plants used in this study were collected randomly from the Cape Flats natural reserve in Western Cape. South Africa, in November 2012/2013. Herbarium was made for each plant collection, and identified by Mr. Franc Wits (Department of Biodiversity and Conservations Biology, UWC). Voucher numbers were deposited at the Chemistry Department (UWC).

3.3 Methods

3.3.1 Preparation of the Methanolic and Ethyl acetate Leaf Extract of the plants

The aerial parts of each plant were harvested and allowed to dry at room temperature and 100g of each plant was blended separately with ~200-300 mL of solvents (methanol or ethyl acetate) (Dhawan and Gupta, 2017), and thoroughly mixed and filtered after 24 h using a Buchner vacuum filter. The extraction process was repeated twice and the filtrates of each plant were combined and evaporated under reduced pressure at 45^{o}C with a Rotary evaporator. The weight of dried methanolic or ethyl

acetate extract of the leaves in grams and percentage yield in relation to the weight of the leaves used were determined and kept cold (-5^{o}C) until further use (Anokwuru et al., 2011).

3.3.2 Maintenance of B16 Melanoma Cells

All tissue culture experiments were performed in a model NU-5510E NuAire DHD autoflow automatic CO_2 air-jacketed incubator and an AireGard NU-201-430E horizontal laminar airflow table-top work station that provides a HEPA-filtered clean work area (NuAire). B16 melanoma cells were a kind gift from Prof Lester Davids of Department of Medical Bioscience, University of the Western Cape, South Africa. The cells were cultured in medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% Penicillin/Streptomycin (100 U/mL Penicillin and 100 U/mL Streptomycin) and 20% heat-inactivated and gamma-irradiated Fetal Bovine Serum (FBS), and grown in at 37^{o}C as monolayer in a humidified incubator (Relative humidity 80-90%) in an atmosphere containing 5% CO_2:95% air (Pintus et al., 2015).

B16 melanoma cells were used having been widely used for this purpose possibly because they are relatively easy to culture in vitro and similarity of their melanogenic mechanisms with normal human melanocytes (Stapelberg et al., 2019).

Frozen cells in cryovials were removed from -80^{o}C freezer and thawed quickly. The cells were introduced into 15 mL conical centrifuge tubes and fresh culture medium added to neutralize the effect of Dimethylsulfoside (DMSO) used for its preservation. The tube containing cells was centrifuged at 3000 rpm at 25^{o}C for 3 min. Supernatant was discarded and the pellet was re-suspended in 5 mL fresh complete culture medium

and introduced into T-25 tissue culture flask (surface area (SA) 2500 mm^2). The flask was placed under a PrimoVert phase-contrast microscope to ascertain presence of cells, and then placed in 5% CO_2 incubator at 37°C. The cells were observed under microscope after 24 h incubation to confirm attachment. The flask was incubated until about 80-90% cell confluence was reached (Pintus et al., 2015).

3.3.3 Subculturing of Cells

Trypsinization of cells was done at 80-90% confluency. Complete medium in culture flask containing confluent cells was aspirated. The cells were washed with 1 mL of PBS for 1 min. PBS was removed and 0.05% trypsin-EDTA added. The flask was placed in incubator for about 2 min in order to allow cells to detach, after which it was transferred to the laminar flow safety cabinet. The action of trypsin was deactivated with the addition of 2 mL of complete medium to the flask. The medium containing detached cells were aspirated and dispensed into 15 mL conical centrifuge tube. It was placed in the centrifuge, spun at 3000 rpm for 3 min at 25°C to separate cells from medium-trypsin solution. After centrifugation, the supernatant was discarded and 4 mL of fresh complete medium was introduced to dislodge the cell pellet at the bottom of the conical centrifuge tube. The cells were mixed gently to ensure homogenous suspension. From the suspension, 1 mL was transferred into T-25 culture flask to maintain stock cultures.

3.3.4 Cell Counting and Viability Testing

TC20™ automated cell counter (Bio-Rad) was used for cell counting according to manufacturer's procedure. It uses an optical method to count cells in liquid medium within counting slide. To assess cell viability, 0.4% of Trypan Blue stain in 1:1 ratio

(v/v) was mixed with cell suspension. Ten µL of trypan blue-stained cell suspension was introduced into the counting chamber of the slide and then inserted into the TC20^{TM} automated cell counter. The total number of cells counted, total number of viable cells together with the percentage viability were displayed on the screen of the counter within 1 min. The desired concentration of viable cells for a particular experiment was then determined based on the displayed viable cells using the cell counter calculator.

Preparation of stock solutions and buffer used in this study is shown in Table 3.1 below.

Table 3.1 Preparation of Stock Solutions and Buffers

Stock solution	Preparation
MTT (5mg/ml)	A stock solution of 5mg/ml of MTT 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide was prepared in PBS.
Phenylmethanesulfonylfluoride (PMSF)	A 1 mM stock solution was prepared in deionized water
100 mm phosphate buffer solution (PH 6.5)	Sodium dihydrogen monophosphate ($NaH_2PO_4.H_20$) 137.99 g of was dissolved in 800 ml distilled water. The pH was adjusted to 6.5 with 75 mM di-sodium hydrogen monophosphate ($NaH_2PO_4.H_20$). The buffer volume was made up to 1000 mL with sodium dihydrogen monophosphate. The buffer is stable for a month at 4°C.
5% Triton x 100	38mL of deionized water was added to 2mL of absolute Triton X
SDS sample buffer	10mL of 10mM Tris-HCl buffer (pH 7.0) containing 1% SDS, 25% - glycerol and 1% Bromophenol blue without β-mercaptoethanol

3.3.5 Morphological Evaluation of Cells

B16 melanoma cell line was cultured in 12-well plates and treated after 24-hour incubation at 37^{o}C in a humidified incubator when cells had attached to the bottom of the plates. Zeiss light microscope was used to study the morphology of cells.

3.3.6 MTT Cytotoxicity Assay

The MTT is a colorimetric assay used to determine viability of cells, and is based on the ability of succinate dehydrogenase enzymes in mitochondria of live cells to metabolize yellow 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple colour insoluble formazan product which can be measured between 500 and 600 nm wavelengths by spectrophotometry (Saravanan et al., 2003). Viable cells were seeded at a density of 6 x 10^4 (100 µL/mL) in 96-well plate and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 24 hours to form a cell monolayer. After 24 h, the supernatant on the monolayer was gently discarded and 100 µL of medium containing serially diluted concentrations of test extracts were added daily and then incubated for a duration of 48 or 72 h under standard incubation settings.

At the end of 72 h incubation, 10 µL of 5 mg/mL MTT in PBS was added to each well and incubated for 3 hours at 37 ˚C in 5 % CO_2 atmosphere. Supernatant was removed and 100 µL of DMSO was added and the plates were shaken for 5 secs to solubilize the formazan while absorbance was being measured at 570 nm using microplate reader (POLARstar Omega BMG LABTECH, Germany).

The percentage inhibition of proliferation was calculated using the formula below:

$$\%\ \text{Inhibition of Proliferation} = \frac{100\ -\ \text{Test}}{\text{Untreated OD}} \times\ 100\%$$

3.3.7 SDS Gel Preparation

Proteins were separated by denaturing SDS-PAGE under reducing conditions (Laemmli, 1970). Briefly, gels were prepared from 40% stock of premixed 37:5:1 acrylamide: bisacryamide and consisted of separating and stacking gels which were

poured between two assembled glass plates separated by a 1.0 mm thick comb spacer. The gels were prepared in 1.0 mm Hoeffer dual gel caster and about 10 mL were enough for one gel. About 4.7mL of the separating gel was poured about 1 cm below the wells of the comb and about 1 mL water saturated 1-butanol was overlaid on top of the separating gel and left to set. Afterwards, the butanol was poured off the gel and rinsed with deionized water. Stacking gel (≈ 1.5 mL) was poured over the running gel. The comb was immediately inserted into the stacking gel to make wells and allow stacking gel to set. After, the comb was removed, the gel was transferred to the Mighty Small apparatus (Hoeffer) containing running buffer.

3.3.8 Melanin Synthesis Assay

Melanin synthesis assay is a colorimetric assay used to determine relative quantity of melanin produced. Melanin is produced by melanocytes after stimulation by hormones either through endocrine or paracrine signalling (Dell'Angelica et al., 2000).

3.3.8.1 Cellular Melanin Content Determination

The experiment was performed according to the technique previously described by Wang *et al*. (2018) with slight modifications. Briefly, B16 melanoma cells were cultured at a density of 6.0 x 10^4 cells/mL and 500 µL of this stock was dispensed into each well of 12-well plates and incubated for 24 hours for adherence. After the incubation, the cells were treated with serially diluted concentrations of extracts in 500 µL of DMEM. Kojic acid was used as positive control. At the end of 48 or 72h of treatment, medium in each well was dispensed into appropriately labelled Eppendorf tubes and spun at 4 000rpm for 3 min at 25^{o}C. The medium was removed and the tubes were rinsed with PBS. The wells were rinsed with 200µL of PBS. Cells in each well

of 12-well plates were then lysed with 400µl of 1M NaOH. The cell suspension was transferred to the respectively labelled Eppendorff tubes and solubilized in heating block (Eppendorf Thermomixer Comfort, Merck Chemicals Ltd, South Africa) at 60^0C with mixing at 300 rpm for 60 min. At the completion of solubilisation, 200µL was dispensed into appropriately labelled 96-well plates and absorbance read at 405nm with microplate reader (POLARstar Omega BMG LABTECH, Germany) for determination of intracellular melanin content. 400µL of the solvent (NaOH) which passed through the processes as the samples was used as blank.

3.3.9 Intracellular Tyrosinase Activity assay

Tyrosinase activity was determined by measuring the rate of L-DOPA oxidation as previously described by Kim *et al.* (2002) with some modifications. Cells were treated with extracts for different time range as described in the previous section. Cells were washed and lysed in 200 µL of 50 mM Sodium phosphate buffer (pH 6.5) containing 1% Triton X-100 and 0.1 mM Phenylmethylsulfonyl fluoride (PMSF). Cells were then frozen at -80 ^{o}C for 30 min. and thawed in waterbath at 37^{o}C for 2 min and mixed. Cellular extracts were clarified by centrifugation at 12, 000 rpm for 30 min. at 4 ^{o}C. Protein content of the cellular extracts was determined using the Nanodrop ND-1000 spectrometer (ThermoScientific). Into a 96-well plate was pipetted 120 µL of 0.067 M PBS (pH 6.8) and 20 µL of cellular extracts added and incubated at 37 ^{o}C for 15 min. At the end of incubation, 40 µL of 2.5 mM L-DOPA dissolved in 0.067 M PBS (pH 6.8) was added into the 96-well plate and immediately monitored for the formation of dopachrome ($\square$ = 3700 $M^{-1}cm^{-1}$) by measuring the linear increase in absorbance at 475 nm for 60 min.

Tyrosinase activity = $\frac{K}{10^{-6} \in \times V \times 2.5 \times 0.1}$

Where K = Slope of the curve

V = Test volume of cell extract

$\in$ = Dopachrome extinction coefficient

3.3.10 Dihydroxyphenylalanine (DOPA) Staining Assay

The DOPA-staining assay was performed as previously reported by Pintus *et al*. (2015) with slight modifications. Cells were treated with extracts for different time range as described in the previous section. After treatment, cells were harvested with lysis bu∈ffer, as described in section **3.3.9** and the supernatant was collected for analysis of the protein content using the Nanodrop ND-1000 spectrometer (ThermoScientific). Protein extracts (5µg) were mixed with 10 mM Tris-HCl buffer, pH 7.0, containing 1%, SDS, 25% glycerol, 1% Bromophenol blue without mercaptoethanol or heating and resolved by 8% SDS-polyacrylamide gel electrophoresis at initially 100V until gel front enters the resolving gel; then at 200V until the end of the run.After running, the gel was rinsed in 0.1 M phosphate buffer (pH 6.8) and equilibrated for 15 min in the same buffer. The gel was then transferred to a staining solution containing 0.1 M phosphate buffer (pH 6.8) with 5 mM L-DOPA incubated in the dark for 4 h at 37 ^{o}C. Tyrosinase activity was visualized in the gel as dark melanin-containing bands.

3.3.11 Evaluation of Reactive Oxygen Species (ROS)

The activity of reactive oxygen species (ROS) activities within cells can be detected by using fluorogenic molecular probe 5- (and 6)-chloromethyl-2', 7'-dichlorofluorescein diacetate actyl ester (CM-H2 DCFDA) (Invitrogen). CM-H2DCFDA permeates into cells freely; within the cells, its intracellular esterases

cleave its acetate groups forming a highly fluorescent compound 2, 7-dichlorofluorescin (DCF). The fluorescent DCF can be detected by fluorescence microscopy and spectroscopy, or flow cytometry. Evaluation of ROS was done as described by Koptyra *et al*. (2006) with slight modifications. Briefly, cells were cultured in a 12-well plate at a density of 6.0 x 10^4/mL. The cells were treated with 100 µg of *Serruria furcellata* extract and 300 µg of Kojic acid for 48 hours, as well as 50 µg of *Otholobium fruticans* extract for 72 hours. After the treatment, cells were washed with PBS and stained with 7.5 µM of CM-H_2 DCFDA prepared in PBS from a DMSO stock solution and incubated for 30 min at 37°C in a humidified CO_2 incubator. The cells were washed twice with ice-cold PBS following which the cells were acquired and 10,000 events analyzed on a flow cytometer (Becton Dickinson FACScan instrument, BD Biosciences Pharmingen, San Diego, CA, USA) fitted with a 488 nm argon laser.

3.3.12 Extraction of RNA

The procedure for the extraction of RNA was performed on the murine B16 melanoma cell line according to the manufacturer's instructions (RNeasy Mini Kit Qiagen, Nertherlands). For the RNA extraction, confluent cells treated in T25 with extracts, kojic acid (as positive control), and untreated cells (as negative control) were trypsinized with trypsin-EDTA and transferred to 15mL centrifuge tubes. Tubes were spun and the supernatant medium was discarded. The pellets were dislodged and then introduced into 2mL eppendorf tubes. Total cell count was determined using TC20™ automated cell counter (Bio-Rad). Cells in Eppedorf tubes were spun at 3000 rpm for 3 min using an Eppendorf 5417R bench top centrifuge; the medium discarded and 500 µL of PBS was added and spun at 3000 rpm for 2 min. Washing with PBS was repeated until there was no trace of pink colour of the medium. The PBS was then decanted.

To the clean pellet of cells in the Eppendorf tube was added 700µL of QIAzol Lysis Reagent and the pellet was disrupted and homogenized with 21 gauge needle and 2mL syringe. The homogenate was incubated at room temperature for 5 min. Then, 140µL of chloroform was added. It was shaken vigorously for 15 s and incubated at room temperature for 2-3 min. After incubation, it was centrifuged at 12,000 x g at 4^{o}C. The upper aqueous phase was transferred carefully to a new collection tube without transferring any interphase. One and a half (1.5) volumes of 100% ethanol was added and mixed thoroughly by pipetting. Up to 700µL sample, including any precipitate, was pipetted into an RNeasy $^{(R)}$ Mini column in a 2mL collection tube. It was centrifuged at 8000 x g for 15 s at room temp and the flow-through was discarded. This process was repeated for the remainder of the sample. Then 700µL Buffer RWT was added to the RNeasy Mini column and centrifuged for 15 s at 8000 x g and the flow-through discarded. Thereafter, 500µL Buffer RPE was added to the RNeasy Mini column and centrifuged for 15 s at 8000 x g and the flow-through discarded. This process was repeated but centrifuged for 2 min at 8000 x g. The RNeasy Mini column was placed into a new 2 mL collection tube and centrifuged at 8000 x g for 1min to further dry the membrane. The RNeasy Mini column was then transferred to a new 1.5 mL collection tube and 40µL RNase-free water was pipetted directly into the RNeasy Mini column membrane and centrifuged for 1 min at 8000 x g to elute RNA. The concentration and quality of RNA was assessed using the Nanodrop ND-1000 spectrometer (ThermoScientific) and all the RNA samples were stored at -80 °C.

3.3.13 Primer Design

Gene specific primers used for qPCR are shown in Table 5.3. The tyrosinase gene (TYR), tyrosinase-related protein-1 gene (TYR-1), tyrosinase-related protein-2 gene (TYR-2), microophthalmia associated transcription factor (MITF) gene (Kwak et al.,

2011), melanocortin-1-receptor gene (MC1R) (Chang et al., 2015), and β-Catenin gene (Jho et al., 2002) were previously reported sequences. Each primer of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was designed to be 20 bp long using the NCBI Primer-BLAST algorithm accessible at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The oligonucleotide sequences were sent for synthesis to Inqaba biotech http://www.inqababiotec.co.za/. The primers were delivered as a lyophilized pellet and a 10 µM working stock solution was prepared by re-suspending the pellet in 1X TE buffer (10 mM Tris, pH 7.5 to 8.0, 1 mM EDTA), the primers were stored at -20 °C.

Table 3.2 Primer sequences for PCR amplification of cDNA

Primers	FWD 5'- 3'	REV 3'- 5'	Ref	Temp/ºC
TYR	GTCGTACCCTGAAAATCCTAACT	CATCGCATAAAACCTGATGGC	Kwak et al., 2011	61
TYR-1	CTTTCTCCCTTCCTTACTGG	TCGTACTCTTCCAAGGATTCA	Kwak et al., 2011	61
TYR-2	TTATATCCTTCGAAACCAGGA	GGGAATGGATATTCCGTCTTA	Kwak et al., 2011	62
MITF	GTATGAACACGCACTCTCGA	GTAACGTATTTGCCATTTGC	Kwak et al., 2011	62
MC1R	TGACCTGATGGTAAGTGTCAGC	ATGAGCACGTCAATGAGGTT	Chang et al., 2015	61
β-Catenin	ATGGCTACTCAAGCTGAC	CAGCACTTTCAGCACTCTGC	Jho et al., 2002	62
GAPDH	ACCCACTCCTCCACCTTTG	CTCTTGTGCTCTTGCTGGG		61/62

3.3.14 Reverse transcription of mRNA to cDNA

The cDNA was synthesized using the Transcriptor First Strand cDNA synthesis kit from Roche diagnostics (South Africa), according to the manufacturer's instructions. All the reagents were kept on ice. The template RNA mixture was prepared with the reagents as shown in Table 3.3 in sterile, nuclease-free, thin-walled PCR tubes to a final volume of 13 µL. The tubes were then incubated at 65 °C for 10 minutes, after

which the cDNA synthesis reagents in Table 3.3 were added to make a final volume of 20 µL.

Table 3.3: Reagents and components for cDNA synthesis

Component/reagent	Volume	Final concentration
RNA	Variable	1 µg
Oligo DT primer	1 µL	2.5 µM
PCR grade water	Variable	
Transcriptor Reverse Transcriptase Reaction Buffer	4 µL	1 X (8mM $MgCl_2$)
Protector Rnase Inhibitor	0.5 µL	20 U
Deoxynucleotide Mix	2 µL	1 mM
Transcriptor Reverse Transcriptase	0.5 µL	10 U
Final Volume	20 µL	

The reaction was incubated at 55 °C for 30 min followed by a transcriptase inactivation step of 5 min at 85 °C. The concentration of the synthesised cDNA was determined with a NanoDrop Spectrophotometer ND1000. The cDNA was diluted to a final working concentration of 250 ng.

3.3.15 Analysis of gene expression profiles of the gene using qPCR

Expression profiles of the genes were analysed via qPCR in the control, extract-treated and Kojic acid- treated B16 melanoma cells. The house keeping genes GAPDH was used as the calibrator. All reactions were performed on the LightCycler® 480 System (Roche diagnostics) instrument. The reactions were prepared as outlined in Table 3.4.

Table 3.4: Reagents for a standard qPCR reaction

Reagents	Final Concentration
SYBR green master mix (10X)	1 X
Forward primer	1 µM
Reverse primer	1 µM
CDNA	25 ng
PCR grade DH_2O	Variable to make 20 µL
Final volume	20 µL

The qPCR reactions were performed by analysis of each of the genes in each group. In addition, reactions for the reference housekeeping genes and a no-template control (water) were also set up for each group. A 18 µL aliquot of reaction master-mix was pipetted into each well of a 96-well plate and a 2 µL aliquot (250 ng) of cDNA from each group was then added as the qPCR template to each well respectively. The 96 well plates were sealed with clear sealing film and a qPCR run set up on the LightCycler® 480 instrument according to the parameters in Table 3.5 and 3.6. The evaluating parameters selected for data analysis were fluorescence (d[F1]/dT), melting temperature (Tm) and crossing point (Cp). The Second Derivative Maximum algorithm was employed for Cp determination where Cp was measured at the maximum increase of fluorescence.

Specificity of real-time PCR primers was determined by amplification plots, melting temperature, and melting curve analysis using LightCycler Software, Version 1.5 (Roche Diagnostics). Standard curves were generated using a dilution series in the concentration range 250 ng to 0.025 ng. The PCR efficiencies were calculated using the REST® software and all threshold cycle (Ct) values were taken into consideration according to the following equation: E=10[-1/slope] (Pfaffl et al., 2002).

Table 3.5: Cycling Protocol for the qPCR

Programme	Cycles	Analysis Mode
Pre-incubation	1	None
Amplification	40	Quantification
Melting Curve	1	Melting Curve
Cooling	1	None

Table 3.6: Specific LightCycler® 480 parameters for the qPCR

Programme Name	Target Temp (°C)	Acquisition Mode	Hold (hh:mm:ss)
Pre-incubation	95	None	00:03:00
Amplification	95	None	00:00:10
	Primer Dependent 50 - 67	One	00:00:20
Melting curve	95	None	00:00:05
	65	None	00:01:00
	97	Continuous	5-10 acquisitions/ºC
Cooling	40	None	00:00:10

Statistical Analysis

Data are expressed as means ± SME of experiments performed in triplicate. The values were analyzed by One-Way ANOVA, followed by Tukey's multiple comparism test using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The level of statistical significance was measured at $p \leq 0.05$.

CHAPTER FOUR

Cytotoxicity and Melanogenesis-Inhibition Potentials of Medicinal Plants

4.1 Introduction

Medicinal plants have been used as traditional treatments for a plethora of human diseases for thousands of years and in many parts of the world. In rural areas of developing countries, plants are still in use as the primary source of medicines (Chitme et al., 2004). There are a number of natural products derived from medicinal plants that have proven to be an abundant source of biologically active compounds and a lot of these have served as the basis for the development of new lead chemicals for pharmaceuticals. However, most developing countries have huge resources of medicinal and aromatic plants mostly in the rural areas and much of these are still not fully explored. The aims of this study were to (1) screen six medicinal plants for their melanin synthesis inhibition potentials on murine B16 melanoma cells, and (2) select plants that inhibit melanin synthesis and determine the mechanism(s) of melanin inhibition.

4.1.1 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Maclura pomifera* Leaves

Cytotoxicity of methanolic extract of *Maclura pomifera* leaves was performed on murine B16 melanoma cells using the MTT assay to assess the viability of the cells after exposure for 48 h (Fig 4.1A) and 72 h (Fig. 4.2A) respectively. Also, melanin

inhibition assay of the methanolic extract was done on murine B16 melanoma cells at 48 h (Fig 4.1B) and 72 h (Fig 4.2B) respectively.

Cytotoxicity was done with a serial dilution of the crude extract starting from 25 µg to 3.125 µg (Fig 4.1A). Untreated cells served as negative control while 6% Dimethylsulphoxide (DMSO) served as positive control. Viability of B16 cells reduced progressively from the least concentration, 3.125 µg/mL, to the highest, 25 µg/mL. Although the reduction in viability of B16 cells at 3.125 µg/mL and 6.25 µg/mL were not significantly different from the control, there was a significant reduction in the viability of the cells at 12.5 µg/mL ($p < 0.05$) and 25 µg/mL ($p < 0.01$). Thus, the two highest concentrations (6.25µg/mL and 12.5µg/mL) of methanolic extract of *Maclura pomifera* leaves used were toxic to the cells. Melanin assay was done with the same serially diluted concentrations used for the cytotoxicity experiments. As shown in Fig 4.1 B there was an apparent increase in the melanin concentration at 3.125µg/mL (141 % of the untreated control) but it was not statistically significant from the untreated control. The higher concentrations of the extract did not reduce melanin synthesis significantly different from the control. However, melanin concentration at 6.25µg/mL and 12.5µg/mL were 90.6% and 76% respectively, and were not significantly different from the untreated control. Because of the cell death that occurred in the cytotoxicity assay, the apparent reduction in melanin concentration caused by 6.25 µg/mL and 12.5 µg/mL concentrations were as a result of corresponding reduction in cell density that synthesized melanin.

Viability of B16 cells reduced progressively as the extract concentration increased at 72 h cytotoxicity testing. The lowest concentration, 3.125µg/mL, and 6.25µg/mL reduced the cell viability and were 73.7 % and 65.9 % respectively. The viability was

dose-dependently reduced to 33.4 % ($p < 0.05$) and 30.7 % ($p < 0.01$) by 12.5 µg and 25 µg extract concentrations. Melanin synthesis at 72 h was dose-dependently reduced as the concentration of the extract increased. The melanin synthesized was 99.2 %, 73.3 % and 50.5 % ($p < 0.05$) of the untreated control for 3.125µg/mL, 6.25µg/mL and 12.5 µg/mL, respectively. The observed melanin reduction could have been caused by corresponding reduction in B16 cell density as the concentration of the crude extract increased.

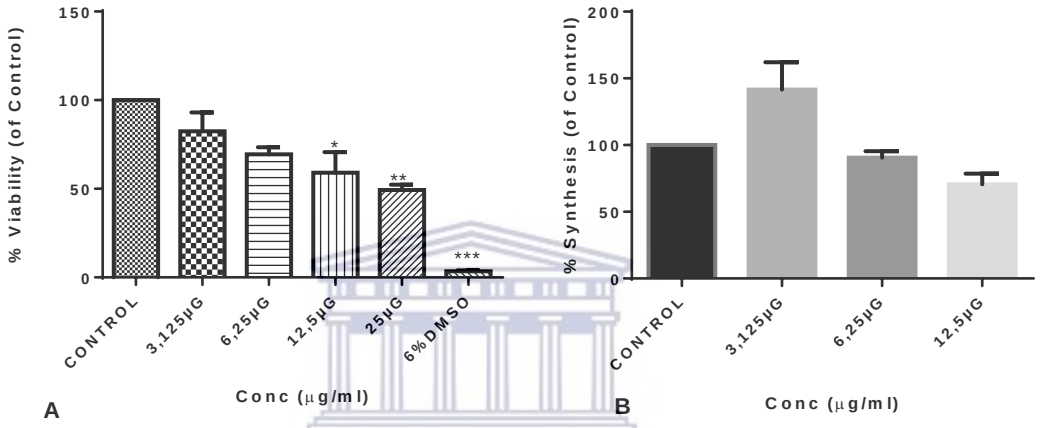


Fig 4.1: Effect of Methanolic Extract of *Maclura pomifera* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

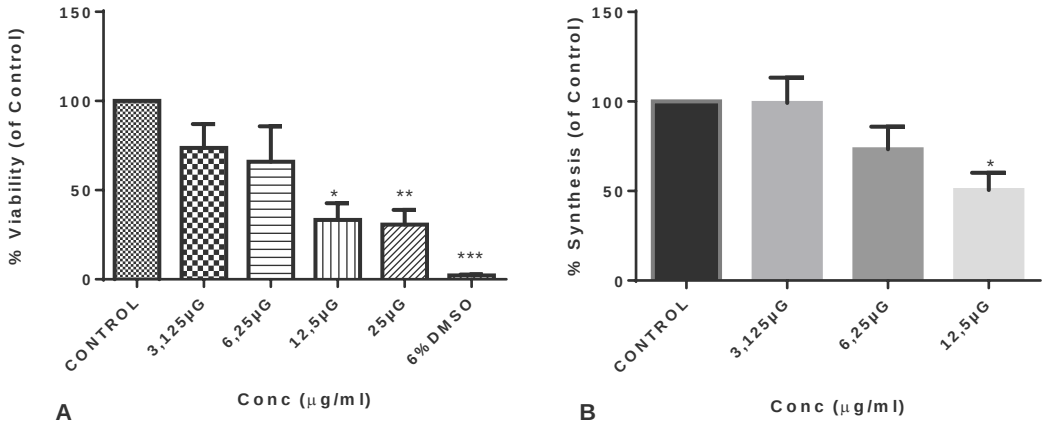


Fig 4.2: Effect of Methanolic Extract of *Maclura pomifera* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

4.2 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Otholobium fruticans* Leaves

Cytotoxicity of methanolic extract of *Otholobium fruticans* leaves was performed on murine B16 melanoma cells and MTT was used to assess the viability of the cells after exposure for 48 h (Fig 4.3A), 60 h (Fig 4.4A) and 72 h (Fig. 4.5A) . Also, melanin inhibition assay with the methanolic extract was done on murine B16 melanoma cells at 48 h (Fig 4.3B), 60 h (Fig 4.4B) and 72 h (Fig 4.5B). Cytotoxicity was done with serial dilution of methanolic extract. The concentrations used were 12.5 µg/mL, 25.0 µg/mL, 50 µg/mL and 100 µg/mL and 6% DMSO was used as the positive control.

At 48 h treatment the viability of the cells reduced dose-dependently as the concentration increased. It was 90.3 %, 61.8 % ($p < 0.01$), 46.5 % ($p < 0.001$), and 40.9 % ($p < 0.001$) respectively (Fig. 4.3A). At the respective extract concentration, the melanin concentration also reduced progressively as the concentration of the treatment extract increased from 12.5µg/mL to 100µg/mL (Fig. 4.3B). The concentrations of melanin were 106.6 %, 94.7 %, 76.7 %, and 53 % ($p < 0.001$) respectively. There was significant reduction in cell viability of B16 melanoma cells from 25.0µg/mL to 100µg/mL (Fig. 4.3A), thus the reduction in melanin synthesized could have been due to corresponding reduction in cell density with increasing concentration of the crude *Otholobium fruticans* extract.

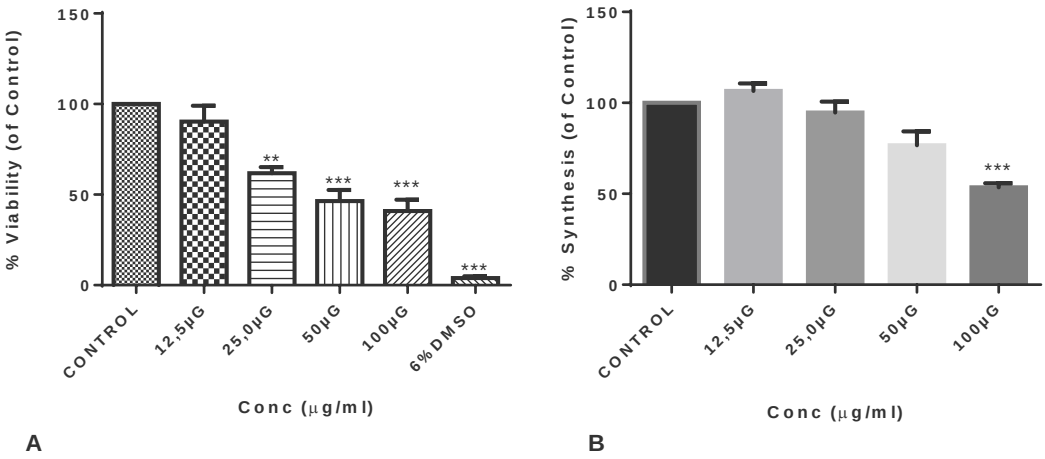


Fig 4.3: Effect of Methanolic Extract of *Otholobium fruticans* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

There was a decrease in the viability of B16 cells at sixty-hour cytotoxicity testing (Fig. 4.4A). The cells were 93.9 %, 86.9 %, 90.2 %, 76.7 %, 74.2 %, 61.3 % ($p < 0.01$), and 17.0 % ($p < 0.0001$) of the untreated control at 1.56 µg/mL, 3.13 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL of the extract concentration respectively. Five different concentrations of the extract were used in the melanin synthesis inhibition assay and were 6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL, and 100µg/mL (Fig. 4.4B). The corresponding melanin concentrations were 100.8 %, 96.6 %, 85.4 %, 58.9 % ($p < 0.05$), and 47.6 ($p < 0.01$) respectively. Due to toxicity of the extract to the cells, the significant reduction in melanin concentration could have been due to cell death and the consequent reduction in cell density that synthesized the melanin.

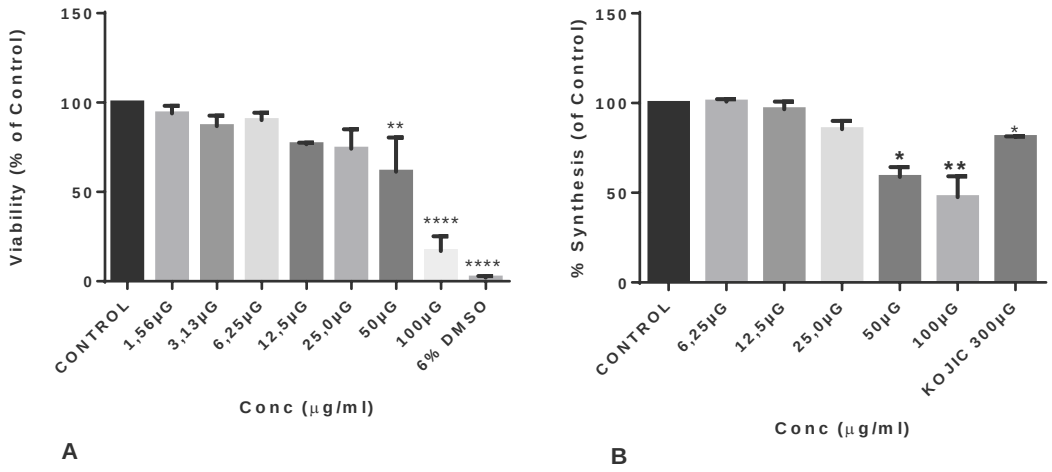


Fig 4.4: Effect of Methanolic Extract of *Otholobium fruticans* on viability (A) and intracellular melanin activity (B) at 60 hours in B16 melanoma cells.

The viability of the cells at 72 h (Fig. 4.5A) using 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL of the extracts showed 99.8 %, 91.9 %, 79.2 %, 32. 6 % ($p < 0.001$) viability compared with the untreated control, respectively. The corresponding melanin concentrations at 72 h treatment were 120.9 %, 115.6 %, 61.1 % ($p < 0.05$) and 34.0 % ($p < 0.001$) of the untreated control (Fig. 4.5B). There was dose-dependent decrease in viability as the concentration of the extract increased but was only significant at 100µg/mL. However, statistical reduction in melanin synthesis was with 50µg/ml (61.1% melanin synthesized) and 100µg/mL (34.0% melanin synthesized). There was no significant cytotoxicity to cells by 50 µg/mL concentration of the extract (79.2% viability) but the corresponding 61.1% melanin synthesized with 50 µg/mL concentration of the extract showed that 50 µg/mL significantly reduced melanin synthesis without being significantly toxic to cells. Thus, 50µg/mL of *Otholobium fruticans* is a safe concentration for melanin synthesis inhibition. Moreover, the significant reduction in melanin synthesis by 100µg/mL of the extract could have been

as a result of cell death by this concentration which led to significant reduction in cell density that synthesized melanin.

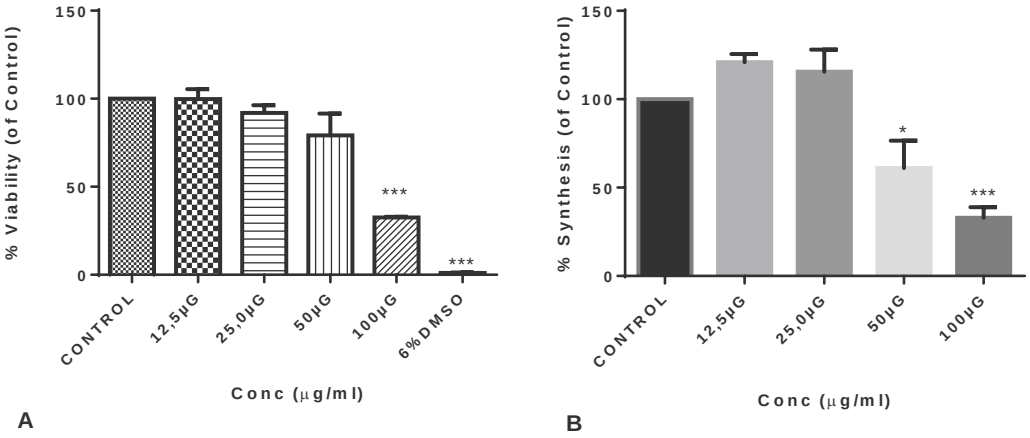


Fig 4.5: Effect of Methanolic Extract of *Otholobium fruticans* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

4.3 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Psoralea aphylla* Leaves

Cytotoxicity of methanolic extract of *Psoralea aphylla*was done on murine B16 melanoma cells. MTT was used to determine cell viability after treatment for 48 h (Fig. 4.6A) and 72 h (Fig. 4.7A). One hundred µg/mL of methanolic extract of *Psoralea aphylla*was serially diluted until 1.56µg/mL. The concentrations were 1.56µg/mL, 3.125µg/mL, 6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL, and 100µg/mL. After 48 h, the viability of the cells relative to the untreated control were 99.8%, 101.8%, 100.6%,102.7%, 86.9% ($p < 0.05$), 71.8% ($p < 0.0001$), and 41.4% ($p < 0.0001$) respectively. Melanin synthesis assay was done after 48 h treatment with serially increasing concentration of the extract (Fig. 4.6B). The concentrations used were

6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL, and 100µg/mL. The concentration of melanin synthesized reduced progressively as the concentration increased.

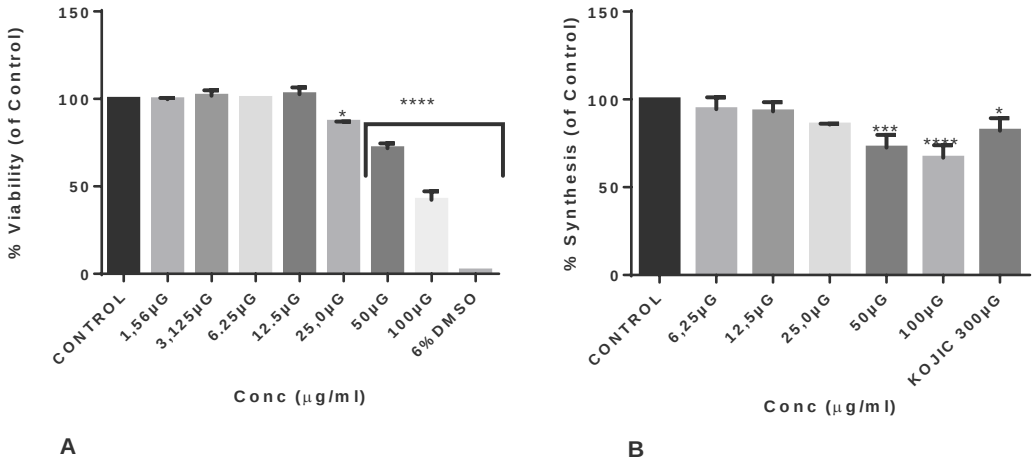


Fig. 4.6: Effect of Methanolic Extract of *Psoralea aphylla* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

Relative to the untreated control, the melanin concentrations were 94.5%, 93.1%, 85.7%, 72.6% ($p < 0.001$), and 66.8% ($p < 0.0001$). Considering the toxicity of 25µg/mL, 50µg/mL, and 100µg/mL to B16 cells causing significant reduction in cell viability, the reduction in melanin synthesized by these concentrations were consequent upon marked reduction in cell density that synthesized the melanin. After 72 h treatment and using the same concentrations (Fig. 4.7A), the viability was 85.4%, 82.5%, 80.8%, 78.6%, 76.9%, 62.9% ($p < 0.05$), and 36.9% ($p < 0.0001$). The melanin assay was done after 72 h treatment (Fig. 4.7B). The concentrations used were 6.25µg/mL, 12.5µg/mL, 25.0µg/mL, 50µg/mL, and 100µg/mL and the corresponding melanin synthesized were 97.6%, 96.8%, 98.1%, 82.3% ($p < 0.01$), 60.0% ($p < 0.0001$) respectively. The significant reduction in melanin concentration by 50.0µg/mL and 100 µg/mL were possibly due toxicity to the cells by these concentrations of the plant extract.

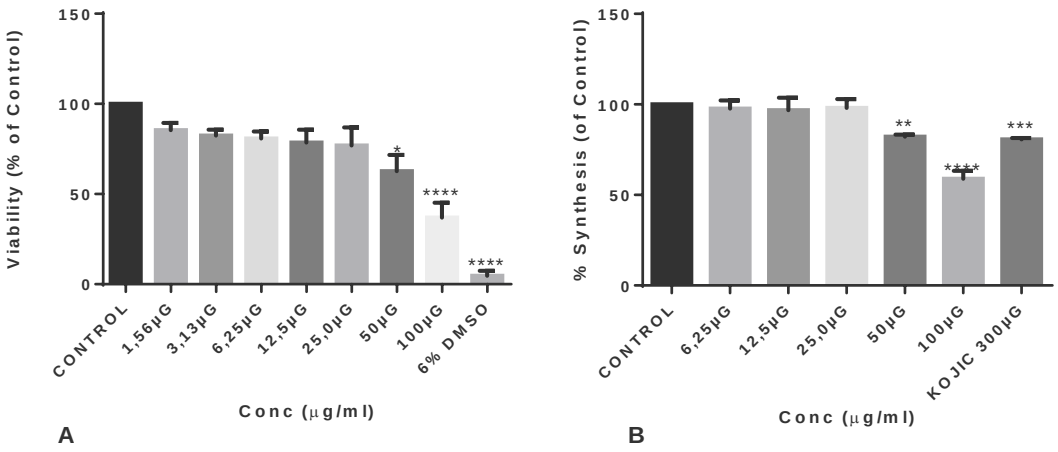


Fig. 4.7: Effect of Methanolic Extract of *Psoralea aphylla* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

4.4 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Phylica ericoides* Leaves

Cytotoxicity of methanolic extract of *Phylica ericoides* leaves was performed on murine B16 melanoma cells for 48 h (Fig. 4.8A) and 72 h (Fig.4.9A). MTT was used to determine the viability of cells. Untreated cells served as negative control and DMSO was used as positive control. Cytotoxicity was determined by using serially diluted concentrations of the extract from 100µg/mL to 12.5µg/mL. After 48 h treatment, the 50µg/mL and 100µg/mL concentrations reduced the viability of the cells significantly to corresponding viability values of 78.2% ($p < 0.05$) and 78.6% ($p < 0.05$). The melanin assay (4.8B) done after 48 h treatment showed that 100µg/ml concentration caused significant reduction in melanin synthesis (75.8% of the control, $p < 0.01$). However, the reduction in melanin synthesis was due to reduction in cell density caused by toxicity of the extract to the cells. With cytotoxicity and melanin assay performed after 72 hour treatment with the same concentrations of the extract,

the concentrations were no longer significantly toxic to the cells (Fig. 4.9A). In the same manner, there was no significant reduction in melanin concentration after 72 h of treatment (Fig. 4.9B).

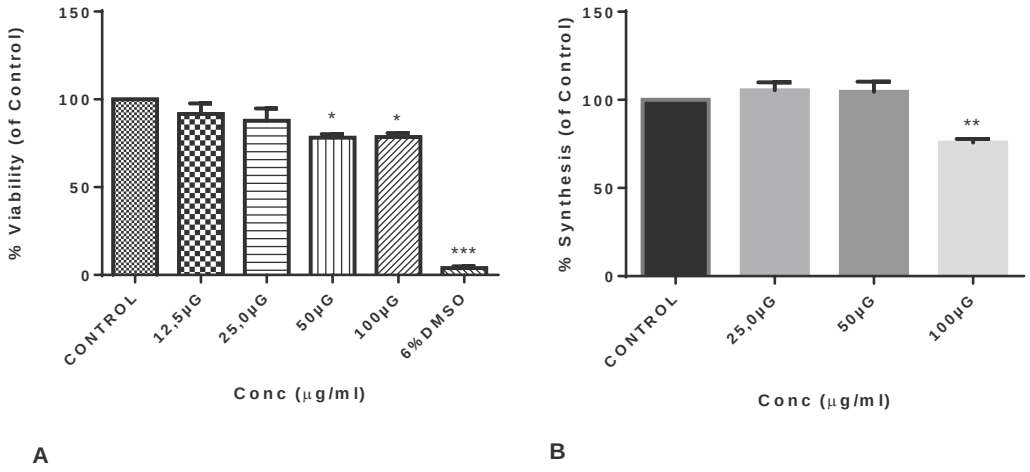


Fig. 4.8: Effect of Methanolic Extract of *Phylica ericoides* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

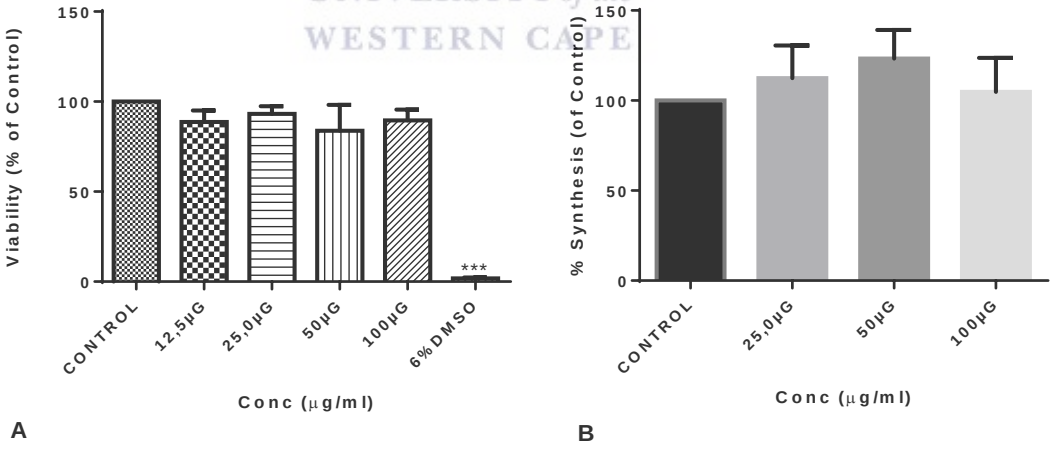


Fig. 4.9: Effect of Methanolic Extract of *Phylica ericoides* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

4.5 Cytotoxicity and Melanin Inhibition of the Ethyl actate Extract of *Rhynchosia villosa* Leaves

Cytotoxicity of ethylacetate extract of *Rynchosia villosa* leaves on murine B16 melanoma cells was determined at 48 h (4.10A) and 72 h (4.11A). Untreated cells were used as the negative control and DMSO as the positive control. Serially increasing concentrations used were 50µg/mL, 100µg/mL, 200µg/mL, and 400µg/mL. At 48 h, none of the concentrations produced any significant difference in the viability of the cells compared with the negative control. The corresponding viability values produced by the concentrations tested were 71.9%, 70.3%, 68.0%, and 69.0% (4.10A). Melanin assay was done at 48 h (4.10B) and 72 h (4.11B). The melanin concentrations produced by the respective concentrations of the extract used for the experiment at 48 h were 99.4 %, 114.0%, 93%, and 57.7% (4.10B). None of the concentrations of the extract used produced any significantly different melanin concentration levels from the untreated control.

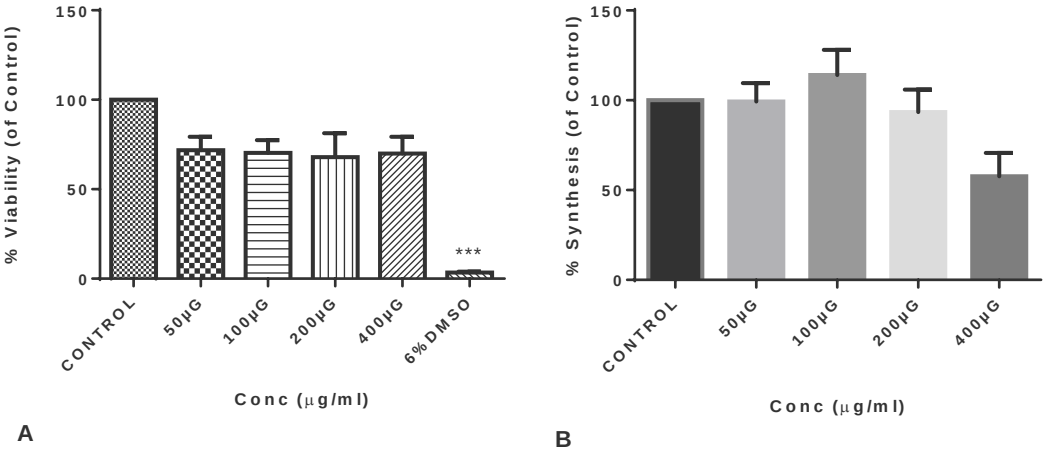


Fig. 4.10: Effect of Ethyl Acetate Extract of *Rhynchosia villosa* on (A) viability and (B) intracellular melanin activity, at 48 hours in B16 melanoma cells.

The values of the viability of B16 cells at 72 h (4.11A) were 81.4%, 98.5%, 87.9%, and 80.9%. None was significantly different from the negative control. On the other hand, the melanin concentrations at 72 h (4.11B) were 120.8%, 132.0%, 99.5% and 52.3% ($p < 0.05$). Thus, 400µg/mL reduced melanin synthesis without significant toxicity to the cells.

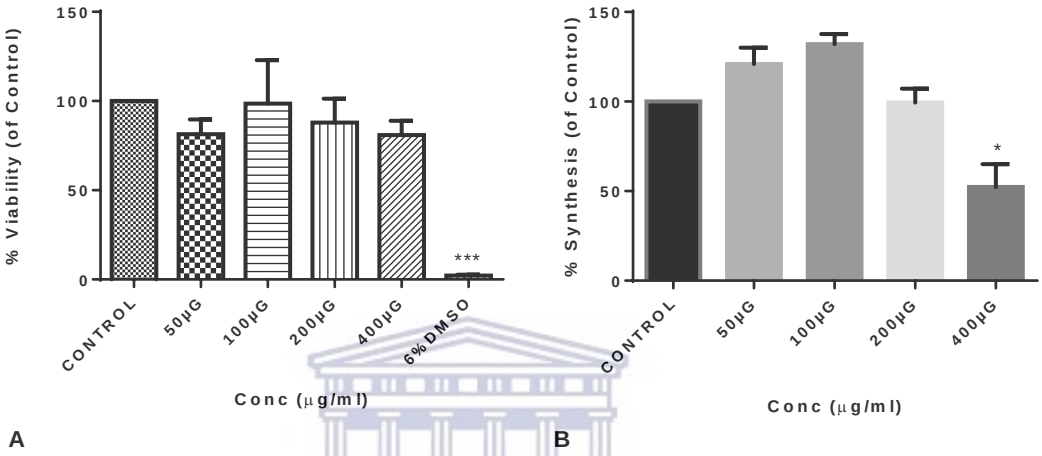


Fig. 4.11: Effect of Ethyl Acetate Extract of *Rhynchosia villosa* on (A) viability and (B) intracellular melanin activity, at 72 hours in B16 melanoma cells.

4.6 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Rhynchosia villosa* Leaves

Cytotoxicity of methanolic extract of *Rhynchosia villosa* was evaluated on murine B16 melanoma cells at 48 h (4.12A) and at 72 h (4.13A). The untreated cells served as negative control and DMSO as positive control. The tested concentrations of the extract were 50µg/mL, 100µg/mL, 200µg/mL, and 400µg/mL. At 48 h, the corresponding viability values of B16 cells were 100%, 98.9%, 94.5%, and 81.8%. None of the tested concentrations was toxic to the cells (4.12A). Melanin assay was performed at 48 h (4.12B) to evaluate the melanin synthesis inhibition of methanolic extract of

Rhynchosia villosa. The corresponding concentrations of melanin synthesized were 98.1%, 95.6%, 82.0%, and 51.6%. However, there was no statistically significant reduction in melanin concentration relative to the untreated control (Fig. 4.12B).

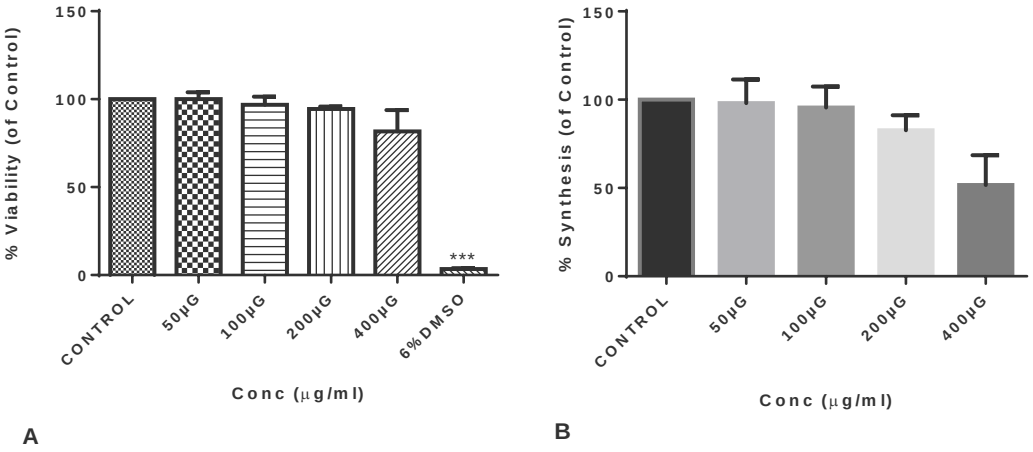


Fig 4.12: Effect of Methanolic Extract of *Rhynchosia villosa* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

The values of the viability at 72 h (4.13A) were 80.2%, 92.8%, 87.3%, and 75.2%. The concentrations tested were not toxic to B16 cells. However, there was progressive reduction in melanin concentration at 72 h (4.13B). Compared with the untreated control, the melanin concentration values were 122.4%, 107.4%, 82.9%, and 47.1% ($p < 0.01$). Thus, 400µg/ml produced significant reduction in melanin concentration at 72 h without significant toxicity to B16 cells.

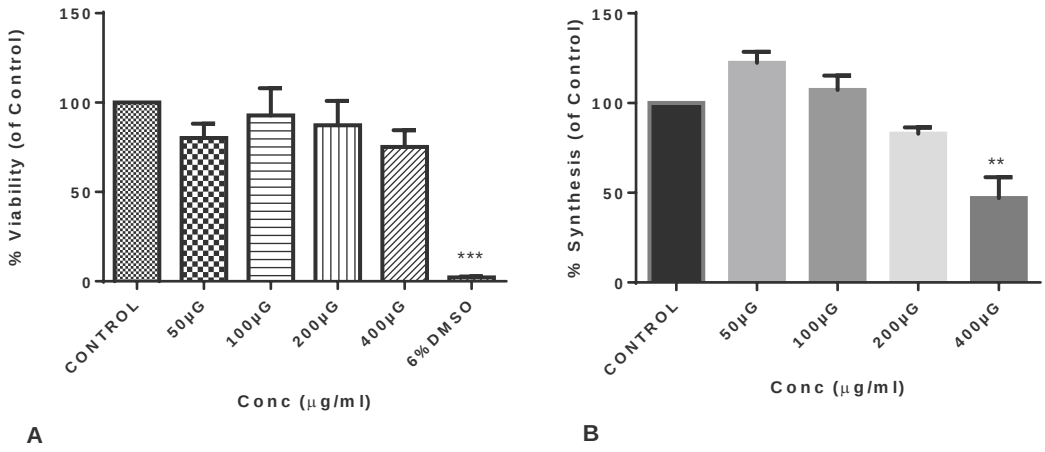


Fig 4.13: Effect of Methanolic Extract of *Rhynchosia villosa* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

4.7 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Serruria furcellata* Leaves

Cytotoxicity of methanolic extract of *Serruria furcellata* leaves was performed on murine B16 melanoma cells using MTT to assess the viability of the cells after exposure for 48 h (Fig 4.14A), 60 h (Fig 4.15A) and 72 h (Fig. 4.16A). Also, melanin inhibition assay with the methanolic extract was done on murine B16 melanoma cells at 48 h (Fig 4.14B), 60 h (Fig 4.15B) and 72 h (Fig 4.16B). Cytotoxicity was done with serial dilution of methanolic extract. The concentrations used were 12.5 µg/mL, 25.0 µg/mL, 50 µg/mL and 100 µg/mL and 6% DMSO was used as the positive control. At 48 h treatment the viability values of the cells were 88.9 %, 93.8 %, 91.4 %, and 89.8 % respectively (Fig. 4.14A). There was no significant reduction in cell viability of B16 melanoma cells from 25.0µg/mL to 100µg/mL. At the respective extract concentration, the melanin concentrations were 101.6 %, 106.1 %, 98.8 %, and 57 % ($p < 0.001$) (Fig. 4.14B) respectively. Thus, 100µg/mL of the extract reduced melanin concentration by 43%.

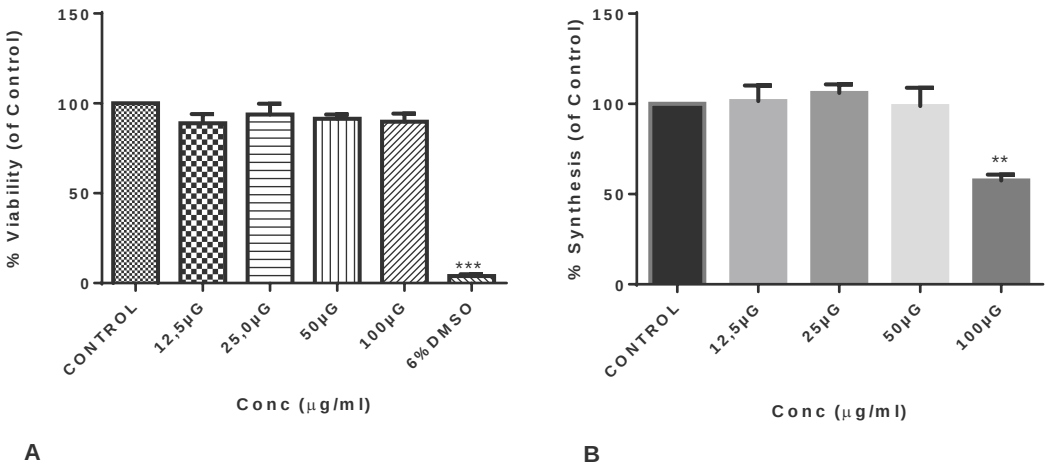


Fig. 4.14: Effect of Methanolic Extract of *Serruria furcellata* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

Cytotoxicity at 60 h was done with 1.56µg/mL, 3.13µg/mL, 6.25µg/mL, 12.5µg/mL, 25.0µg/mL, 50µg/mL and 100µg/mL concentrations, and 6% DMSO was used as the positive control. There was decrease in viability of B16 cells for all the concentrations tested. The cells were 91.3%, 86.2 %, 78.5 %, 76.8 %, 73.9 %, 75.2 %, 70.3% ($p < 0.05$), and 65.4 % ($p < 0.05$) of the untreated control respectively. Five different concentrations of the extract were used in the melanin synthesis inhibition assay and were 12.5µg/mL, 25µg/mL, 50µg/mL, 100µg/mL, and 200µg/mL, (Fig. 4.15A). The corresponding melanin concentrations were 97.5 %, 88.3 %, 88.2 %, 88.7 % ($p < 0.05$), and 79.7 ($p < 0.05$) respectively. Due to toxicity of the extract to the cells, the significant reduction in melanin concentration could have been due to cell death and the consequent reduction in cell density that synthesized the melanin.

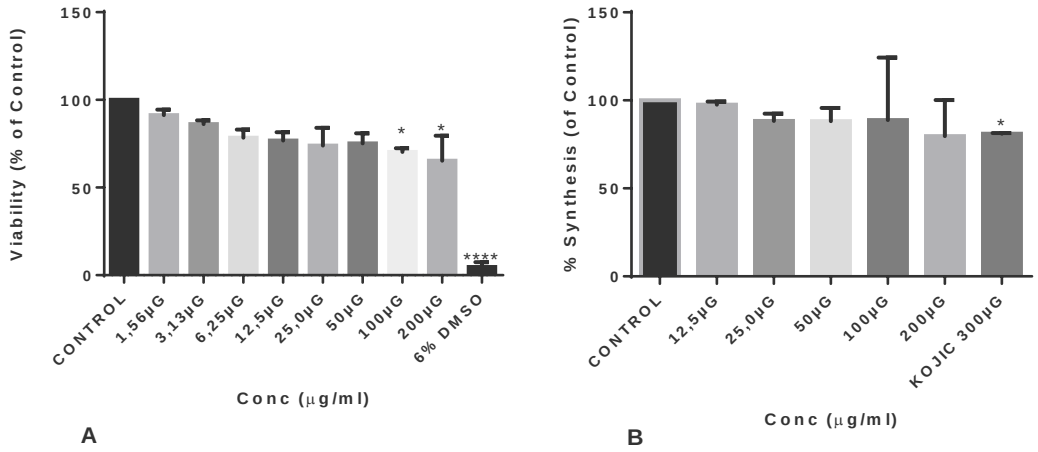


Fig. 4.15: Effect of Methanolic Extract of *Serruria furcellata* on (A) viability and (B) intracellular melanin activity at 60 hours in B16 melanoma cells.

The viability of the cells at 72 h (Fig. 4.16A) using 12.5 µg/mL, 25 µg/mL,50 µg/mL, and 100 µg/ml of the extracts showed 89.1 %, 86.1 %, 83.2 %, 90.7 % viability compared with the untreated control, respectively. The corresponding melanin concentrations at 72 h treatment were 110.6 %, 115.0 %, 127.0 % ($p < 0.05$) and 90.2 % ($p < 0.001$) of the untreated control (Fig. 4.16B). There was no significant reduction in viability with increasing concentration of the extract. Also, there was no reduction in melanin concentration compared with the untreated control (Fig. 4.16B).

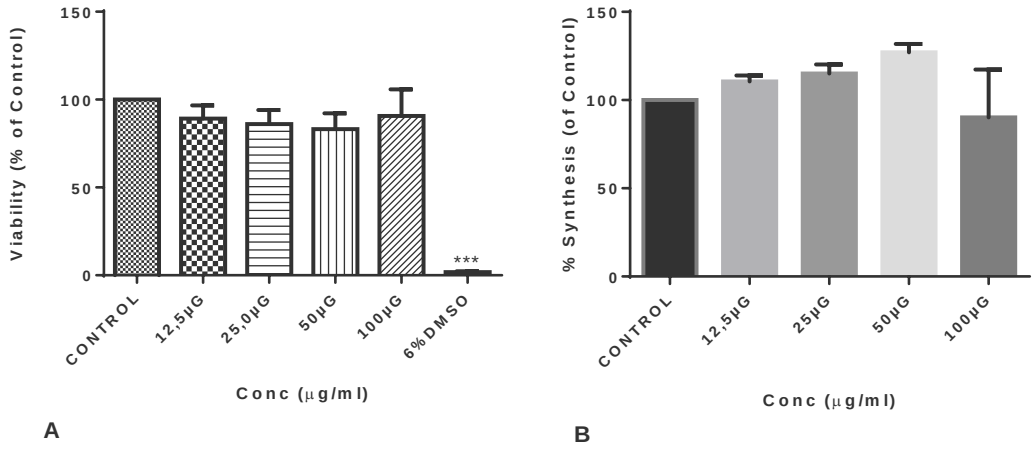


Fig. 4.16: Effect of Methanolic Extract of *Serruria furcellata* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

4.8 Cytotoxicity and Melanin Inhibition of Kojic Acid

Kojic acid is a known depigmentation agent used as positive control in this study (Pintus et al., 2015). To determine nontoxic concentration to be used in this study, cytotoxicity was done with serially diluted concentration from 200 µg/mL to 12.5 µg/mL and 6%DMSO served as the positive control. The treatment times were 48 h (Fig. 4.17A and Fig. 4.18A), 60 h (Fig. 4.19A), and 72 h (Fig. 4.20). Cytotoxicity was evaluated on murine B16 melanoma cells and the concentrations used were 12.5µg/mL, 25.0 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL (Fig. 4.17A). The concentrations tested were not toxic to the cells. Compared with the untreated control, the values were 100.3%, 96.5%, 97.8%, 98.6%, and 104.8%. Four different concentrations of Kojic acid used for melanin synthesis inhibition assay were 25.0µg/mL, 50 µg/mL, 100µg/mL, and 200 µg/mL (Fig. 4.17B). The corresponding melanin concentrations were 101.1%, 104.4%, 98.5%, and 91.5% ($p < 0.05$). Significant reduction in melanin synthesis (8.5%) was possibly produced by 200µg/mL concentration of Kojic acid.

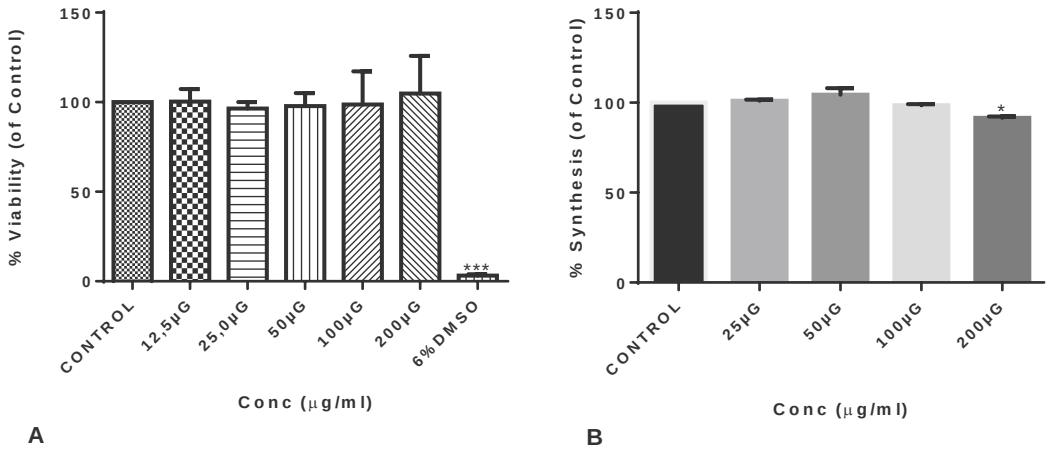


Fig. 4.17: Effect of Kojic acid (200 µg/mL) on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

To determine whether there would be a higher percentage of a reduction in melanin synthesis, 300 µg/mL of Kojic acid was tested over 48 h and the viability was 87.7% of the untreated control (Fig 4.18A). Melanin synthesis was not significantly different from the control, hence, not toxic to the cells. Melanin inhibition assay showed 17.6% reduction in melanin concentration (82.4% of the control) (Fig. 4.18B).

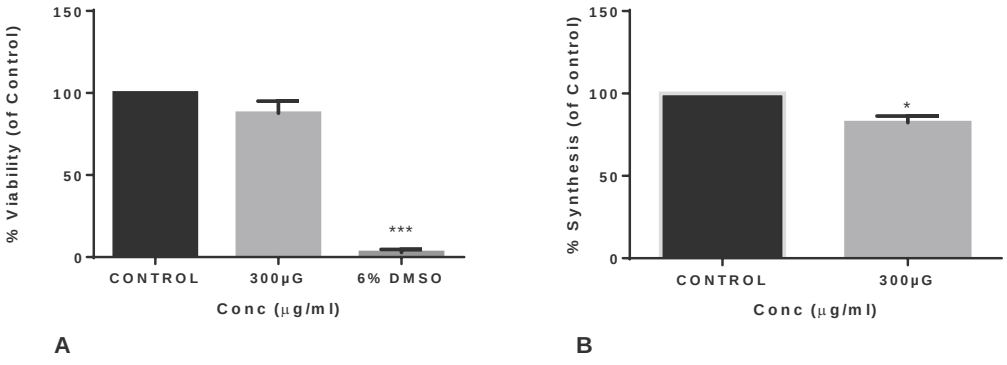


Fig. 4.18: Effect of Kojic acid (300 µg/mL) on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

The cytotoxicity and melanin inhibition potential of 200 µg/mL and 300µg/mL were determined at 60 h exposure time (Fig. 4.19) and the viability values of cells was 104% and 98% respectively (Fig. 4.19A). The corresponding concentrations of the synthesized melanin were 86.5% and 68.7% ($p < 0.01$) respectively (4.19B). Thus, there was 31.3% reduction in the concentration of melanin by 300µg/mL of Kojic acid without toxicity to murine B16 melanoma cells.

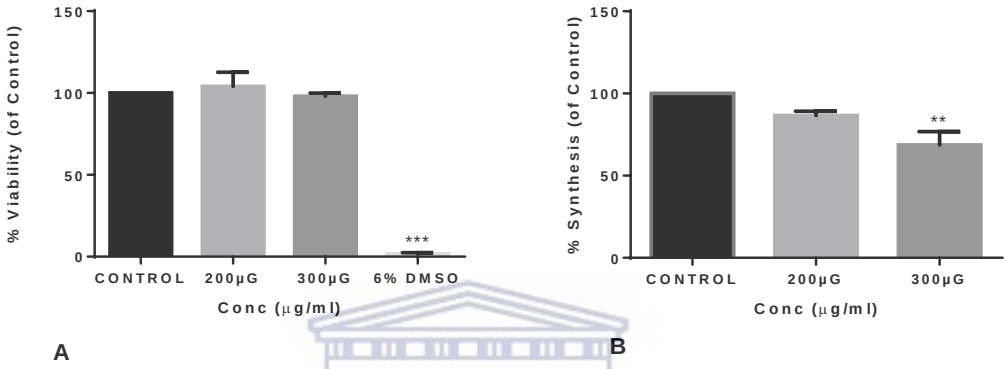


Fig. 4.19: Effect of Kojic acid (200 and 300 µg/mL) on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

The viability of the cells at 72 h (Fig. 4.20A) using 12.5 µg/mL, 25 µg/mL,50 µg/mL, 100 µg/mL, and 200 µg/mL of Kojic acid showed 82.8 % ($p < 0.05$), 86.3 %, 78.2 % ($p < 0.01$), 67.3 % ($p < 0.001$), and 69.5% ($p < 0.001$) viability compared with the untreated control, respectively. All the concentrations of Kojic acid tested over the 72-hour treatment duration were toxic to B16 cells except 25 µg/mL.

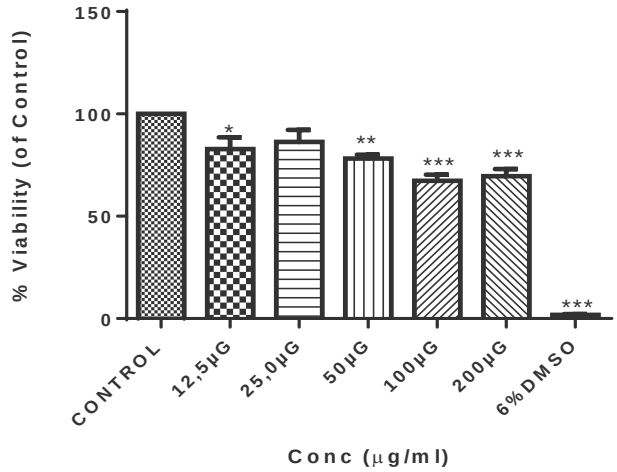


Fig. 4.20: Viability of Kojic acid at 72 hours

Chapter Conclusion

The results of viability and melanin inhibition potentials of the methanolic and/or ethylacetate extracts of six plants belonging to different genera were provided in this chapter. All the concentrations tested in a few plants did not reduce melanin synthesis without significant toxicity to cells. However, *S. furcellata* and *O. fruticans* inhibited melanin synthesis significantly at 100µg and 50µg respectively. Thus, because of their comparatively low effective concentrations, the methanolic extracts of *S. furcellata* and *O. fruticans* were investigated further in this study.

The next chapter provides results of further studies on *S. furcellata*. It contains the results of cell viability and melanin inhibitionstudies on this plant and describes the effects of the extract on the cell morphology, noncancerous cell line, tyrosinase activity, DOPA staining, intracellular reactive oxygen species and relative melanogenesis gene expression, and ends with discussion of the results and chapter conclusion.

CHAPTER FIVE

EFFECT OF METHANOLIC EXTRACT OF *SERRURIA FURECELLATA* ON MELANIN SYNTHESIS IN B16 MELANOMA CELLS

5.1 Introduction

Melanogenesis is a physiological process that leads to the synthesis of melanin pigments, which are responsible for skin pigmentation and provide a beneficial effect in preventing skin damage under normal condition. Therefore, skin pigmentation is an important variable phenotype in humans, and makes cutaneous pigmentation disorders frequent symptoms presenting in different forms. Dyspigmentation, particularly hyperpigmentation is one of the most common dermatological presenting complaints from patients with darker skin colour, particularly hyperpigmentation (Vashi et al., 2017). The prevalence of skin pigmentation disorders varies with regions of the world (Davis and Callender, 2010). However, of all the diseases encountered globally, epidemiological data has shown that skin diseases account for almost 34% of all diseases and the prevalence of the dermatological disorders have gotten worse over time (Chahal et al., 2013, Leo and Sivamani, 2014).

Various modalities have been adopted in the management of hyperpigmentation including topical and oral medications, as well as non-invasive procedures. The gold standard for the topical management of this condition is the hydroxyphenolic compound known as hydroquinone (Woolery-Lloyd and Kammer, 2011). Despite its

efficacy, hydroquinone is associated with some side effects including exogenous ochronosis, skin irritation, and contact dermatitis in people of darker skin complexion (Zhu and Gao, 2008). Because of crucial function of tyrosinase in melanin pathway, its inhibitors have become increasingly important for medicinal and cosmetic products that may be used as powerful skin-whitening agents for treating skin disorders (Kim and Uyama, 2005).

Many plants of different genera belonging to the Proteaceae family have been used in folk medicine for treating different ailments in human body. Tyrosinase inhibitors from natural sources are present in several chemical classes like flavonols, phenolics, flavanols, isoflavanoids, flavonones, terpenes, steroids, alkaloids, chalcones, flavonoids, coumarins, stilbenes, long chain fatty acids, biscoumarins, bipiperidines, oxadiazole, tetraketones, etc (Loizzo et al., 2012). Masevhe *et al.* reported that *Faurea Saligna* Harv (Proteaceae) is used traditionally for treating epilepsy in Venda area of South Africa (Masevhe et al., 2015). *S. furcellata* is a member of Proteaceae family but has not been investigated before. Hence, the aim of the present study was to evaluate the melanin inhibition potential and mechanisms of action of *S. furcellata* in B16 melanoma cells.

5.2 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Serruria furcellata* Leaves

The cytotoxicity evaluation of the methanolic extract of *S. furcellata* leaves was performed on murine B16 melanoma cells using MTT to assess the viability of the cells after 48 h exposure (Fig. 5.1A). In addition, the melanin inhibition assay with the methanolic extract was done at 48 h (Fig 5.1B) while cytotoxicity was done with serial dilutions of the methanolic extract. The test concentrations used were 12.5, 25.0, 50

and 100 µg/mL and 6% DMSO was used as the positive control. At 48 h treatment the corresponding cell viability values were 88.9 %, 93.8 %, 91.4 %, and 89.8 % respectively (Fig. 5.1A). There was no significant reduction in cell viability of B16 melanoma cells from the concentration 25.0 to 100µg/mL. At the respective extract concentration, the melanin concentrations were 101.6 %, 106.1 %, 98.8 % and 57 % respectively ($p < 0.001$) (Fig. 5.1B). Thus, 100µg/mL of the extract reduced melanin concentration by 43%.

Furthermore, the cytotoxicity of 100µg of this extract was evaluated with non-cancerous cell line, HaCaT, which is a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin (Fig 5.2). There was no significant difference between the untreated control and extract-treated cells while treatment with the positive control, 6% DMSO, resulted in cell death. In the skin, one melanocyte interacts with 30-40 keratinocytes making epidermal melanin units at the basal layer of the skin (Haass and Herlyn, 2005). Hence, 100 µg/mL of the methanolic leaf extract of of *S. furcellata* is safe for both the melanocytes and the surrounding keratinocytes.

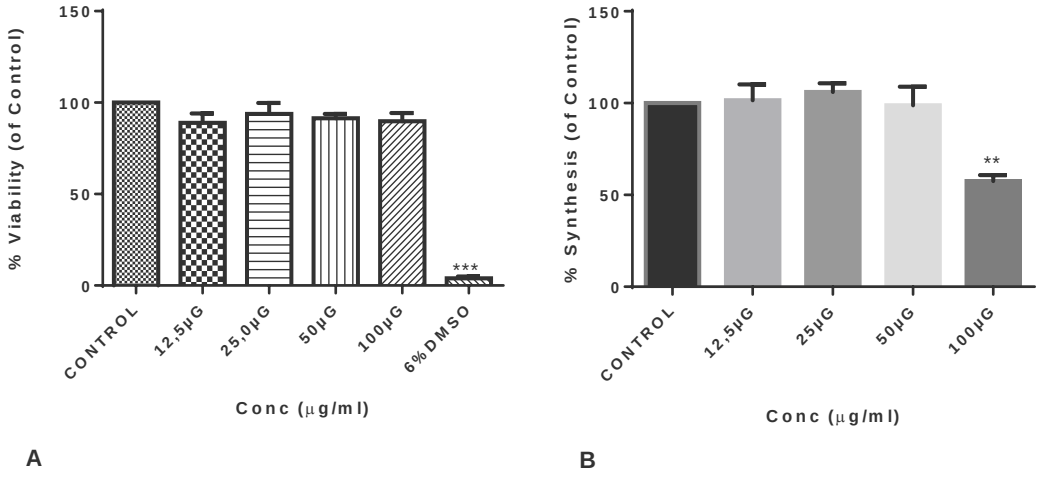


Fig. 5.1: Effect of Methanolic Extract of *S. furcellata* on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

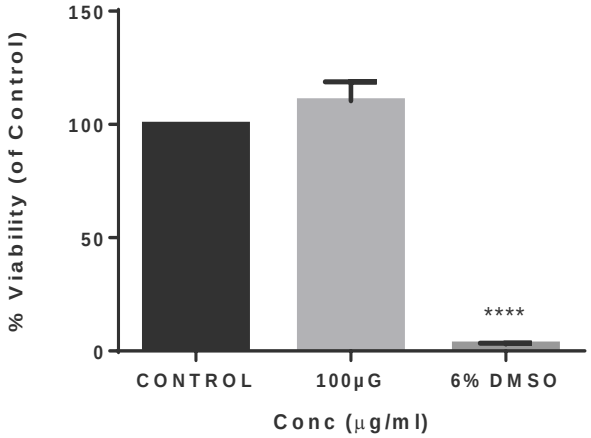


Fig. 5.2: Effect of 100 µg/mL of *S. furcellata* methanolic extract on HaCaT cell line

5.3 Cytotoxicity and Melanin Inhibition of Kojic Acid

Kojic acid is often used as positive control in melanin inhibition experiments due to its potent inhibition of melanin synthesis (Wang et al., 2018). Thus, Kojic acid was used in this study as positive control while cell viability and melanin inhibition potentials were tested over 48 h using 300 µg/mL Kojic acid concentration. Theresults obtained showed that although cell viability was 87.7% of the untreated control (Fig. 5.3A), it was not significantly different from the control, hence, Kojic acid could be said to be non-toxic to the cells. The melanin inhibition assay on the other hand showed 17.6% reduction in melanin concentration (82.4% of the control) (Fig. 5.3B).

The 43% reduction in melanin concentration by 100 µg/mL concentration of *S. furcellata* extract (Fig 5.1B) compared to the 17.6% reduction by the 300 µg/mL concentration of Kojic acid (Fig. 5.3B) tends to suggest that *S. furcellata* reduced melanin synthesis better than Kojic acid.

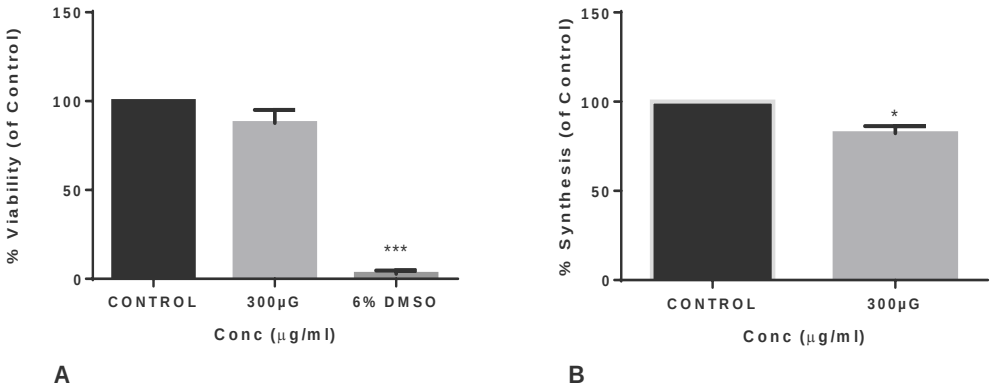


Fig. 5.3: Effect of Kojic acid (300 µg/mL) on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

5.4 Effect of Methanolic Extract of *S. furcellata* on the Morphology of Murine B16 Melanoma Cells

Murine B16 melanoma cells were treated with the 100 µg/mL concntration of *S. furcellata* extract for over 48 h and 6% DMSO was used as a positive control. As shown below (Fig. 5.4), there was no apparent difference in the morphology of cells treated with *S. furcellata* (Fig. 5.4B) and those of the untreated control (Fig. 5.4A). Thus, the 100 µg/mL concentration of *S. furcellata* extract did not appear to affect the morphology of the cells. Cells treated with 6% DMSO (Fig. 5.4C) appeared completely different from those of the control; theyappeared rounded or ovoid shaped, and were shrunk without the dendrites that are typical of melanocytes. Hence, treatment with 6% DMSO appeared to affect the morphology of the cells.

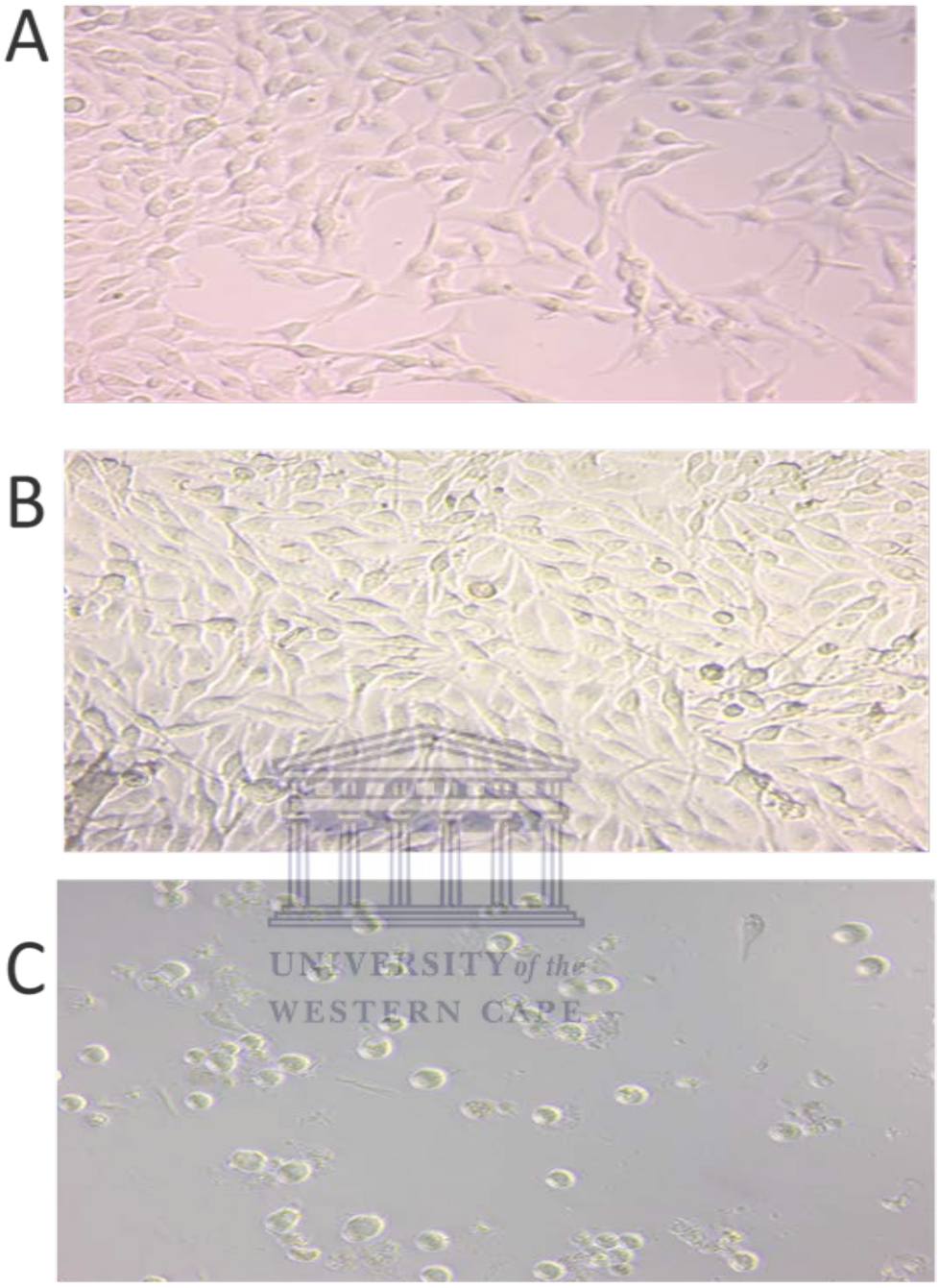


Fig. 5.4: Photomicrographs of B16 Melanoma Cells showing effect of *S. furcellata* extract treatment for 48 h. (Mag. x 200). A: Untreated Control B: 100 µg/mL *S. furcellata* C: 6% Dimethylsulphoxide.

5.5 Effect of *S. furcellata* on Tyrosinase Enzyme Activity

The intracellular tyrosinase enzyme activity of B16 melanoma cells was determined by measuring the rate of L-DOPA oxidation by tyrosinase in B16 cell lysates. The Km and V_{max} values were obtained from the graph plotted with GaphPad Prism 6 version. Kojic acid was used as positive control, while PBS + L-DOPA and PBS only were run together with treatment samples to serve as controls to monitor the kinetics of the enzyme-substrate reactions. An active tyrosinase enzyme oxidized L-DOPA to pink-coloured dopachrome and the absorbance was monitored every min for 60 min. The tyrosinase enzyme activity of the control lysate was 1440 ± 70 µmol/min (Table 5.1) and is represented by the blue hyperbolic plot in Fig. 5.5. The enzyme activity of lysates from B16 cells treated with 100 µg/mL of *S. furcellata* for 48 h was 2267 ± 23 µmol/min and is represented by red hyperbolic Michaelis-Menten plot (Fig. 5.5).

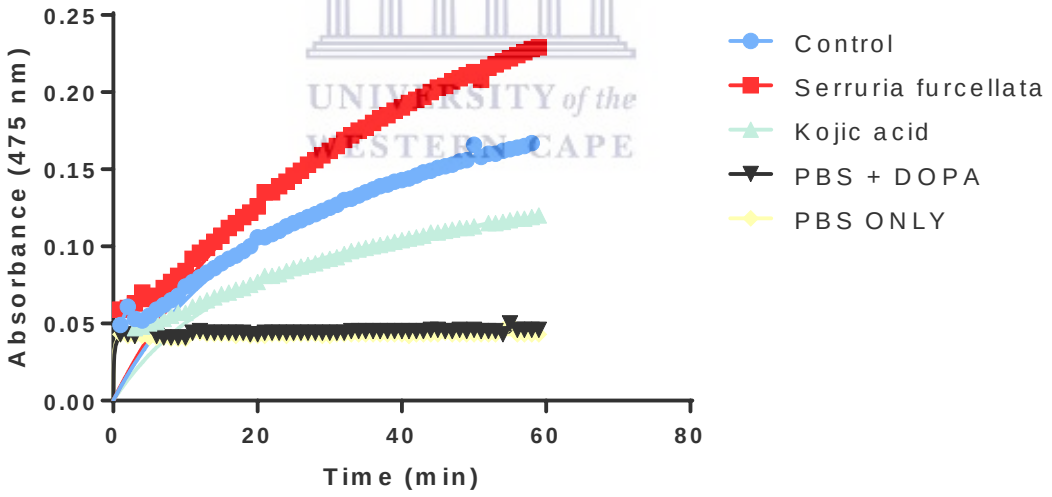


Fig 5.5: Michaelis-Menten plot for inhibition of *S. furcellata* extract on B16 melanoma intracellular tyrosinase activity using L-DOPA as substrate

Table: 5.1: Inhibition of Tyrosinase activity by *S. furcellata* extract

INHIBITOR	Conc	Km(mM)	Vmax(µmol/min)	Slope K(Km/Vmax)	Enzyme Activity
Control	0µg	5.71 ± 0.30	0.2144 ± 0	26.65 ± 1.34	1440 ± 70
Serruria furcellata	100µg	13.6 ± 0.14	0.3242 ± 0	41.6 ± 0.85	2267 ± 23
Kojic acid	300µg	2.55 ± 0.07	0.1416 ± 0	18.02 ± 0.49	974 ± 26

When compared with the untreated control, it could be concluded that the more evident tyrosinase enzyme activity observed in the lysate of cells treated with *S. furcellata* was stimulated by the treatment ($p < 0.001$). The enzyme activity of lysate of B16 cells treated with Kojic acid was 994 ± 26 µmol/min ($p < 0.01$) and is represented by a green hyperbolic Michaelis-Menten plot (Fig. 5.5). Because there was no tyrosinase enzyme in PBS+DOPA and PBS ONLY groups, pink colour was not formed at the end of the 60 min reaction time (see the black and brown horizontal Michaelis-Menten plots in Fig. 5.5). The tyrosinase activity of cells treated with *S. furcellata* extract was higher than the untreated control and Kojic acid indicating that the 100 µg/mL concentration of *S. furcellata* did not inhibitbut rather stimulated intracellular tyrosinase activity in B16 melanoma cells. However, Kojic acid inhibited the enzyme when compared with the untreated control (Fig.5.5 and 5.6; Table: 5.1).

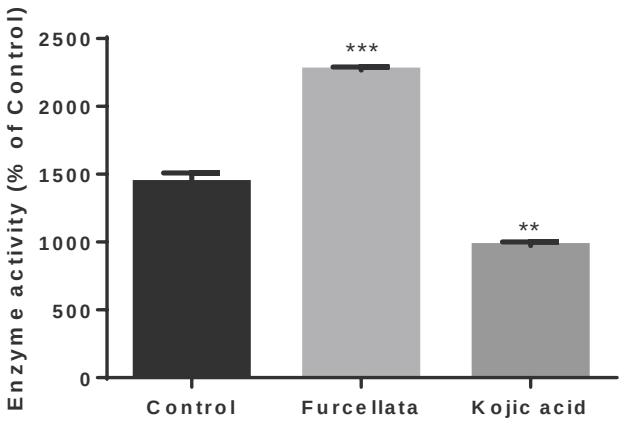


Fig.5.6: Effect of *S. furcellata* extract on inhibition of B16 melanoma intracellular tyrosinase activity using L-DOPA as substrate.

5.6 Dihydroxyphenylalanine (DOPA) Staining

The effect of methanolic extract of *S. furcellata* on intracellular tyrosinase activity was also confirmed by tyrosinase zymography. B16 melanoma cells were treated with 100 µg of methanolic extract of *S. furcellata* for 48 h and 300 µg/mL of Kojic acid, positive control, for 48 h. Tyrosinase activity in the untreated control was low compared with *S. furcellata* (Fig. 5.7A and B). The tyrosinase activity of Kojic acid was the least. Results were in agreement with the data of intracellular tyrosinase inhibition, confirming the stimulatory effect of the extract on tyrosinase activity.

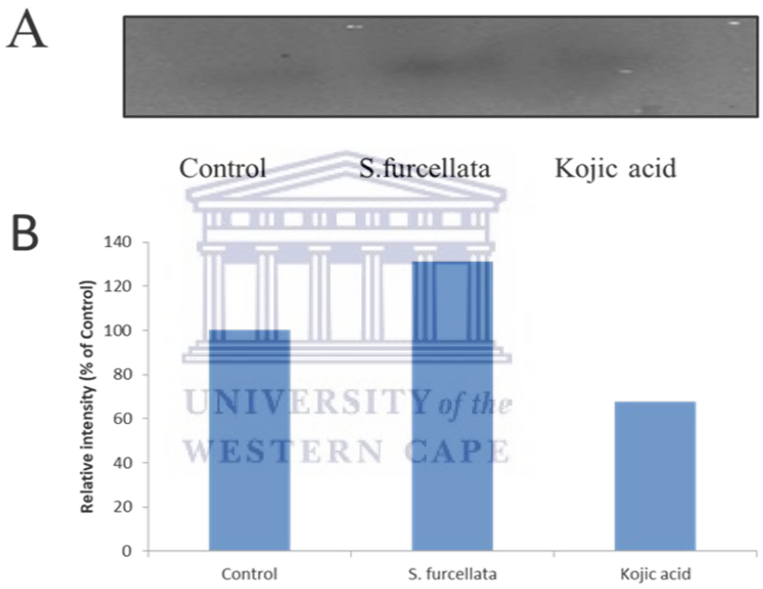


Fig. 5.7: Effect of methanolic extract of *S. furcellata* on B16 melanoma cells by L-DOPA staining. Tyrosinase activity was estimated by zymography (A) and the relative band intensity was determined with Image J software (B)

5.7 Effect of Methanolic Extract of *S. furcellata*on on Interacellular ROS

The effect of the treatment of with *S. furcellata* extract on intracellular ROSin B16 melanoma cells is shown in Fig. 5.8. ROS was evaluated using the cell permeant dye chloromethyl-2', 7'-dichlorofluorescindiacetate (CM-H_2DCFDA). The histogram depicted black represents the unstained control while the red histogram represents stained control. There was no shift of histogram to the right in the extract-treated cells (green), implying there was no increase in intracellular ROS. Also in the Kojic acid-treated cells, there was no shift in the histogram (green),which indicates that Kojic acid did not cause an increase in the generation of intracellular ROS. These results tend to suggest that the inhibitory effect of *S. furcellata* on melanin synthesis was not mediated by increased generation of reactive oxygen species.

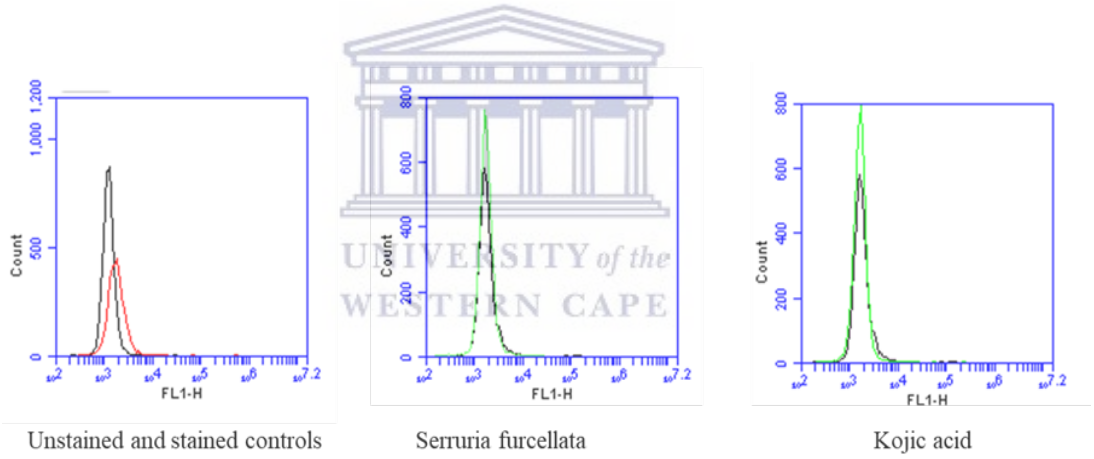


Fig. 5.8: Representative histograms of cells stained with CM-H2DCFDA dye and evaluated by flow cytometry.

5.8 Effect of Methanolic Extract of *S. furcellata*on on Relative Expression of Melanogenesis Genes

To determine the mechanism by which *S. furcellata* inhibited melanin synthesis, expression of six different genes involved in melanogenesis pathways was evaluated using real-time quantitative PCR. The PCR sample of each gene was done in triplicate. Relative expression of each gene was normalized to glyceraldehyde-3- phosphate dehydrogenase (GAPDH). Compared with the untreated control TYR (1.077 ± 0.047), TRP-1 (2.933 ± 1.995), TRP-2 (1.103 ± 1.192), and MC1R (1.703 ± 0.679) were up-regulated, while MITF (-6.850 ± 7.478) and β-CATENIN (-10.787 ± 806) were supressed. Thus, TYR, TRP-1, TRP-2 and MITF were up-regulated.

TYR is an important enzyme in the melanin synthesis pathway as it catalyzes the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA), which is promptly oxidized to DOPAquinone (DQ) which is immediately converted into the intermediate DOPAchrome (Hearing, 2011). TYR, TRP-1 and TRP-2 are downstream in the melanogenesis pathways; MITF is a major transcription factor which regulates TYR, TRP-1, and TRP-2 expression (Baek and Lee, 2015). Thus the binding of α-MSH to MC1R transduces a signal to increase the expression of microphthalmia-associated transcription factor (MITF) by activating cAMP (Bertolotto et al., 1998). MC1R is up-regulated but MITF was down-regulated. The β-Catenin gene was down-regulated by treatment with *S. furcellata* (Fig. 5.9). Wnt/β-catenin pathway is one of the regulatory pathways for melanin synthesis. In this pathway, MITF is targeted through activation of frizzled receptor, inhibition of glycogen synthase kinase 3β (GSK3β), and stimulation of β-catenin accumulation (Shin et al., 2015). Thus, *S. furcellata* reduced melanin synthesis in murine B16 melanoma cells through neither the cAMP-mediated

signal pathway nor the Wnt/β-catenin pathway but possibly through the degradation of the melanin pigment.

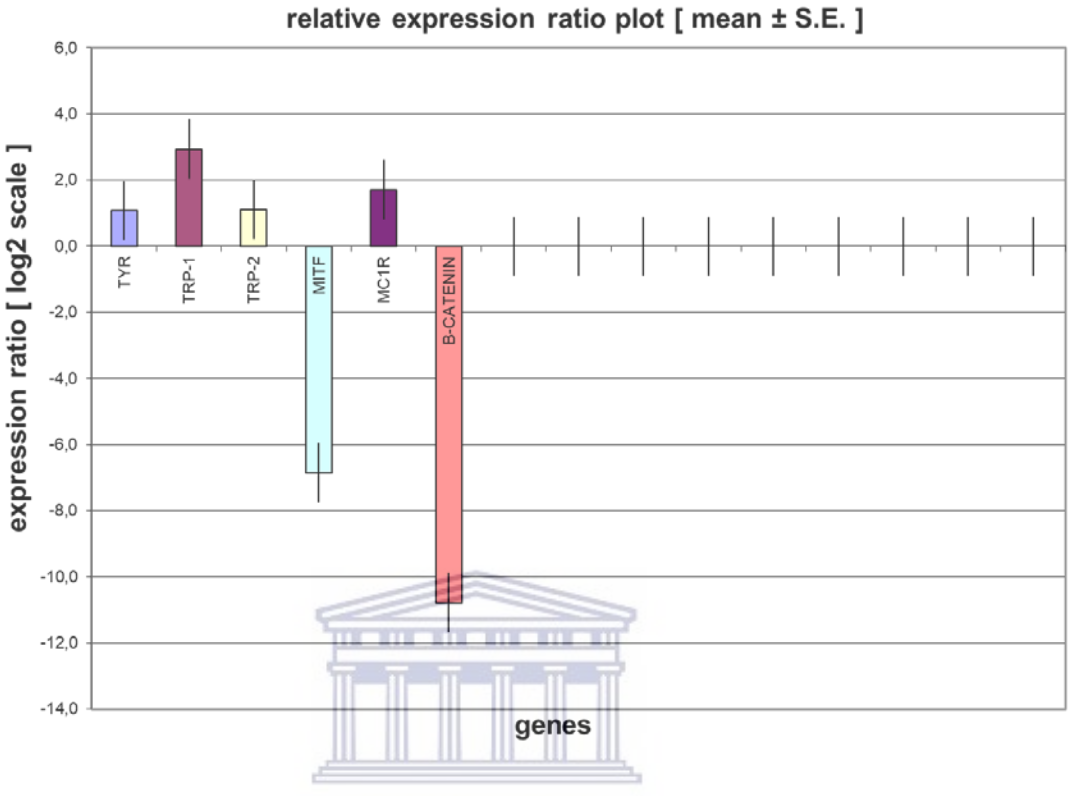


Fig. 5.9: Quantitative Real-time PCR relative expression ratio plot of genes for *S. furcellata* compared with untreated control

Kojic acid is used as positive control in melanin inhibition experiments due to its inhibitory effects on melanin synthesis (Wang et al., 2018). Unlike in cells treated with *S. furcellata*, treatment with Kojic acid resulted in up-regulation of TYR (3.050 ± 2.225), TRP-1 (4.037 ± 3.278), MC1R (2.057 ± 1.176), and MITF (1.183 ± 0.755) and the down-regulation of TRP-2 (-1.400 ± 1.546) and β-Catenin (-3.617 ± 3.15) (Fig. 5.10).

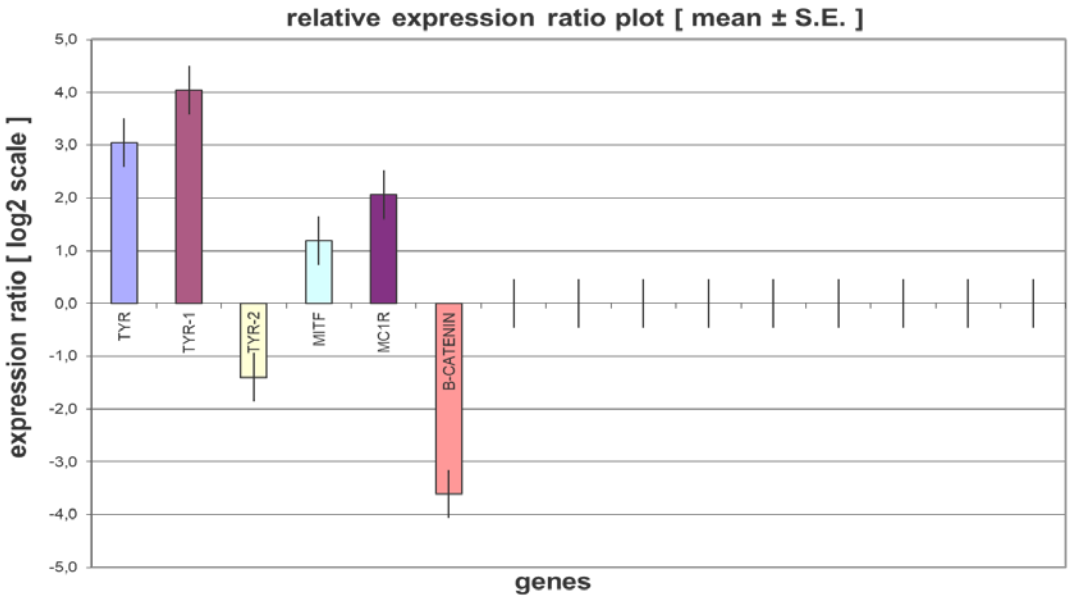


Fig. 5.10: Quantitative Real-time PCR relative expression ratio plot of genes for Kojic acid compared with untreated control

5.9 Discussion

5.9.1 Introduction

Wild plants have always been an important source of primary medicare and other necessities of daily life for tribal communities all over the world and in most rural areas of developing countries, plants remain a primary source of medicine (Chitme et al., 2004). Traditional medicinal plants are used for the management of diseases like essential hypertension and type-2 diabetes mellitus in many parts of the world including Africa(Davids et al., 2016).

Cosmeceuticals are increasingly popular alternatives to standard depigmenting agents (Levin and Momin, 2010) and cosmeceuticals made from plant-derived phytochemicals have been documented to exhibit various degrees of cellular actions for various skin pigmentation-related diseases (Fisk et al., 2014, Leo and Sivamani,

2014). There are currently no known published studies on the biological effects of *S. furcellata* on skin pigmentation,thus, this study was done to evaluate the melanin synthesis inhibition potential as well as the mechanism(s) of action of the methanolic leaf extract of this plant.

5.9.2 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *S.furcellata* Leaves

To determine the antimelanogenic effects of the *S. furcellata* extract, murine B16 melanoma cells were treated with different concentrations of the extracts of this plant while Kojic acid was used as a positive control that inhibit melanin synthesis as previously reported (Wang et al., 2018). The result obtained showed that *S. furcellata* inhibited melanin synthesis better than Kojic acid (Fig. 5.1).For safe depigmentation, the therapeutic agent should not be toxic to both the target cells and the surrounding tissues. There is interaction between one melanocyte and 30-40 keratinocytes making epidermal melanin units at the basal layer of the skin (Haass and Herlyn, 2005). The effective concentration of the *S. furcellata* that reduced melanin synthesis was not toxic to noncancerous cell line, HaCaT, which was the normal keratinocytes used in this study. This was also confirmed with the effect of the extract on the cell morphology (Fig. 5.4). There was no apparent changes on extract-treated cells compared with the control.

S. furcellata belongs to Protaceae family. The genera of Proteaceae are very diversified, however, *Banksia* sp. provides a striking example of adaptive radiation in plants (Mast and Givnish, 2002). Plants in this family have been reported to inhibit melanin synthesis. Polar extracts of root of *Protea madiensis* Oliv. inhibited

melanogenesis when used traditionally as herbal medicine for treatment of skin diseases in Rwanda (Kamagaju et al., 2013). *Protea neriifolia* (Proteaceae) contains a bioactive compound 2,4-dihydroxyphenyl-(6′-O-benzoyl)-O-β-D-glucoside with structural similarity to arbutin which has been used as a whitening agent in cosmetics (Sarkar et al., 2013b, Maeda and Fukuda, 1996). As at the time of this study, there has not been phytochemical data on *S. furcellata* to foretell its possible mechanism of melanin inhibition. Having established that it reduced melanin synthesis, further experiments were done.

5.9.3 Effect of *S.furcellata* on Tyrosinase Enzyme Activity

To further elucidate the molecular mechanism(s) by which *S. furcellata* reduced melanin synthesis in murine B16 melanoma cells, effect of the crude extract on tyrosinase activity was evaluated. Tyrosinase (TYR) is a crucial enzyme in melanin synthesis pathway (Demirkiran et al., 2013). Tyrosinase is the primary enzyme that catalyzes the first two different chemical reactions which are the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone (Nishio et al., 2016). The activity was evaluated by measuring the rate of L-DOPA oxidation by tyrosinase in B16 cell lysates. The extract stimulated the tyrosinase enzyme more than the control (Fig 5.5). To further confirm the effect of the extract on murine B16 melanoma intracellular tyrosinase, tyrosinase activity was estimated by zymography (Fig 5.6). The zymography result was in agreement with tyrosinase kinetic activity as it further confirmed the stimulatory effect of the extract on the tyrosinase activity. The reference standard, Kojic acid, reduced the enzyme activity. Many plant metabolites have been reported to modulate the activity of enzymes involved in the ageing processes or hyperpigmentation, most importantly,

tyrosinase (Mukherjee et al., 2011, Cefali et al., 2016). However, among the many ways of melanin synthesis reduction by bioactive compounds, it has been documented that melanin synthesis reduction can occur by degradation of melanin pigment in B16 melanoma cells without affecting the tyrosinase activity or tyrosinase expression (Hosoya et al., 2012). Stimulation of tyrosinase enzyme activity should lead to increased levels of melanin synthesis; in this study, methanolic extract of *S. furcellata* leaves generated an increase in the tyrosinase activity and there was a reduction in melanin synthesis. Therefore, it was assumed that the extract possibly caused degradation of melanin pigment in B16 melanoma cells.

5.9.4 Effect of Methanolic Extract of *S. furcellata* on Intracellular ROS

The result in this study showed that the extract of *S. furcellata* did not increase ROS in B16 melanoma cells (5.8). ROS are continuously being produced by many normal cellular events, and majorly from aerobic respiration. The ROS generated during these events are constantly counteracted by several antioxidant proteins (Chang and Chuang, 2010). In line with this, the unstained control in Fig. 5.8 did not have increased intracellular ROS. Intracellular production of ROS has been proposed as an aetiological factor for natural depigmentation processes which include greying of hair and many other pathological conditions such as vitiligo (Jiménez-Cervantes et al., 2001). It has been reported in previous studies that MITF is phosophorylated and then degraded in response to ROS stimulation (Liu et al., 2009, Kim et al., 2014). In addition, inhibition of hyperpigmentation has been demonstrated to occur by degradation of MITF through ROS-induced ERK phosphorylation (Ko and Cho, 2018). In the present study, because the extract did not cause obvious increase in the intracellular ROS production, it can then be further assumed that the mechanism by

which it brought about reduction in melanin synthesis was possibly not by increased intracellular ROS.

5.9.5 Effect of Methanolic Extract of *S.furcellata* on Relative Expression of Melanogenesis Genes

To further elucidate the molecular mechanism(s) by which *S. furcellata* reduced melanin synthesis in murine B16 melanoma cells, the effect of the methanolic extract on relative expression of melanogenic enzymes was evaluated by performing real-time quantitative PCR on 6 different genes involved in melanogenesis pathways (Fig. 2.2). The result showed that tyrosinase gene (TYR), tyrosinase-related protein-1 gene (TRP-1), tyrosinase-related protein-2 gene (TRP-2)/dopachrome tautomerase gene, and melanocortin-1-receptor gene (MC1R) were all up-regulated while microphthalmia associated transcription factor gene (MITF) and β-Catenin gene were down-regulated (Fig. 5.9).

Tyrosinase is the primary enzyme that catalyzes the first two different chemical reactions which are the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone (Nishio et al., 2016). However, the rearrangement of DOPA to dihydroxyphenylindolecarboxylic acid (DHICA) is catalysed by TRP-2 (Kim et al., 2015). Furthermore, microphthalmia-associated transcription factor (MITF) has been documented to be the master regulator of melanocyte differentiation, proliferation, pigmentation, and survival (Levy et al., 2006). As a major transcription factor, it regulates TYR, TRP-1, and TRP-2 expression (Baek and Lee, 2015). Decrease in MITF expression leads to the downregulation of differentiation markers and inhibits melanogenesis (Jiménez-Cervantes et al., 2001).

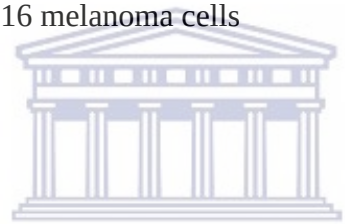
In the present study, compared with the control, MITF was suppressed but downstream genes, TYR, TRP-1, and TRP-2 (Fig. 2.2) were minimally expressed (Fig. 5.9). Another signalling pathway involved in melanin synthesis includes Wnt/β-catenin pathway. β-catenin is phosphorylated by a complex of many proteins comprising casein kinase 1 (CK1), axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) when this signal pathway is not activated, and then affected by ubiquitin and degraded via ubiquitin proteasomes (Wang et al., 2018). However, on activation of Wnt pathway, interactions of Wnt 1, Wnt 3a, and Wnt 8 with frizzled receptors and low density lipoprotein receptor-related (Lrp) 5/6 co-receptors leads to GSK3β being negatively regulated (Giles et al., 2003). Then, cytoplasmic β-catenin will translocate into the nucleus and bind to the MITF promoter, causing transcriptional activation of MITF (Latres et al., 1999, Bellei et al., 2008, Shin et al., 2015). In this study, MITF and β-Catenin were suppressed but TYR, TRP-1, TRP-2, and MC1R were up-regulated minimally. From above, down-regulation of β-Catenin and consequent suppression of MITF suggested that Wnt/β-catenin pathway was not activated by the extract. In addition, intracellular cyclic adenosine monophosphate (cAMP)-mediated pathway is an important pathway that induces melanogenesis in melanocytes. Binding of α-Melanocyte-stimulating hormone (α-MSH) to MC1R activates cAMP (Suzuki et al., 1996, Bertolotto et al., 1998) and causes an increase in the expression of MITF via activation of the cAMP-response element binding protein (CREB) transcription factor (Jung et al., 2009). Although MC1R was expressed with associated minimal up-regulation of TYR, TRP-1 and TRP-2, MITF which is the mater regulator was down-regulated. Thus, it suggested that cAMP-mediated pathway was not activated by the extract of *S. furcellata.*

Therefore, it could be concluded that methanolic extract of *S. furcellata* leaves reduced melanin synthesis possibly through degradation of melanin pigment in B16 melanoma cells.

5.9.6 Chapter Conclusion

This chapter described the melanin inhibition potential of the crude extract of *S. furcellata*, a member of the Proteaceae family, on murine B16 melanoma cells and the molecular mechanism of the process was established. It is the first time biological potential has been investigated for this plant.

The next chapter describes the melanin inhibition potential of methanolic extract of *O. fruticans* extract on B16 melanoma cells



CHAPTER SIX

OTHOLOBIUM FRUTICANS REDUCED MELANIN SYNTHESIS IN MURINE B16 MELANOMA CELLS THROUGH INHIBITION OF cAMP-MEDIATED PATHWAY AND INCREASED INTRACELLULAR ROS

6.1 Introduction

Many plants of different genera belonging to the *Fabaceae* family have been used in folk medicine for treating different ailments in human body. Tyrosinase inhibitors from natural sourcesmay be present in many plants including the *Fabaceae* family.Allam et al. demonstrated the melanin synthesis inhibition potentials of the methanol extract and bioactive compounds isolated from the flowers of *Vicia fabaL* (broad bean)(Allam et al., 2018).

The *Otholobium* genus belongs to the family Fabaceae with limited numbers of species distributed mainly on the South-Eastern and Eastern parts of Africa, also extending into the Mediterranean climate areas of South Africa (the Great Cape Flora region). The recentmigration of many *Psoralea* species under *Otholobium*(Grimes, 1990) indicates the close similarity of both generaand is expected to be reflected on the chemical constituents (and biological action) of both these plants.The health benefits of *Otholobium* genus have not been extensively studied as yet, however many species of *Psoralea* have been studied and shown to contain many natural drugs directly related to skin diseases like psoralen which affected melanin biosynthesis.

Since *Otholobium fruticans* is a poorly studied plant hence, the present study aims to evaluate the melanin inhibition potential and mechanisms of action of *Otholobium fruticans* in B16 melanoma cells.

6.2 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Otholobium fruticans* Leaves

Cytotoxicity of methanolic extract of *Otholobium fruticans* leaves was performed on murine B16 melanoma cells and MTT was used to assess the viability of the cells after exposure for 72 h (Fig. 6.1A). Also, melanin inhibition assay with the methanolic extract was done on murine B16 melanoma cells for 72 h (Fig. 6.1B).

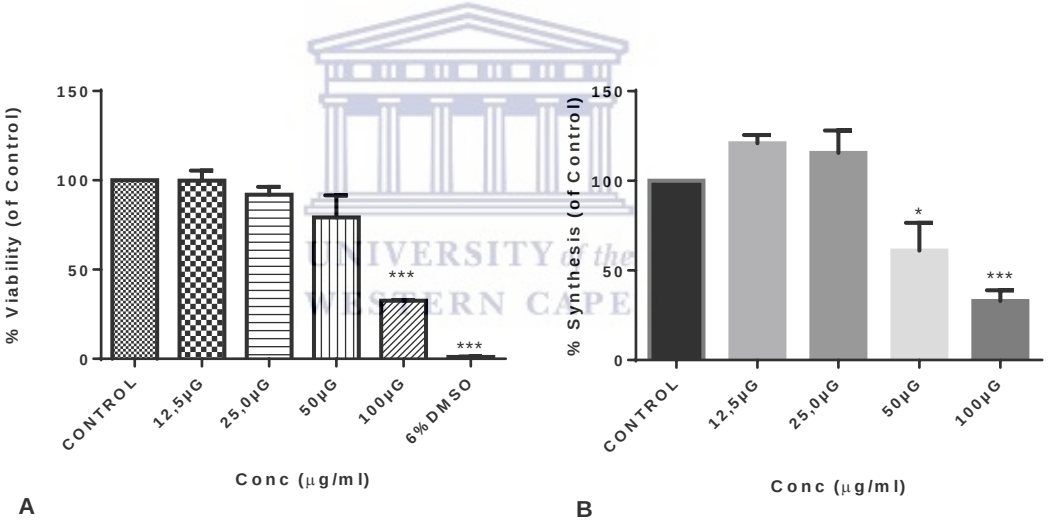


Figure 6.1: Effect of Methanolic Extract of *O. fruticans* on viability (A) and intracellular melanin activity (B) at 72 hours in B16 melanoma cells.

Cytotoxicity was done with serial dilution of the methanolic extract. The concentrations used were 12.5 µg/mL, 25.0 µg/mL, 50 µg/mL and 100 µg/mL and 6% DMSO was used as the positive control. The viability of the cells at 72 h (Fig. 6.1A) using 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL of the extracts showed 99.8

%, 91.9 %, 79.2 %, 32. 6 % ($p < 0.001$) viability compared with the untreated control, respectively. The corresponding melanin concentrations at 72 h treatment were 120.9 %, 115.6 %, 61.1 % ($p < 0.05$) and 34.0 % ($p < 0.001$) of the untreated control (Fig. 6.1B). There was dose-dependent decrease in viability as the concentration of the extract increased but was only significant at 100µg/mL. However, statistical reduction in melanin synthesis was with 50µg/mL (61.1% melanin synthesized) and 100µg/mL (34.0% melanin synthesized). There was no significant cytotoxicity to cells by 50 µg/mL concentration of the extract (79.2% viability) but the corresponding 61.1% melanin synthesized with 50 µg/mL concentration of the extract showed that 50 µg/mL significantly reduced melanin synthesis (38.9% decrease) without being significantly toxic to cells. Thus, the 50µg/mL concentration of *O. fruticans* is a safe concentration for melanin synthesis inhibition. Moreover, the significant reduction in melanin synthesis by 100µg/mL of the extract was as a result of cell death by this concentration which led to significant reduction in cell density that synthesized melanin.

Furthermore, the cytotoxicity of the 50µg/mL concentration of this extract was evaluated with non-cancerous cell line, HaCaT, which is a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin (Fig 6.2). No significant difference was observed between the untreated control and extract-treated cells. The positive control, 6% DMSO, was however seen to result in cell death. There is interaction between one melanocyte and 30-40 keratinocytes making epidermal melanin units at the basal layer of the skin (Haass and Herlyn, 2005). Consequently, 50µg/mL concentration of methanolic extract of leaves of *O. fruticans* could be safe for both the melanocytes and the neighbouring keratinocytes.

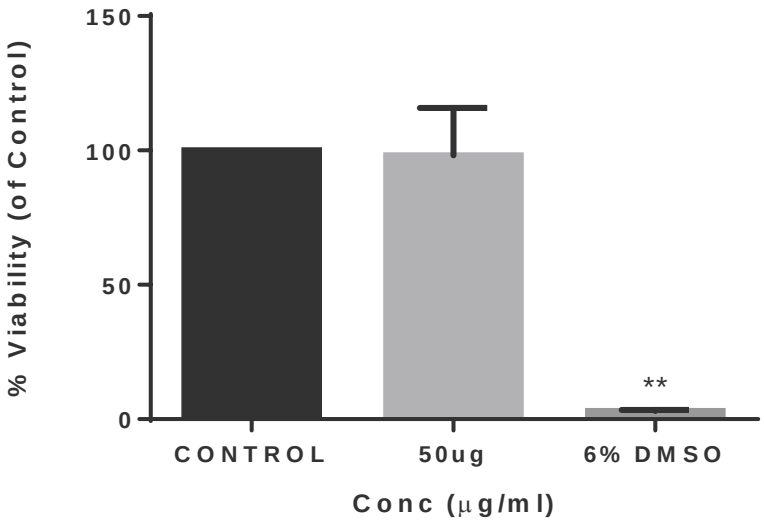


Figure 6.2: Effect of 50 µg of *O. fruticans* on HaCaT

6.3 Cytotoxicity and Melanin Inhibition of Kojic Acid

Kojic acid is used widely as a positive control in melanin inhibition experiments due to its inhibitory effects on melanin synthesis (Wang et al., 2018). Thus, Kojic acid was used in this study as positive control. Viability and melanin inhibition potentials were tested over 48 h using 300µg/ml. The viability was 87.7% of the untreated control (Fig. 6.3A) and was not significantly different from the control. Hence, Kojic acid is not toxic to the cells. Melanin inhibition assay showed 17.6% reduction in melanin concentration (82.4% of the control).

Reduction in melanin concentration of 38.9% by 50µg/mL of *O. fruticans*extract compared with 17.6% reduction by 300 µg/mL of Kojic acid tends to suggest that *O. fruticans* reduced melanin synthesis better than Kojic acid. The cytotoxicity testing and melanin assay with Kojic acid was done for 48 h in this study because the least concentration that produced significant reduction in melanin synthesis compared with the control was significantly toxic to cells at 72 h as seen in section **4.7** (Fig. 4.20).

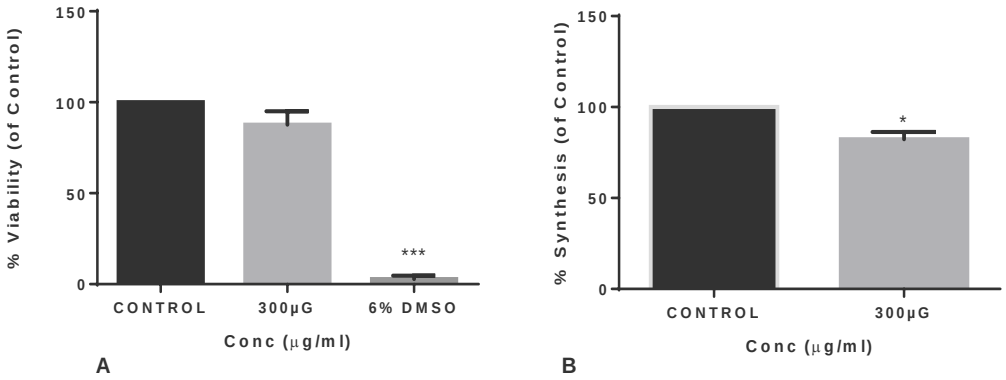


Figure 6.3: Effect of Kojic acid (300 µg/mL) on viability (A) and intracellular melanin activity (B) at 48 hours in B16 melanoma cells.

6.4 Effect of Methanolic Extract of *Otholobium fruticans* on the Morphology of Murine B16 Melanoma Cells

Murine B16 melanoma cells were treated with 50µg/mL of *O. fruticans* extract over 72 h and 6% DMSO was used as positive control. As shown below (Fig. 6.4) there was no apparent difference in the morphology of cells treated with *O. fruticans* (Fig. 6.4B) from those of the untreated control (Fig. 6.4A). However, the morphology of cells treated with 6% DMSO (Fig. 6.4C) appeared completely different from those of the controland of the extract-treated cells; shrunk without the dendrites typical of melanocytes and with round or ovoid shapes. The variation in cell shapes relative to the control cells may be related to toxicity effects.

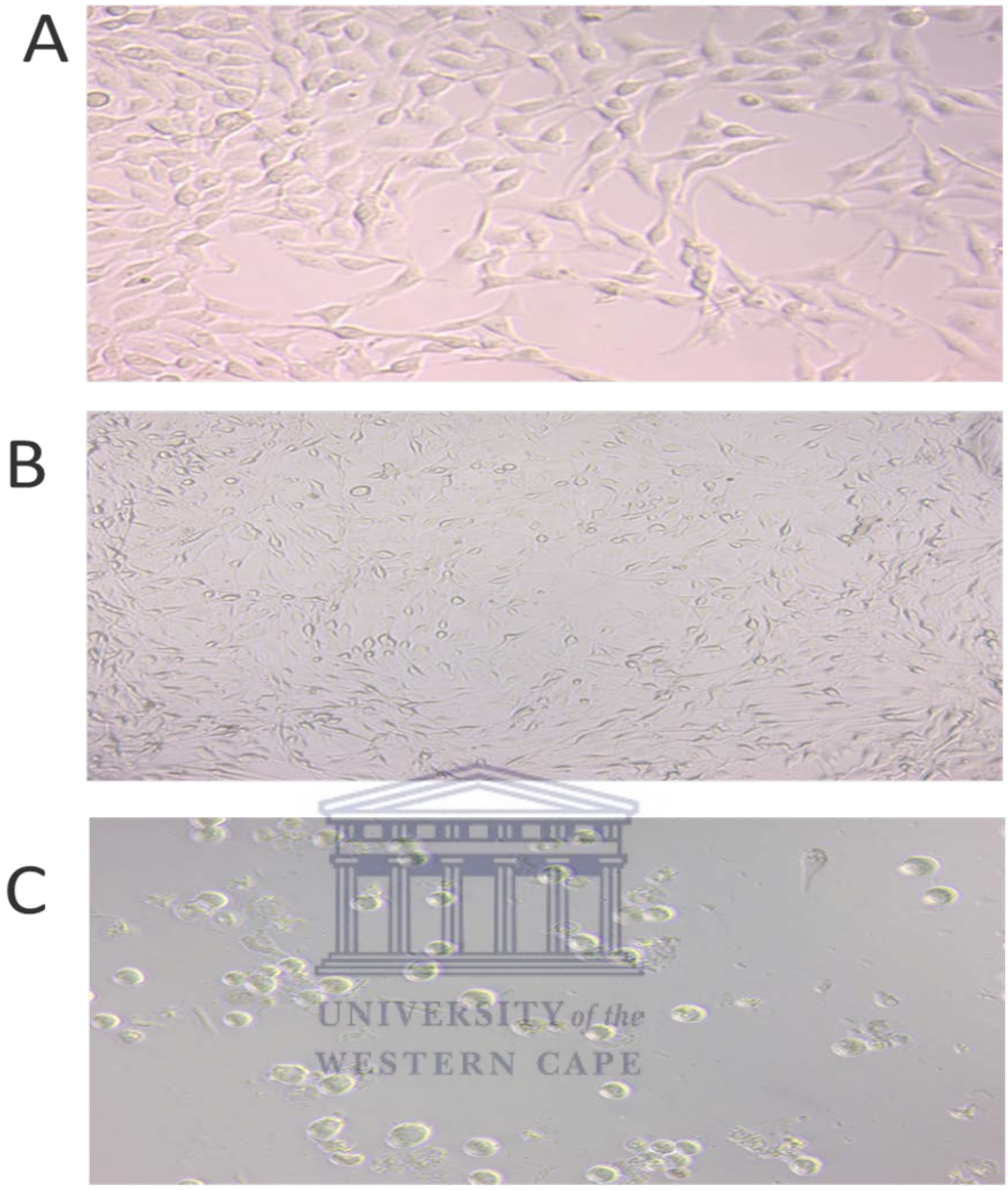


Figure 6.4: Photomicrographs of B16 Melanoma Cells showing effect of *O. fruticans* extract treatment for 72 h. (Mag. x 200). A: Untreated Control B: 50 µg/mL *O. fruticans* C: 6% Dimethylsulphoxide (Photomicrographs were taken in A where there were discrete cells, but B was fully confluent with no area with discrete cells, while all cells in C appeared ovoid)

6.5 Effect of *Otholobium fruticans* on Tyrosinase Enzyme Activity

The intracellular tyrosinase enzyme activity of B16 melanoma cells was determined by measuring the rate of L-DOPA oxidation by tyrosinase in B16 cell lysates. The Km and Vmax values were obtained from the graph plotted with GaphPad Prism 6 version. Kojic acid was used as positive control. However, mixture of PBS and L-DOPA and PBS only were run together with treatment samples to serve as controls to monitor the kinetics of the enzyme-substrate reactions. An active tyrosinase enzyme oxidized L-DOPA to pink-coloured dopachrome and the absorbance was monitored every min for 60 min. The tyrosinase enzyme activity of the control lysate was 512.15 ± 17µmol/min (Table 6.1) and is represented by the blue hyperbolic plot in Fig. 6.5. The enzyme activity of lysates from B16 cells treated with 50 µg/mL of *O. fruticans* for 72 h was zero and is represented by a pink horizontal Michaelis-Menten plot in Fig. 6.5. Compared with the untreated control, it implied that the tyrosinase enzyme in lysate treated with *O. fruticans* was inhibited. The enzyme activity of lysate of B16 cells treated with Kojic acid was zero and is represented by a red horizontal Michaelis-Menten plot (Fig. 6.5). This showed that the tyrosinase enzyme present in the lysate was inhibited by Kojic acid. Because there was no tyrosinase enzyme in the mixture of PBS and L-DOPA and PBS ONLY groups, pink colour was not formed at the end of the 60 min reaction time. They are represented by yellow and green horizontal Michaelis-Menten plots (Fig. 6.5). This experiment showed that *O. fruticans* and Kojic acid inhibited tyrosinase enzyme activity in B16 melanoma cells.

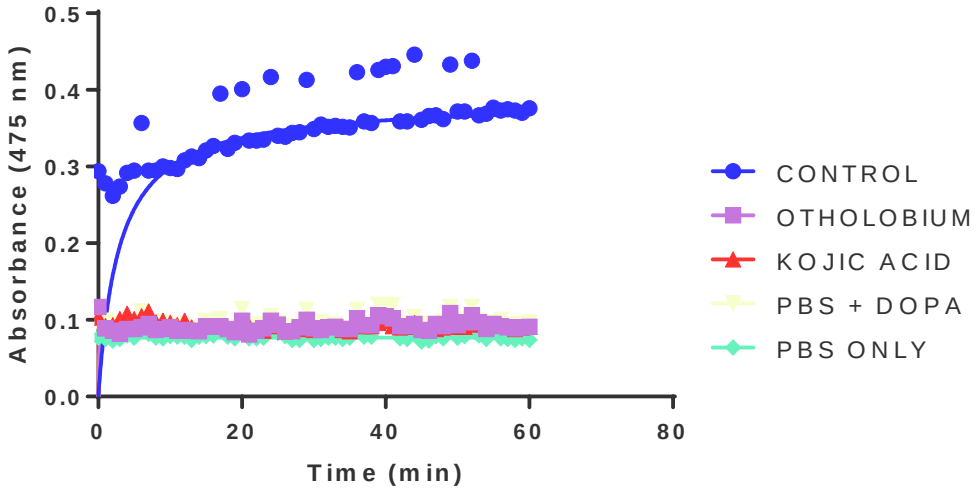


Figure 6.5: Michaelis-Menten plot for inhibition of *O. fruticans* extract on B16 melanoma intracellular tyrosinase activity using L-DOPA as substrate

Table: 6.1: Inhibition of Tyrosinase by *O. fruticans* extract

INHIBITOR	Conc	Km(mM)	Vmax(µmol/min)	Slope K(Km/Vmax)	Enzyme Activity
Control	0µg	2.36 ± 0.36	0.2494 ± 0.05	9.48 ± 0.32	512 ± 17
Otholobium fruticans	50µg	-0.108	0.1065 ± 0	0	0
Kojic acid	300µg	-0.0508	0.1065 ± 0	0	0

6.6 Dihydroxyphenylalanine (DOPA) Staining

The effect of methanolic extract of *O. fruticans* on intracellular tyrosinase activity was also confirmed by tyrosinase zymography. B16 melanoma cells were treated with 50 µg/mL of methanolic extract of *O. fruticans* for 72 h while 300 µg/mL of Kojic acid, positive control, was for 48 h. The tyrosinase activity in the untreated control was detectable with visible band (Fig. 6.6A and B). There was no visible band for *Otholobium fruticans* and almost nonvisible band for kojic acid. Results were in agreement with data of intracellular tyrosinase inhibition, confirming the possible anti-melanogenic effect of the extract.

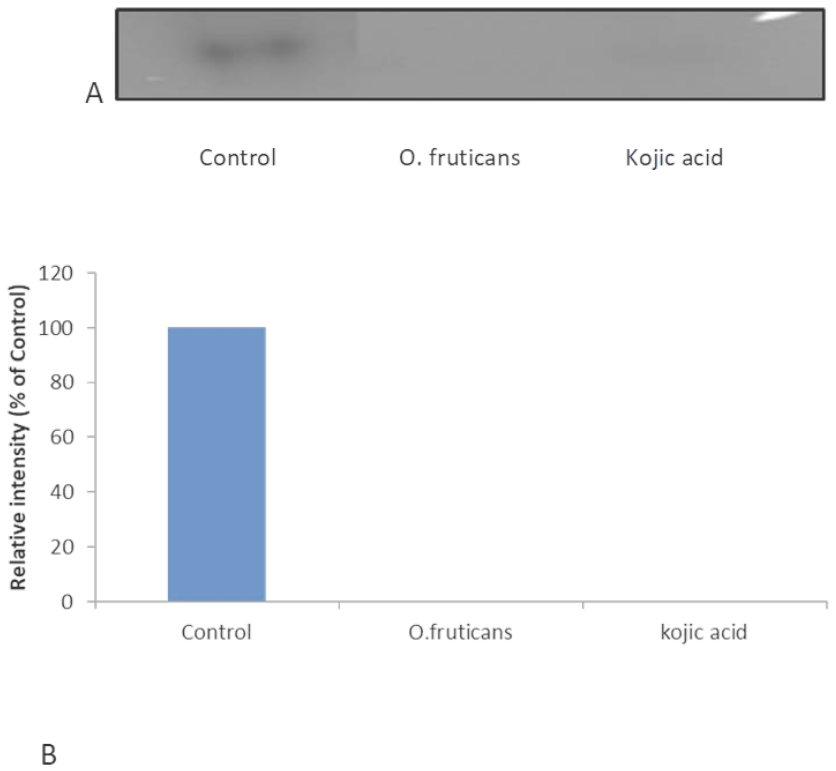


Fig. 6.6: Effect of methanolic extract of *O. fruticans* leaves on B16 melanoma cells by L-DOPA staining Tyrosinase activity was estimated by zymography (A) and the relative intensity of band was determined with Image J software (B)

6.7 Effect of Methanolic Extract of *Otholobium fruticans* on Intracellular ROS

The effect of treatment of B16 melanoma cells with *O. fruticans* extract on intracellular ROS is shown in Fig. 6.7. ROS was evaluated using the cell permeant dye chloromethyl-2', 7'-dichlorofluorescindiacetate (CM-H2DCFDA). The black and blue histograms represent unstained control while red represent stained control. There was a shift of histogram to the right in extract-treated cells, implying increased intracellular ROS. Unlike in *O. fruticans* extract-treated cells, there was no shift in the histogram for Kojic acid (green) and the unstained cells. Hence, Kojic acid did not cause increased intracellular ROS. Therefore, methanolic extract of *O. fruticans* produced its melanin inhibitory effect possibly through increased generation of reactive oxygen species.

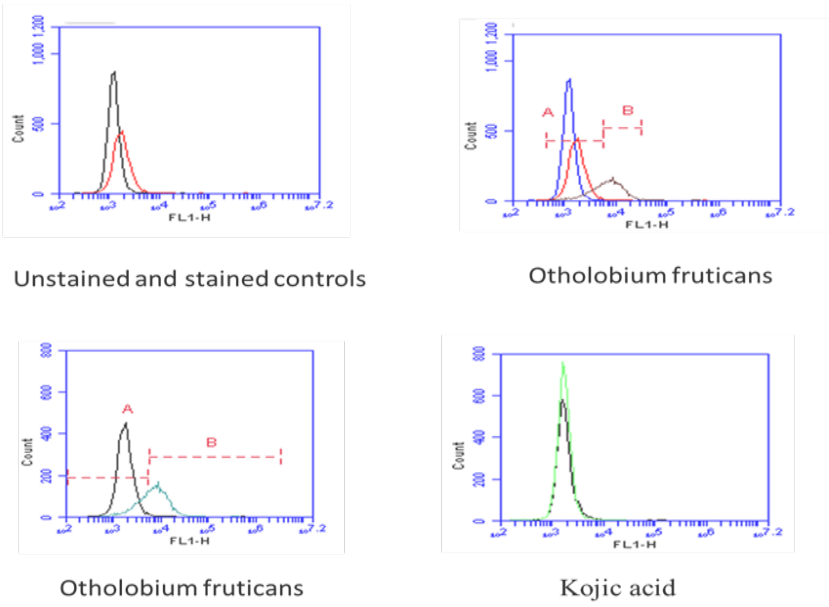


Figure 6.7: Representative histograms of cells stained with CM-H2DCFDA dye and evaluated by flow cytometry

6.8 Effect of Methanolic Extract of *Otholobium fruticans* on Relative Expression of Melanogenesis Genes

To determine the mechanism by which *O. fruticans* inhibited melanin synthesis, expression of six different genes involved in melanogenesis pathways was evaluated using real-time quantitative PCR. The PCR sample of each gene was done in triplicate. Relative expression of each gene was normalized to glyceraldehyde-3- phosphate dehydrogenase (GAPDH). Compared with the untreated control all the genes, TYR (-1.557 ± 1.826), TRP-1(-2.987 ± 3.237), TRP-2 (-1.740 ± 1.265), MITF (-2.943 ± 3.099), and MC1R (-0.540 ± 4.837) were down-regulated except β-Catenin (5.093 ± 4.837) (Fig. 6.8). TYR is an important enzyme in the melanin synthesis pathway as it catalyzes the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA), which is promptly oxidized to DOPAquinone (DQ) which is immediately converted into the intermediate DOPAchrome (Hearing, 2011). TRP-1 and TRP-2 were also not

expressed , and together with TYR, are downstream enzymes in the melanogenesis pathways. MITF and MC1R were also down-regulated. MITF is a major transcription factor which regulates TYR, TRP-1, and TRP-2 expression (Baek and Lee, 2015). Thus binding of α-MSH to MC1R transduces a signal to increase the expression of microphthalmia-associated transcription factor (MITF) by activating cAMP (Bertolotto et al., 1998). β-Catenin gene was well expressed by treatment with *O. fruticans*. Furthemore, Wnt/β-catenin pathway is one of the regulatory pathways for melanin synthesis. In this pathway, MITF is targeted through activation of frizzled receptor, inhibition of Glycogen synthase kinase 3β (GSK3β), and stimulation of β-catenin accumulation (Shin et al., 2015). Thus, due to down-regulation of MC1R, MITF, and consequently, TYR, TRP-1, and TRP-2, *O. fruticans* reduced melanin synthesis in murine B16 melanoma cells possibly through inhibition of cAMP-mediated pathway.

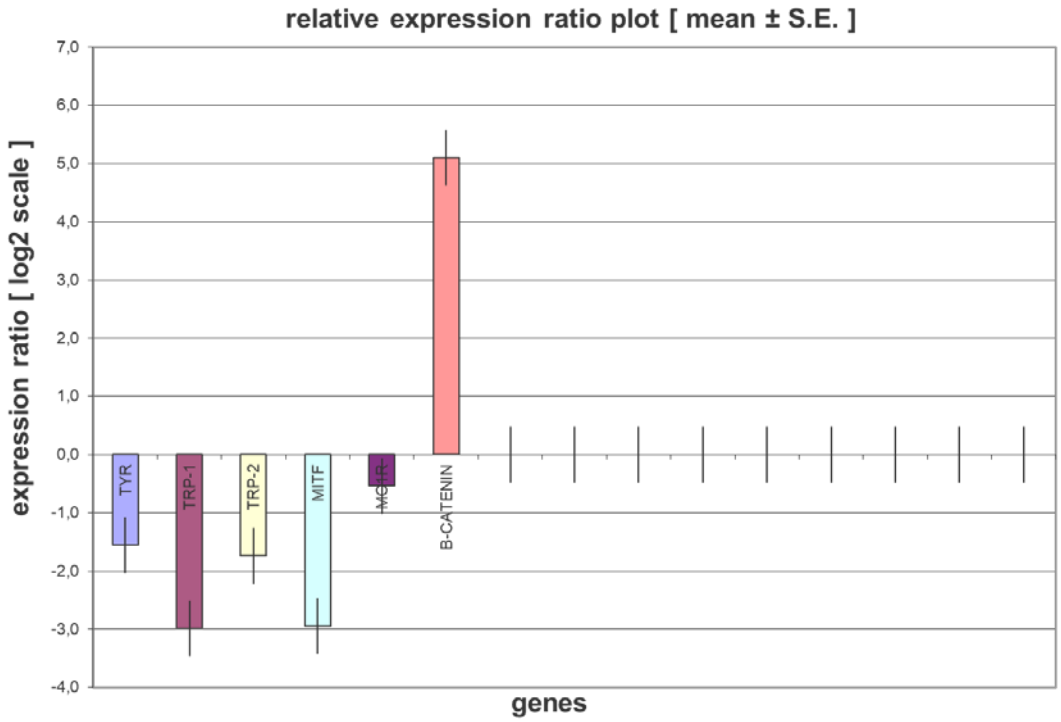


Figure 6.8: Real-time quantitative PCR relative expression ratio plot of genes for *Otholobium fruticans* compared with untreated control

Kojic acid is used as positive control in melanin inhibition experiments due to its inhibitory effects on melanin synthesis (Wang et al., 2018). Unlike in *O. fruticans* treatment group, TYR (3.810 ± 3.593), TRP-1 (3.077 ± 2.835), and MC1R (4.567 ± 4.494) were up-regulated. TRP-2 (-3.847 ± 3.284) and MITF (-2.307 ± 2.48) were down-regulated while β-Catenin was up-regulated (0.817 ± 1.404) (Fig. 6.9).

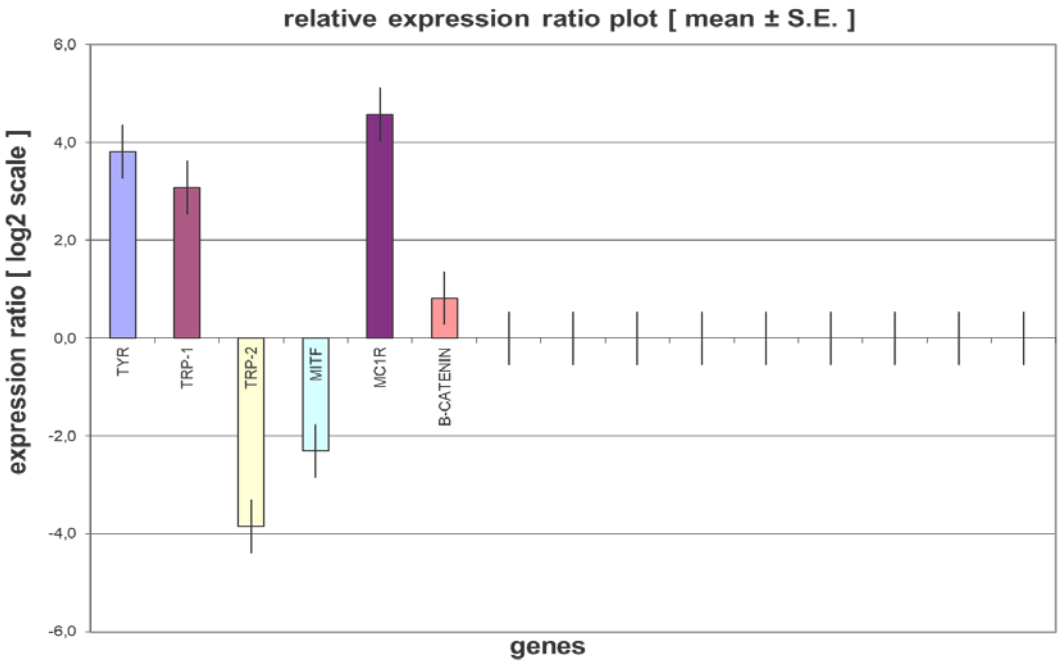


Figure 6.9: Real-time quantitative PCR relative expression of genes ratio plot of genes for Kojic acid compared with untreated control

6.9 Discussion

6.9.1 Introduction

Medicinal plants have been used as traditional treatments for a plethora of human diseases for thousands of years and in many parts of the world and in most rural areas of developing countries, plants remain a primary source of medicine (Chitme et al., 2004). There are a number of natural products derived from medicinal plants that have proven to be an abundant source of biologically active compounds and a lot of these have served as the basis for the development of new lead chemicals for pharmaceuticals. However, most developing countries have huge resources of medicinal and aromatic plants mostly in the rural areas and much of these are still not fully explored.

Cosmeceuticals are increasingly popular alternatives to standard depigmenting agents (Levin and Momin, 2010).They are preferred alternatives as they are often not costly and easily available over the counter (Fisk et al., 2014) and cosmeceuticals made from plant-derived phytochemicals, have been documented to exhibit various degrees of cellular actions for various skin pigmentation-related diseases (Fisk et al., 2014, Leo and Sivamani, 2014).

There are currently no known published studies on the biological effects of *O. fruticans* on skin pigmentation, thus, this study was done to evaluate the melanin synthesis inhibition potential as well as the mechanism(s) of action of the methanolic leaf extract of this plant.

6.9.2 Cytotoxicity and Melanin Inhibition of the Methanolic Extract of *Otholobium fruticans* Leaves

To determine the antimelanogenic effects of the *O. fruticans* extract, murine B16 melanoma cells were used, having been widely used for this purpose possibly because they are relatively easy to culture *in vitro* and similarity of their melanogenic mechanisms with normal human melanocytes. As a positive control, Kojic acid was used which is widely used in similar experiments due to its inhibitory effects on melanin synthesis (Wang et al., 2018). The result showed that *O. fruticans* inhibited melanin synthesis better than Kojic acid. In depigmentation, it is expected that the therapeutic agent must not be toxic to both the target cells and the surrounding tissues. There is interaction between one melanocyte and 30-40 keratinocytes making epidermal melanin units at the basal layer of the skin (Haass and Herlyn, 2005). The effective concentration of the *O. fruticans* that reduced melanin synthesis was not toxic

to noncancerous cell line, HaCaT, which was the normal keratinocytes used in this study. This was also confirmed with the effect of the extract on the cell morphology (Fig. 6.4). There was no apparent changes on extract-treated cells compared with the control.

Otholobium fruticans is a member of Fabaceae family. From chemical and pharmacological point of view, Fabaceae is one of the most studied groups of plants (Wink, 2013, Neves et al., 2017). Valuable chemical components such as flavonoids, alkaloids, coumarins etc. used for treatment of various diseases are derived from this family (Wink, 2013). Plants in this family have been reported to inhibit melanin synthesis. Clinical trial done on *Glycyrrhiza glabra* (Liquorice), a member of Fabaceae, showed that 2.5% g applied to 100 females for four weeks produced significantly improved symptoms of melasma without any side effects compared to the placebo group (Badria, 2015). On B16 murine melanoma cells, it was observed that Glabridin, a bioactive compound derived from Liquorice, inhibited tyrosinase activity at 1.0 µg/mL without affecting DNA synthesis (Yokota et al., 1998). Similarly, bakuchiol, a bioactive compound isolated from *Otholobium pubescens*, another species of Otholobium, has been reported to have hypoglycaemic effects in mice (Krenisky et al., 1999). However, Chaudhuri, and Bojanowski have demonstrated that bakuchiol can function as an anti-ageing compound through retinol-like regulation of gene expression (Chaudhuri and Bojanowski, 2014). Since there is yet no data on phytochemicals and compounds isolated from *Otholobium fruticans*, it is possible that compounds present in the family Fabaceae or genus Otholobium may also be present in the crude extract of *Otholobium fruticans* which is used in this study.

6.9.3 Effect of Methanolic Extract of *Otholobium fruticans* on Tyrosinase Enzyme Activity

To further elucidate the molecular mechanism(s) by which *O. fruticans* reduced melanin synthesis in murine B16 melanoma cells, the effect of the crude extract of *O. fruticans* on tyrosinase activity was evaluated. Tyrosinase (TYR) is a very important enzyme in the melanogenesis pathway (Demirkiran et al., 2013). It is the primary enzyme that catalyzes the first two different chemical reactions viz, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone (Nishio et al., 2016). TYR activity was evaluated by measuring the rate of L-DOPA oxidation by tyrosinase in B16 cell lysates. The extract was found to inhibit tyrosinase enzyme activity as did Kojic acid (Fig 6.5) and this was further confirmed by zymography results (Fig 6.6) which appeared to corroborate the findings on tyrosinase activity. Thus, tyrosinase activity was inhibited by both the extract and Kojic acid. It was reported previously that *Glabridin*, a bioactive compound derived from Liquorice, inhibited tyrosinase activity at 1.0 µg/mL without affecting DNA synthesis in B16 melanoma cells (Yokota et al., 1998) and Clinical trial done on *Glycyrrhiza glabra* (Liquorice), a member of Fabaceae, showed that 2.5% g applied to 100 females for four weeks produced significantly improved symptoms of melasma without any side effects compared to the placebo group (Badria, 2015).

Many plant metabolites have been reported to modulate the activity of enzymes involved in the ageing processes or hyperpigmentation, most importantly, tyrosinase (Mukherjee et al., 2011, Cefali et al., 2016). However, as a constituent of skin lightening preparations, Kojic acid is known to cause skin irritation with such side effects as cytotoxicity, dermatitis, and skin cancer (Busca and Ballotti, 2000).

Cosmeceuticals are increasingly popular alternatives to standard depigmenting agents (Levin and Momin, 2010).Thus, results from this study tend to suggest that methanolic extract of *O. fruticans* could be considered a better alternative to Kojic acid as an inhibitor of melanin synthesis.

6.9.4 Effect of Methanolic Extract of *Otholobium fruticans* on Intracellular ROS

The results from this study showed that unlike the positive control Kojic acid and the unstained control, the extract of *O. fruticans* increased ROS (Fig 6.7). ROS are continuously being produced by many normal cellular events, and majorly from aerobic respiration. The ROS generated during these events are constantly counteracted by several antioxidant proteins (Chang and Chuang, 2010). In line with this, the unstained control in Fig. 6.7 did not have increased intracellular ROS. Depending on the levels produced, ROS are essential to cell signalling and regulation or can also be injurious to cell survival as by-products of metabolic processes. Previous studies have implicated ROS in melanogenesis (Cunha et al., 2012, Liu et al., 2012). It causes various effects in melanocyets. In addition, ROS generation has been proposed as one of the causes of such natural depigmentation processes as hair greying and many other pathological conditions including vitiligo (Jiménez-Cervantes et al., 2001). Also, previous studies have reported that MITF is phosphorylated and thereafter degraded in response to ROS stimulation (Liu et al., 2009, Kim et al., 2014), consequently leading to depigmentation. Moreover, a short pulse of H_2O_2 applied to B16 murine melanoma and human melanoma cells induced a transient repression of melanogenesis by mechanisms that include MITF-dependent downregulation of melanogenic enzymes (Jiménez-Cervantes et al., 2001). It can therefore be hypothesized that increased intracellular ROS in B16 melanoma cells caused by methanolic extract of *O. futicans*

leaves resulted in a reduction in melanin synthesis via a mechanism that includes MITF-dependent down-regulation of melanogenic enzymes.

6.9.5 Effect of Methanolic Extract of *Otholobium fruticans* on Relative Expression of Melanogenesis Genes

In order to either accept or reject the hypothesis above, the effect of the methanolic extract of *O. frutican* on relative expression of melanogenic enzymes was evaluated by performing real-time quantitative PCR on 6 different genes involved in the melanogenesis pathways (Fig. 2.2). The result showed that tyrosinase gene (TYR), tyrosinase-related protein-1 gene (TRP-1), tyrosinase-related protein-2 gene (TRP-2)/dopachrome tautomerase gene, microphthalmia associated transcription factor gene (MITF), and melanocortin-1-receptor gene (MC1R) were all down-regulated relative to the control with only the β-Catenin gene being up-regulated.

Tyrosinase is the primary enzyme that catalyzes the first two different chemical reactions which are the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone (Nishio et al., 2016). However, the rearrangement of DOPA to dihydroxyphenylindolecarboxylic acid (DHICA) is catalysed by TRP-2 (Kim et al., 2015). Furthermore, microphthalmia-associated transcription factor (MITF) has been documented to be the master regulator of melanocyte differentiation, proliferation, pigmentation, and survival (Levy et al., 2006). As a major transcription factor, it regulates TYR, TRP-1, and TRP-2 expression (Baek and Lee, 2015). Decrease in MITF expression leads to the downregulation of differentiation markers and inhibits melanogenesis (Jiménez-Cervantes et al., 2001). In this study, MITF was downregulated which, as a master regulator of TYR, TRP-1,

and TRP-2, possibly caused downregulation of these genes. In addition, intracellular cyclic adenosine monophosphate (cAMP)-mediated pathway is an important pathway that induces melanogenesis in melanocytes. Binding of α-Melanocyte-stimulating hormone (α-MSH) to MC1R activates cAMP (Bertolotto et al., 1998, Suzuki et al., 1996), and causes an increase in the expression of MITF via activation of the cAMP-response element binding protein (CREB) transcription factor (Jung et al., 2009). But in this study, MC1R was suppressed which possibly suggests that cAMP-mediated pathway was inactivated.

Another signalling pathway involved in melanin synthesis is the Wnt/β-catenin pathway. β-catenin is phosphorylated by a complex of many proteins comprising casein kinase 1 (CK1), axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) when this signal pathway is not activated, and then affected by ubiquitin and degraded via ubiquitin proteasomes (Wang et al., 2017). However, on activation of the Wnt pathway, interactions of Wnt 1, Wnt 3a, and Wnt 8 with frizzled receptors and low density lipoprotein receptor-related (Lrp) 5/6 co-receptors leads to GSK3β being negatively regulated (Giles et al., 2003). Then, cytoplasmic β-catenin will translocate into the nucleus and bind to the MITF promoter, causing transcriptional activation of MITF (Latres et al., 1999, Bellei et al., 2008, Shin et al., 2015). β-Catenin gene was up-regulated in this study while MITF was suppressed. Suppression of MITF suggested that Wnt/β-catenin pathway was not activated expression of β-Catenin gene notwithstanding. It then implies that inactivation of the cAMP pathway was predominant. These results are in line with previous findings by Yokota et al. *Glabridin*, a bioactive compound derived from Liquorice, inhibited tyrosinase activity at 1.0 µg/mL without affecting DNA synthesis

in B16 melanoma cells (Yokota et al., 1998). Also, the findings of Stapelberg et al. showed that *Vachellia karroo*, a member of Fabaceae family, showed significant down-regulation of tyrosinase gene expression (Stapelberg et al., 2019).

Unlike in *Otholobium fruticans* treatment, Kojic acid produced different results. TYR, TYR-1 and MC1R were up-regulated while TYR-2 and MITF were suppressed. In addition, the β-Catenin gene was up-regulated but not as high as observed with *O. fruticans* extract.

Therefore, due to downregulation of MC1R, MITF, TYR, TRP-1, and TRP-2, *Otholobium fruticans* could be said to cause a reduction in melanin synthesis in murine B16 melanoma cells via two mechanisms, namely, inhibition of cAMP-mediated pathway and increased intracellular ROS. Findings from this study tends to suggest that *Otholobium fruticans* could be a candidate anti-melanogenic agent and it might be effectively used inthe treatment of hyperpigmentation disorders.

6.10 Chapter Conclusion

This chapter sought to describe the depigmenting potential and the molecular mechanisms involved in the activity of *Otholobium fruticans*.

The next chapter is the “synthesis”of all previous chapters which will give a summary of the results.

CHAPTER SEVEN

Conclusions and Recommendations

7.1 General Conclusions

This study was designed to screen selected South African medicinal plants in order to identify novel medicinal plants that could inhibit melanin synthesis,which is often anomalous in hyperpigmentation disorders. The selected plants were *Otholobium fruticans, Rhynchosia villosa, Phylica erricoidesPsoralea aphylla, Serruria furcellata,* and*Maclura pomifera*.The study also sought to evaluate the molecular mechanism(s) of action by which the different extracts of the selected plants inhibit melanin synthesis. Crude extracts of the plants were made with methanol or ethylacetate and evaluated by performing cytotoxicity profiling on murine B16 melanoma cells, and human HaCaT (control) cells.

The rationale for this study is the prevalence of epidermal hyperpigmentation in most parts of the world, often caused by increased melanin synthesis. The current treatment modalities for hyperpigmentation of various aetiologies have associated side effects which necessitated the development of safe novel cosmeceuticals from medicinal plants. The gold standard for topical treatment is Hydroquinone but apart from other side effectsassociated with this medication, itsuse has alsobeen associated with hypopigmentation of the normal surrounding tissues.

Chapter 4 described the results of screening of the 6 selected South African plants for melanogenesis inhibition activity. The results showed that *Maclura pomifera, Phylica*

ericoides and *Psoralea aphylla* did not possess melanogenesis inhibition activity at the experimental conditions of this study. However, *Otholobium fruticans*, *Serruria furcellata*, and both methanolic and ethylacetate extracts of *Rhynchosia villosa* showed various degrees of melanin synthesis inhibition. While 400 µg/mL of ethylacetate extract of *Rhynchosia villosa* produced reduction in melanin synthesis at a $p < 0.05$, the same concentration of the methanolic extract of *Rhynchosia villosa* resulted in a reduction of melanin synthesis at a $p < 0.01$ level compared with the untreated control. Thus, methanolic extract of *Rhynchosia villosa* had higher inhibition activity than ethylacetate extract. One hundred µgTreatment with both the 100 µg/mL and 50 µg/mL of methanolic extract of *Serruria furcellata* and *Otholobium fruticans*, respectively, reduced resulted in significant reduction in melanin synthesis compared with the untreated control (*S. furcellata* at $p < 0.01$, *O. fruticans* at $p < 0.05$). Because of the relatively lower concentrations of the mthanolic extracts of *S. furcellata* and *O.fruticans* that inhibited melanin synthesis,these two plant extracts were further investigated for their molecular mechanism(s) of actions.

Chapter 5 described the results of the evaluation of the molecular mechanisms of melanin synthesis inhibition by the methanolic extract of *S. furcellata* and the results showed that this extract of *S. furcellata* leaves reduced melanin synthesis possibly through the degradation of the melanin pigment secreted by B16 melanoma cells.

Chapter 6 described the results of the evaluation of the molecular mechanisms of melanin synthesis inhibition by the methanolic extract of *Otholobium fruticans*. The results showed that *O. fruticans* reduced melanin synthesis in murine B16 melanoma cells through the inhibition of the cAMP-mediated pathway and increased intracellular

ROS. This study suggests that *O. fruticans* is a possible candidate anti-melanogenic agent and might be effective in hyperpigmentation disorders.

7.2 Future perspective and recommendations

- Phytochemical studies of *S. furcellata* and *O. fruticans* highly recommended to identify the possible bioactive constituents and further bio-evaluation of the individual active compounds to determine their quality as new anti-pigmenting agents.
- Green nanoparticles could be synthesised with these two plants and melanin synthesis reduction activity evaluated in future.
- Western blotting could be done in lieu of qPCR profiling to assess gene expression in melanogenesis.
- The melanin synthesis inhibition activity of these extracts could be evaluated on a simulated 3D epidermal structure.
- Ultrastructural changes in the cells could be studied using scanning and transmission electron microscopic techniques.
- An appropriate in vivo model could be used to study the progression and induced modulation of melanogenesis in a living system.

7.3 Chapter Conclusions

Chapter seven is the last in this thesis. It is a synthesis of results and findings of this study and gave the general conclusions, future perspectives and future recommendations and is followed by the list of all the references used in this thesis.

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