Ethnopharmacological study on plants used for skincare and beauty by some Xhosa communities

by

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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy

In the

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal, Pietermaritzburg

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STUDENT DECLARATION

Ethnopharmacological study on plants used for skincare and beauty by some Xhosa communities

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VUYISILE SAMUEL THIBANE

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We hereby declare that we acted as supervisors of this PhD student:

Student full name: Vuyisile Samuel Thibane

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Thesis title: Ethnopharmacological studies of plants used for skincare and beauty by some Xhosa communities

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed examiners.

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DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part of and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1:

Thibane, V.S., Ndhlala, A.R., Abdelgadir, A.H., Finnie, J.F., Van Staden, J., 2018. The cosmetic potential of plants from the Eastern Cape Province traditionally used for skincare and beauty. South African Journal of Botany. **(*In press*)** (DOI: https://doi.org/10.1016/j.sajb.2018.05.003).

Contributions: Laboratory analysis and manuscript preparation were performed by the first author under the supervision of the four supervisors.

Publication 2:

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CONFERENCE CONTRIBUTIONS

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ABSTRACT

The paraphrase "*beauty lies in the eyes of the beholder"* by Plato, a Greek philosopher, has echoed through the fabrics of time and still echoes in our generation. The statement simply ought to refer to that the observer gets to decide what is "*beautiful"* in their eyes. The decision on what and who is beautiful is heavily influenced by one's surroundings. In a digital-market environment the concept of beauty enhancement is paraded by bigger forces to drive their own agenda. The desire to enhance one's facial appearance has significantly contributed to the observed growth of the beauty industry. The notable growth of the industry has in some cases resulted in unpleasant consequences due to the side effects of some of the products in the market. In this global-blooming industry products are traded and exchanged at a rapid rate. Introduction of safer natural beauty enhancement products are required for the South African market if we were to alleviate those with undesirable side effects and could combat some socio-economic challenges through job creation.

The use of plants as the source of natural compounds has proven to be a reliable strategy in ethnopharmacological applications. The Eastern Cape Province has a rich plant biodiversity and the communities have immense indigenous knowledge (IK) on the use of these plants. There is a need to explore the pharmacological application of these plants by introducing beauty enhancement product formulations made from local resources. The project was aimed at documenting and conducting

ethnopharmacological evaluation of plants used for skincare and beauty for their potential in formulation of beauty enhancement products.

An ethnobotanical survey was conducted to document plants that are used by communities in the Raymond Mhlaba municipality for skincare and beauty. Knowledge holders were identified by purpose sampling method and the interviews were conducted in *isiXhosa* using a structured questionnaire. Information on demographics, names of the plant, type of plant, plant part used, method of preparation and administration and frequency of use was collected and captured in the questionnaires. The Asphodelaceae and Asteraceae were the most represented families of the plants used for skincare and beauty. The communities used sustainable harvesting practices as the leaves were the most utilized plant parts. The most reported beauty enhancement uses were for achieving desired skin complexion and for skin smoothness, with both accounting up to 50% of the reported plant usages.

Sixteen plants with the highest frequency index (FI) were selected from the ethnobotanical survey for ethnopharmacological studies related to beauty enhancement. This included *Acokanthera oblongifolia* (Hochst.) Codd, *Aloe ferox* Mill, *Arctotis arctotoides* (L.f) O.Hoffm, *Bulbine frutescens* (L.) Willd, *Cassipourea flanaganii* (Schinz) Alston, *Chenopodium album* L, *Clausena anisata* (Willd.) Hook.f ex Benth, *Haemanthus albiflos* Jacq, *Marrubium vulgare* L, *Ilex mitis* (L.) Radlk, *Plantago lanceolata* L, *Rorippa nasturtium-aquaticum* (L.) Hayek, *Sonchus asper* L, *Symphytum officinale* L, *Ruta graveolens* L and *Urtica urens* L.

The antimicrobial activity of plant extracts was assessed using the microdilution bioassay to determine the minimum inhibitory concentrations (MIC). The antimicrobial activity of petroleum ether (PE), dichloromethane (DCM), 70% aqueous ethanol (v/v) and water extracts of the selected plants were assessed against infectious skin microorganisms including *Bacillus subtilis* ATCC 6051*, Brevibacillus agric* ATCC 51663*, Staphylococcus aureus* ATCC 12600*, Staphylococcus epidermidis* ATCC 12228*, Escherichia coli* ATCC 11775*, Klebsiella pneumoniae* ATCC 13883, *Candida albicans* ATCC 10231 and the dermatophytes *Microsporum canis* ATCC 36299*, Trichophyton mentagrophytes* ATCC 9533 and *Trichophyton tonsurans* ATCC 28942. The majority of the tested plant extracts were effective and inhibited the skin commensal bacteria *E. coli* with MIC values less than 100 µg/mL. Prolonged infections by commensal bacteria can condition the skin environment and provide favourable conditions for more opportunistic bacteria such as the *Staphylococci* genus. Ethanol extracts of *C. flanaganii* and *U. urens* expressed high antibacterial activity against *S. aureus* with MIC values less than 100 µg/mL. Ethanol extracts of *R. graveolens* and dichloromethane extracts of *A. arctotoides* were effective at inhibiting *S. epidermidis* and *S. aureus*, respectively. Inhibition of two opportunistic bacteria has a positive effect on skin tone, due to the scarring and darkening associated with infection by the *Staphylococci* genus. There was notable activity recorded against *C. albicans* and dermatophytes *M. canis, T. mentagrophytes* and *T. tonsurans* by extracts of *A. oblongifolia*, *A. arctotoides, C. flanaganii, I. mitis* and *R. graveolens* at different polarities with MIC's less than 1000 µg/mL.

The phenolic content and antioxidant activity of the plants were determined by assessing 50% aqueous methanol extracts (v/v) for their total phenolic and flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP) and the coupled oxidation of linoleic acid and bleaching of *β*-carotene. The antioxidant mechanism of phenolic compounds associated with beauty enhancement has been proposed to be due to their free radical chain breaking capabilities, metal chelation, oxidant quenching and inhibition of enzymatic activity. The total phenolic content of *A. ferox*, *I. mitis* and *C. flanaganii* were significantly high with recorded values ranging from 37.87 to 50.34 mgGAE/g. The flavonoid content of *C. flanaganii, A. oblongifolia* and *P. lanceolata* were significantly high. Methanol extracts of *R. graveolens* and *C. flanaganii* expressed the highest antioxidant activity, with IC_{50} values comparable to the standard antioxidant when assessed for their DPPH radical scavenging activity. The presence of antioxidants in the skin structural layers has a positive effect on the health and function of the skin. Extracts of *U. urens, A. ferox, C. flanaganii, B. frutescens, P. lanceolata, H. albiflos, M. vulgare, C. anisata, S. officinale* and *R. nasturtium-aquaticum* expressed good metal chelating potential. The highest oxidative protection in the *β*-carotene linoleic acid model with comparative oxidation rate ratio (ORR) to the positive control was observed for *C. flanaganii*, *S. officinale* and *U. urens*. The results indicate the ability of the plant extracts to provide protection against increased levels of lipid peroxidation in the skin, an important factor in beauty enhancement, due to delaying the age process.

The photo-protective effect of the plant extracts was measured by calculating the sun protection factor (SPF). The SPF is the ratio of ultraviolet (UV) radiation required to produce minimal erythema dose (MED) in protected skin to unprotected skin with higher values indicative of increased protection from photo damage. Ethanol extracts of *P. lanceolata, C. flanaganii, A. oblongifolia, I. mitis* and *A. arctotoides* exhibited SPF values of more than 15, which translated to photo protection of the skin against UVB radiation by more than 93.3%. The plant extracts demonstrated the highest absorbance of UVB radiation at a wavelength region between 300 – 305 nm. These will protect the skin against UV-induced oxidative damage and enhance the skin's health and function.

The inhibition of enzymes with beauty enhancement potential by the plant extracts was assessed against tyrosinase, secretory phospholipase A_2 ($sPLA_2$), lipoxygenase (15-LOX) and cyclooxygenase (COX-1 and COX-2). Ethanol extracts of *R. nasturtium-aquaticum*, *C. anisata*, *S. officinale* and *C. flanaganii* expressed good anti-tyrosinase activity. The coupled protection against UV-induced damage and modulation of the tyrosinase enzyme activity can be exploited to achieve the desired skin complexion. The anti-inflammatory studies revealed the potential of extracts of *C. flanaganii, P. lanceolata* and *R. nasturtium-aquaticum* to serve as dual inhibitors of 15-LOX and COX-2 enzymes. The inhibition of 15-LOX and COX-2 is effective at resolving psoriasis, a skin-inflammatory associated disease which has a negative effect on the health and beauty of the skin.

The effectiveness of ethanol extracts of *C. flanaganii, C. album, C. anisata* and *R. nasturtium-aquaticum* in maintaining the cells health and function was examined on human epidermal melanocytes (HEM) cell lines. Ethanol extracts of *C. flanaganii, C. album, C. anisata* and *R. nasturtium-aquaticum* were able to inhibit cellular tyrosinase activity and therefore reduce melanin production. The effective concentrations of the extracts were further reported as non-toxic to melanocytes. The observed anti-tyrosinase activity of the extracts against HEM cell lines contribute positively in achieving the desired skin complexion while providing photo protection against UV-induced damage. Therefore, plant extracts that are efficient and safe to use can be incorporated into formulations intended for beauty enhancement and further analysed under clinical trials. The study further suggests that the model undertaken be promoted to individuals and corporations interested in formulation of cosmeceuticals to ensure the safety and efficiency of their products.

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ANOVA	Analysis of variance	DPPH	2,2-diphenyl-β-picrylhydrazyl
ANT	Antioxidant activity	DPPH-H	2,2-diphenyl-β-picrylhydrazine
ARC	Agricultural Research Council	EC	Effective concentration
ATCC	American type culture collection	EtOH	Ethanol
ATM	African traditional medicine	FBS	Fetal bovine serum
ATP	Adenosine triphosphate	FC	Flavonoid content
BHT	Butylated hydroxytoluene	FDA	Food and drugs administration
CE	Catechin equivalent	FI	Frequency index
CLAMS	*β*-carotene-linoleic acid model system	FRAP	Ferric reducing antioxidant power
COX	Cyclooxygenase	GAE	Gallic acid equivalent
DCM	Dichloromethane	GDP	Gross domestic products
DHI	5,6-dihydroxyindole	GIS	Geographic information system
DHICA	5,6-dihydroxyindole-2-carboxylic acid	HCl	Hydrochloric acid
DMSO	Dimethyl sulfoxide	HEM	Human epidermal melanocytes
DNA	Deoxyribonucleic acid	HETE	Hydroxyeicosatetraenoic
DOPA	3,4-dihydroxy-L-phenylalanine	HSD	Honest significant difference

IA	Initial activity	PE	Petroleum ether
IC	Inhibitory concentration	PG	Phosphatidylglycerol
IK	Indigenous knowledge	PGs	Prostaglandins
INT	*p*-iodonitrotetrazolium chloride	PMA	Phorbol 12-myristate 13-acetate
LD	Lethal dose	RCPGD	Research Centre for Plant Growth and Development
LOX	Lipoxygenase	RMEDA	Raymond Mhlaba Economic Development Agency
LSD	Least significant difference	ROS	Reactive oxygen species
MED	Minimal erythema dose	RSA	Radical scavenging activity
MH	Mueller-Hinton	SAAB	South African Association of Botanists
MIC	Minimum inhibitory concentration	SC	Stratum corneum
MMP	Matrix metalloproteinase	SPF	Sun protection factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	$sPLA_2$	Secretory phospholipase A_2
NDGA	Nordihydroquairetic acid	SPSS	Statistical package for social science
NSAIDs	Nonsteroidal anti-inflammatory drugs	TAC	Tricarboxylic acid cycle
NU	Bews herbarium	TCA	Trichloroacetic acid
ORR	Oxidation rate ratio	TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
PC	Phosphatidylcholine	TNBA	5-thio-2-nitrobenzoic acid
PD	Potato dextrose	TPC	Total phenolic content

TUT	Tshwane University of Technology	US	United States
TRP	Tyrosinase-related protein	UV	Ultraviolet
UKZN	University of KwaZulu-Natal	YM	Yeast malt

Chapter 1

Introduction and Literature Review

1.1. Introduction

The use of plants in primary healthcare systems, beauty enhancement and in daily rituals and ceremonies in African Traditional Medicine (ATM) dates as far back as ancient Egyptian times. Egyptian records document plant materials, seed oils and spices being used to treat various ailments and as part of their daily lives **(DOLD and COCKS, 2005)**. In South Africa, there are records of the Khoi-San community using medicinal plants for their healthcare maintenance **(REYNOLDS, 1950)**. The use of plants and plant materials is prevalent throughout the African continent for the livelihood of different communities **(GURIB-FAKIM, 2006)**.

A number of South African communities still rely on the use of medicinal plants as a form of primary healthcare and for skincare. The Xhosa communities around the Raymond Mhlaba municipality in the Eastern Cape are examples of such communities. The use of medicinal plants in these communities has been practiced for generations to fight ailments, as a form of social status, healthcare and for beauty enhancement. The Eastern Cape Province has a rich plant biodiversity and a strong cultural belief on the usage of various medicinal plants **(DOLD and COCKS, 2002)**. Recently, research and case studies have focused on the exploitation of this rich biodiversity in an attempt to combat some of the socio-economic challenges facing the province. In some of these communities initiation of localized projects based on processing of these medicinal plants can aid in poverty alleviation, job creation and boosting the economy **(GELDENHUYS and VAN WYK, 2002; MELIN et al., 2017)**.

A number of products produced from local medicinal plants already exist and can be found at local shops and at street herbal markets. However, there is a need for scientific validation of some of the medical plants currently being used for formulation of these products and to ensure their safe uses. Some of the plants are believed to enhance beauty and improve healthcare and are very common amongst Xhosa men and women **(DOLD and COCKS, 2005)**. A number of articles and review papers now exist on medicinal plants used for skincare, skin treatment and those that can combat skin-related diseases which in turn contribute to the enhancement of beauty and healthcare **(JOHNSY et al., 2012; SHARMA et al., 2013)**. These studies have highlighted that the knowledge on medicinal plant usage is limited to specific members in rural communities. The indigenous knowledge on the various uses of plants in these communities is held by few individuals and it is important that the knowledge is disseminated for the purpose of documentation, preservation and creation of novel bio-economies.

1.2. Literature review

1.2.1. The skin

1.2.1.1. The structure and biological function of the skin

The skin is a complex multi-layered organ which has a significant effect on the appearance and beauty of an individual **(JIANG and DELACRUZ, 2011)**. The skin further serves as a barrier for the body and can be readily repaired from external and internal assaults **(EL-DOMYATI et al., 2002; BARONI et al., 2012)**. It is divided into three structural compartments, namely the epidermis, dermis and the hypodermis (Figure 1.1). The interaction of these three layers is important in the skin exerting its barrier function, achieving a desired complexion, regulating body temperature, protecting against microbial invasions and the harmful effects from external UV radiation.

The epidermis consists of keratinocytes, melanocytes, Langerhans and Merkel cells. Keratinocytes are the most abundant cell type, accounting for approximately 90% of the epidermal cells **(BROHEM et al., 2011)**. In keratinocytes, keratin is the main component and provides structural integrity to the epidermis and therefore is responsible for the firmness of the skin **(RAMMS et al., 2013)**. A firm skin results in a more radiant looking appearance, a trait that is mostly associated with beauty and skin health.

A close relationship exists in the epidermis between melanocytes and keratinocytes, with each melanocyte surrounded by a number of keratinocytes **(LEI et al., 2002)**. Melanocytes are melanin-producing cells of the epidermis and are positioned at the

basal layer of the epidermis and in the hair bulb **(HAAKE and SCOTT, 1991)**. Once melanocytes are established in the epidermis they extend their dendrites towards keratinocytes which allows them to transfer melanin to a large number of keratinocytes. The melanin produced by melanocytes protects the skin against UV radiation and oxidative damage **(SEIBERG, 2001; BRENNER and HEARING, 2008; DELEVOYE, 2014)**.

The dermis is the connective tissue component of the skin and provides it with pliability, elasticity and tensile strength. It protects the body from mechanical injury, binds water, aids in thermal regulation and includes receptors of sensory stimuli. The dermis interacts with the epidermis in maintaining the properties of both the tissues **(KOLARSICK et al., 2011)**. The dermis is mainly composed of fibrous and amorphous extracellular matrix with collagen and elastic connective tissue being the main type of fibrous tissues of the dermis. Non-fibrous glycol-proteins, proteoglycan and glycosaminoglycan form part of the dermal connective tissue **(HAAKE et al., 2001)**. Collagen is the major dermal constituent and accounts to approximately 75% of the skin's dry weight and provides both tensile strength and elasticity **(AGREN et al., 2015)**. The elasticity of the skin is a major contributing factor in wrinkle formation. Formation of wrinkles is one of the hallmarks of the skin's age process with a skin free of wrinkles and lines more desired. Furthermore, the tissue of the hypodermis insulates the body and serves as a supply of energy and protects the skin.

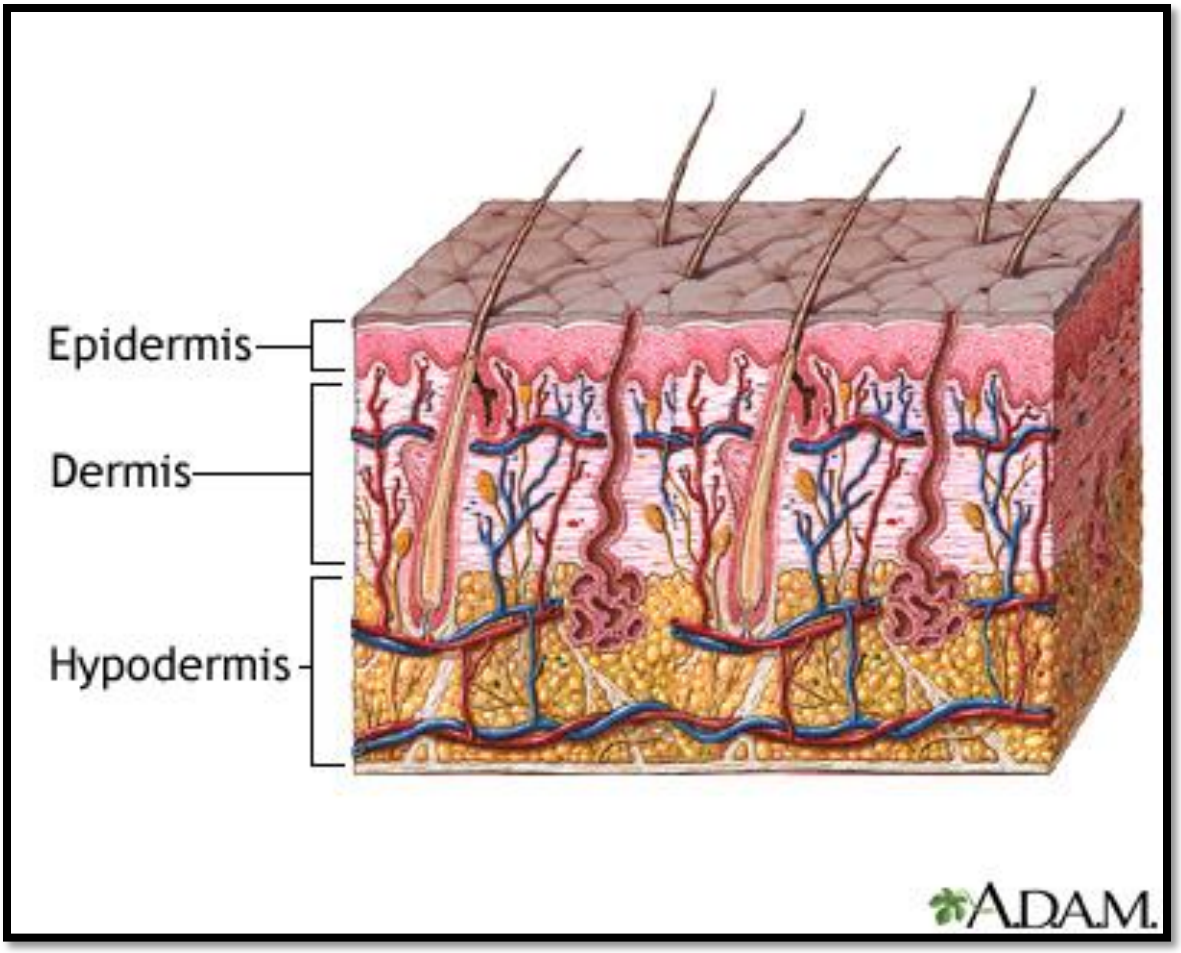

Figure 1.1: The skin structural compartments of epidermis, dermis and hypodermis. Upper-layer of epidermis mainly constructed of the stratum corneum (SC), keratinocytes in the middle layer and melanocytes at the basal layer of the epidermis. The dermis is mainly composed of collagen and the hypodermis serves as an insulator. *Adapted from University of Maryland (http://www.umm.edu/imagepages/8912.htm).*

1.2.1.2. The role of the skin in beauty

To look beautiful and healthy is a long held need in human beings and can date as far back as the cave dwellers. A combination of qualities together contributes to a person feeling and being perceived beautiful. Looking beautiful is desired by many people and to some extent is worth pursuing. Natural beauty is a blessing and a sign of a healthy

lifestyle; hence beauty and healthcare go hand in hand. To some a perfectly smooth face with no skin blemishes, without any skin eruptions and a desired complexion completes a list of attributes that contribute to a person's looks and appearance and in turn classifies them beautiful **(KHAN, 1996)**. To a large extent, the perception of many people will contribute significantly to classifying a person as beautiful. Beauty is about being content with your own looks and appearance, with efforts to achieving the desired beauty level differing from person to person. Beauty is therefore interpreted differently by various groups because of the variation in people's perception and views.

The skin is the largest organ surrounding the body and is exposed to both intrinsic and extrinsic factors. The skin plays a major role in the facial beauty features of a person. A skin that is firm, smooth, with an even complexion and is radiant contributes to a person being perceived as beautiful. However, changes resulting from those intrinsic and extrinsic factors affect the beauty of the skin by altering the structure of the epidermal and dermal layers **(FARAGE et al., 2008)**. Extrinsic factors relate to the environment and are brought about by the detrimental effects of UV radiation. Ultraviolet light penetrates the epidermis and dermis layers of the skin and cause damage to the skin cellular constituents **(HOFFMANN et al., 2000; HOCKBERGER, 2002; CÉSARINI et al., 2003; RAHIMPOUR and HAMISHEHKAR, 2012)**. In the skin, the antioxidant capacity of the epidermis is much greater than that of the dermis.

The enzymatic activity in the epidermis is due to the action of superoxide dismutase, glutathione peroxidase and glutathione reductase with the non-enzymatic activity due to

the action of reduced glutathione, α-tocopherol, ubiquinol and ascorbic acid. Intrinsic factors relate to the accumulative effect of high concentrations of reactive oxygen species (ROS) generated through aerobic respiration. Therefore, accumulation of these damaged biomolecules in the skin further result in damage in dermal collagen density. Collagen is the major structural protein and associated damages will initiate losses in skin integrity **(CALLEJA-AGIUS et al., 2013)**, aging process **(LONGO et al., 2013)**, wrinkle formation **(KUWAZURU et al., 2012)** and skin darkening **(SCHARFFETTER-KOCHANEK et al., 2000)** all of which are the hallmarks of beauty reduction. The expression of the matrix metalloproteinase (MMP) plays a significant part in accelerated degradation of collagen **(QUAN et al., 2013)**. This can be coupled with the reduction of the synthesis of new extracellular matrix components by the dermal fibroblasts which generally replace the degraded matrix.

The physical barrier function of the skin is mainly attributed to the stratum corneum **(SIMPSON et al., 2011; LULEVICH et al., 2010)**. The formed barrier prevents invasion of pathogens, contributes to regulation of water and solutes loss, as well as contributing to mechanical protection **(KIRSCHNER et al., 2013)**. A breach of the skin will lead to skin dryness and proliferation of skin disease further escalating the beauty reduction process **(WHITE-CHU and REDDY, 2011)**. The stratum corneum consists mainly of protein-enriched cells and lipid-enriched intracellular domains and is composed of multiple layers of non-viable differentiated corneocytes **(ECKHART et al., 2013)**.

Corneocytes are the largest of the keratinocytes, characterized by a flattened-polyhedral shape. The shape and features of the corneocytes are adapted to maintain

the integrity of the stratum corneum yet allow for the desquamation process **(HAAKE et al., 2001)**. The modification in epidermal differentiation and lipid composition results in altered function. Alteration of the physical barrier function of the stratum corneum may lead to skin dryness and wrinkling due to subsequent water loss and the skin's loss of structural integrity. This loss of structural integrity further contributes to beauty deterioration and alterations in skin's appearance due to associated-diseases **(HARDING, 2004; PROKSCH et al., 2006; PROKSCH et al., 2008)**. It is therefore important that the skin is protected from all the factors negatively affecting its beauty and health.

1.2.1.3. The health and care of the skin

The concept of one caring for their skin contributes significantly to the perception of their beauty as the skin is always the first place one looks at. The health and attractiveness of the skin is largely influenced by nutrition, where in essence you get what you put in. The oral intake and topical application of a number of nutrient-rich products is known to have a positive effect on the skin's health due to their interactions with the skin **(POLEFKA et al., 2012; MEINKE et al., 2013)**. However, it is important to note that both therapeutic methods might have different pharmokinetics.

Orally supplemented nutrient-rich products first have to overcome metabolism associated absorption before being absorbed into the bloodstream, while topically applied products only have to overcome transdermal absorption **(LODÉN et al., 2011; STAMFORD, 2012)**. This has to be taken into consideration when deciding on effective

therapeutic intervention that can ensure optimal skin health. Throughout history, a lot of strategies have been utilized to treat the reduction of skin health and beauty. The use of plants, essential oils and spices has been extensively reported across the world with the use of plants being a more favoured method. Plants are more diverse and are represented by different families with varying nutritional content. Topical and oral application of plant-derived metabolites may reinforce the skin constituents and protect against factors known to negatively impact on the health and beauty of the skin.

1.2.2. Medicinal plant metabolites

Medicinal plants are represented by different families, genera and species and these variations results in varying uses of the plants. Plants have been reported for their application in medicine, nutrition and cosmetics **(VRANESIĆ-BENDER, 2010; MUKHERJEE et al., 2011)**. These applications of the plant have been credited to the variety of metabolites from the plants. Plants are a source of a number of metabolites whose structure, function and usability have only been partially explored **(SCHWAB, 2003)**. Although the focus on natural product research has been on-going for decades there is still room for improvement in this area. The variation of plant metabolites in-plants and between-plants assures continued exploitation of these plants.

Plant metabolites contribute significantly to its metabolism and survival. Primary metabolites are mainly involved in the growth and development of the plant with the energy, adenosine triphosphate (ATP), required for their synthesis generated through

three pathways; glycolysis, tricarboxylic acid cycle (TAC) and mitochondrial electron transport chain **(FERNIE et al., 2004)**. Secondary metabolites are generally defined as chemical compounds found in plants that are not involved in primary metabolism but influence interaction of the plant with the environment **(JULKUNEN-TIITTO, 2005)**.

Secondary metabolites are produced by plants in response to pathogens, herbivory and environmental stresses as a protective mechanism and are classified into three major groups; terpenes, phenolic compounds and nitrogen containing compounds (Figure 1.2) **(HARTMANN, 2007; VERMA and SHUKLA, 2015)**. Therefore, it is sensible to highlight that there is variation in the content and concentration of secondary metabolites for the different plants. This plant adaptation mechanism has been exploited for decades in natural products research, contributing to formulation of novel products for new and existing industries **(HUSSAIN et al., 2012)**. The beauty and health industry is one where these adaptations by plants can be exploited to formulate products to address the ever growing needs of the consumers.

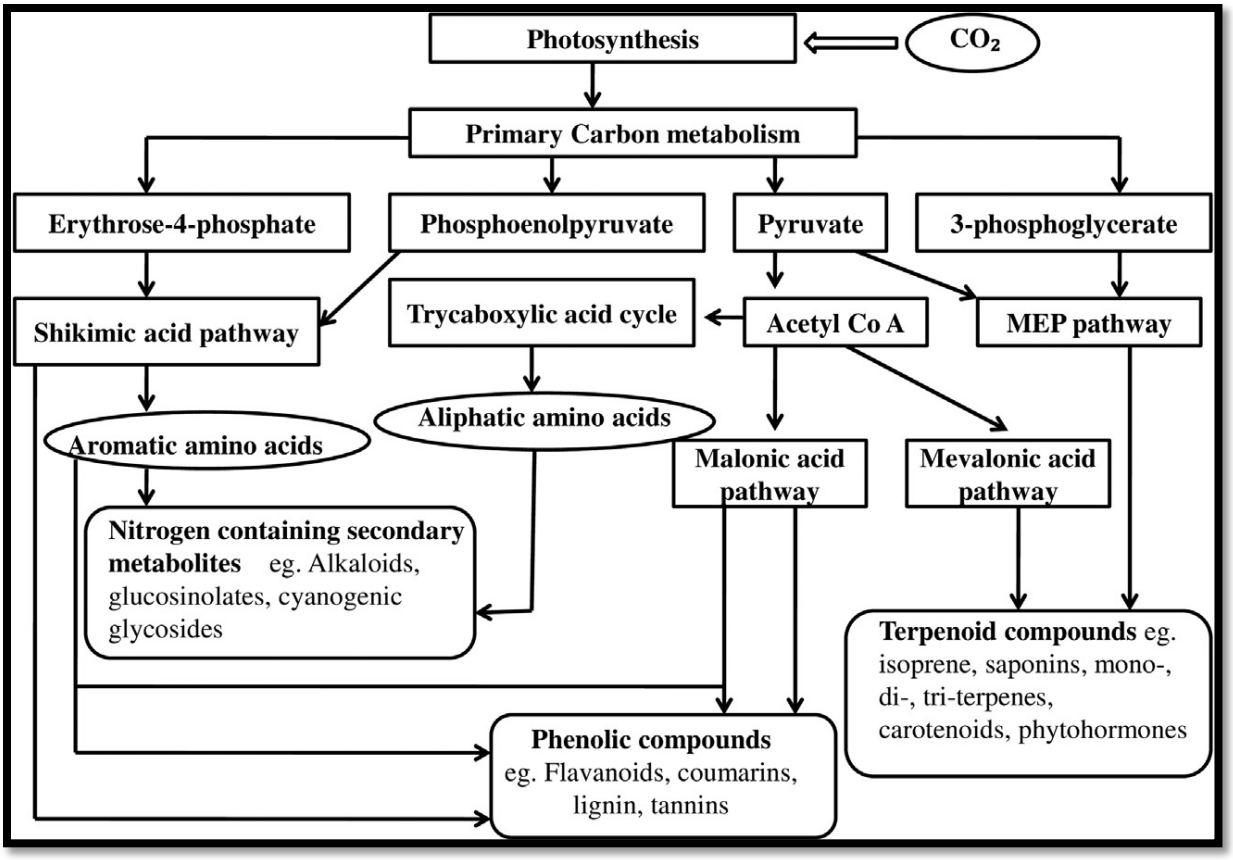

Figure 1.2: Cellular respiration and biosynthetic pathways of secondary metabolites in plants. Secondary metabolites are not involved in primary metabolism and are characterized by three major groups of phenolic, terpenoid and nitrogen compound containing compounds. *Adapted from VERMA and SHUKLA, (2015)*.

1.2.3. Interaction of plant metabolites with the skin

1.2.3.1. Effect of antioxidants on the beauty of the skin

The imbalance of oxidants and antioxidants has undesired consequences in any biological system. It is the antioxidants that are responsible for maintaining the oxidative stress level in the skin by keeping the ROS levels low and thus avoiding cellular damages. In the skin, proteins, deoxyribonucleic acid (DNA), lipids and fatty acids and sugars are all vulnerable to oxidative damage **(HALLIWELL and CHIRICO, 1993;**

DIZDAROGLU et al, 2002; BENOV and BEEMA, 2003; LOBO et al., 2010). However, mechanisms are in place to constantly get rid of oxidized biomolecules and maintain the skin's homeostasis. The antioxidant system is categorized into two major groups of enzymatic antioxidants and non-enzymatic antioxidants. The activity of antioxidants is effective through various strategies, such as preventing formation of free radicals, acting as reducing agents neutralizing free lipid radicals, interrupting propagation of auto-oxidation chain reactions, singlet oxygen quenchers, acting as metal chelators and inhibiting activity of enzymes involved in free radical formation **(RAHMAN, 2007; GODMAN et al., 2011; CAROCHO and FERREIRA, 2013)**.

The excessive excitation by UVB radiation on the skin is one of the leading causes of skin mutation due to oxidation of nucleic acids in keratinocytes and melanocytes. The characterized UV-induced damage further leads to premature aging of the skin as a result of the production of proteolytic enzymes breaking down proteins and sugars. The phenolic hydroxyl groups attached to ring structures of flavonoids and phenolic acids neutralize the formed ROS and protect the skin from UV-induced damage. Protection of the skin against UV-associated damages effectively maintains the membrane structure and integrity intact, positively influencing the beauty of the skin **(FISHER et al., 1997; KURTMANN and SCHROEDER, 2009; PROCHÁZKOVÁ et al., 2011; WATSON et al., 2014)**.

1.2.3.2. Effect of proteins on structural integrity of the skin

Collagen peptides are natural bioactives with skincare and beauty benefits. Collagen peptides are of different length with an abundance of hydroxyproline, glycine and proline. Hydroxyproline is unique to collagen and can be used to differentiate collagen from other proteins. Collagen peptides are efficiently digested to di- and tri-peptides which are resistant to further intracellular hydrolysis. Collagen peptides induce collagen and hyaluronic acid production, improving the dermal collagen fragmentation and therefore counter the hallmarks of the age process and beauty deterioration **(ASSERIN et al., 2015)**.

1.2.3.3. Effects of vitamins and minerals on the health and function of the skin

The interaction of mineral salts and vitamins with the skin has some recognizable benefits to the skin's appearance. Some of the noticeable attributes include improvement of the skin tone, colour and texture with reduced wrinkles, which all contributes to the beauty of the skin. The catalytic function of enzymes in the skin layers further requires the presence of metal ions as co-factors. The presence of macronutrients, micronutrients and trace elements all contribute significantly to the health and function of skin. Nutrients that are integral to skin health and function includes vitamins A, C and E, copper (Cu), zinc (Zn), calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg). Supplementation of the skin with these nutrients forms part of what is now referred to as nutricosmetics **(TAEYMANS et al., 2014)**.

Vitamin A

Vitamin A (retinol) protects the skin against UV-related damages such as wrinkle formation and dark spots formation. Retinoic acid derived from retinol reduces the degradation of collagen by modulating the activity of the matrix metalloproteinase involved in wrinkle and dark spots formation **(KAFI et al., 2007)**. Vitamin A further protects the skin from oxidative damage by preventing lipid radical formation through reactions with peroxyl radicals **(JEE et al., 2006)**.

Vitamin C

Vitamin C (L-ascorbic acid) is effective at scavenging ROS and therefore stimulates the synthesis of collagen through the reduction of oxidative damage to the skin. This impacts positively on the skin firmness due to the association of collagen with the skin's structural integrity function. Vitamin C further inhibits tyrosinase activity, resulting in depigmentation of the skin. Effective depigmenting compounds are those with a coupled photo-protective function to ensure the desired skin complexion is achieved without any side effects **(FARRIS, 2005; GASPAR and CAMPOS, 2007)**.

Vitamin E

Vitamin E protects the skin against UV-induced oxidative damage and consequently reduces erythema, tanning and photo aging. The photo-protective effect of vitamin E to the skin is due to its ability to halt lipid peroxidation and form unreactive tocopheroxyl radicals. The resultant protection of the skin against UV radiation contributes

significantly to the beauty and health of the skin **(MAALOUF et al., 2002; THIELE et al., 2007)**.

Copper (Cu)

The presence of Cu in the skin is critical in the function of enzymes involved in cross-linking collagen and therefore enhancing the skin's structural integrity. The function of the enzyme tyrosinase is also enhanced by the presence of Cu in the skin. Tyrosinase plays a critical role in the complexion and tone of the skin through the melanisation process **(HIGDON and DRAKE, 2012)**.

Zinc (Zn)

Zinc is a micronutrient in the skin and also functions as a co-factor for enzymes involved in regulation of oxidative stress in the skin **(SCHWARTZ et al., 2005)**.

Calcium (Ca)

Calcium is a macronutrient and plays a key role in the differentiation of basal keratinocytes to corneocytes, creating the tough outer layer of the skin. This outer layer plays a key role in wound healing and hydrating the skin **(LANSDOWN, 2002)**.

Potassium (K), Sodium (Na) and Magnesium (Mg)

Potassium, Na, and Mg function as co-factors for enzymes found in the epidermal and dermal layers of the skin. These minerals further acts as key factors in the maintenance

of the cellular membrane through the action of electrolyte balancing **(HIGDON and DRAKE, 2012)**.

1.2.4. The state of the natural product industry in South Africa

South Africa's plant biodiversity has been reported in many studies to be amongst one of the richest globally **(VAN WYK and GERICKE, 2000; VAN WYK and WINK, 2004)**. However, even with the observed biodiversity the country has witnessed numerous calls for more to be done on the commercialization of some of these plants **(OKOLE and ODHAV, 2002; LALL and KISHORE, 2014)**. The commercialization process will aid in combating some of the socio-economic challenges faced by communities in rural areas through job creation and benefit-sharing schemes.

It is worth highlighting that a number of success stories have been reported regarding product formulation of some of the plants endemic to South Africa **(JOUBERT and DE BEER, 2011; VAN WYK, 2011)**. A number of established researchers have paved a way for upcoming young researchers to navigate and launch successful products into the market by learning from their experiences **(GERICKE, 2011; MYBURGH, 2011)**.

The natural product research market is an ever expanding market and is influenced significantly by trends, innovation and societal awareness **(RAMLI, 2015)**. The market is always in need of novel natural product formulations. The South African market, being a developing market, has benefited from this upward global trend with a slight increase in

the number of natural products formulated from medicinal plants **(KOMANE et al., 2015)**.

Some of the drivers contributing to the expansions are consumer preferences and more reliable products attending to the market needs. Therefore, it is sensible to relook at the rich plant biodiversity of South Africa to meet this demand and address some of the challenges encountered with some plant products. For instance, other products would offer benefits of skin lightening without any photo-protective properties and therefore leaving the skin vulnerable to photo-damage **(DLOVA et al., 2015)**. New product interventions could be formulating products that can enhance skin complexion and offer photo-protective properties simultaneously. Product formulations are needed that are safe, tailored for South Africans and formulated from South African plants.

1.2.5. Adverse reactions associated with some products used in the health and beauty industry

In the past decade South Africa has seen a spiralling increase of formulations specifically for beauty enhancement and health maintenance of the skin. Some of the major contributing factors have been modernization and societal awareness of one's appearance. However, many have fallen into the trap of too many products that have swamped the South African market with some studies flagging them for containing potent banned compounds. The target markets of most of the products were western to Asian and not for African skin **(MANELI et al., 2015)**.

The undesired side effects of a number of these products include, but are not limited to, skin burning, skin peeling, skin darkening and formation of skin lesions **(DAVIDS et al., 2016)**. Unfortunately, all these have an undesired consequence on the beauty and health of the skin. Furthermore, these failed products have negatively tainted the cosmeceutical industry. What is more concerning is the fact that despite a number of warnings issued against the usage of these products, a number of people still pursue their usage **(OLUMIDE et al., 2008; MANELI et al., 2015)**. The reluctance of people to not use these products is partly due to not enough alternatives being available on the South African market.

1.3. Aims and objectives of the study

The aim of this project was to document and evaluate the indigenous knowledge on the use of medicinal plants for skincare and beauty by some Xhosa communities in the Eastern Cape Province. The documented knowledge regarding the usages of plants for skincare and beauty will contribute to the national knowledge system of useful plants to the cosmeceutical platform. Plants were evaluated for their ethnopharmacological efficacy on bioassays with relevance to beauty enhancement. The selected plants were assessed for their antimicrobial activity, phenolic content, antioxidant capabilities, photo-protective effect, anti-tyrosinase activity, anti-inflammatory activity and lastly their safety and efficiency was evaluated on a HEM cell line model system. The results of the study will be a guide to the potential of some of the plants for incorporation into cosmeceutical formulations.

Chapter 2

Ethnobotanical survey on plants used for skincare and beauty by Xhosa communities in the Raymond Mhlaba municipality, Amathole district, Eastern Cape Province

2.1. Introduction

The skin, the largest organ surrounding the body, covers all major organs and is greatly influenced by internal and external stimuli, with the face always exposed to these stimuli **(FINK and NEAVE, 2005)**. The health and beauty of the face is affected by the skins structural integrity, skin barrier functions and regenerative potential **(LIAKOU et al., 2013)**. The functional failure of these processes sets in motion a string of events that will lead to beauty deterioration and eventually lead to the aging process **(TIGGES et al., 2014; KRUTMANN et al., 2017)**. Skin health is the principal determinant of individual and societal perception of what constitutes beauty **(SYNNOTT, 1993)**.

Over the last two decades, the global beauty industry has transformed and grown significantly and is estimated to have reached $382 billion in 2010 **(ŁOPACIUK and ŁOBODA, 2013)**. Innovation of new products to meet the demands of consumers and preference towards the use of natural beauty products contributed significantly to the growth **(KUMAR, 2005; LOPES and CASSON, 2007)**. The industry has had an average growth of over 1.5% a year in emerging markets, despite the global economic crisis in 2008. The growth in emerging markets has been influenced heavily by a growing number of the middle class with increased disposable income, market globalization, emerging trends, politics and societal awareness **(RAMLI, 2015)**.

South Africa is one of the emerging markets in the health and beauty industry with many of the leading global companies having satellite centres in the country. The South African cosmetic and beauty industry in 2010 reached roughly around $1.84 billion and

contributed to 1% of the country's gross domestic product (GDP) and has since been amongst the countries top performers in a struggling economy **(INDUSTRIAL DEVELOPMENT, 2017)**. However, many of the beauty products sold in South Africa are mainly imported from other markets **(BOSIU et al., 2015)**. In South Africa, researchers have been reporting on commercialization developments **(STREET and PRINSLOO, 2013)** and the urgent need for full exploration of South African plants to contribute in the global beauty market **(LALL and KISHORE, 2014)**.

The Eastern Cape Province has previously been documented to possess a large biodiversity of plants. Ethnobotanical surveys have been conducted on the different plant uses in the area over the last few decades **(DOLD and COCKS, 2005; OTANG et al., 2012)**. However, there is still a gap in the investigation of the ethnopharmacological application of many of these plants. Therefore, the current ethnobotanical study was aimed at recording plants used for skincare and beauty by Xhosa communities in the Raymond Mhlaba municipality, in the Amathole district.

2.2. Materials and Methods

2.2.1. Study area

The Eastern Cape Province covers an area of approximately 168 966 km^2, and has a population of 6 996 976. It is the second largest province in South Africa by surface area and has the third largest population. The province is one of the poorest in South Africa, with an estimated 72% of the population living below the poverty line **(WESTAWAY, 2012)**. The province is predominantly inhabited by *AmaXhosa* with *isiXhosa* being the main medium of expression. Other clans such as *AmaBhaca, AmaBomvana, AmaPondo, AmaPondomise* and *AbaThembu* also use *isiXhosa* as their medium of expression. A large portion of the province incorporates the former homelands of Transkei and Ciskei. The province is divided into six district municipalities, which are further subdivided into 31 local municipalities.

An ethnobotanical survey on the use of plants for skincare and beauty was conducted in villages in and around the Raymond Mhlaba municipality in the Amathole district in the Eastern Cape Province (Figure 2.1). The Raymond Mhlaba municipality was established by the amalgamation of the Nkonkobe and Nxuba local municipalities following the 2016 municipal elections. The study was conducted in 9 different villages including Alice, Debenek, Fort Beaufort, Hogsback, Khayalethu, KwaMqayisa, Middledrift, Ntselamazi and Seymour.

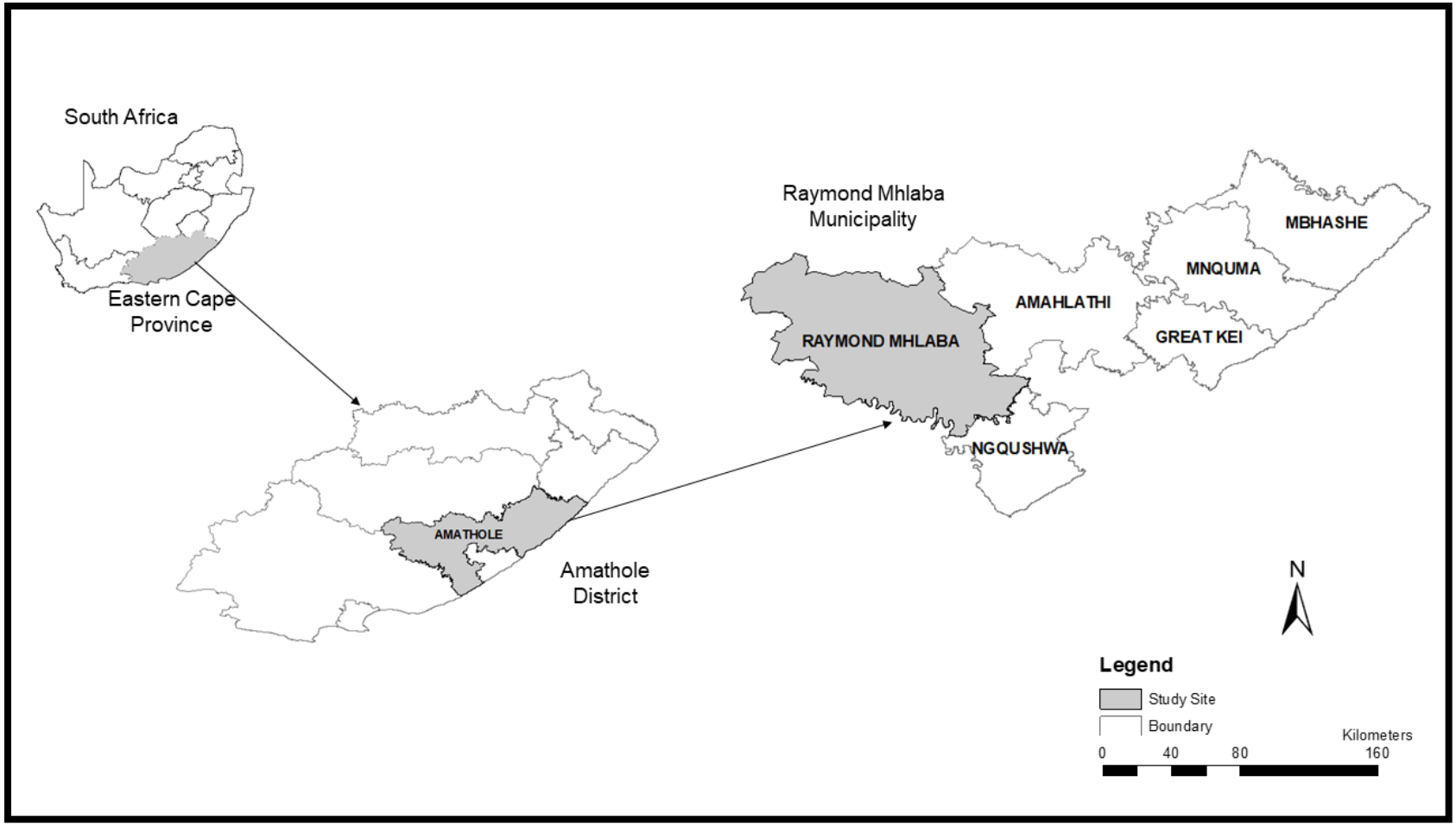

Figure 2.1: Study area map of Raymond Mhlaba municipality in the Amathole district, Eastern Cape Province, South Africa.

2.2.2. Ethnobotanical survey

Knowledge holders were identified by the purposive sampling method, where only participants with knowledge on plants used for beauty and skincare were selected for the study **(PALINKAS et al., 2015)**. Key informants were identified and together with representatives from the local economic development agency (Raymond Mhlaba Economic Development Agency) other participants in the communities were identified. Permission from the office of the local chief was requested prior to the commencement of the survey. Participants signed a consent form prior to the interview process indicating their willingness to participate in the study.

The interviews were conducted in *isiXhosa* using a structured questionnaire with assistance from an interpreter fluent in both *isiXhosa* and English **(ZOHRABI, 2013)**. The data captured on the questionnaires was later translated to English. Where consent was given, audio and video assistance were used during the interview process (Figure 2.2). The interview process was designed such that information on the demographics, names of plants used for skincare and beauty, type of plant, plant part used, method of preparation and administration and frequency of use were collected. The interviews were conducted at the participant's homesteads and included walks to the garden and field showing the mentioned plants (Figure 2.3).

The survey was conducted between May 2015 and November 2016 around villages in the Raymond Mhlaba municipality. From the survey, plants were identified scientifically from their vernacular names according to scientific works of **BHAT and JACOBS**

(1995), VAN WYK et al. (1997) and DOLD and COCKS (1999). The identified plants were validated together with botanist and curator Tony Dold (Rhodes University). Voucher specimens were prepared and deposited at the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg Campus (NU-Herbarium).

2.2.3. Data collection and storage

The plants were grouped according to their family names, type of plant and reported usages. The frequency index (FI) was calculated using the formula:

$$FI = FC/N \times 100$$

Where, FC is the total number of times a plant was mentioned by the knowledge holders and N is the total number of knowledge holders that participated in the study. The data collected from the questionnaire was stored according to the UKZN and Agricultural Research Council (ARC) policies for data storage.

Figure 2.2: Interview process with knowledge holders conducted at their homes (A, C), RMEDA boardroom (B) and outside (D) with data captured on questionnaires and audio devices.

Figure 2.3: Interview process included walks to the garden (A), fields (B) and demonstration (C) with the knowledge holders on method of preparation of some of the plants.

2.3. Results and Discussion

2.3.1. Ethnobotanical survey

A total number of 50 indigenous knowledge holders were interviewed in this study. The participants were aged between 28 and 83 years, with females accounting for 60% and males 40% of the knowledge holders. Results from the study indicate women possess more knowledge on the use of plants for skincare and beauty. The youngest participant in the study was a 28-year-old female who claimed that the knowledge on the use of plants for beauty was passed on to her by the grandmother.

The results can be validated by the fact that women in households are the care givers and therefore likely to pass on the knowledge to their daughters. Other studies have shown that females are more entrusted in passing on the indigenous knowledge than the males **(MADIKIZELA et al., 2012)**. Recent studies have further shown that females are more prone to venture into the beauty industry as they have the advantage of having been passed on knowledge on how to care for their skin **(BOYD, 2000)**.

Table 2.1 represents results for plants used for skincare and beauty by Xhosa communities in the Raymond Mhlaba municipality, Amathole district, Eastern Cape Province. Plants with high frequency index, selected for bioassays, are reported in Table 2.2. A total number of 37 plants from 24 different families were reported to be used for skincare and beauty (Table 2.1). The most represented families were the Asphodelaceae (16%) and the Asteraceae (11%). The Amaranthacea, Amaryllidaceae,

Brassicaceae, Lamiaceae and Rutaceae were represented by 5% for each family (Figure 2.4).

The Asphodelaceae are succulent plants used for various purposes and an important family within the Eastern Cape flora known to thrive in different climatic conditions **(VAN WYK et al., 1997)**. The Asteraceae is an important family with previous reported usage on skin against sores, wounds and burns **(HUTCHINGS, 1989)**. The Asteraceae family includes widely distributed herbaceous plants, with bright and very aromatic flowers **(ACHIKA et al., 2014)**. The plants characteristics are significant when formulating beauty products that are appealing and attractive.

The other families included the Apiaceae, Apocynaceae, Aquifoliaceae, Asparagaceae, Balanophoraceae, Boraginaceae, Cannabaceae, Caprifoliaceae, Caricaceae, Euphorbiaceae, Gunneraceae, Hypoxidaceae, Lauraceae, Moringaceae, Plantaginaceae, Polygonaceae and Rhizophoraceae which were evenly represented at 3% of the reported families (Figure 2.4).

Table 2.1: Plants used for skincare and beauty by Xhosa communities in the Raymond Mhlaba municipality, Amathole district, Eastern Cape Province.

Family name/Scientific name	Xhosa name	Type of plant	Parts used	Method of preparation	Method of administration	Recorded traditional usage	FI
Amaranthaceae *Amaranthus hybridus* L.	Utyuthu	Herbaceous	Leaves	Infusion	Oral ingestion	Improves skin smoothness.	2
Amaranthaceae *Chenopodium album* L.	Imbikicane	Herbaceous	Leaves	Infusion	Topical application	Reduce inflammation.	8
Amaryllidaceae *Allium sativum* L.	Ivimbampunzi	Herbaceous	Bulb	Infusion	Oral ingestion	Improves skin smoothness.	4
Amaryllidaceae *Haemanthus albiflos* Jacq.	Umathunga	Herbaceous	Bulb	Decoction	Oral ingestion	Fleshy bulb taken for wound treatment.	6
Apiaceae *Alepidea amatymbica* Eckl. & Zeyh.	Iqwili	Herbaceous	Leaves	Infusion	Oral ingestion	Treatment of wounds.	2
Apocynaceae *Acokanthera oblongifolia* (Hochst.) Codd	Ubuhlungu	Herbaceous	Leaves	Infusion	Oral ingestion	Improves skin smoothness.	6
Aquifoliaceae *Ilex mitis* (L.) Radlk.	Isidumo	Tree	Bark	Mashing	Topical application	Used as sunblock and for desired skin complexion. Treatment of pimples.	6
Asparagaceae *Albuca setosa* Jacq.	Inqwebeba	Herbaceous	Leaves	Infusion	Oral ingestion	Used for cleansing.	4
Asphodelaceae *Gasteria bicolor* Haw.	Intelezi	Succulent	Leaves	Maceration	Oral ingestion	Leaf juice used for laxative effect.	2
Asphodelaceae *Bulbine abyssinica*	Uyakayakana	Herbaceous	Leaves	Infusion	Oral ingestion	Treatment of wounds.	2

Family name/Scientific name	Xhosa name	Type of plant	Parts used	Method of preparation	Method of administration	Recorded traditional usage	FI
A.Rich.							
Asphodelaceae *Bulbine frutescens* (L.) Willd.	Itswela le nyoka	Herbaceous	Leaves	Infusion	Topical application	Fresh leaf juice applied for wound treatment and skin smoothness.	8
Asphodelaceae *Aloe ferox* Mill.	Ikhala	Succulent	Leaves, oil	Maceration	Oral ingestion, enema	Improves skin complexion, constipation and skin disorders.	20
Asphodelaceae *Aloe ciliaris* Haw.	Ikhala	Succulent	Leaves, oil	Maceration	Oral ingestion, enema	Treatment of constipation.	6
Asphodelaceae *Aloe tenuior* Haw.	Ikhala	Succulent	Leaves	Maceration	Oral ingestion	Improves skin complexion.	6
Asteraceae *Arctotis arctotoides* (L.f.) O.Hoffm	Ubushwa	Herbaceous	Leaves	Mashing	Topical application	Leaf paste applied directly on skin for wound treatment and smoothness.	4
Asteraceae *Sonchus asper* (L.) Hill	Ihlaba	Herbaceous	Leaves	Infusion	Oral ingestion	Improves skin smoothness and inflammation.	4
Asteraceae *Artemisia afra* Jacq. ex Willd.	Umhlonyana	Herbaceous	Leaves	Infusion	Oral ingestion, Inhalation	Improves skin smoothness and used for cough treatment.	2
Asteraceae *Helichrysum petiolare* Hilliard & B.L.Burtt	Impepho	Herbaceous	Leaves	Burning	Inhalation	Bad luck.	4
Balanophoraceae *Sarcophyte sanguinea* Sparrm.	Umavumbuka	Herbaceous	Seed	Mashing	Topical application	Improves skin complexion.	4
Boraginaceae							

Family name/Scientific name	Xhosa name	Type of plant	Parts used	Method of preparation	Method of administration	Recorded traditional usage	FI
Symphytum officinale L.	Izicwe	Herbaceous	Leaves	Infusion	Oral ingestion	Used to reduce inflammation.	6
Brassicaceae *Brassica oleracea* L.	Kale	Herbaceous	Leaves	Infusion	Oral ingestion	Improves skin smoothness.	2
Brassicaceae *Rorippa nasturtium-aquaticum* (L.) Hayek	Uwatala	Herbaceous	Leaves	Infusion	Oral ingestion, topical application	Leaf paste applied for a smooth skin.	6
Cannabaceae *Cannabis sativa* L.	Intsangu	Shrub	Leaves	Infusion	Oral ingestion	Improves skin smoothness and complexion. Treatment of skin cancer.	4
Caprifoliaceae *Scabiosa albanensis* R.A.Dyer	Isilawu	Herbaceous	Roots	Decoction	Bath	Bad luck.	2
Caricaceae *Carica papaya* L.	Paw Paw	Tree	Seeds	Mashing	Topical application	Improves skin smoothness.	4
Euphorbiaceae *Spirostachys africana* Sond.	Umthombothi	Tree	Bark	Mashing	Topical application	Improves skin complexion.	6
Gunneraceae *Gunnera perpensa* L.	Iphuzi	Herbaceous	Roots	Decoction	Oral ingestion	Used for cleansing.	4
Hypoxidaceae *Hypoxis argentea* Harv. ex Baker	Inongwe	Herbaceous	Bulb	Decoction	Oral ingestion	Bad luck.	8
Lamiaceae *Marrubium vulgare* L.	Umhlonyane	Herbaceous	Leaves	Infusion	Oral ingestion	Reduce inflammation.	12
Lamiaceae *Stachys* sp.	Unopepilana	Herbaceous	Roots	Decoction	Topical	Used as sunblock.	2

Family name/Scientific name	Xhosa name	Type of plant	Parts used	Method of preparation	Method of administration	Recorded traditional usage	FI
					application		
Lauraceae *Persea americana* Mill.	Avocado Tree	Tree	Seed	Mashing	Topical application	Used for desired skin complexion.	6
Moringaceae *Moringa oleifera* Lam.	Moringa	Tree	Leaves, seeds	Infusion	Oral ingestion	Good health.	2
Plantaginaceae *Plantago lanceolata* L.	Ubendlela	Herbaceous	Leaves	Infusion	Oral ingestion	Treatment of wounds.	8
Polygonaceae *Emex australis* Steinh.	Inkunzane	Shrub	Leaves	Decoction	Inhalation	Used for cleansing.	4
Rhizophoraceae *Cassipourea flanaganii* (Schinz) Alston.	UmMemezi	Tree	Bark	Mashing	Topical application	Paste used as sunblock and for desired skin complexion.	18
Rutaceae *Clausena anisata* (Willd.) Hook.f. ex Benth.	Iperipes	Shrub	Leaves	Infusion	Oral ingestion	Treatment of constipation.	10
Rutaceae *Ruta graveolens* L.	Ivendrithi	Herbaceous	Leaves	Infusion	Oral ingestion	Treatment of inflammation.	8
Urticaceae *Urtica urens* L.	Uralijan	Herbaceous	Leaves	Infusion	Oral ingestion	Used for treatment of burns.	4

FI - Frequency index

Table 2.2: Plants used for skincare and beauty with high frequency index (FI), selected for bioassays.

Family name/Scientific name	Xhosa name	Voucher specimen	Plant part used	FI
Amaranthaceae *Chenopodium album* L.	Imbikicane	THIBANE 6 NU	Leaves	8
Amaryllidaceae *Haemanthus albiflos* Jacq	Umathunga	THIBANE 9 NU	Bulb	6
Apocynaceae *Acokanthera oblongifolia* (Hochst.) Codd	Ubuhlungu	THIBANE 1 NU	Leaves	6
Aquifoliaceae *Ilex mitis* (L.) Radlk	Isidumo	THIBANE 11 NU	Bark	8
Asphodelaceae *Bulbine frutescens* (L.) Willd	Itswela le nyoka	THIBANE 4 NU	Leaves	8
Asphodelaceae *Aloe ferox* Mill	Ikhala	THIBANE 2 NU	Leaves	20
Asteraceae *Arctotis arctotoides* (L.f) O.Hoffm	Ubushwa	THIBANE 3 NU	Leaves	4
Asteraceae *Sonchus asper* L.	Ihlaba	THIBANE 14 NU	Leaves	4
Boraginaceae *Symphytum officinale* L.	Izicwe	THIBANE 8 NU	Leaves	6
Brassicaceae *Rorippa nasturtium-aquaticum* (L.) Hayek	Uwatala	THIBANE 13 NU	Leaves	6
Lamiaceae *Marrubium vulgare* L.	Umhlonyane	THIBANE 10 NU	Leaves	12

Family name/Scientific name	Xhosa name	Voucher specimen	Plant part used	FI
Plantaginaceae *Plantago lanceolata* L.	Ubendlela	THIBANE 12 NU	Leaves	8
Rhizophoraceae *Cassipourea flanaganii* (Schinz) Alston	UmMemezi	THIBANE 5 NU	Bark	18
Rutaceae *Clausena anisata* (Willd.) Hook.f. ex Benth.	Iperipes	THIBANE 7 NU	Leaves	10
Rutaceae *Ruta graveolens* L.	Ivendrithi	THIBANE 15 NU	Leaves	8
Urticaceae *Urtica urens* L.	Uralijan	THIBANE 16 NU	Leaves	4

NU: Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg

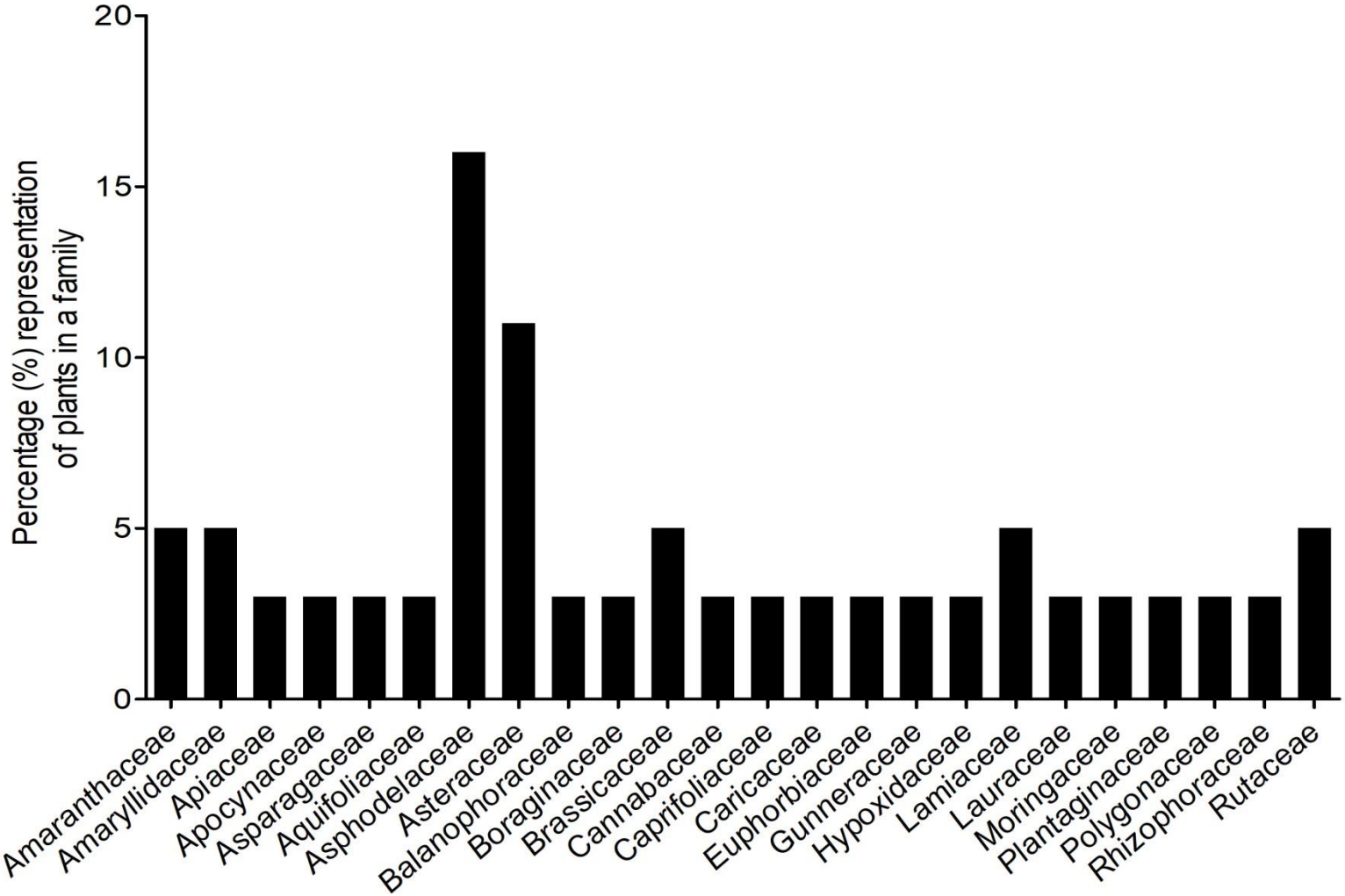

Figure 2.4: Percentage (%) representation of total recorded plants from a family used for skincare and beauty in the Raymond Mhlaba municipality, Eastern Cape Province.

The results from the survey on plant types and plant parts used for skincare and beauty are represented in Figure 2.5. Herbaceous plants were the most used plant type (65%) for skincare and beauty (Figure 2.5B). The second highest plant type reported to be used for skincare and beauty were trees (16%) followed by succulents (11%) and shrubs (8%). The observed variations of plant types used can be accredited to the province having a complex biome made up of the Cape fynbos, shrub lands, subtropical thicket and Karoo semi-desert with a large biodiversity **(LUBKE et al., 1986; PHILLIPSON, 1987)**.

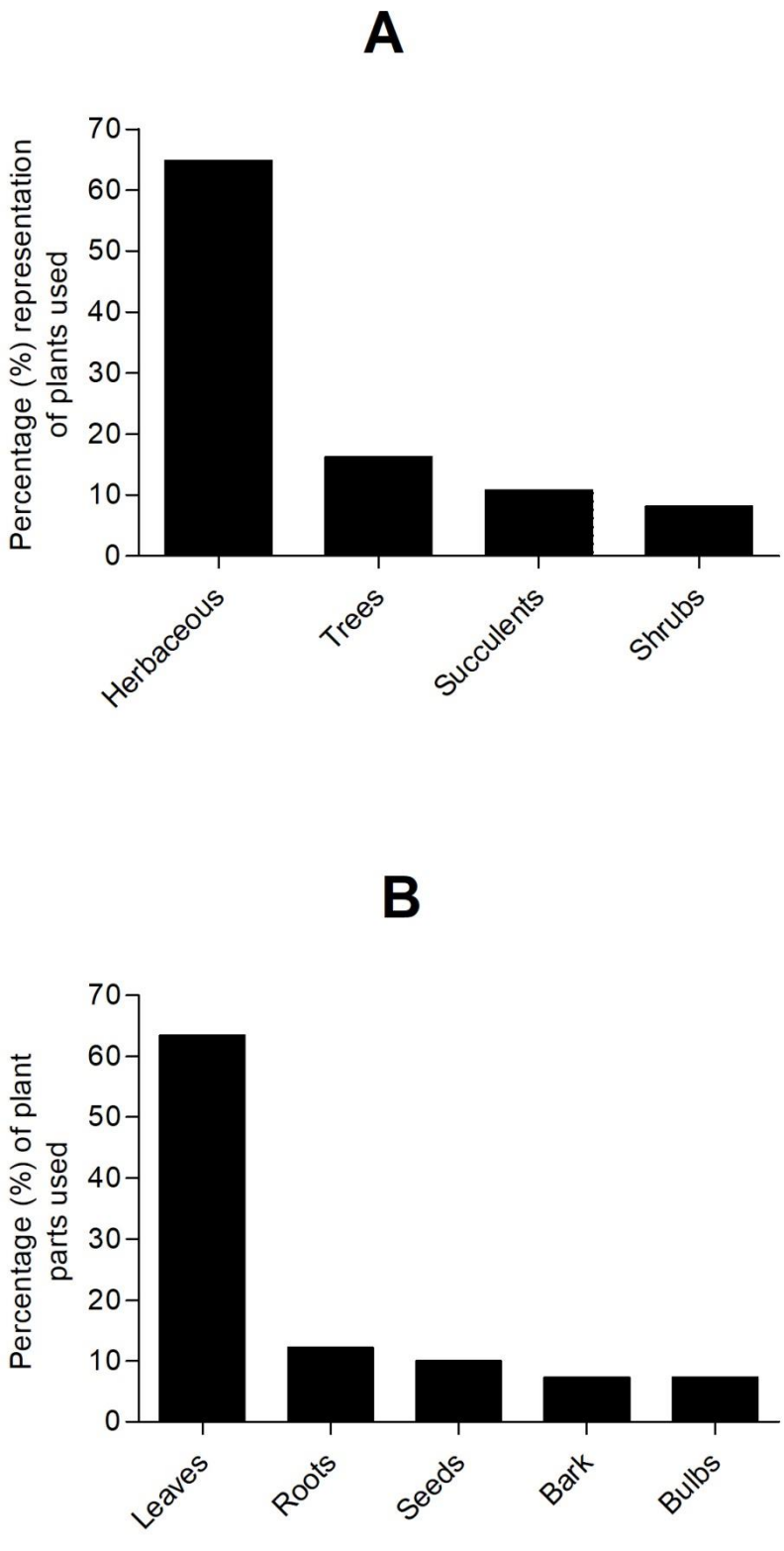

Figure 2.5: Percentage (%) representation of total number of plants (A) recorded during survey, and % number of plant parts used (B) for skincare and beauty.

The leaves (64%) were the most utilized plant parts for skincare and beauty (Figure 2.5B). The observed high usage of leaves can be due to photosynthetic reactions that are localized at the leaves, with primary and secondary metabolites produced during photosynthesis playing a crucial role in the photo-protection abilities of the plant **(EDREVA et al., 2008; BARTWAL et al., 2013)**. Therefore, the favoured use of the leaves for skincare and beauty could be due to the high concentration of these metabolites. Furthermore, the use of leaves is a more sustainable harvesting method and is less destructive to the plant as compared to the use of roots and bark **(WILLIAMS et al., 2013)**. Sustainable methods of harvesting, preparation and administration are important to maintain the activity of the active compounds.

The roots were the second highest (12%) plant part used for skincare and beauty (Figure 2.5B). During osmotic and abiotic stress, secondary metabolites are produced by the plant and are accrued in the roots to offer protection to the plant **(XIONG and ZHU, 2002)**. The observed usage of roots for skincare and beauty can be credited to the accumulated metabolites that can relieve the skin from intracellular oxidative stresses **(MUNNÉ-BOSCH and PEÑUELAS, 2003)**.

Other plant parts used for skincare and beauty included the seeds (10%), bark (7%) and bulbs (7%). Secondary metabolites may be produced during mechanical damage of the plant and are accumulated at the plant's outer cell wall to offer protection **(LEÓN et al., 2001)**. The bulbs and seeds have a storage function in the plant with carbohydrates, proteins, fatty acids and trace elements concentrated at this location. These stored

nutrients have been reported to have health benefits to the skin and therefore the usage of bulbs and seeds for skincare and beauty can be supported.

The highest reported usage of plants was for plants used for improving skin complexion (29%), skin smoothness (21%) and treatment of wounded skin (20%) (Figure 2.6). Plants are used to tone skin complexion and serve as a sunblock and skin lighteners. These were followed by plants used against constipation (10%), bad luck (6%), cleansing (6%), skin disease (4%), skin cancer (2%) and other usages (2%). It was interesting to note that plants used for constipation were mentioned to have health and beauty effects. It was also noted from the survey that plants used against bad luck or misfortunes are believed to enhance a person's look and appearance.

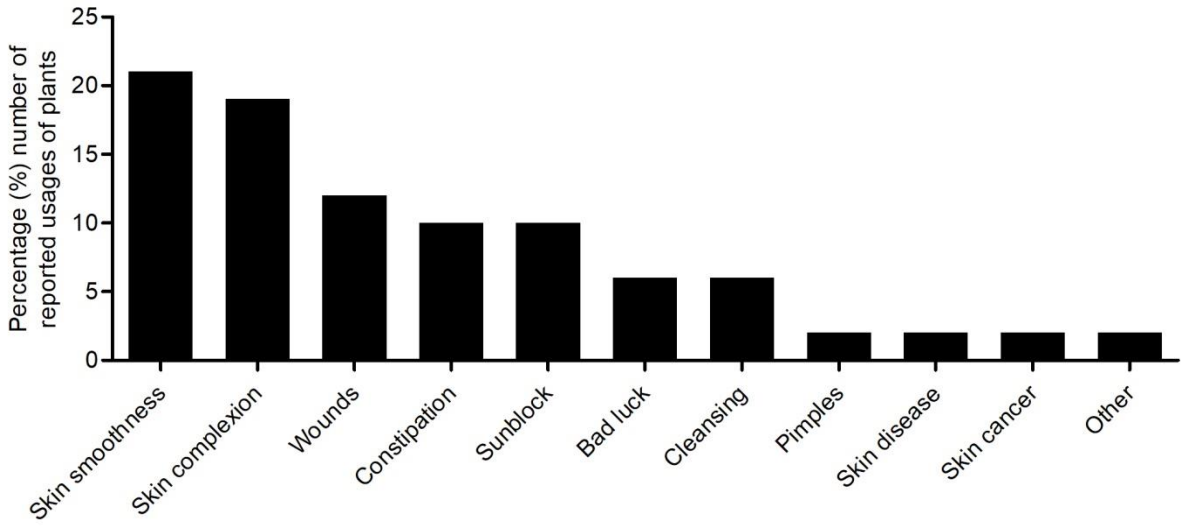

Figure 2.6: Percentage (%) number of reported usages of plants used for skincare and beauty.

Infusion was the method of preparation that was mostly and commonly mentioned to be used (50%), followed by mashing (19%), decoction (17%), maceration (11%) and burning (3%) (Figure 2.7). The observed high use of the infusion method can be linked to more of the cited plants being herbaceous. The infusion method is advantageous as it does not result in the destruction of delicate compounds that can have skincare and beauty effects. The oral ingestion method of administration was the most popular method (59%) followed by topical application (27%), inhalation (7%), enema (5%) and bathing (2%) (Figure 2.8).

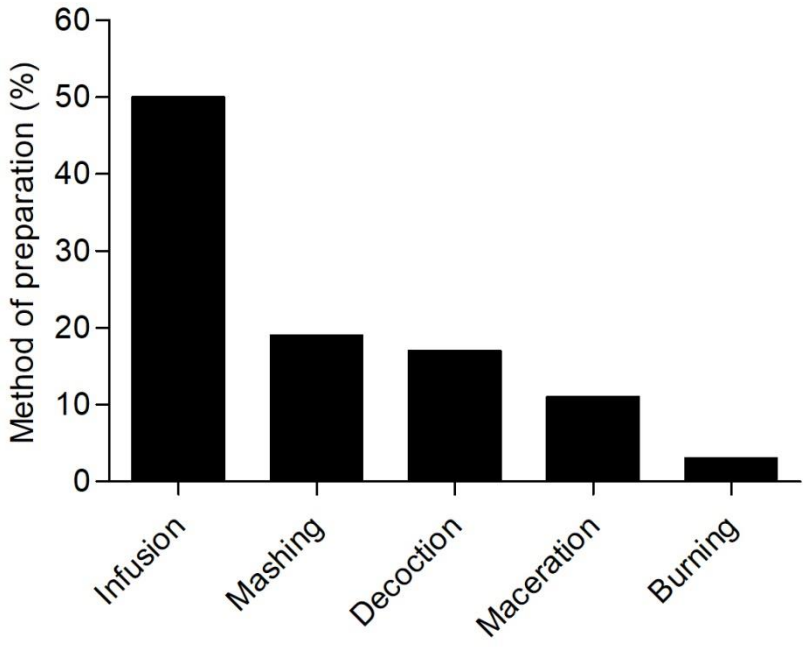

Figure 2.7: Preparation methods of plants used for skincare and beauty as a % recorded.

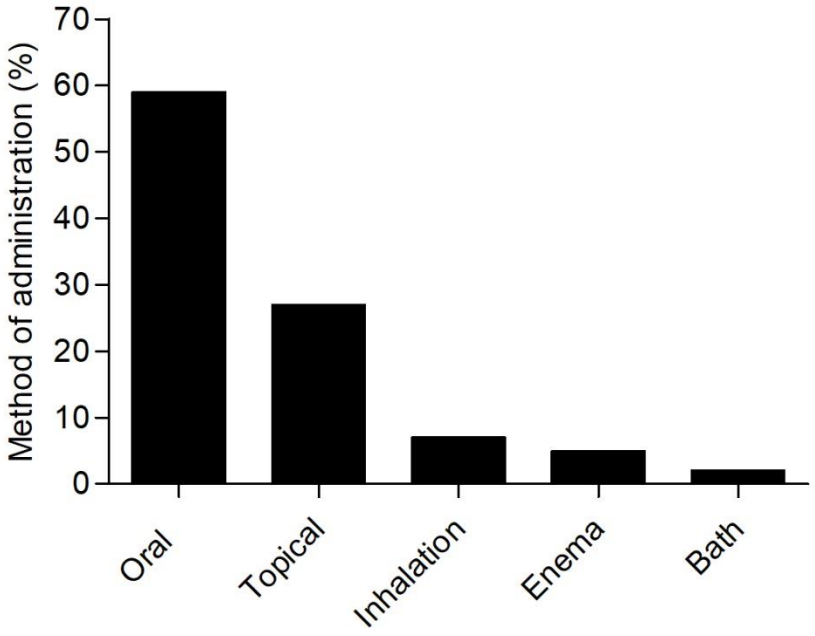

Figure 2.8: Administration methods for plants used for skincare and beauty as a % recorded.

2.4. Conclusions

The stability and success of the beauty industry, even though a global economic meltdown, makes it an attractive industry for resource investment. Novel beauty formulations can be developed from the natural flora of the Eastern Cape Province. This development could alleviate some of the socio-economic challenges facing the province. The province is amongst one of the poorest in South Africa with many living below the breadline. A total of 24 different families of plants used for skincare and beauty were reported. There are a variety of active compounds responsible for the beauty effects of the plants, due to variation in biomolecules between species. The Asteraceae family was the second most represented family and the family includes some widely distributed herbaceous plants, with bright and very aromatic flowers. The plants characteristics are significant when formulating beauty products that are appealing and attractive.

The results from the study indicated leaves as the most utilized plant parts for skincare and beauty related problems. The use of leaves is a more sustainable harvesting method and is less destructive to the plant as compared to the roots and bark. Sustainable methods of harvesting, preparation and administration are important to maintain the activity of the active compounds. It was interesting to note that plants used for skin complexion and smoothness accounted for 50% of the reported plant usages. Formulation of novel beauty products that can be used to tone the skin and offer the desired complexion is of significance since the beauty market is populated with products that have adverse effects on the skin. Many of these products have been reported to

contain compounds that are cancerous and bad for the skin with some banned by the United States (US) food and drugs administration (FDA) agency. Therefore, findings from this study will contribute positively to the South African beauty market in formulating novel natural beauty formulations. The desire to look “beautiful” is desired by many and a safe reliable beauty formulation made from natural products will reduce the risk of encountering products in the market that have adverse effects on the skin.

Chapter 3

Antimicrobial activity of plants used for skincare and beauty

3.1. Introduction

The skin plays host to various microorganism which includes bacteria, fungi and viruses, all of which form part of the microbiota on a healthy skin **(ROSENTHAL et al., 2011)**. The skin is made up of the epidermal and dermal layers with sebaceous glands that are connected to the hair follicles **(LAI-CHEONG and MCGRATH, 2009)**. These soft tissues are mostly the initial infection sites when the delicate balance that exists between the skin and these microorganisms is disturbed **(CHRISTENSEN and BRÜGGEMANN, 2014)**. The disturbance may be due to a breach of the skin surface or when environmental conditions favour one microorganism in the microbiota **(CHILLER et al., 2001; GRICE and SEGRE, 2011)**. The results of when one of the skin commensals outgrows the rest in the microbiota become noticeable on the surface, altering the health and beauty of the skin. Generally, a flaw less skin free of noticeable lesions, eruptions and infections is acceptable as it contributes significantly to a person being perceived as “beautiful” **(FINK and NEAVE, 2005)**.

Infections caused by dermatophytes and infectious skin yeast and bacteria result in skin-related diseases that consequently lead to alterations in skin health and beauty. Dermatophytes belonging to three genera of *Epidermophyton*, *Microsporum* and *Trichophyton*, which invade keratinized tissues of the skin, are responsible for causing skin diseases such as dermatophytosis or ringworm **(NWEZE and EKE, 2017)**. The development of dermatophytosis on the human skin is classified according to the infected body region with tinea faciei indicative of infections to the face and is characterized by a ring-like rash on the skin **(GUPTA et al., 2003)**. *Candida* species

such as *Candida albicans* are commensals on healthy skin and mucosal surfaces, forming part of the skin microflora. However, when the immune system is suppressed the opportunistic yeast is responsible for development of candidiasis, which is characterized by a superficial skin infection **(SAMARANAYAKE, 2009)**.

Bacterial cultures are found both as commensals and pathogens on healthy skin in the same microbiota **(COGEN et al., 2008; GONZALEZ et al., 2011)**. Furthermore, *Bacillus* species are known commensals of the skin **(EARL et al., 2008)**, while bacteria belonging to the genus *Staphylococcus* have been reported to infect the skin and cause skin-related diseases such as cellulitis, erysipelas, bullous impetigo, folliculitis and furunculosis **(LAUBE, 2004; LWASE et al., 2010; OTTO, 2010).** Therefore, the aim of this study was to examine the antimicrobial activity of plants used for skincare and beauty against skin dermatophytes and infectious skin yeasts and bacteria, by determining their minimum inhibitory concentration (MIC) values using the microdilution plate assay.

3.2. Materials and Methods

3.2.1. Plant extract preparation

Plant material (Table 2.2) was dried in an oven at 50 °C for 72 h, or until constant dryness was achieved, and stored at room temperature in brown paper bags until extraction. The dried plant materials were ground into powders and sequentially extracted in 20 mL/g (v/w) with petroleum ether (PE), dichloromethane (DCM), 70% aqueous ethanol (v/v) and water for 1 h in a sonication bath. Extracts were filtered through Whatman No. 1 filter paper and the crude extracts concentrated under vacuum and subsequently dried under a constant flow of cool air.

3.2.2. Antibacterial activity assay

Different solvents of varying polarities were used to determine the antibacterial activity of selected plants used for skincare and beauty. Skin commensals and infectious skin bacteria responsible for causing superficial skin infections and diseases used in the study included the Gram-positive *Bacillus subtilis* ATCC 6051, *Brevibacillus agri* ATCC 51663, *Staphylococcus aureus* ATCC 12600 and *Staphylococcus epidermidis* ATCC 12228, the Gram-negative *Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883. The antibacterial activity of the plant extracts was assessed using the micro-dilution bioassay to determine the minimum inhibitory concentration (MIC) **(ELOFF, 1998a)**.

Plants extracted with organic solvents were re-suspended in 10% dimethyl sulfoxide (DMSO) and ethanol and aqueous extracts were re-suspended in their respective extraction solvent. Bacterial cultures were grown overnight at 37 °C in Mueller-Hinton (MH) broth. The bacterial inoculum (1 x 10^6 colony forming units/mL) was used in the assay. A volume of 100 µL of each extract was added in the first well of the microtiter plate and subsequently diluted down with 100 µL of sterile distilled water to prepare different extract concentrations down the plate. After dilution, 100 µL of bacterial inoculum was added into each well and the plate was incubated at 37 °C for 24 h. After incubation, 50 µL (0.02% w/v) of the growth indicator, *p*-iodonitrotetrazolium chloride (INT), was added in each well and the plate was further incubated at 37 °C for 2 h. A neomycin solution (20 mg/mL) was used as a positive control and 10% DMSO was used as the negative control. The MIC was assayed as the lowest concentrations where the INT remained colourless. The assay was performed in duplicates and repeated three times.

3.2.3. Antifungal activity assay

The infectious skin yeast *Candida albicans* ATCC 10231 and skin dermatophytes *Microsporum canis* ATCC 36299, *Trichophyton mentagrophytes* ATCC 9533 and *Trichophyton tonsurans* ATCC 28942 responsible for causing diseases such as dermatophytosis or ringworm and candidiasis were used in the study. The antifungal activity was determined by the micro-dilution bioassay as detailed by **MASOKO et al. (2007)**.

Fungal yeast cells were grown in yeast malt (YM) broth and incubated at 37 °C for 48 h and the skin dermatophytes were grown in potato dextrose (PD) broth at 35 °C for 4 days **(CLSI, 2008)**. The fungal inoculum (1 x 10^6 colony forming units/mL) prepared with sterile YM and PD broth was used in the assay. A volume of 100 µL of each extract was added in the first well of the microtiter plate and subsequently diluted down with 100 µL of sterile distilled water to prepare different extract concentrations. After dilution, 100 µL of the yeast cells and dermatophytes were added into each well and the plates were incubated at 37 °C for 48 h and 35 °C for 4 days, respectively. After incubation, 50 µL (0.02% w/v) of INT was added to each well with the yeast cells and the plate was further incubated at 37 °C for 2 h. The INT was added at the start of incubation with the skin dermatophytes. Amphotericin B (25 mg/mL) was used as a positive control and the respective extract solvents were used as negative controls. The MIC was assayed as the lowest concentrations where INT remained colourless. The assay was performed in duplicate and repeated three times.

3.2.4. Statistical analysis

The results were calculated as means ± standard deviation. The data was subjected to one-way analysis variance (ANOVA) using IBM Statistical Package for Social Science (SPSS) and means were separated using Fisher's multiple range tests ($p < 0.05$). Means were compared to each other with Fisher's least significant difference (LSD) test.

3.3. Results and Discussion

3.3.1. Antibacterial activity assay

The MIC's results for the antibacterial activity of plants used for skincare and beauty are presented in Table 3.1. In this study, MIC values less than or equal to 1000 µg/mL were considered as having notable activity **(ALIGIANNIS et al., 2001; VAN VUUREN, 2008)**. However, with the recent developments in the antimicrobial activity research, considerations of even lower MIC values are encouraged. To keep up with the latest trends, MIC values less than or equal to 100 µg/mL were considered as having good activity **(VAN VUUREN and HOLL, 2017)**.

Staphylococcus aureus is a known virulent infectious skin bacteria and *S. epidermidis* is an opportunistic bacterium with both being responsible for causing skin-related diseases **(VUONG and OTTO, 2002)**. Ethanol extracts of *C. flanaganii* and *U. urens* expressed good antibacterial activity against the virulent *S. aureus* with MIC values of 98 µg/mL for each. Dichloromethane extract of *A. arctotoides* was also effective in inhibiting *S. aureus* with an MIC value of 98 µg/mL. However, there was notable inhibition of *S. aureus* by DCM extracts of *P. lanceolata* and *R. graveolens* with MIC values of 781 and 390 µg/mL, respectively. Similarly, PE and water extracts of *A. arctotoides* and *C. flanaganii* expressed notable activity with MIC values of 195 and 781 µg/mL, respectively.

The results presented show extracts of *A. arctotoides* and *C. flanaganii* are potent against the virulent *S. aureus.* The infectious *S. epidermidis* was susceptible to ethanol

extract of *R. graveolens* with an MIC value of 98 µg/mL. There was notable activity recorded for ethanol extracts of *A. oblongifolia, A. ferox, A. arctotoides, C. flanaganii, C. album, C. anisata,* and *S. officinale* against *S. epidermidis* with MIC values ranging between 195 and 781 µg/mL. Furthermore, there was notable inhibition of *S. epidermidis* by DCM extracts of *C. flanaganii* and *S. asper* with MIC values of 390 and 781 µg/mL, respectively.

The susceptibility of *S. aureus* and *S. epidermidis* to DCM and ethanol extracts of different plant extracts is significant in skincare and beauty since some of the Staphylococci have been reported to be methicillin-resistant and responsible for the prevalence of skin-related diseases **(OTTO, 2010)**. These can include redness of the skin and warmth under the skin (cellulitis and erysipelas) to deeper infection of the hair follicle and inflammation of the hair nodule (furunculosis) that results in scarring and skin darkening when it heals **(LAUBE, 2004; GUNDERSON and MARTINELLO, 2012)**.

The infectious skin bacterium *E. coli* was observed to be highly susceptible to ethanol extracts of the majority of the tested plants with MIC values less than 100 µg/mL and the only recorded resistance towards *M. vulgare* and *R. nasturtium-aquaticum*. Water extracts of 75% of the tested plants expressed good antibacterial activity against *E. coli* with MIC values less than 100 µg/mL. It was interesting to note that even though *K. pneumoniae* was resistant to ethanol extracts by the majority of the tested plants it was susceptible to PE and DCM extracts of *A. arctotoides* with MIC values of 98 µg/mL for each extract. Petroleum ether extract of *R. graveolens* also expressed good

antibacterial activity against *K. pneumoniae* with an MIC value of 98 µg/mL. However, there was notable activity against *K. pneumoniae* by PE and DCM extracts of *C. anisata* and *H. albiflos* with MIC values of 781 µg/mL for each extract. Dichloromethane extracts of *A. oblongifolia, I. mitis, M. vulgare* and *U. urens* expressed notable activity against *K. pneumoniae*. Similarly, water extracts of *C. flanaganii* and *I. mitis* expressed notable activity against *K. pneumoniae*. Furthermore, ethanol extracts of *A. arctotoides* and *C. flanaganii* expressed notable activity against *K. pneumoniae* with MIC values of 781 µg/mL for each extract.

The infectious skin bacterium *B. agri* was inhibited by ethanol extract of *C. flanaganii* with an MIC value of 98 µg/mL. There was notable antibacterial activity exhibited by ethanol extracts of *A. oblongifolia, A. ferox, A. arctotoides, C. anisata, I. mitis, P. lanceolata, R. nasturtium-aquaticum* and *R. graveolens* against *B. agri* with MIC values ranging between 195 and 781 µg/mL. Water extracts of *A. ferox* and *C. flanaganii* were recorded to have noteworthy antibacterial activity with MIC values of 781 and 390 µg/mL, respectively.

The growth of the skin commensal *B. subtilis* was inhibited by water extracts of *A. ferox* and *C. anisata* with MIC values of 781 µg/mL for each extract. There was notable inhibition of the skin commensal bacteria by DCM extracts of *C. album, P. lanceolata* and *R. graveolens* with MIC values ranging from 195 to 781 µg/mL. Petroleum ether extracts of *A. arctotoides* and *R. graveolens* expressed noteworthy antibacterial activity with MIC values of 390 and 195 µg/mL, respectively. However, *B. subtilis* was inhibited

by ethanol and water extracts of both *C. flanaganii* and *U. urens* with MIC value of 98 µg/mL for each extract. Similarly, DCM extracts of *A. arctotoides* and *C. flanaganii* expressed good antibacterial activity against the skin commensal *B. subtilis* with MIC values of 98 µg/mL for each extract.

The observed susceptibility of *E. coli* and *K. pneumoniae* could be partly attributed to their thin cellular peptidoglycan and lipopolysaccharide layer thereby allowing penetration of the extracts into the cell, being Gram-negative bacteria. Furthermore, the resultant resistance of *B. subtilis, S. aureus* and *S. epidermis* to some of the extracts could be attributed to the thick cellular peptidoglycan with linear polysaccharide chains cross-linked by short peptides associated with Gram-positive bacteria and thereby limiting penetration of active compounds into the cell **(SELTMANN and HOLST, 2002)**. The observed antibacterial activity by different extracts of varying polarities is significant when combating microbial infections and disease as penetration of active compounds depends on their interaction with the bacterial cell structure **(NAZZARO et al., 2013)**.

Combination of some of the extracts with noteworthy antibacterial activity might result in their reduced MIC values and therefore enhance their antibacterial activity. The observed antibacterial activity of some of the extracts at low concentrations will surely reduce the possibility of resistance developing against them. Penetration of the active compounds from the plant extracts through the cell membrane will be beneficial when formulating skin care and beauty products like hygiene soaps, creams and sanitizers to combat the microbial load and invasion on the skin. The results may well highlight the

different plant extracts that can be used for varying formulations for skincare and beauty products.

Table 3.1: Antibacterial activity (MIC values) results of plants used for skincare and beauty.

Medicinal plant	Plant part	Extract	Gram-positive				Gram-negative	
			B.a	B.s	S.a	S.e	E.c	K.p
Acokanthera oblongifolia	Leaves	PE	12500	6250	3125	6250	12500	6250
		DCM	12500	3125	3125	6250	12500	**781**
		Ethanol	**390**	1562	1562	**390**	**98**	1562
		Water	1562	1562	12500	6250	**98**	6250
Aloe ferox	Leaves	PE	6250	6250	12500	6250	6.125	3125
		DCM	6250	6250	12500	6250	12500	6250
		Ethanol	**195**	3125	3125	**781**	**98**	1562
		Water	**781**	**781**	12500	6250	**98**	3125
Arctotis arctotoides	Leaves	PE	6250	**390**	**195**	1562	**390**	**98**
		DCM	3125	**98**	**98**	1562	**781**	**98**
		Ethanol	**390**	**781**	**781**	**781**	**98**	**781**
		Water	3125	3125	3125	1562	**98**	3125
Bulbine frutescence	Leaves	PE	12500	3125	1562	6250	6.125	3125
		DCM	12500	3125	1562	6250	12500	12500
		Ethanol	3125	6250	3125	1562	**98**	3125
		Water	6250	3125	12500	6250	**98**	6250
Cassipourea flanaganii	Bark	PE	3125	6250	6250	1562	3125	1562
		DCM	3125	**98**	6250	**390**	12500	1562
		Ethanol	**98**	**98**	**98**	**781**	**98**	**781**
		Water	**390**	**98**	**781**	6250	**98**	**390**
Chenopodium album	Leaves	PE	6250	3125	6250	6250	12500	3125
		DCM	12500	**781**	3125	6250	12500	3125
		Ethanol	3125	1562	1562	**195**	**98**	3125
		Water	12500	1562	12500	6250	12500	3125
Clausena anisata	Leaves	PE	6250	3125	12500	6250	12500	**781**
		DCM	3125	1562	12500	6250	12500	**781**
		Ethanol	**781**	3125	6250	**781**	**98**	1562
		Water	12500	**781**	12500	6250	12500	3125
Haemanthus albiflos	Bulb	PE	12500	3125	6250	6250	6250	**781**
		DCM	12500	1562	3125	6250	12500	**781**
		Ethanol	6250	6250	6250	6250	**98**	1562
		Water	3125	6250	6250	6250	**98**	3125

Medicinal plant	Plant part	Extract	Gram-positive				Gram-negative	
			B.a	**B.s**	**S.a**	**S.e**	**E.c**	**K.p**
Illex mitis	Bark	PE	6250	6250	6250	6250	6250	6250
		DCM	12500	6250	12500	6250	12500	**781**
		Ethanol	**390**	6250	6250	1562	**98**	1562
		Water	6250	1562	12500	6250	**98**	**781**
Marrubium vulgare	Leaves	PE	6250	6250	6250	6250	6250	1562
		DCM	12500	3125	3125	6250	12500	**781**
		Ethanol	12500	6250	6250	6250	3125	1562
		Water	12500	6250	12500	6250	**98**	3125
Plantago lanceolata	Leaves	PE	12500	3125	6250	6250	6250	12500
		DCM	12500	**781**	**781**	6250	**781**	12500
		Ethanol	**781**	6250	6250	1562	**98**	12500
		Water	3125	3125	6250	6250	**98**	12500
Rorippa nasturtium-aquaticum	Leaves	PE	6250	12500	6250	6250	6250	12500
		DCM	12500	6250	12500	6250	12500	6250
		Ethanol	**781**	6250	6250	1562	3125	1562
		Water	3125	6250	12500	6250	12500	3125
Ruta graveolens	Leaves	PE	6250	**195**	1562	6250	3125	**98**
		DCM	12500	**195**	**390**	6250	**781**	3125
		Ethanol	**195**	3125	3125	**98**	**98**	1562
		Water	6250	3125	12500	6250	**98**	1562
Sonchus asper	Leaves	PE	6250	6250	6250	6250	6250	3125
		DCM	6250	1562	3125	**781**	12500	1562
		Ethanol	1562	6250	3125	1562	**98**	1562
		Water	1562	3125	12500	6250	**98**	3125
Symphytum officinale	Leaves	PE	12500	1562	12500	6250	3125	12500
		DCM	12500	1562	3125	6250	12500	3125
		Ethanol	3125	6250	3125	**781**	**98**	3125
		Water	1562	3125	12500	6250	**98**	6250
Urtica urens	Leaves	PE	12500	6250	3125	6250	12500	6250
		DCM	12500	1562	1562	6250	12500	**781**
		Ethanol	1562	**98**	**98**	1562	**98**	1562
		Water	12500	**98**	3125	6250	6250	6250
Neomycin			**39**	**39**	**39**	**39**	**39**	**39**

PE – Petroleum ether, DCM – Dichloromethane. B.a – *Brevibacillus agri*, B.s – *Bacillus subtilis*, S.a – *Staphylococcus aureus*, S.e – *Staphylococcus epidermidis*, E.c – *Escherichia coli*, K.p – *Klebsiella pneumoniae*. Results expressed as µg/mL and extracts with MIC values highlighted in bold are considered to have notable activity (MIC ≤ 1000 µg/mL) and good activity (MIC ≤ 100 µg/mL).

3.3.2. Antifungal activity assay

The MIC results for the antifungal activity of selected plant extracts are presented in Table 3.2. Infectious yeasts and skin dermatophytes lead to skin-related diseases such as candidiasis and dermatophytosis and thereby alter the health and beauty of the skin **(LAUBE, 2004)**. Ethanol extracts of *A. arctotoides* and *C. flanaganii* were observed to be potent and yielded notable antifungal activity against the infectious skin yeast *C. albicans* and *dermatophytes, M. canis, T. mentagrophytes* and *T. tonsurans* with MIC values ranging between 390 and 781 µg/mL.

Interestingly, water extracts of *C. flanaganii* expressed noteworthy antifungal activity against infectious skin yeast *C. albicans* and dermatophyte *T. mentagrophytes* with MIC values of 390 and 781 µg/mL, respectively. Water extracts of *I. mitis* also inhibited the growth of dermatophyte *M. canis* with an MIC value of 390 µg/mL. Traditionally, the ground powder of *C. flanaganii* and *I. mitis* are mixed with water and applied topically. The results from this study have been able to validate the plants traditional method of preparation and successful use **(ELOFF, 1998b; DOLD and COCKS, 2005)**.

Extracts of *A. arctotoides* have been previously reported to be active against the opportunistic infectious skin yeast *C. albicans* **(OTANG et al., 2012)**. The potency of the plant in inhibiting *C. albicans* was further presented in the current study with MIC values of 781 and 390 µg/mL for PE and DCM extracts, respectively. There was notable inhibition of the growth of *C. albicans* by DCM extracts of *R. graveolens* with an MIC value of 781 µg/mL.

Furthermore, *M. canis* was susceptible to treatment by water extracts of *A. arctotoides* with MIC an value of 781 µg/mL. Extracts of *A. arctotoides* were observed to have notable activity against infectious skin yeast and dermatophytes at varying polarities. Dichloromethane extracts of *A. oblongifolia* and *C. flanaganii* inhibited the growth of *M. canis* and *T. tonsurans* with MIC values of 781 µg/mL for each extract. The growth of *M. canis* could further be inhibited by ethanol extracts of *A. oblongifolia* with an MIC value of 781 µg/mL. Petroleum ether extracts of *C. flanaganii* inhibited the growth of *M. canis* with an MIC value of 781 µg/mL. The observed antifungal activity by different extracts of varying polarities is significant when formulating products that can combat fungal invasions and assist in maintaining the skin health and beauty.

In general, the infectious skin yeast and skin dermatophytes were less susceptible to the tested plant extracts with inhibition mostly observed at higher concentrations. The antifungal activity was observed to be more notable as the tested plant extracts could not inhibit the infectious yeast and dermatophytes with an MIC less than 100 µg/mL. Combination of plant extracts with notable antifungal activity might contribute to lower antifungal concentrations being observed.

Table 3.3: Antifungal activity (MIC values) results of plants used for skincare and beauty.

Medicinal plant	Plant part	Extract	C.a	M.c	T.m	T.t
Acokanthera oblongifolia	Leaves	PE	6250	3125	1562	3125
		DCM	1562	**781**	1562	**781**
		Ethanol	3125	**781**	1562	1562
		Water	1562	6250	12500	1562
Aloe ferox	Leaves	PE	12500	1562	3125	1562
		DCM	6250	6250	3125	3125
		Ethanol	1562	6250	1562	6250
		Water	6250	3125	3125	1562
Arctotis arctotoides	Leaves	PE	**781**	1562	3125	6250
		DCM	**390**	1562	1562	6250
		Ethanol	**781**	**781**	**781**	**781**
		Water	1562	**781**	1562	3125
Bulbine frutescence	Leaves	PE	6250	6250	6250	3125
		DCM	3125	6250	6250	3125
		Ethanol	1562	1562	1562	3125
		Water	1562	12500	3125	6250
Cassipourea flanaganii	Bark	PE	3125	**781**	1562	1562
		DCM	1562	**781**	1562	**781**
		Ethanol	**390**	**390**	**390**	**390**
		Water	**390**	1562	**781**	1562
Chenopodium album	Leaves	PE	6250	3125	3125	6250
		DCM	3125	3125	3125	3125
		Ethanol	3125	3125	1562	1562
		Water	1562	6250	12500	12500
Clausena anisata	Leaves	PE	3125	3125	6250	3125
		DCM	1562	6250	6250	1562
		Ethanol	1562	1562	3125	1562
		Water	1562	12500	12500	6250
Haemanthus albiflos	Bulb	PE	3125	3125	3125	3125
		DCM	1562	1562	3125	1562
		Ethanol	1562	1562	6250	3125
		Water	3125	6250	12500	12500
Illex mitis	Bark	PE	6250	3125	6250	6250
		DCM	3125	6250	3125	3125
		Ethanol	3125	6250	**781**	3125
		Water	1562	**390**	1562	3125
Marrubium vulgare	Leaves	PE	3125	3125	6250	6250
		DCM	3125	6250	6250	6250
		Ethanol	3125	6250	6250	3125
		Water	1562	12500	12500	6250
Plantago lanceolata	Leaves	PE	3125	3125	3125	6250
		DCM	3125	3125	3125	3125
		Ethanol	1562	1562	1562	3125
		Water	3125	3125	3125	12500
Rorippa nasturtium-aquaticum	Leaves	PE	3125	3125	3125	3125
		DCM	3125	6250	6250	1562
		Ethanol	1562	6250	1562	1562
		Water	1562	6250	12500	6250

Medicinal plant	Plant part	Extract	C.a	M.c	T.m	T.t
Ruta graveolens	Leaves	PE	3125	3125	3125	6250
		DCM	**781**	6250	1562	1562
		Ethanol	1562	3125	3125	1562
		Water	3125	3125	12500	1562
Sonchus asper	Leaves	PE	6250	6250	6250	3125
		DCM	3125	1562	1562	1562
		Ethanol	1562	3125	3125	1562
		Water	1562	3125	6250	6250
Symphytum officinale	Leaves	PE	6250	6250	6250	6250
		DCM	3125	6250	6250	6250
		Ethanol	1562	1562	1562	3125
		Water	1562	3125	3125	12500
Urtica urens	Leaves	PE	6250	3125	3125	3125
		DCM	3125	1562	1562	1562
		Ethanol	3125	3125	3125	3125
		Water	1562	6250	6250	6250
Amphotericin B			**48**	**48**	**48**	**48**

PE – Petroleum ether, DCM – Dichloromethane. C.a – *Candida albicans*, M.c – *Micrococcus canis*, T.m – *Trichophyton mentagrophytes,* T.t – *Trichophyton tonsurans*. Results expressed as µg/mL and extracts with MIC values highlighted in bold are considered to have notable activity (MIC ≤ 1000 µg/mL) and good activity (MIC ≤ 100 µg/mL).

3.4. Conclusions

The results from this study indicated ethanol extracts of *A. arctotoides* and *C. flanaganii* as potent extracts for inhibiting activity of all tested microorganisms. The inhibitory activity was recorded both at lower and higher concentrations. At concentrations less than 100 µg/mL, ethanol and water extracts of *A. arctotoides* could inhibit the growth of *E. coli.* Ethanol extracts of *C. flanaganii* inhibited growth of *B. subtilis, S. aureus, E. coli* and *B. agri.* Inhibition by other extracts was observed at concentrations more than 100 µg/mL for the other microorganisms. The observed potency of these extracts is significant when combating microorganisms that can alter the health and beauty of the skin through proliferation of diseases.

Novel natural compounds can be isolated from these plant extracts for the skincare and beauty industry. It was interesting to note that water extracts of *C. flanaganii* could inhibit infectious skin bacteria *B. subtilis, B. agri, E. coli, K. pneumoniae* and *S. aureus* with MIC values ranging between 98 and 781 µg/mL. Water extracts of *C. flanaganii* were not only active against skin infectious bacteria but also against the infectious yeast *C. albicans* and dermatophyte *T. mentagrophytes*. In general, dermatophytes *M. canis, T. mentagrophytes* and *T. tonsurans* were resistant to many of the tested plant extracts while the skin commensal, *E. coli* was susceptible to ethanol extracts of 87% of the tested plants. The antimicrobial activity of the plant extracts varied with the different polarities of the extracts.

Chapter 4

Evaluation of plants for their phenolic content, antioxidant activity, photo-protective effect and anti-tyrosinase activity

4.1. Introduction

Phenolic compounds are secondary metabolites produced by plants and are found in different parts of the plant, which includes the leaves, flowers, fruits, seeds, roots and bark layers **(GHASEMZADEH and GHASEMZADEH, 2011)**. The main function of these secondary metabolites is to protect the plant against internal and external stresses, infections and disease, ultraviolet (UV) radiation, assist in environmental adaptation and form part of cellular signal transduction **(BECKMAN, 2000; VERVERIDIS et al., 2007; BARTWAL et al., 2013)**. The diversity of plant phenols in terms of structure, substitutes and their degree of hydroxylation and polymerization determines their activity in the plant **(RICE-EVANS et al., 1995; KÄHKÖNEN et al., 1999; KATALINIC et al., 2006)**. Phenolic compounds are classified according to their chemical structure and are divided into phenols and polyphenols **(LIMA et al., 2014)**. Phenols are simple structures with low molecular weight, while polyphenols are characterized by multiple linked phenol groups with relatively high molecular weight **(QUIDEAU et al., 2011)**.

The prolonged exposure of the skin to UV radiation has undesirable consequences and contributes significantly to alterations in the health and beauty of the skin **(FARAGE et al., 2008)**. Ultraviolet radiation is divided into 3 categories which includes UVA (320 – 400nm), UVB (280 – 320nm) and UVC (200 – 280nm). The UVC radiation is mostly absorbed by the ozone layer and will not reach the earth's surface and therefore, has no significant biological implications. Ultraviolet-B has more energy than UVA and mostly contributes to the harmful effect of UV radiation. The UVB radiation has an immediate effect on the skin as it directly reacts with proteins, lipids and nucleic acids in the

epidermis while UVA penetrates deeper into the skin and initiates oxidative damage through generation of ROS **(GIL and KIM, 2000; KAPOOR and SARAF, 2009; MISHRA et al., 2011)**.

The oxidative damage as a result of UV-induced damage and the disturbance of the delicate balance that exists between oxidants and antioxidants is responsible for proliferation of many skin-associated health and beauty problems. **(BICKERS and ATHAR, 2006; SHINDE et al., 2012; KAMMEYER and LUITEN, 2015)**. Furthermore, the accumulation of damaged lipids, proteins and nucleic acids due to oxidative damage has a negative impact on the skin **(FARAGE et al., 2008; LORENCINI et al., 2014)**. These damaged cellular components are highly reactive and can further react with other components forming a never-ending cycle and eventually leading to formation of wrinkles, skin infections, dark spots through enhancing melanin biosynthesis and the premature aging of the skin **(DRÖGE, 2002; CÉSARINI et al., 2003)**.

Phenolic compounds have a beneficial impact on biological systems due to the subsequent stability they bring about in the oxidant-antioxidant phenomenon **(ATOUI et al., 2005; DAI and MUMPER, 2010; MUKHERJEE et al., 2011)**. The presence of antioxidants from plants has been shown to reduce the undesirable consequences of oxidants generated within the skin bilayer. Antioxidants derived from phenolic acids and flavonoids play a significant role by influencing physiological processes to maintain skin beauty and health **(RATZ-ŁYKO et al., 2015; TUNDIS et al., 2015)**. These plant secondary metabolites impart their beneficial activity to the skin due to their ability to act

as oxidant quenchers, lipid peroxide decomposers, metal chelators, oxidative enzyme inhibitors and absorbers of UV radiation **(PISOSCHI and POP, 2015)**. Therefore, the aim of the current study was to evaluate plants used for skincare and beauty for their phenolic content, antioxidant potential, photo-protective effect and anti-tyrosinase activity.

4.2. Materials and Methods

4.2.1. Plant extract preparation

Plant material (Table 2.2) was dried in an oven at 50 °C for 72 h, or until constant dryness was achieved, and stored at room temperature in brown paper bags until extraction. The dried plant material was ground into powders and extracted in 20 mL/g (v/w) of different solvents in a sonication bath at room temperature, based on the assay. Plant extracts for the phytochemical content determination and antioxidant activity assays were prepared by extracting with 50% aqueous methanol (v/v) for 30 min. For photo protective effect and anti-tyrosinase activity assays, extracts were prepared by extracting with 70% aqueous ethanol (v/v) for 1 h. All extracts were filtered through Whatman No. 1 filter paper and the crude extracts concentrated under vacuum and subsequently dried under a constant flow of cool air.

4.2.2. Phenolic content determination

4.2.2.1. Total phenolic content determination

Total phenolic content was determined using the Folin Ciocalteu (Folin C) method as described by **MAKKAR (1999)**. Briefly, 950 µL of sterile distilled water was added to 50 µL of extract in a test tube to a final volume of 1 mL. A volume of 500 µL 1N Folin C reagent and 2.5 mL 2% sodium carbonate solution (w/v) was added to the solution and incubated at room temperature for 40 min. Absorbance readings were measured at 725 nm using a spectrophotometer (Spectroquant® Pharo 300, Darmstadt). Gallic acid was used as a positive control and a reaction mixture containing 50% aqueous methanol

(v/v) instead of the sample served as the negative control. The total phenolic content was expressed as Gallic acid equivalents (GAE). The assay was performed in duplicate and repeated three times.

4.2.2.2. Flavonoid content determination

Flavonoid content was determined as described by **MAKKAR (1999)**. Briefly, 950 µL of sterile distilled water was added to 50 µL of extract in a test tube to a final volume of 1 mL. A volume of 2.5 mL methanol:HCl (5:1 v/v) and 2.5 mL vanillin reagent (1%, w/v) were added to the solution and incubated in a 30 °C water bath for 20 min. Absorbance readings were measured at 500 nm using a spectrophotometer (Spectroquant® Pharo 300, Darmstadt). Catechin was used as a positive control and a reaction mixture containing 50% aqueous methanol (v/v) instead of the sample served as the negative control. The flavonoid content was expressed as catechin equivalents (CE). The assay was performed in duplicate and repeated three times.

4.2.3. Antioxidant activity assay

4.2.3.1. DPPH (2,2-diphenyl-β-picrylhydrazyl) radical scavenging activity assay

The antioxidant potential of the plant extracts was performed by evaluating their DPPH radical scavenging capabilities as described by **KARIOTI et al. (2004)**. The plant extracts were re-suspended in 50% aqueous methanol (v/v) to an initial concentration of 50 mg/mL and two-fold serially diluted down a 96-well microtiter plate. Fifteen microliters of the plant extract was diluted with 735 µL of methanol and then added to a

DPPH solution (750 µL, 0.1 mM) to give a DPPH solution reaction mixture (1.5 mL, 500 µM). The reaction mixtures were prepared under dim light and incubated in the dark at room temperature for 30 min. L-Ascorbic acid was used as a positive control and a reaction mixture without any plant extract served as the negative control. The absorbance of the reaction mixture was read at 492 nm using a microplate reader (Optic Iveymen® System 2100-C). The free radical scavenging activity (RSA) as determined by the discoloration of the DPPH solution was calculated according to the formula:

$$\% \text{ RSA} = 100 \times (1 - A_E/A_D)$$

Where, A_E is the absorbance of the reaction mixture containing the sample extract/standard antioxidant and A_D is the absorbance of the DPPH solution only. The percentage RSA was plotted against the plant extract concentration and the IC_{50} determined from the normalized logarithmic regression curve. The assay was performed in duplicate and repeated three times.

4.2.3.2. Ferric reducing antioxidant power (FRAP) activity assay

The ferric reducing power of the plant extracts was determined using the method outlined by **LIM et al. (2009)**. The reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence hydrogenated atoms was used to measure the antioxidant potential of the plant extracts. A volume of 30 µL of plant extracts at an initial concentration of 6.25 mg/mL was added into a 96-well microtiter plate and two-fold serially diluted down the plate. A volume of 40 µL potassium phosphate buffer (0.2 M, pH 7.2) and 40 µL

potassium ferricyanide (1% in phosphate buffer, w/v) were added to each well of the microtiter plate with 50% aqueous methanol (v/v). The microtiter plate was covered with aluminium foil and incubated at 50 °C for 20 min.

After the incubation period, 40 µL trichloroacetic acid (TCA) (10% in phosphate buffer, w/v), 150 µL distilled water and 30 µL ferric chloride ($FeCl_3$) (0.1% in phosphate buffer, w/v) were added to the microtiter plate and the plate further incubated at room temperature for 30 min in the dark. L-Ascorbic acid was the standard antioxidant used as a positive control and methanol was used as a negative control. The absorbance was measured at 630 nm using a microplate reader (Optic Iveymen® System, Model 2100-C). The FRAP percentage inhibition was plotted against the plant extract concentration and the IC_{50} determined from the normalized logarithmic regression curve. The assay was performed in duplicate and repeated three times.

4.2.3.3. *β*-carotene-linoleic acid model system (CLAMS)

The coupled inhibition of *β*-carotene bleaching and linoleic acid oxidation was measured using the CLAMS as detailed by **AMAROWICZ et al. (2004)**. The CLAMS measure the ability of a test solution to prevent the coupled bleaching and oxidation of *β*-carotene and linoleic acid, respectively in emulsified aqueous systems. The emulsion loses its orange colour due to the reaction with radicals but this process can be inhibited by antioxidants. Plant extracts and a standard antioxidant BHT (2,6-di-tert-butyl-4-methylphenol) at an initial concentration of 6.25 mg/mL were used.

A stock solution of *β*-carotene-linoleic acid was prepared in a brown Schott bottle by dissolving 10 mg *β*-carotene in 10 mL chloroform. Excess chloroform was evaporated under vacuum, leaving a thin film of *β*-carotene and immediately adding 200 µL linoleic acid and 2 mL Tween 20. Aerated distilled water (497.8 mL) was added and vigorously mixed to form an orange-coloured emulsion with a final *β*-carotene concentration of 20 mg/mL. A 4.8 mL aliquot of the emulsion was dispensed into each test tube with 200 µL of the plant extracts/antioxidant added, giving a final concentration of 250 µg/mL in the tube. Absorbance for each reaction was measured immediately (t = 0) at 470 nm and incubated at 50 °C and measurements taken every 30 min for 2 hours using a microplate reader (Optic Iveymen® System, Model 2100-C). BHT was used as positive control and a solution with methanol instead of plant extract served as the negative control. Tween 20 solution was used to blank the spectrophotometer. The assay was performed in duplicate and repeated three times. The rate of *β*-carotene bleaching was calculated using the formula:

$$\text{Rate of bleaching } (R) = \ln (A_{t=0}/A_{t=t}) \times 1/t$$

Where, $A_{t=0}$ is the absorbance of the emulsion at 0 minute; and $A_{t=t}$ is the absorbance of the emulsion at 30, 60, 90 and 120 min. The average rates of *β*-carotene bleaching were then calculated based on rates at 30, 60 and 90 min. The calculated average rates were used to determine the antioxidant activity (ANT) of the plant extracts and where expressed as percentage inhibition of the rate of *β*-carotene bleaching using the formula:

$$\% \text{ ANT} = (R_{control} - R_{sample})/R_{control} \times 100$$

Where, $R_{control}$ and R_{sample} represent the respective average *β*-carotene bleaching rates for the negative control and plant extracts. Antioxidant activity was further expressed as the oxidation rate ratio (ORR) using the formula:

$$ORR = R_{sample}/R_{control}$$

4.2.4. Photo-protective effect studies

The photo-protective effect of the plant extracts was determined according to **MALSAWMTLUANGI et al. (2013)**, with slight modifications. The photo protective effect of the plant extracts effectively measures the sun protection factor (SPF) which is the ratio of UV radiation required to produce minimal erythema dose (MED) in protected skin to unprotected skin. The MED is the amount of UV radiation that will produce minimal erythema or sunburn on unprotected skin. The higher the SPF values the more protection from sunburn.

Extracts were re-suspended in ethanol to a concentration of 0.5 mg/mL (500 ppm). The UV light absorption capacity of the extracts was measured between 290 – 320 nm at 5 nm intervals using a UV-vis light spectrophotometer (Spectroquant® Pharo 300, Darmstadt). Ethanol was used as a blank for the spectrophotometer. The absorbance

readings were used to calculate the SPF values according to Mansur's mathematical equation below **(MANSUR et al., 1986)**.

$$SPF = CF \times \Sigma 290^{320} \times EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where CF, is the correction factor (10); EE (λ) is the erythmogenic effect of radiation with wavelength λ; I (λ) is the value at wavelength λ solar intensity spectrum. EE × I are constants determined by **SAYRE et al. (1979)**. The assay was performed in duplicate and repeated three times.

4.2.5. Anti-tyrosinase activity assay

The inhibition of tyrosinase activity was determined using the dopachrome method with 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a substrate as detailed by **MOMTAZ et al. (2008)**, with slight modifications. Briefly, plant extracts were re-suspended in dimethyl sulfoxide (DMSO) to a final concentration of 20 mg/mL. The extracts were diluted to an initial concentration of 400 µg/mL in potassium phosphate buffer (0.1 M, pH 6.5) and two-fold serially diluted down the plate. In a 96-well microtiter plate, 70 µL of the plant extract was added followed by 30 µL of the mushroom tyrosinase enzyme (333 units/mL in potassium phosphate buffer, 0.1 M; pH 6.5).

The reaction mixture was incubated for 10 min at room temperature. After incubation, 110 µL of 12 mM L-DOPA was added to each well to initiate the reaction. Extracts with all compounds, except for the substrate, were used as negative control and kojic acid

was used as a positive control. The microtiter plate was further incubated for 45 min at room temperature and the absorbance was read at 492 nm on a microplate reader (Optic Iveymen® System, Model 2100-C). The percentage inhibition of the tyrosinase enzyme was calculated using the formula;

$$\% \text{ tyrosinase inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

The % tyrosinase inhibition was plotted against the plant extract concentration and the IC_{50} determined from the normalised logarithmic regression curve. The experiment was performed in duplicate and repeated three times.

4.2.6. Statistical analysis

The results were calculated as means ± standard deviation. The data was subjected to one-way analysis variance (ANOVA) using IBM Statistical Package for Social Science (SPSS) and means were separated using Fisher's multiple range tests ($p < 0.05$). Means were compared to each other with Fisher's least significant difference (LSD) test.

4.3. Results and Discussion

4.3.1. Phenolic content determination

The total phenolic content assay specifically measures the total number of phenolic compounds present in the extract which includes simple phenols and polyphenols. Flavonoids are a group of polyphenolic compounds and share the same C_6-C_3-C_6 structural backbone consisting of two aromatic rings and a heterocyclic ring that contains one oxygen moiety **(WINK and SCHIMMER, 2010)**. Polyphenols are structurally more diverse than simple phenols with linked phenol groups and therefore ought to have relatively high molecular weight **(SCALBERT et al., 2002)**. The biochemical activities of flavonoids depend on their chemical structure, hydroxylation, substitutes and degree of polymerization. The presence of these diverse structures results in polyphenolic compounds like flavonoids with increased antioxidant capabilities **(HEIM et al., 2002; KUMAR and PANDEY, 2013)**.

The total phenolic content for *C. flanaganii, I. mitis* and *A. ferox* was significantly higher when compared to all the other extracts (Figure 4.1). The recorded values were 50.34 ± 1.27, 41.28 ± 2.07 and 37.87 ± 2.17 mgGAE/g, respectively. The results for *A. ferox* would be expected since the plant has been extensively studied and reported to have significant levels of anthrones (Aloin A and B), chromones (Aloesin and Aloesin A) and other phenolic compounds **(FRUM and VILJOEN, 2006; VAN WYK et al., 2009; CHEN et al., 2012; KANAMA et al., 2015)**. The bark of *C. flanaganii* and *I. mitis* have been extensively used in cosmeceuticals with the phenolic content of the plants not fully

described **(HUTCHINGS et al., 1996; DOLD and COCKS, 2002; DOLD and COCKS, 2005)**.

Phenolic compounds are secondary metabolites produced by plants and are found in different parts of the plant, including the bark layers **(GHASEMZADEH and GHASEMZADEH, 2011)**. In biological systems like the skin these polyphenolic compounds are reduced by free radicals resulting in a more stable system with less ROS **(SAIJA et al., 1995; SELVARAJ et al., 2015)**.

The flavonoid content of *P. lanceolata, A. oblongifolia* and *C. flanaganii* was significantly higher when compared to all the other extracts with values recorded at 5.71 ± 0.21, 5.48 ± 0.06 and 4.75 ± 0.04 mgCE/g, respectively (Figure 4.1). Flavonoids inhibit xanthine oxidase activity and prevent the reaction of xanthine oxidase with molecular oxygen which prevents the release of the superoxide radical and hydrogen peroxide **(COS et al., 1998)**. Reduction of superoxide radical and hydrogen peroxide significantly contributes to a reduced oxidative stress in a biological system. The antioxidant mechanism of flavonoids has been reported to be due to their free radical chain breaking capabilities, metal chelation, singlet oxygen quenching and inhibition of enzymatic activity **(KELLY et al., 2002; LEOPOLDINI et al., 2006)**. Understanding the phenolic content of the plant will contribute significantly in elucidating the mechanism of action with which the plants are able to exert their reported cosmeceutical properties. Interestingly, there were significant levels of flavonoids in extracts of *C. flanaganii*. The

reported cosmeceutical potential of *C. flanaganii* may be due to their strong antioxidant activity attributed to their flavonoid content **(COCKS and DOLD, 2002)**.

There were high levels of total phenolic compounds recorded for *R. graveolens, R. nasturtium-aquaticum, C. anisata, H. albiflos* and *A. oblongifolia* of 28.72 ± 1.81, 24.79 ± 1.30, 18.52 ± 1.85, 17.10 ± 0.47 and 15.05 ± 1.28 mgGAE/g, respectively. Furthermore, the flavonoid content for *S. officinale, C. anisata, R. nasturtium-aquaticum, C. album, H. albiflos* and *U. urens* was high with values recorded at 2.29 ± 0.09, 1.80 ± 0.02, 1.35 ± 0.08, 0.16 ± 0.06, 1.10 ± 0.07 and 1.02 ± 0.03 mgCE/g, respectively. The total phenolic content and flavonoid content for *C. flanaganii, A. oblongifolia, C. anisata, R. nasturtium-aquaticum* and *H. albiflos* were significant in both measurement assays.

It was interesting to note that although extracts of *P. lanceolata* and *S. officinale* were recorded to have low levels of total phenolic acid content, the flavonoid content was high. Similarly, extracts of *A. ferox, I. mitis* and *R. graveolens* were recorded to have low levels of flavonoid content with the total phenolic content high. Extracts with high content of phenolics and flavonoids will have a positive effect on the health and beauty of the skin. The results are important as this widens the number of different plant extracts that can be used, based on their phenolic compounds for skincare and beauty. However, further studies on the different groups of phenols present in the plant extracts are required to have a better understanding on the mode of action for their proposed function.

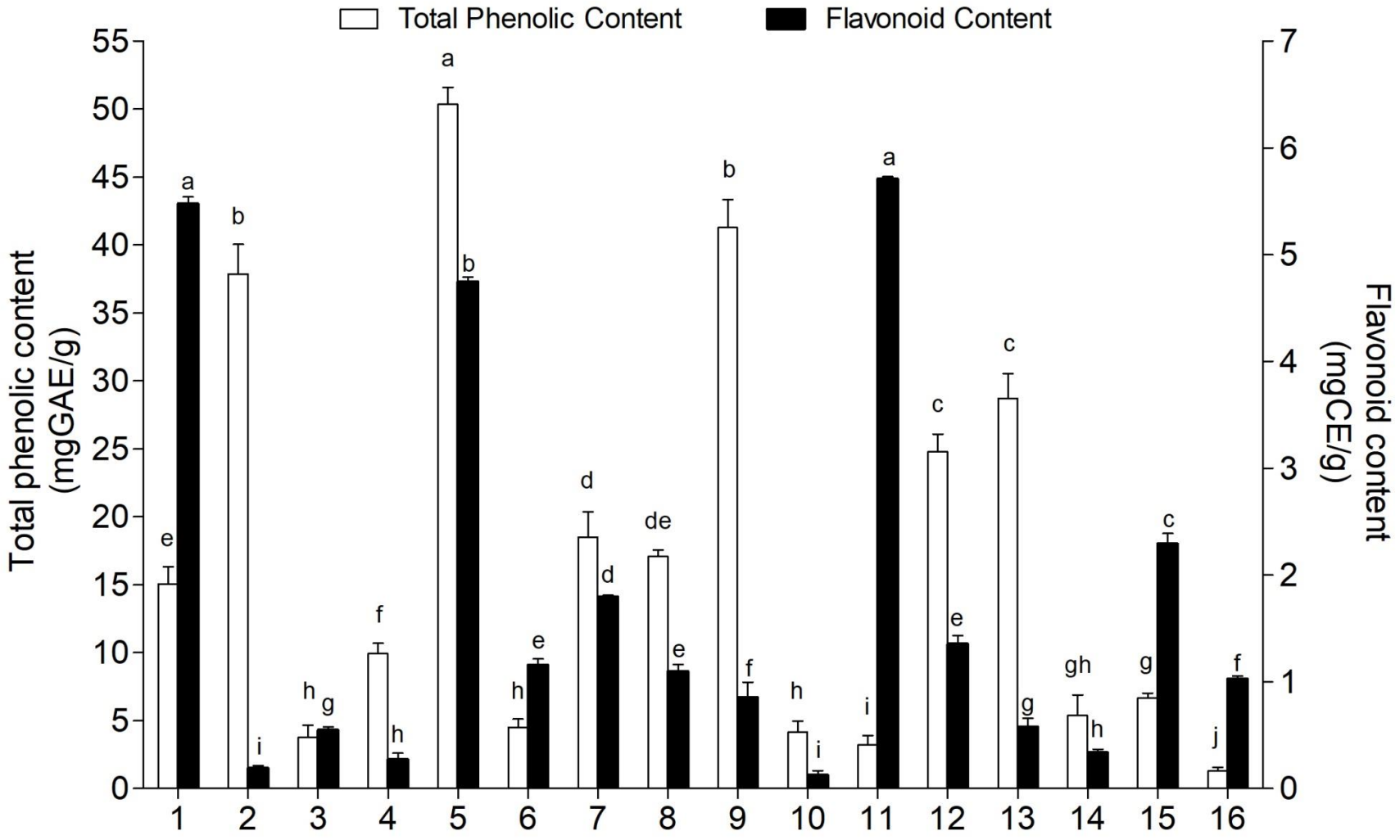

Figure 4.1: Phenolic content of plants (1). *Acokanthera oblongifolia,* (2). *Aloe ferox,* (3). *Arctotis arctotoides,* (4). *Bulbine frutescens,* (5). *Cassipourea flanaganii,* (6). *Chenopodium album,* (7). *Clausena anisata,* (8). *Haemanthus albiflos,* (9). *Illex mitis,* (10). *Marrubium vulgare,* (11). *Plantago lanceolata,* (12). *Rorippa nasturtium-aquaticum,* (13). *Ruta graveolens,* (14). *Sonchus asper,* (15). *Symphytum officinale,* (16). *Urtica urens,* used for skincare and beauty. Left Y-axis (Total phenolic content). Right Y-axis (Flavonoid content). Results indicate mean and standard deviation with $p < 0.05$; n=3. Letters indicate Fisher's least significant difference (LSD). GAE – Gallic acid equivalents; CE – Catechin equivalents.

4.3.2. Antioxidant activity assay

4.3.2.1. DPPH radical scavenging activity assay

The ability of plant extracts to quench free radicals was determined using the DPPH radical scavenging activity assay. The discoloration of the purple-coloured DPPH free radical to the colourless 2,2-diphenyl-β-picrylhydrazine (DPPH-H) was used as an indication of the antioxidant potential of the tested plant extracts. The antioxidant activity results are represented in Table 4.1 as DPPH IC_{50} values. The expressed IC_{50} results of the plant extracts are compared to those of ascorbic acid, a known antioxidant agent with high antioxidant potential **(ARRIGONI and DE TULLIO, 2002)**.

The IC_{50} results for *R. graveolens* (0.361 ± 0.017 µg/mL) and *C. flanaganii* (0.375 ± 0.033 µg/mL) were comparable to that of the standard antioxidant of 0.331 ± 0.004 µg/mL. The results indicate that extracts from the two plants have a large portion of antioxidants to quench free oxidants in a biological system, such as the skin. The presence of antioxidants in biological systems has a positive influence on physiological processes like maintaining skin structural integrity, delaying the ageing process and the reduction of disease proliferation **(VALKO et al., 2007; RATZ-ŁYKO et al., 2015)**. Therefore, plants with higher antioxidant activity are required for maintaining physiological processes affecting the beauty and health of the skin.

The IC_{50} results for *C. album, U. urens, A. ferox, S. officinale, M. vulgare, A. arctotoides, I. mitis* and *B. frutescens* exhibited noticeable antioxidant potential, quenching a significant proportion of the oxidants (Table 4.1). The results indicate that the plants

were not able to quench all the free oxidants in the system. However, there are cosmeceutical benefits of maintaining a fine balance of both oxidants and antioxidants in the system as the presence of oxidants at low levels are responsible for initiating cell signalling pathways **(KLOTZ et al., 2001)**. Cell signalling pathways play an important role in the removal of UV-damaged cells and therefore contribute significantly in the generation of new skin cells **(HANCOCK et al., 2001; LÓPEZ-ALARCÓN and DENICOLA, 2013)**. However, the presence of antioxidants from exogenous supplementation such as plants, food and vegetables has been reported to have more health and cosmeceutical benefits **(SARIKURKUCU et al., 2008)**.

Table 4.4: DPPH radical scavenging activity results of plants used for skincare and beauty.

Medicinal plant	Plant Part	DPPH IC_{50} (µg/mL)
Acokanthera oblongifolia	Leaves	1.185 ± 0.073d
Aloe ferox	Leaves	**0.462 ± 0.042b**
Arctotis arctotoides	Leaves	**0.487 ± 0.012b**
Bulbine frutescence	Leaves	**0.497 ± 0.033b**
Cassipourea flanaganii	Bark	**0.375 ± 0.033a**
Cassipourea album	Leaves	**0.419 ± 0.025b**
Clausena anisata	Leaves	0.556 ± 0.041c
Haemanthus albiflos	Bulb	2.459 ± 0.585e
Illex mitis	Bark	**0.491 ± 0.029b**
Marrubium vulgare	Leaves	**0.472 ± 0.045b**
Plantago lanceolata	Leaves	0.548 ± 0.049c
Rorippa nasturtium-aquaticum	Leaves	0.527 ± 0.054c
Ruta graveolens	Leaves	**0.361 ± 0.017a**
Sonchus asper	Leaves	0.522 ± 0.028c
Symphytum officinale	Leaves	**0.469 ± 0.031b**
Urtica urens	Leaves	**0.452 ± 0.058b**
Ascorbic acid		**0.331 ± 0.004a**

Results expressed as IC_{50} (µg/mL). Means and standard deviation highlighted in bold were significant with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

4.3.2.2. FRAP activity assay

The ability of antioxidants to reduce the Fe^{3+} complex to the Fe^{2+} form was used to determine the antioxidant activity of the plant extracts. The formation of the Perl's Prussian blue Fe^{2+} ion was measured with a spectrophotometer and the EC_{50} values presented in Table 4.2 were calculated from the absorbance readings. Extracts from *U. urens, A. ferox, C. flanaganii, B. frutescens, P. lanceolata, H. albiflos, M. vulgare, C. anisata, S. officinale* and *R. nasturtium-aquaticum* expressed good metal chelating potential that were comparable to that of the positive control.

The antioxidant activity of the plant extracts observed through their metal chelation capabilities, prevented formation of free radicals and lipid peroxides. Formation of free radicals and lipid peroxides play a significant role in development of skin diseases **(BICKERS and ATHAR, 2006)**. Therefore, preventing the formation of these oxidants will have a positive impact on skin beauty.

Table 4.5: Ferric reducing antioxidant power results for plants used for skincare and beauty.

Medicinal plant	Plant Part	FRAP EC_{50} (µg/mL)
Acokanthera oblongifolia	Leaves	1.329 ± 0.101c
Aloe ferox	Leaves	**0.801 ± 0.025ab**
Arctotis arctotoides	Leaves	1.703 ± 0.103d
Bulbine frutescence	Leaves	**0.969 ± 0.082b**
Cassipourea flanaganii	Bark	**0.813 ± 0.048ab**
Chenopodium album	Leaves	1.393 ± 0.024c
Clausena anisata	Leaves	**1.195 ± 0.097bc**
Haemanthus albiflos	Bulb	**1.115 ± 0.114bc**
Illex mitis	Bark	1.625 ± 0.148d
Marrubium vulgare	Leaves	**1.145 ± 0.118bc**
Plantago lanceolata	Leaves	**0.979 ± 0.058b**
Rorippa nasturtium-aquaticum	Leaves	**1.198 ± 0.152bc**
Ruta graveolens	Leaves	2.924 ± 0.312e
Sonchus asper	Leaves	1.335 ± 0.054c
Symphytum officinale	Leaves	**1.169 ± 0.031bc**
Urtica urens	Leaves	**0.726 ± 0.010a**
Ascorbic acid		**0.987 ± 0.001b**

Results expressed as EC_{50} (µg/mL). Means and standard deviation highlighted in bold were significant with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

4.3.2.3. *β*-carotene linoleic acid model

The coupled oxidation of linoleic acid and bleaching of *β*-carotene assay was used to determine the antioxidant potential of the plants extract as described by **PAJERO et al. (2002)**. The delay in the discoloration of the *β*-carotene chromophore, which is characterized by the orange colour, is due to the presence of antioxidants. Table 4.3 represents the results as *β*-carotene oxidation rate ratio (ORR) with lower *β*-carotene ORR indicative of the antioxidant potential of the plant extracts. There was a notable increase in the antioxidant potential of the tested plant extract between 60 and 90 min. The ORR for *C. flanaganii* and *S. officinale* were significantly lower than that of the positive control (BHT). The ORR_{60min} was 0.0346 ± 0.003, 0.0469 ± 0.001 and the ORR_{90min} was 0.0335 ± 0.001, 0.0446 ± 0.001 for *C. flanaganii* and *S. officinale,* respectively. The ORR for *U. urens* was comparable to that of the positive control with ORR_{60min} of 0.0554 ± 0.002 and ORR_{90min} of 0.0533 ± 0.004. The ORR of the other plant extracts was significantly higher when compared to the positive control, an indication of their susceptibility to lipid peroxidation **(GUTTERIDGE and HALLIWELL, 1990)**. The results indicate the ability of the plant extracts to provide protection against increased levels of lipid peroxidation of the cellular membrane. Protection against peroxides formed during lipid peroxidation plays an important role in delaying the aging process due to their destructive potential to the skin **(KAMMEYER and LUITEN, 2015)**. However, the liposolubility property of the extracts is important when trying to inhibit lipid peroxidation in cell membranes. Therefore, antioxidants should have appropriate liposolubility to be incorporated into the membrane and react with reactive species **(NIKI, 2014)**.

Table 4.6: *β*-carotene linoleic acid model results of plants used for skincare and beauty.

Medicinal plant	Plant Part	$ORR_{60\ min}$	$ORR_{90\ min}$
Acokanthera oblongifolia	Leaves	0.1072 ± 0.001e	0.1045 ± 0.003f
Aloe ferox	Leaves	0.1395 ± 0.003h	0.1170 ± 0.012g
Arctotis arctotoides	Leaves	0.1301 ± 0.009g	0.1084 ± 0.001f
Bulbine frutescence	Leaves	0.1378 ± 0.004h	0.1363 ± 0.002e
Cassipourea flanaganii	Bark	**0.0346 ± 0.003a**	**0.0335 ± 0.001a**
Chenopodium album	Leaves	0.1272 ± 0.005g	0.1207 ± 0.001g
Clausena anisata	Leaves	0.1050 ± 0.004e	0.1046 ± 0.005f
Haemanthus albiflos	Bulb	0.2222 ± 0.006j	0.1885 ± 0.002g
Ilex mitis	Bark	0.1684 ± 0.004i	0.1581 ± 0.002f
Marrubium vulgare	Leaves	0.0896 ± 0.003d	0.0830 ± 0.005d
Plantago lanceolata	Leaves	0.1021 ± 0.001e	0.0919 ± 0.007e
Rorippa nasturtium-aquaticum	Leaves	0.1206 ± 0.002f	0.1073 ± 0.001f
Ruta graveolens	Leaves	0.2160 ± 0.004j	0.1965 ± 0.002h
Sonchus asper	Leaves	0.0796 ± 0.004c	0.0679 ± 0.004c
Symphytum officinale	Leaves	**0.0469 ± 0.001a**	**0.0446 ± 0.001a**
Urtica urens	Leaves	**0.0554 ± 0.002b**	**0.0533 ± 0.004b**
BHT		**0.0642 ± 0.001b**	**0.0534 ± 0.001b**

Results expressed as ORR_{60min} and ORR_{90min}. Means and standard deviation highlighted in bold were significant with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

4.3.3. Photo-protective effect studies

Sun protection factor (SPF) values effectively compares the amount of time needed to cause sunburn on protected skin to the amount of time needed to cause sunburn on unprotected skin. Higher SPF values indicate protection from sunburn due to the absorption, reflection and scattering of harmful UV radiations **(MISHRA et al., 2011)**. Importantly, formulations are categorized according to their SPF values, with minimal protection (SPF < 12), moderate protection (SPF 12 – 30) and high protection (SPF > 30). Consequently, formulations with SPF 15, SPF 30 and SPF 60+ may protect the skin from UVB radiation by 93.3%, 96.7% and 98.35%, respectively **(STEVANATO et al., 2014)**.

The photo-protective effect of *Aloe ferox* extracts illustrated in Figure 4.2 is widely accepted to be due to Aloesin, previously reported to absorb UVB radiation and offer protection against eczema and sunburn **(GROLLIER et al., 1987; VAN WYK and GERICKE, 2000; ZAHN et al., 2008)**. The SPF values for *P. lanceolata, C. flanaganii, A. oblongifolia, I. mitis* and *A. arctotoides* were recorded to be higher than those of *A. ferox* with SPF values of 22.19 ± 2.177, 20.80 ± 0.696, 20.293 ± 0.417, 19.67 ± 2.144 and 17.61 ± 0.329, respectively (Figure 4.2). Interestingly, there were significant levels of phenolic compounds recorded for the plant extracts, except for *A. arctotoides* (Figure 4.1).

The total phenolic content and flavonoid content of extracts of *C. flanaganii* and *A. oblongifolia* were significantly high. Furthermore, the flavonoid content of *P. lanceolata*

and the total phenolic content of *I. mitis* were also recorded to be high. The plant extracts demonstrated the highest absorbance of UV radiation at a wavelength region between 300 – 305 nm (Figure 4.3a, Figure 4.3b). The apparent absorbance of the extracts at this region could therefore be attributed to the absorption, reflection or scattering of harmful UVB radiation associated with phenolic compounds.

The successful incorporation of these extracts into sunscreens and body lotion formulations may provide moderate protection against UVB radiation due to their achieved SPF values **(STEVANATO et al., 2014)**. Application of formulations incorporated with the extracts may protect the skin by 93.3% during the period of application. Protection of the skin effectively protects the skin from the UVB radiation that penetrates the epidermis and dermal layers of the skin causing direct damage to proteins, lipids and nucleic acids therefore negatively impacting the appearance of the skin **(KAMMEYER and LUITEN, 2015)**.

The SPF results from Figure 4.2 for *C. anisata*, *M. vulgare*, *H. albiflos*, *U. urens*, *S. officinale*, *R. nasturtium-aquaticum*, *S. asper*, *B. frutescens*, *C. album* and *R. graveolens* showed the ability of the plant extracts to provide minimal protection with SPF < 12, if they were to be successfully incorporated in beauty formulations.

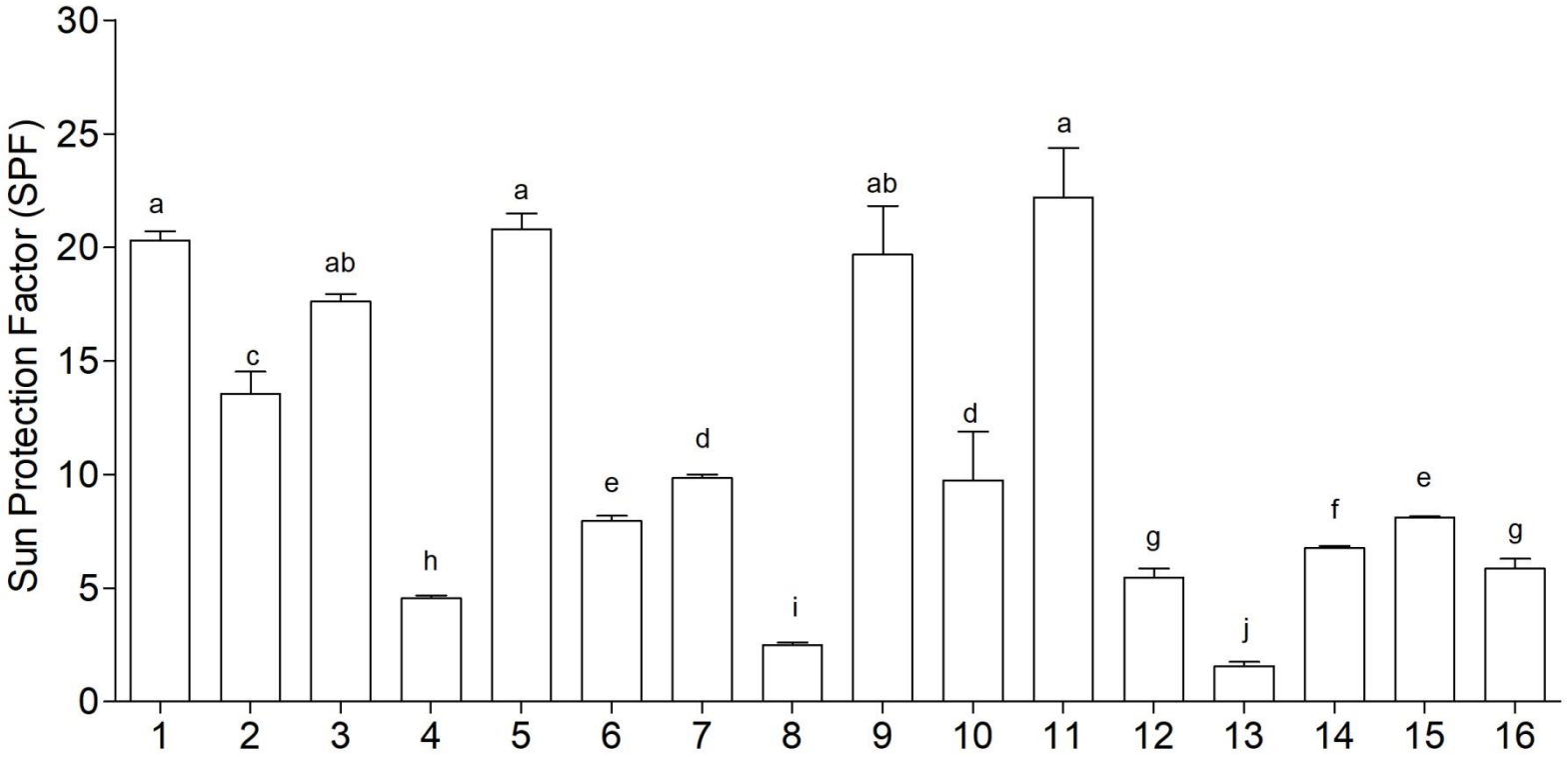

Figure 4.2: Photo protective effect results of plants (1). *Acokanthera oblongifolia,* (2). *Aloe ferox,* (3). *Arctotis arctotoides,* (4). *Bulbine frutescens,* (5). *Cassipourea flanaganii,* (6). *Chenopodium album,* (7). *Clausena anisata,* (8). *Haemanthus albiflos,* (9). *Illex mitis,* (10). *Marrubium vulgare,* (11). *Plantago lanceolata,* (12). *Rorippa nasturtium-aquaticum,* (13). *Ruta graveolens,* (14). *Sonchus asper,* (15). *Symphytum officinale,* (16). *Urtica urens,* used for skincare and beauty. Results indicate mean and standard deviation with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

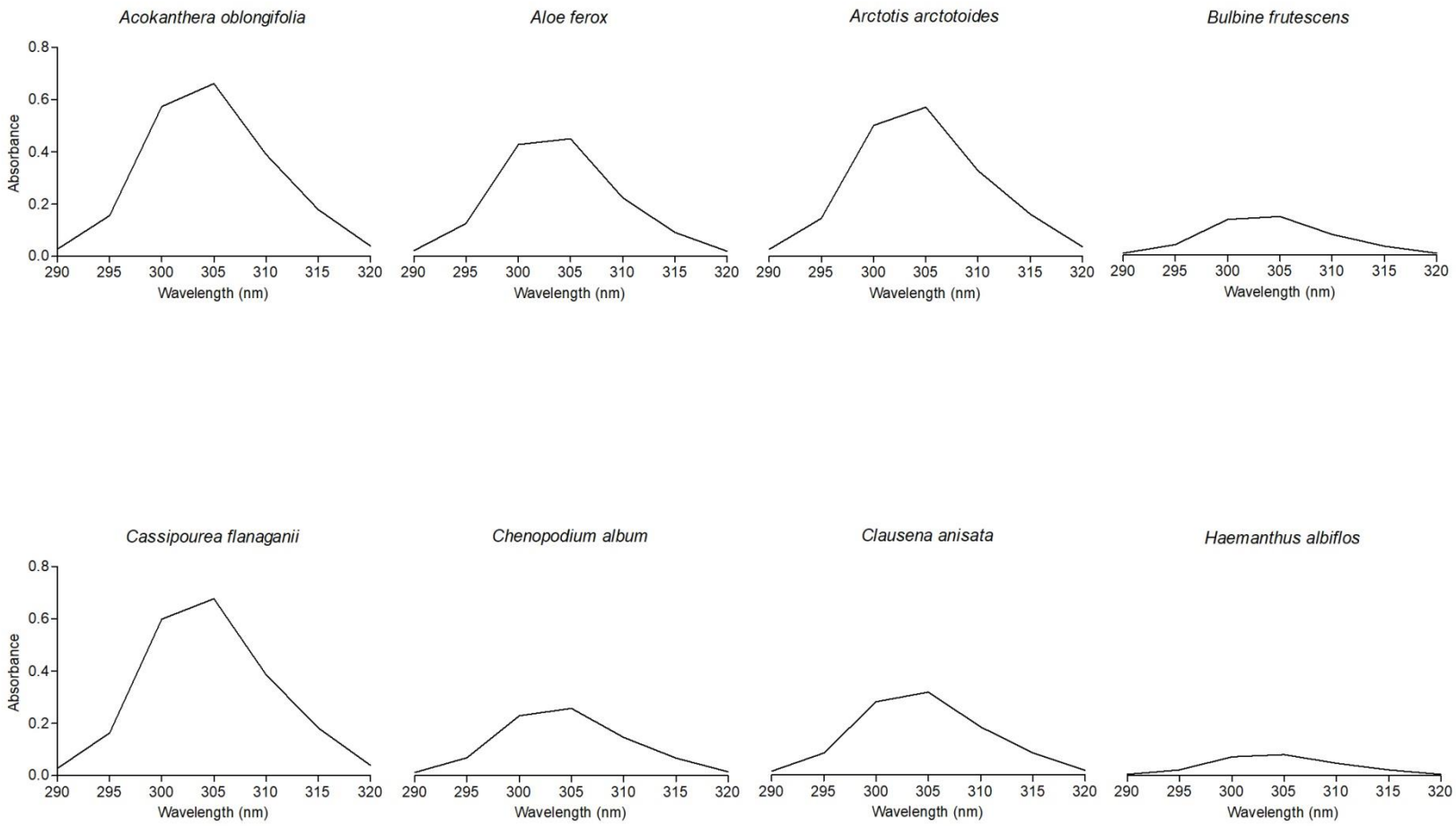

Figure 4.3a. Photo-protective effect results of plants used for skincare and beauty expressed as absorbance readings at a wavelength of 290 – 320 nm.

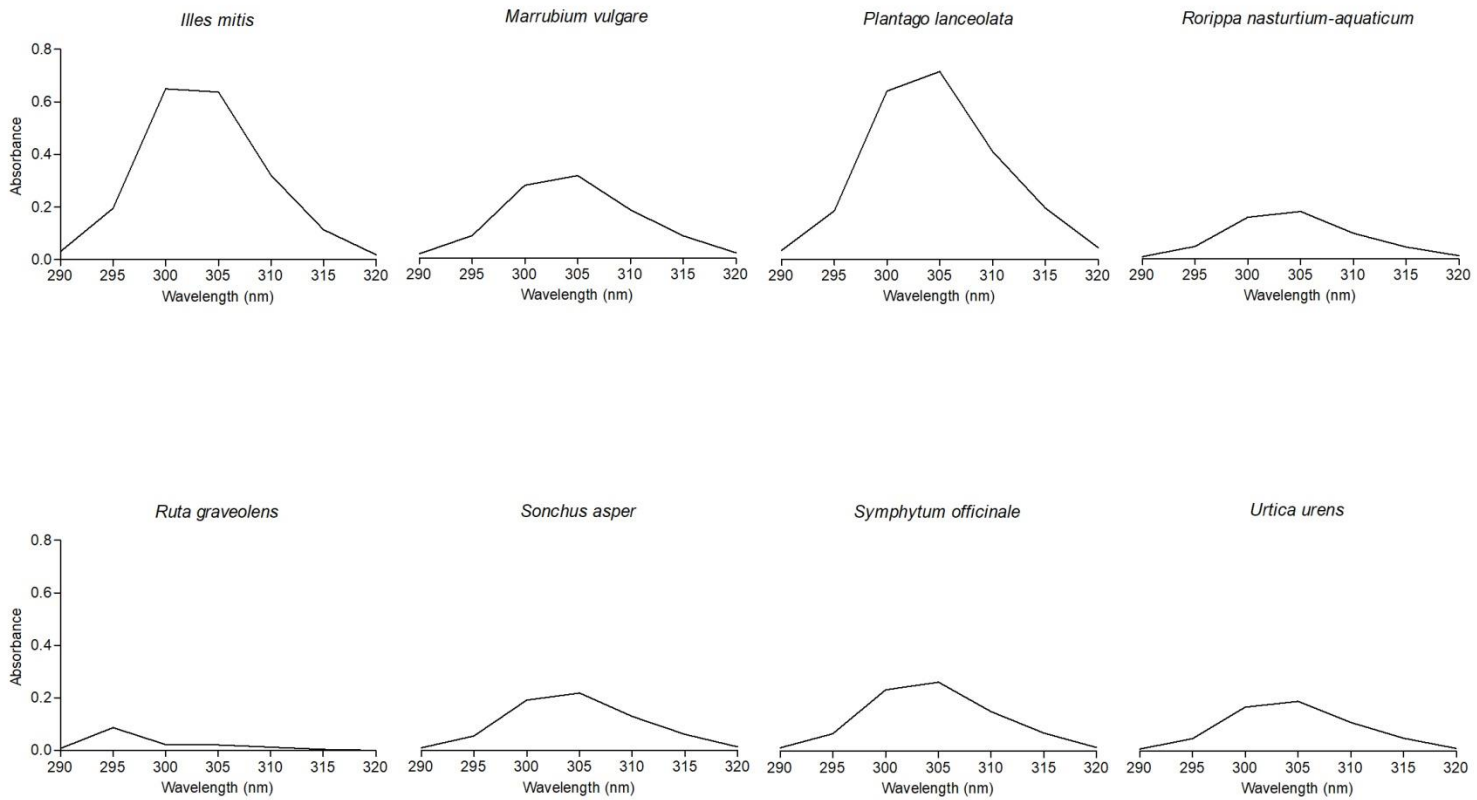

Figure 4.3b: Photo-protective effect results of plants used for skincare and beauty expressed as absorbance readings at a wavelength of 290 – 320 nm.

4.3.4. Anti-tyrosinase activity assay

Tyrosinase is a multifunctional copper-containing enzyme with a key role in the melanisation process and the enzyme is widely distributed in fungi, plants and animals **(GERMANÒ et al., 2012)**. Tyrosinase is involved in the first two steps of the melanisation process where it catalyses the ortho-hydroxylation of tyrosine to L-DOPA (monophenolase activity) and the subsequent oxidation of L-DOPA to dopaquionone (diphenolase activity) in the presence of reactive oxygen species **(WOOD and SCHALLREUTER, 1991; HEARING, 2011)**. Dopaquinone is a precursor of melanin and is responsible for the formation of dark spots and darkening of the skin due to oxidative damage.

Table 4.4 presents the anti-tyrosinase activity of the plant extracts, in which the results are expressed as IC_{50} values. Ethanol extracts of *R. nasturtium-aquaticum*, *C. anisata*, *S. officinale* and *C. flanaganii* expressed good anti-tyrosinase activity comparable to that of the positive control with IC_{50} values of 19.64 ± 1.43, 21.83 ± 0.15 and 22.05 ± 1.87 and 22.24 ± 1.32 µg/mL, respectively. The action with which tyrosinase inhibitors exert their activity has previously been reported to be as reducing agents, reducing *o*-dopaquionone back to L-DOPA, as *o*-dopaquionone scavengers or competitive inhibitors to tyrosinase active site, all preventing the melanisation process **(KIM and UYAMA, 2005; CHANG, 2009; KHAN, 2012)**. Interestingly, extracts of *R. nasturtium-aquaticum*, *C. anisata*, *S. officinale* and *C. flanaganii* significantly expressed good antioxidant activity when assessed for their radical scavenging activity and metal chelation capacity. Therefore, the observed anti-tyrosinase activity by these extracts

could be due to their antioxidant potential through the reduction of *o*-dopaquionone back to L-DOPA as previously elucidated with other plants. L-DOPA is oxidized to *o*-dopaquionone in the presence of ROS.

Furthermore, there was good anti-tyrosinase activity for *B. frutescens*, *C. album*, *A. ferox* and *P. lanceolata* with IC_{50} values ranging between 23.94 and 24.78 µg/mL. Incorporation of these extracts in formulations that can be used against developed UV-induced dark-spots will be significant in the skincare and beauty industry. Aloesin from *Aloe ferox* has previously been reported to inhibit tyrosinase activity **(JONES et al., 2002; YAGI and TAKEO, 2003)** with the action further being illustrated to be synergistic with other compounds **(JIN et al., 1999)**. The inhibition of the tyrosinase enzyme by phenolic compounds can further be the mechanism of action by the other plant extracts. However, studies on the mechanism with which these plant extracts exert their anti-tyrosinase activity still need to be explored.

Table 4.4: Anti-tyrosinase activity results for plants used for skincare and beauty.

Medicinal Plant	Plant Part	Tyrosinase IC_{50} (µg/mL)
Acokanthera oblongifolia	Leaves	41.44 ± 2.16g
Aloe ferox	Leaves	24.71 ± 2.35bc
Arctotis arctotoides	Leaves	32.11 ± 2.96e
Bulbine frutescens	Leaves	23.94 ± 1.76bc
Cassipourea flanaganii	Bark	**22.24 ± 1.32ab**
Chenopodium album	Leaves	24.11 ± 0.52b
Clausena anisata	Leaves	**21.83 ± 0.15a**
Haemanthus albiflos	Bulb	79.58 ± 1.21i
Illex mitis	Bark	38.38 ± 1.69f
Marrubium vulgare	Leaves	43.54 ± 2.68g
Plantago lanceolata	Leaves	24.78 ± 0.35bc
Rorippa nasturtium-aquaticum	Leaves	**19.64 ± 1.43a**
Ruta graveolens	Leaves	35.61 ± 0.16e
Sonchus asper	Leaves	31.19 ± 1.85d
Symphytum officinale	Leaves	**22.05 ± 1.87ab**
Urtica urens	Leaves	55.13 ± 2.31h
Kojic acid		**19.38 ± 2.72a**

Means and standard deviation values highlighted in bold were significant with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

4.4. Conclusions

The antioxidant mechanism of phenolic compounds has been proposed to be due to their free radical chain breaking capacity, metal chelation, free radical quenching and inhibition of enzymes responsible for free radical generation. Extracts of *C. flanaganii, A. oblongifolia, C. anisata, R. nasturtium-aquaticum* and *H. albiflos* expressed significant levels of total phenolic content and flavonoid content of all the tested plants. Extracts of *C. flanaganii* expressed good overall antioxidant activity, exhibiting radical quenching, metal chelation and free radical chain breaking capabilities.

There was significant metal chelation activity by extracts of *C. anisata, R. nasturtium-aquaticum,* and *H. albiflos.* Interestingly, the flavonoid content of *S. officinale* was high exhibiting radical quenching and metal chelation capabilities. Extracts of *P. lanceolata* had high flavonoid content but only exhibited metal chelation capabilities. However, the observed correlation of the phenolic content and antioxidant activity was not observed for all the extracts. Extracts of *A. oblongifolia,* expressed high levels of total phenolic content and flavonoid content but poor antioxidant activity. Similarly, extracts of *U. urens* expressed good overall antioxidant activity, exhibiting radical quenching, metal chelation and free radical chain breaking capabilities, but the phenolic content was low. The chemical structure, hydroxylation, substitutes and degree of polymerization of phenolic compounds are known to influence the antioxidant activity of plant extracts. The results indicate the ability of some plant extracts to provide protection against an increased level of oxidative stress leading to protection against formation of wrinkles, disease and delaying the age process.

The phenolic compound content and antioxidant activity of some of the plants further played a significant role in the photo-protection and enzyme inhibition of the extracts. Extracts of *C. flanaganii* and *A. oblongifolia* expressed high levels of phenolic compounds and moderate protection against UV radiation with SPF values above 15. Interestingly, even though extracts of *P. lanceolata* and *I. mitis* only expressed high levels of flavonoid content and total phenolic content, respectively, their photo-protective capability was also moderate with SPF values above 15. However, it should be noted that the photo-protective effect employed by extracts of *A. arctotoides* might not be due to their radical scavenging capabilities but their phenolic content. Other metabolites might be responsible for the proposed absorption, reflection and scattering of the harmful UV radiation.

The phenolic content and antioxidant activity of plant extracts does have a significant impact on the enzyme inhibition capacity of some of these extracts. This was evident from the inhibition of tyrosinase activity by extracts of *C. flanaganii, C. anisata, R. nasturtium-aquaticum* and *S. officinale.* The results from the study were able to show the beneficial effect of plants with elevated phenolic compounds and antioxidant activity in beauty enhancement through skin photo-protection and achieving desired skin complexion through their anti-tyrosinase activity.

Chapter 5

Evaluation of plants used for skincare and beauty for their anti-inflammatory activity

5.1. Introduction

The activation of the inflammatory response in the skin is a beneficial response system to dermal tissue injury, since the skin has a barrier function and contributes significantly to a person's appearance. The release of arachidonic acid from the membrane bilayer serves as the starting point for a general inflammatory response **(NIJVELDT et al., 2001)**. Phosphoglycerides are the main components of membrane bilayers and consist of a three-carbon backbone glycerol, two long-chain fatty acids esterified to the hydroxyl group on carbon-1 and carbon-2 of the glycerol and a phosphoric acid esterified to the carbon-3 hydroxyl group of glycerol **(POLLARD et al., 2017)**. The alcohol group provides phosphoglycerides with their name, with one carrying a glycerol head group termed phosphatidylglycerol (PG) and one with a choline head group termed phosphatidylcholine (PC) **(MARSH, 2013)**. Phosphatidylcholine are the most abundant phospholipids with a saturated fatty acid at *sn*-1 and an unsaturated fatty acid at *sn*-2, and plays a significant role in membrane integrity **(BHAGAVAN and HA, 2015)**.

Phospholipase A_2 (PLA_2) has high affinity for PC and hydrolyses the ester bond at the *sn*-2 position releasing free arachidonic acid and lysophospholipids **(MURAKAMI and KUDO, 2004)**. The released free arachidonic acid serves as precursor molecules for eicosanoids, which are responsible for skin inflammation **(DENNIS et al., 2011)**. Therefore, inhibitors that can inhibit phospholipase A_2 activity are desired to reduce the progression of inflammation.

Lipoxygenase (LOX) enzymes are non-heme iron containing dioxygenases that catalyse the addition of molecular oxygen to arachidonic acid to form hydroxyeicosatetraenoic acid (HETE). Lipoxygenase enzymes play a significant role in progression of inflammation through the metabolism of arachidonic acid to leukotrienes **(RADMARK et al., 2015)**. The 15-LOX enzyme activity is associated with atherosclerotic plaque formation due to elevated levels of oxidized lipids **(KOBE et al., 2014)**. The formation of plaques in skin blood vessels has undesired consequences on the health and function of the skin, resulting in morphological changes at the inflammation sites such as psoriasis **(CHEN and LYGA, 2014)**. Psoriasis is an inflammatory skin disease associated with increased accumulation of atherosclerotic plaques and is characterized by thickened and scaling of the skin. Progression of psoriasis is undesired as it affects the health and beauty of the skin **(ALEXANDROFF et al., 2009)**.

The two main isoforms of cyclooxygenase (COX), COX-1 and COX-2, are responsible for initiation of eicosanoids production and progression of inflammation at sites of inflammation **(WARNER and MITCHELL, 2004)**. The enzymes are responsible for conversion of arachidonic acid to a hydroperoxy endoperoxide (PGG_2) which is further reduced to corresponding prostaglandin H_2 (PGH_2) through peroxidase activity. Prostaglandin H_2 serves as a precursor of prostaglandins (PGs) which are responsible for acute inflammation of the skin **(RICCIOTTI and FITZGERALD, 2011)**. Traditionally, non-steroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of localized pain and swelling associated with PGs synthesis. However, most NSAIDs used are non-selective for COX enzymes, inhibiting both COX-1 and COX-2.

The enzyme activity of COX-1 has been reported to initiate the production of PGs responsible for maintenance and protection of the intestinal mucosal layer **(DENNIS and NORRIS, 2015)**. Therefore, long-term usage of NSAIDs will result in inhibition of COX-1 enzyme activity leading to gastric ulcerations. Studies have reported phenolic compounds from plants to have anti-inflammatory activity **(FERRANDIZ and ALCARAZ, 1991)**. The inhibition of PLA_2, LOX and COX enzyme's activity has been suggested as one of the action phenolic compounds induce as their anti-inflammatory activity **(LAUGHTON et al., 1991; SON et al., 2005)**. Therefore, the aim of the study was to test plants used for skincare and beauty for their anti-inflammatory activity by evaluating their inhibition of $sPLA_2$, 15-LOX, COX-1 and COX-2 enzyme activity.

5.2. Materials and Methods

5.2.1. Plant extract preparation

The reduced selection of plants in the current study was based on the ethnobotanical usage of the selected plants. Plants selected for the study included, *Aloe ferox*, *Cassipourea flanaganii*, *Chenopodium album*, *Clausena anisata*, *Haemanthus albiflos*, *Plantago lanceolata*, *Rorippa nasturtium-aquaticum*, *Ruta graveolens*, *Symphytum officinale* and *Urtica urens*. Plants were dried in an oven at 50 °C for 72 h, or until constant dryness was achieved, and stored in the dark at room temperature in brown paper bags until extraction. The dried plant material was ground into powders and extracted in 20 mL/g (v/w) with acetone and water in a sonication bath for 1 h at room temperature. Acetone as a solvent has been reported to extract a wide range of inhibitors, from polar to non-polar, while water is the most used solvent in ethnobotanical extract preparations. Extracts were filtered through Whatman No. 1 filter paper and the plant crude extracts were concentrated under vacuum and subsequently dried under a constant flow of cool air.

5.2.2. Anti-inflammatory activity assay

5.2.2.1. Secretory phospholipase A_2 ($sPLA_2$) inhibitor screening assay

The anti-inflammatory activity of selected plants was evaluated by examining the inhibition of the human $sPLA_2$ enzyme using the $sPLA_2$ (Type V) inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) as described by **GEORGE et al. (2014)**. The assay measures free 5-thio-2-nitrobenzoic acid (TNBA) following hydrolysis of

diheptanoyl thio-phosphatidylcholine (PC) by $sPLA_2$. Briefly, 10 µL of the $sPLA_2$ enzyme was dissolved in assay buffer solution (25 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM KCl, 0.3 mM Triton X-100, pH 7.5) and 10 µL of plant extracts at concentrations of 50, 25, 12.5 and 6.25 µg/mL were added into a 96-well microtiter plates. The reaction was initiated by adding 200 µL of a substrate solution (diheptanoyl thio-PC, 1.66 mM), covered with aluminium foil and incubated at 25 °C for 15 min. After incubation, 10 µL of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid; 10 mM, 0.4 M Tris-HCl, pH 8.0) was added into each well. Reaction mixture containing the assay buffer and solvent served as the blank and the mixture with $sPLA_2$ enzyme and solvent served as the 100% initial activity (IA). Thioetheramide-PC served as the positive control. The hydrolysed diheptanoyl thio-PC was measured at 420 nm using a microplate reader (Optic Iveymen® System, Model 2100-C). The percentage inhibition was calculated using the formula below. The percentage inhibition was plotted against plant extract concentration and the IC_{50} determined from the normalized logarithmic regression curve.

$sPLA_2$ % Inhibition = [(100% IA – Inhibitor)/100% IA x 100]

5.2.2.2. Lipoxygenase (LOX) inhibitor screening assay

The anti-inflammatory activity of selected plants was evaluated by examining the inhibition of the 15-LOX enzyme using the LOX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) as described by **BOUDJOU et al. (2013)**. The assay measures hydroperoxides produced in the lipoxygenation reaction using purified 15-LOX. Briefly, 90 µL of 15-LOX enzyme was dissolved in assay buffer solution (0.1 M

Tris-HCl, pH 7.4) and 10 µL plant extracts at a concentration of 50, 25, 12.5 and 6.25 µg/mL were added into each well of a 96-well microtiter plate. The plate was incubated at 25 °C for 5 min. After incubation, the reaction was initiated by adding 10 µL substrate solution of arachidonic acid (1 mM) and mixed on a shaker for 10 min. The reaction was stopped by adding 100 µL of the chromogen into each well. Reaction mixture containing the assay buffer served as the blank and the mixture with 15-LOX enzyme and solvent served as the 100% IA. Nordihydroguairetic acid (NDGA) served as a positive control. The absorbance was measured at 490 nm using a microplate reader (Optic Iveymen® System, Model 2100-C). The percentage inhibition was calculated using the formula below. The percentage inhibition was plotted against plant extract concentration and the IC_{50} determined from the normalized logarithmic regression curve.

LOX % Inhibition = [(100% IA – Inhibitor)/100% IA x 100]

5.2.2.3. Cyclooxygenase (COX-1 and COX-2) inhibitor screening assay

The anti-inflammatory activity of the selected plants was evaluated by examining the inhibition of the ovine COX-1 and human COX-2 enzyme using the COX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) as described by **BOUDJOU et al. (2013)**. The assay measures the peroxidase activity of ovine COX-1 and human COX-2, by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Briefly, 150 µL assay buffer (0.1 MTris-HCl, pH 8), 10 µL heme and 10 µL ovine COX-1 enzyme were added to each well of the 96-well microtiter plate. The same procedure was repeated with the human COX-2 enzyme. A volume of

10 µL plant extract at a concentration of 50, 25, 12.5 and 6.25 µg/mL was added to each well. The plate was carefully mixed by shaking for 30 sec and followed by incubation at 25 °C for 5 min. After incubation, 20 µL of TMPD was added to each well of the 96-well microtiter plate and the reaction was initiated by addition of 20 µL arachidonic acid. The plates were further incubated at 25 °C for 5 min. Reaction mixture containing assay buffer and heme served as the blank and a mixture with COX-1 or COX-2 enzyme, assay buffer and heme served as the 100% IA. Indomethacin served as a positive control. The absorbance of oxidized TMPD was read at 490 nm using a microplate reader (Optic Iveymen® System, Model 2100-C). The percentage inhibition was calculated using the formula below. The percentage inhibition was plotted against plant extract concentration and the IC_{50} determined from the normalized logarithmic regression curve.

COX % Inhibition = [(100% IA – Inhibitor)/100% IA x 100]

5.2.3. Statistical analysis

The results were calculated as means ± standard deviation. The data was subjected to one-way analysis variance (ANOVA) using IBM Statistical Package for Social Science (SPSS) and means were separated using Fisher's multiple range tests ($p < 0.05$). Means were compared to each other with Fisher's least significant difference (LSD) test.

5.3. Results and Discussion

5.3.1. Anti-inflammatory activity assay

5.3.1.1. Secretory phospholipase A_2 ($sPLA_2$) inhibitor screening assay

Alterations of the skin due to inflammatory responses affect the beauty and health of the skin if left untreated due to the development of scarring and dark spots. The IC_{50} results for the anti-inflammatory activity of plant extracts through the inhibition of $sPLA_2$ enzymes are expressed in Table 5.1. The results indicated that the tested extracts were unable to significantly inhibit the $sPLA_2$ activity with IC_{50} values lower than the positive control. The reduced activity of the $sPLA_2$ will result in lower levels of free arachidonic acid in the skin. The free arachidonic acid is metabolized into eicosanoids by LOX and COX which are responsible for proliferation of acute inflammation **(DENNIS et al., 2011)**. Therefore, the anti-inflammatory activity of extracts inhibiting the $sPLA_2$ activity will be as a result of the reduced level of arachidonic acid.

Water extracts of *C. flanaganii* expressed high activity against $sPLA_2$ with a low IC_{50} value of 12.34 ± 0.78 µg/mL when compared to the other extracts. The lowest activity of water extracts against $sPLA_2$ was recorded for *H. albiflos* with the highest IC_{50} value of 28.09 ± 1.58 µg/mL. Acetone extracts of *C. album* had a significant higher activity with a low IC_{50} value of 10.75 ± 0.85 µg/mL when compared to other acetone extracts. This result further validated the reported ethnobotanical usage of *C. album* for reducing inflammation **(BHAT, 2013)**. Furthermore, acetone extracts of *R. graveolens* had the lowest activity against $sPLA_2$ enzyme activity with the highest IC_{50} value of 38.53 ± 2.26 µg/mL. In general, water extracts were more active at inhibiting the enzyme activity of

$sPLA_2$ with IC_{50} values ranging between 12.34 and 28.09 µg/mL when compared to acetone extracts with IC_{50} values ranging between 10.75 and 38.53 µg/mL. The anti-inflammatory activity of acetone extracts of *C. album* and water extracts of *C. flanaganii* were notable. The observed lower activity of the other extracts against $sPLA_2$ enzyme will result in the release of free arachidonic acid, contributing to the progression of skin inflammation during pathological conditions **(MAGRIOTI and KOKOTOS, 2010)**.

Table 5.1: Enzyme inhibition results of the $sPLA_2$, 15-LOX, COX-1 and COX-2 by plants used against skin inflammation.

Scientific name	$sPLA_2$ IC_{50} (µg/mL)		15-LOX IC_{50} (µg/mL)		COX-1 IC_{50} (µg/mL)		COX-2 IC_{50} (µg/mL)	
	Acetone	Water	Acetone	Water	Acetone	Water	Acetone	Water
Aloe ferox	17.28 ± 0.42d	24.21 ± 1.30f	32.23 ± 2.67c	53.17 ± 1.53g	**2.81 ± 0.44a**	**1.81 ± 0.20a**	**4.36 ± 0.69b**	**6.49 ± 0.74b**
Cassipoure flanaganii	18.91 ± 1.22e	**12.34 ± 0.78b**	**21.59 ± 0.92b**	**16.72 ± 0.69b**	116 ± 22.55c	30.13 ± 2.99c	**7.87 ± 0.48c**	**6.50 ± 1.03b**
Chenopodium album	**10.75 ± 0.85b**	21.46 ± 1.77e	48.39 ± 1.70d	18.99 ± 0.78c	176 ± 26.87d	130 ± 16.48f	39.85 ± 2.50h	9.14 ± 0.59c
Clausena anisata	24.82 ± 0.54f	24.61 ± 0.86f	67.62 ± 4.13h	41.45 ± 2.21f	**4.29 ± 1.17a**	**3.13 ± 1.20a**	15.18 ± 1.56e	**6.89 ± 0.91b**
Haemanthus albiflos	15.75 ± 0.35c	28.09 ± 1.58g	64.62 ± 3.20h	35.36 ± 2.98d	**5.28 ± 1.34a**	**1.89 ± 0.45a**	14.87 ± 1.41e	**4.25 ± 0.95a**
Plantago lanceolata	16.73 ± 0.35cd	16.18 ± 1.17c	**23.50 ± 0.99b**	58.49 ± 1.63i	60.12 ± 14.17b	28.94 ± 1.82c	**3.82 ± 0.27b**	11.20 ± 1.16d
Rorippa nasturtium-aquaticum	16.55 ± 0.88cd	16.68 ± 0.82c	70.39 ± 3.05i	**16.68 ± 1.78b**	61.85 ± 13.66b	94.33 ± 9.79e	**2.69 ± 0.17a**	10.33 ± 1.06d
Ruta graveolens	38.53 ± 2.26h	21.23 ± 2.09e	57.97 ± 6.15f	54.49 ± 2.55h	**12.25 ± 0.99a**	**2.32 ± 0.34a**	35.48 ± 2.18g	**4.48 ± 0.27a**
Symphytum officinale	35.06 ± 1.81g	18.56 ± 0.58d	53.55 ± 2.19e	35.36 ± 3.05d	**3.89 ± 0.33a**	82.85 ± 4.40d	23.74 ± 2.23f	23.40 ± 2.23e
Urtica urens	17.10 ± 0.60d	18.14 ± 0.54d	61.56 ± 2.46g	38.87 ± 2.49e	**2.96 ± 0.30a**	10.01 ± 3.78b	12.67 ± 0.64d	44.01 ± 3.83f
Thioetheramide-PC	**1.47 ± 0.44a**							
Nordihyroquairetic acid			**10.83 ± 0.45a**					
Indomethacin					**0.597 ± 0.17a**		**8.80 ± 0.92c**	
LSD value	1.348	0.657	2.674	1.103	12.11	6.111	1.007	1.187

Mean and standard deviation values highlighted in bold were significant and notable with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

5.3.1.2. Lipoxygenase (LOX) inhibitor screening assay

The inhibition of the 15-LOX enzyme was used to determine the anti-inflammatory activity of the plant extracts with their results expressed as IC_{50} in Table 5.1. The results indicated that the tested extracts were unable to significantly inhibit the 15-LOX activity with IC_{50} values lower than the positive control. The free arachidonic acid released by the activity of $sPLA_2$ is metabolized by 15-LOX to form HETE, which results in proliferation of atherosclerotic plaque formation. This build-up of oxidized fatty acids inside the arteries leads to inflammation, resulting in accumulation of the skin at the inflammation sites (psoriasis).

Water and acetone extracts of *C. flanaganii* expressed notable activity when compared to other extracts in inhibiting 15-LOX with IC_{50} values of 16.72 ± 0.69 and 21.59 ± 0.92 µg/mL, respectively. Extracts of *C. flanaganii* would be effective at combating the skin inflammatory-associated disease psoriasis. Proliferation of psoriasis has a negative impact on the health and beauty of the skin in affected individuals **(ALEXANDROFF et al., 2009)**. Furthermore, water extracts of *C. album* and *R. nasturtium-aquaticum* also exhibited notable activity when compared to other extracts against 15-LOX with IC_{50} values of 18.99 ± 0.78 and 16.68 ± 1.78 µg/mL, respectively. Acetone extracts of *P. lanceolata* inhibited notable activity against 15-LOX with an IC_{50} value of 23.50 ± 0.99 µg/mL. Water extracts were more active with IC_{50} values ranging from 16.68 to 58.49 µg/mL compared to acetone extracts with IC_{50} values between 21.59 and 70.39 µg/mL. *Cassipourea flanaganii* was the only plant recorded with the ability to display some form

of inhibition with both acetone and water. Therefore, extracts of *C. flanaganii* would be effective at combating the skin inflammatory-associated disease psoriasis.

5.3.1.3. Cyclooxygenase (COX-1 and COX-2) inhibitor screening assay

The inhibition of COX-1 and COX-2 was used to determine the anti-inflammatory activity of the extracts with the results expressed as IC_{50} (Table 5.1). The COX-1 activity was significantly inhibited by the positive control and none of the tested plant extracts were able to inhibit the enzyme with IC_{50} values lower than the positive control. Extracts with reduced inhibitory activity against COX-1 are encouraged due to reported beneficial effects associated with COX-1 activity **(SULEYMAN et al., 2007)**. However, it was interesting to note that there was notable activity recorded for water extracts of *A. ferox, C. anisata, H. albiflos* and *R. graveolens* inhibiting COX-1 with IC_{50} values ranging between 1.81 and 3.13 µg/mL. Acetone extracts of *A. ferox, S. officinale* and *U. urens* also yielded notable activity against COX-1 with IC_{50} values ranging between 2.81 and 3.89 µg/mL. The enzyme activity of COX-1 initiates the production of beneficial prostaglandins responsible for maintenance and protection of the intestinal mucosal layer **(DENNIS and NORRIS, 2015)**. Therefore, extracts from these plants are unlikely candidates to serve as natural inhibitors due to persistent ulcerations associated with the inhibition of COX-1 activity.

It was interesting to note that acetone and water extracts of the different plants were able to inhibit COX-2 with IC_{50} values lower than the positive control. Water extracts of *H. albiflos, R. graveolens, A. ferox, C. flanaganii* and *C. anisata* significantly inhibited

COX-2 activity with IC_{50} values ranging from 4.25 to 6.89 µg/mL. Acetone extracts of *R. nasturtium-aquaticum, P. lanceolata* and *A. ferox* significantly inhibited the activity of COX-2 with IC_{50} values ranging from 2.69 to 4.36 µg/mL. Furthermore, there was notable activity recorded for water extracts of *C. album* and acetone extracts of *C. flanaganii* with IC_{50} values comparable to the positive control.

There was an observed selective inhibition of the COX-2 activity by extracts of *C. flanaganii, P. lanceolata* and *R. nasturtium-aquaticum*. This is significant when screening for natural inhibitors with less risk of developing gastro-intestinal health effects. Therefore, the selective inhibition of COX-2 activity is beneficial in modulation of skin inflammation during pathological conditions **(MARTEL-PELLETIER et al., 2003)**. Although a number of extracts could not inhibit the COX-1 activity with low IC_{50} values comparable to the positive control, their activity was notable. Extracts of *A. ferox, C. anisata*, *H. albiflos* and *R. graveolens* have a potential to serve as non-selective inhibitors of COX-1 and COX-2 enzyme activity due to their observed notable activity against COX-1. Incorporation of extracts selective for COX-2 and 15-LOX into product formulation will enhance the beauty of the skin due to the reduced scarring, dark spots and uneven skin associated with proliferation of chronic inflammation.

5.4. Conclusions

The loss of skin function due to a prolonged inflammatory response is an undesired consequence with potential to affect the health and beauty of the skin. Therefore, successful resolution of inflammation through the removal of pro-inflammatory mediators to restore normal tissue structure and function is desired. Interestingly, water extracts from *C. flanaganii* and *C. album* showed potential to serve as dual inhibitors of 15-LOX and COX-2. Inhibition of both enzymes is desired as the enzymes are known to act as pro-inflammatory mediators of the skin. The same could be noted with acetone extracts from *C. flanaganii* and *P. lanceolata*. However, it was observed that some of the compounds extracted with acetone might interfere with the assay kit. In the future, ethanol extracts will be preferred.

Dual inhibitors for 15-LOX and COX-2 are desired for effective treatment of skin inflammation under pathological conditions. However, the inability of some of these extracts to inhibit the $sPLA_2$ enzyme activity during progressed inflammation will result in increased levels of arachidonic acid. The free arachidonic acid will be further metabolized by pro-inflammatory enzymes resulting in the progression of inflammation. Therefore, a triple-inhibition of $sPLA_2$, 15-LOX and COX-2 enzyme activity can prove to be an effective way of modulating skin inflammation. The results from the study showed extracts from *C. flanaganii* and *C. album* as potential natural inhibitors of the three enzymes with reduced side effects. However, compounds responsible for the observed anti-inflammatory activity still need to be identified.

Chapter 6

Evaluation of plant extract efficiency on the cells health and function using a human epidermal melanocytes (HEM) cell line

6.1. Introduction

Melanocytes are melanin-producing cells of the epidermis and are positioned at the basal layer of the epidermis with a black skin typically characterized by a large content of melanin **(HIROBE, 2004).** Different types of melanin are produced by the activities of the melanogenic enzymes located inside melanocytes, tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) **(NAGATA et al., 2004)**. Tyrosinase is responsible for the rate-limiting reaction of melanin production, tyrosine hydroxylation, and therefore it is a common therapeutic target against hyperpigmentation **(ITO, 2003)**.

Tyrosinase related-protein 1 and TRP-2 are involved in the synthesis of the different types of melanin. Tyrosinase initiates melanin synthesis by catalysing the hydroxylation of tyrosine to L-DOPA and the subsequent oxidation of L-DOPA to dopaquinone. The formed dopaquinone is a highly reactive ortho-quinone which can further be oxidized to form dopachrome. Dopachrome serves as the substrate for the production of eumelanin and pheomelanin. 5,6-dihydroxyindole-2-carboxylic acid (DHICA) is therefore formed from the tautomerase reaction of dopachrome by TRP-2 followed by a catalysation reaction by TRP-1, while 5,6-dihydroxyindole (DHI) is formed from the reaction of TRP-2 only. Subsequent polymerization reactions of DHICA and DHI will form eumelanin, the most common form of melanin. However, the production of pheomelanin will be as a result of the formation of a cysteinyldopa conjugate from the reaction of dopachrome and cysteine **(COSTIN and HEARING, 2007)**.

The produced melanin is transferred into melanosome structures of the melanocyte cell. The cell extends their melanosome-contained dendrites towards the keratinocytes which allows them to transfer pigment to a large number of keratinocytes **(JOSHI et al., 2007)**. Melanosome are distinctive organelles of the melanocytes and their size, number and orientation plays a significant role in pigmentation. Melanosomes that are involved in the synthesis of black or brown eumelanin are elliptical and have an internal organization of longitudinally oriented, concentric lamellae **(ABDEL-MALEK and SWOPE, 2011)**. Melanosomes that synthesize red and yellow pheomelanin pigment have a spheroidal shape and micro vesicular internal structures. Eumelanin contributes to a more dark skin while pheomelanin contributes in much lighter skin pigmentation **(ITO and WAKAMATSU, 2008)**. Consequently, pigmentation of the skin by melanin is determined by the rate of synthesis of eumelanin and pheomelanin, the relative eumelanin and pheomelanin contents and the rate of transfer of melanosomes into keratinocytes.

Melanin produced by these melanosomes plays a significant role in the photo-protection of the skin against UV-induced oxidative damage **(ABDEL-MALEK et al., 2010)**. The produced melanin protects the skin from UV damage due to its ability to absorb and reflect UV energy and its ability to neutralize oxidative damage **(LEI et al., 2002)**. Protection of the skin against UV-related damage contributes to achieving a desired skin complexion, an attribute that significantly contributes to a person's beauty **(SCHÄFER et al., 2010)**. Plant metabolites have previously been reported to protect the skin from UV-induced oxidative damage, through the regulation of melanin production

(CHOI and SHIN, 2016). The activity and health of melanocytes contributes significantly to the skin's ability to protect against photo damage, age dark spots, post inflammatory scarring and hyperpigmentation. Therefore, the aim of the study was to evaluate the safety and efficiency of plant extracts in maintaining the cells health and function using a HEM cell line.

6.2. Materials and Methods

6.2.1. Plant extract preparation

Plants were selected for their efficiency on human epidermal melanocytes (HEM) cell lines. The selection was based on their reported antioxidant, photo-protective effect and anti-tyrosinase activity. Plants selected for the study included, *Cassipourea flanaganii*, *Chenopodium album*, *Clausena anisata* and *Rorippa nasturtium-aquaticum*. Plant materials were dried in an oven at 50 °C for 72 h, or until constant dryness was achieved, and stored at room temperature in brown paper bags until the extraction. The dried plant material was ground into powder form and extracted at 20 mL/g (v/w) with 70% aqueous ethanol. Extracts were filtered through Whatman No. 1 filter paper and the plant crude extracts were concentrated under vacuum and subsequently dried under a constant flow of cool air.

6.2.2. Human epidermal melanocytes (HEM) cell culture

The HEM cell line (PCS-200-013) was purchased from ATCC (American Type Culture Collection, USA) and stored in liquid nitrogen, until the assay. Cells were retrieved from the liquid nitrogen prior to use, thawed and the cell suspension was centrifuged at 150 *g* for 7 min to remove storage media. The cell pellet was re-suspended in complete growth medium of dermal cell basal medium (PCS-200-030) supplemented with appropriate adult melanocyte growth kit (PCS-200-042) containing 5 µg/mL rh insulin; 50 µg/mL ascorbic acid; 6 mM L-glutamine; 1.0 µM epinephrine; 1.5 mM calcium chloride; peptide growth factor; M8 supplement (a proprietary formulation containing

fetal bovine serum (FBS) and other growth factors); 0.5 mL Penicillin-Streptomycin-Amphotericin B solution). A volume of 5 mL HEM cell suspension was seeded in T25 cell culture flask with an initial concentration of 1 x 10^5 cell/mL and incubated in a humidified incubator at 37 °C with 5% CO_2. Cells were removed from the incubator and checked for percent cellular confluency under the light microscope mounted with a Leica DM IL LED camera and LAS X Software (Wetzlar, Germany) at 10x magnification. Cells were cultured for 7 – 9 days, with fresh media added after 24 – 48 h to achieve confluency of > 70% suitable for biological assays.

6.2.3. Cell monolayer detachment and trypan blue exclusion test

The cells were detached from the culture flask surface by initially removing spent media aseptically without disturbing the cell monolayer. The monolayer was rinsed with 5 mL Dulbecco's phosphate buffered saline (D-PBS) to remove residual medium. A volume of 5 mL trypsin-EDTA (0.05% trypsin; 0.02% EDTA) was added into the culture flask and incubated for 3 min at room temperature to initiate detachment of the cells from the culture flask. After detachment, cells were trypsinized by adding 5 mL of trypsin neutralizing solution. The detached-trypsinized cells were transferred into a sterile conical tube and culture flask was rinsed with D-PBS to recover excess cells. The cell suspension was centrifuged at 150 *g* for 7 min and the cell pellet re-suspended in complete growth medium. Viability of the cells was determined by trypan blue test (Bio-Rad Laboratories, CA, USA), where 10 µL of cell suspension was mixed with 10 µL trypan blue (0.4% w/v) and allowed to stand for 5 min at room temperature **(LOUIS and SIEGEL, 2011)**. The mixture was then loaded in an automated haemocytometer (Bio-

Rad Laboratories, CA, USA) and viable cells were calculated to an initial concentration of 1 x 10^4 cells/mL.

6.2.4. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay

The MTT CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Anatech Instruments) was used to assess toxicity of the plant extracts by evaluating their effect on proliferation of the HEM cells. The CellTiter 96® assay is a modification of the MTT assay described by **MOSMANN (1983)**. Briefly, 200 µL of viable confluent monolayer HEM cell suspension (1 x 10^4 cells/mL) was added in each well of a 96-well microtiter plate and incubated in a humidified incubator at 37 °C with 5% for 24 h. After incubation, cells were treated with plant extracts at concentrations of 0, 5, 15, 50, 100 and 200 µg/mL and further incubated at 37 °C with cell proliferation recorded after 24 h. After incubation, 15 µL of the MTT dye solution was added in each well of the plate and further incubated at 37 °C for 4 h. The MTT solubilisation solution was added and the plate further incubated at 37 °C for 1 h. The amount of MTT reduction was measured immediately by detecting absorbance of formazan (E,Z)-5-[4,5-dimethylthiazol-2-yl]-1,3-diphenylformazan) using a microplate reader at a wavelength of 570 nm. The well containing the medium with no cells served as the blank and the well with untreated cells served as the negative control while L-ascorbic acid was used as a positive control. The assay was performed in duplicate and repeated three times. The lethal dose (LD_{50}) values were determined as the concentration required for reducing the activity by more than 50% when compared to untreated cells.

6.2.5. Cellular anti-tyrosinase activity assay

The efficiency of plant extract at inhibiting cellular tyrosinase enzyme activity using L-DOPA as a substrate was assayed as described by **NAGATA et al. (2004)** with slight modification. Briefly, cells were seeded at a density of 1 x 10^4 cells/mL in a 96-well microtiter plate and incubated in a humidified incubator at 37 °C with 5% for 24 h. After incubation, cells were treated with plant extracts at a concentration of 5, 15, 50 and 100 µg/mL and further incubated at 37 °C for 24 h. Cells were washed with D-PBS and lysed with 45 µL 1% Trixon X-100-D-PBS using a sonicator. After sonication, 5 µL 20 mM L-DOPA was added to each well to initiate the reaction. The microtiter plate was further incubated at 37°C for 1 h and absorbance was measured at 475 nm in a plate reader. Kojic acid was used as a positive control. The percentage inhibition of the cellular tyrosinase enzyme was calculated using the formula:

$$\% \text{ tyrosinase inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

The % tyrosinase inhibition was plotted against the plant extract concentration and the IC_{50} determined from the normalised logarithmic regression curve. The experiment was performed in duplicate and repeated three times.

6.2.6. Statistical analysis

The results were calculated as means ± standard deviation. The data was subjected to one-way analysis variance (ANOVA) using IBM Statistical Package for Social Science (SPSS) and means were separated using Fisher's multiple range tests ($p < 0.05$). Means were compared to each other with Fisher's least significant difference (LSD) test.

6.3. Results and Discussion

6.3.1. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay

The HEM cells were observed to achieve the desired cellular confluency of 60 to 80% required for bioassay (Figure 6.1D). The growth of melanocytes was characterized by formation of a monolayer with increased number of di- and tri-polar dendrites between day 4 and 7. The absence of phorbol 12-myristate 13-acetate (PMA) in the growth medium promoted formation of dendrites and cell proliferation with no signs of contact inhibition during the formation of the monolayer. There was an observed increase in melanin-containing melanosomes inside the cells (Figure 6.1B, C and D). Melanocytes cells displayed a darker character due to the accumulation of melanin inside the cells. In a keratinocyte-melanin unit model, the formed melanin granules would be transferred to the surrounding keratinocytes where they influence colour formation and contribute to photo protection **(ABDEL-MALEK et al., 2010)**.

The efficiency of the selected plant extracts on cell health was assessed on confluent melanocytes and results were expressed as percentage (%) cellular activity (Figure 6.2). All the extracts at concentrations ranging between 5 and 15 µg/mL enhanced cellular proliferation with recorded activity of ± 100%. Fully differentiated melanocytes have a long proliferation period with a doubling time of about 1.5 – 4 days **(HSU et al., 2005)**. Therefore, an effective internal protective system is required by the cell to avoid initiation of early apoptosis during the growth period. Treatment with these extracts can therefore enhance the health of the cell, effectively protecting them from physiological

programmed cell death. These results from the study can be used to determine effective workable concentrations of the extracts on HEM cell lines.

Furthermore, reaction of melanocytes to the treatment was recorded to be dose-dependent, with the highest sensitivity recorded at higher concentrations. Melanocytes were more sensitive to treatment by extracts of *C. flanaganii* with a recorded LD_{50} value of 50 µg/mL, when compared to untreated cells. Inhibition of melanocytes activity by more than 90% by extracts of *C. flanaganii* will significantly reduce the total number of melanocytes, impacting on the photo-protective effect of the skin.

The HEM cells were less sensitive to treatment by extracts of *C. album, C. anisata* and *R. nasturtium-aquaticum* and maintained the health of the cells during the study period. Therapeutic agents that positively modulate cell viability are desired in beauty enhancement formulations due to their projected effect on the function of the cells. The results from the study were used to determine effective workable concentrations of the extracts on bioassay investigating the efficiency of extracts on the function of melanocytes.

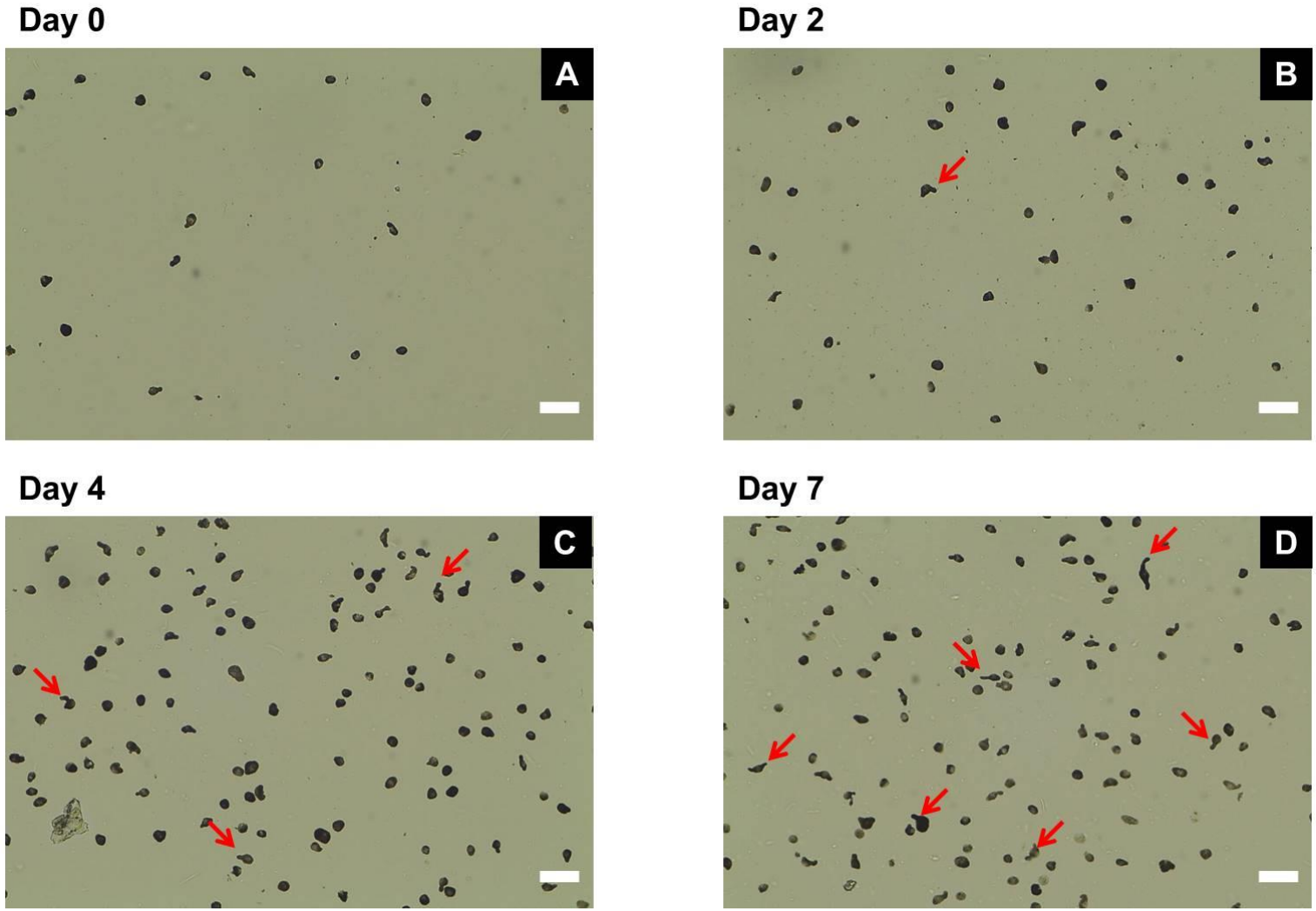

Figure 6.1: Proliferation of human epidermal melanocyte (HEM) cells at day 0 (A), 2 (B), 4 (C) and 7 (D) grown in complete dermal basal medium supplemented with melanocyte growth supplements. Confluency was observed by formation of dendrites (red arrows) between day 4 and 7. Scale bar: 20 µm.

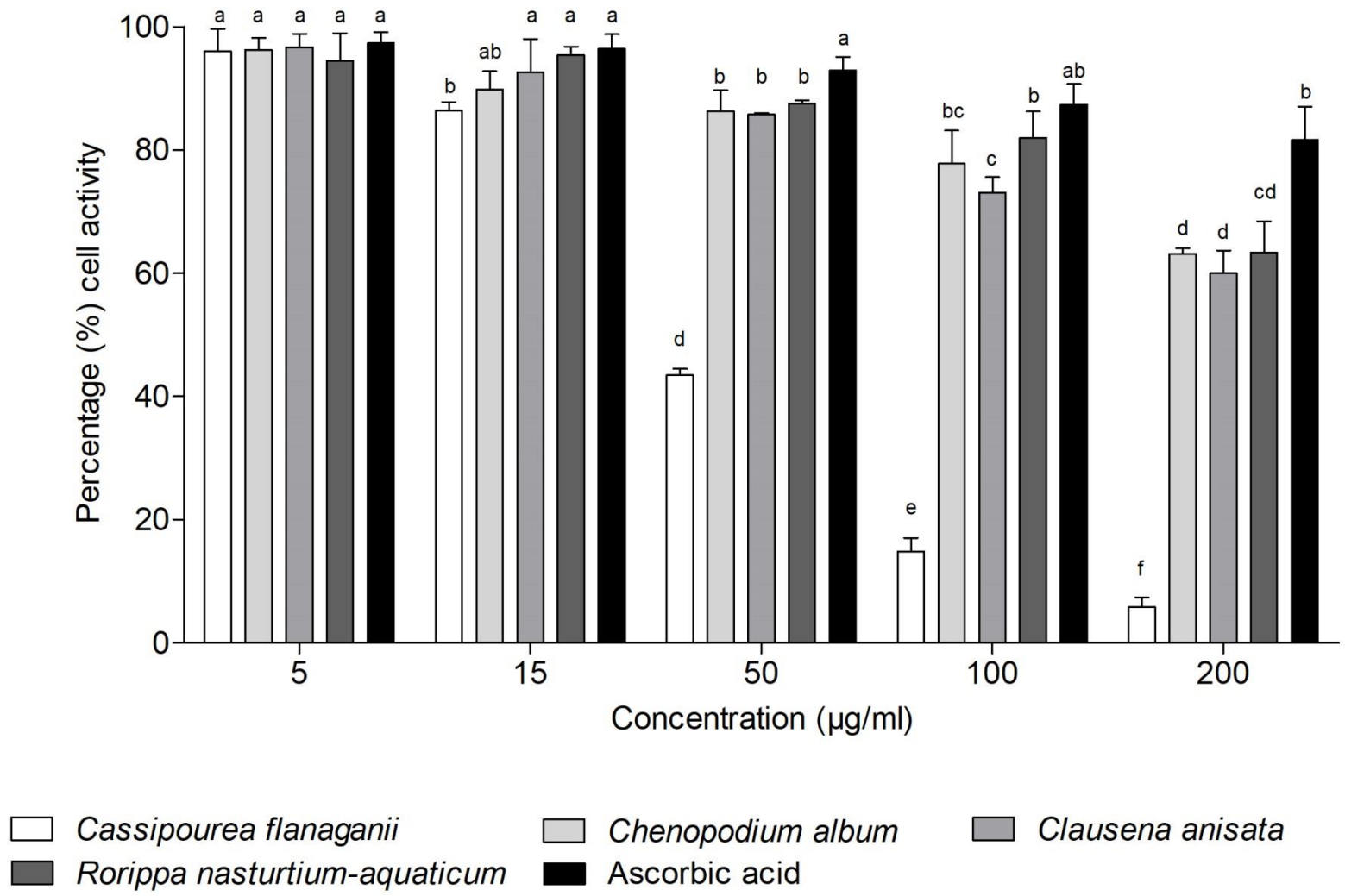

Figure 6.2: MTT activity assay of *Cassipourea flanaganii, Chenopodium album, Clausena anisata* and *Rorippa nasturtium-aquaticum* on HEM cell lines grown in complete dermal basal medium and supplemented with melanocyte growth supplements expressed as LD_{50} (µg/mL). Results indicate the mean and standard deviation values with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

6.3.2. Cellular anti-tyrosinase activity assay

The inhibition of the tyrosinase enzyme inside melanocytes was used to assess the efficiency of plant extracts in modulating the function of HEM cells with results expressed as IC_{50} µg/mL (Figure 6.3). The *in vitro* assay reported in Chapter 4 screened for inhibitors of mushroom tyrosinase with L-DOPA used as a substrate. However, the inhibitory effect of tyrosinase might be affected in a biological system like melanocytes. The cellular anti-tyrosinase activity assay would serve as a more rigorous method for determining the efficiency of these plant extracts.

There was no significant difference between the activity of the extracts and the positive control. Extracts of *C. flanaganii, C. album, C. anisata* and *R. nasturtium-aquaticum* were reported to be effective at inhibiting cellular tyrosinase with IC_{50} values ranging between 1.42 and 1.55 µg/mL. These concentrations are not toxic to the cells and will not result in significant reduction of the total number of melanocytes. The efficiency of the extracts in modulating the function of the tyrosinase enzyme will contribute positively to achieving the desired skin complexion and protecting melanocytes from UV-induced oxidative damage by ROS. The results of the study confirmed the efficiency of the plant extracts in a biological system like epidermal melanocytes when examining for their beauty enhancement potential.

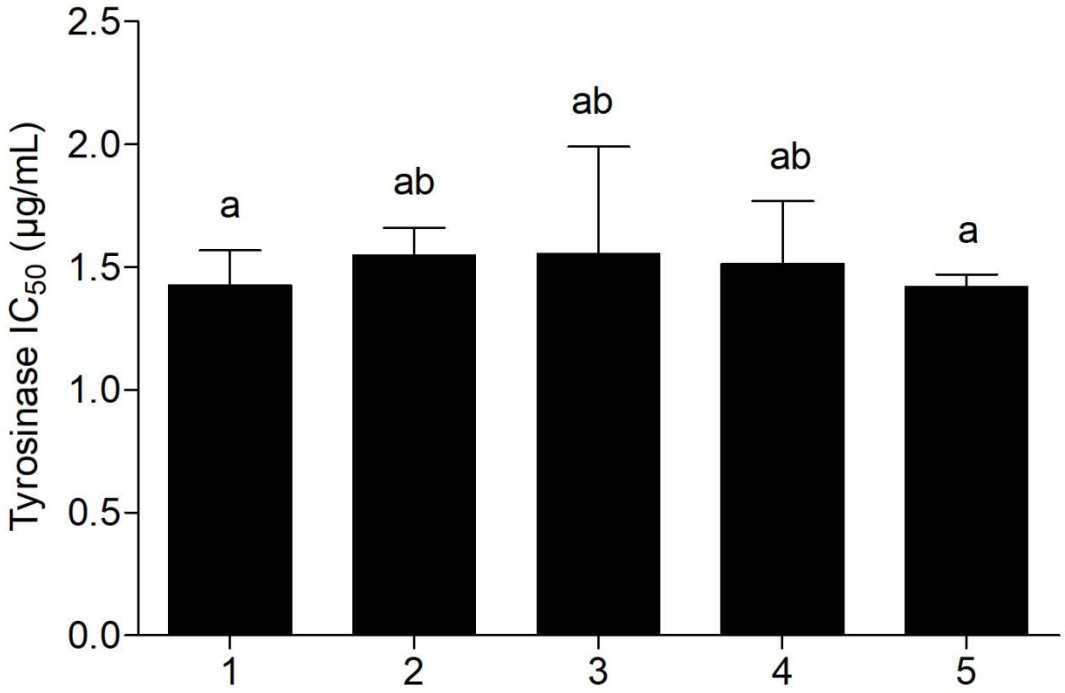

Figure 6.3: Cellular anti-tyrosinase activity results of plant extracts (1). *Cassipourea flanaganii,* (2). *Chenopodium album,* (3). *Clausena anisata,* (4). *Rorippa nasturtium-aquaticum* and (5). Kojic acid on HEM cell lines grown in complete dermal basal medium and supplemented with melanocyte growth supplements expressed as IC_{50} (µg/mL). Results indicate mean and standard deviation values with $p < 0.05$; n=3. Letters indicate Fisher's LSD.

6.4. Conclusions

The production of melanin by melanocytes has a dual function on the maintenance of the skin due to melanin's photo-protective effect and contribution towards achieving desired skin complexion. The health and function of melanocytes play a significant role in the ability of the cell to achieving this. Treatment of the HEM cells with all the extracts at concentrations of 5 – 15 µg/mL prolonged cell proliferation and consequently enhancing the function of the cell. However, the observed toxicity of extracts of *C. flanaganii* at higher concentrations can be correlated with the data collected from the ethnobotanical survey. The plant was recorded as used for hypopigmentation by some community members. However, the survey results further reported undesired darkening of the skin with prolonged usage of the plant. The conventional topical application of the extract by mixing the ground bark with water results in higher concentrations being administered. One of the reported active mechanisms that skin lightening agents are known to induce their activity is through the reduction of the number of melanocytes. This mechanism has a drawback in that once all the melanocytes are depleted, the skin becomes exposed to UV damage and hence the reported darkening. Lower concentrations of the plant are therefore encouraged in order to observe the beauty enhancement properties reported with earlier bioactivity assays. The results from this study are significant when developing formulations that are efficient and not toxic to melanocytes. Clinical studies on the transdermal effect and delivery of the required concentrations of formulations incorporated with these extracts will be advantageous to ensure their safe usage.

Chapter 7

General conclusions

The desire to look beautiful is an emotion many people have in common. The use of medicinal plants in ATM has many reported usages, including beauty enhancement. The cosmeceutical industry is not stagnant; the success of the industry thrives on constant innovation and introduction of novel formulations to meet market demand. One of the drawbacks of innovation and big industries is that it is easy to “go overboard” and as a result formulations with undesired consequences end up in the market. In an effort to address some of the challenges to the cosmeceutical market, this study aimed at tapping into indigenous knowledge on how beauty enhancement issues are addressed.

However, one has to be cognisant to the fact that plant metabolites function in synergism to induce their reported activity when contemplating isolating active compounds. The aspect of quality assurance of whole extract preparations can also be questioned, as studies have shown plants extracted within the same environmental community possessing varying concentrations of the active compounds. Whole extract preparations were given preference with care taken to use the same plant material for all the assays.

This pharmacological study was guided by the data collected from the ethnobotanical survey. The ethnobotanical data reported on the most favoured plants used by communities for toning the skin and achieving the desired skin complexion and smoothness with no visible eruptions. Sixteen medicinal plants were selected from the results of the survey and evaluated for their cosmeceutical potential by assessing their skincare and beauty enhancement properties. The skincare properties were

demonstrated by assessing the plants for their antimicrobial activity against common skin commensal bacteria, infectious skin microorganisms and skin dermatophytes. The results from the study highlighted the potential of ethanol extracts of *A. arctotoides* and *C. flanaganii* in inhibiting the activity of all tested microorganisms. Novel products can be formulated using these two plants, specific for skincare, since the tested microorganisms are known to cause skin-related infections and diseases.

The beauty enhancement properties of the plants were further demonstrated by assessing the plant extracts against a number of bioactivity assays. The plants were assessed for their phenolic compound content, antioxidant potential and photo-protective effects. The extracts were further evaluated for their inhibition activity against the skin-complexion related tyrosinase enzyme and skin-inflammation related $sPLA_2$, 15-LOX, COX-1 and COX-2 enzymes. Lastly, the efficiency of four extracts were assessed for their safety and maintenance of cell health and function using an HEM skin cell line model.

Plants are naturally a good source of antioxidants due to the environment in which they are constantly exposed to UV-induced oxidative stress and mechanical damages. It is interesting when the antioxidant activity derived from the phenolic content of the plants can be linked to other biological activities. This was observed with extracts of *R. nasturtium-aquaticum, C. anisata, S. officinale* and *C. flanaganii*, which were able to reduce the activity of the tyrosinase enzyme. Reduction of the tyrosinase enzyme is significant, especially when formulating products that can treat UV-induced skin

darkening and dark spots associated with the proliferation of inflammation by skin diseases.

The absorption, reflection and scattering of harmful UV radiation were measured for the photo-protective effect of the extracts. Higher SPF values are indicative of the effectiveness of the plant extracts against UV radiation. Sun protection factor values above 15 were observed for extracts of *P. lanceolata, C. flanaganii, A. oblongifolia, I. mitis* and *A. arctotoides*. There were high levels of phenolic and flavonoid compounds recorded for these plant extracts with the exception being *A. arctotoides*. Application to the skin with formulations incorporating these extracts may protect the skin by 93.3% against UVB radiation for up to 2 hours.

The inhibition of pro-inflammatory enzymes of $sPLA_2$, 15-LOX COX-1 and COX-2 is a significant strategy in reducing skin inflammation. The $sPLA_2$ enzyme, responsible for the release of arachidonic acid, was resistant to 80% of the tested plant extracts with notable activity only expressed by extracts of *C. album* and *C. flanaganii*. Therefore, the inability of the extracts to inhibit the $sPLA_2$ enzyme will result in the release of free arachidonic acid. Extracts of *C. flanaganii, C. album, P. lanceolata* and *R. nasturtium-aquaticum* expressed notable activity against 15-LOX, therefore inhibiting the enzyme from further metabolizing the released free arachidonic acid. The two COX isoenzymes (COX-1 and COX-2) were also inhibited by some extracts in metabolizing the free arachidonic acid into pro-inflammatory mediators. Extracts of *A. ferox, C. anisata, H. albiflos, R. graveolens, S. officinale* and *U. urens* expressed notable activity in inhibiting

the COX-1 enzyme. Furthermore, extracts of *A. ferox, C. flanaganii, C. anisata, H. albiflos, P. lanceolata* and *R. nasturtium-aquaticum* inhibited the activity of the COX-2 enzyme. However, dual inhibitors of 15-LOX and COX-2 are desired for effective treatment of inflammatory associated skin diseases with reduced health side effects. Extracts of *C. flanaganii, P. lanceolata* and *R. nasturtium-aquaticum* were the most effective dual inhibitors of 15-LOX and COX-2.

The observed effectiveness of extracts of *C. flanaganii* on bioactivity assays related to beauty enhancement can be corroborated by the ethnobotanical survey data collected from the study. *Cassipourea flanaganii* was the second highest cited plant after *A. ferox*. Formulation of beauty enhancement products with *C. flanaganii* have to be incorporated with other plant extracts with photo-protective capabilities in order to enhance the usage of the product. The use of the bark of *C. flanaganii* illustrates that sustainable harvesting techniques will be required to prevent total destruction of the plant. The study further highlighted other plants that can be used effectively for beauty enhancement which were previously reported for other traditional medicine uses.

The anti-tyrosinase activity of *C. album, C. anisata, C. flanaganii* and *R. nasturtium-aquaticum* was previously reported (Chapter 4) with IC_{50} values comparable to the positive control. The efficiency of these plant extracts was further reported with the inhibition of cellular tyrosinase of melanocytes with IC_{50} values ranging between 1.42 and 1.55 µg/mL which were comparable to the positive control. All the tested plant extracts were reported to enhance cellular proliferation at these lower concentrations

which were not toxic to cells. However, high concentrations of *C. flanaganii* should be avoided as the plant was determined to be toxic to melanocytes. Interestingly, extracts of *C. album, C. anisata* and *R. nasturtium-aquaticum* were not reported for their hypopigmentation activity in the literature and from the survey. The results from this study indicated for the first time their potential as beauty enhancement agents through their ability to enhance skin complexion.

The results from this study provide a full ethnopharmacological profiling of *C. flanaganii* as a model plant with beauty enhancement potential. The potency of this plant in bioactivity assays reporting on beauty enhancement was covered in depth and the efficiency further tested on a skin cell line model. This approach further identified new applications of some of the other plants in beauty enhancement and ascertained their efficacy and safe utilization. The beauty industry requires the highest ethical approach to ensure the protection of the consumers. Such approaches need to be upheld by individuals and entities interested in formulation of products intended for application on the skin. Future work related to this study should include incorporation of plant extracts with skincare and beauty enhancement potential into formulations for product development. Stability and efficacy studies will also have to be conducted on the formulated products to ensure their shelf life.

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