PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON SOME SOUTH AFRICAN PLANTS USED IN TRADITIONAL MEDICINE FOR SKIN HYPERPIGMENTATION.

UNIVERSITY *of the*
WESTERN CAPE

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Sciences at the University of the Western Cape.

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ABSTRACT

The regulation of the Western beauty care products is currently inadequate and a huge concern to consumers throughout the world, as most of the products manufactured by personal care industry are made of synthetic additives, while the traditional skin-care products are safe products made from natural elements. Usage of traditional medicinal plants is perceived as a superior alternative to achieve improvement of the gradually deteriorating standard of manufacturing beauty care products. Therefore, the present study assesses the suitability of several South African plant species conventionally used in traditional medicine for skin hyperpigmentation treatment. Ten plant species, viz. *Cassine peragua*, *Cassipourea gummiflua*, *Clivia miniata*, *Cryptocarya myrtifolia*, *Gunnera perpensa*, *Kigelia africana*, *Protorhus longifolia*, *Rapanea melanophloeos*, *Rhynchosia villosa*, and *Senecio serratuloides* were obtained after interviewing fifteen traditional healers within the Eastern Cape Province.

The methanolic extracts of the ten suggested plants were subjected to a preliminary bio-evaluation against the tyrosinase enzyme. The results showed that *Rhynchosia villosa* root was the most active extract and hence, the *R. villosa* extract was subjected to further chromatography and studied. Five known flavonoids were isolated for the first time from *R. villosa*. The isolated constituents include genistein (**C1**), cajanin (**C2**), 2′-hydoxygenistein (**C3**), catechin (**C4**), and gallocatechin (**C5**). The tyrosinase inhibitory activity of the isolated compounds showed the highest activity for **C1** (31.45 µM), followed by **C4** (36.86 µM), **C2** (38.97 µM), **C5** (60.40 µM), and lastly **C3** (69.49 µM).

To the best of our knowledge, and according to an extensive search of the Sci-Finder database, the dissertation is the first scientific report to be carried out on the phytochemical and tyrosinase inhibition of *R. villosa* plant species. The *R. villosa* showed a high tyrosinase inhibitory activity, which makes it a likely candidate as a tyrosinase inhibitor that may be used as depigmenting agent for the treatment, or prevention, of pigmentation disorders in relation to hyperpigmentation. Furthermore, the results obtained also justify the traditional uses of the plant for skin depigmentation.

Keywords: tyrosinase enzyme, hyperpigmentation, traditional medicine, *Rhynchosia villosa*.

DECLARATION

I, **Mtandazo Rondo**, hereby declare that the dissertation entitled **'Phytochemical and biological studies on some South African plants used in traditional medicine for skin hyperpigmentation'** is a bona fide record of my independent research work and has not been previously submitted for any academically purposes in any other institution. In the scope of the dissertation I have not used anybody's work as my own; the used sources have been ascribed in the correct way, thus, by means of citation, quotation, and complete reference. I further declare that ethical guidelines were complied with in conducting the study.

Signature: M. Pama

Date: **May 2017**

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Justice would not be served without thanking **Mr Solomon Kuca** and **Ms Noluvo Ndzoyiya** as they took over the role of **Mr Zekanzima Ndzoyiya** after his death, making sure that I receive the necessary information to complete the work. I extend thanks to my one and only friend, **Mr Zuko Mtlomelo**, for his endless encouragement and support throughout my life journey.

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Before the closure of the acknowledgment chapter, I wish to articulate how obliged I'm to my late grandmother, **Ms Thalitha Rondo**, I thank her for her words of wisdom and teachings. Last but not least, I also give many thanks to my mother, **Ms Nomlindelo Rondo**, for nurturing and raising me to become a better person in life. I truly believe that without her I wouldn't have made the prodigious and triumphant strides that I have made in my life.

The benevolence of the above-mentioned individuals made me to affirm with the words of the Brazilian novelist, Coelho Paulo, that "*when you want something, all the universe conspires in helping you to achieve it*." I wish that the **Mighty God** may bless all of them and their families!

DEDICATION

To the late **Mr Zekanzima Jourbert Ndzoyiya** (*15 July 1949 — 3 April 2016*)

A leading light traditional healer from Lusikisiki in the Eastern Cape Province (S. Africa).

He started his healing journey in year 1985.

In his lifetime as a traditional healer, he practiced in various

Southern African countries, which include Botswana, Namibia, Lesotho, and Swaziland.

He was a dignified man, with great wisdom, persistence, and compassion.

I truly believe that the study wouldn't have been feasible without him.

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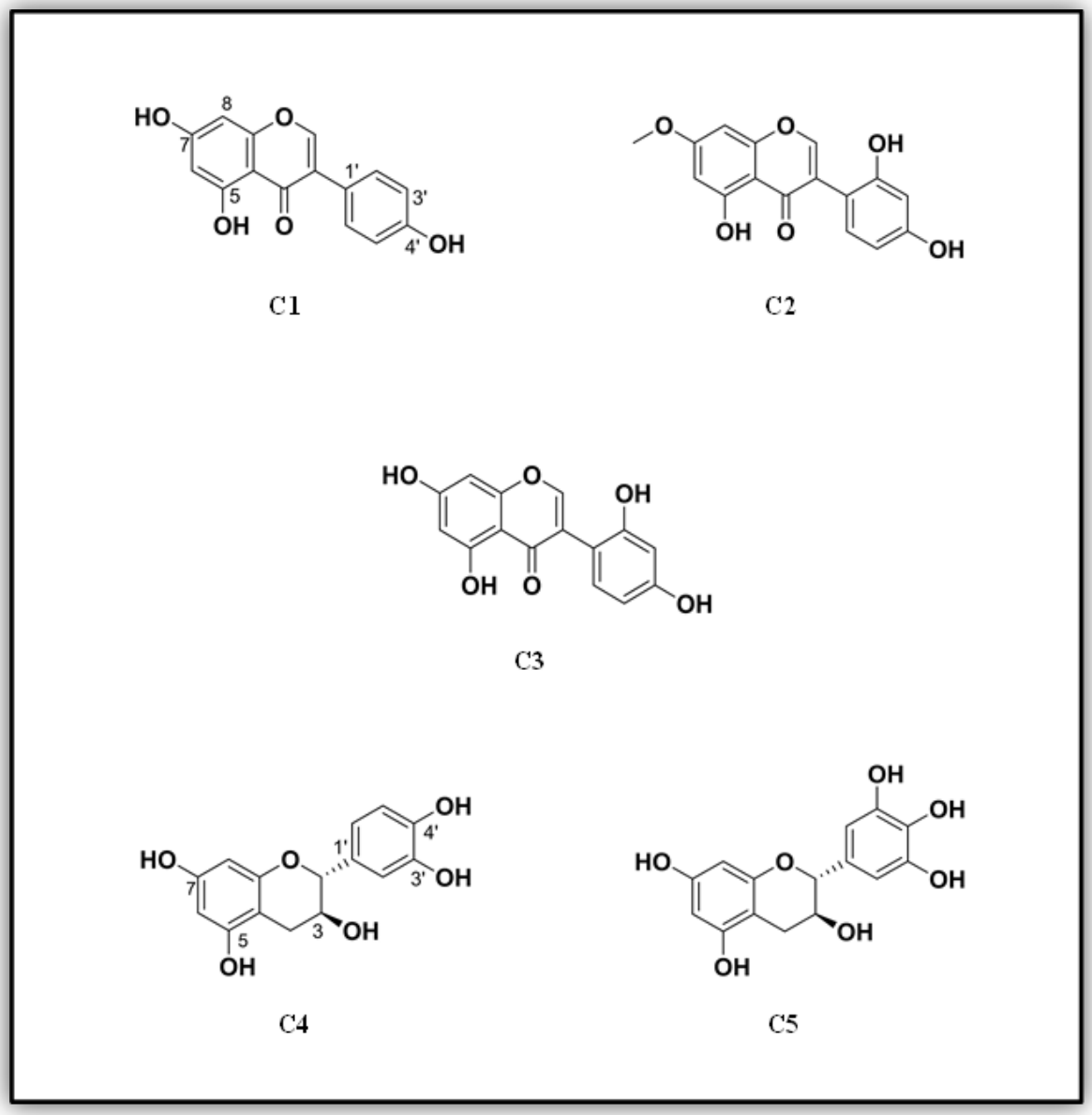
LIST OF ABBREVIATIONS

λ	Wavelength
β	Beta (equatorial position)
α	Alpha (axial position)
δ_C	Carbon chemical shift
δ_H	Proton chemical shift
1D NMR	One-dimensional nuclear magnetic resonance
2D NMR	Two-dimensional nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon 13 nuclear magnetic resonance
BBO	Broadband observe
CD_3OD	Deuterated methanol
CFR	Cape floristic region
d	doublet
DCM	Dichloromethane
dd	doublet of doublets
DEPT	Distortionless enhancement by polarization transfer
DIW	Deionized water
DMSO	Dimethyl sulphoxide
EtOH	Ethanol
EtOAc	Ethyl acetate
GTPs	Green tea polyphenols
Hex	Hexane
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome
HIV-OMH	HIV-associated oral mucosal melanin hyperpigmentation

HMBC	Heteronuclear multiple-bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single-quantum coherence
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
IDRC	International Development Research Centre
J	Coupling constant
m	multiplet
MeOH	Methanol
MHz	Megahertz
min	minutes
mM	millimolar
NMR	Nuclear magnetic resonance spectroscopy
nm	nanometre
NOESY	Nuclear overhauser effect spectroscopy
OHGs	*ortho*-hydroxygenisteins
pH	Potential hydrogen
PIH	Post-inflammatory hyperpigmentation
ppm	parts per million
s	singlet
S/No.	Structure number
SANBI	South African National Biodiversity Institute
TLC	Thin layer chromatography
UV	Ultraviolet
viz.	*videlicet* (that is to say; namely)

LIST OF ISOLATED COMPOUNDS

The *Rhynchosia villosa* methanolic extract afforded the following five compounds:



CHAPTER ONE

INTRODUCTION

WHY NATURAL PRODUCTS STUDY IN COSMETICS?

"*The perception is that natural ingredients are more pure and kinder to skin than something made in the lab, but nothing could be farther from the truth*" — Dr. Joel Schlessinger

1.1 THE PROBLEM

Epidemiological data shows that about 35 % of women in South Africa use skin whitening products, which consist of synthetic chemicals, to bleach their skin (United Nation Environmental Programme, 2008). The inadequate regulation of beauty care products is a global issue. This is attested to by the dialectical claims emanating from various environment extremist groups, such as the Campaign for Safe Cosmetics and the Environmental Working Group, who proclaim that many of the cosmetics used by consumers worldwide are poisonous (Gattuso, 2011). According to their research, it has been found that a number of cosmetics consist of paraben compounds; such compounds are linked to breast cancer and hormone dysfunction (Gray, 2010; Environmental Working Group, 2016). The most common dermatological disorders in S. Africa are associated with hyperpigmentation; this is due to the fact that 91 % of the S. African population has a dark complexion. Dermatological studies conducted in Durban amongst 900 women with dark complexions showed that 67 % of the women who frequently use skin whitening products have genuine hyperpigmentation disorders (Webmaster, 2014).

1.2 SOUTH AFRICAN FLORA USE IN TRADITIONAL MEDICINE

It is clear that more work needs to be done in enhancing the standard of cosmetics. Hence, the use of traditional medicinal plants which consist of natural products is regarded as an efficient way to ameliorate the standard of manufacturing beauty care products. In the context of the

dissertation, the phrase ‘natural products’, refers to substances or compounds that are produced by nature and they are directly extracted from plants (Dorland’s Illustrated Medicinal Dictionary, 2000). On that note, the abundance of plant species in S. Africa is exceptional. S. Africa is one of the most plant fecund countries, consisting of approximately 23 420 plant species of about 368 families, with more than 50 % of such flora found nowhere else in the world (Cowling and Hilton-Taylor, 1994; Willis, 2006).

Nonetheless, apart from the profusion of plant species in S. Africa, knowledge regarding the phytochemistry and efficacy of such plant species remains insufficient. Thus, out of the enormous number of plant species found within the country, only 50 plant species are presently traded to any substantial level (Welford and Le Breton, 2008). This therefore necessitates more, efficient research, which will lead to innovative use of the S. African plants. The approach of using plant species in the discovery of therapeutics remains an ultimate reservoir for the effective usage of plants (Hostettmann, 1998).

Although the exploitation of plant species is quite meager when it comes to commercialization in the African continent as a whole, it is certain that the traditional medicinal practice and use of remedies made from indigenous plants play a significant role in the primary health care of millions of sub-Saharan African natives. This claim is attested to by the International Development Research Centre (IDRC), and according to the IDRC survey, about 85 % of Africans occupying the sub-Saharan African regions depend on traditional healers and their remedies for primary health care (Stanley, 2004). This claim is also supported by the large number of traditional medicinal practitioners within each sub-Saharan African country. In S. Africa alone, there are about 200 000 traditional healers (Willis, 2006).

Furthermore, the use of traditional medicinal plants as a primary health care by a variety of culturally diverse African populations has existed for thousands of years, long before the introduction of the Western medicine (Veilleux and King, 1996). Traditional medicinal remedies are used to treat a variety of ailments and infections, which include tuberculosis, nervous complaints, depression, headache, fever, rebalancing the digestion system, and skin disorders, to mention but a few. Therefore, the current study focuses on the treatment of skin disorders, particularly the treatment of skin hyperpigmentation by the usage of medicinal plants.

1.3 NATURAL COSMETICS

The usage of traditional medicinal plants in curing dermatological disorders is perceived as the most efficient approach, to such an extent that beauty care manufacturers predominantly adopt this method to produce 'friendly' skin-care products (Draelos, 2008). This approach of using natural products in the manufacturing of cosmeceuticals is encouraged by the fact that, unlike synthetic drugs which are harmful to the skin, they result in improvement of the skin appearance through the delivery of nutrients which are necessary for a healthy skin (Rebeiro *et al.*, 2015). Scientific findings show that traditional medicinal plants were amongst the first beauty care products, and, interestingly, the use of natural products in manufacturing cosmeceuticals is still perceived as an efficient method, but with substantial advancements. Thus, with new formulation techniques, control of raw materials and greater standardization are achieved, the aforementioned advances have led to innovative cosmeceuticals (Kirk-Othmer, 2013).

1.4 AIM OF THE STUDY

The ultimate aim of the current study is to evaluate several plants traditionally used for the treatment of skin disorders, as tyrosinase inhibitors, and then select the most active extract for further investigation.

1.5 OBJECTIVES OF THE STUDY

The purpose of the research project is to:

- ✓ Collect various plant species which are conventionally used for skin hyperpigmentation from their natural habitats, identify and document them.
- ✓ Assess the extracts of *Cassine peragua* (bark), *Cassipourea gummiflua* (bark), *Clivia miniata* (root rhizome), *Cryptocarya myrtifolia* (stem bark), *Gunnera perpensa* (rhizome), *Kigelia africana* (fruit), *Protorhus longifolia* (bark), *Rapanea melanophloeos* (bark), *Rhynchosia villosa* (root and leaves), and *Senecio serratuloides* (leaves) against tyrosinase activity.
- ✓ Use various chromatographic methods to separate bioactive compounds and to use bioassays to detect activity from the most active extract against the tyrosinase enzyme.
- ✓ Thereafter, the active compounds would be characterized, identified, and evaluated against the tyrosinase enzyme.

CHAPTER TWO

LITERATURE REVIEW

2.1 GENERAL REVIEW

2.1.1 SOUTH AFRICAN FLORA

The world comprises of approximately 260 000 plant species (Miller and Spoolman, 2008). As mentioned previously, S. Africa is a country that encompasses a large number of plant species; coming sixth after Brazil (56 215), Columbia (51 220), China (32 200), Indonesia (29 375) and Mexico (26 071), with 23 420 plant species (Butler, 2016). S. Africa is the only country in the world with an entire plant kingdom within its boundaries, and around 10 % of the world's flowering plant species (angiosperms) are found in S. Africa (Fry, 2010; Germishuizen and Meyer, 2003). The world is divided into six floral kingdoms, namely, Boreal Kingdom (20 million km^2), Antarctic Kingdom, Neotropical Kingdom, Australian Kingdom, Paletropical Kingdom, and the smallest, but ironically the most diverse of them all, is the Cape Floral Kingdom of the Western Cape Province of S. Africa, with only a 90 000 km^2 surface area (Charters and Madre, 2015).

The Cape floristic region (CFR) is the most fertile region on Earth, as it consists of the highest density of plant species globally with 31.9 % of the species being endemic (Takhtajan, 1986). This kingdom comprises roughly 8 700 plant species, with approximately 6 000 (69 %) plant species found nowhere else in the world (Goldblatt, 1978; Bond and Goldblatt, 1984). The CFR ranges from Cape Point (W. Cape Province) to Grahamstown (Eastern Cape Province) and minimally into the Northern Cape Province (Figure 2.2). Although the CFR only represents less than 0.5 % of the African continent's land surface area (Figure 2.1), it is home to nearly 20 % of

the continent's flora, making the CFR rank number seven in the biodiversity 'hotspots' of the world (Goldblatt and Manning, 2000).

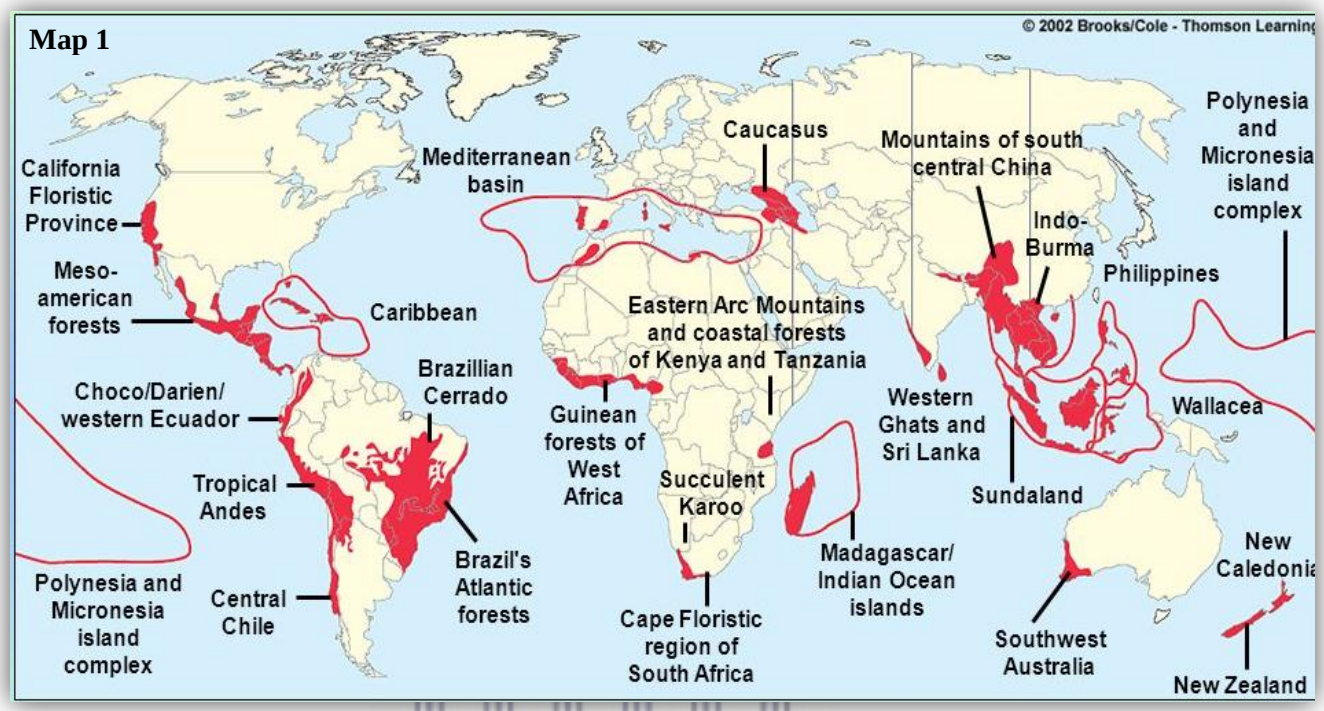


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Figure 2.1: Floristic regions throughout the world.

The CFR is one of S. Africa's eight World Heritage sites and is made up of eight protected areas (Figure 2.2) that are considered to be the most important examples of the CFR, namely, Cape Peninsula National Park (Table Mountain), Boland Mountain Complex, Groot Winterhoek Wilderness Area, Cederberg Wilderness Area, De Hoop Nature Reserve, Boosmansbos Wilderness Area, Swartberg Mountains, and Baviaanskloof Wilderness Area, such areas spans from the Cape Peninsula to the E. Cape Province (Anon, 1999).

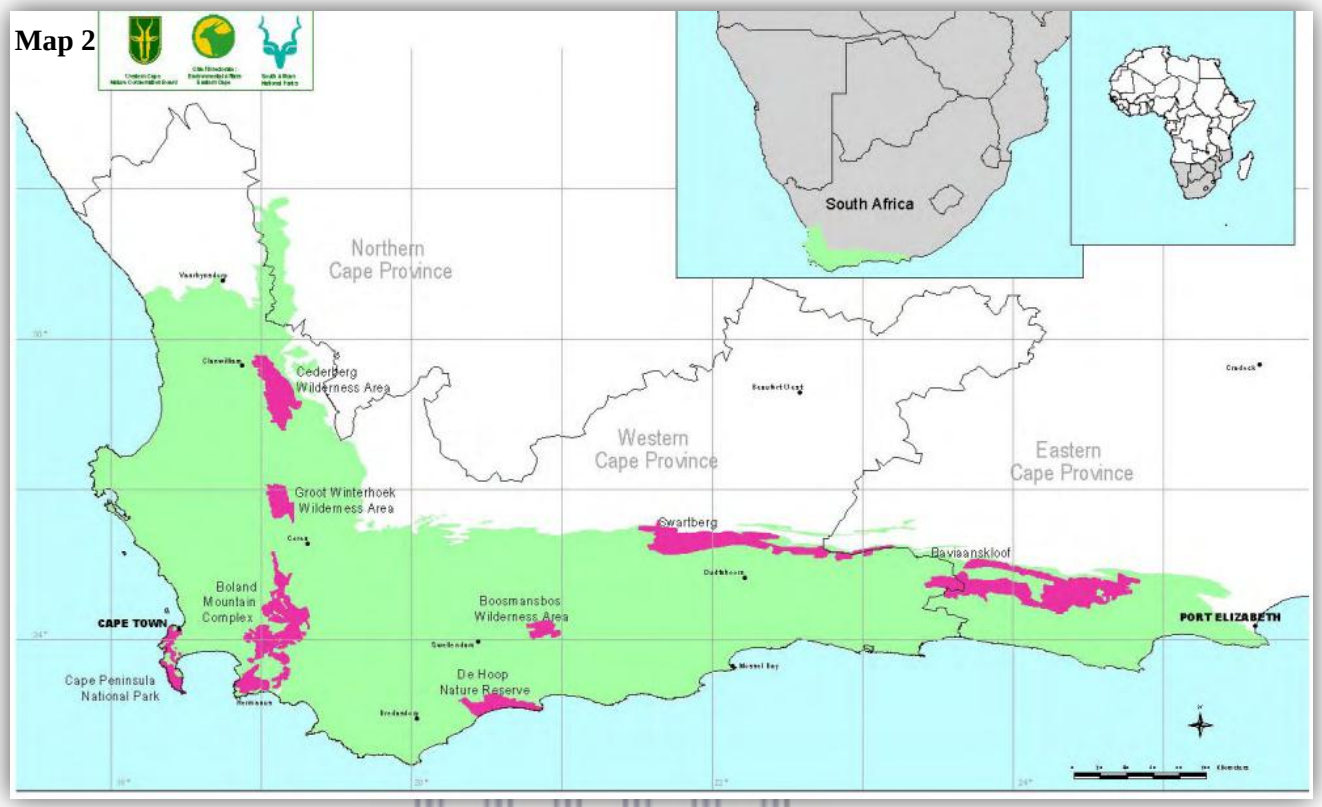

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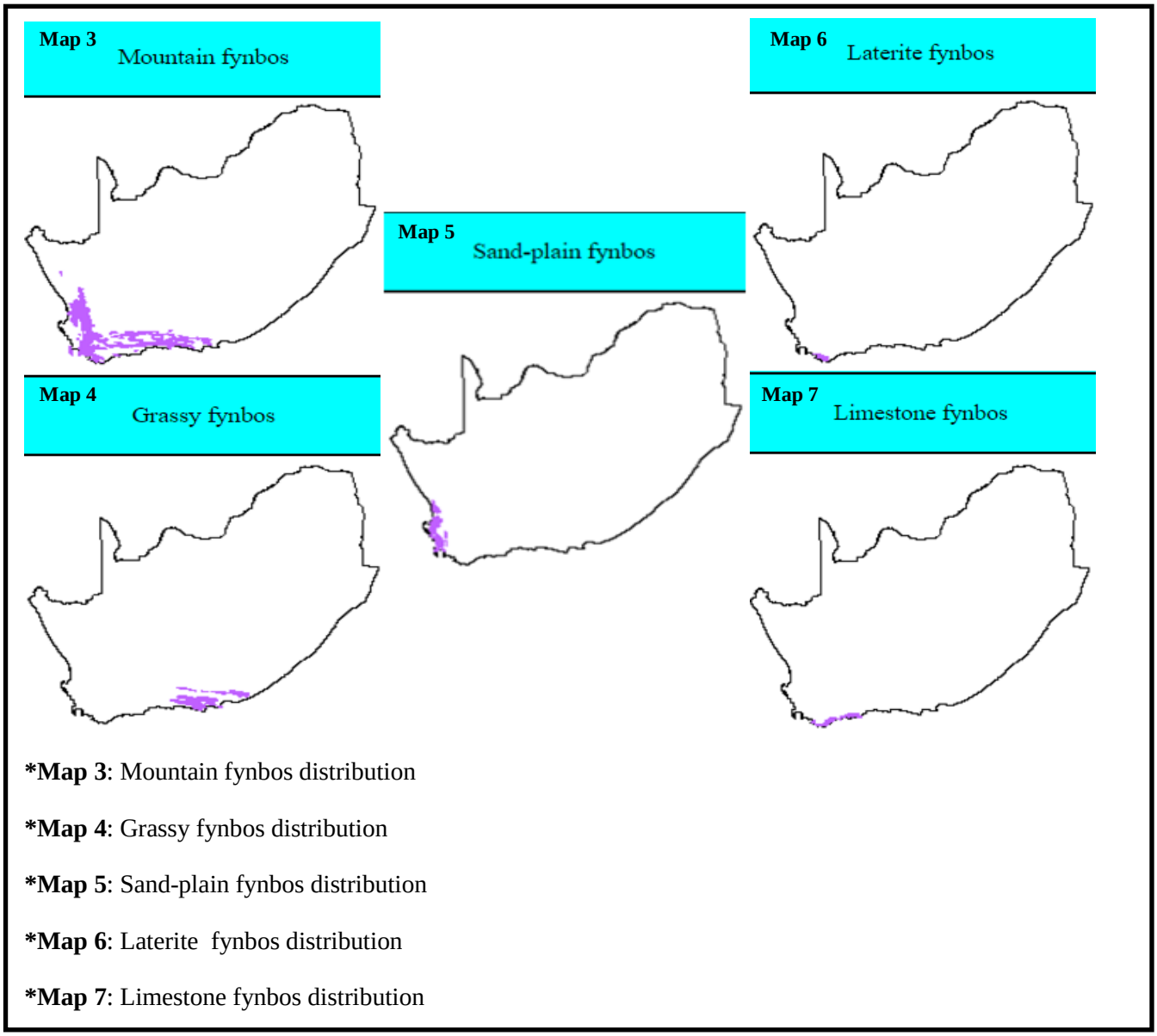
Figure 2.2: Cape floristic region.

Moreover, the primary vegetation type of the W. Cape is 'fynbos' (word derived from Afrikaans meaning 'fine bush'), and this distinctive vegetation of sclerophyllous shrubland constitute about 80 % of all the vegetation of the Cape Floral Kingdom, while 20 % is shared amongst other vegetation such as Thicket biomes, Nama Karoo and Succulent Karoo (Anon, 1999). There are five main vegetation types of fynbos; mountain fynbos being the largest, followed by grassy fynbos, sand-plain fynbos, laterite (coastal) fynbos, and limestone fynbos, and their distribution within the CFR is depicted in Figure 2.3 (Low and Rebelo, 1996).



***Map 3**: Mountain fynbos distribution

***Map 4**: Grassy fynbos distribution

***Map 5**: Sand-plain fynbos distribution

***Map 6**: Laterite fynbos distribution

***Map 7**: Limestone fynbos distribution

Retrieved from *Calflora.net* website: http://www.calflora.net/southafrica/capeflora.html

Figure 2.3: Main fynbos vegetation types.

Moreover, the fynbos biome is mainly made up of fine-leaved shrubs such as heaths (Ericaceae), larger-leaved showy flowered (Proteaceae), bunches of reed-like (Restionaceae), and fan-like (Iridaceae) plants (Bond and Goldblatt, 1984). The plant taxa recorded for S. Africa varies over time, due to new discoveries and taxonomic research. For instance, 268 new species of S. African plants have been described between 1994 and 2004 (Willis, 2006), hence the information in Table 2.1 below may have changed slightly as it was documented in 1984.

Table 2.1: The largest families and genera in the Cape Flora Kingdom

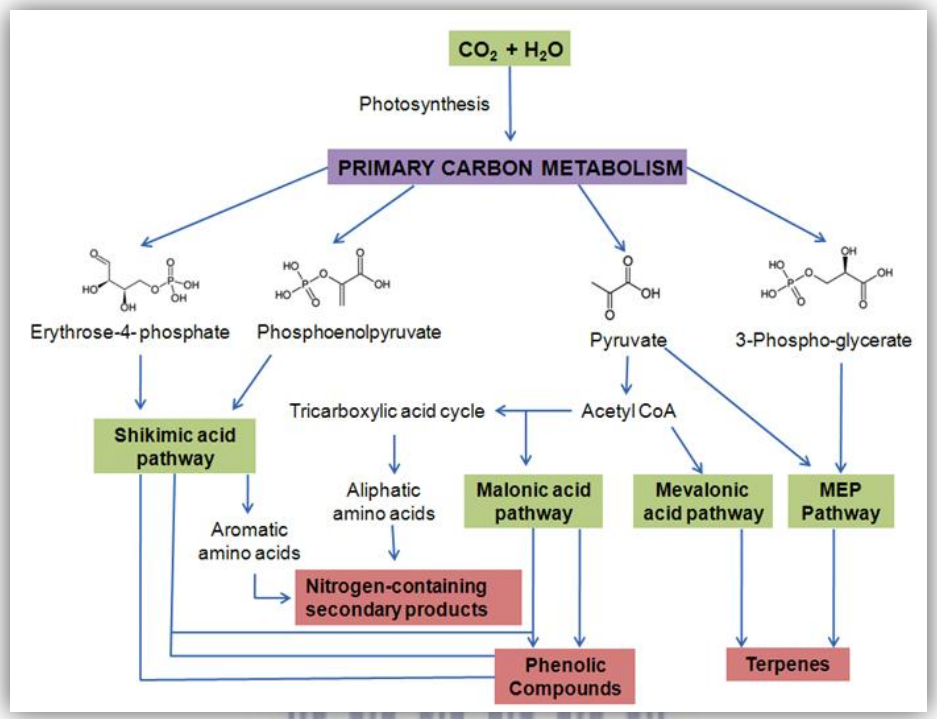
Family	Total species	Endemic species	Largest genus	Species
Daisy: Asteraceae	986	608	*Senecio*	133
Heath: Ericaceae	722	700	*Erica*	550
Vygle: Mesembryanthemaceae	660	507	*Ruschia*	138
Pea: Fabacea	644	525	*Aspalathus*	245
Iris: Iridaceae	612	485	*Gladiolus*	88
Protea: Proteaceae	320	306	*Leucadendron*	80
Cape reed: Restionaceae	310	290	*Restio*	85
Figwort: Scrophulariaceae	310	160	*Selago*	59
Buchu: Rutaceae	259	242	*Agathosma*	130
Bellflower: Campanulaceae	222	157	*Lobelia*	42
Orchid: Orchidaceae	206	124	*Disa*	52
Sedges: Cyperaceae	203	124	*Ficinia*	57
Milkwort: Polygalaceae	139	117	*Muraltia*	106
Jujube: Rhamnaceae	136	122	*Phylica*	133
Storkbill: Geraniaceae	133	67	*Pelargonium*	125
Sorrel: Oxalidaceae	129	90	*Oxalis*	129
Rose: Rosaceae	114	97	*Cliffortia*	106

***Source**: Bond and Goldblatt (1984)

2.1.2 NATURAL PRODUCTS AND THEIR USE IN COSMETICS

As stated above, although there is a surplus of plant species in S. Africa, these plants have not been effectively used. It has been identified that all plant species produce natural products. These plant-derived substances, also known as 'natural ingredients' or 'secondary metabolites', can be found in different parts of the plant, i.e., the roots, flowers, stems, barks, fruits, leaves and seeds (Chugh *et al*., 2012). Each and every plant species has a distinctive mechanism for biosynthesizing secondary metabolites, referred to as secondary metabolism (Scheme 2.1) and such mechanism is responsible for the uniqueness of a plant species (Maplestone *et al.*, 1992). It is very important to note that natural products or secondary metabolites are unnecessary for the growth of the plant species, although they contribute to its colour, aroma and flavour (Fraenkel, 1959; Saxena *et al.*, 2013). They come into existence in plant species due to the environmental conditions under which the plant germinates (Colegate and Molyneux, 2008). Natural products are also produced to protect the plant species against predators, pollution, stress, drought, ultraviolet (UV) exposure and pathogenic attacks (Schafer and Wink, 2009).

In terms of the chemistry, the secondary metabolites are classified based on their chemical structures, composition and their solubility in various solvents or the pathway in which they are synthesized (Kabera *et al*., 2014). The various groups of secondary metabolites include alkaloids, steroids, terpenoids, phenols, flavonoids, tannins *etc.* and they are differentiated by the functional groups present (Krishnaiah *et al.*, 2007). Such compounds are a subdivision of three chemically distinct groups, namely, nitrogen-containing secondary products, phenolic compounds and terpenes (Van Wyk and Wink, 2004).



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Scheme 2.1: Major pathways of secondary metabolites biosynthesized from primary metabolites (Secondary metabolism).

It has been observed that each plant species produces a characteristic mix of these bioactive compounds, which are regarded to be high value products compared to their precursors, the primary metabolites (Veberič, 2010). Secondary metabolites are considered to be fine chemicals, and are extensively used as pharmaceuticals, flavourants, pesticides, cosmetics, *etc*. (Balandrin *et al*., 1985). The focal point of the present study is based on the latter, i.e., the use of natural products in cosmetics. The use of natural products in cosmetics has existed for centuries and has evolved drastically in recent years. As mentioned previously, natural products are perceived as components that have less adverse effects compared to synthetic drugs; thus, they are observed

as natural and free from all the harmful synthetic chemicals, with anti-tyrosinase, antioxidant and antimicrobial activities (George, 2011; Rebeiro *et al.*, 2015).

On the same note, being beautiful and feeling young are qualities that give the sense of pleasure and are, perhaps, the desire of every human being throughout the world. Hence the prevalent use of cosmetics (Kapoor, 2005). A huge problem is that many cosmetics consist of synthetic additives which are harmful or poisonous, leading to a rising number of consumers being concerned about marketed cosmetics. Thus the demand for cosmetics with traceable and more natural ingredients, free from harmful chemicals and with an emphasis on the rejuvenative properties of botanicals is instigated (Ferrer *et al*., 2012).

It is crucial to define the term 'cosmetics' in order to avoid misperceptions. According to the European Parliament and the Council of the European Union (2009: 64), 'cosmetic' refers to:

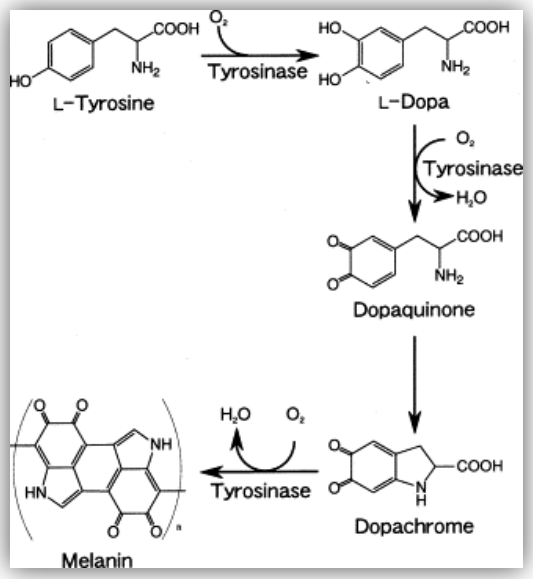
> Any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view to exclusively, or mainly, to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours.

Also, a misconception between the term 'cosmetics' and 'cosmeceuticals' is frequently discerned. The term 'cosmeceuticals,' particularly refers to cosmetics that are known to have healing properties. Thus, it serves as a combination between drug effect and cosmetic effect; cosmeceuticals are intended to improve both the health and beauty of the skin by external application (Elsner and Maibach, 2000). The research is based on examining the tyrosinase inhibitory effect of natural products from medicinal plants which are traditionally used as cosmeceuticals.

2.1.3 TYROSINASE EFFECT

Tyrosinase, also known as polyphenol oxidase (PPO), is defined in The American Heritage Dictionary of an English Language (2016) as "any of a family of copper-containing enzymes found in animal and plant tissues, fungi and bacteria, that catalyze the oxidation of phenolic compounds and are responsible for the production of the pigment melanin from tyrosine and for the browning of fruits, vegetables, and mushrooms when cut and exposed to air". The interest of the current research is mainly centered on inhibition of the tyrosinase enzyme, to avoid extra production of the human pigment melanin. Melanin is the pigment accountable for the colour of the eyes, hair and skin; its occurrence in human skin, takes place in a cell called melanocyte via a process known as melanogenesis (Sturm *et al.*, 1998). Melanogenesis is in control of the biological reactions that defend the skin from free radical attacks which lead to cellular injury of the skin (Narayanaswamy *et al.*, 2011).

Scheme 2.2 portrays the biosynthesis of melanin (melanogenesis). In the process, tyrosinase increases the rate of the transformation reaction of L-tyrosine to L-dopa by hydroxylation and it also leads to the formation of dopaquinone from L-dopa by oxidation (Mason, 1955). A fast transformation of dopaquinone to dopachrome occurs via series of non-enzymatic reactions (Seiji, 1967). Tyrosinase appears once again in the process, catalyzing the rate of transformation from dopachrome to the formation of melanin. Dopachrome is regarded as a possible control point of melanin biosynthesis (Körner and Pawelek, 1980). However, not all substances that can inhibit the formation of dopachrome are tyrosinase inhibitors, e.g., thymol (Satooka and Kubo, 2011). Melanocyte cells are capable of producing two types of melanin pigments viz.'eumelanin' (black or brown) and 'pheomelanin' (red or yellow) as depicted in Scheme 2.3 (Commo *et al.*, 2004).



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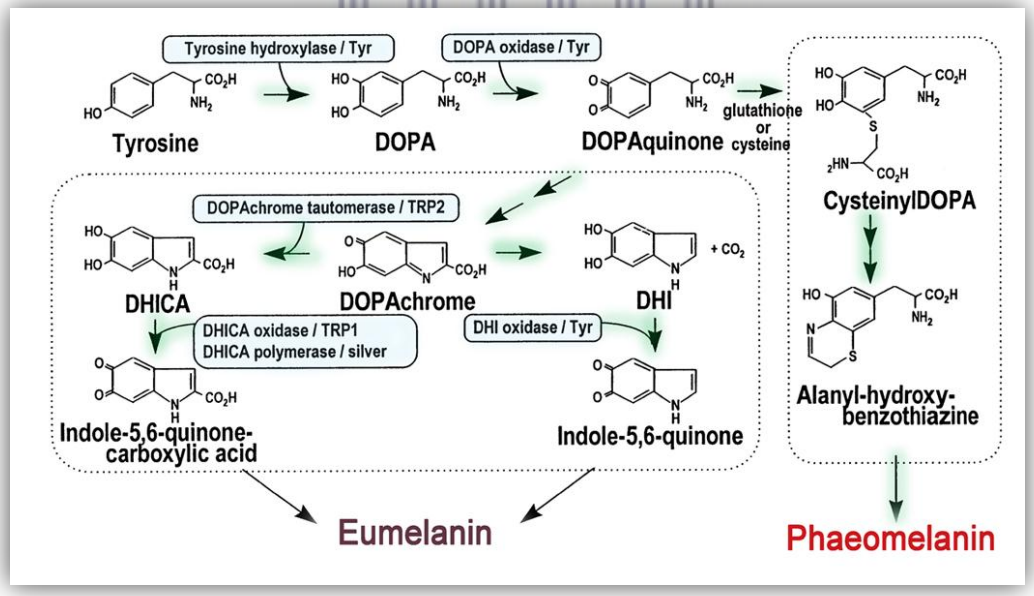
Scheme 2.2: Melanin biosynthesis (Melanogenesis process).



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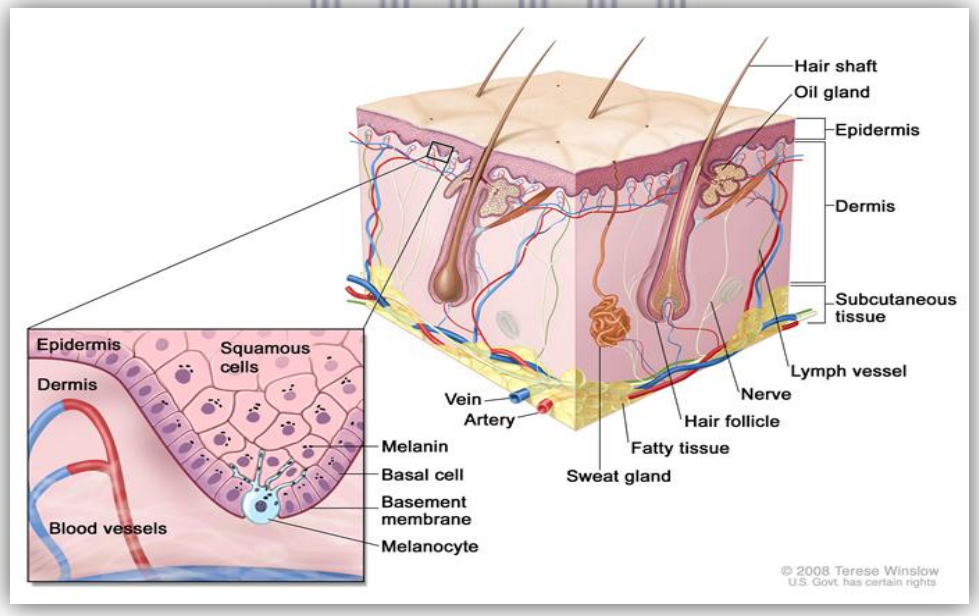
Scheme 2.3: Production of different pigments by melanocytes.

Since melanogenesis is a series of biological reactions which proceeds through a free radical mechanism, it could be disrupted by the selective use of antioxidants which are effective enough to alter these reactions (Momtaz *et al*., 2008). The unavoidable production of free radicals, such as different reactive oxygen species (ROS) of aerobic metabolism, leads to cellular injury of the skin (Devasagayam *et al*., 2004). These harmful oxidants are mainly due to environmental pollutants and UV radiation from sunlight and together with other free radicals damage the skin, instigating skin disorders (Silva *et al*., 2010). ROS increase the melanin biosynthesis, which is detrimental to deoxyribonucleic acid (DNA) and they are likely to cause proliferation of melanocytes (Yasui and Sakurai, 2003).

As stated above, the enzymatic effects of tyrosinase are also found during browning of food when stored or processed in air. Such effects lead to a loss of nutritional quality, decrease in digestibility and formation of anti-nutritional and toxic compounds in the food (Friedman, 1996). Hence, tyrosinase inhibitors are also needed to inhibit browning reactions and preventing adverse effects of browning during food storage, processing and consumption (Rao *et al.*, 2013).

2.1.4 SKIN DISEASE; HYPERPIGMENTATION

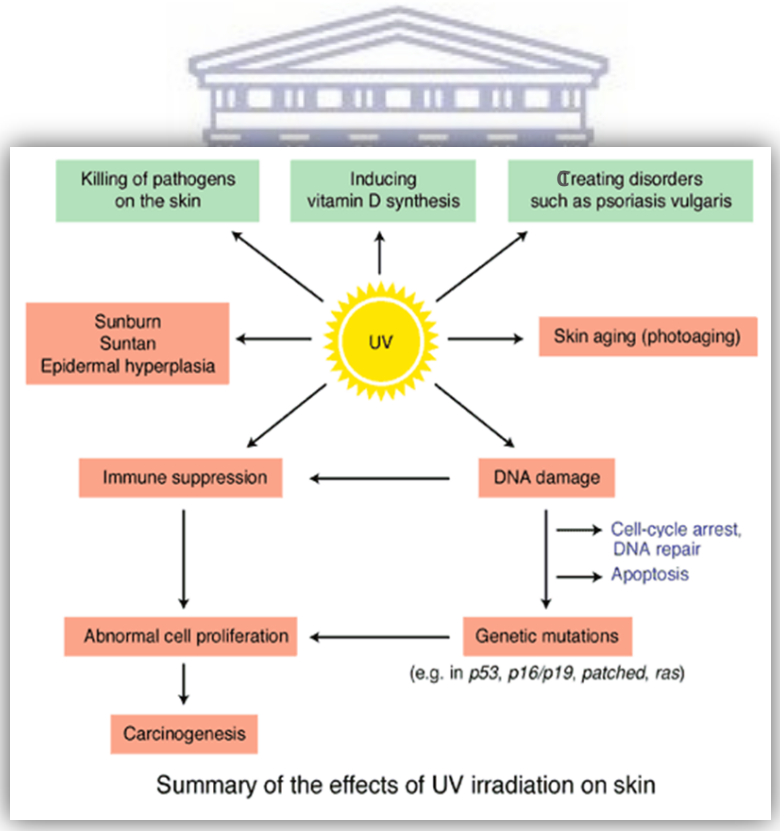
The largest and outermost organ in the human body is the skin. It has a surface area of approximately 1.8 m^2 and accounts for about 16 % of the body mass in the average adult (Thomas, 1993; D'Orazio *et al.*, 2013). This organ is made up of three layers (Figure 2.4), viz. the epidermis (which acts as a barrier to external influences and water loss from the inside), dermis (contains blood vessels to supply oxygen and nutrients), and subcutaneous tissue also known as hypodermis, which consists of connective tissue and fat (Weller *et al.*, 2008). Generally, the skin protects the body from the external environment, regulating temperature, aiding in fluid balance, prohibiting dangerous microbes and chemicals from the body and defending the body from the sunlight (Rebeiro *et al.*, 2015). Thus, the skin is the most vulnerable organ to invasion by bacteria, viruses, and other toxic elements (Narayanaswamy *et al.*, 2011).



Retrieved from *National Cancer Institute* website:http://www.cancer.gov/types/skin/hp/skin-genetics-pdq

Figure 2.4: Three-dimensional diagram of the skin.

It is known that 34 % of all occupational diseases are skin disorders (Spiewak, 2000). Skin disorders have a great variation, particularly when it comes to symptoms and severity, thus, they can be classified as genetic or circumstantial, permanent or temporary, lethal or harmless, painful or painless (Brind'Amour, 2016). UV radiation from the sun is seen as the major cause of skin diseases. Penetration of UV radiation leads to variations in the composition of the skin, including accumulation of the elastic fibers, collagen reduction and degeneration, and the deposition of glycosaminoglycan (Braverman and Fonferko, 1982; Olikarnin and Kallionen, 1989; Smith *et al*., 1962). It also leads to various skin disorders (Scheme 2.4) such as, skin cancer, age spots, photosensitivity, moles, polymorphous light eruption (PMLE), solar elastosis (wrinkles), *etc*. (Kennedy, 2015).



Retrieved from *Healthy Fellow* website: http://www.healthyfellow.com/220/skin-aging-and-ellagic-acid/

Scheme 2.4: Summary of the effects of UV irradiation on skin.

Skin darkening (i.e., hyperpigmentation) is a result of extra production of melanin, which is mainly associated with UV radiation. However, UV radiation is not the only cause of hyperpigmentation, other causes include genetic disposition, aging, hormonal influences, air pollution and skin injuries or inflammatory skin conditions (Drakaki *et al.*, 2014). Hyperpigmentation is also a side effect of certain hormone treatments, chemotherapy drugs, antibiotics, anti-seizure drugs, and other medications (Butler and Elston, 2016).

Hyperpigmentation disorders are differentiated based on the multiple mechanisms that produce various types of discolourations, spots, or marks. The different types of hyperpigmentation include ephelide (freckles), sun spots (age spots), stretch marks, moles, melanoma, post-inflammatory hyperpigmentation (PIH), melasma (chloasma), seborrheic keratoses, acanthosis nigricans, diabetic dermopathy, tinea versicolor (pityriasis versicolor), *etc*. (Just About Skin, 2014; Stulberg *et al*., 2003). However, not all of the above-mentioned forms of hyperpigmentation are explained in the scope of the dissertation, thus, only the common forms of hyperpigmentation, particularly in S. Africa is discussed.

Melasma is one of the most common types of hyperpigmentation. It is also known as chloasma or 'pregnancy mask'. Although the latter name and the fact that an estimated 70 % of women who get affected by this hyperpigmentation during their pregnancy might suggest that melasma is only for pregnant women, this is simply not true, as studies revealed that 10 % of men have such skin discolouration too (Moin *et al*., 2006; Vazquez *et al*., 1988). Of course, such hyperpigmentation is common to pregnant women, particularly those with a darker complexion, but, it also affects adolescents and older women as a result of side effects of certain medications (Jadotteand Schwartz, 2010). Melasma appears as dark patches or hyperpigmented macules on the face, neck and rarely the upper limbs (Bagherani *et al.*, 2015). It is somewhat idiopathic,

especially to men, thus, its pathogenesis is unclear, but it is anticipated to be due to UV radiation, anti-seizure medications, hormonal variations of pregnancy and thyroid disease (Cayce *et al.*, 2004).

Another common type of hyperpigmentation is that of post-inflammatory hyperpigmentation (PIH). PIH is a skin condition that results from preceding cutaneous disorders, such as trauma (e.g., burns), inflammatory diseases (e.g., lichen planus, lupus erythematosus, atopic dermatitis), infections, allergic reactions, mechanical injuries, reactions to medications, and phototoxic eruptions (Schwartz and Elston, 2015). Lentigo is a common hyperpigmentation disorder; this hyperpigmentation is a circumscribed brown spot resulting from an increased number of melanocytes at the dermoepidermal junction (Ortonne, 2003). Lentigines can occur in any area of the skin, and they are classified in accordance with aetiological factors, thus, some lentigines are associated with too much exposure to UV radiation (e.g., lentigo simplex, solar lentigo, photochemotherapy (PUVA) lentigo, reticular/ink-spot lentigo, and sunbeds lentigo), some are not associated with overexposure to UV radiation (e.g., genital lentiginosis, labial lentigo, and oral lentiginosis), and other lentigines are characteristic feature of multisystem syndromes (Carli and Salvini, 2009).

Care is needed in differentiating these spots and moles, as they are subtle to those which are symptoms of Melanoma. Melanoma is the most dangerous form of skin cancer, it develops in the melanocyte, when unrepaired DNA damage due to UV radiation causes mutations (genetic defects), making the skin cells multiply rapidly, forming malignant tumours (Skin Cancer Foundation, 2016). Melanoma tumours can grow anywhere in the skin, yet they are likely to begin in the trunks (chest and back) in men and on the legs in women. They are brown or black in colour, however some can appear pink, tan, or white (American Cancer Society, 2016).

Melanoma is regarded as the most harmful skin cancer type since it is likely to spread to other parts of the body if not caught early and due to its reluctance to chemotherapy (Marieb and Hoehn, 2013).

Furthermore, pigmentation disorders in relation to hyperpigmentation are closely associated with Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS). It has been shown that HIV infected people are more likely to have dermatological disorders; the prevalence is revealed to approach 100 % (Koehler *et al*., 1992). The most common type of hyperpigmentation or PIH disorder affecting approximately 75 % of the people with HIV in S. Africa is that of morbilliform eruption (Jordaan, 2008). Other hyperpigmentation disorder associated with HIV-affected people is that of HIV-associated oral mucosal melanin hyperpigmentation (HIV-OMH). In S. Africa, HIV-OMH was found to be 18.5 %; relatively high when compared to other sub-Saharan African countries, e.g., Tanzania 4.7 % and Kenya 6 % (Chandran *et al*., 2016; Chandran *et al*., 2014). Moreover, some hyperpigmentation or PIH associated with HIV include Kaposi's sarcoma, seborrheic dermatitis, psoriasis, cutaneous vasculitis, thrombocytopenic purpura, pemphigoid, autoimmune blistering, *etc.* (Knott, 2015).

2.2 REVIEW OF *RHYNCHOSIA VILLOSA*

2.2.1 FABACEAE

Fabaceae is the predominant family of the plant order Fabales, and it constitutes nearly 95 % of this order, while 5 % is shared among Polygalaceae, Surianaceae, and Quillajaceae families (Turner, 2016). It is also one of the largest families of flowering plants (angiosperms), following Asteraceae and Orchidaceae, respectively, with about 19 400 recognized plant species of approximately 751 genera; such plant species amount to almost 7 % of angiosperms (Judd *et al*., 2002; Christenhusz and Byng, 2016). Fabaceae is also scientifically known as Leguminosae or Papilionaceae, but, often called legume, pea or bean family (International Association for Plant Taxonomy, 2012). This family has three distinct subfamilies, namely, Faboideae, Caesalpinioideae, and Mimosoideae which have been recognized to be the only phylogenetic from this family 55–50 MYA; these subfamilies used to be treated at the family level (Herendeen *et al*., 1992).

2.2.2 *RHYNCHOSIA*; *RHYNCHOSIA VILLOSA*

Rhynchosia is one of the ten genera of the subtribe *Cajaninae* of tribe *Phaseoleae*; *Phaseoleae* is amongst the 28 tribes of subfamily Faboideae (Moteetee *et al*., 2012). *Rhynchosia* genus has approximately 230 plant species which are prevalent the most in Africa, with approximately 60 of such species occurring in S. Africa (Schrine, 2005; Germishuizen 2006). They are also found in other continents, particularly in climates characterized by warm temperatures, such as tropical Asia, northern Australia, and also in tropical and subtropical America (Schrine, 2005). The plant species of *Rhynchosia* which occur in S. Africa were separated based on morphological structures into five sections by Baker in 1923; such sections include *Cyanospermum* (Wight and

Arnott) Benth. (1 species), *Arcyphyllum* Torrey and Gray (2 species), *Polytropia* Presl. (2 species), *Chrysoscias* E. Mey (4 species), and *Eurhynchosia* Baker (50 species) (Baker, 1923).

Rhynchosia belongs to the *Eurhynchosia* section and is closely related to *Eriosema*, which also falls within the same section, and is also one of the *Cajaninae* genera. The two genera have similar pinnately compound leaves and axillary racemes (Doyle and Doyle, 1993; Lackey, 1981). Such close relation between the two genera is attested to by the similarity of the morphological structure of the plant species of interest, *Rhynchosia villosa*, to that of *Eriosema cordatum*. Both plant species develop a perennial underground organ, an erect rootstock. Also, both plant species have stems and leaves with observable pubescence, and flowers of the inflorescences are yellow in colour with red veins, but the size and shape of the leaves of these two species are different, as the leaves of *R. villosa* adopt a peltate shape, while those of *E. cordatum* assume an ovate shape (Stirton and Gordon-Gray, 1978; Amrisha, 2015). However, the lack of anatomical and developmental studies on these plant species makes it problematic to precisely differentiate these plant species, as it is sometimes unclear to tell where root gives place to stem (Stirton and Gordon-Gray, 1978).

Moreover, the synonym of *R. villosa*, which is *Eriosema villosum* also substantiate the close relation between these two genera. *Eriosoma villosum* is not the only synonym of *R. villosa*; other known synonyms are *Rhynchosia sigmoides* and *Sigmodostyles villosa*. Nonetheless, *R. villosa* is endemic to S. Africa and widely distributed in KwaZulu-Natal (KZN), Mpumalanga Province, and E. Cape Province (Foden and Potter, 2005). This plant species is known as '*Uphuzi lobala'* or '*Uphuzi lentaba'* by the people of the Pondoland, its Xhosa name is derived from that of *Gunnera perpensa*, which is'*Uphuzi lomlambo*,' under the misapprehension that the two plants are phylogenetical and/or morphological related.

2.2.3 TRADITIONAL USES OF *R. VILLOSA*

The similarity of *R. villosa* (*E. villosum*) and *E. cordatum* is not merely based on their morphological structures and names, but also on their pharmacological activities. Thus, the root of *R. villosa* is posited to be traditionally used and interchangeable with that of *E. cordatum*, and other two *Eriosoma* species, *E. salignum* and *E. kraussianum* by the people of KZN for male sexual disorders, which include erectile dysfunction and/or male potency, hence this, suggests that they consist of some aphrodisiac activity (Drewes *et al*., 2013). The above-mentioned plant species are collectively known by the people of KZN as '*Ubangalala*,' and they have been used by the S. African men or Zulu warriors for centuries to enhance their sexual energy and potency (Hutchings, 1996).

Alternatively, the *R. villosa* root is utilized by the people of the Pondoland as the main constituent in the fusion or decoction that helps women with fertility problems. Therefore, this suggests that the plant species of interest has estrogenic properties, which is attributed to its use as the ovulatory stimulant. Moreover, the people of Lusikisiki mainly use *R. villosa* root in cosmetic preparations. It is used externally and internally to enhance the skin texture and tone, while it also aids in treating swollen parts of the body when washed with water that has soaked leaves of this plant. The pharmacological and ethnopharmacological uses of some *Rhynchosia* species are presented in Table 2.2.

Table 2.2: Pharmacological and ethnopharmacological uses of some *Rhynchosia* species

Plant Source	Location	Pharmacological and ethnopharmacological use(s)	Reference(s)
R. rothii	India	Tonic	(Gauri and Sharma, 2011)
R. rufescens	India	Abortion	(Ajesh *et al*., 2012)
R. beddomei	India	Antioxidant, anti-inflammatory, analgesic, diuretic activity, and microbial activity	(Sridharamurthy *et al*., 2013) (Eswaraiah *et al*., 2013) (Bakshu and Raju, 2001)
R. scarabaeoides	India	Antibacterial activity	(Challa *et al*., 2011)
R. minima	India, USA and Sri Lanka	Anthelmintic activity	(Mali and Mahale, 2008)
R. volubilis	Japan and Korea	Antiproliferative activity and used as cosmeceuticals	(Kinjo *et al*., 2001) (Hong *et al*, 2016)
R. hirta	Kenya	Malaria	(Pascaline *et al*., 2011)
R. nulubilis	Korea	Antioxidant, and antinociceptive	(Yim *et al.*, 2007; Kim *et al.*, 2009)
R. molubils	Korea	Antioxidant and anti-tyrosinase activity	(Ha, 2006)
R. precatoria	Mexico	Tuberculosis	(Coronado-Aceves *et al*., 2016)
R. caribaea	Namibia	Analgesics	(Dushimemaria *et al.*, 2015)
R. capitata	Pakistan	Gastric problems flatulence, constipation, purgative, headache, nausea and vomiting	(Wariss *et al.*, 2014)
R. reniformis	Pakistan	Antiglycation, platelet aggregation, antioxidant, cytotoxic and phytotoxic	(Ahmad *et al*., 2011)
R. viscosa	Tanzania	Inflammatory skin disorders and insect bites	(Crawford *et al*., 2010)
R. recinosa	Tanzania, and Zimbabwe	Peptic ulcers, and antileishmanial activity	(Haule *et al.*, 2012) (Monzote *et al*., 2014)
R. heterophylla	Zambia	Fermentation of beverage and anti-inflammatory	(Zulu *et al*., 1997)
R. insignis	Zimbabwe	Diarrhea	(Chimponda and Mukanganyama, 2010)

2.2.4 CHEMISTRY OF *RHYNCHOSIA*

The chemistry of the *Rhynchosia* genus is not fully complete, since not many compounds have been identified from this genus. The insufficient information available about the chemistry and biology of this genus is due to the lack of work that has been done in examining its species in the many regions in which they occur, except in India. For instance, S. Africa has a huge number of *Rhynchosia* species (58), 57 % more than that of India (25), but information about their pharmacological activities is limited (Germishuizen, 2006; Baker, 1976; Sanjappa, 1992). As demonstrated by Tables 2.3.1–2.4.6, that show summary of the main compounds of *Rhynchosia*, intensive and novel work in this genus has been done in India by Adinarayana and core-workers. A comprehensive phylogenetic study of S. African *Rhynchosia* species was carried out by Baker in 1923. The literature available indicates that more pharmacological studies need to be done on the S. African *Rhynchosia* species.

The class of *Rhynchosia* consists of herbs, commonly climbing or trailing, sometimes woody stock, rarely shrubs (Bisby, 1994). A large number of isolated constituents from the species of this genus have been obtained from leaves. The organic compounds which are most abundant in *Rhynchosia* are flavones, mainly *C*-glycosylflavones (Rao *et al*., 1991). Such constituents are distributed amongst edible fruits, cereals and leaves; they have important biological potential, such as anti-inflammatory, anti-diabetic, anti-spasmodic, anxiolytic, and hepatoprotective activities, hence their excellence use in human diet (Court and Williams, 2015). Also, a numerous number of compounds isolated from *Rhynchosia* are known as pro-antioxidant molecules. Such compounds include the flavones (**1**, **2**, **6**, **11**, and **12**), isoflavones (**14** and **15**), flavonols (**29**, **30**, **32**, **33**, and **39**), flavan-3-ol (**40**), flavanonols (**42** and **43**), phenolic acids and/or benzonoic acid derivatives (**45** – **48**), xanthone (**53**) and sterol (**55**) (Goufo and Trindade,

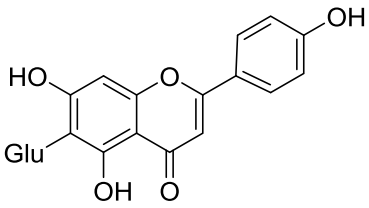
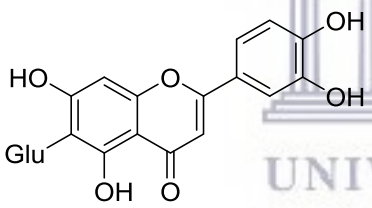
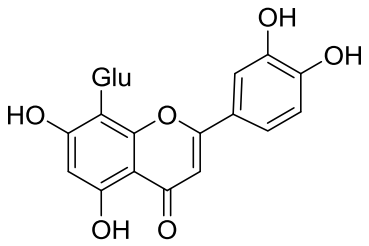
2014; Mathangi and Sudha, 2012; Banerjee *et al*., 2012; Battu *et al.*, 2011; Rammohan *et al.*, 2015). Such compounds are found in food, suggesting that many species of *Rhynchosia* like other legumes also act as a potential source of edibles.

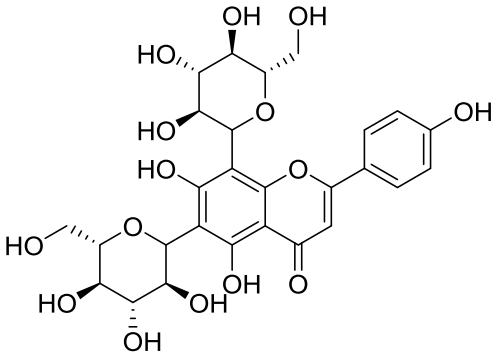
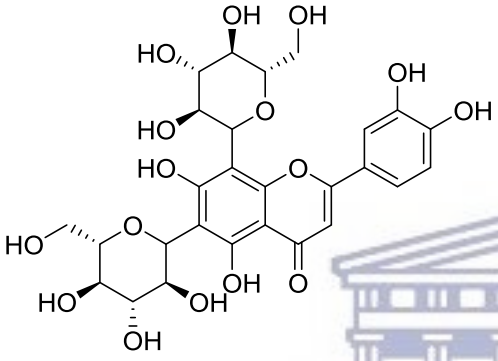
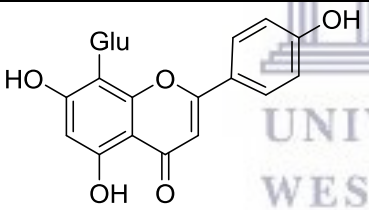
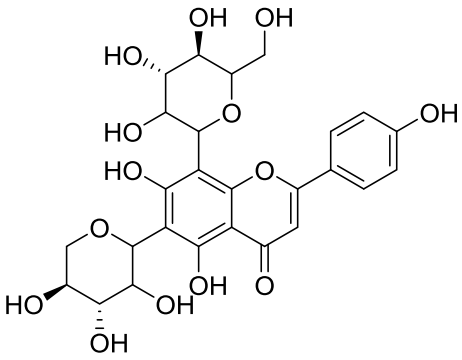
The above-mentioned compounds substantiate the use of some of the *Rhynchosia* species as cosmeceuticals. As in the case of *R. molubilis*, which is reported to consist of antioxidants that lead to its ability to block UV radiation, automatically prevent oxidation and remove free-radicals, it is also able to carry out skin-whitening action such as the inhibition of melanin formation (Ha, 2006). Another *Rhynchosia* species which has been found to be beneficial in depigmenting the skin and treating solar elastosis (wrinkles), is that of *R. nolubilis*, it has been revealed to be excellent in inhibiting melanin generation, while it can treat skin wrinkles through reducing the expression of collagenase MMP-1 and increasing the generation of collagen (Yim *et al*., 2009; Hong *et al*., 2016). On the other hand, Koreans also use *R. volubilis* as a major ingredient in local cosmetic formulations, as it is known to possess anti-inflammatory, antibacterial, anti-aging, skin whitening activities as well as prevention of wrinkles (Hong *et al*, 2016; Lai *et al*., 2012).

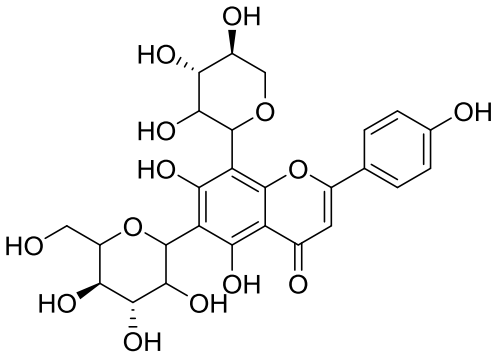
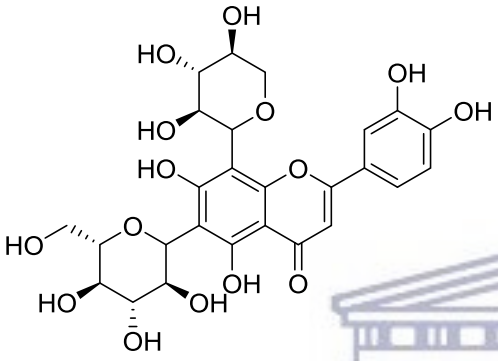
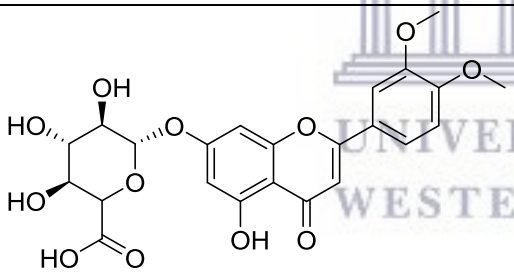
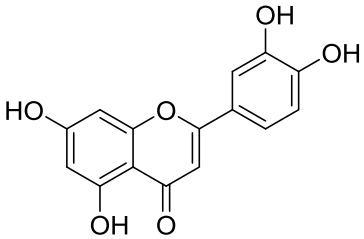
Generally, the extensive occurrence of the different classes of flavonoids (Table 2.3.1–2.3.6) in *Rhynchosia* supports the significance of this genus in cosmetic and pharmaceutical fields as anti-inflammatory and antioxidant agents (Malinowska, 2013). Gallic acid (**45**) and the pyrogallol-type tannins (**41**, **49**, **51**, **52**, and **58**) isolated from *R. volubilis* showed high antiproliferative activities against murine melanoma (B16F10) cells, hence this also promotes the cosmeceutical effects of the *Rhynchosia* species, i.e., *R. volubilis*, *R. nolubilis* and *R. molubilis* in particular (Kinjo *et al*., 2001).

Group 1: Flavonoids;

Table 2.3.1: Flavones and flavone glycosides

S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
1	 Isovitexin	*R. suaveolens* *R. heynei* *R. capitate* *R. beddomei* *R. rufescens* *R. sericea* *R. rothii* *R. cana* *R. jacobii* *R. bracteata* *R. minima*	Radical scavenging activity	(Rammohan *et al.,* 2015) (Adinarayana *et al.*, 1985) (Rao and Gunasekar, 1987) (Adinarayana *et al*., 1980c) (Adinarayana *et al*., 1979) (Adinarayana and Ramachandraiah, 1986) (Seetharamamma *et al.*, 1989) (Nia *et al.*, 1992) (Besson *et al*., 1977)
2	 Isoorientin	*R. suaveolens* *R. rufescens* *R. beddomei* *R. rothii* *R. capitate* *R. cana* *R. jacobii*	Radical scavenging activity	(Rammohan *et al.,* 2015) (Adinarayana *et al*, 1985) (Adinarayana *et al*., 1979) (Adinarayana *et al*., 1980c) (Adinarayama and Ramachandraiah, 1986) (Adinarayana and Chetty, 1985) (Rao and Gunasekar, 1987) (Seetharamamma *et al.*,1989)
3	 Orientin	*R. cana* *R. jacobii* *R. heynei* *R. rufescens* *R. beddomei* *R. hirsuta* *R. rothii*		(Adinarayana and Ramachandraiah, 1986) (Seetharamamma *et al.*,1989) (Adinarayana *et al*., 1979) (Adinarayana *et al*., 1980c) (Adinarayana *et al*., 1985) (Rao and Gunasekar, 1987)

4	 Vicenin-2	*R. rothii* *R. heynei* *R. hirsuta* *R. rufescens* *R. beddomei* *R. cana* *R. jacobii* *R. minima*	Anti-inflammatory	(Rao and Gunasekar, 1987) (Adinarayana and Ramachandraiah, 1986) (Adinarayana *et al*., 1985) (Adinarayana *et al*., 1979) (Adinarayana *et al*., 1980c) (Seetharamamma *et al.*, 1989) (Besson *et al*., 1977)
5	 Lucenin-2	*R. cana* *R. beddomei* *R. rufescens* *R. rothii*		(Adinarayana and Ramachandraiah, 1986) (Adinarayana *et al*., 1980c) (Adinarayana *et al*., 1980b) (Adinarayana *et al*., 1979) (Adinarayana *et al*., 1985) (Rao and Gunasekar, 1987)
6	 Vitexin	*R. cana* *R. capitate* *R. heynei* *R. rufescens* *R. beddomei*		(Adinarayana and Ramachandraiah, 1986) (Adinarayana *et al*., 1985) (Adinarayana *et al*., 1979) (Adinarayana *et al*., 1980c)
7	 Vicenin-1	*R. jacobii*		(Seetharamamma *et al.*,1989)

8	 Vicenin-3	*R. minima*		(Besson *et al*., 1977)
9	 Schaftoside	*R. minima*		(Besson *et al*., 1977)
10	 3′,4′-di-*O*-methylluteolin-7-*O*-glucuronide	*R. beddomei*		(Adinarayana *et al*., 1980c) (Adinarayana *et al*., 1985)
11	 Luteolin	*R. suaveolens*		(Adinarayana *et al*., 1985)

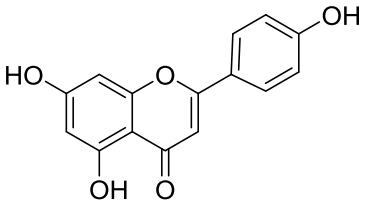
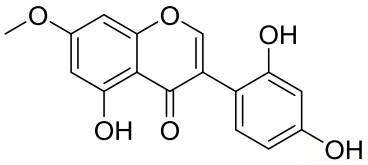
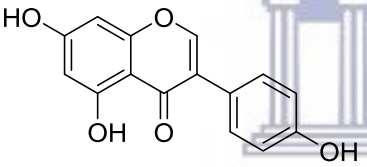
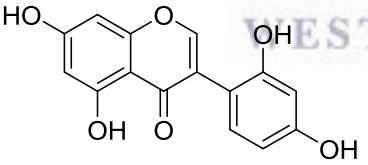
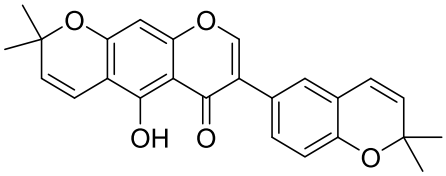
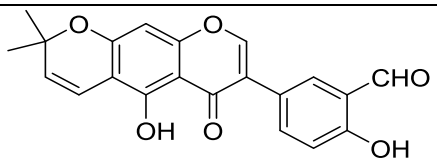
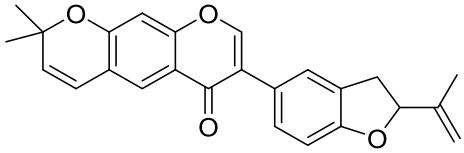
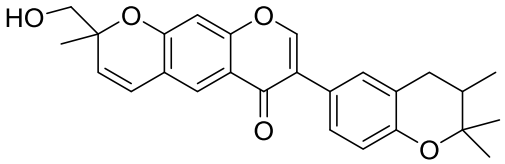
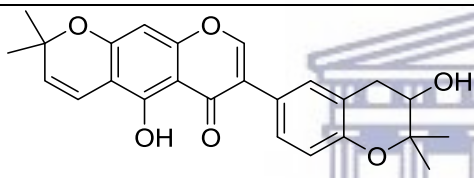
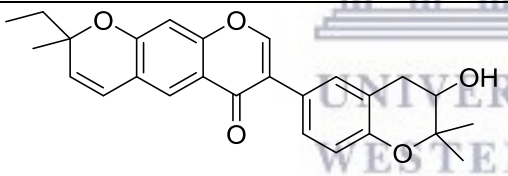
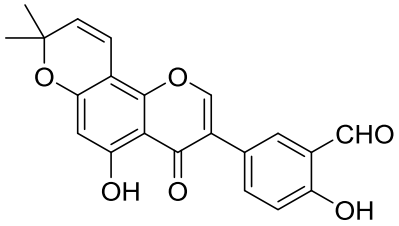
12	 Apigenin	*R. beddomei*		(Adinarayana *et al*., 1985)

Table 2.3.2: Isoflavones

S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
13	 Cajanin	*R. edulis* *R. densiflora* *R. carabaea* *R. hirsuta*		(Ogungbe *et al.,* 2011) (Ingham, 1990)
14	 Genistein	*R. densiflora* *R. carabaea* *R. hirsuta*		(Ingham, 1990)
15	 2′-hydoxygenistein	*R. densiflora* *R. carabaea* *R. hirsuta*		(Ingham, 1990)
16	 Ulexin B	*R. edulis*		(Ogungbe *et al.,* 2011)

17	 Scandenal	*R. edulis*		(Ogungbe *et al.*, 2011)
18	 Rhynedulin A	*R. edulis*		(Ogungbe *et al.*, 2011)
19	 Rhynedulin B	*R. edulis*		(Ogungbe *et al.*, 2011)
20	 Rhynedulin C	*R. edulis*		(Ogungbe *et al.*, 2011)
21	 Cyclochandalone	*R. edulis*		(Ogungbe *et al.*, 2011)
22	 Rhynedulina	*R. edulis*		(Ogungbe *et al.*, 2011)

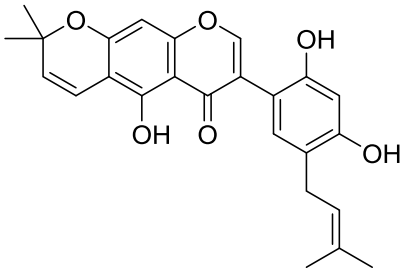
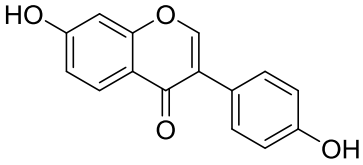
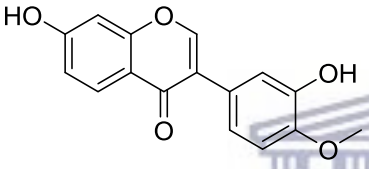
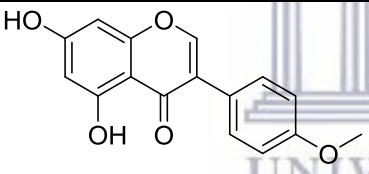
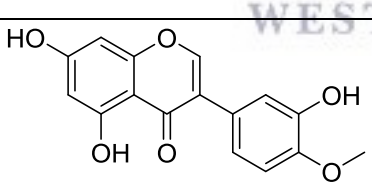
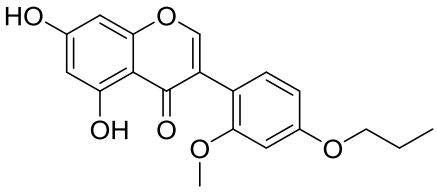
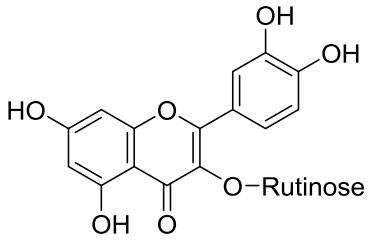
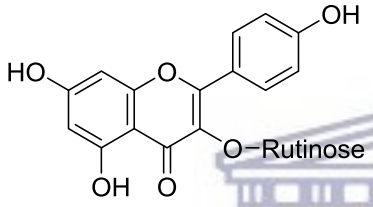
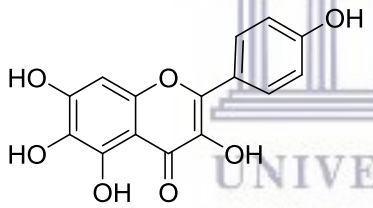
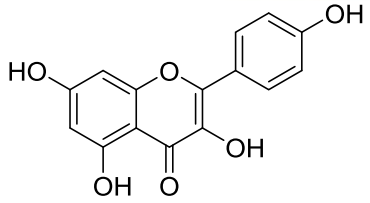
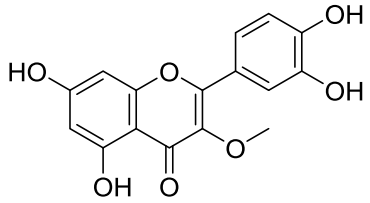
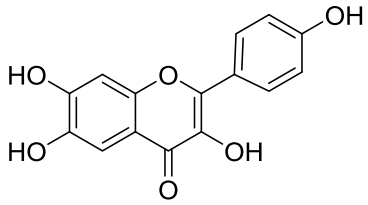
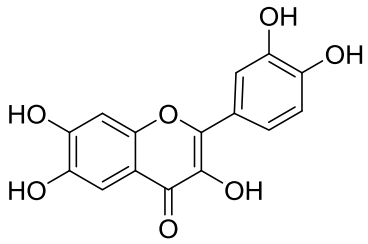
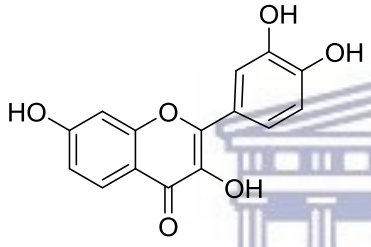
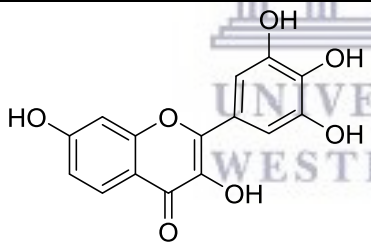
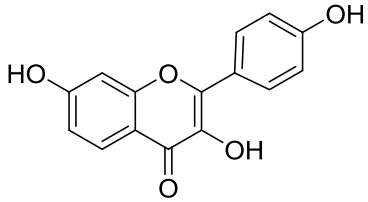
23	 Cajanone	*R. edulis*		(Ogungbe *et al.*, 2011)
24	 Daidzein	*R. volubilis*		(Li and Xiang, 2011)
25	 Calycosin	*R. volubilis*		(Li and Xiang, 2011)
26	 Biochanin A	*R. volubilis*		(Li and Xiang, 2011)
27	 Pratensein	*R. volubilis*		(Li and Xiang, 2011)
28	 3-[4′-(1′-methoxy)-propoxy]-phenyl-5,7-dihydroxyisoflavone	*R. volubilis*		(Li and Xiang, 2011)

Table 2.3.3: Flavonols

S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
29	 Rutin/Rutoside	*R. beddomei* *R. hirta*		(Adinarayana *et al*., 1985) (Adinarayana *et al*., 1980c) (Adinarayana *et al*., 1980a)
30	 Kaempferol-3-*O*-rutinoside/Nicotiforin	*R. hirta*		(Adinarayana and Chetty, 1985) (Adinarayana *et al*., 1980a)
31	 6,7,4′-trihydroxyflavonol	*R. beddomei*		(Adinarayana *et al*., 1980b)
32	 Kaempferol	*R. rufescens*		(Adinarayana and Ramachandraiah, 1984)
33	 3-*O*-methylquercetin	*R. rufescens*		(Adinarayana and Ramachandraiah, 1984)

34	 5-deoxygaletin	*R. beddomei*		(Adinarayana *et al*., 1980b)
35	 5-deoxyquercetagetin/Rhynchosin	*R. beddomei*		(Adinarayana *et al*., 1980b)
36	 Fisetin	*R. beddomei*		(Adinarayana *et al*., 1980b)
37	 Robinetin	*R. beddomei*		(Adinarayana *et al*., 1980b)
38	 7,4′-dihydroxyflavonol	*R. beddomei*		(Adinarayana *et al*., 1980b)

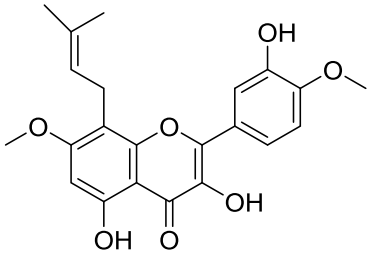
39	 8-*C*-prenylquercetin-7,4′-dimethyl ether/Rhynchospermin	*R. hirta*		(Adinarayana *et al.*, 1981)

Table 2.3.4: Flavan-3-ols

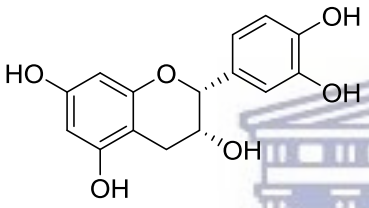
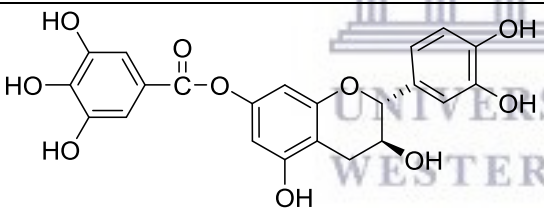
S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
40	 Epicatechin	*R. volubilis*		(Li and Xiang, 2011)
41	 7-*O*-galloylcatechin	*R. volubilis*	Antiproliferative activity	(Kinjo *et al*., 2001)

Table 2.3.5: Flavanonols

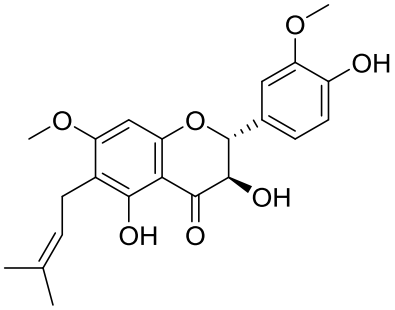
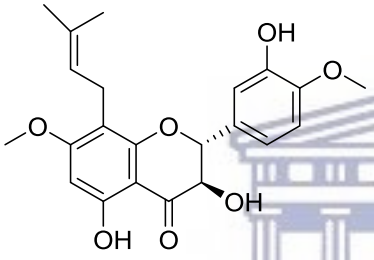
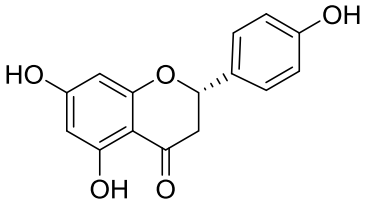
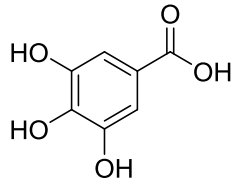
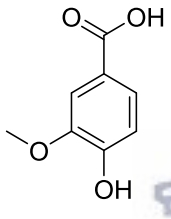
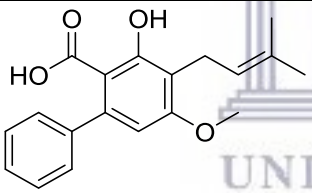
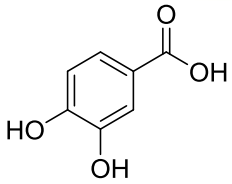
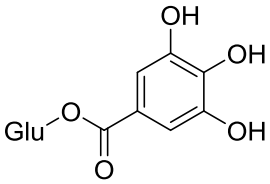
S/No.	Chemical structure and name of isolatedcompound	Plant source(s)	Biological activity	Reference(s)
42	 6-*C*-prenyltaxifolin-7,3′-dimethylether/Isotirumalin	*R. densiflora* *R. hirta*		(Rao and Gunasekar, 1998) (Rao and Gunasekar, 1988)
43	 8-*C*-prenyltaxifolin-7,4'-dimethyl ether/Tirumalin	*R. hirta*		(Adinarayana *et al.*, 1985) (Adinarayana *et al.*, 1980a)

Table 2.3.6: Flavanone(s)

S/No.	Chemical structure and name of isolatedcompound	Plant source(s)	Biological activity	Reference(s)
44	 Naringenin	*R. beddomei*		(Adinarayana *et al.*, 1980c)

Group 2: Miscellaneous;

Table 2.4.1: Phenolic acids

S/No.	Chemical structure and name isolated compound	Plant source(s)	Biological activity	Reference(s)
45	 Gallic aid	*R. volubilis* *R. minima*	Antiproliferative activity	(Kinjo *et al*., 2001) (Krishnamurty *et al*., 1975)
46	 4-hydroxy-3-methoxybanzoic acid/Vanillic acid	*R. hirta*		(Adinarayana *et al*., 1985)
47	 3-hydroxy-5-methoxy-4-(3-methyl-2-butenyl)-2-biphenylbenzoic acid	*R. suaveolens*	Antibacterial	(Khan and Shoeb, 1984)
48	 Protocatechuic acid	*R. minima*		(Krishnamurty *et al*., 1975)
49	 Glucogallin	*R. volubilis*	Antiproliferative activity	(Kinjo *et al*., 2001)

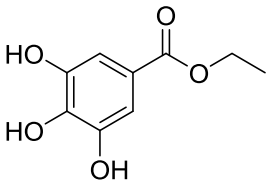
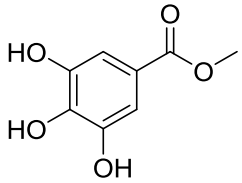
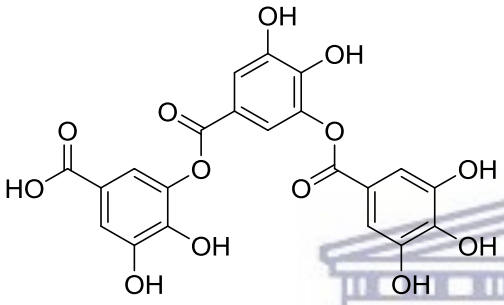
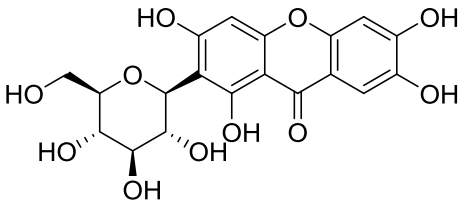
50	 Gallic acid ethyl ester	*R. minima*		(Krishnamurty *et al.*, 1975)
51	 Gallic acid methyl ester	*R. volubilis*	Antiproliferative activity	(Kinjo *et al*., 2001)
52	 Trigallic acid	*R. volubilis*	Antiproliferative activity	(Kinjo *et al*., 2001)

Table 2.4.2: Xanthones

S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
53	 Mangiferin	*R. suaveolens*	Radical scavenging activity	(Rammohan *et al.*, 2015) (Adinarayana and Chetty, 1985)

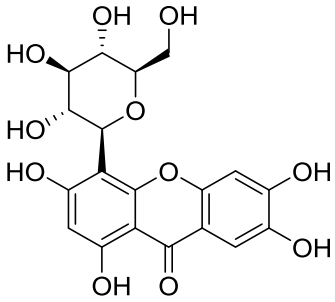
54	 Isomangiferin	*R. suaveolens*		(Adinarayana and Chetty, 1985)

Table 2.4.3: Sterols

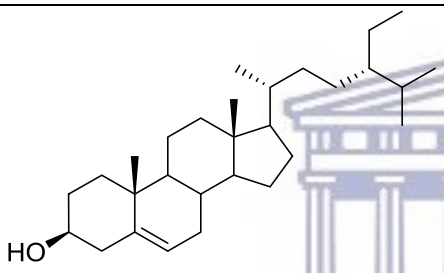
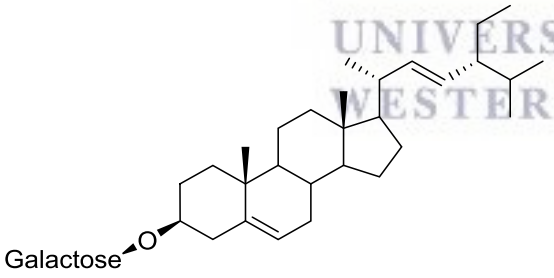
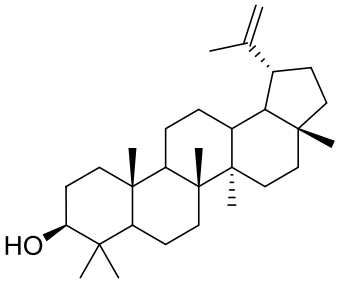
S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
55	 *β*-sitosterol	*R. minima*		(Ahmed *et al.*, 1992) (Krishnamurty *et al*., 1975)
56	 Stigmasteryl galactoside	*R. minima*		(Ahmed *et al.*, 1992)
57	 Lupeol	*R. minima*		(Ahmed *et al.*, 1992)

Table 2.4.4: Galloyl derivative(s)

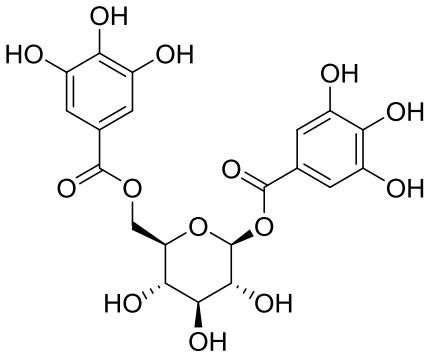
S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
58	 1,6-digalloly glucose	*R. volubilis*	Antiproliferative activity	(Kinjo *et al.*, 2001)

Table 2.4.5: Simple aromatic natural products

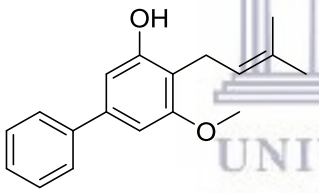
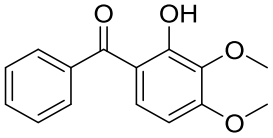
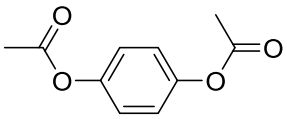
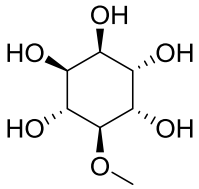
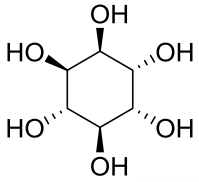
S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
59	 3-hydroxy-5-methoxy-4-(3-methyl-2-butenyl)biphenyl	*R. suaveolens*	Antibacterial	(Khan and Shoeb, 1984)
60	 2-hydroxy-3,4- dimethoxybenzophenone	*R. suaveolens*		(Rammohan *et al.*, 2015)
61	 Hydroquinone diacetate	*R. minima*		(Krishnamurty *et al.*, 1975)

Table 2.4.6: Simple non-aromatic natural products

S/No.	Chemical structure and name of isolated compound	Plant source(s)	Biological activity	Reference(s)
62	 D-pinitol	*R. beddomei* *R. hirta*		(Adinarayana *et al*., 1979) (Adinarayana *et al*., 1980a)
63	 D-inositol	*R.* spp. *R. beddomei*		(Allen and Allen, 1981) (Adinarayana *et al*., 1980c)

R: *Rhynchosia*, **Glu**: glucose, ***R. hirta*** = *R. cyanosperma*, **spp**: unspecified species

CHAPTER THREE

COLLECTION/EXTRACTION AND CHEMICAL CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM TRADITIONALLY USED HERBAL MEDICINE FOR TREATMENT OF HYPERPIGMENTATION

3.1 METHODS AND MATERIALS

3.1.1 CHEMICAL REAGENTS

Methanol (MeOH), ethyl acetate (EtOAc), ethanol (EtOH), hexane (Hex), dichloromethane (DCM), and dimethyl sulfoxide (DMSO) were secured from Merck (S. Africa). The mushroom tyrosinase (EC 1.14.18.1) 5771 Unit/mg, L-tyrosine, kojic acid and deuterated methanol (CD_3OD) were purchased from Sigma Aldrich (S. Africa).

3.1.2 THIN LAYER CHROMATOGRAPHY (TLC)

Pre-coated TLC plates of silica gel 60 F_{254} [Merck (Darmstadt; Germany)] were used for TLC analysis using a UV lamp of $\lambda_{254}/\lambda_{366}$ nm [CAMAG (Muttenz; Switzerland)] to visualize the 'spots' or bands after developing the TLC plates in various solvent systems. Chemical detection was performed after spraying with vanillin/H_2SO_4 reagent and heating at 110 ℃.

3.1.3 COLUMN CHROMATOGRAPHY (CC)

Silica gel 60 (0.040 – 0.063 mm) (Merck Millipore; S. Africa) and sephadex LH-20 (Sigma Aldrich; S. Africa) were used in various sizes of glass columns.

3.1.4 HIGH PERFOMANCE LIQUID CHROMATOGRAPHY (HPLC)

For semi-prep HPLC purification, a HPLC '1200 series' (Agilent Technologies) comprising a variable wavelength UV/Vis detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), column compartment (G1316A) and reversed phase C_{18} column SUPELCO (25 × 2.1 cm, 5 μm) was used. The used mobile phase was MeOH-deionized H_2O (DIW) (55:45 to 70 % in 30 min, then to 100 % in 5 min) and the flow rate employed was 1.0 mL/min.

3.1.5 NUCLEAR MAGENETIC RESONANCE SPECTROSOPY (NMR)

Deuterated methanol (CD_3OD) was used to prepare the samples for NMR. NMR spectra were recorded at 25 ℃ on a Bruker Avance III HD 400 MHz NMR spectrometer (Germany) using a 5 mm BBO probe. Standard 1D and 2D NMR pulse sequences were used to acquire 1D and 2D data respectively. The ^{1}H (δ_H) and ^{13}C (δ_C) chemical shifts were measured comparatively to CD_3OD signals at δ 3.23, 4.78 (^{1}H NMR) and δ 48.3 (^{13}C NMR) in ppm.

3.1.6 TYROSINASE ENZYME INHIBITION

The skin enzymatic inhibitory assay was executed in the course of the study following the approach used by Vanni *et al*. (1990), with slight modification (Popoola *et al*., 2015). Initially, a stock solution of 10 mg/mL was prepared by dissolving the plant extracts to be tested in dimethyl sulfoxide (DMSO), then 5 dilutions were performed using 50 mM sodium phosphate buffer (pH 6.5) for all working solutions to obtain the working concentrations of 100.00, 50.00, 25.00, 12.50, and 6.25 µg/mL, respectively. Kojic acid was used as a positive control in this assay. Each sample working solution of 70 µL was combined with 30 µL of tyrosinase [from mushroom, 500

Unit/mL in sodium phosphate buffer (pH 6.5)] in triplicate, in a 96-well plate. After incubation at room temperature for 5 min, 110 µL of the substrate (2 mM L-tyrosine) was added to each well. Final concentrations of the positive control, crude extracts and/or isolated compounds ranged from 1.0 - 100 µg/mL. The sample control was made up of each sample using sodium phosphate buffer (pH 6.5) only. The enzymatic activity was determined by measuring the absorbance at 490 nm using AccuReader M965 microplate reader (Metertech; Taiwan). The measurements were recorded immediately after incubation was carried out at room temperature (25℃) for approximately 30 min. Equation 1 was employed in determining the percentage of tyrosinase inhibition.

Equation 1: Percentage of tyrosinase inhibition;

$$\% \text{ of tyrosinase inhibition} = \frac{(A_{control} - A_{blank\,1}) - (A_{sample} - A_{blank\,2})}{(A_{control} - A_{blank\,1})} \times 100\ \%$$

Where $\boldsymbol{A_{control}}$ is the absorbance of the control with the enzyme, $\boldsymbol{A_{blank\,1}}$ is the absorbance of the control without the enzyme, $\boldsymbol{A_{sample}}$ is the absorbance of the test sample with the enzyme and $\boldsymbol{A_{blank\,2}}$ is the absorbance of the test sample without the enzyme.

3.1.7 TLC BIOAUTOGRAPHY

TLC bioautograpy was carried out as described by Wangthong *et al*. (2007), with slight changes. Initially, the tyrosinase solution was prepared by dissolving 1 mL of the 1000 Unit enzyme in 1 mL of 50 mM sodium phosphate buffer (pH 6.5). The substrate was also prepared by dissolving 0.0036 g of L-tyrosine in 10 mL of 50 mM sodium phosphate buffer (pH 6.5). Then, the positive control, crude extracts and/or main fractions were dissolved in methanol to prepare a stock

solution of 1 mg/mL. The kojic acid, crude extracts and/or main fractions solutions were then spotted on the TLC plate and eluted with DCM-MeOH [(95:5) and/or (90:10)]. The developed plate was then sprayed with tyrosinase enzyme and incubated at a room temperature for 5 min. After incubation, it was sprayed with L-tyrosine (20 mM) and once again incubated at room temperature for 30 min. Indication of the presence of tyrosinase inhibitory activity amongst the investigated samples was shown by clear white zones as the background of the plate assumed a purple-greyish colour.

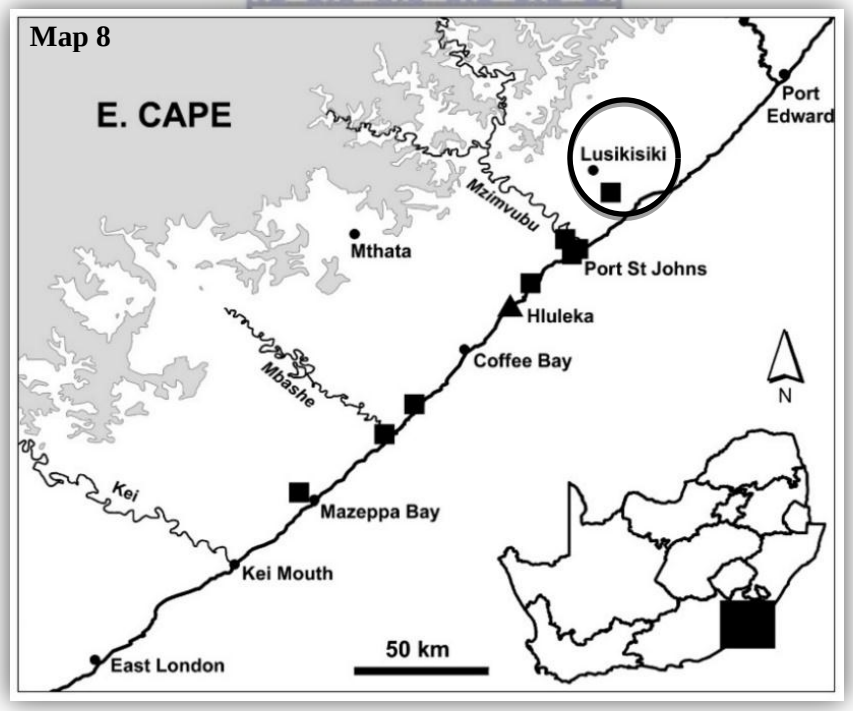
3.1.8 STATISTICAL ANALYSIS

The tyrosinase enzyme inhibitory effect of the plant extracts, isolated compounds and kojic acid were assayed in triplicate. The final values of the tyrosinase skin enzymatic inhibition assay were expressed as IC_{50} values and determined using the GraphPad Prism 5.0 software. For all analyses, $p < 0.05$ was considered to indicate a statistically significant difference.

3.2 ACQUISITION AND AUTHENTICATION OF PLANT MATERIALS

3.2.1 COLLECTION OF PLANT MATERIALS

The collection of the plant materials examined in the study was done by the late Mr Zekanzima Ndzoyiya and Mr Solomon Kuca from Ntsubane forest complex in Lusikisiki (E. Cape Province), during November 2015. Lusikisiki is located approximately 40 km north of Port St. Johns (Figure 3.1); it is characterized by spectacular views of natural vegetation and waterfalls (South African Traveler, 2015). The Ntsubane forest complex is a perpetual evergreen forest, situated on the periphery of the Indian Ocean. The voucher specimens examined in the study were authenticated by Dr. Christopher Cupido (SANBI; Kirstenbosch, S. Africa).



Retrived from *Zoologische mededelingen* wbsite:http://www.zoologischemededelingen.nl/83/nr03/a06

Figure 3.1: Location of Lusikisiki in the E. Cape Province (S. Africa).

3.2.2 INVESTIGATED PLANT SPECIES AND RELATIVE INFORMATION

Fifteen interviews were conducted with fifteen traditional healers within the E. Cape Province, particularly in the Pondoland (Mthatha, Libode, Port St. Johns, and Lusikisiki), to obtain ethnomedicinal knowledge and relative importance of various indigenous medicinal plants which are used in healing skin disorders and hyperpigmentation disorders, in particular. Ten plant species from various families were commonly suggested by the interviewed traditional healers. Such plant species and the relative information are shown in Table 3.1.

Table 3.1: Investigated plant species and their relative information

No.	Scientific name	Family	Herbarium number	Local name	English name	Part used	Endemism	Traditional uses	Reference(s)
1	*Cassine peragua* L.	Celastraceae	NBGSLD0001440	Umbomvane	Cape saffron	Bark	Endemic to Southern Africa	Traditionally used for treating burns.	—
2	*Cassipourea gummiflua* Tul.	Rhizophoracea	NH0006334-0	Umemezi	Large-leaved onionwood	Bark	Not endemic to South Africa	Is used as a substitute of *Cassipourea malosana* as skin lightener, and to treat skin ailments and sunburn. Also used for protection from evil spirits.	(Brink, 2006)
3	*Clivia miniata* (Lindl.) Bosse	Amaryllidaceae	PREART0003332	Umayime	Bush lily	Root rhizome	Endemic to Southern Africa	The rhizome is taken for snake-bite, wounds and pain relief. While whole plant is used to help with childbirth and to hasten parturition.	(Tait, 2001)
4	*Cryptocarya myrtifolia* Stapf	Lauraceae	SAM0043557-0	Umthungwa	Camphor tree	Stem bark	Endemic to South Africa	Used interchangeable with the bark of *Ocotea bullata* internally as in form of tea or applied as steam (external use) to treat pimples.	(Hutchings *et al*., 1996) (Van Wyk and Van Wyk, 1997)
5	*Gunnera perpensa* L.	Gunneraceae	K000350081	Uphuzi lomlambo	River pumpkin	Rhizome	Not endemic to South Africa	Externally used as a wound dressing. Infusions may be taken internally to treat psoriasis.	(Mabona and van Vuuren, 2013)

6	*Kigelia africana* (Lam.) Benth.	Bignoniaceae	PRE0181079-0	Umfongothi	Sausage tree	Fruit	Not endemic to South Africa	Used for various skin disorders from fungal infections, boils, acne and psoriasis, dressing wounds and sores.	(Saini *et al.*, 2009)
7	*Protorhus longifolia* (Bernh.) Engl.	Anacardiaceae	BNRH0010636	Uzintlwa	Red beech	Bark	Not endemic to South Africa	Used for blood-clotting related diseases.	(Mosa *et al.*, 2011)
8	*Rapanea melanophloeos*(L.) Mez	Myrsinaceae	PRE0822924-0	Maphipha	Cape beech	Bark	Endemic to Southern Africa	The plant species is mainly used as a charm to protect against evil spirits. Its powder form bark is internally used (chewed) and/or externally used to treat sore throats and wounds.	(Van Wyk *et al.*, 1997)
9	*Rhynchosia villosa* (Meissner) Druce	Fabaceae	PREART0003226	Uphuzi lobala	—	Root	Endemic to South Africa	It is used externally and internally to enhance the skin texture and tone, while it also aid in treating swollen parts of the body when washed with its soaked leaves. Also used in hormone-dependent diseases.	—
10	*Senecio serratuloides* DC.	Asteraceae	NHSLD0002401	Ntsukumbini	Two-day cure	Leaves	Not endemic to South Africa	Tea made from the leaves is taken in case of infections; leaves are applied directly to purulent sores. Leaf decoction is taken as a blood purifier in case of skin eruptions, while powdered leaves or roots are applied to burns or sores.	(De Wet *et al.*, 2013) (Kelmanso *et al.*, 2000)

*The plants were collected from Lusikisiki by Mr Z. Ndzoyiya and Mr S. Kuca, except *Kigelia african* (**10**) which was collected from Eerste Rivier by Ms Luveni Sonka.

3.3 INVESTIGATION OF TYROSINASE INHIBITION OF CRUDE EXTRACTS

3.3.1 TYROSINASE ENZYME INHIBITION OF CRUDE EXTRACTS

The tyrosinase enzyme inhibition assay was used for the preliminary bio-evaluation of the crude extracts and was carried out as described in Section 3.1.6. Kojic acid was used as a positive control in the assay since the inhibition exerted by kojic acid is well established due to its ability to chelate to copper in the active sites of the enzyme (Kubo *et al*., 2000). Table 3.2 and Figure 3.2 show the average percentages of tyrosinase inhibition of the investigated plant species screened at various concentrations.

Table 3.2: Average percentages of tyrosinase inhibition (%) and detectable anti-tyrosinase IC_{50} values of the different plant extracts

No.	Plant extract code	Plant extract name	Average percentages of tyrosinase inhibition (%) in various concentrations (µg/mL) and detectable anti-tyrosinase IC_{50} value of the plant extracts					
			100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	IC_{50} (µg/mL)
1	CM	*Clivia miniata*	29.00	10.27	8.14	7.84	6.93	NA
2	PL	*Protorhus longifolia*	41.63	38.96	33.33	32.88	31.28	NA
3	RV	*Rhynchosia villosa* (roots)	56.40	55.78	49.16	45.28	36.00	15.67
4	GP	*Gunnera perpensa*	14.31	9.59	6.09	5.18	3.20	NA
5	CR	*Cryptocarya myrtifolia*	27.40	23.82	19.63	18.65	18.26	NA
6	RM	*Rapanea melanoploes*	52.51	47.79	38.89	37.21	30.82	26.30
7	Ko	Kojic acid	98.47	97.40	97.33	89.48	86.98	0.9
8	SS	*Senecio serratuloides*	40.87	39.80	24.73	20.70	16.82	NA
9	RV_{leaves}	*Rhynchosia villosa* (leaves)	34.55	17.12	16.02	13.85	10.27	NA
10	CG	*Cassipourea gummiflua*	17.05	16.29	12.18	6.01	5.72	NA
11	CP	*Cassine peragua*	29.30	24.80	24.12	16.82	15.91	NA
12	KA	*Kigelia africana*	39.04	28.39	22.83	20.47	17.50	NA

***NA**: Not active in tested concenration range

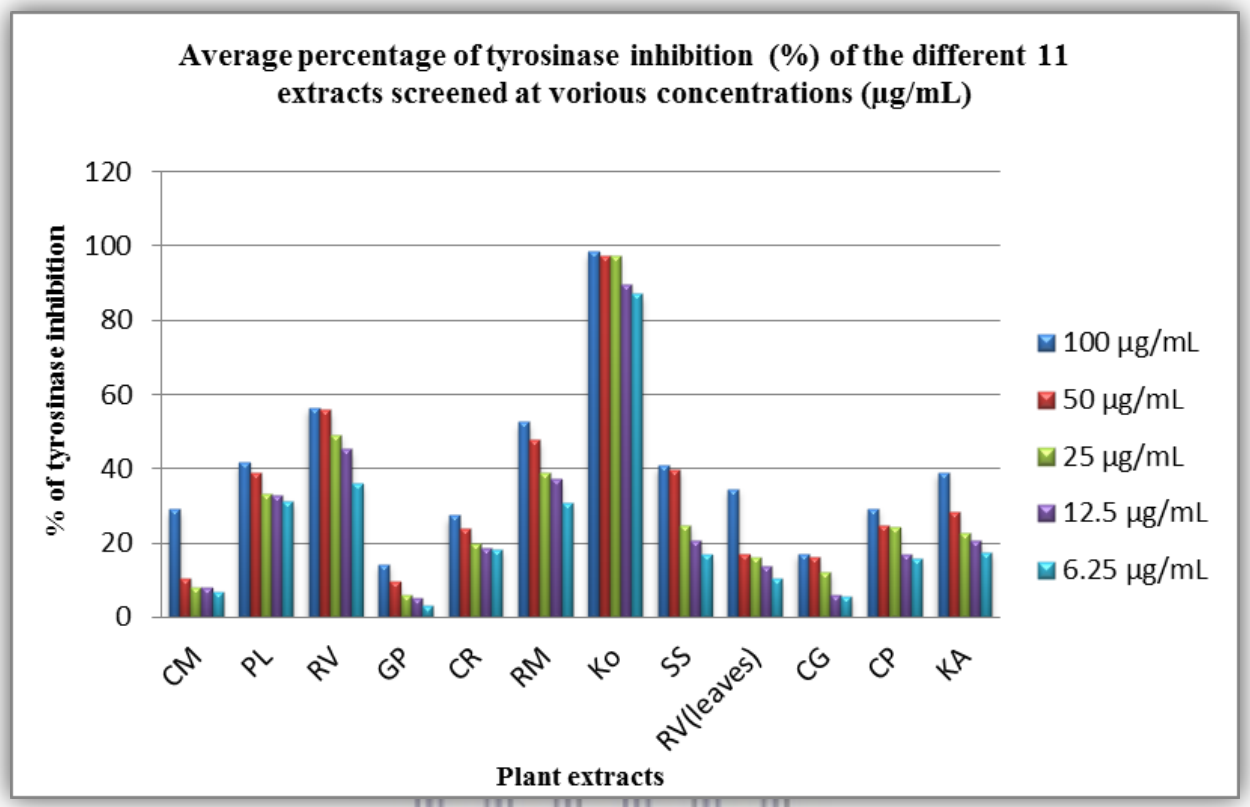


Figure 3.2: Bar graph depicting average percentages of tyrosinase inhibition (%) of *Clivia miniata* (CM), *Protorhus longifolia* (PL), *Rhynchosia villosa* root (RV), *Gunnera perpensa* (GP), *Cryptocarya myrtifolia* (CR), *Rapanea melanophloeos* (RM), kojic acid (Ko), *Senecio serratuloides* (SS), *Rhynchosia villosa* leaves [RV(leaves)], *Cassipourea gummiflua* (CG), *Cassine peragua* (CP), and *Kigelia africana* (KA) extracts screened at various concentrations (µg/mL).

3.3.2 TLC BIOAUTOGRAPHY OF CRUDE EXTRACTS

To confirm the results obtained in Section 3.3.1, a qualitative approach was performed, i.e., that of TLC bioautography. This is a method that combines chromatographic separation and *in situ* biological activity determination. It was employed in the preliminary screening of natural products which are anticipated to have the tyrosinase enzyme inhibitory effect and for the

bioactivity-directed fractionation and isolation of active constituents from complex fractions (Cheng and Wu, 2013). The assay was executed as described in Section 3.1.7.

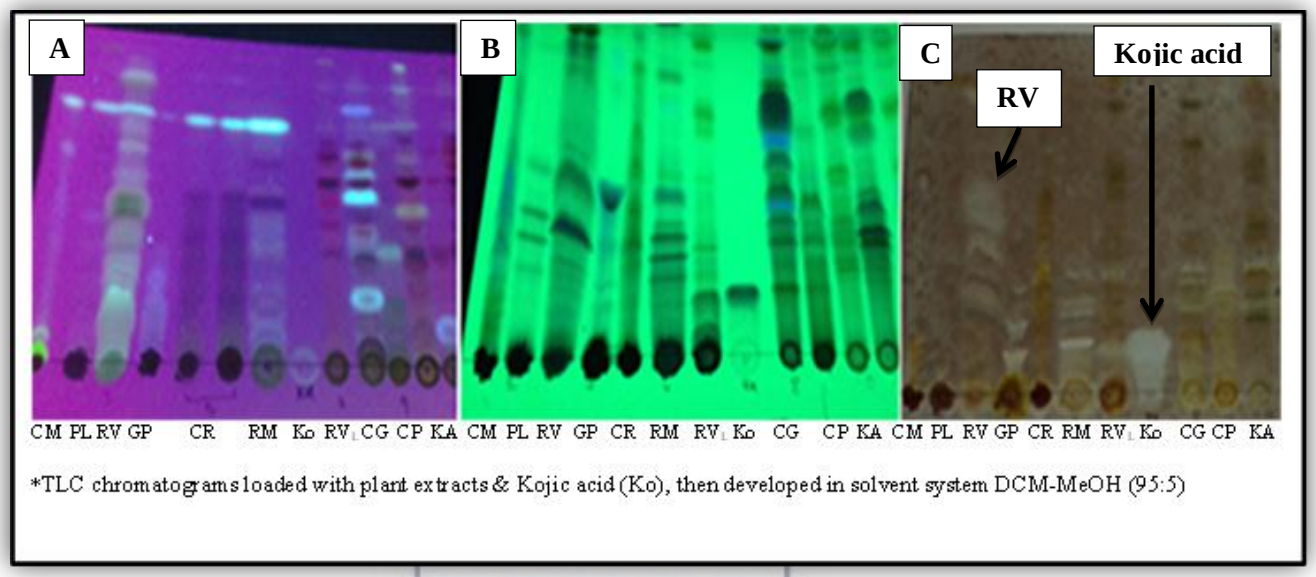


Figure 3.3: TLC chromatograms of *Clivia miniata* (CM), *Protorhus longifolia* (PL), *Rhynchosia villosa* root (RV), *Gunnera perpensa* (GP), *Cryptocarya myrtifolia* (CR), *Rapanea melanophloeos* (RM), *Rhynchosia villosa* leaves (RV_L), kojic acid (Ko), *Cassipourea gummiflua* (CG), *Cassine peragua* (CP), and *Kigelia africana* (KA) extracts developed in solvent system DCM-MeOH (95:5), **A**: viewed under UV (366 nm), **B**: after spraying with vanillin/H_2SO_4, heating at 110℃ and viewed under UV (254 nm), **C**: after spraying with tyrosinase enzyme and tyrosine.

According to Figure 3.3, it became apparent that the plant extract of RV (*R. villosa* root) was the most active, since it had clearly developed white zones of tyrosinase inhibition compared to other extracts, after spraying with both tyrosinase enzyme and tyrosine (**C**), respectively. This was the indication of the presence of bioactive constituents that are capable of inhibiting tyrosinase enzyme. Hence, *R. villosa* was chosen for further chromatographic study to isolate bioactive compounds.

3.3.3 DISCUSSION OF THE ANTI-TYROSINASE INHIBITION OF THE INVESTIGATED PLANT EXTRACTS

Collection of the nine plant species **1**–**9** (Table 3.1), which are traditionally used for maintaining the skin structural integrity and healing skin hyperpigmentation was done in Lusikisiki (Ntsubane forest complex) within the E. Cape Province, while plant species **10** (Table 3.1) was collected in Eerste Rivier, which is situated in the W. Cape Province. Initial steps taken prior to the actual execution of the experiments were carried out using the extracts obtained of the suggested plants which included the bio-evaluation of the plant extracts against the tyrosinase enzyme (Figure 3.2 and Figure 3.3).

The results of the skin enzyme inhibitory assay showed that two plant species, namely, *Rhynchosia villosa* root and *Rapanea melanoploes* bark inhibited tyrosinase enzyme (Figure 3.2 and Figure 3.3). *R. villosa* was found to be the most active extract, as it inhibited mushroom tyrosinase with IC_{50} value of 15.67 µg/mL, while *R. melanoploes* inhibited mushroom tyrosinase with IC_{50} value of 26.3 µg/mL. Hence *R. villosa* was chosen for further chromatographic study, in order to isolate the bioactive compounds.

3.4 DESCRIPTION AND DISTRIBUTION OF *R. VILLOSA*

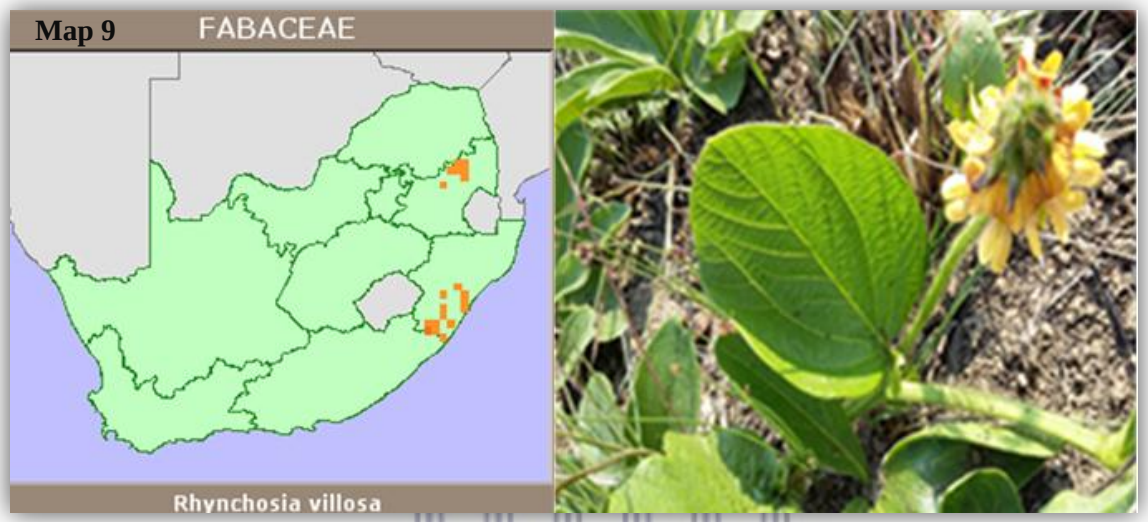
3.4.1 DESCRIPTION OF *R. VILLOSA*

As mentioned previously, *R. villosa* is one of the Fabaceae species. Like many legumes, *R. villosa* is a trailing plant species with hairy petioles, stems and leaves. The flowers of the inflorescence are yellow in colour with red veins, its pinnately compound leaves are light-green and they assume peltate shape. It consists of perennial underground system, an erect rootstock (Figure 3.4), which is the traditional used part for skin depigmentation. Both the rootstock and the leaves of *R. villosa* were bioevaluated in the study against the tyrosinase enzyme, where the rootstock showed higher tyrosinase inhibitory activity than the leaves (Section 3.3); this substantiated its preference in traditional use for skin depigmentation instead of the leaves.

Figure 3.4: Illustration of the rootstock of *R. villosa*.

3.4.2 DISTRIBUTION OF *R. VILLOSA*

As denoted in Table 3.1, *R. villosa* is endemic to S. Africa and widely distributed in KZN, Mpumalanga Province and E. Cape Province (Figure 3.5) (Foden and Potter, 2005).



Map 9: Retrieved from *Red List of South Africa Plants* website: http://redlist.sanbi.org/species.php?species=370-90

Figure 3.5: Distribution of *R. villosa* and its description.

3.5 EXTRACTION AND FRACTIONATION OF *R. VILLOSA* ROOT

3.5.1 EXTRACTION OF *R. VILLOSA* ROOT

The *R. villosa* root (RV) with a mass of 131.2 g was initially grated to small shreds before it was allowed to extract in 500 mL of MeOH for 48 hours. The mixture was then filtered and the combined filtrates were concentrated under reduced pressure at 40 ℃. The crude extract was then weighed [15.11 g (11.5 % of the raw material)] and kept at approximately 0 ℃.

3.5.2 COLUMN CHROMATOGRAPHY OF THE CRUDE EXTRACT

The crude extract of *R. villosa* (15 g) was applied to a silica gel column and eluted with Hex:EtOAc (6.6 L:7.4 L) and EtOAc:MeOH (3.85 L:1.15 L) gradient mixtures of increasing polarity as shown in Table 3.3.

Table 3.3: Fractionation of the methanolic extract of *R. villosa* (15 g)

SOLVENT SYSTEM Hex:EtOAc	VOLUME (L)	FRACTIONS
100:0	0.5	1-2
90:10	1	3-6
80:20	1	7-10
70:30	2	11-18
60:40	2	19-26
50:50	2	27-34
30:70	2	35-42
10:90	2	43-50
0:100	1.5	51-56
EtOAc:MeOH		
95:5	1	55-58
90:10	1	59-62
80:20	1	63-66
70:30	1	67-70
50:50	1	71-74

Fractions of 250 mL were collected (Figure 3.6). Similar fractions were combined according to their TLC profile to obtain 20 main fractions (Table 3.4).

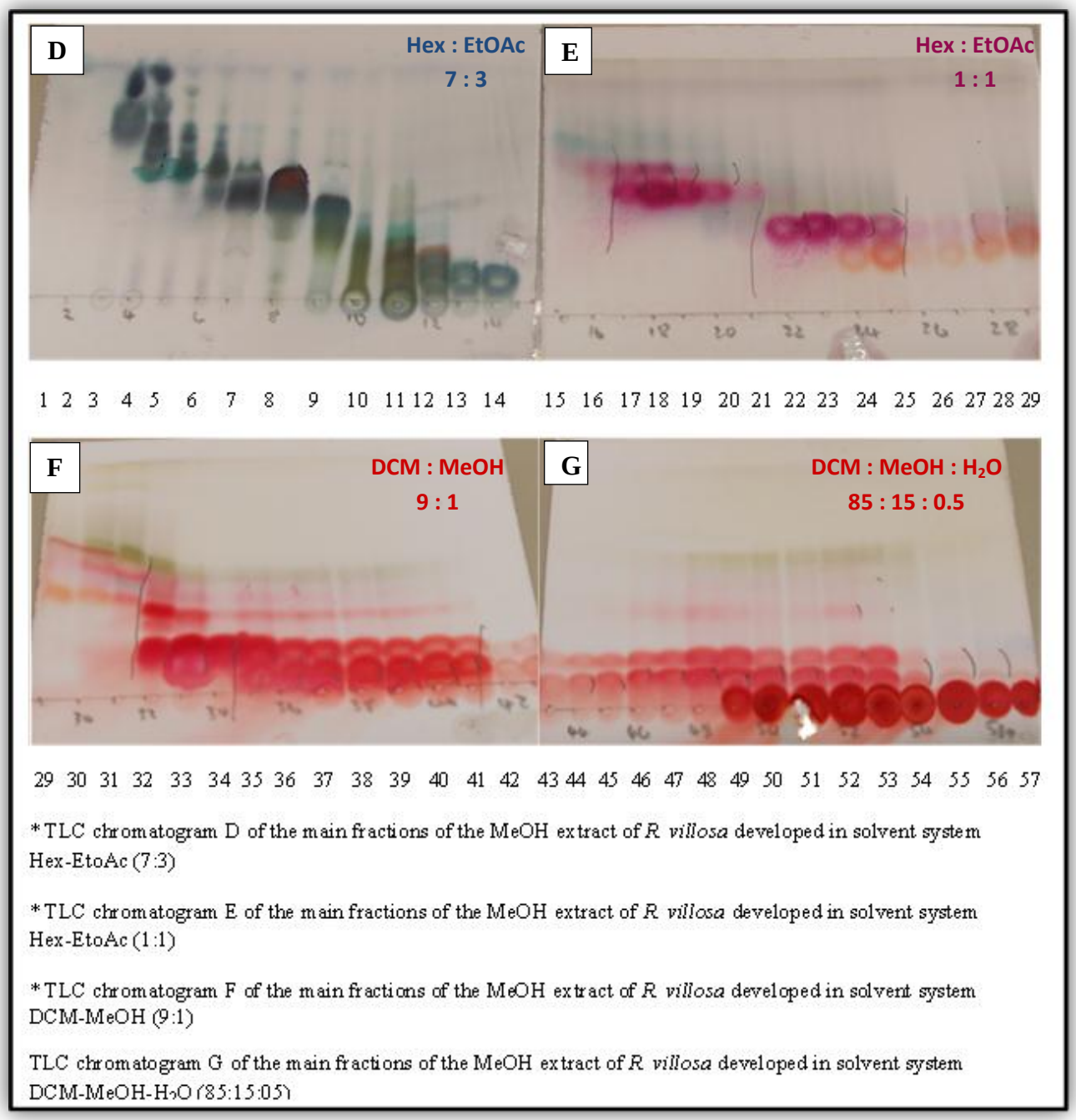


Figure 3.6: TLC chromatograms of fractions obtained from column chromatography of *R. villosa* methanolic extract,

adsorbent: silica gel
solvent systems: **D** [Hex:EtOAc (7:3)]; **E** [Hex:EtOAc (1:1)]; **F** [DCM:MeOH(9:1)]; **G** [DCM:MeOH:H2O (85:15:0.5)]
detection: sprayed with vanillin/H_2SO_4 and heated at 110 ℃.

Table 3.4: Main fractions of *R. villosa* methanolic extract

COMBINED FRACTIONS	DESIGNATED CODE	MASS (mg)
1-2	I	5.1
3	II	75.7
4-5	III	79.7
6-7	IV	94.5
8-9	V	46.3
10-12	VI	153.9
13-14	VII	28.8
15-16	VIII	1.2
17-21	IX	118.3
22-25	X	83.0
26-28	XI	10.0
29-31	XII	61.5
32-34	XIII	324.2
35-41	XIV	242.0
42-48	XV	163.8
49-53	XVI	612.4
54-59	XVII	4399.4
60-63	XVIII	5295.7
64-67	XIX	583.4
68-70	XX	288.4

3.5.3 QUALITATIVE SCREENING OF THE MAIN FRACTIONS USING TLC BIOAUTOGRAPHY

The main fractions (I-XV) obtained from the main column were spotted on TLC plates (2 copies) and developed in DCM:MeOH (9:1). One of the plates (**H)** was sprayed with vanillin/H_2SO_4 and other plate (**I**) was sprayed with the tyrosinase enzyme and tyrosine, as explained in Section 3.1.7. Figure 3.7 shows the two plates and the inhibition zones in plate **I**, which indicated bioactivity, showing that fractions IX-XV contained the most active compounds.

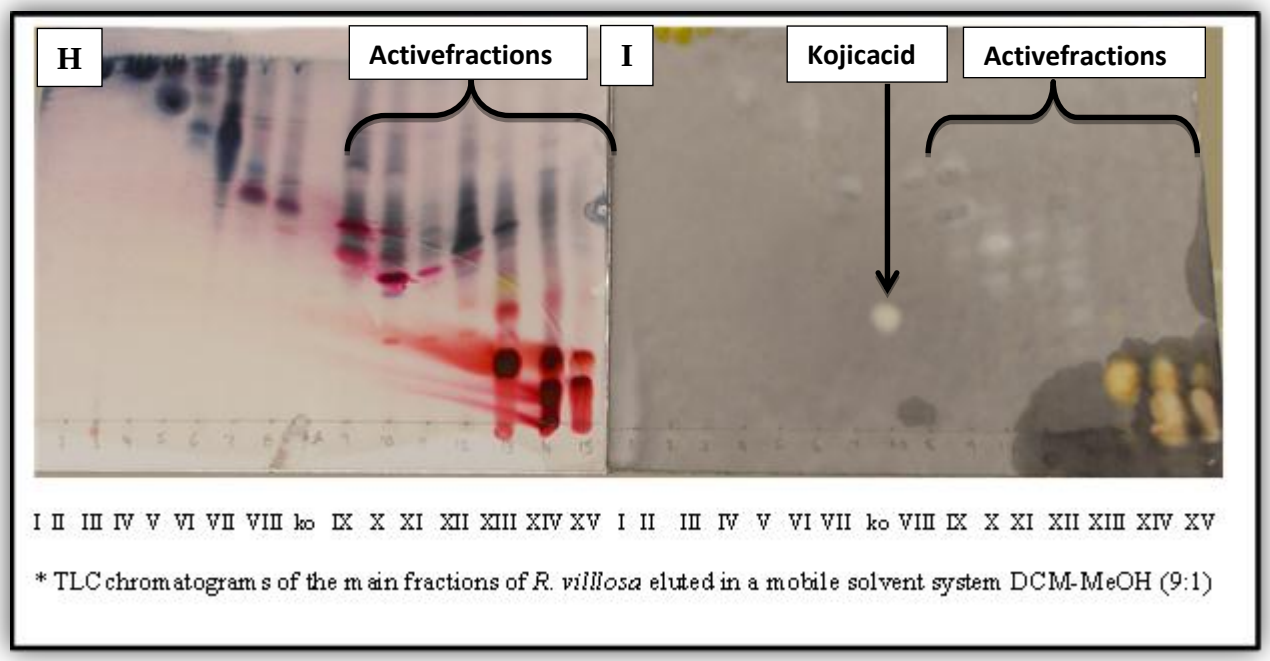


Figure 3.7: TLC chromatograms of the main fractions (I-XV) spotted on TLC plates and developed in DCM:MeOH (9:1), plate **H**: sprayed with vanillin/H_2SO_4 and heated at 110 ℃, plate **I**: sprayed with tyrosinase and L-tyrosine.

3.6 ISOLATION OF THE CHEMICAL CONSTITUENTS OF *R. VILLOSA*

3.6.1 COLUMN CHROMATOGRAPHY OF MAIN FRACTION IX

Fraction IX (118.3 mg) was applied onto a sephadex column and eluted with a 5 % aqueous EtOH solution. Similar fractions were combined together to give the sub-fractions IX_1 – IX_5 (Table 3.5; Figure 3.8).

Table 3.5: Collective sub-fractions from the fractionation process of the main extract IX

COMBINED FRACTIONS	DESIGNATED CODE	MASS (mg)
1-12	Discarded	—
13-23	IX_1	1.1
24-31	IX_2	15.2
32-34	IX_3	46.1
36-37	IX_4	49.8
38-44	IX_5	6.1

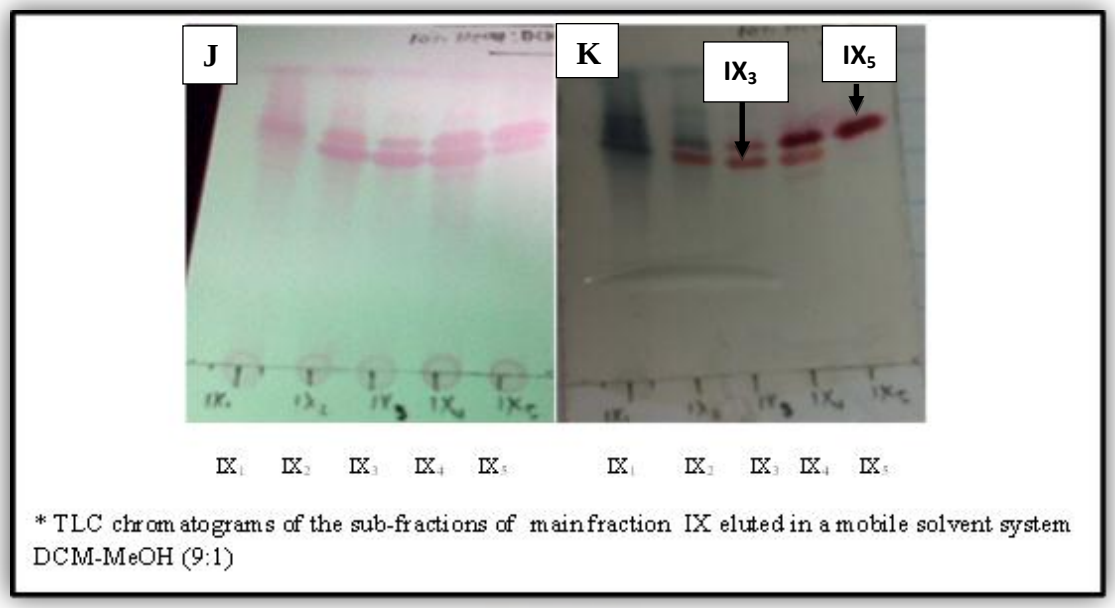


Figure 3.8: TLC chromatogram of the sub-fractions of main fraction IX viewed under UV (254 nm; **J**) and after vanillin spraying and heating at 110 ℃ (**K**).

In order to determine the content and purity of the obtained active compounds and their molecular structures, the samples were subjected to NMR spectroscopy. Thus, it was confirmed from the NMR spectrometer that sub-fraction IX_3 and IX_5 were pure compounds. The samples were isolated with a mass of 46.1 mg (IX_3; 0.035 %) and 6.1 mg (IX_5; 0.0046 %), respectively.

3.6.2 HPLC CHROMATOGRAPHY OF MAIN FRACTION X

As indicated in Figure 3.7, the main fraction X was amongst the fractions that showed the highest tyrosinase inhibitory activity. This fraction was initially dissolved in MeOH, before it was injected in the HPLC. A gradient solvent system of MeOH:DIW (55:45 to 70 % in 30 min, then to 100 % in 5 min) was used as the eluent. HPLC of fraction X (83 mg) afforded one prominent peak (Figure 3.9), which was collected and labelled X_1 (4 mg; 0.0030 %).

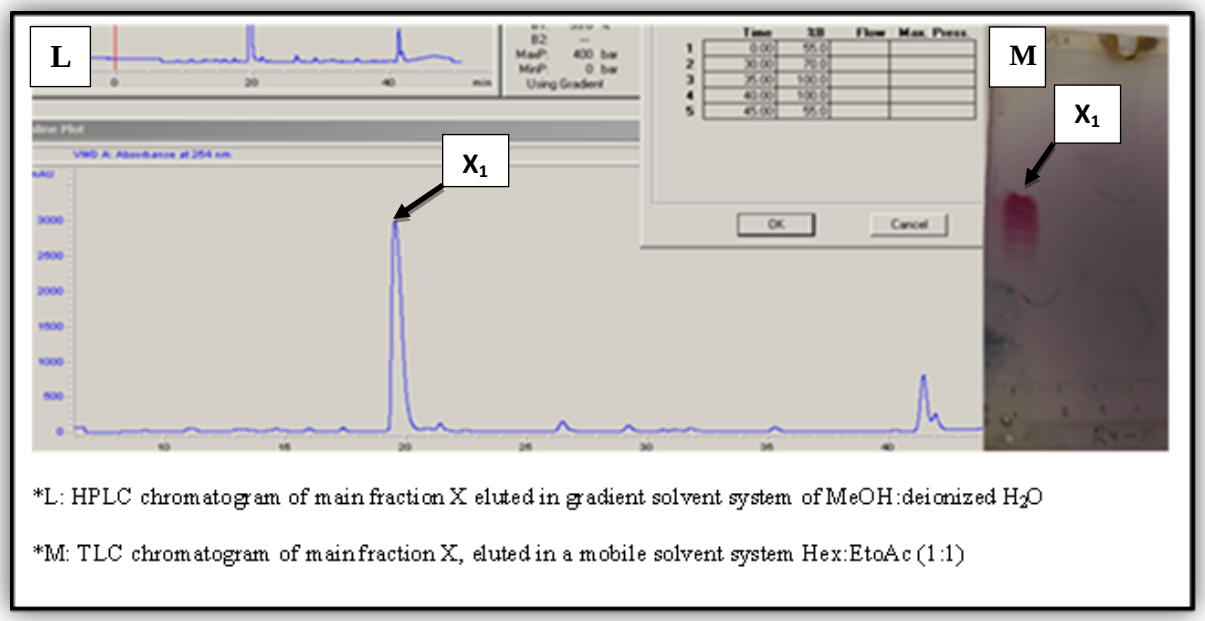


Figure 3.9: HPLC chromatogram (**L**) of the afforded peak X_1, and TLC chromatogram after spraying with vanillin/H_2SO_4 and heating at 110 ℃ (**M**).

***Conditions used**:

Solvent	MeOH:DIW (55:45 to 70 % in 30 min, then to 100 % in 5 min)
Column	SULPECO, RP-18 (25 × 2.1 cm)
Flow rate	1.0 mL/min
Detection	UV at 254 nm

3.6.3 COLUMN CHROMATOGRAPHY OF MAIN FRACTIONS (XIV AND XV)

Sephadex fractionation of the two main fractions, viz. XIV and XV with a combined mass of 406 mg was performed. According to the results of the TLC bioautography method which was employed for bioactivity-directed fractionation of the main fractions of *R. villosa* (Figure 3.7), these two polar fractions were found to be amongst active fractions against the tyrosinase enzyme. The combined fraction was applied to a sephadex column using 5 % aqueous EtOH. Two pure compounds labelled XIV_4 (5.9 mg; 0.0045 %) and XIV_6 (287.4 mg; 0.22 %) (Table 3.6; Figure 3.10) were isolated.

Table 3.6: Collective sub-fractions from fractionation of the combined main fractions (XIV and XV)

COMBINED FRACTIONS	DESIGNATED CODE	MASS (mg)
1-30	Discarded	—
31-38	XIV_1	6.0
39-42	XIV_2	8.5
43 -54	XIV_3	68.6
55-58	XIV_4	5.9
59-61	XIV_5	20.1
62-75	XIV_6	287.4

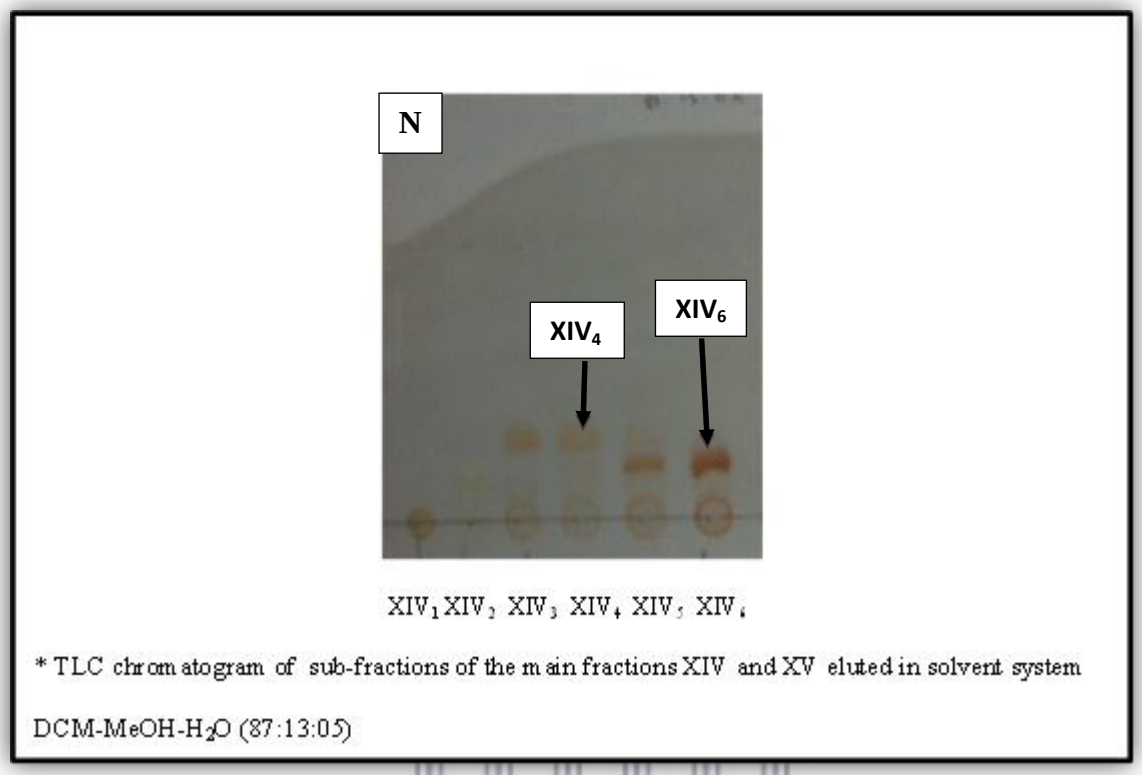
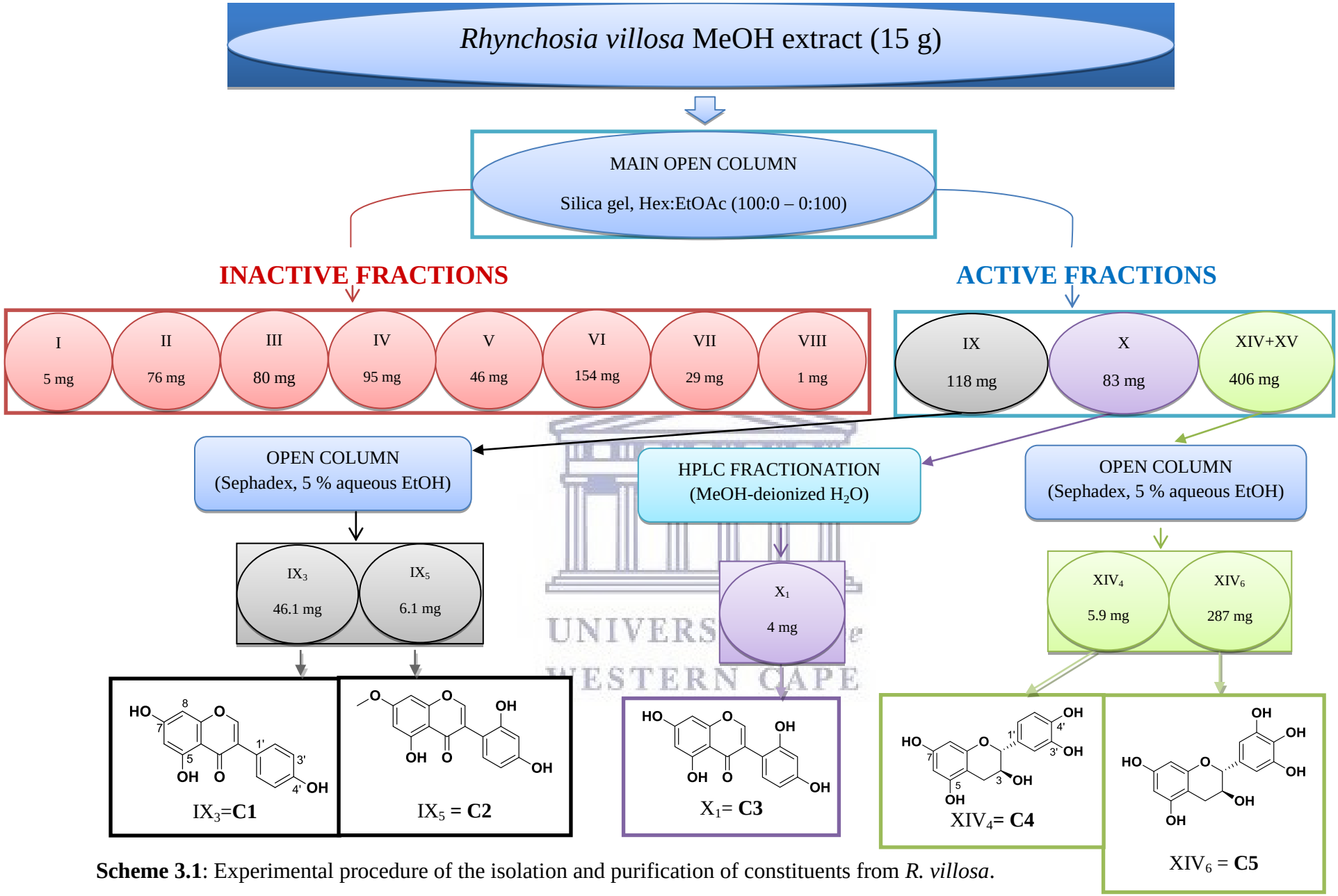


Figure 3.10: TLC chromatogram of the sub-fractions of combined main fractions (XIV and XV) after spraying with vanillin/H_2SO_4 and heating at 110℃ (**N**).

The compounds were thoroughly characterized using NMR spectroscopy. According to the ^{1}H and ^{13}C NMR data obtained, the two polar sub-fractions (XIV_4 and XIV_6) indicated in Figure 3.10 were found to be pure compounds.

3.6.4 SUMMARY OF THE ISOLATION AND PURIFICATION OF CONSTITUENTS FROM *R. VILLOSA*

As pointed out in Section 3.6.1 – 3.6.3, five compounds viz. IX_3, IX_5, X_1, XIV_4 and XIV_6 were isolated from four active main fractions (IX, X, and combined fractions XIV and XV; Figure 3.7) of *R. villosa*. To avoid inconvenience, the isolated compounds (IX_3, IX_5, X_1, XIV_4 and XIV_6) were labelled **C1**, **C2**, **C3**, **C4** and **C5** from here on respectively. Scheme 3.1 shows the summary of the isolation and purification of constituents from *R. villosa*. Hence both **C1** and **C2** were isolated from fractionation of main fraction IX through sephadex open column. Same applies with **C4** and **C5**; they were isolated from fractionation of combined main fractions (XIV and XV) with aid of sephadex open column. While **C3** was afforded from fractionation of main fraction X via semi-prep HPLC.



Scheme 3.1: Experimental procedure of the isolation and purification of constituents from *R. villosa*.

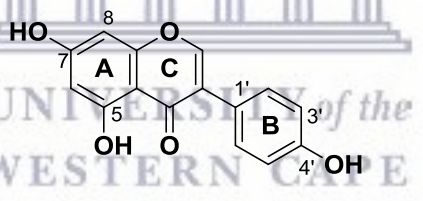
CHAPTER FOUR

STRUCTURE ELUCIDATION AND ANTI-TYROSINASEACTIVITY OF *R. VILLOSA* CONSTITUENTS

4.1 STRUCTURE ELUCIDATION OF ISOLATED ISOFLAVONES

Structure elucidation of genistein (**C1**):

One-dimensional NMR experiments are fundamental in determining the resonance frequency of each ^{1}H or ^{13}C nucleus in the molecule, thus, both ^{1}H and ^{13}C NMR experiments were employed the most in elucidating the structures of the isolated compounds in the current study. Moreover, the chemical shifts obtained gave important information about the nature and number of protons and carbons in the compounds of interest. The recorded chemical shifts were then validated by comparing them with existing literature data (Pinheiro and Justino, 2012).



Genistein (**C1**)

Figure 4.1: Chemical structure of genistein (**C1**).

Compound IX_3 (46.1 mg; 0.035 %), was isolated as an amorphous yellow powder, showing a characteristic flavonoid nature using UV-Vis light (TLC) and the NMR data obtained. Its spectral data was correlated with the existing literature data (Yoon *et al*., 2016). The ^{1}H NMR spectrum (Figure 4.2; Table 4.1) showed aromatic protons of ring A at δ_H 6.02 [(H-6, *d*) 2.0 Hz] and 6.12 [(H-8, *d*) 2.0 Hz], while another deshielded olefinic proton was observed and is attributed to ring C at δ_H 7.86 (H-2, *s*). Signals typically observed for ring B, revealed two pairs

of equivalent aromatic protons, one pair resides at δ_H 6.72 and 6.74 [(H-3' and H-5', *d*) 8.8 Hz], while the other pair resides at δ_H 7.23 and 7.25 [(H-2' and H-6', *d*) 8.8 Hz], which are characteristic of a 1,4-disubstituted ring B.

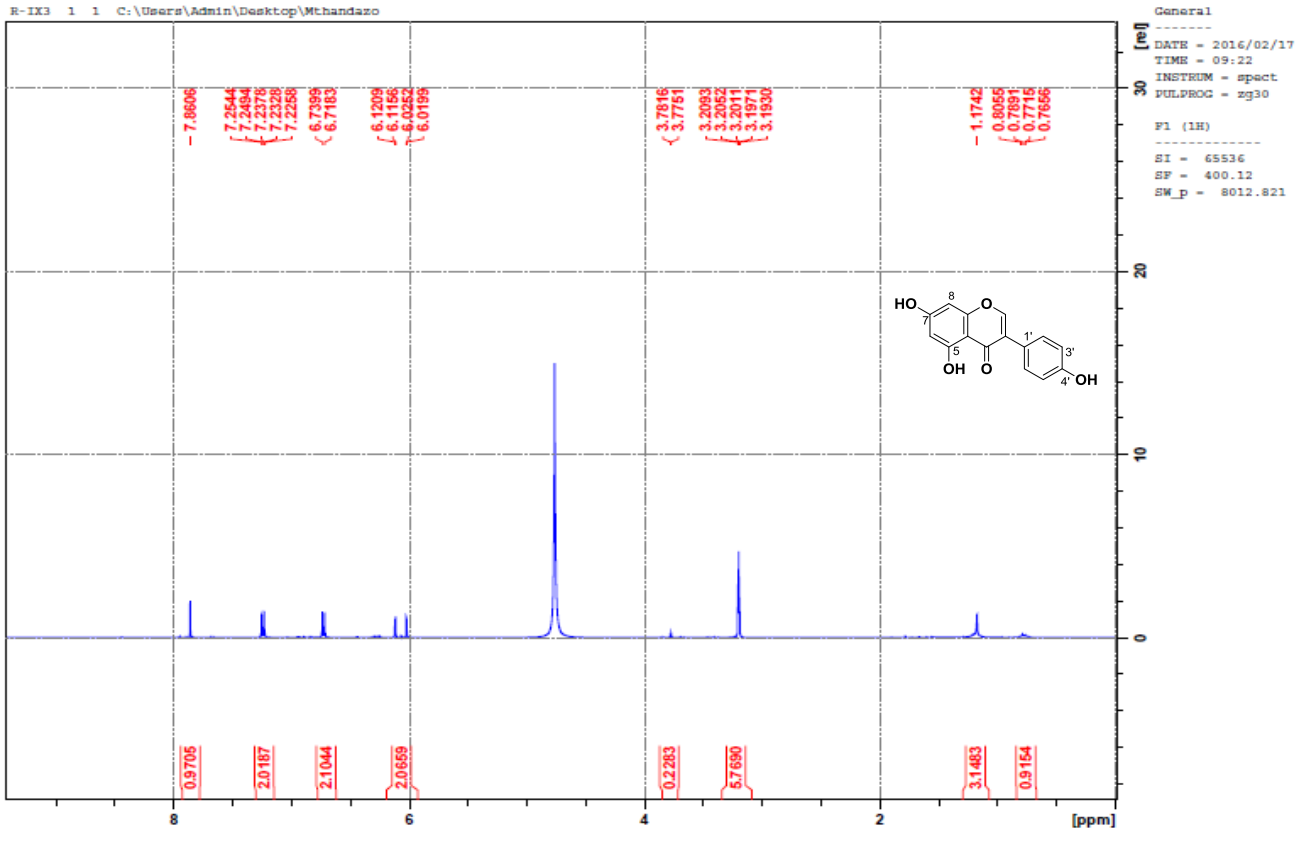


Figure 4.2:[1]H NMR spectrum of compound **C1** in CD_3OD.

The [13]C NMR spectrum (Figure 4.3; Table 4.1) depicted twelve aromatic carbons δ_c 162.2 (C-5), 100.2 (C-6), 169.1 (C-7), 94.8 (C-8), 157.3 (C-9), 103.6 (C-10), 122.3 (C-1'), 158.6 (C-4'), 130.0 (C-2' and C-6'), 114.8 (C-3' and C-5') belonging to ring A and B. An olefinic carbon at δ_c 152.9 (C-2), quaternary carbon at δ_c123.0 (C-3) and carbonyl carbon at δ_c 180.3 (C-4, C=O) were observed and are attributed to ring C. The high intensity signals at δ_C 114.8 and 130.0 were

evidence of the presence of the two pairs of equivalent carbons, i.e., C-3' with C-5' and C-2' with C-6' positions, respectively.

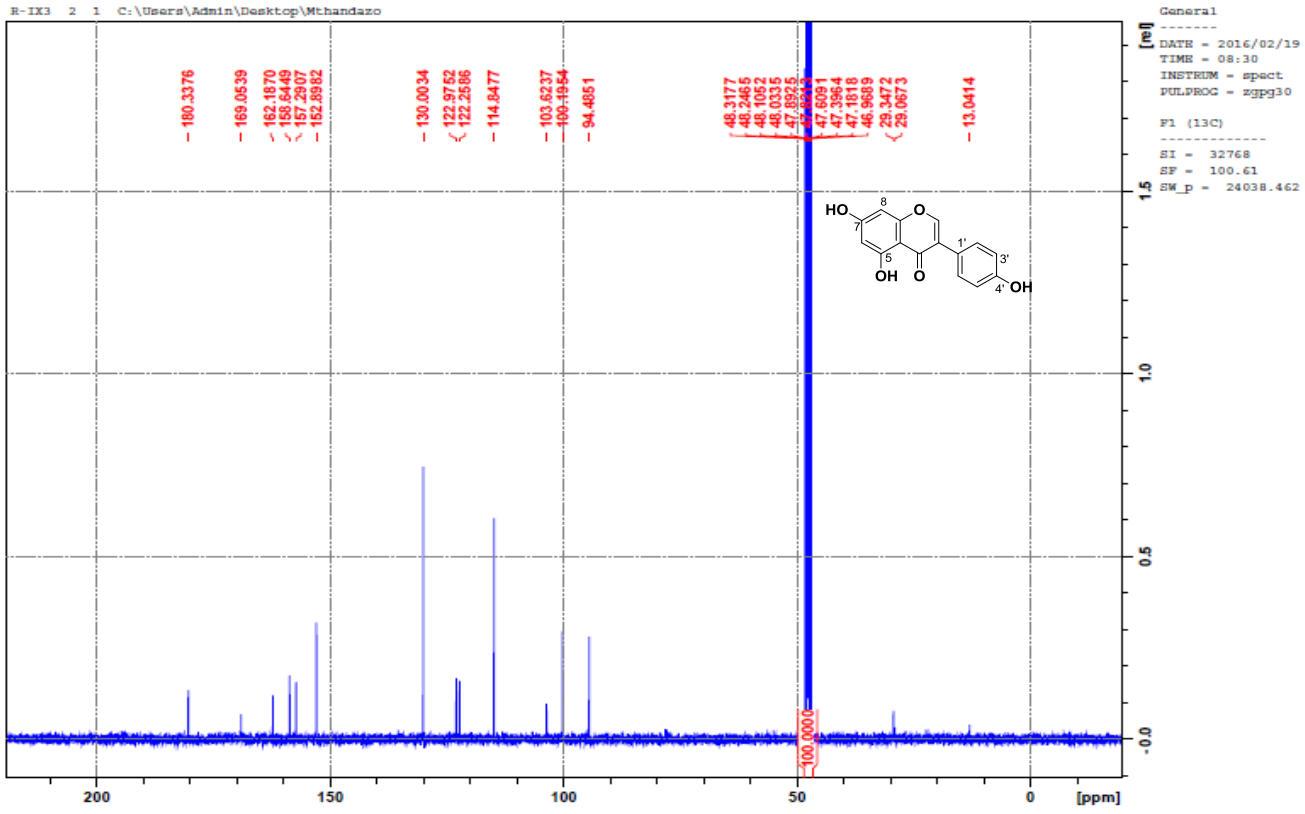
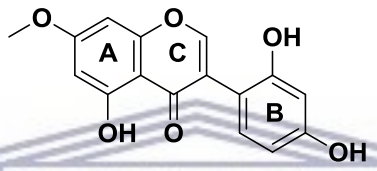


Figure 4.3:^{13}C NMR spectrum of compound **C1** in CD_3OD.

These data implied the presence of an isoflavone nucleus functionalized with a 5,7,4'-trihydroxy group. Careful review of the literature thus confirmed the compound to be genistein, a common isoflavone isolated from soybean and widely distributed in Leguminosae. Figure 4.1 shows the chemical structure of genistein (**C1**).

Structure elucidation of cajanin (**C2**):

Elucidation of the chemical structure of **C2** was achieved by employing heteronuclear 2D NMR experiments (HSQC and HMBC), which generate contour maps that show the correlations between proton and carbon nuclei, hence providing an opportunity to observe the ^{1}H and ^{13}C NMR data directly (Claridge, 1999). Two-dimensional NOESY data were also acquired and these data are useful at establishing non-bonded connectivity, or connectivity through space (Pinheiro and Justino, 2012).



Cajanin (**C2**)

Figure 4.4: Chemical structure of cajanin (**C2**).

Compound IX_5 (6.1 mg; 0.0046 %) was isolated as an amorphous yellow powder. The ^{1}H NMR spectrum (Figure 4.5; Table 4.1) showed two aromatic protons attributed to ring A δ_H 6.26 [(H-6, *d*) 2.2 Hz], 6.46 [(H-8, *d*) 2.2 Hz] and three methyl protons δ_H 3.81 (MeO-7, *s*) attributed to a methoxy functional group. An additional three protons were observed for ring B at δ_H 6.30 [(H-3', *d*) 2.4 Hz], 6.28 [(H-5', *dd*) 2.4 Hz, 8.4 Hz], and 6.96 [(H-6', *d*) 8.4 Hz], while one deshielded proton was observed to resonate at δ_H 7.91 (H-2, *s*) and is attributed to ring C.

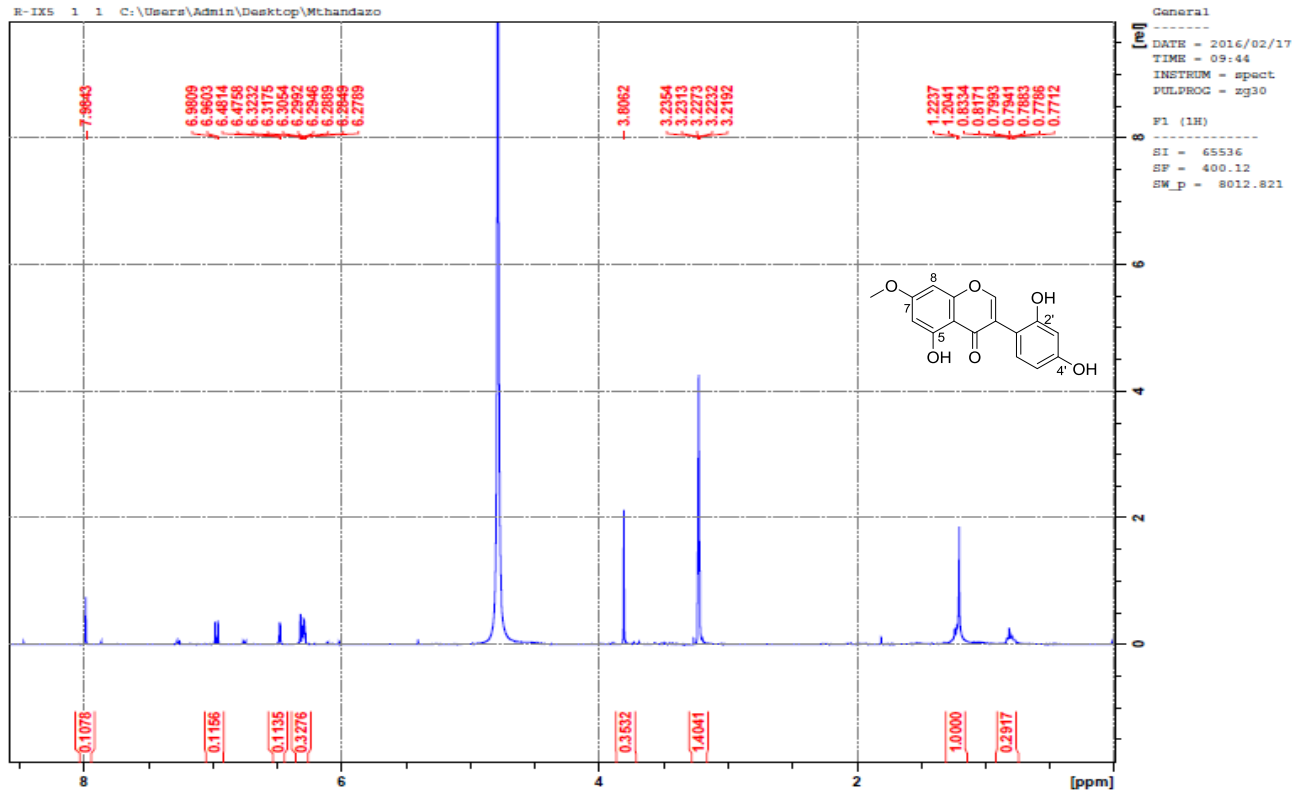


Figure 4.5:[1]H NMR spectrum of compound **C2** in CD_3OD.

Significant differences were observed in the ^{13}C NMR spectrum (Figure 4.6; Table 4.1) of **C2** when compared to that of **C1** as some of the ring B carbons were found to be shielded and this is likely due to additional electron density imparted by the hydroxyl group occurred at C-2' position. Two carbon signals residing at δ_c 102.8 (C-3'; *ortho*) and 106.6 (C-5'; *para*) were highly shielded in comparison to **C1**, while both C-6' (δ_c 131.8; *meta*) and C-4' (δ_c 158.6; *meta*) were found in the same positions as for **C1**. As mentioned above, unlike in **C1**, a deshielded carbon signal occurring at δ_c 156.2 (C-2') indicated the presence of a hydroxyl functional group at the C-2' position. Additional carbon signals were observed to resonate at δ_c 155.6 (C-2), 121, 4 (C-3), 181.4 (C-4), 162.1 (C-5), 97.9 (C-6), 165.9 (C-7), 91.8 (C-8), 158.3 (C-9), 105.6 (C-10)

109.2 (C-1') and 55.1 (MeO-7) accounting for all the expected signals for the structure attributed to **C2** (Figure 4.4).

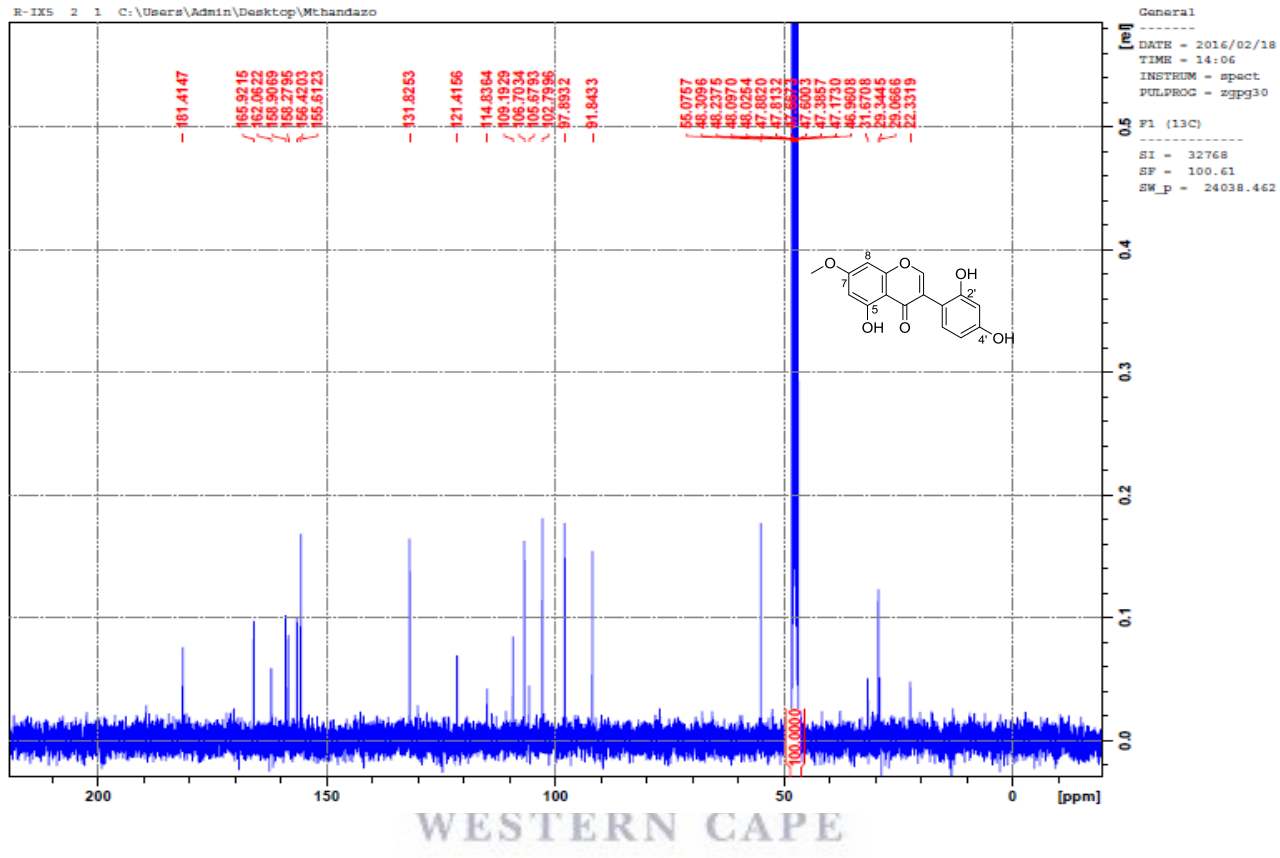


Figure 4.6:^{13}C NMR spectrum of compound **C2** in CD_3OD.

The NMR spectra of **C2** was found to be similar to that of **C1**, with the main difference being the presence of the methoxy signals at δ_H 3.81/δ_c 55.1 in ^{1}H and ^{13}C NMR spectra, respectively, indicating the presence of the methoxy group (OMe) at C-7. The position of the methoxy group was additionally evidenced from a 2D HMBC spectrum (Figure 4.7) which showed a correlation between the *O*-methyl protons at δ_H 3.81, to C-7 (δ_C 165.9).

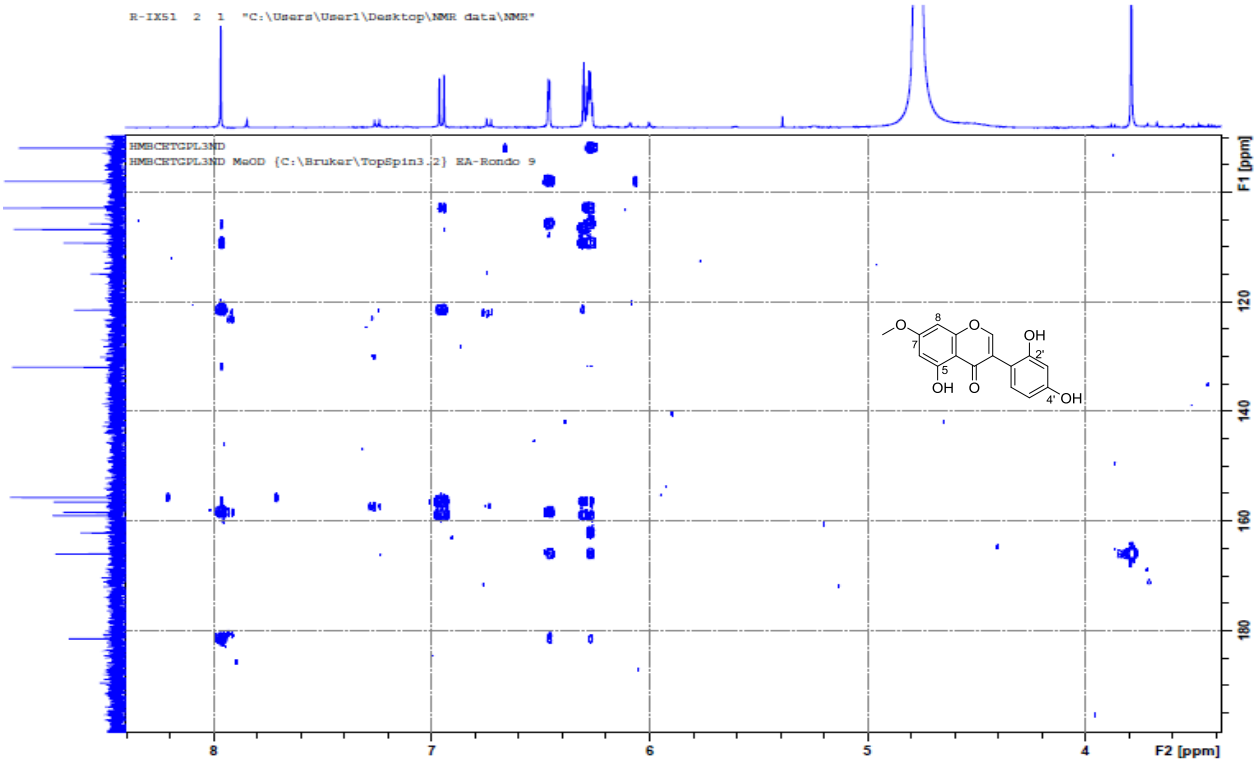


Figure 4.7: HMBC NMR spectrum of compound **C2** in CD_3OD.

Through ^{13}C NMR (Figure 4.6) and 2D HSQC spectra (Figure 4.8), we were able to assign unambiguously the proton/carbon correlations and other unprotonated signals.

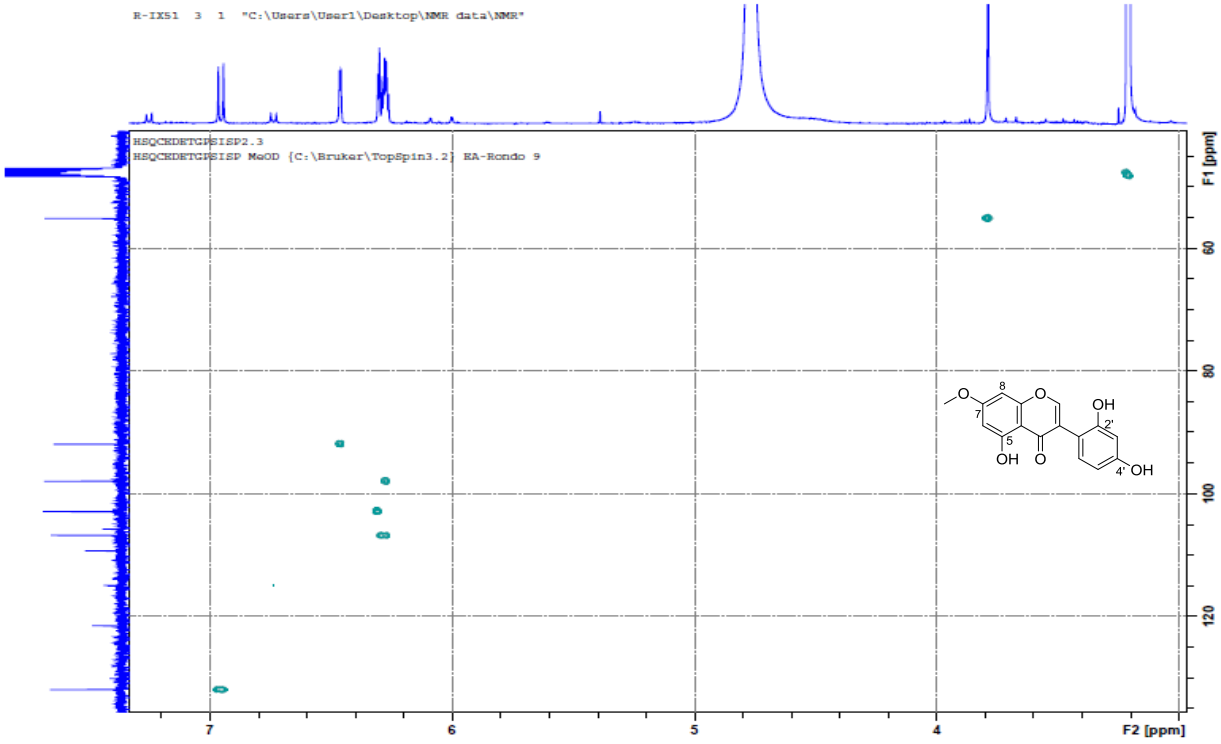


Figure 4.8: HSQC NMR spectrum of compound **C2** in CD_3OD.

In addition, the methoxy group in the 2D NOESY spectrum (Figure 4.9) showed cross peaks with H-6 and H-8. The chemical structure of **C2** was finally confirmed by comparison with the previously reported data (Waffo *et al*., 2000). Compound **C2** was identified as cajanin, a major constituent of one of the closely related species to that of *Rhynchosia*, *Cajanus cajan*.

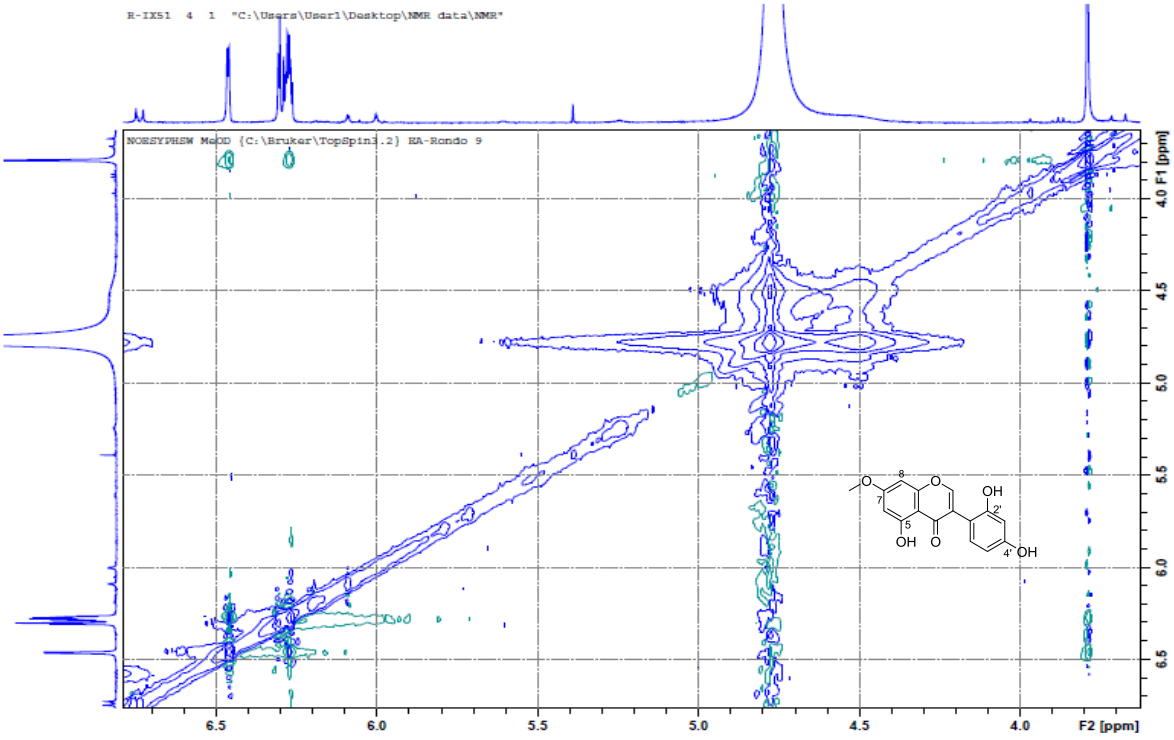
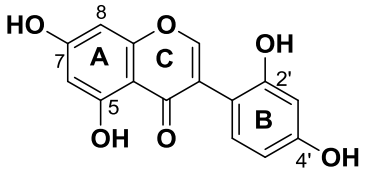


Figure 4.9: NOESY NMR spectrum of compound **C2** in CD_3OD.

Structure elucidation of 2'-hydroxygenistein (**C3**):



2'-hydroxygenistein (**C3**)

Figure 4.10: Chemical structure of 2'-hydroxygenistein (**C3**).

Compound X_1 (4mg; 0.0030 %) was obtained as a pale-yellow powder, and its spectral data was found to be very similar to that of **C2**, except the methoxy signals were absent. The structure elucidation of **C3** (Figure 4.10) was carried out by comparing its spectral data with the existing, documented data (Awouafack *et al*., 2011; Lo *et al*., 2002). The ^{1}H-NMR spectrum (Figure 4.11; Table 4.1) showed the two deshielded protons residing at δ_H 6. 14 [(H-6, *d*) 2.0 Hz], and 6.27 [(H-8, *d*) 2.0 Hz] to be a *meta* coupled pair that revealed free hydroxyl groups at the C-5 and C-7 positions. While the protons in ring B were observed at δ_H 6.31 [(H-3', *d*) 2.4 Hz], 6.28 [(H-5', *dd*) 2.0 Hz, 8.4 Hz], and 6.97 [(H-6', *d*) 8.4 Hz]. A deshielded singlet that is characteristic of all isoflavones, signifying the olefinic proton in ring C, was observed at δ_H 7.93 (H-2, *s*).

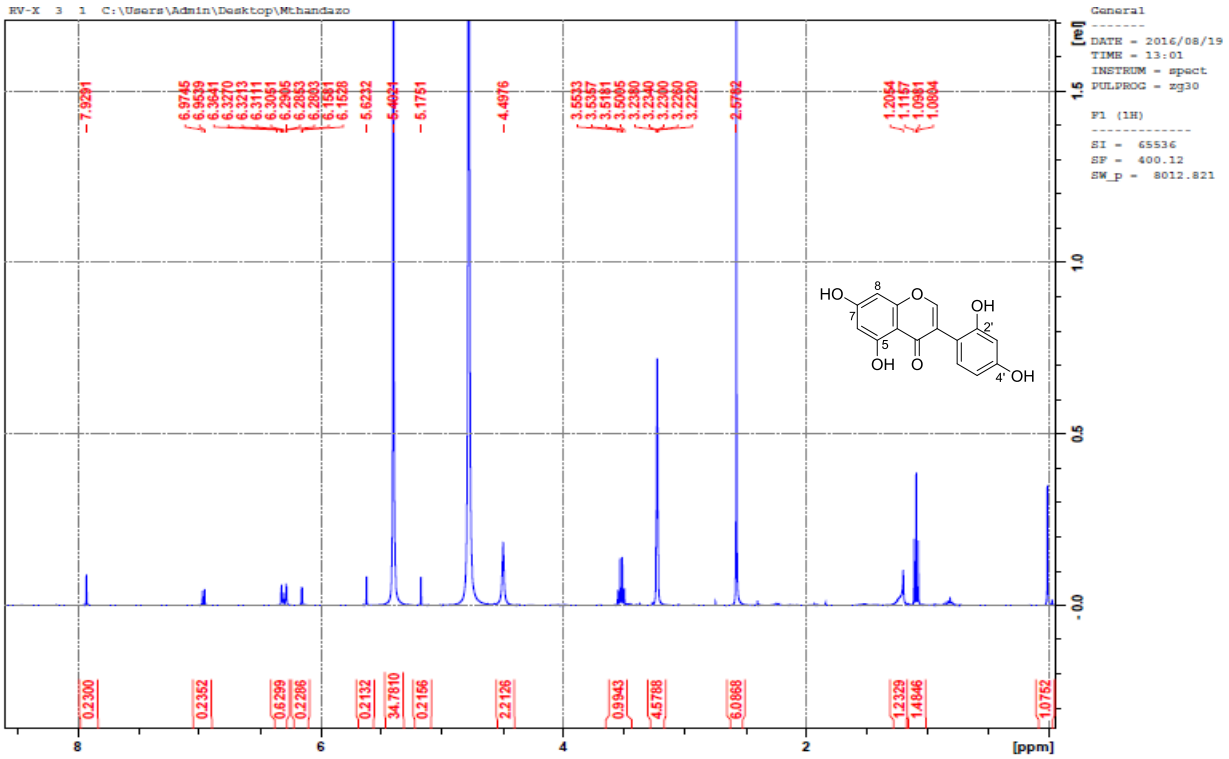


Figure 4.11:[1]H NMR spectrum of compound **C3** in CD_3OD.

The [13]C NMR spectrum (Figure 4.12; Table 4.1) showed twelve aromatic carbons δ_c 163.6 (C-5), 100.2 (C-6), 162.2 (C-7), 94.8 (C-8), 159.7 (C-9), 106.1 (C-10), 110.8 (C-1'), 160.2 (C-2'), 114.8 (C-3'), 157.7 (C-4'), 108.1 (C-5') and 133.1 (C-6') of ring A and B. An olefinic carbon residing at δ_c 156.6 (C-2), quaternary carbon at δ_c 122.5 (C-3) and carbonyl carbon at δ_c 182.5 (C-4, C=O) were observed and attributed to ring C.

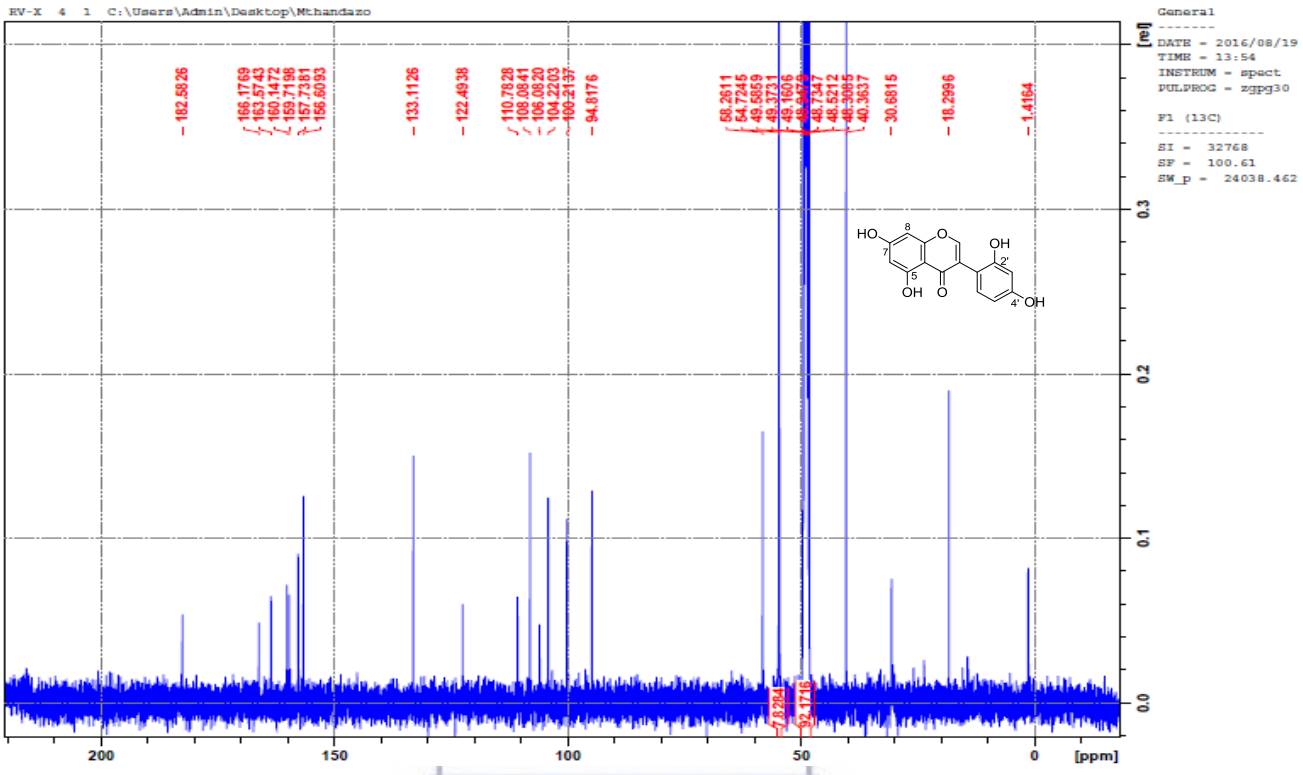


Figure 4.12:[13]C NMR spectrum of compound **C3** inCD_3OD.

Moreover, DEPT-135 spectrum (Figure 4.13) aided in assigning protonated carbons and proton/carbon correlations. From the spectral data of **C3** and through to careful literature review, the compound was thus identified as 2'-hydroxygenistein (Figure 4.10).

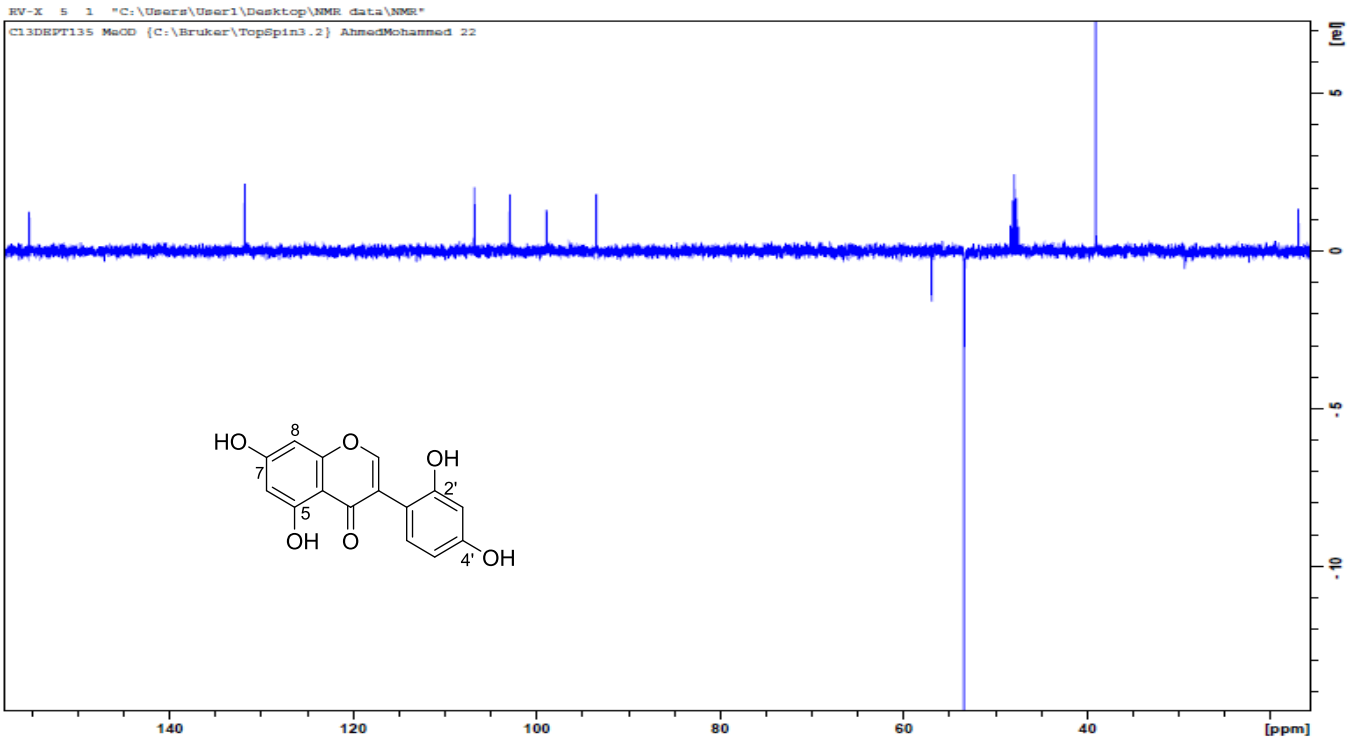
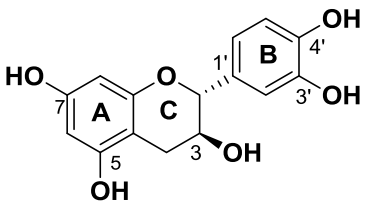


Figure 4.13: DEPT-135 NMR spectrum of compound **C3** in CD_3OD.

4.2 STRUCTURE ELUCIDATION OF THE ISOLATED FLAVAN-3-OLS

Structure elucidation of catechin (**C4**):



Catechin (**C4**)

Figure 4.14: Chemical structure of catechin (**C4**).

Compound XIV_4 (5.9 mg; 0.0045 %) was obtained as a dark yellow amorphous powder, and it was identified by comparing the obtained spectral data with the previously reported data (Hye *et al.*, 2009). The ^{1}H-NMR spectrum (Figure 4.15; Table 4.1) showed peaks at δ_H 4.51 [H-2, *d*, *J*(H-2α, H-3*α*) 6.1 Hz]; 3.91 (H-3, *m*); 2.79 [H-4*α*, *dd*, *J*(H-4*α*, H-3*α*) 8 Hz, *J*(H-4α, H-4β) 16 Hz]; 2.44 [H-4*β*, *dd*, *J*(H-4*β*, H-3*α*) 5.6 Hz, *J*(H-4*β*, H-4*α*) 16 Hz]; 5.78 [H-6, *d*, *J*(H-6, H-8) 2.4 Hz]; 5.84 [H-8, *d*, *J*(H-8, H-6) 2.4 Hz]; 6.75 [H-2', *d*, *J*(H-2', H-6') 1.8 Hz]; 6.69 [H-5', *d*, *J*(H-5', H-6') 8 Hz] and 6.64 [H-6', *dd*, *J*(H-6', H-2') 1.8 Hz, *J*(H-6', H-5') 8 Hz]. A strong coupling constant (*J*) between δ_H 4.51 (H-2α) and 3.91 (H-3α) of 6.1 Hz was observed, supporting a *trans*-configuration between these two protons (Foo, 1987).

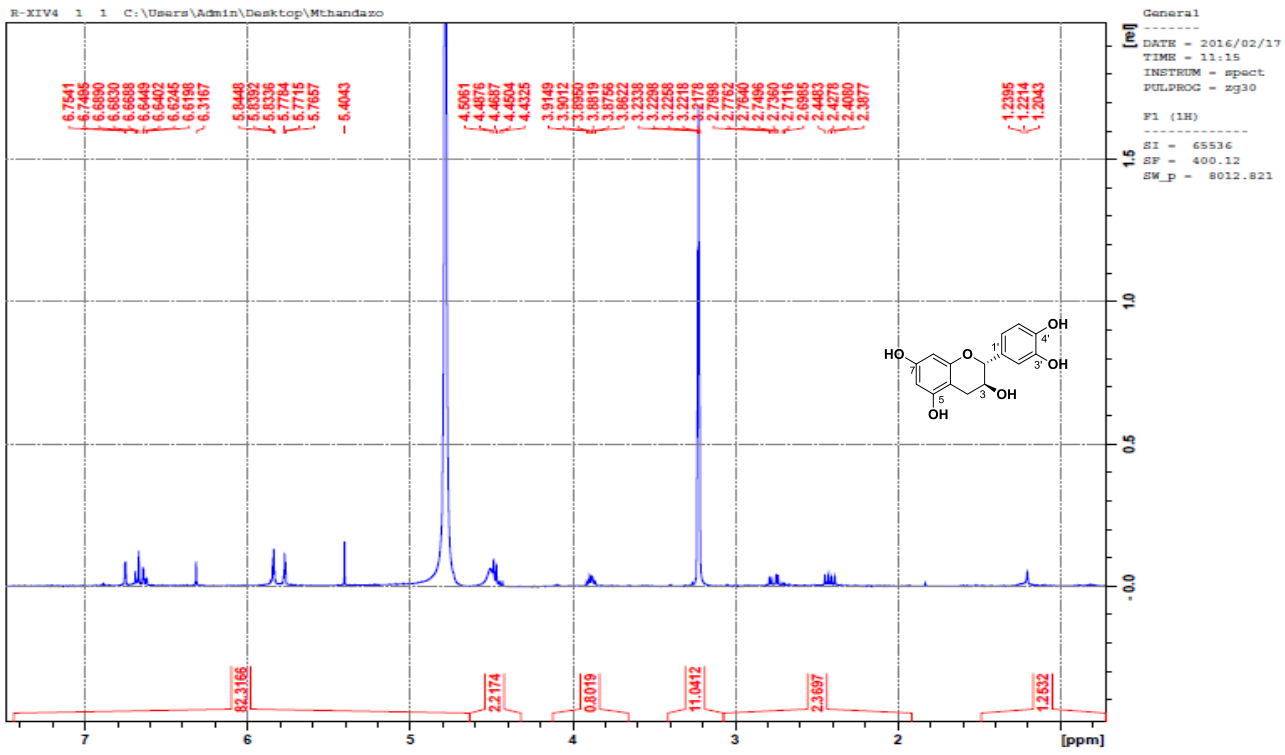


Figure 4.15:[1]H NMR spectrum of compound **C4** in CD_3OD.

The [13]C NMR spectrum (Figure 4.16; Table 4.1) of **C4** showed carbon signals at δ_C 27.1 (C-4), 67.4 (C-3), 81.5 (C-2), 94.1 (C-6), 94.9 (C-8), 99.4 (C-10), 113.9 (C-2'), 114.7 (C-5'), 118.6 (C-6'), 130.8 (C-1'), 144.8 (C-3'), 144.9 (C-4'), 155.5 (C-5), 156.2 (C-9) and 156.4 (C-7). The above data showed that **C4** consists of a flavan-3-ol backbone, through a thorough literature review; it was identified as catechin, a common procyanidin monomer typically isolated from green tea (Jeong and Kong, 2004).

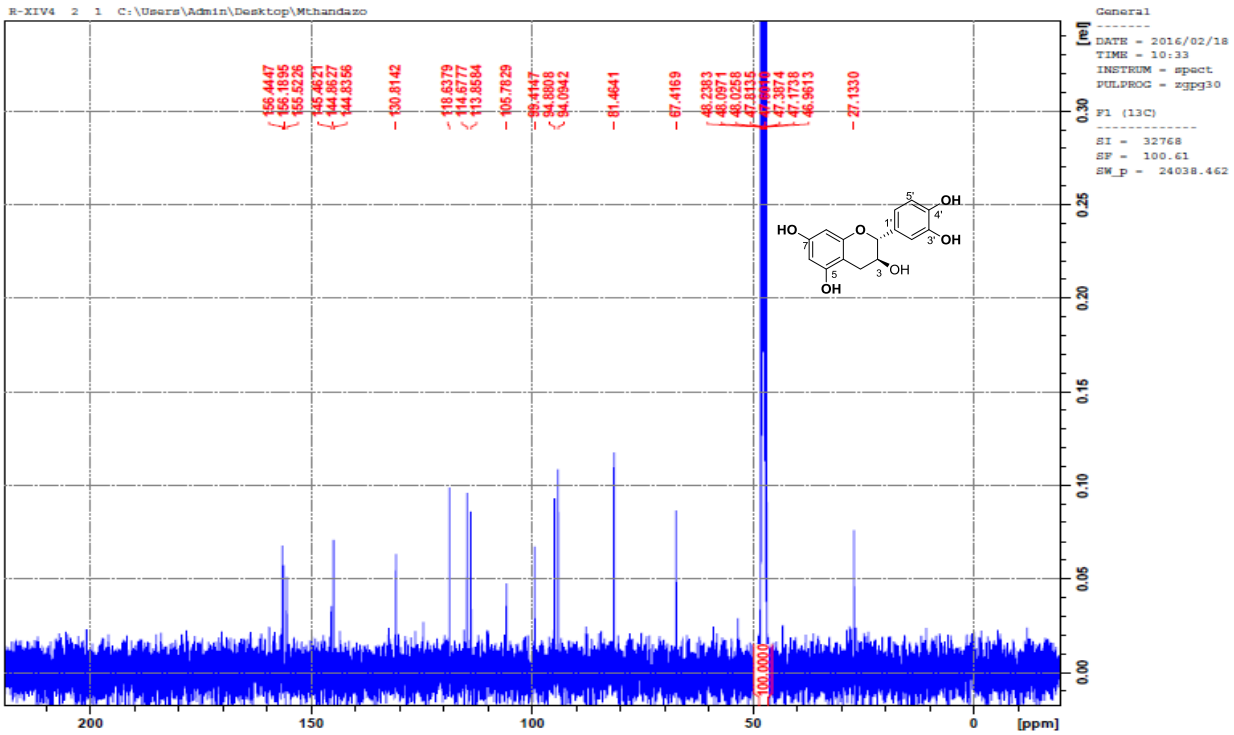
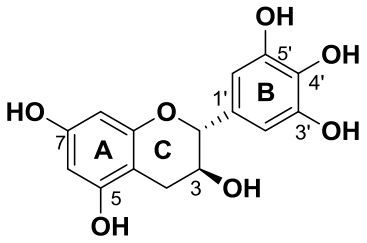


Figure 4.16:[13]C NMR spectrum of compound **C4** in CD_3OD.

Structure elucidation of gallocatechin (**C5**):



Gallocatechin (**C5**)

Figure 4.17: Chemical structure of gallocatechin(**C5**).

Conversely, compound XIV_6 (287.4 mg; 0.22 %) was obtained as a brownish-red powder, and it demonstrated very similar NMR spectra to that of **C4**, with only slight differences. The ^{1}H-NMR spectrum (Figure 4.18; Table 4.1) showed proton signals at δ_H 4.48 [H-2, *d*, *J*(H-2α, H-3*β*) 7.2 Hz]; 3.93 (H-3, *m*); 2.46 [H-4*α*, *dd*, *J*(H-4*α*, H-3*α*) 7.8 Hz, *J*(H-4*α*, H-4*β*) 16.2 Hz]; 2.75 [H-4*β*, *dd*, *J*(H-4*β*, H-3*α*) 5.2 Hz, *J*(H-4β, H-4β) 16.2 Hz]; 5.86 [H-6, *d*, *J*(H-6, H-8) 2.0 Hz]; 5.84 [H-8, *d*, *J*(H-8, H-6) 2.0 Hz] and 6.33 (H-2' and H-6', *s*). The intense signal at δ_H 6.33 signifies the presence of two equivalent aromatic protons at the C-2' and C-6' positions in ring B. This peak was found to be absent in the case of **C4**. Likewise as in the case of **C4**, a strong coupling constant of 7.2 Hz was observed between δ_H 4.48 (H-2α) and 3.93 (H-3α), signifying a *trans*-configuration between the two protons. In the case of both **C4** and **C5** epimers, the coupling constant between these signals would be very weak (Foo, 1987).

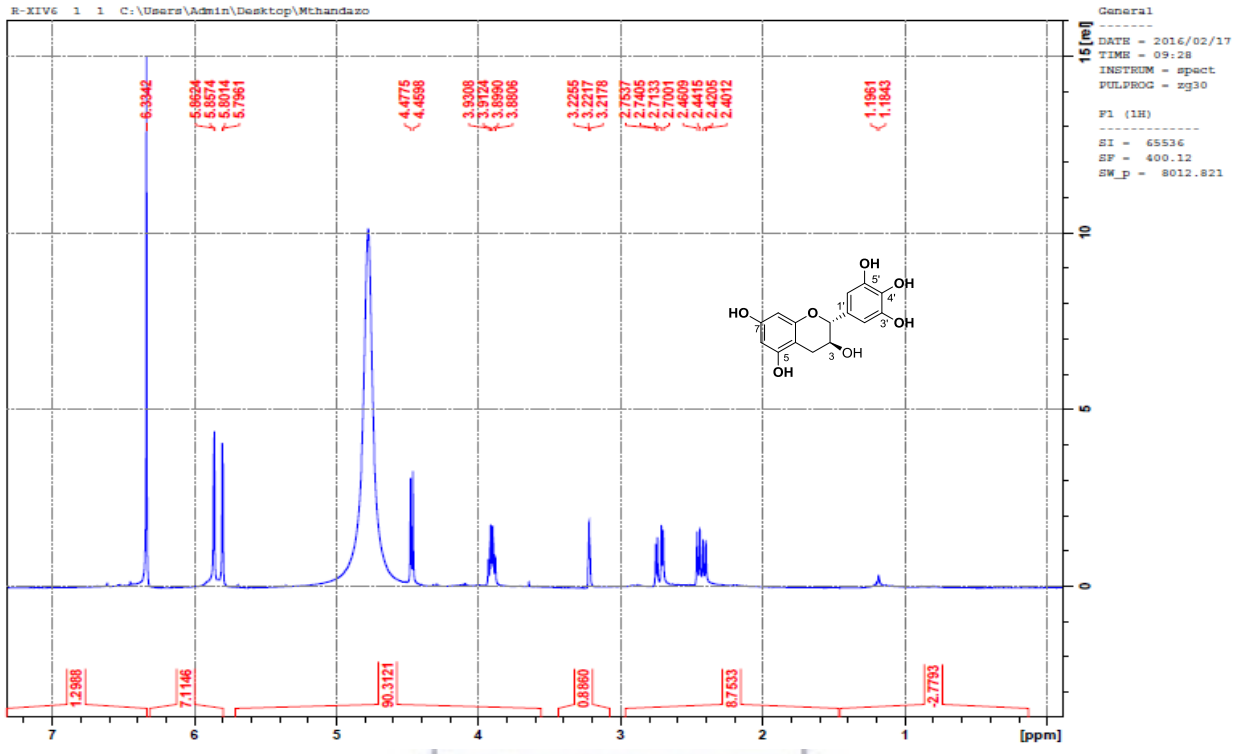


Figure 4.18:[1]H NMR spectrum of compound **C5** in CD_3OD.

The [13]C NMR spectrum (Figure 4.19; Table 4.1) revealed carbon signals at δ_C 26.6 (C-4), 67.3 (C-3), 81.4 (C-2), 94.2 (C-6), 95.0 (C-8), 99.4 (C-10), 105.9 (C-2' and C-6'), 130.2 (C-1'), 132.6 (C-4'), 145.4 (C-3' and C-5'), 155.4 (C-5), 156.2 (C-9) and 156.3 (C-7). The high intensity signals at δ_C 105.9 and 145.4, reveals the presence of two pairs of equivalent carbons i.e., C-2' with C-6', and C-3' with C-5', respectively. Another distinction for the spectra of **C5**, was a carbon signal residing at δ_C 132.6 signifying that C-4' was highly shielded in comparison to the **C4** spectra, where the C-4' signal was found at δ_C 144.9. This is likely due to the additional electron density provided by the hydroxyl group at the C-5' position.

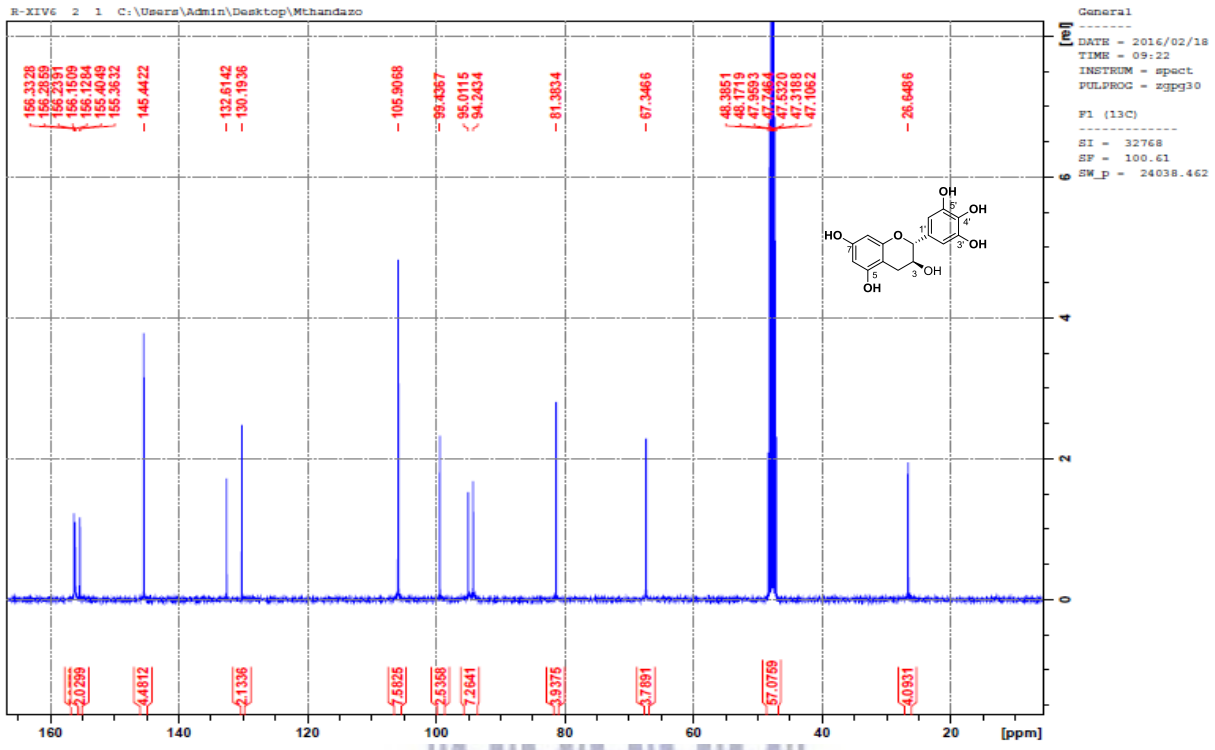
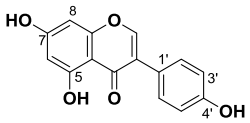
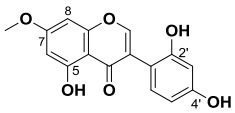
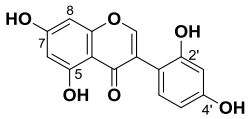
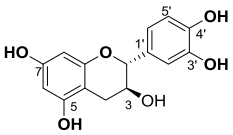
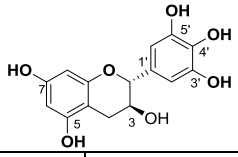


Figure 4.19:[13]C NMR spectrum of compound **C5** in CD_3OD.

Moreover, the unusual dark brown colour of this pure compound (**C5**) is thought to be due to the presence of pyrogallol moiety (ring B) (Kumada and Kato, 1970). Such compounds are typically a white solid, but its samples and derivative samples (e.g., **C5**) are typically brownish because of its sensitivity toward oxygen (Fiege *et al.*, 2014). The chemical structure of **C5** was finally confirmed by comparison with the previously reported data (Medimagh *et al.*, 2010) and identified as gallocatechin (Figure 4.17).

Table 4.1: ^{1}H [400 MHz: *m*, *J*(Hz)] and ^{13}C (100 MHz) NMR spectral data of isolated compounds (**C1 - C5**) in CD_3OD

Code & No.	IX_3 (**C1**)		IX_5 (**C2**)		X_1 (**C3**)		XIV_4 (**C4**)		XIV_6 (**C5**)	
Chemical structure										
	^{13}C	^{1}H, *m*, *J*	^{13}C	^{1}H, *m*, *J*	^{13}C	^{1}H, *m*, *J*	^{13}C	^{1}H, *m*, *J*	^{13}C	^{1}H, *m*, *J*
2	152.9	7.86, *s*	155.6	7.91, *s*	156.6	7.93, *s*	81.5	4.51, *d*, 6.1	81.4	4.48, *d*, 7.2
3	123.0		121.4		122.5		67.4	3.91, *m*	67.3	3.93, *m*
4	180.3		181.4		182.5		27.1	H-4α: 2.44, *dd*, 8.0, 16.0 H-4β: 2.79, *dd*, 5.6, 16.0	26.6	H-4α: 2.46, *dd*, 7.8, 16.2 H-4β: 2.75, *dd*, 5.2, 16.2
5	162.2		162.1		163.6		155.5		155.4	
6	100.2	6.02, *d*, 2.0	97.9	6.26, *d*, 2..2	100.2	6.14, *d*, 2.0	94.1	5.77, *d*, 2.4	94.2	5.80, *d*, 2.0
7	169.1		165.9		166.2		156.4		156.3	
8	94.8	6.12, *d*, 2.0	91.8	6.46, *d*, 2.2	94.8	6.27, *d*, 2.0	94.9	5.84, *d*, 2.4	95.0	5.86, *d*, 2.0
9	157.3		158.3		159.7		156.2		156.2	
10	103.6		105.6		106.1		99.4		99.4	
1'	122.3		109.2		110.8		130.8		130.2	
2'	130.0	7.23, *d*, 8.8	156.4		157.7		113.9	6.75, *d*, 1.8	105.9	6.33, *s*
3'	114.8	6.72, *d*, 8.8	102.8	6.30, *d*, 2.4	104.2	6.31, *d*, 2.4	144.8		145.4	
4'	158.6		158.9		160.2		144.9		132.6	
5'	114.8	6.74, *d*, 8.8	106.5	6.28, *dd*, 2.4, 8.4	108.1	6.28, *dd*, 2.4, 8.4	114.7	6.69, *d*, 8.0	145.4	
6'	130.0	7.25, *d*, 8.8	131.8	6.96, *d*, 8.4	133.1	6.97, *d*, 8.4	118.6	6.64, *dd*, 1.8, 8.0	105.9	6.33, *s*
OMe			55.1	3.81, *s*						

4.3 ANTI-TYROSINASE ACTIVITIES OF THE ISOLATED COMPOUNDS

Tyrosinase inhibitors play an important role in healing and preventing pigmentation disorders in relation to hyperpigmentation, as they inhibit the overproduction of the tyrosinase enzyme, consequently leading to the adequate regulation of the production of melanin by the melanocytes. The effect of browning of food, due to the tyrosinase enzymatic effects has already been discussed, as within the broader aspects of the present study. Thus, the tyrosinase inhibitors are also required to inhibit and prevent the adverse effects of browning during food storage, processing and consumption, so as to maintain the commercial value or quality of such products. It has been shown that the free 1,3-dihydroxy system (ring A) in the isoflavone structure strongly affects both the inhibitory strength and the inhibitory mode of the isoflavones to mushroom tyrosinase (Chang, 2014). Therefore, the present study warrants the study of the anti-tyrosinase activity, as all the isolated compounds in this study consisted of a free 1,3-dihydroxy system and were all indeed found to be active against the tyrosinase enzyme. The IC_{50} values ranged between 31.5 and 69.5 µM (Figure 4.20; Table 4.2), showing the anti-tyrosinase effectiveness of the isolated compounds to be in the following order; **C1** >**C4** >**C2** >**C5** > **C3**.

It was extrapolated that substitution of hydroxy group by a methoxy group has a significant effect on the tyrosinase activity as shown by cajanin (**C2**), which is an *O*-methylated derivative of 2'-hydroxygenistein (**C3**), as it revealed a higher activity than its hydroxylated analogue. Therefore, our study correlates the existing data, which has proven that methoxylation play a vital role in inhibition strength of the certain methoxylated compound to the mushroom tyrosinase activity (Kim *et al.,* 2002). The tyrosinase inhibitory assay was performed as described in Section 3.1.6. Tyrosinase inhibitory effects of the isolated constituents from *R. villosa* are shown in Table 4.2 and portrayed in Figure 4.20.

Table 4.2: Tyrosinase inhibition of *R. villosa* constituents

Compound code & No.	Name of the isolated compound	Average percentages of tyrosinase inhibition (%) in various concentrations (µg/mL) and anti-tyrosinase IC_{50} values (µg/mL &µM) of the isolated compounds						
		100.0 µg/mL	**50.0 µg/mL**	**25.0 µg/mL**	**12.5 µg/mL**	**6.25 µg/mL**	**IC_{50} (µg/mL)**	**IC_{50} (µM)**
IX_3 (**C1**)	Genistein	69.47	62.56	59.40	42.99	36.52	**8.5**	**31.45**
IX_5 (**C2**)	Cajanin	66.27	59.46	50.31	42.12	36.95	**11.7**	**38.97**
X_1 (**C3**)	2'-hydroxygenistein	64.38	47.80	42.62	32.09	19.67	**19.9**	**69.49**
XIV_4 (**C4**)	Catechin	75.83	68.10	51.81	41.24	28.77	**10.7**	**36.86**
XIV_6 (**C5**)	Gallocatechin	72.46	52.30	37.52	21.03	19.09	**18.5**	**60.40**
Ko	Kojic acid	98.48	97.74	94.15	89.32	87.02	**0.9**	**6.33**

***Ko**: Kojic acid was used as a positive control

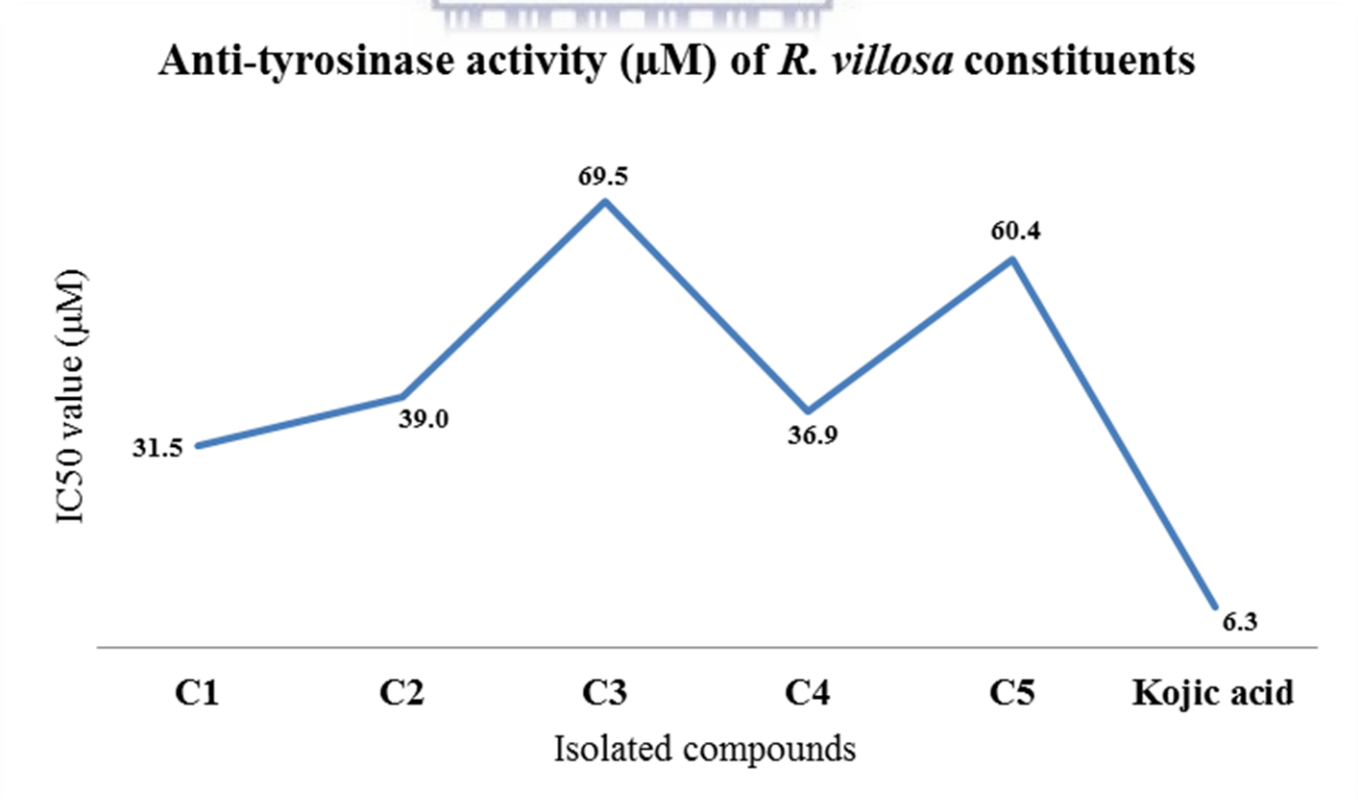


Figure 4.20: Effects of *R. villosa* constituents on inhibition of tyrosinase activity.

4.4 DISCUSSION ON THE ANTI-TYROSINASE ACTIVITIES OF THE ISOLATED COMPOUNDS

The tyrosinase enzyme assay and its qualitative method (TLC bioautography), were used for the bioactivity-directed fractionation and isolation of the five active constituents in the present study. Through TLC bioautography (Figure 3.7) it was observed that main fractions IX – XV were the most active main fractions as they depicted clear white zones of tyrosinase inhibition. The isolated constituents were also bio-evaluated against the tyrosinase enzyme, where compound **C1** showed the highest tyrosinase inhibitory activity out of the isolated compounds. Generally, all the isolated compounds showed high activity against the mushroom tyrosinase, with IC_{50} value differences fluctuating from 4 – 8.5 fold less than that of kojic acid (positive control).

Furthermore, the Fabaceae species are widely used in pharmaceutical and/or cosmetics, one of such species, which consists of the beneficial effects on skin, through its anti-tyrosinase, antioxidant and anti-inflammatory activities is that of *Glycine max*, well-known as soybean. It has been found that the effects of soybean are due to existence of isoflavones within it (Shamani, 2008). From the results of the present study, a similarity is distinguished between the soybean and *R. villosa*, since *R. villosa* also showed a large number of its constituents to be isoflavones; hence this supports its use as a traditional cosmeceutical.

Genistein (**C1**) (Perkin and Newbury, 1899) is said to be one of the most common isoflovones found in skin care products that are made from soybeans. Such skin care products brighten the skin, decrease redness, boost collagen production and improve skin tone (Jaliman, 2011). Unlike many synthetic substances that are applied on the skin, topically applied soybean does not exert any hormonal effects (Schmid *et al*., 2008). Our findings confirm the existing data, which demonstrated that genistein (**C1**) is a natural tyrosinase inhibitor, as it was found to inhibit

mushroom tyrosinase activity with an IC_{50} value of 31.5 µM, only 4-fold less than that of kojic acid, an accepted standard tyrosinase inhibitor (6.3 µM).

Scientific findings attest to the fact that isoflavones consists of estrogenic hormonal activity, since they have a similar structure with that of the human female hormone 17-β-estradiol. They can bind to both α and β estrogen receptors and they can imitate the action of the endogenous estrogens on target organs, thereby exerting many health benefits when used in some hormone-dependent diseases (Vitale *et al*., 2013). Hence, post-menopausal women, whose estrogen production has dropped dramatically, may see obvious results when using soybean-containing moisturizing lotions, since the soybean natural phytoestrogens work to repair the thinness of the skin and the decreased collagen production caused by the lack of estrogen (Schmid *et al*., 2001). This confirms the use of soybean and *R. villosa* as a form of natural relief of estrogenic symptoms and/or other hormone-dependent diseases. Also, the effectiveness of soybean isoflavones in healing dermatological and hormone-dependant disorders validates the fact that the two plant species (*Glycine max* and *R. villosa*) can be used interchangeably, as they have similar biological and pharmacological activities.

Genistein (**C1**) consists of derivatives such as the well-known *ortho*-hydroxygenisteins (OHGs) (Chang, 2014; Chang, 2009). These derivatives are typically isolated from fermented soybean foods, microbial fermentation broth feeding with soybean meal or produced by genetically modified microorganisms and they are rarely found in plant species (Chang, 2014). The *ortho*-hydroxylated derivatives of genistein (**C1**) differ in structure-activity relationships, thus, the number and positions of hydroxyl groups in the chemical structures of such isoflavones intensely affect their function (Scotti *et al*., 2012). For instance, in the current experiment, 2'-hydroxygenistein (**C3**) (Ingham, 1977), which is one of the OHGs, showed a lower activity when

compared to other isolated constituents, with an IC_{50} value of 69.5 µM, although it comprises of a free 1,3-dihydroxy system (ring A), together with a resorcinol moiety (ring B). Its analogue, 3'-hydroxygenistein, however, has been reported to consist of an anti-tyrosinase IC_{50} value of 15.9 µM, merely 1.5 fold less than that of the control, kojic acid (Ding *et al*., 2015).

Cajanin (**C2**) (Bilyard *et al*., 1984) is the major constituent of *Cajanus cajan*, a species from *Cajanus* genus; *Cajanus* is one of the *Cajaninae* genera and it is very closely related to *Rhynchosia* and other two genera from this subtribe, *Atylosia* and *Dunbaria* (Pundir and Singh, 1985; Pundir and Singh, 1986). The species *Cajanus cajan* has been found to have potent antioxidant, antiplasmodial, anti-inflammatory and hypocholesterolemic activities (Rerk-am *et al*., 2013, Pal *et al*., 2011). Rerk-am *et al*. (2013) revealed that although *C. cajan* seeds had a slight anti-tyrosinase activity with EC_{50} value of 267 µg/mL against the mushroom tyrosinase, it quenched DPPH radicals with EC_{50} value of 44.36 µg/mL, while it demonstrated substantial activity against the inhibition of the Fe^{2+}-induced microsomal lipid peroxidation with EC_{50} of 25.97 µg/mL. The latter data substantiates the high potential of *C. cajan* constituents and it also proves that such constituents can be beneficial in healing hyperpigmentation related disorders, particularly acne vulgaris due to the plant species' high inhibition of the Fe^{2+}-induced microsomal lipid peroxidation (Bowe and Logan, 2010). The current study validated this postulation as the cajanin (**C2**) isolated from *R. villosa* was revealed to inhibit the mushroom tyrosinase with a substantial IC_{50} value of 39.0 µM.

On the other hand, the present study totally disagrees with that of Meyer (1960), which revealed that catechin (**C4**) (Roux and Maihs, 1958) has a tanning property with respect to the human skin, and suggested that it may be a useful active ingredient for the treatment of hypopigmentation, leukoderma. Our study revealed catechin (**C4**) to have a high anti-

melonogenesis effect with an IC_{50} value of 36.9 µM against the mushroom tyrosinase, proving that it may perform adequately as a depigmenting agent. Our results are substantiated by that of Sato and Toriyama (2009), as they found that catechin (**C4**) inhibited melanin synthesis in B16 melanoma cells. Furthermore, Western blotting analysis was performed in their study which further indicated characteristics of the catechin (**C4**) inhibition of tyrosinase.

The current study is consistent with the previous data (Ko *et al*., 2011), as they found out that gallocatechin (**C5**) (Saayman and Roux, 1965) has a tyrosinase inhibition percentage of 66.8 %, when screened at 100 µg/mL, and 55.7 % at 50 µg/mL. Therefore, in our study gallocatechin (**C5**) had a comparable tyrosinase inhibition percentage of 72.5 % when screened at 100 µg/mL, and 52.3 % at 50 µg/mL. Its epimer, epigallocatechin was reported to consist of an anti-tyrosinase IC_{50} value of 0.7 mM by Ondonbayar *et al*. (2016), 11-fold less active when compared to that of gallocatechin (**C5**), which was found to be 60.4 µM in the current study. It can be postulated that the difference between catechin (**C4**; 36.9 µM) and gallocatechin (**C5**; 60.4 µM) anti-tyrosinase activities of nearly 23.5 µM (0.55-fold) is due to the presence of pyrogallol moiety (ring B) in gallocatechin (**C5**).

Generally, the above-mentioned types of flavonoids are frequently referred to as green tea polyphenols (GTPs) due to their abundance in green tea made from *Camellia sinensis* leaves (Rahmani *et al*., 2015). Research has revealed that the modes of action of GTPs for the photo protection of the skin include ROS scavenging and regulation of genes to inhibit apoptosis, reduce inflammation, improve the skin barrier, and to repair DNA (Hsu, 2005). Our results are consistent with previous documented data, since both compound **C4** and **C5** were found to be active against the mushroom tyrosinase enzyme. Additionally, all the isolated compounds were

active against mushroom tyrosinase. Hence this supports the traditional uses of *R. villosa* for skin depigmentation.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Preliminary screening of the ten investigated plant species, viz. *Cassine peragua*, *Cassipourea gummiflua*, *Clivia miniata*, *Cryptocarya myrtifolia*, *Gunnera perpensa*, *Kigelia africana*, *Protorhus longifolia*, *Rapanea melanophloeos*, *Rhynchosia villosa*, and *Senecio serratuloides* against the tyrosinase enzyme showed that *R. villosa* was the most active plant species, as its root extract inhibited mushroom tyrosinase activity with 15.67 µg/mL. Therefore, further chromatographic studies were carried out on the plant extract leading to the isolation of 5 known flavonoids, namely, genistein (**C1**), cajanin (**C2**), 2′-hydoxygenistein (**C3**), catechin (**C4**), and gallocatechin (**C5**). Furthermore, the tyrosinase inhibitory activity of the isolated compounds was also tested, and the purified constituents showed the highest activity for **C1** (31.45 µM), followed by **C4** (36.86 µM), **C2** (38.97 µM), **C5** (60.40 µM), and **C3** (69.49 µM).

Although there are a large number of *Rhynchosia* species occurring in S. Africa, research available for their biological and pharmacological importance is insufficient. For instance, there is no documented scientific or ethnomedicinal information that justifies the traditional uses of *R. villosa*. Therefore, to the best of our knowledge and according to the Sci-Finder database, the current investigation is the first scientific report to be carried out on *R. villosa*. In the present study, tyrosinase inhibitory activity demonstrated by *R. villosa* and examination of its constituents and their possible biological application in cosmetic products as skin lighteners was carried out.

To extrapolate, our research work has substantiated the projected hypotheses of the *R. villosa* potential in healing dermatological anomalies. According to our findings, it is possible that the *R.*

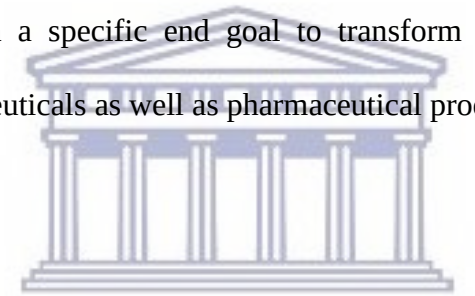
villosa root can bring about a transformation in the manufacturing of skin-care products by the personal care industry. Thus, its tyrosinase inhibitory effects makes it a candidate tyrosinase inhibitor that can be used as depigmenting agent for the treatment or prevention of pigmentation disorders in relation to hyperpigmentation. Moreover, obtained results justify the traditional use of *R. villosa* for skin depigmentation.

5.2 RECOMMENDATTIONS

From the above, it is suggested that:

- ✓ A reasonable amount of raw material of the examined plant species must be used in future, so to be able to obtain the available constituents in the plant of interest in adequate amounts; which in return will allow further relative biological studies on such isolated constituents.
- ✓ Appropriate action must be taken to guarantee that potential active constituents are not lost or destroyed during extraction of the plant to be examined. If the plant was chosen on the basis of traditional uses then it is needed to prepare the extract exactly as described by traditional healer in order to simulate as closely as possible the traditional herbal remedy.
- ✓ Since melanogenesis is a series of biological reactions which proceeds through a free radical mechanism, it could be disrupted by selective use of antioxidants. Hence to test the isolated constituents' antioxidant potential would do justice in confirming their uses in manufacturing of cosmetics.

- ✓ Also, since the isolated compounds are active on the biological activity of interest, a rapid search to verify their structural integrity and binding effect to targets of the tyrosinase activity via a commonly used *in silico* or technique of structure-based virtual screening known as 'docking' may be beneficial.
- ✓ Furthermore, uses of these isolated constituents in beauty care products and pharmaceutical industry will require confirmation of their capability to reduce undesirable skin enzymatic effects in *in vivo* models. Additional investigation, for instance, cytotoxicity determination, reduction of cellular melanin formation and anti-tyrosinase activities in B16F10 melanocytes and dermatological related clinical tests are suggested with a specific end goal to transform the present discoveries into conclusive cosmeceuticals as well as pharmaceutical product formulations.



REFERENCES

Adinarayana, D., and Chetty, P. R. (1985). Chemical investigation of some medicinal plants occurring in southern India. *Indian Journal of Chemestry*, *24B*, 453.

Adinarayana, D., and Ramachandraiah, P. (1984). Methylated flavonols of *Rhynchosia rufescens*. *Pharmazie, 39*, 714-715.

Adinarayana, D., and Ramachandraiah, P. (1986). *C*-glycosides of *Rhynchosia cana*. *Journal of Natural Product, 49(6)*, 1158-1159.

Adinarayana, D., Gunasekar, D., and Ramachandraiah, P. (1979). *C*-glycosides of *Rhynchosia* species. *Current Science*, *48*(*16*), 726.

Adinarayana, D., Gunasekar, D., Ramachandraiah, P., Seligmann, O., and Wagner, H. (1980a). Tirumalin, a new prenylated dihydroflavonol from *Rhynchosia cyanosperma. Phytochemistry, 19(3).* 478-480.

Adinarayana, D., Gunasekar, D., Seligmann, O., and Wagner, H. (1980b). Rhynchosin, a new 5-deoxyflavonol from *Rhynchosia beddomei*. *Phytochemistry, 19*, 483-484.

Adinarayana, D., Gunasekar, D., Seligmann, O., and Wagner, H. (1980c). Flavone *O*- and *C*-glycosides of *Rhynchosia beddomei*. *Phytochemistry, 19(3),* 480-481.

Adinarayana, D., Ramachandaiah, P., and Rao, K. N. (1985). Flavonoid profiles of certain species of *Rhynchosia* of the family Leguminosae (Fabaceae). *Experientia, 41(2)*, 251-252.

Adinarayana, D., Ramachandraiah, P., Seligmann, O., and Wagner, H. (1981). Rhynchospermin. A prenylated flavanol from *Rhynchosia cyanosperma*. *Phytochemistry, 20(8)*, 2058-2059.

Ahmad, N., Shinwari, Z. K., Hussain, J., Ahmad, S., Abbas, G., Zada, M., Ahmed. N., and Iqbal, J. (2011). Antiglycation, platelet aggregation, antioxidant, cytotoxicity and phytotoxic activities of the crude extract/fractions of *Rhynchosia reniformis*. *Journal of Pharmacy Research, 4(10),* 3721-3724.

Ahmed, W., Ahmad, Z., and Malik, A. (1992). Stigmasteryl galactoside from *Rhynchosia minima*. *Phytochemistry, 31(11)*, 4038-4039.

Ajesh, T. P., Krishnaraj, M. V., Prabu, M., and Kumuthakalavalli, R. (2012). Herbal abortifacients used by Mannan Tribes of Kerala, India. *International Journal of PharmTech Research, 4(3)*, 1015-1017.

Allen O. N., and Allen, E. K. (1981). *The Leguminosae*: *A source book of characteristics, uses and nodulation*. Wisconsin: The University of Wisconsin Press.

American Cancer Society (2016, May 5). *Melanoma skin cancer: What is Melanoma skin cancer?* Retrieved from American Cancer Society website: http://www.cancer.org/acs/groups/cid/documents/webcontent/003120-pdf.pdf

Amrisha. (2015, October 10). *Observations: Yellow flower with red veins*. Retrieved from iSpot website: http://www.ispotnature.org/node/737191

Anon. (1999). *Nomination proposal for the Cape Floristic Region. Phase 1. Cape Peninsula protected environment to be listed as a World Heritage Site*. Report prepared by South African National Parks for Department of Environment Affairs and Tourism, South Africa.

Awouafack, M. D., Spiteller, P., Lamshoft, M., Kusari, S., Ivanova, B., Tane, P., and Spiteller, M. (2011). Antimicrobial isopropenyl-dihydrofuranoisoflavones from *Crotalaria lachnophora*. *Journal of Natural Products*, *74*, 274-282.

Bagherani, N., Gianfaldoni, S., and Smoller, B. (2015). An overview on Melasma. *Pigmentary Disorders*, *2*, 216.

Baker, E. G. (1923). Revision of South African species of *Rhynchosia*. *Bothalia*, *1*, 113-138.

Baker, J. B. (1976). *Rhynchosia*. In: Hooker, J.D. (Ed.). *Flora of British India volume II*. London: L Reeve and Company, 5, Henrietta Street, Covent Garden.

Bakshu, L. M., and Raju, R. R. V. (2001). Antimicrobial activity of *Rhynchosia boddemei*. *Fitoterapia*, *72*, 579-582.

Balandrin, M. F., Klocke, J. A., Wurtele, E. S., and Bollinger, W. H. (1985). Natural plant chemicals: Sources of industrial and medicinal materials. *Science, 228(4704)*, 1154-1160.

Banerjee, S., Sanjay, K. R., Chethan, S., and Malleshi N.G. (2012). Finger millet (*Eleusine coracana*) polyphenols: Investigation of their antioxidant capacity and antimicrobial activity. *African Journalof Food Science*, *6*, 362–374.

Barnes, S., and Kim, H. (2010). Cautions and research needs identified at the Equol, Soy, and Menopause Research Leadership conference. *Journal of Nutrition, 140(7),* 1390-1394.

Battu, G., Male, C. K. V. L. S. N., Haripriya, T., Malleswari, V. N., and Reeshma, S. (2011). A phyto-pharmacological review on vigna species. *Pharmanest*, *2*, 62-67.

Besson, E., Chopin, J., Krishnaswami, L., and Krishnamurty, H. G. (1977). C-glycosylflavones from *Rhynchosia minima*. *Phytochemistry, 16(4)*, 498.

Bilyard, K. G., Cooksey, C. J., Dahiya, J. S., Garratt, P. J., and Strange, R. N. (1984). Two isoprenylated isoflavone phytoalexins from *Cajanuscajan*. *Phytochemistry, 23(4)*, 871-873.

Bisby, F. (1994). Phytochemical dictionary of the Leguminosae. UK: Taylor Francis Ltd.

Bond, P., and Goldblatt, P. (1984). Plants of the Cape Flora - A descriptive Catalogue. *Journal of South African Botany, Supplement 13*, 1-455.

Bowe, W. P., and Logan, A. C. (2010). Clinical implications of lipid peroxidation in acne vulgaris: Old wine in new bottles. *Lipids in Health and Disease, 9,* 141.

Braverman, I. M., and Fonferko, E. (1982). Studies in cutaneous aging: In the elastic fiber network. *The Journal of Investigative Dermatology, 78(5),* 434-443.

Brind'Amour, K. (2016, April 16). *Get the skinny on skin disorders*. Retrived from Healthline website: http://www.healthline.com/health/skin-disorders#Overview1

Brink, M., 2006. *Cassipourea malosana* (Baker) Alston. Retrieved from PROTA4U (Plant Resources of Tropical Africa) website: http://www.prota4u.org/search.asp

Butler, D. F., and Elston, D. M. (Ed.). (2016, March 8). *Drug-induced pigmentation clinical presentation*. Retrieved from Medscape website: http://emedicine.medscape.com/article/1069686-clinical

Butler, R. A. (2016, May 21). *The top 10 most biodiverse countries*. Retrieved from Mongabay website: https://news.mongabay.com/2016/05/top-10-biodiverse-countries/

Carli, P., andSalvini, C. (2009). Melanocyte nevi. In: Williams, H. (Ed.). *Evidence-based dermatology*, 2nd Edition. USA: John Wiley and Sons, Incorporated.

Cayce, K. A., McMichael, A. J., and Feldman, S. R. (2004). Hyperpigmentation: An overview of the common afflictions. *Dermatology Nursing, 16(5),* 401-406.

Challa, S., Rajam, K. K., Kasapu, V. V. V. S., Tanneeru, S. K., and Kantamreddi, V. S. S. (2011). Antibacterial properties of an Indian traditional medicinal plant, *Rhynchosia scarabaeoides* (L.) DC. (Fabaceae). *Alternative Medicine Studies, 1(17),* 70-72.

Chandran, R., Feller, L., Lemmer, J., and Khammissa R. A. G. (2016). HIV-Associated oral mucosal melanin hyperpigmentation: A clinical study in a South African population sample. *AIDS Research and Treatment, 2016,* 1-5.

Chandran, R., Lemmer, J., Khammissa R. A. G., and Feller, L. (2014). HIV-associated oral melanin hyperpigmentation. *Journal of the Dental Association of South Africa, 69(8),* 370-371.

Chang, T. S. (2009). An updated review of tyrosinase inhibitors. *International Journal of Molecular Sciences, 10,* 2440-2475.

Chang, T. S. (2014). Isolation, bioactivity, and production of *ortho*-hydroxydaidzein and *ortho*-hydroxygenistein. *International Journal of Molecular Science, 15,* 5699-5716.

Charters, M., and Madre, S. (2015). *Flora of South Africa*. Retrieved from Calflora.net website: http://www.calflora.net/index.html

Cheng, Z., and Wu, T. (2013). TLC bioautography: High throughput technique for screening of bioactive natural products. *Combinatorial Chemistry and High Throughput Screening, 16(7),* 531-549.

Chimponda, T., and Mukanganyama, S. (2010). Antimycobacterial activities of selected medicinal plants from Zimbabwe against mycobacterium aurum and corynebacterium glutamicum. *Tropical Biomedicine, 27(3),* 595-610.

Chugh, C. A., Mehta S., and Dua, H. (2012). Phytochemical screening and evaluation of biological activities of some medicinal plants of Phagwara, Punjab. *Asian Journal of Chemistry, 24(12),* 5903-5905.

Christenhusz, M. J. M., and Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa, 261(3),* 201-217.

Claridge, T. (1999). *High-resolution NMR techniques in organic chemistry*, 1st Edition. Oxford, England: Elsevier.

Colegate, S. M., and Molyneux, R. J. (2008). *Bioactive natural products: Detection, isolation, and structure determination*. Boca Rotan, FL, USA: CRC Press.

Commo, S., Gaillard, O., and Bernard, B. A. (2004). Human hair greying linked to a specific depletion of hair follicle melanocytes affecting both the bulb and the outer root sheath. *British Journal of Dermatology, 150,* 435-443.

Coronado-Aceves, E. W., Sanchez-Escalante, J. J., Lopez-Cervantes, J. Robles-Zepeda, R. E., Velazquez, C., Sanchez-Machado D. I., and Garibay-Escobar, A. (2016). Antimycobacterial activity of medicinal plants used by the Mayo people of Sonora, Mexico. *Journal of Ethnopharmacology, 190,* 106-115.

Court, F. L., and Williams G. (2015). The occurrence, fate, and biological activities *C*-gylcosyl flavonoids in the human diet. *Critical Reviews in Food Science and Nutrition, 55(10),* 1352-1367.

Cowling, R. M., Hilton-Taylor, C., and Huntley, B.J. (Ed.). (1994). *Patterns of plant diversity and endemism in Southern Africa: An overview*. Pretoria, South Africa: Botanical Diversity in Southern Africa, National Botanical Institute.

Crawford, A., Bohni, N., Maes, J., Kamuhabwa, A., Moshi, M., Esguerra, C., de Witte, P., and Wolfender, J. (2010). Zebrafish bioassay-guided microfractionation combined with cap NMR a comprehensive approach for the identification of anti-inflammatory and anti-angiogenic constituents of *Rhynchosia viscosa*. *Planta Medica, 76,* 544.

D'Orazio, J., Jarrett, S., Amaro-Ortiz, A., and Scott, T. (2013). UV radiation and the skin. *International Journal of Moleculer Sciences, 14*, 12222-12248.

Devasagayam, T, Tilak. J. C., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. D. (2004). Free radicals and antioxidants in human health: Current status and future prospects. *Journal of Association of Physicians of India, 52*, 794-804.

De Wet, H., Nciki, S., and Van vuuren, S. (2013). Medicinal plants used for the treatment of various skin disorders by a rural community in northern Maputaland, South Africa. *Journal of Ethnobiology and Ethnomedicine, 9(1),* 51.

Ding, H. Y., Chiangb, C. M., Tzengc, W. M., and Chang, T. S. (2015). Identification of 3'-hydroxygenistein as a potent melanogenesis inhibitor from biotransformation of genistein by recombinant *Pichiapastoris*. *Process Biochemistry, 50*, 1614-1617.

Dorland's illustrated medical dictionary, 29th Edition. (2000). Philadelphia, PA., USA: W.B. Saunders Company.

Doyle J. J., and Doyle, J. L. (1993). Chloroplast DNA phylogeny of the *Papilionoid* legumes tribe *Phaseoleae*. *Systematic Botany, 18*, 309-327.

Draelos, Z. D. (2008). The cosmeceutical realm. *Clinics in Dermatology, 26(6)*, 627-632.

Drakaki, E., Dessinioti, C., and Antoniou C. V. (2014, May 15). *Air pollution and the skin*. Retrieved from Frontier in Environmental Science website: http://journal.frontiersin.org/article/10.3389/fenvs.2014.00011/full

Drewes, S. E., Selepe M. A., Van Heerden, F. R., Archer R. H., and Mitchell, D. (2013). Unravelling the names, origins and chemistry of "muthis" used for male sexual disorders in KwaZulu-Natal, South Africa. *South African Journal of Botany, 88*, 310-316.

Dushimemaria, F., Mumbengegwi, D. R., and Bock, R. (2015). Indigenous knowledge of medicinal plants used for the treatment of cancer, chapter 3. In: Chinsembu, K. C.,Cheikhyoussef, A., Mumbengengwi, D., Kandawa-Schulz, M., Kasanda, C. D., and Kazembe, L. (Eds.). *Indigenous knowledge of Namibia.* Namibia: University of Namibia Press.

Elsner, P., and Maibach, H. I. (Eds.). (2000). *Cosmeceuticals*: *Drugs vs. cosmetics*. New York, USA: Marcel Dekker.

Environmental Working Group (2016). *EWG's 2016 sunscreen guide: A decade of progress, but safety and marketing concerns remain*. Retrieved from EWG website: https://www.ewg.org/sunscreen/report/executive-summary/

Eswaraiah, M. C., Elumalai, A., Roa, N., Parasanthi, P., and Kumar, C. P. (2013). Diuretic activity of *Rhynchosia beddomei* leaves extract in rats. *International Journal of Pharmacy and Pharmaceutical Sciences, 5(3),* 273-274.

Ferrer, A., Hidalgo, C., Kaps, R., and Kougoulis, J. S. (2012). *Revision of European ecolabel criteria for soaps, shampoos and hair conditioners*. Markert analysis for the joint research centre of the European Union and institute for prospective technological studies.

Fiege, H., Heinz-Werner, V., Hamamoto, T., Umemura, S., Iwata, T., Miki, H., Fujita, Y., Buysch, H. J., and Garbe, D., Paulus, W. (2014). *Ullmann's Encyclopedia of Industrial Chemistry* (7th Ed.). Weinheim, Germany: Wiley-VCH.

Foden, W., and Potter, L. (2005, June 30). *Rhynchosia villosa* (Meisn.) Druce. National assessment: Red list of South African plants. Retriveved from Red List of South African Plants website: http://redlist.sanbi.org/species.php?species=370-90

Foo, L. Y. (1987). Phenylpropanoid derivatives of catechin, epicatechin, and phylloflavan from phyllocladus trichomanoides. *Phytochemistry, 26(10),* 2825-2830.

Fraenkel, G. S. (1959). The raison d'Être of secondary plant substances; these odd chemicals arose as a means of protecting plants from insects and now guide insects to food. *Science, 129(3361),* 1466-1470.

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

Fry, C. (2010). *The World of Kew*. London: Royal Botanic Gardens at Kew.

Gauri, R. D., and Sharma, J. (2011). Indigenous knowledge on the utilization of medicinal plant diversity in the Siwalik Region of Garhwali Himalaya, Uttarakhand. *Journal of Forest Science, 47(1),* 23-31.

Gattuso, D. J. (2011). True story of cosmetics. *Competitive enterprise institute, issue analysis 2011(6),* 1-29.

George, P. (2011). Concerns regarding the safety and toxicity of medicinal plants - An overview. *Journal of Applied Pharmaceutical Science, 1(6),* 40-44.

Germishuizen, G. (Ed.). (2006). *Rhynchosia*. In: Germishuizen, G., Meyer, N. L., Steenkamp, Y., and Keith, M. (Eds.). *A checklist of South African plants.* Pretoria, South Africa: South African Biodiversity Network (SABONET) Report no. 41.

Germishuizen, G., and Meyer, N. L. (Eds.). (2003). *Plants of Sourthern Africa: An Annotated Checklist*. Pretoria, South Africa: South Africa National Botanical Institute.

Goldblatt, P. (1978). An analysis of the flora of Southern Africa: Its characteristics, relationships and origins. *Annals of the Missouri Botanical Gardens, 65,* 369-436.

Goldblatt, P., and Manning, J. C. (Eds.). (2000). *Cape Flora. A Conspectus of the Cape Flora of South Africa. Strelitzia 9*. Cape Town, South Africa: National Botanical Institute, and Missouri Botanic Gardens.

Goufa, P., and Trindade, H. (2014). Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. *Food Science and Nutrition, 2(2),* 75-104.

Gray, J. (2010). *State of the evidence: The connection between breast cancer and the environment*, 6th Edition. San Francisco, California: Breast Cancer Fund.

Ha, J. B. (2006). Effects of *Rhynchosia molubilis* saponin on hepatotoxicity and pathology. *Jornal of Life Science, 16(2),* 186-191.

Haule, E. E., Moshi, M. J., Nondo, R. S., Mwangomo, D. T., and Mahunnah R. L. (2012). A study of antimicrobial activity, acute toxicity and cytoprotective effect of a polyherbal extract in a rat ethanol-HCl gastric ulcer model. *BMC Research Notes, 5,* 546.

Herendeen, P. S., Crepet, W. L., and Dilcher, D. L. (1992). The Fossil History of the Leguminosae: Phylogenetic and biogeographic implications. In: Herendeen P. S., and Dilcher, D. L. (Eds.). *Advances in legume systematics: The fossil record (part 4).* UK: Royal Botanic Gardens, Kew.

Hong, S. H., Sim, M. J., and Kim Y. C. (2016). Melanogenesis-promoting effects of *Rhynchosia nulubilis* and *Rhynchosia volubilis* ethanol extracts in melan-a cells. *Toxicology Research, 32(2),* 141-147.

Hosny, M., and Rosazza, J. P. N. (1999). Microbial hydroxylation and methylation of genistein by *Streptomycetes*. *Journal of Natural Products, 62,* 1609-1612.

Hostettmann, K. (1998). Strategy for the biological and chemical evaluation of plant extracts. *Pure Applied Chemistry, 70,* 1- 9.

Hsu, S. (2005). Green Tea and the Skin.*Journal of the American Academy of Dermatology, 52(6),* 1049-1059.

Hutchings, A., Scott, A. H., Lewis, G., and Cunningham, A. (1996). *Zulu medicinal plants: A inventory*. Pietermaritzburg, South Africa: Natal University Press.

Hye, M. A., Taher, M. A., Ali, M. Y., Ali, M. U., andZaman, S. (2009). Isolation of (+)-catechin from *Acacia catechu* (Cutch tree) by a convenient method. *Journal of Scientific Research, 1(2),* 300-305.

Ingham, J. L. (1977). Phytoalexins of hyacinth bean (*Lablab niger*), *Zeitschrift für Naturforschung C, 32c(11-12),* 1018-1020.

Ingham, J. L. (1990). Systematic aspects of phytoalexin formation within tribe *Phaseoleae* of the Leguminosae (subfamily Papilionoideae). *Biochemical Systematics and Ecology, 18(5),* 329-343.

International Association for Plant Taxonomy (IAPT). (2012). *Nomenclature of taxa according to their rank: Names of families and subfamilies, tribes and subtribes* (*article 18*). Retrieved from BRATISLAVA IAPT website: http://www.iapt-taxon.org/nomen/main.php?page=art18

Jadotte, Y. T., and Schwartz, R. A. (2010). Melasma: Insights and perspectives. *Acta Dermatovenerologica Croatica, 18(2),* 124-129.

Jaliman, D. (2011, November 4). *Healthy skin.the soy effect*. Retrieved from WebMD website: http://blogs.webmd.com/healthy-skin/2011/11/the-soy-effect.html

Jeong, W. S., and Kong, A. N. T. (2004). Biological properties of monomeric and polymeric catechins: Green tea catechins and procyanidins.*Pharmaceutical Biology, 42*, 84-93.

Jordaan, H. F. (2008). Common skin and mucosal disorders in HIV/AIDS. *South African Family Practice, 50(6),* 14-23.

Judd, W. S., Campbell, C. S., Kellogg, E. A., Stevens, P. F., and Donoghue, M. J. (2002). *Plant systematics: A phylogenetic approach*, 2nd Edition. Sunderland, MA: Sinauer Associates.

Just About Skin. (2014, March 27). *Know what kind of spots you have? There are different types of hyperpigmentation*. Retrieved from Just About Skin website: http://www.justaboutskin.com/2014/03/types-of-hyperpigmentation/

Kabera, J. N., Semana, E., Mussa, A. R., and He, X. (2014). Plant secondary metabolites: Biosynthesis, classification, function and pharmacological properties. *Journal of Pharmacy and Pharmacology, 2,* 377-392.

Kapoor, V. P. (2005). Herbal cosmetics for skin and hair care. *National Botanical Research Institute, 4(4),* 306-314.

Kelmanson, J. E., Jäger, A. K., and van Staden, J. (2000). Zulu medicinal plants with antibacterial activity. *Journal of Ethnopharmacology, 69*, 241-246.

Kennedy E. (2015, August 15). *Ten Common skin conditions caused by sun exposure*. Retrieved from Empowher website: http://www.empowher.com/skin-hair-amp-nails/content/10-common-skin-conditions-caused-sun-exposure?page=0,1

Khan, K. A., and Shoeb, A. (1984). Two antibacterial biphenyls from *Rhynchosia suaveolens* (L. f.) DC. *Phytochemistry*, *23*, 765 -766.

Kim, S. J., Shin, J. Y., Cho, M. H., Oh, Y. S., Park, N. Y., and Lee, S. H. (2007). Antioxidant activity and isoflavone profile of *Rhynchosia nolubilis* seeds pickled in vinegar (chokong). *Food Science and Biotechnology, 16(3),* 444-450.

Kim, Y. M., Yun, J., Lee, C. K., Lee, H., Min, K. R., and Kim, Y. (2002). Oxyresveratrol and hydroxystilibene compounds inhibitory effect on tyrosinase and mechanism of action. *The Journal of Biological Science*, *277(18),* 16340-16344.

Kinjo, J., Negao, T., Tanaka, T., Nonaka, G., and Okabe, H. (2001). Antiproliferative constituents in the plant 8.seeds of *Rhynchosia volubilis*. *Biological Pharmacology Bulletin, 24(12),* 1443-1445.

Kirk-Othmer, (2013). *Kirk-Othmer chemical technology of cosmetics*. Hoboken, New Jersey, USA: Wiley.

Knott, L. (2015, April 24). *HIV and skin disorder*. Retrieved from Patient website: http://patient.info/doctor/hiv-and-skin-disorders

Ko, R. K., Kim, G. O., Hyun, C. G., Jung, D. S., and Lee, N. S. (2011). Compounds with tyrosinase inhibition, elastase inhibition and DPPH radical scavenging activities from the branches of *Distyliumracemosum* Sieb.et Zucc. *Phytotherapy Research*, *25(10),* 1451-1456.

Koehler, J. E., Quinn, F. D., Berger, T. G., Le Boit, P. E., and Tappero J. W. (1992). Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. *The New England Journal of Medecine, 327(23),* 1625-1631.

Körner, A. M., and Pawelek, J. (1980). Dopachrome conversion: A possible control point of melanin biosynthesis. *The Journal of Investigative Dermatology, 75(2),* 192-195.

Krishnaiah, D., Sarbatly R., and Bono A. (2007). Phytochemical antioxidants for health and medicine – A move towards nature.*Biotechnology and Molecular Biology Review, 1(4),* 97-104.

Krishnamurty, H. G., Krishnaswamy, L., and Rangaswamy, N. S. (1975). Hydroquinone diacetate from *Rhynchosiaminima*. *Phytochemistry, 14(11),* 2518-2519.

Kubo, I., Kinst-Hori, I., Chaudhuri, S. K., Kubo, Y. (2000). Flavonols from*Heterotheca inuloides*: Tyrosinase inhibitory activity and structural criteria. *Bioorganic and Medicinal Chemistry, 8,* 1749-1755.

Kumada, K., and Kato, H. (1970). Browning of pyrogallol as affected by clay minerals. *Soil Science and Plant Nutrition, 16(5),* 195-200.

Lackey, J. A. (1981). Tribe 10.*Phaseoleae*. In: Polhill, R. M., and Raven P. H (Eds.*). Advances in legume systematics, I.* UK: Royal Botanic Gardens, Kew.

Lai, J., Xin, C., Zhao, Y., Feng, B., He, C., Dong, Y., Fang, Y., and Wei, S. (2012). Study of active ingredients in black soybean sprouts and their safety in cosmetic use. *Molecule*, *17*, 11669-11679.

Li, S. H., and Xiang, Q. L., (2011). Chemical constituents in *Rhynchosia volubilis*. *Chinese Traditional and Herbal Drugs*, *42(7),* 1276-1278.

Lo, W. L., Chang F. R., Hsieh T. J., and Wu, Y. C. (2002). The Constituents of *Euchresta formosana*. *Journal of the Chinese Chemical Society*, *49*, 421-426.

Low, A. B. and Rebelo, A.G. (1996). *Vegetation of South Africa, Lesotho and Swaziland. A companion to the vegetation map of South Africa, Lesotho, and Swaziland.*Pretoria, South Africa: Department of Environment Affairs and Tourism, South Africa.

Mabona, U., and van Vuuren, S. (2013). Southern African medicinal plants used to treat skin diseases. *South African Journal of Botany*, *87*, 175-193.

Mali, R. G., and Mahale, N. B. (2008). Evaluation of *Rhynchosia minima* (Linn.) DC leaves for anthelmintic activity. *International Journal of Pharmaceutical Science and Nanotechnology*, *1(2),* 91-93.

Malinowska, P. (2013). Effect of flavonoids content on antioxidant activity of commercial cosmetic plant extracts. *Herba Polonica Journal, 59(3),* 63-75.

Maplestone, R. A., Stone, M. J., and Williams, D. H. (1992).The evolutionary role of secondary metabolites – A review.*Gene, 115(1-2),* 151-157.

Marieb N. E., and Hoehn K. (Eds.). (2013). Chapter 5: The integumentary system. In: Marieb N. E., and Hoehn K. (Eds.). *Human anatomy and physiology*, 9th Edition. Boston, USA: Pearson.

Mason, H. S. (1955). Comparative biochemistry of the phenolase complex. *Advances in Enzymology*, *16*, 105-184.

Mathangi, S. K., and Sudha, K .(2012). Functional and phytochemical properties of finger millet (*Eleusine coracana* L.) for health. *International Journal of Pharmaceutical, Chemical and Biological Sciences , 2(4),* 431-438.

Medimagh, S., Hammami, S., Faidi, K., Hajji, N., Abreu, P. J. M., and Mighri, Z. (2010). Gallocatechin and *trans* syringing from *Limoniastrum guyonianum bois* growing in Tunisia. *Journal de la Société Chimique de Tunisie*, *12*, 207-210.

Meyer, L. H. (1960). *Food chemistry*. New York, USA: Reinhold Publishing Corporation.

Miller, G. T., and Spoolman , S. (Eds.). (2008). Sustaining biodiversity: The species approach. In Miller, G. T., and Spoolman , S. (Eds.). *Living in the environment: Principles, connections, and solutions*. Canada: Brooks/Cole, Cengage Learning.

Moin, A., Jabery, Z., and Fallah, N. (2006). Prevalence and awareness of melasma during pregnancy. *International Journal of Dermatology, 45(3),* 285-288.

Momtaz, S., Mapunya, B.M., Houghton, P.J., Edgerly, C., Hussein, A., Naidoo, S., and Lall, N., (2008). Tyrosinase inhibition by extracts and constituents of *Sideroxylon inerme* L. stem bark, used in South Africa for skin lightening. *Journal of Ethnopharmacology, 119,* 507-512.

Monzote, L., Pinon, A., and Setzer, W. N. (2014). Antileishmanial potential of tropical rainforest plant extracts. *Medicines, 1(1),* 32-55.

Mosa, R.. A., Oyedeji, A. O., Shode, F. O., Singh, M., and Opoku, A. R. (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit anti-platelet aggregation activity. *African Journal of Pharmacy and Pharmacology, 5(24),* 2698-2714.

Moteetee, A. N., Boatwright J. S., and Jaca, T. P. (2012). A review of *Rynchosia* section *cyanospermum* (*Phaseoleae, Fabaceae*) in South Africa. *South African Journal of Botany, 81,* 124-127.

Nia, M. A., Sreeramulu, K., and Gunasekar, D. (1992). Constituents of *Rhynchosia bracteata* and *Rhynchosia sublobata* leaves. *Fitoterapia, 63,* 283.

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

Ogungbe, I. V., Hill, G. M., Crouch, R. A., Vogler, B., Nagarkoti, M., Haber, W. A., and Setzer, W. N. (2011). Prenylated isoflavonoids from *Rhynchosia edulis*. *Natural Product Communications, 6(11),* 1637-1644.

Olikarnin, A., and Kallioinen, M. (1989). A biochemical and immunohistochemical study of collagen in sun-exposed and protected skin. *Photo-dermatology, 6(1),* 24-31.

Ondobayar, B., Muruta, T., Batkhuu, J., Yasunaga, K., Goto, R., and Sasaki, K. (2016). Antioxidant flavonols and phenolic compounds from *Atraphaxis frutescens* and their inhibitory activities against insect phenoloxidase and mushroom tyrosinase. *Journal of Natural Products, 79,* 3065-3071.

Ortonne, J. P. (2003). Clinical response to alefacept: Results of a phase 3 study of intramuscular administration of alefacept in patients with chronic plaque psoriasis. *Journal of the European Academy of Dermatology and Venereology, 17(Supplement 2)*, 12-16.

Pal, D., Mishra, P., Sachan, N., and Ghosh, A. K. (2011). Biological activities and medicinal properties of *Cajanuscajan* (L) Millsp. *Journal of Advanced Pharmaceutical Technology and Research, 2(4),* 207-214.

Pascaline, J., Charles, M., George, O., and Lukhoba, C. (2011). An inventory of medicinal plants that the people of Nandi use to treat Malaria. *Journal of Animal and Plant Sciences, 9(3),* 1192-1200.

Perkin A. G., and Newbury F. G. (1899). The colouring matters contained in Dyer's broom (*Genista tinctoria*) and heather (*Calluna vulgaris*). *Journal of Chemical Society, 75*, 830-839.

Pinheiro, P. F., and Justino, G. C. (2012). Structural analysis of flavonoids and related compounds – A review of spectroscopic applications. In: Rao, V. (Ed.). *Phytochemicals – A global perspective of their role in nutrition and health*. Ontario, Canada: InTech, Chapters.

Popoola, O. K., Marnewick J. L., Rautenbach, F., Ameer, F., Iwuoha, E. I., and Hussein, A. A. (2015). Inhibition of oxidative stress and skin aging–related enzymes by prenylated chalcones and other flavonoids from *Helichrysum teretiforium*. *Molecules, 20,* 7143-7155.

Pundir, R. P. S., and Singh, R. B. (1986). Karyotypic analysis of *Cajanus, Atylosia,* and *Rhynchosia* species. *Theory and Applied Genetics, 72*, 307-313.

Pundir, R. P. S., and Singh, R. B. (1985). Biosystematic relationships among *Cajanus, Atylosia,* and *Rhynchosia* species and evolution of pigeonpea [*Cajanuscajan* (L.) Millsp.]. *Theory and Applied Genetics, 69*, 531-534.

Rahmani, A. H., Al shabrmi, F. M., Allemailem, K. S., Aly, S. M., and Khan M., A. (2015). Implications of green tea and its constituents in the prevention of cancer via the modulation of cell signalling pathway. *BioMed Research International, 2015*, 1-12.

Rammohan, A., Gunasekar, D., Reddy, N. V., Vijaya, T., Devillee, A., and Bodo, A. (2015). Evaluation of antioxidant activity of phenolic compounds isolated from *Rhynchosia suaveolens* (Fabaceae). *Natural Product Communications, 10(4),* 609-611.

Rao, C. V., and Gunasikar, D. (1987). *C*-glycosides from *Rhynchosia rothii*. *Acta Ciencia Indica* (*Chemistry*), *13(3),* 187-189.

Rao C. V., and Gunasekar, D. (1988). Isotirumalin, a new prenylated dihydroflavonol from *Rhynchosiacyanosperma* (Benth). *Indian Journal of Chemistry, 19(39),* 383-384.

Rao, C. V., and Gunasekar, D. (1998). *C*-prenylated dihydroflavonol from *Rhynchosia densiflora*. *Phytochemistry, 48(8),* 1453-1455.

Rao, K. R. S. S., Tripathy, N. K., Rao, D. S. and Prakasham R. S. (2013). Production, characterization, catalytic, and inhibitory activities of tyrosinase. *Research Journal of Biotechnology, 8(1),* 87-99.

Rao, K.V., Sreeramulu, K., and Gunasekar, D. (1991). Chemical constituents of the genus *Rhynchosia*. *Indian Journal of Natural Products, 7(1),* 3-12.

Rerk-am, U., Kongsombat, B., Tangsatirapakdee, S., and Banchonglikitkul, C. (2013). Biological activities of *Cajanus cajan* ethanolic extracts. *Thai Journal of Pharmaceutical Sciences, 38,* 112-114.

Ribeiro, A. S., Estanqueiro M., Oliveira, M. B., and Lobo, J. M. S. (2015). Main benefits and applicability of plant extracts in skin care products. *Cosmetics, 2*, 48-65.

Roux, D. G., and Maihs, A. E. (1958). Black wattle catechin, *Nature, 182*, 1798.

Saayman, H. M., and Roux, D. G. (1965). The origins of tannins and flavonoids in black-wattle barks and heartwoods, and their associated 'non-tannin' components. *Biochemical Journal, 97*, 794.

Saini, S., Kaur, H., Verma, B., Ripudamn, and Singh, S. K. (2009). *Kigelia africana* (Lam.) Beth. – An overview. *Natural Product Radiance, 8(2),* 190-197.

Sanjappa, M. (1992). *Legumes of India.* India: Debra Dun: Bishen Singh Mahendra Pal Singh.

Sato, K., and Toriyama, M. (2009). Depigmentation effects of Catechins. *Molecules, 14*, 4425-4432.

Satooka, H., and Kubo, I. (2011). Effects of thymol on mushroom tyrosinase-catalyzed melanin formation. *Journal of Agriculture and Food Chemestry, 59*, 8908–8914.

Saxena, M., Saxena, J., Nema, R., Singh, D., and Gupta, A. (2013). Phytochemistry of medicinal plants. *Journal of Pharmacognosy and Phytochemistry, 1(6),* 168-182.

Schafer, H., and Wink, M., (2009). Medicinally important secondary metabolites in recombinant microorganisms or plants: Progress in alkaloid biosynthesis. *Biotechnology Journal, 4(12),* 1684-1703.

Schmid, D., Belser, E., and Meister, S. (2008). *Use of soy isoflavones for stimulation of skin collagen synthesis*. Switzerland: Mibelle Biochemistry.

Schmid D., Muggli R., and Zull E. (2001). Dermatological application of soy isoflavones to skin ageing in post-menopausal women. *Cosmetics and Toiletries, 1,* 146-151.

Schrine, B. D. (2005). *Phaseoleae*. In: Lewis, G. P., Schrire, B., Mackinder, B., and Lock, M. (Eds.). *Legumes of the world*. UK: Royal Botanic Gardens, Kew.

Schwartz, R. A., and Elston D. M. (Ed.). (2015, June 22). *Postinflammatory hyperpigmentation*. Retrieved from Medscape website: http://emedicine.medscape.com/article/1069191-overview

Scotti, L., Bezerra M. F. J. Jr., Magalhaes M. D.R., da Silva, M.S., Pitta, I.R., and Scotti, M.T. (2012). SAR, QSAR and docking of anticancer flavonoids and variants: A review. *Current Topics in Medicinal Chemistry, 12*, 2785-2809.

Seetharamamma, B., Rao, C. V., and Gunasekar, D. (1989). Flavone *C*-glycosides of *Rhynchosia jacobii. Indian Journal of Natural Products, 5(1)*, 22-23.

Seiji, M. (1967), Melanogenesis. In: Zelickson, A. S. (Ed.). *Ultrastructure of normal and abnormal skin*. Philadelphia: Lea and Febiger.

Shamani. (2008, March 21). *Effects of soy on topical skin*. Retrieved from Pharnarcymix Blog: http://blog.pharmacymix.com/effects-of-topical-soy-on-skin

Silva, A. R., Menezes, P. F. C., Martinello, T., Novakovich, G. F. L., De Oliveira Praes, C. E., and Feferman, I. H. S. (2010). Antioxidant kinetics of plant-derived substances and extracts. *International Journal of Cosmetic Science, 32(1)*, 73–80.

Singh, S. K. (2010). *Handbook on cosmetics (processes, formulae, with testing methods*). Delhi, India: Asia Pacific Business Press Incl.

Skin Cancer Foundation. (2016). *Melanoma*: *What is Melanoma?* Retrieved from Skin Cancer Foundation website: http://www.skincancer.org/skin-cancer-information/melanoma

Smith, J. G., Davidson, E. A., Tindall, J. P., and Sams, W. M. (1962). Alterations in human dermal connective tissue with age and chronic sun damage. *The Journal of Investigative Dermatology, 39*, 347-350.

South African Traveler. (2015, June 25). *Lusikisiki*. Retrived form StayZA website: http://www.stayza.com/lusikisiki/

Spiewak, R. (2000). Occupational skin diseases among farmers. In: Zagorski, J. (Ed.). *Occupational and para-occupational diseases in agriculture*. Lublin, Poland: Institute of Agricultural Medicine.

Sridharamurthy, N. B., Dhiman, V., and Yogananda, R. (2013). Extraction and screening of antioxidant, antiinflammatory, and analgesic activity of *Rhynchosia beddomei* leaves. *Research Journal of Pharmacology and Pharmacodynamics, 4(5)*, 319-327.

Stanley, B. (2004, February 13). *Recognition and respect for African traditional medicine*. Retrieved from Canada's International Development. Research Centre (IDRC) website:https://www.idrc.ca/en/article/recognition-and-respect-african-traditional-medicine

Stirton C. H., and Gordon-Gray, K. D. (1978).The *Eriosema cordatum* complex. I. The *Eriosema populifolium* group. *Bothalia, 12(3),* 395-340.

Stulberg, D. L., Clark, N., and Tovey, D. (2003). Common hyperpigmentation disorders in adults: Part II. Melanoma, seborrheic keratoses, acanthosis nigricans, melasma, diabetic dermopathy, tinea versicolor, and postinflammatory hyperpigmentation. *American Family Physician, 68(10),* 1963-1968.

Sturm, R. A., Box, N., and Ramsay, M. (1998). Human pigmentation genetics: The difference is only skin deep. *BioEssays*, *20*, 712–721.

Tait, A. (2001, January). *Medicinal properties of clivia*: *Clivia miniata*. Retrieved from the website: http://www.clivias.com/Articles/Article013.htm

Takhtajan, A. (1986). *Floristic regions of the world*. Berkeley: University of California Press.

The American Heritage dictionary of an English language: 5th Edition. (2016). Boston, Massachusetts, USA: Houghton Mifflin Harcourt Publishing Company.

The European Parliament and the Council of the European Union. (2009). Regulation (EC) No 1223/2009 of the European parliament and the council of 30 November 2009 on cosmetic products.*Official Journal of the European Union, 52,* 59-209.

Thomas, C. L. (1993). *Taber's cyclopedic medical dictionary, Edition 18*. Philadelphia, Pennsylvania, USA: F.A Davis Company.

Turner, B. L. (2016). *Fabales*: *Plant order*. Retrieved from Encyclopaedia Britannica website: https://global.britannica.com/plant/Fabales

United Nation Environmental Programme (2008). *Mercury in products and wastes*. Retrieved from the United Nations Environment Programme website: http://www.unep.org/chemicalsandwaste/Portals/9/Mercury/AwarenessPack/English/UNEP_Mod1_UK_Web.pdf

Vanni, A., Gastaldi, D., and Giunata, G. (1990). Kinetic investigations on the double enzymatic activity of the tyrosinase mushroom. *Annali di Chimica, 80*, 35-60.

Van Wyk, B., and Van Wyk, P. (1997). *Field guide to the trees of Southern Africa*. Cape Town, South Africa: Struik Publishers.

Van Wyk, B-E, and Wink, M. (2004). *Medicinal plants of the world*. Pretoria, South Africa: Briza Publications.

Van Wyk, B-E., Van Oudtshoorn, B. and Gericke, N. (1997). *Medicinal plants of South Africa.* Pretoria, South Africa: Briza Publications.

Vazquez, M., Maldonado, H., Benmaman, C., and Sanchez, J. L. (1988). Melasma in men. A clinical and histologic study. *International Journal of Dermatology, 27(1),* 25-27.

Veberič, R. (2010). *Bioactive compounds in fruit plants*. Ljubljana, Slovenia: CIP - Catalogue Publication National and University Library, Ljubljana.

Veillux, C., and King, S. R. (1996). *An introduction to ethnobotany*. Retrieved from Access excellence website: http://www.accessexcellence.org

Vitale, D. C., Piazza, C., Melilli, B., Drago, B., and Salomone, S. (2013). Isoflavones: Estrogenic activity, biological effect and bioavailability. *European Journal of Drug Metabolism and Pharmacokinetics, 13(1),* 15-25.

Waffo, A. K., Azebaze, G. A., Nkengfack, A. E., Fomum, Z. T., Meyer, M., Bodo, B., and van Heerden, F. R. (2000). Indicanines B and C, two isoflavonoid derivatives from the root bark of *Erythrinaindica*. *Phytochemistry, 53,* 981-985.

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

Wariss, H. M., Ahmad, S., Anjum, S., Khurshid and Alam, K. (2014). Ethnobotanical studies of dicotyledonous plants of Lal Suhanra National Park, Bahawalpur, Pakistan. *International Journal of Science and Research, 3(6),* 2319-7064.

Webmaster. (2014, October 10). A responsible approach to treating skin pigmentation: An interview with dermatologist, Dr. Ncoza C. Dlova (MBChB, FCDerm). *Frontshop Pharmacy Magazine*. Retrieved from Frontshop website: http://www.frontshop.co.za/responsible-approach-treating-skin-pigmentation/

Welford, L., and Le Breton, G. (2008). Bridging the gap: Phytotrade Africa's experience of certification of natural products. *Forest, Trees, and Livelihoods, 18,* 69-79.

Weller, R. P. J. B., Hunter, J. A. A., Savin, J. A., and Dahl, M. V. (Eds.). (2008). The function and structure of the skin. In: Weller, R. P. J. B., Hunter, J. A. A., Savin, J. A., and Dahl, M. V. (Eds.). *Clinical dermatology*, 4th Edition. Oxford, UK: Blackwell Publishing Ltd.

Willis, C. K. (Ed.). (2006). *A South African response to the global strategy for plant conversation*. Pretoria, South Africa: South African National Biodiversity Institute.

Yanyan, G., Weiping, Y., Pu, L., Ya, H., Mengjie, Y., and Xiangqian, S. (2011). Extraction and separation of isoflavonoids from *Rhynchosia volubilis* Lour using ionic liquid. *Chinese Journal of Applied Chemistry, 28(5),* 537-541.

Yasui, H., and Sakurai, H. (2003). Age-dependent generation of reactive oxygen species in the skin of live hairless rats exposed to UVA light. *Experimental Dermatology, 12(5),* 655-661.

Yim, J. H., Lee, O., Choi, U., and Kim, Y. (2009). Antinociceptive and anti-Inflammatory effects of ethanolic extracts of *Glycine max* (L.) Merr and *Rhynchosia nulubilis* seeds. *International Journal of Molecular Sciences, 10(11),* 4742-4753.

Yoon, K. H., Park, K. J., Yin, J., Yoon, K. H., Lee, J. Y., Hwang, Y. J., Lee, D. I., Choi, Y. W., and Lee, M. (2016). Antioxidative and antitumor effects of isoflavones isolated from the leaves of *Maackia fauriei*. *Record of Natural Products, 10(4),* 441-451.

Zulu, R. M., Dillon, V. M., and Owens, J. D. (1997). Munkoyo beverage, a traditional Zambian fermented maize gruel using *Rhynchosia* root as amylase source. *International Journal of Food Microbiology, 34(3),* 249-258.