

**MUSHROOM TYROSINASE ACTIVITY OF NOVEL
AND KNOWN PHENOLIC COMPOUNDS
ISOLATED FROM *GREYIA FLANAGANII* (BOLUS)**

By

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ABSTRACT

Hyper-pigmentation of the skin is a common problem that is prevalent in middle aged and elderly people. It is caused by over production of melanin, the pigment that is responsible for the colour of hair and skin in humans. Tyrosinase is known to be the key enzyme in melanin production. Forty plants belonging to different families were tested for their activity on tyrosinase using L-tyrosine as a substrate.

Ethanol leaf extract of *Greyia flanaganii* (Bolus) showed significant ($p < 0.05$) inhibitory activity exhibiting the 50% inhibitory concentration (IC_{50}) of 32.62 $\mu\text{g/ml}$. It was further investigated for its toxicity and effect on melanin production by melanocytes cells. *G. flanaganii* showed significant ($P < 0.05$) 20% reduction of melanin at 6.25 $\mu\text{g/ml}$ while 80% of cells were found to be viable, thus indicating low levels of cytotoxicity ($IC_{50} < 400 \mu\text{g/ml}$). The 1,2-diphenyl-2-picrylhydrazyl (DPPH) method was used to quantify the total radical scavenging capacity (RSC) of the extract. The amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) by the ethanol extract of the leaves of *G. flanaganii* was found to be 22.01 $\mu\text{g/ml}$.

‘Acne vulgaris’ is a chronic inflammatory follicular disorder of the skin that affects areas containing the largest oil glands including face, neck, back and chest. It affects up to 80%

of adolescents to some extent and less commonly occurs in infants (Shaw and Kennedy, 2007). *Propionibacterium acnes* is an obligate anaerobic gram-positive bacterium that is associated with or linked to 'Acne vulgaris'. It is largely commensal and is present on most people's skin and live on fatty acids in the sebaceous glands on sebum secreted by pores. The effect *Greyia flanaganii* (Bolos) against acne causing bacteria, *P. acnes* was investigated using microdilution assay. The minimum inhibitory concentration (MIC) of the extract of *G. flanaganii* was found to be 250 µg/ml.

A bioassay guided fractionation of the ethanolic leaf extract of *G. flanaganii* led to the isolation of six known compounds and one novel phenolic compound. Isolated compounds were; 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (**C1**), 2',4',6'-trihydroxydihydrochalcone (**C2**), 2',6'-dihydroxy-4',4-dimethoxydihydrochalcone (**C3**), (2*R*,3*R*)-3,5,7-trihydroxy-3-*O*-acetylflavanone (pinobanksin-3-acetate/dihydrogalangin-3-acetate) (**C4**), 2',6'-dihydroxy-4'-methoxydihydrochalcone (**C5**), 5,7-dihydroxyflavanone [(2*S*)-pinocembrin] (**C6**) and 4-hydroxyphenethyl 3-hydroxy-5-phenylpentanoate (**C7**). **C1-C6** are well known compounds whereas **C7** is a novel compound. The isolated compounds were tested for their cytotoxicity, antioxidant, tyrosinase inhibitory and antibacterial activities. **C3** and **C4** were only tested on tyrosinase due to low yield of these compounds during isolation. **C2** exhibited significant ($p < 0.05$) antityrosinase activity exhibiting the IC_{50} of 69.15 µM. **C1**, **C4**, **C5**, **C6** and **C7** also showed significant ($p < 0.05$) inhibitory activity of the tyrosinase enzyme exhibiting 11.5, 35, 24, 22 and 31 % enzyme inhibition respectively at 200 µg/ml. **C3** did not show any significant inhibition of the enzyme at

the highest concentration tested (200 µg/ml). **C6** showed low toxicity effect on melanocytes cells, IC_{50} of 373 µM was observed and about 10% reduction of melanin content was found at 1.5 µg/ml. All the compounds tested for antioxidant activity exhibited good radical scavenging activity. The EC_{50} (concentration of extract which decreases the initial DPPH absorbance by 50%) values of **C2**, **C1**, **C5** and **C7** was found to be ranging from 3.46 to 76.1 µM. The EC_{50} of the positive control used (vitamin C) was found to be 11.3 µM. The isolated compounds were also tested for their activity against *P. acnes*. **C2**, **C7** and **C1** exhibited MIC values of 968, 1590 and 418 µM respectively. The positive drug control; erythromycin exhibited an MIC of 136 µM. The cytotoxicity, antityrosinase, antioxidant and antibacterial activity of *G. flanaganii* extract are being reported for the first time. Other flavonoid compounds such as pinistrobin, 7-methylethiodictyol, dihydrogalangin, chrysin etc have been previously isolated from this plant but all the isolated compounds in this study have been isolated for the first time from *G. flanaganii*.

C2 which showed the best antityrosinase activity (IC_{50} value of 69.1 µM) as compared to the other samples tested in the present study. This sample also showed low cytotoxicity on melanocytes cells (IC_{50} 219 µM) and resulted in 10% reduction of melanin at 3.125 µg/ml. Therefore, this compound can be considered for its use for the treatment of skin hyper-pigmentation problems. **C1** which showed low MIC (418 µM) as compared to positive drug should be considered for further studies in clinical trials for a possible anti-acne agent. **C2** and **C1** which exhibited an EC_{50} value of 3.46 and 6.69 µM respectively seem to have good antioxidant activity and can be considered for their use as UV

protecting agents. Based on these results, it shows that the leaf extract of *Greyia* *flanaganii* and **C2** have some potential to be considered as a possible anti-tyrosinase agent though their mechanism studies need to be done in detail to support their possibilities of being used to treat hyper-pigmentation problems.

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List of abbreviations

ANOVA	Analysis of variance
^{13}C-NMR	Carbon nuclear magnetic resonance
DMSO	Dimethyl sulphoxide
DOPA	1-3,4-dihydroxyphenylalanine
DPPH	1,2-diphenyl-2-picrylhydrazyl
DCM	Dichloromethane
EtOAc	Ethylacetate
EtOH	Ethanol
FCS	Foetal calf serum
HEX	Hexane
HQ	Hydroquinone
^1H-NMR	Proton nuclear magnetic resonance
H_2O_2	Hydrogen peroxide
MEM	Minimal Essential Medium
MeOH	Methanol
NMR	Nuclear magnetic resonance spectroscopy
PBS	Phosphate buffer



ROS	Reactive oxygen species
RSC	Radical scavenging capacity
TLC	Thin layer chromatography
TRP-1	Tyrosinase related protein-1
UV	Ultra violet
WHO	World Health Organization
XTT	Sodium 3-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitrobenzene sulfonic acid hydrate) labelling reagent)



CHAPTER 1

Introduction

Chapter 1

Introduction

1.1 Background

The use of plants as medicines is dated back to early man (Phillipson, 2001). Humans relied on nature for their basic needs such as food, shelter, clothing, fertilizers, flavours, fragrances and last but not least medicines. Plants have formed the sophisticated traditional medicine systems that have been in existence for thousands of years (Anon, 1998, Yelisida, 2005; Cunningham, 1993).

Traditional medicine refers to the health practices, approaches, knowledge and beliefs incorporating plants, animals and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in-combination to treat, diagnose and prevent illnesses or maintain well-being of individuals. Medicine, in several developing countries, using local traditions and beliefs, is still the mainstay health care till to date. The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and South Africa etc (Hoareau and Dasilva, 1999; Coetzee et al, 1999; Diederichs, 2002, Nair, 2005). In China 40% of the total medicinal consumption is attributed to traditional tribal medicines. In Japan, herbal medicine preparations are more in demand than mainstream pharmaceutical products (Hoareau and Dasilva, 1999).

Africa is a rich source of medicinal plants (Hoareau and Dasilva, 1999). They are an integral part of African culture, which is the oldest and most diverse in the world.

Plants have been used in African medicine to treat fever, asthma, constipation, hypertension, skin diseases etc (Medical news press). About 80% of the black population uses traditional medicine as the primary healthcare system (Bussman and Sharon, 2006, Van wyk et al 1997).

1.2 Uses of plants

Plants are a source of fuel, building material, craft material, dyes, food supplements and medicine all over the world. Approximately 80% of black population make use/rely on plants for these services (Light, 2005). Traditional medicine can be viewed as a parallel system to western health care. Approximately 3000 species are used by an estimated 200,000 indigenous traditional healers (Van wyk et al. 1997). Plants can be used for different purposes, some of them are mentioned below:

1.2.1 Plants as a source of food

Food is any substance composed primarily of carbohydrates, fats, water, and /or proteins that can be drunk or eaten by human beings or animals for nutrition or pleasure (figure 1.1). Food sources include plants, animals or other categories such as fungus or fermented products like alcohols (Davidson, 2006).

Many plant or plant parts are eaten as food. There are around 2000 plants species which can be cultivated for food, and many have several distinct cultivars (Davidson, 2006; Mander, 1999). Fruits are ripened extensions of plants including seeds within. Many plants have evolved fruits that are attractive as a food source to animals as well. Fruits, therefore, make up a significant part of the diets of most cultures. Some botanical fruits such as tomatoes, pumpkin etc are eaten by humans as vegetables.



Figure 1.1 Food Pyramid (<http://dietmotion.com/images/food-pyramid.jpg>)

1.2.2 Drug discovery

Although natural products, particularly secondary metabolites, have formed the basis of medicines, the presence of these compounds in the biochemistry of the plant is very often difficult to justify. It has been suggested that these compounds may have been synthesized by the plant as part of the defence system of the plant, e.g. plants are known to produce phytoalexins as a response to attack by bacteria and fungi (Louw et al, 2002). The presence of highly toxic natural products has also been highlighted in some animals namely the Amazonian frogs so as to deter predation by other animals. Whatever the reasons for the presence of these compounds in nature could be, it is needless to state that they provide invaluable resources that have been used to find new drug molecules. Table 1.1 gives an indication of the development of new drugs from natural products. Spectroscopic methods coupled with good extraction



techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. A sound isolation strategy has helped in the isolation and characterisation of many bioactive molecules. Nowadays, bioassay-guided fractionation of medicinal plants is a feature of routine in the attempt to isolate components from natural sources. These techniques are not only being restricted to plant sources but they are also being applied to microbial and even fungal sources of metabolites. In practice as soon as the material is collected, in the case of plants, it needs to be identified by a taxonomist so as to ascertain the correct identity of the material. Voucher specimens for herbarium specimens are kept. Various parts of the plant are collected separately (leaves, flowers, stem, wood, bark, root, root bark etc.) and are dried quickly in drying cabinets preparing them for isolation of active compounds (Gurib-Fakim, 2006).

Table 1.1 Plant based drugs and medicines (Taylor, 2000)

Drugs/ chemical	Action	Plant source
Acetyldigoxin	Cardiotonic	<i>Digitalis lanata</i> (Grecian foxglove, woolly foxglove)
Adoniside	Cardiotonic	<i>Adonis vernalis</i> (pheasant's eye, red chamomile)
Caffeine	CNS stimulant	<i>Camellia sinensis</i> (tea, also coffee, cocoa and other plants)
Camptothecin	Anticancerous	<i>Camptotheca acuminata</i>
Colchicine	Antitumor, antigout	<i>Colchicum autumnale</i> (autumn crocus)
Gаланthamine	Cholinesterase inhibitor	<i>Lycoris squamigera</i> (magic lily, resurrection lily, naked lady)
Glycyrrhizin	Sweetener, treatment for Addison's disease	<i>Glycyrrhiza glabra</i> (licorice)
Irinotecan	Anticancer, antitumor agent	<i>Camptotheca acuminata</i>
Lapachol	Anticancer, antitumor	Tabebuia species (trumpet tree)
Monocrotaline	Topical antitumor agent	<i>Crotalaria sessiliflora</i>

1.2.3 Plants as a source of cosmetics

Cosmetic product refers to any substance or preparation intended for application to any external surface of the human body (i.e. the epidermis, hair system nails, lips and external genitals organs) or teeth or buccal mucosa wholly or mainly for the purpose of cleaning, perfuming or protecting them or keeping them in good conditions or changing their appearance or combating body odour or perspiration except where such cleaning, perfuming, protecting, keeping and changing is wholly for the purpose of treating or preventing diseases (Aburjai and Natsheh, 2003; Dweck, 1996). Novel bioactive ingredients for cosmetics are derived from sea, earth and plant kingdom. Popular ingredients include Chinese herbs, Vitamins, minerals, antioxidants, enzymes, hormones and naturals.

Plants have been used for cosmetic purposes since time immemorial. They have once been the main source and foundation of all cosmetics before various methods were discovered of synthesizing substances with similar properties (Aburjai and Natsheh, 2003). The first cosmetic derived from plant is dated about 3100-2907 BC which was used in Egypt (Dold and Cocks, 2002; Cocks et al, 2003).

There are many plants which are being used for cosmetics. Ginseng is a traditional drug used for more than 2000 years. It activates the skin metabolism (Tanaka and Okada, 1991), reduces keratinisation (Kim et al, 1989), provides moisture and softens, alleviate wrinkling and enhance skin whiteness (Dweck, 1997). Other plants that have been used in cosmetics preparations are *Artemisia vulgaris* and *Artemisia absinthum* which are used for skin diseases. The entire plant is made into decoction and is used as a wash for many kinds of wounds and skin ulcers (Dweck, 1997). *Salvia officinalis* (L) also called common sage, true sage or garden sage is used as a lotion to improve

the condition of hair and skin. The major *S. officinalis* constituents responsible for the effect of hair are tannins, saponins as well as borneol and camphor (Aburjah and Natsheh, 2003; Boiceanu et al, 1986). Majority of South African plants such as *Calodendrum capensis*, are being used traditionally as cosmetics but they have not been scientifically validated.

1.3 Research question:

Can we obtain valuable products from plants grown in South Africa which can be used for skin-hyperpigmentation problems or skin-lightening purposes?

1.4 Structure of the thesis

Chapter 1 In this chapter an overview of traditional medicine and its use in cosmetics is given.

Chapter 2 The structure and function of the skin, skin hyper-pigmentation disorders, their causes and treatments are discussed in detail.

Chapter 3 In this chapter description, distribution and uses of the selected plants in this study are briefly discussed.

Chapter 4 Inhibitory effect of selected plant extracts on tyrosinase activity has been discussed.

Chapter 5 Inhibitory effect of *Greyia flanaganii* (Bolus) leaf extract on tyrosinase activity, radical scavenging activity, melanin production and its toxicity on melanocytes cell have been mentioned. The antibacterial activity of the extract against *Propionibacterium acnes* is also discussed.



Chapter 6 Isolation of active compounds from *Greyia flanaganii* (Bolus) using different isolation procedures such as column chromatography etc is discussed together with the inhibitory activity of tyrosinase, radical scavenging activity, melanogenesis and antibacterial activity against *Propionibacterium acnes* of all the isolated compounds.

Chapter 7 General discussions and conclusions

Chapter 8 Acknowledgements

Chapter 9 Appendix

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CHAPTER 2

Skin disorders, causes and treatment

2.1 Background

Hyper-pigmentation of skin is a common problem that is prevalent in middle aged and elderly people. Hyper-pigmentation can be caused by excessive exposure to UV light, drug reaction and can also occur during ageing. Dermatological disorders associated with hyper-pigmentation include age spots, melasma and site of actinic damage to mention the few (Pandya and Guevera, 2000).

Melanin is the pigment responsible for the colour of skin in humans. It also occurs in bacteria, fungi and plants. Tyrosinase is known to be the key enzyme in melanin biosynthesis (Nerya et al, 2003). Over-activity of this enzyme leads to overproduction of melanin leading to hyper-pigmentation of the skin and under-activity leads to disorders such as vitiligo (depigmentation spots that occurs on the skin) and whitening of hair. Overproduction of melanin can be prevented by avoiding excessive UV light exposure and can be treated with skin-lightening agents such as bleaching hydroquinone, kojic acid and retinoids (Halder et al, 2004). Inhibition of tyrosinase can also lead to reduced melanin production. Some commercially available chemical and fungal derived skin-lightening agents have been proven to have chronic, cytotoxic, mutagenic effects in humans (Nerya et al, 2003; Wang et al, 2006; Wu et al, 2003). Therefore, there is a need for alternative herbal derived and pharmaceutical agents for the treatment of hyper-pigmentation of human skin.

2.2 Rationale of the study

To test the activity of different plant extracts on tyrosinase inhibition and identify bioactive principle(s) of the best extract.

2.3 Objectives

- Select plants based on their ethnobotanical and phytochemical knowledge.
- Preparation of ethanolic extracts of the selected plants.
- Test each extract for their activity on tyrosinase using L-tyrosine as the substrate.
- Select effective plant extracts and further test their effect on melanin biosynthesis by melanocytes cells and investigate the best extract's antioxidant activity and antibacterial activity against *Propionibacterium acnes*.
- Isolation of active compounds from the plants which shows good antityrosinase activity and comparatively less cytotoxicity than the other plants.
- Test all the isolated compounds for antityrosinase activity and evaluate their cytotoxicity on melanocytes cells.
- Test antibacterial activity of the isolates and active extracts against *Propionibacterium acnes*
- Test the antioxidant activity of the isolates.

2.4 Structure and function of the skin

The skin is one of the heaviest body organs. It covers between 1.5 and 2 m, comprising about one sixth of the body's total weight. The skin performs several important physiological functions; this includes regulation of body temperature and metabolism, excretion (via sweat glands), synthesis of vitamin D in the epidermal layer when exposed to UV rays, uses specialised cells to protect us from UV rays of the sun etc (Murphy, 1995). The skin consists of three layers (figure 2.1) namely:

- i. Epidermis-** It is the outer layer of the skin which also consists of several layers- the basal cell layer, the spinous cell layer, the granular cell layer

and the stratum corneum. Cells in the epidermis include the keratinocytes which are the most abundant cells in this layer, melanocytes which constitutes about 5% of the living cells in this layer (Murphy, 1995).

ii. Dermis- This layer is just below the epidermis. It consists of fats, collagen and also elastin fibres that provide strength and flexibility to the skin. In older persons the elastin fibres fragments and much of the skin's elastic quality is lost. This, along with the loss of subcutaneous fat, results in wrinkles (Murphy, 1995).

iii. Subcutaneous layer or hypodermis- This is the inner most layer of the skin. It serves as storage for fats. The fats stored in this layer represent an energy source for the body and helps to insulate the body against changes in the outside temperature (Murphy, 1995).

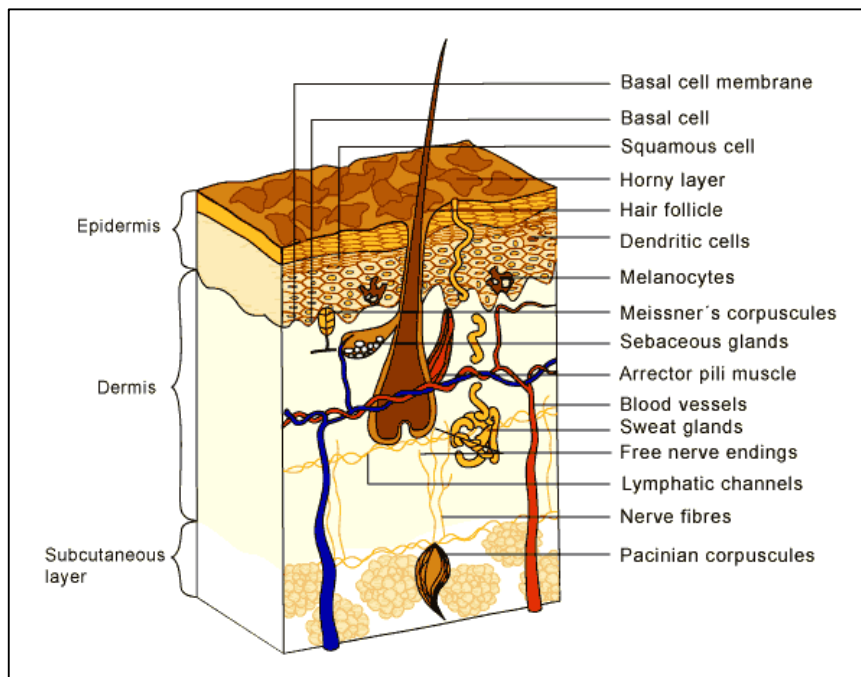


Figure 2.1 Structure of the normal skin

(http://skincare.dermis.net/content/e01aufbau/e660/e661/e700/013_haut_aufbau_eng.

[gif](#))

2.5 Melanin biosynthesis and its importance

Melanin is a class of compounds found in the plant, animal and protista kingdoms, where it serves predominantly as a pigment (Kim and Uyama, 2005). In humans, melanin is the primary determinants of the color of the skin, hair and eyes. It is synthesized within melanosomes, membrane-bound granules, from melanocytes and is then transferred to keratinocytes through a physiological process called melanogenesis (figure 2.2) (Ancans et al, 2003; Kim and Uyama, 2005). Tyrosinase is known to be the key enzyme in melanin biosynthesis (figure 2.2). It catalyses two distinct reactions: hydroxylation of the amino acid tyrosine to 3,4 dihydroxyphenylalanine (DOPA) by monophenolase action and oxidation of DOPA in to o-dopaquinone by diphenolase action. This o-quinone is transformed into melanins in a series of non-enzymatic reactions (Baurin et al, 2002; Wang et al, 2006).

There are two types of melanin pigments that can be produced by the melanocytes cells namely: 'eumelanin' (black or brown) and 'pheomelanin' (red or yellow) (figure 2.3) (Commo et al, 2004; Summers, 2006). The color of hair, skin etc in human is determined by the type, distribution and degree of melanin pigment synthesized. Each individual of different racial group have more or less the same number of melanocytes cells, thus the type of melanin produced depends on the functioning of the melanocytes e.g. people with darker skin are just genetically programmed to constantly produce higher levels of melanin even without exposure to UV light and the melanosomes remains singular (figure 2.4) (Commo et al, 2004; Baurin et al, 2002; Kim and Uyama, 2005; Summers, 2006; Sturm et al, 1998). In individuals with fair or lighter skin colour, melanosomes cluster in membrane bound organelles (figure 2.4) (Sturm et al, 1998). The role of melanin is to protect the skin against UV light



damage by absorbing UV sunlight and by removing reactive oxygen species (Kim and Uyama, 2005; Summers, 2006).

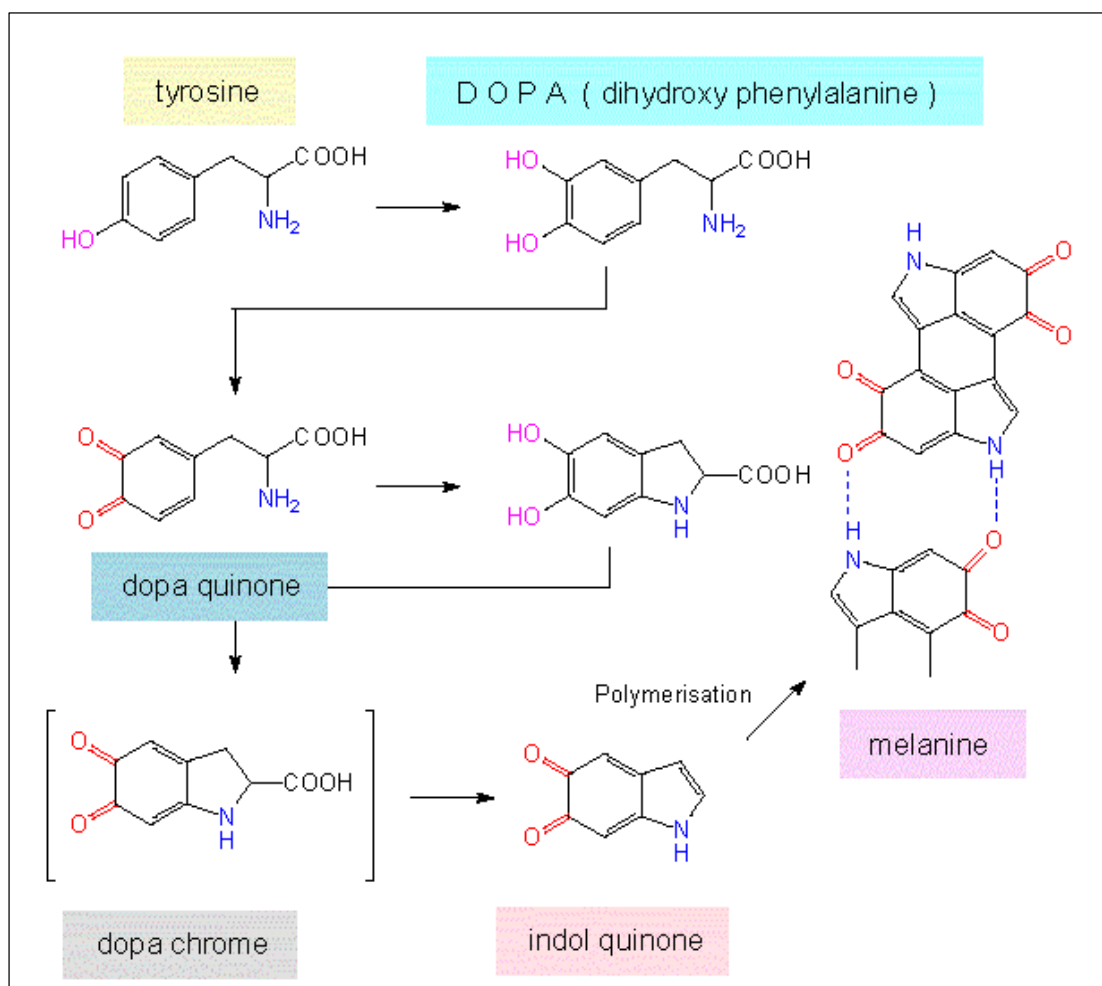


Figure 2.2 Melanin biosynthesis pathway (Moss, 2005)

(<http://www.chem.qmul.ac.uk/iubmb/enzyme/reaction/AminoAcid/melanin.html>)

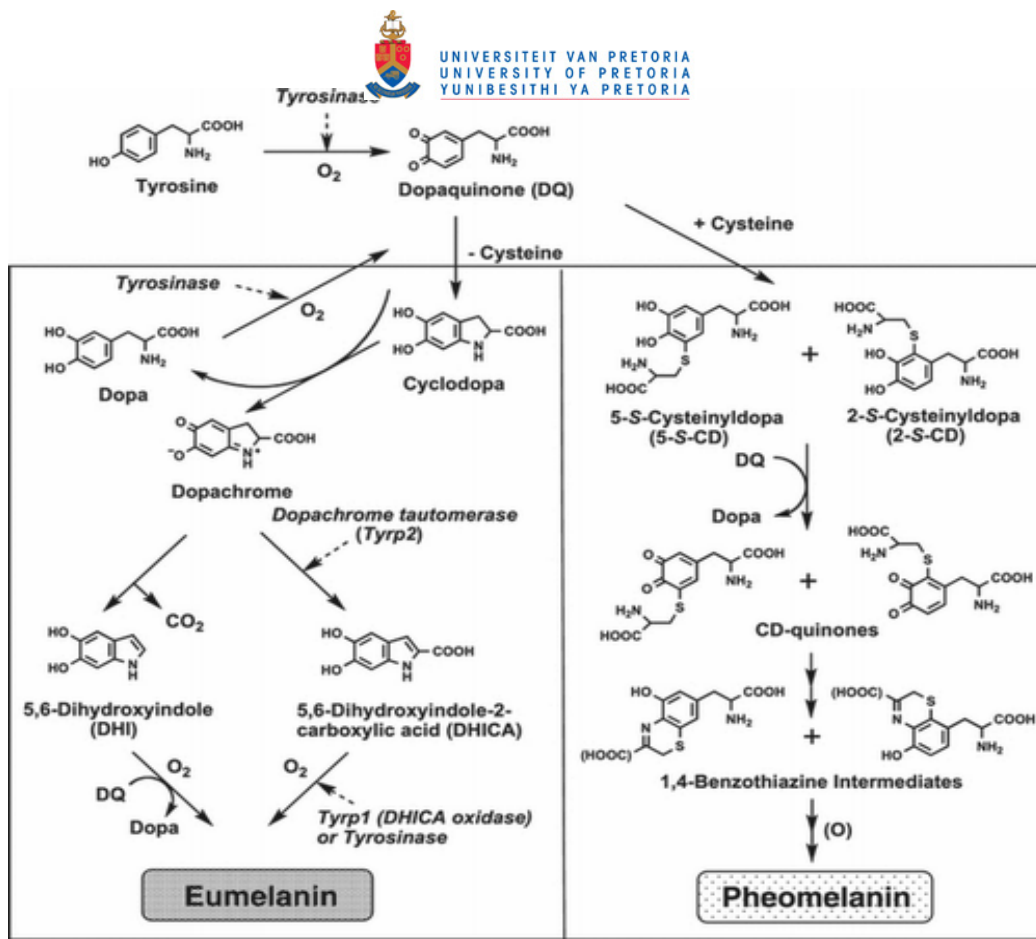


Figure 2.3 Production of different pigments by melanosomes (Simon et al, 2008)

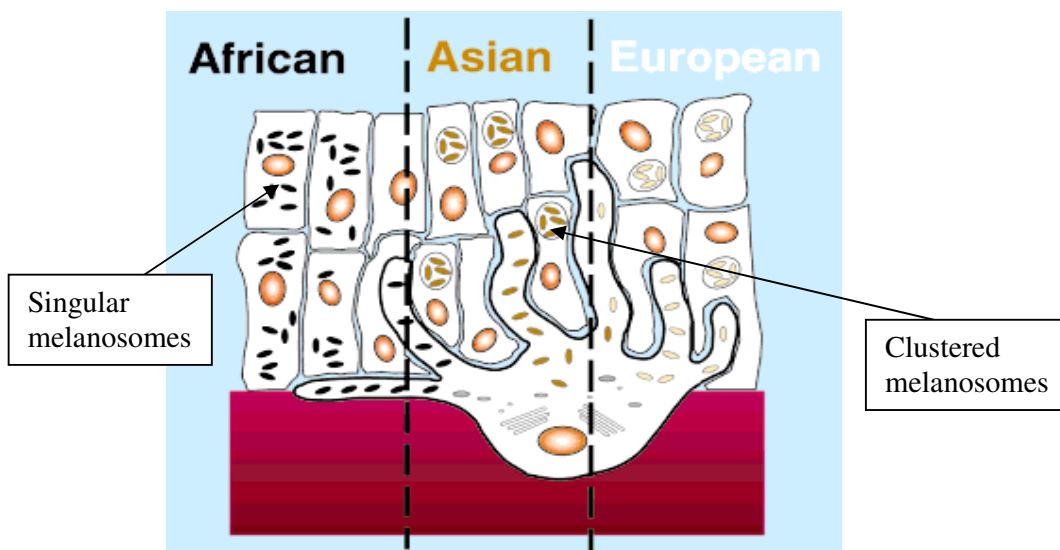


Figure 2.4 Structure of melanosome distribution for different racial groups (Sturm et al, 1998)

2.6 Factors affecting skin pigmentation

The process of melanogenesis is affected by a variety of environmental, hormonal and genetic factors. Environmental factors include excessive exposure to UV light, which induces immediate pigment-darkening due to swelling and re-alignment of melanosomes (Rendon, 2003, Schulleuter et al, 1998; Simon et al, 2008). Example for hormonal factors is evident in pregnant women whereby during this period, dark brown patches occur on the forehead, face, cheeks and it can occur due to reactions caused by usage of drugs. Genetic factors are evident in cases like albinism whereby individuals loses the TPR1 gene which disrupts the correct formation of the melanosomal complex and thereby inhibits melanin synthesis, resulting in hypopigmented phenotype (Sturm et al, 1998)

2.7 Hyper-pigmentation disorders

Hyper-pigmentation is a common and distressing problem. Females report more often, although not rare in males. These disorders have a tremendous psychological impact especially in females. There are different kinds of hyper-pigmentation disorders that are encountered during life. Some of them are as follows:

2.7.1 Age spots

This is also known as brown spots, lentigines or liver spots. They usually appear on the hands, but can also appear on the face, arm and feet as brown patches. They are harmless and are caused by excessive exposure of the skin to the sun. It may also be caused by nutritional deficiency, impaired liver function dietary and ageing (Moss, 2005, Murphy, 1995).

2.7.2 Melasma

Melasma appears as blotchy, brownish pigmentation on the face of adults. Both sides of the face are usually affected. The most common sites of involvement are the cheeks, bridge of nose, forehead, and upper lip. The precise cause of melasma is unknown. People with a family history of melasma are more likely to develop melasma themselves. A change in hormonal status may trigger melasma. It is commonly associated with pregnancy and is also called chloasma, or the "mask of pregnancy." Birth control pills may also cause melasma, however, hormone replacement therapy used after menopause has not been shown to cause the condition. Sun exposure also contributes to melasma. While there is no cure for melasma, many treatments have been developed. Melasma may disappear after pregnancy; it may remain for many years, or a lifetime. Sunscreens are essential in the prevention of melasma (American Academy of Dermatologists press 2008; Montemarano, 2008; Daniel et al, 2003).

2.7.3 Freckles

Freckles are clusters of concentrated melanin which are most often visible on people with a fair complexion. A freckle is also called an "ephelis". They can be found on anyone no matter the background; however, having freckles is genetic and is related to the presence of the dominant melanocortin-1 receptor (MC1R) gene variant. The formation of freckles is triggered by exposure to sunlight. The exposure to UV-B radiation activates melanocytes to increase the melanin production, which causes freckles to become darker. Freckles are predominantly found on the face, although they may appear on any skin exposed to the sun. Freckles are rare on infants and more common on children before puberty; they are less common on adults (Hanson et al, 2006; Daniel et al, 2003).

2.8 Treatment of hyper-pigmentation

Like other disorders hyper-pigmentation of the skin can be treated. Depending on the type and depth of pigmentation different treatment procedures are followed. The use of sun screens is important and this helps to reduce the development of pigmentation disorders. Skin lighteners are also used for the treatment of hyper-pigmentation this includes chemicals such as bleaching hydroquinone, retinoid etc which are available commercially (Cayce et al, 2004, Tiedtke et al, 2004).

2.9 Commercially available skin-lighteners

There are different types of skin-lightening agents that are available commercially to combat dark marks or skin hyper-pigmentation. Some of these chemically derived compounds have shown to have poor penetration of the skin and shows some cytotoxic and mutagenic effect on people. Among treatments the use of chemicals such as 'Kojic acid', 'Hydroquinone', 'Arbutin', 'Aloesin' etc are the most common ones. Descriptions of some of these chemicals are as follows:

2.9.1 Hydroquinone (HQ)

It is a hydroxyphenolic chemical that inhibits the conversion of L-DOPA to melanin by inhibiting the tyrosinase enzyme. Some of the possible mechanisms of action are the destruction of melanocytes, degradation of melanosomes and inhibition of the synthesis of DNA and RNA (Mashhood, 2006). Concentrations of HQ in commercially available formulations vary from 2% to 4%. Clinically and historically evaluation demonstrated clear improvements in most patients during the study done by Pandya and Guevara 2003. However, during this study 72% of the patients showed to develop irritant dermatitis over time, despite the low frequency of application.

2.9.2 Kojic acid (5-hydroxy-2-[hydroxyl methyl]-4-pyrone)

It is a naturally occurring hydrophilic, 'fungal derivative', evolved from certain species of *Acetobacter*, *Aspergillus* and *Penicillium*. It acts by inhibiting the production of free tyrosinase with efficacy similar to hydroquinone. In Japan, kojic acid has been increasingly used in skin products. This is because until recently, topically applied kojic acid at 1% concentration had not exhibited any sensitizing activity. However, more recent long term Japanese studies have shown that kojic acid has a potential of causing 'contact dermatitis' and 'erythema' (Nakagawa et al, 1995; Mashhood, 2006).

2.9.3 Arbutin (b-D-glucopyranoside derivative of hydroquinone)

It is a naturally occurring plant derived compound that has been used for post-inflammatory hyper-pigmentation. The action of arbutin depends on its concentration (Maeda and Fukuda, 1996). Higher concentrations are more efficacious than lower concentrations. A dose-dependent reduction in tyrosinase activity, as well as melanin content in melanocytes, is demonstrated. It was reported that it may cause paradoxical hyper-pigmentation (Mashhood, 2006).

2.9.4 Aloesin

It is a natural derivative of *Aloe vera* that inhibits tyrosinase at noncytotoxic concentrations. Aloesin acts as an inhibitor of DOPA oxidation. It is an experimental product and thus it is not available clinically (Rendon & Gaviria, 2005).

2.10 Cosmeceutical formulations from plants

Due to instability, poor penetration of the skin, irritation and mutagenic effects of chemical and fungal derived compounds in cosmetical applications, humans opt for herbal products for the treatment of different types of skin problems. There are many South African plants which are being used in herbal cosmetics. Rooibos (*Aspalathus*

linearis) is famous for being naturally caffeine free (Morton, 1983) and its low tannin content minimizes the risk of reduced iron absorption, a phenomena frequently found in tea drinkers due to iron-tannin complexation. Rooibos is rich in flavonoids, polyphenols, phenolic acids, oligosaccharides and polysaccharides (Dos et al, 2005). The major flavonoids found in Rooibos tea are aspalathin, iso-orientin, orientin and rutin (Shimamura et al, 2006).

The skin is the largest organ of the body and is exposed to environmental oxidative stress. The incorporation of Rooibos extracts in topical cosmetic formulations has become very popular in recent years because it can target the site of action to reduce ultraviolet radiation damage and photo-aging (Mavon et al, 2005; Van Niekerk and Viljoen, 2008). Rooibos proved to exhibit anti-inflammatory and antimicrobial properties in cosmetic applications. According to earlier reports, hair growth was found to improve with the use of hair care products containing Rooibos. There are many herbal products derived from rooibos (fig 2.5).

Artemisia afra is also popular for skin ailments. An infusion or decoction is used as a lotion by natives in South Africa to bathe hemorrhoids, herpes and venereal sores, while a hot bath in the decoction is used to bring out the rash in measles, mumps and chickenpox. The plant is also held in the mouth to ease the pain of boils and to hasten their bursting. It also has similar uses to *Artemisia herba-alba* and used externally on boils, carbuncles, and large acne pimples. A poultice of the leaf is applied locally to relieve neuralgia, to the swellings in mumps, and to any glandular or skin inflammation. A lotion is also made from the plant for washing the body and rejuvenating the skin (Dweck, 1996).



Figure 2.5 Cosmeceutical products from rooibos (*Aspalathus linearis*)

(<http://www.inheritanceskincare.com/>)

Due to the above mentioned side effects and poor skin penetration ability of the existing agents which are used for the treatment of hyper-pigmentation, it is imperative to find alternative agents from plants.

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CHAPTER 3

Description of selected of plants

3.1 Introduction

The traditional use of plants for the treatment of skin diseases is a common practice in many cultures. Most of the plants such as lavender, cucumber, chamomile etc are used in the modern phytocosmetics (Pieroni et al 2004). In the present study plants were selected to investigate their potential for depigmentation purpose. Forty plants were selected based on ethnobotany and phytochemical information. Plants such as *Ocimum basilicum* Lin var. *Pilosum* (wild) Beth, *Calodendrum capense* (L.F) and *Tanacetum vulgare* L. are known to be used traditionally for the treatment of skin diseases and for skin lightening.

The table below depicts a short description of each plant:

3.2 Description of the selected plants

Plant name		Description and uses
<i>Albezia harveyi</i> E. Forurn.		<i>A. harveyi</i> is about 10 to 15 m high. Young branches have a grey pubescence and leaves have a prominent gland on the upper side of the petiole. It occurs in Kenya, Tanzania, Zambia, and southward to Botswana and the Transvaal in deciduous Combretum. No medicinal properties recorded (Hyde and Wursten, 2008)
<i>Ocimum basilicum</i> Linn.var. <i>pilosum</i> (Willd.) Benth.		Basil is a tender low-growing herb that is grown as a perennial in warm, tropical climates. It is originally native to Iran, India and other tropical regions of Asia. Basil is most commonly recommended to be used fresh; in cooked recipes it is generally added at the last moment, as cooking quickly destroys the flavour. Scientific studies have established that compounds in basil oil have potent antioxidant hence anti-aging, anti-cancer, anti-viral, and antimicrobial properties. (Bozin et al 2006)

<i>Laurus nobilis</i> L.		herb often used to flavor soups, stews, and 6–12 cm long and 2–4 cm broad, with a characteristic finely serrated and wrinkled margin. Some evidence from the medical literature supports Bay having these uses: Antioxidative, analgesic and anti-inflammatory, Anticonvulsant (antiepileptic) (Simic et al, 2003).
<i>Bowkeria verticillata</i> (Eckl. & Zeyh.) Schinz		<i>B. verticillata</i> belongs to the family of Scrophulariaceae. Plants in this family are known to treat hemorrhoids and scrofula (a form of tuberculosis carried in the milk of the infected cows). http://montana.plant-life.org/families/Scrophulariaceae.htm
<i>Calodendrum capensis</i> (L.F.) thunb		<i>C. capense</i> is a member of the Rutaceae, the buchu & citrus family. It occurs along the south and east coast of Southern Africa from around Swellendam in the Western Cape through the Eastern Cape, KwaZulu-Natal, Mpumalanga, Gauteng, North West and Northern Province. The bark is used as an ingredient of skin ointments and is sold at traditional medicine markets (Leistner, 2000).
<i>Cassinopsis ilicifolia</i> Hochst.		<i>C. ilicifolia</i> is an evergreen, scrambling shrub that can grow up to four metres high and four metres wide. The plant grows naturally in Montane forest, and along streams from the southern and eastern Cape to the eastern Free State, Lesotho, Kwa-Zulu Natal, Swaziland, Gauteng, Mpumalanga and Zimbabwe. The bark of this plant is used medicinally by some Zulu people for treating dysentery (Venter and Venter, 1996).

<i>Cinnamomum verum</i> J. Presl		UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA vergreen tree 10–15 metres (32.8–49.2 feet) tall. It is native to southern China, India, Sri Lanka, Bangladesh, and Nepal. The bark is widely used as a spice due to its distinct odour. It is principally employed in cookery as a condiment and flavouring material. It is also used in the preparation of chocolate, especially in Mexico, which is the main importer of true cinnamon. (Agricultural magazine research, 2000)
<i>Callistemon citrinus</i> (Curtis) Skeels.		<i>C. citrinus</i> also called Lemon bottlebrush is a small tree or large shrub that gets 6-12 ft (2-4 m) tall and 6-9 ft (2-3 m) wide. The leaves are narrow, lance shaped, and leathery, with a distinctly citrus aroma. <i>Callistemon citrinus</i> is native to Australia. It may be used for a tropical effect in a pool side planting. http://en.wikipedia.org/wiki/Callistemon_citrinus
<i>Cryptocarya woodii</i> Engl		This is a small to medium size tree usually 5 to 10 m in height. The leaves are small shiny bright green above and slightly paler green below. It is widely distributed from the Eastern Cape, Kwa-Zulu Natal and Swaziland to the eastern side of Gauteng and further north on the eastern side of the continent. <i>Cryptocarya woodii</i> is said to be used as traditional medicine for magical and medicinal purposes. An infusion of the powdered bark is used as remedy for diarrhea (Mbambezeli, 2005).
<i>Murraya koenigii</i> (L.) Sprengel		It is a small tree, growing 4-6 m tall, with a trunk up to 40 cm diameter. Its leaves are highly aromatic and are used as a herb. Their form is small and narrow and they somewhat resemble the leaves of the Neem tree; therefore they are also referred to as <i>Kari Baavu</i> (translated to <i>Black Neem</i>) in the Kannada language. They are commonly used as seasoning in Indian and Sri Lanka. The leaves are also used as a herb in Ayurvedic medicine. Their properties include much value as an antidiabetic, antioxidant, antimicrobial, anti-inflammatory, and hepatoprotective, anti-hypercholesterolemia. http://en.wikipedia.org/wiki/Curry_Tree

<i>Dodonea angustifolia</i> L.F.		or small tree that occurs naturally from Arabia, as well as in Australia and New Zealand. Although naturally occurring in rocky areas it is also cultivated to stabilise moving sand and to prevent erosion. Extracts are used as medicine for fever, sore throats, chest complaints, influenza, stomach disorders and cancer (Agro forestry database) (http://en.wikipedia.org/wiki/Dodonaea_angustifolia).
<i>Dyschoriste fischeri</i> Lindau		It is a perennial shrub or dwarf shrub, growing to height of 1.75 m. It is distributed in southern Africa: Mpumalanga and Limpopo (Joffe, 2001).
<i>Euclea crispa</i> (Thunb.) Gürke subsp. <i>crispa</i> (Thunb.) Gürke		It is an evergreen shrub or small tree 1.8-9(-15) m. tall; bark grey-brown, red-brown, pale mauve-grey. It is widely distributed from Ethiopia and Sudan to South Africa and Namibia. Its antimicrobial activity has reported in a study done by Magama et al (2003)
<i>Euclea divinorum</i> Hiern		<i>E. divinorum</i> is a shrub or small tree up to about 6 m tall, often branching from the base or sometimes with a single stem. Bark is grey, fairly smooth in young trees but fissured in older specimens. It is native to: Botswana, Kenya, Namibia, South Africa, Swaziland, Tanzania etc. The roots are chewed to impart red colour to the mouth. The bark extract is used in dyeing baskets woven from <i>Hyphaene petersiana</i> (Mokola palm) leaves. Its branches are used as chewing sticks for oral care (Mebe et al 1998).

<i>Euclea natalensis</i> A.DC.		at reaches a maximum height of 10 m. The bark of young specimens is white, becoming darker and fissured with age. The roots of this plant are being used for curing bronchitis, pleurisy, chronic asthma and urinary tract infections by the Zulus. The roots are also used as a dye, for skin infections caused by <i>Mycobacterium leprae</i> and to relieve headache and toothache by the local inhabitants of South Africa (Lall et al 2006).
<i>Greyia flanaganii</i> (Bolus)		It is an evergreen shrub endemic to Eastern Cape. This plant is very frost tolerant. It is traditionally believed to have the ability to ward off sickness (Mambezeli et al 2002). During a study done by Bohn et al (1992) it was shown to contain a mixture of flavonoid compounds.
<i>Lantana montevidensis</i> (Spreng) Briq.		Weeping lantana is a low woody shrub growing to only 18-24 in (45-61 cm) high. It is native to south America, but is present nearly world wide as an introduced species. It is also kept as an ornamental plant for its plentiful colorful flowers. http://en.wikipedia.org/wiki/Lantana_montevidensis
<i>Levisticum officinale</i> L. Koch.		The leaves, seeds and fruits of Lovage (<i>Levisticum officinale</i>) are used to flavor food, especially in South European cuisine. Lovage tea can be applied to wounds as an antiseptic, or drunk to stimulate digestion. In the UK, Lovage cordial is traditionally mixed with brandy in the ratio of 2:1 as a winter drink. Lovage is second only to capers in its quercetin content. http://en.wikipedia.org/wiki/Lovage

<i>Macleaya cordata</i> - (Willd.)R.Br.		ng to 2 m by 1m at a fast rate. The whole plant is antioedemic, carminative, depurative and diuretic. The juice from the stems of the leaves is used to treat insect bites. A decoction of the leaves and stems is used in the treatment of ringworm. The poisonous sap is used to counter poisonous sores (Duke and Ayensu, 1985).
<i>Melianthus cosmos</i> (L)		It is a South African evergreen shrub often used in the gardens, with alternate, pinnate leaves often a foot long and having 9-t leaflets. The winged leaf petiole and the stipules are joined into one piece 2 inches long. (http://www.gardeningideas2you.com/homeandgardening/melianthus.html).
<i>Melianthus major</i> (L)		<i>Melianthus major</i> or Kruidjie-roer-my-nie, which means herb-touch-me-not, is a well known plant in the south Western Cape where it occurs naturally; usually along streams and roadside ditches. Although toxic when taken internally, it is used medicinally by the local people. They mostly use the leaves to make poultices and decoctions that are applied directly to wounds, bruises, backache and rheumatic joints (Van der walt, 2000).
<i>Pavonia praemorsa</i> (L.F) Cav.		<i>P. praemorsa</i> is a shrub that grows up to 7 feet belonging to the Malvaceae family. The flowers fade from orange to yellow as they open.http://www.smgrowers.com/products/plants/plantdisplay.asp?plant_id=1207
<i>Polygala myrtifolia</i> L		<i>P. myrtifolia</i> varies in form as it changes to adapt to different areas it grows in. It is an evergreen shrub, the most common forms reach about 1.8 m in height. It occurs naturally from the Bokkeveld Mountains near Clanwilliam in the Western Cape to Kwazulu-Natal. In KwaZulu-Natal it is one of the many plants known for its antibacterial, antimicrobial and antifungal properties. Tests run by the University of Natal at Pietermaritzburg have found that aqueous extracts of <i>P. myrtifolia</i> showed activity against <i>Candida albicans</i> (Van der walt, 2003).

<i>Psychotria capensis</i> (Eckl.) Vatke		University of Pretoria UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA ergreen shrub or small tree, 3-8 m, with a dense network of branches and pale brown bark. It is found on the eastern side of the country from Knysna through the Eastern Cape and KwaZulu-Natal to Mpumalanga and Limpopo. Medicinally <i>P. capensis</i> is used for gastric complaints and root infusions are taken to cause vomiting (Badenhost, 2004).
<i>Rhus undulate</i> Jacq.		It is an evergreen, dense shrub. Bark grey-brown; young branchlets covered in waxy scales; occasionally spiny, especially older branches. Rare in Namibia, but more widespread in South Africa. Have medicinal uses. In the study done by Fourie and Snackers (2003) they isolated a flavone from the roots of this plant which showed anti-inflammatory activity.
<i>Senecio medley-woodii</i> Hutch		<i>Senecio medley-woodii</i> belongs to the genus ‘<i>Senecio</i>’ and family ‘<i>Asteraceae</i>’. It has an upright woolly stem which is grayish to white and it has large yellow flowers. http://www.shoalcreeksucculents.com/newshop/index.php?categoryID=209
<i>Stachys tubulosa</i> macOwan.	No image	Belongs to the Lamiaceae family. Sometimes occurs as subshrubs or shrubs they can annual or perennial. Stems and branches are usually 4-angled. http://zipcodezoo.com/Plants/s/Stachys_tubulosa/Default.asp
<i>Thunbergia natalensis</i> Hook		It is a shrub up to 1m in height. The slender stems arise from a woody base and are mostly unbranched. The natural distribution is throughout the eastern regions of South Africa, from the Eastern Cape, through Kwazulu-Natal, Mpumalanga, Swaziland, Northern Province, Zimbabwe and north into east Africa. It has been reported that to ensure a happy marriage life a Zulu bride is given an infusion of the roots on her wedding day (Aubrey, 2001).

<i>Withania somnifera</i> (L.) Dunal.		UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA b that reaches a height of 170 cm. This herb adaptogen which is a nontoxic herb that works on a nonspecific basis to normalize physiological function, working on the neuroendocrine system. The roots and berries of the plant are used in herbal medicine. In Ayurveda, the fresh roots are sometimes boiled in milk, prior to drying, in order to leach out undesirable constituents. The Japanese patent applications are related to the use of the herb as a skin ointment and for promoting reproductive fertility. (http://www.medicinenet.com/ashwagandha_withania_somnifera-oral/article.htm)
<i>Becium obovatum</i> (E.Mey. ex Benth) N.E.Br		It is a low-growing herb, up to 300 mm in height. <i>Becium obovatum</i> plants are commonly found growing in grassland from the Eastern Cape, KwaZulu-Natal, and Free State to Limpopo in South Africa and from Botswana, Zimbabwe to eastern tropical Africa. These plants are reputedly used in African traditional medical practice, for stomach complaints in children as enemas, and generally as a hair-restorer (Mukoma, 2005)
<i>Coddia rudis</i> (E.Mey. ex Harv.) Verdc		It is multi-stemmed tree with arching branches and small, light green, glossy, spatulate leaves. It belongs to the Rubiaceae family. http://www.zimbabweflora.co.zw/speciesdata/species.php?species_id=155300
<i>Garuleum sonchifolium</i> (DC.) Norl.		Belongs to the Asteraceae family. It is one of eight genera of Calenduleae, with its center of diversity in Africa. Calendula is known in homeopathic remedies and as a common ornamental. http://en.wikipedia.org/wiki/Calenduleae

<i>Kniphofia pauciflora</i> (Dainty poker)		UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA consist of slender pokers with coral buds opening to flared tubular flowers of yellow for a long period. The species is a native of Natal and it belongs to the Asphodalaceae family.
<i>Metarungia pubinervia</i> (T. Anderson) Baden		It is also commonly known as the Red Sunbird Bush. It is perennial, with a rhizome, bulb, or corm, rarely shrubby or treelike. http://www.birdinfo.co.za/botanical/nectar/26_red_sunbird_bush.htm
<i>Salvia sonomensis</i> E. Greene.		It is a creeping flat perennial sage that can spread to 10 m across. Its flowers are blue-violet from May to June. It is native to dry slopes in the coast ranges and Sierra Nevada foothills. This plant is temperamental. At its best one plant will cover an area 10 by 15 feet, but the plant may die back to the crown in one day when conditions vary. http://www.laspilitas.com/nature-of-california/plants/salvia-sonomensis
<i>Chrysanthemum maximum</i> hort		This is small shrub belonging to the Asteraceae family. It is favored by most people due their white flowers. http://www.missouriplants.com/Whitealt/Chrysanthemum_maximum_page.html

<i>Asystasis gangetica</i> (L.)		UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA er, fast-growing, spreading, herbaceous stems from 300 mm to 600 mm in height. The stems root easily at the nodes. <i>A. gangetica</i> is widely distributed from tropical Asia to southern Africa. The subspecies found in South Africa differs from the Asian plant which usually has large pink flowers (Lithudza, 2004).
<i>Tanacetum vulgare</i> L.		Tansy is a flowering herbaceous plant with finely divided compound leaves and yellow, button-like flowers. Common tansy was used as a face wash and was reported to lighten and purify the skin (Lestrange, 1977)
<i>Hydrangea macrophylla</i> (Thunb.) Ser.		<i>Hydrangea macrophylla</i> is a species of <i>Hydrangea</i> native to Japan. Common names include Big leaf Hydrangea, French Hydrangea etc. <i>Hydrangea macrophylla</i> fit quite naturally into woodsy locations, snuggling under and around trees such They fit quite naturally into woodsy locations, snuggling under and around trees such as dogwoods and other shrubs. http://en.wikipedia.org/wiki/Hydrangea_macrophylla
<i>Eranthemum pulchellum</i> Andrews		This spreading shrub is exceptional for its rich blue flowers which occur in terminal spikes during the early winter season. The 3- to 6-inch-long flower spikes are borne above the large, rounded and prominently veined leaves. The plant even looks great in foliage without flowers. Blue Sage will attain a height of 4 to 6 feet and has a 1 to 3 foot spread. This plant may be used in mass color plantings and herbaceous borders. It is also striking as a specimen or container plant on a patio. (Gilman, 1999)

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CHAPTER 4

Antityrosinase activity of plants extracts

4.1 Introduction

Plants have been used traditionally for the treatment of different ailments. Tyrosinase inhibition is a common approach for depigmentation purposes. Due to side effects caused by chemical and fungal derived depigmenting agents, the search for new herbal compounds for the treatment of hyper-pigmentation is of great interest. The goal of the present study was to find or identify agents from plants, which can aid in the treatment of skin-pigmentation problems (Nerya et al, 2003; Curto et al, 1999; Halder et al, 2003).

Forty plants belonging to 22 families were selected for the present study, based on phytochemistry and ethnobotany. In some cases the family is known to be rich in phenolic compounds which have been previously known to have an inhibitory activity on tyrosinase enzyme where as in other instances some plants belonging to a particular family have been proved to have tyrosinase inhibitory activity. The hypothesis of the study is plants selected based on ethnobotanical information (plants used for skin-depigmentation) and based on phytochemistry should have tyrosinase inhibitory activity. The aim was to evaluate the activity of plants extracts on tyrosinase enzyme by using tyrosine as a substrate.

4.2 Materials and methods

4.2.1 Plants and chemicals

Plant materials were collected during December 2006 from the botanical garden of University of Pretoria. Plants were identified at H.G.W.J Schwelckerdt Herbarium of the University of Pretoria. All the samples were air dried, shielded from the sun, at

ambient temperatures. L-tyrosine, tyrosinase, kojic acid, hydroquinone were obtained from Sigma-Aldrich (South Africa).

4.2.2 Extraction

The air dried plant materials (leaves) were grounded to a fine powder. Twenty grams of powdered plant material were each extracted with 200 ml absolute ethanol. Mixtures were left overnight on a shaker at room temperature and then filtered through a Whatman filter paper (15cm). Extraction was repeated three times for each extract and the solvent was evaporated under a vacuum (BUCHI, Rotavapor, R-200) to yield dry extracts (figure 4.1).



Figure 4.1 BUCHI Rotavapor (R-200) used for preparation of extracts

4.2.3 Colorimetric tyrosinase inhibition assay

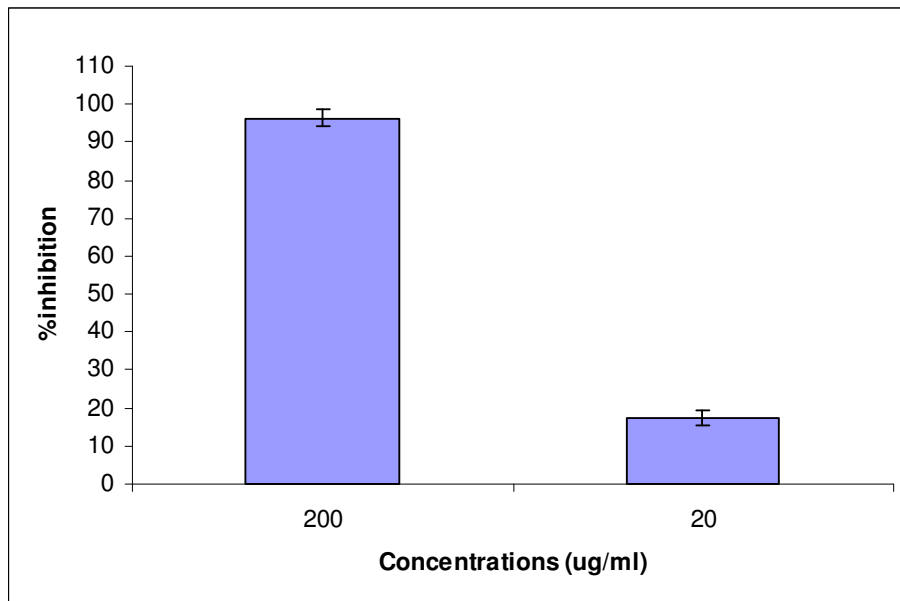
This assay was performed using methods described by Curto et al (1999) and Nerya et al (2003). Extracts were dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 20 mg/ml (stock solution). This stock solution was then diluted to 600 µg/ml in 50 mM potassium phosphate buffer (pH 6.5). Kojic acid was used as a control drug (Lee et al 1997). In a 96 well microtitre plate, 70 µl of each sample solution of different concentrations were combined with 30 µl of tyrosinase (333 Units/ml in phosphate buffer, pH 6.5) in triplicates. After incubation at room temperature for 5 minutes, 110µl of substrate (2 mM L-tyrosine) was added to each well. Final concentrations of the extracts were 200 and 20 µg/ml as this was a screening test to select the plant with good antityrosinase activity. Final concentration of positive controls ranged from 400 to 3.13 µg/ml. Microtitre plates were incubated for 30 min at room temperature. Optical densities of the wells were then determined at 492 nm with the BIO-TEK power Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa).

4.3 Statistical analysis

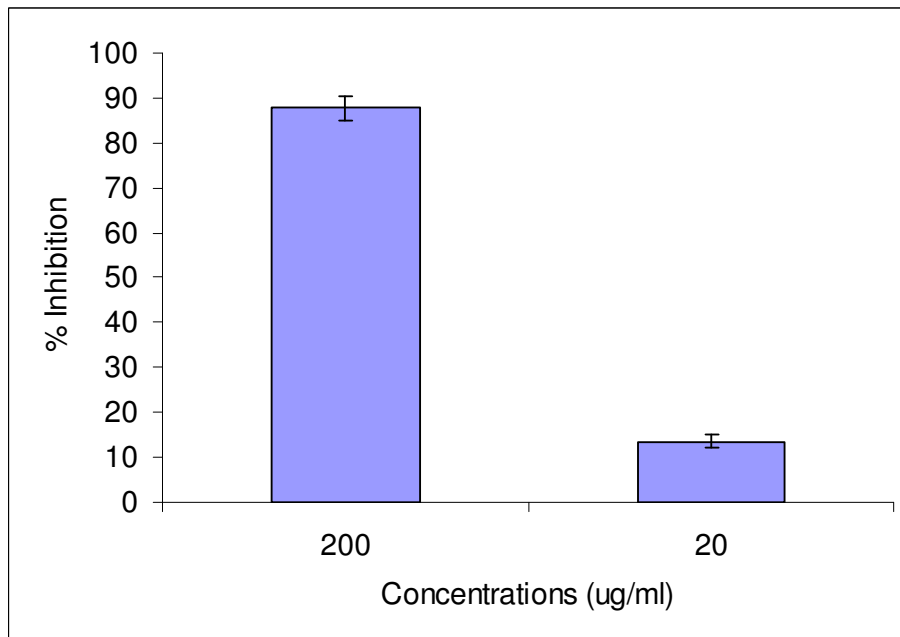
The results were analyzed statistically using one-way analysis of variance (ANOVA) and the least significant differences ($P < 0.05$) were determined according to Duncan's t-test. Results were significant where $P < 0.05$.

4.4 Results and discussions

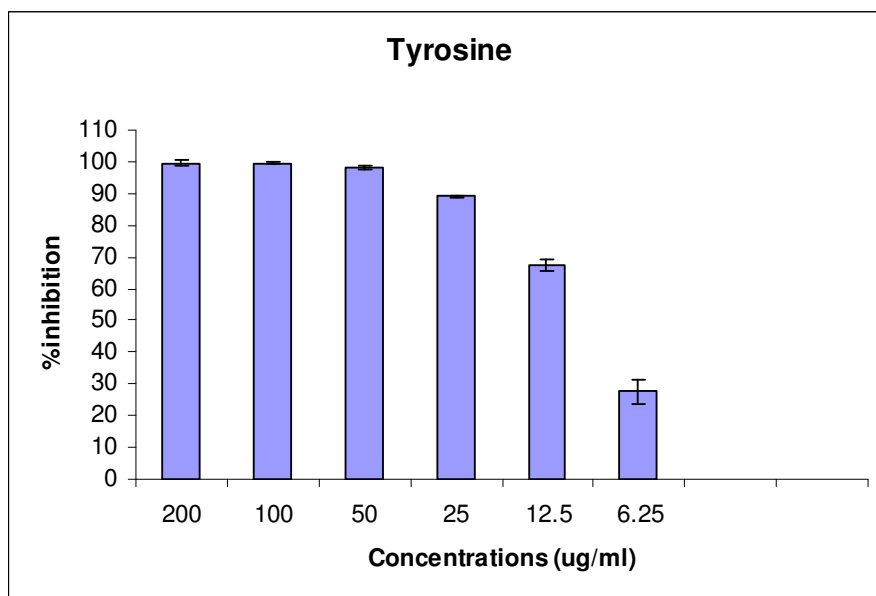
Ethanol extracts of 40 plants were tested for their tyrosinase activity using L-tyrosine as the substrate. The results showed that the plants tested in this study belonging to Acanthaceae, Asteraceae, Asphodelaceae, Apiaceae, Icacinaceae, Lauraceae, Lamiaceae, Myrtaceae, Papaveraceae, Polygalaceae, Solanaceae and Verbenaceae families did not show any significant inhibitory activity of the enzyme even at the highest concentrations tested when tyrosine was used as the substrate. Plants which belonged to Mimosaceae, Anacardiaceae, Ebenaceae, Melianthaceae, Malvaceae, Rubiaceae, Rutaceae, Sapindaceae, Scrophulariaceae and Greyiaceae showed some inhibitory activity of the enzyme at 200 µg/ml. *Greyia flanaganii* showed significant ($P < 0.05$) inhibitory activity of the enzyme exhibiting 95% inhibition at 200 µg/ml (figure 4.2A). *Euclea divinorum* also exhibited 85% tyrosinase inhibition at 200 µg/ml (figure 2.2B). *Bowkeria verticillata* (Eckl. & Zeyh.) Schinz, *Melianthus cosmos* (L.) and *Pavonia praemorsa* (L.F) Cav exhibited 52, 51 and 56% inhibition at 200 µg/ml (figure 4.2 D, E, and F). Results of extracts that exhibited less than 50% inhibition of the enzyme at 200 µg/ml are represented in table 2.1.



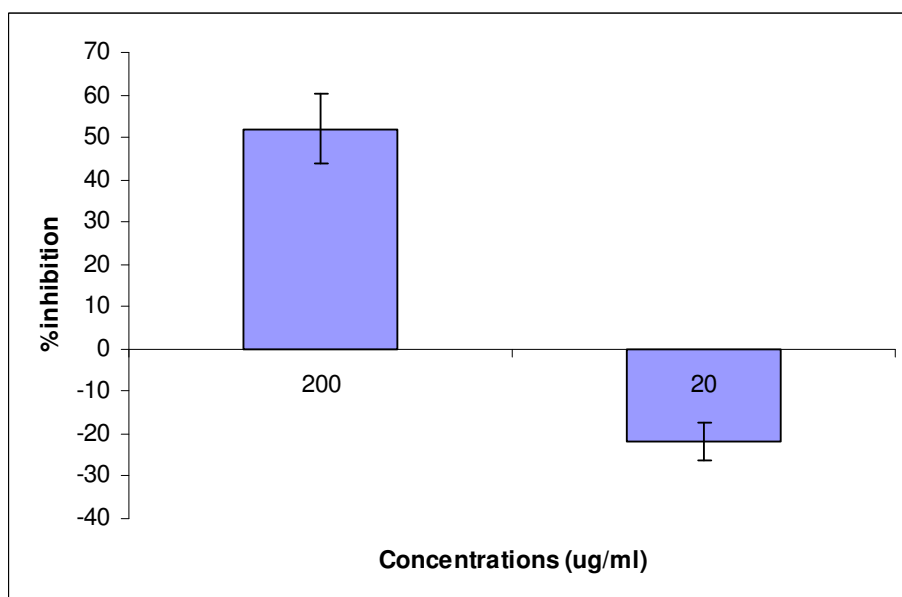
(A)



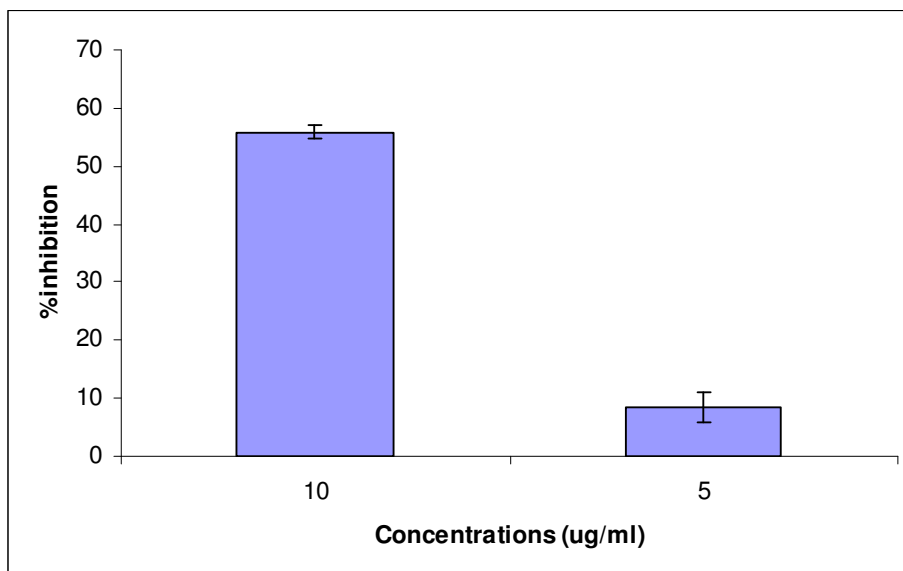
(B)



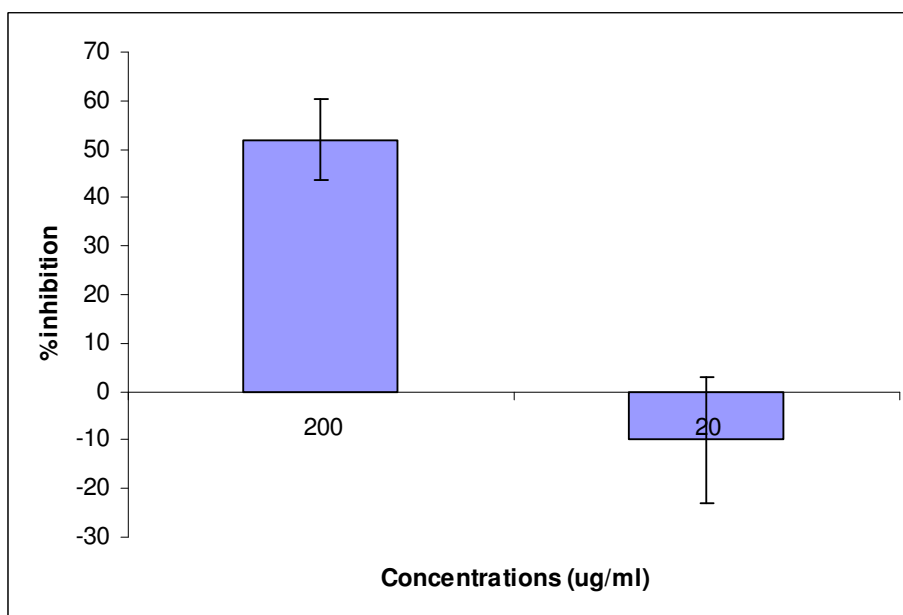
(C)



(D)



(E)



(F)

Figure 4.2 Antityrosinase activity of *Greyia flanaganii* (A), *Euclea divinorum* (B), kojic acid (C), *Bowkeria verticillata* (D), *Melianthus cosmos* (F) and *Pavonia praemorsa* (E) when tyrosine was used as a substrate

Table 4.1 Antityrosinase activity of plant extracts when L-tyrosine was used as a substrate

No	Scientific name	Family	Part used	% Inhibition of Tyrosinase (200 µg/ml)	% Inhibition of Tyrosinase (20 µg/ml)	Voucher specimen no
1	<i>Albezia harveyi</i> E. Fourn.	Mimosaceae	Leaves	23.08		MBM 123
2	<i>Asystasis gangelica</i>	Acanthaceae	Leaves	*	-	I Felton 40
3	<i>Metarungia pubinervia</i>	Acanthaceae	Leaves	*	-	MBM 125
4	<i>Thunbergia natalensis</i> Hook.	Acanthaceae	Leaves	*	-	H.P.V.D. Schyff 5895
5	<i>Hydrangea macrophylla</i>	Acanthaceae	Leaves	*	-	MBM 126
6	<i>Eranthemum pulchellum</i>	Acanthaceae	Leaves	*	-	MBM 127
7	<i>Dyschoriste fischeri</i>	Acanthaceae	Leaves	*	-	A.E Van Wyk 13091
8	<i>Chrysanthemum maximum</i> hort	Asteraceae	Leaves	*	-	MBM 128
9	<i>Senecio medley-woodii</i> Hutch	Asteraceae	Leaves	*	-	A. Abott 2583
10	<i>Garuleum sonchifolium</i> (DC.) Norl.	Asteraceae	Leaves	*	-	MBM 129

11	<i>Tanacetum vulgare</i> (Tansy)	Asteraceae	Leaves	*	-	MBM 130
12	<i>Kniphofia pauciflora</i>	Asphodelaceae	Leaves	*	-	S.P Bester 28
13	<i>Levisticum officinale</i> (Lovage)	Apiaceae	Leaves	*	-	MBM 131
14	<i>Searsia undulata</i>	Anacardiaceae	Leaves	32.69	*	R. Allardice 1516
15	<i>Euclea crispa</i> (Thunb)	Ebenaceae	Leaves	*	-	P. Van Wyk 2139
16	<i>Euclea divinorum</i>	Ebenaceae	Leaves	85.90	12	N. Hahn 188
17	<i>Euclea natalensis</i>	Ebenaceae	Leaves	*	-	N. Hahn 152
18	<i>Cassinopsis ilicifolia</i> Hochst.	Icacinaceae	Leaves	*	-	B. De Winter 9441
19	<i>Laurus nobilis</i> (Bay)	Lauraceae	Leaves	*	-	MBM 132
20	<i>Cryptocarya wodii</i> Engl	Lauraceae	Leaves	*	-	P. Van Wyk 743
21	<i>Cinnamomum verum</i>	Lauraceae	Leaves	*	-	MBM 133
22	<i>Becium obovatum</i> (E.Mey. ex Benth)	Lamiaceae	Leaves	*	-	MBM 134
23	<i>Salvia sonomensis</i>	Lamiaceae	Leaves	*	-	MBM 135
24	<i>Stachys tubulosa</i>	Laminaceae	Leaves	*	-	P.P Swartz 43
25	<i>Ocimum basilicum</i> (Basil)	Lamiaceae	Leaves	*	-	M. Nel 8

26	<i>Melianthus cosmos</i> (L)	Melanthaceae	Leaves	51.92	*	A.E Van Wyk 8842
27	<i>Melianthus major</i> (L)	Melanthaceae	Leaves	16.03	-	E.J Du Plessis 212
28	<i>Pavonia praemorsa</i> (L.F) Cav.	Malvaceae	Leaves	55.77	8	A.E van Wyk 5834
29	<i>Callistemon citrinus</i>	Myrtaceae	Leaves	*	-	MBM 136
30	<i>Macleaya cordata</i> - (Willd.)R.Br.	Papaveraceae	Leaves	*	-	MBM 136
31	<i>Polygala myrtifolia</i> L	Polygalaceae	Leaves	*	-	P. Van Wyk 279
32	<i>Psychotria capensis</i> (Eckl.) Vatke	Rubiaceae	Leaves	*	-	P. Van Wyk 1262
33	<i>Coddia rudis</i> (E.Mey. ex Harv.) Verdc	Rubiaceae	Leaves	43.59	*	A.E Van Wyk 12423
34	Curry tree	Rutaceae	Leaves	*	-	MBM 137
35	<i>Calodendrum capensis</i> (L.F.) thunb	Rutaceae	Leaves	*	-	A.E Van Wyk 3002
36	<i>Dodoneae angustifolia</i>	Sapindaceae	Leaves	32.05	*	N. Hahn 320
37	<i>Bowkeria verticillata</i> (Eckl. & Zeyh.)	Scrophulariaceae	Leaves	52.56	*	A.E Van Wyk 7099
38	<i>Withania somnifera</i>	Solanaceae	Leaves	*	-	F. Du Plessis 97
39	<i>Lantana montevidensis</i>	Verbenaceae	Leaves	*	-	MBM 138
40	<i>Greyia flanaganii</i> Bolus	Greyiaceae	Leaves	95	20	P. Van Wyk 2274

*= no inhibition at the highest concentration tested 200 µg/ml, -=not tested

4.5 Conclusions

Euclea divinorum and *Greyia flanaganii* exhibited the best inhibitory activity of the tyrosinase enzyme. *E. divinorum* has been studied and evaluated for many biological activity earlier (antibacterial, TB etc) but antityrosinase activity has never been reported before. No biological activities have been reported before on *G. flanaganii* (our plant of interest). *G. flanaganii* belongs to the family of Greyiaceae. There are three species that are available in this family, *G. flanaganii*, *G. sutherlandii* and *G. radlkofen*. This family has been investigated before phytochemically and it has been found that it contains a lot of flavonoids compounds. Flavonoids are a group of compounds which have been proven to have antityrosinase and antioxidant activity. Having obtained good antityrosinase activity using tyrosine as a substrate it was decided to investigate the ethanol extract of *G. flanaganii* for antityrosinase activity using L-dopa as the substrate to check if the extract inhibits both the monophenolase and diphenolase activity of the enzyme. Further it was decided to evaluate toxicity (to check if a decrease in melanin production is due to a decreasing number of melanocytes cells or the inhibition of the enzyme itself) and antioxidant activity (since melanin formation proceeds through a free radical mechanism, it could be disrupted by selective antioxidant, potent enough to inhibit this reaction).

4.6 References

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CHAPTER 5

Antityrosinase activity of *Greyia flavaganii* (Bolus)

5.1 Introduction

Inhibition of tyrosinase is one of the major strategies employed to treat hyperpigmentation disorders. Limitations are associated with many of these inhibitors, such as high cytotoxicity, poor skin penetration and low stability in formulations (Nerya et al, 2004). During screening of different plant extracts in this study for tyrosinase inhibition it was found that *Greyia flanaganii* exhibited the best inhibitory activity of the tyrosinase enzyme when tyrosine was used as the substrate. Therefore, it was selected for further investigation for its efficacy of tyrosinase inhibition. *G. flanaganii* was chosen based on its chemical components i.e. it is known to be rich in flavonoids compounds which are a group of compounds that are well known/studied to be tyrosinase inhibitors. The ethanol extract of *G. flanaganii* was evaluated at different concentrations for its antityrosinase activity by using both L-tyrosine and L-DOPA as substrates. The extract was also investigated for its antioxidant activity.

‘Acne vulgaris’ is a chronic inflammatory follicular disorder of the skin that affects areas containing the largest oil glands including face, neck, back and chest. It affects up to 80% of adolescents to some extent and less commonly occurs in infants (Shaw and Kennedy, 2007). *Propionibacterium acnes* is an obligate anaerobic gram-positive bacterium that is associated with or linked to ‘Acne vulgaris’. It is largely commensal and is present on most people’s skin and live on fatty acids in the sebaceous glands on sebum secreted by pores. The accumulation of bacteria (*Propionibacterium acnes* which feeds on the clogs in the hair follicle) in acne could trigger our body's defense mechanism. The white blood cell antibodies would enter the clogged up follicle and kill off the bacteria. Only in the process of destroying the bacteria, the antibodies may severely affect other skin

organisms and cells (e.g. melanocytes) and may cause the formation of free radicals (ROS). ROS (Reactive Oxygen Species) causes enhanced melanin biosynthesis, DNA damage and induced proliferation of melanocytes (Wang et al, 2006). This is how scars (hyper-pigmentation of the skin) develop.

Antibiotics are usually employed to inhibit inflammation of the skin or kill bacteria. Examples of commercially available antibiotics include triclosan, benzoyl peroxide, azeliac acid, erythromycin, retinoid, clindamycin etc (Park et al, 2004). However these antibiotics have been known to induce side effects. Benzoyl peroxide and retinoids have been reported in the study done by Zesch (1988) that they bring about xerosis cutis and skin irritation if they are used excessively as treatment. Wawruch et al (2002) reported that tetracycline, erythromycin, macrolide and clindamycin showed several side effects such as appearance of resistant bacteria organ damage and immunohypersensitivity if they are taken for a long time. The effect *Greyia flanaganii* (Bolus) against acne causing bacteria, *Propionibacterium acnes* was investigated using microdilution assay.

5.2 Materials and Methods

5.2.1 Materials and chemicals

Plant material was collected from the botanical garden of the University of Pretoria in June 2007. Identification was done at the H.G.W.J Schwelckerdt herbarium (PRU) of the University of Pretoria. Tyrosinase, Kojic acid and hydroquinone (positive controls), L-tyrosine and L-Dopa were purchased from Sigma-Aldrich. Foetal calf serum (FCS), trypsin, L-glutamine and penicillin/streptomycin/fungizone, were purchased from

Highveld Biological. The cell proliferating kit II (XTT) (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitrobenzene sulfonic acid hydrate) labelling reagent) was purchased from Roche Diagnostics II. Vitamin C and 1,2-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Highveld Biological.

5.2.2 Extraction

The ethanol extract was prepared as mentioned in chapter 4 section 4.4.1.

5.2.3 Colorimetric tyrosinase inhibition assay

Tyrosinase inhibition assay was performed as described in chapter 4 (section 4.4.3). The concentration of the extract ranged from 3.9 to 500 µg/ml. while positive controls were tested in concentrations ranging from 1.56 to 200 µg/ml. The IC₅₀ values were determined by the use of MSTATC software (East Lansing, MI, US).

5.2.4 Melanogenesis of *Greyia flanaganii* (Bolus)

5.2.4.1 Preparation of melanocytes cell cultures

Mouse melanocyte cell line, B16-F10, was cultured in complete Minimal Essential Eagle's Medium (MEM) containing 10% foetal bovine serum, 1.5 g/L NaHCO₃, 2mM L-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were sub-cultured in a ratio of 1:3 on every third or fourth day.

5.2.4.2 Counting of cells using a hemacytometer

A 1:10 dilution of the cell suspension in trypan blue solution was prepared (e.g. 10 μ l cells in 90 μ l trypan blue) in an eppendorf tube. About 10 μ l of this dilution was transferred in to the two chambers of the hemacytometer. A hand-held tally was used to count the cells under the microscope. The number of cells was counted in the number of squares.

Cell concentration was determined by using the following formula:

$$\begin{aligned}\text{Cell concentration (cell suspension)} &= \# \text{ cells counted per square} \times 10 \times 10000 \\ &= \text{cells per ml}\end{aligned}$$

$$\text{Cell number (cell suspension)} = \text{cell concentration} \times \text{volume of cell suspension}$$

$$\begin{aligned}\text{Volume (cell suspension)} &= \frac{\text{Cell concentration wanted} \times \text{volume wanted}}{\text{Concentration of cells in suspension}}\end{aligned}$$

5.2.4.3 Determination of extract toxicity and melanin content in melanocytes

Mouse melanocytes (B16-F10) cells in complete MEM medium were dispensed into the wells of a 96-well plate (10^4 cells per well) and 24-well plate (10^5 cells per well). After an overnight incubation at 37 °C in 5% CO₂ and a humidified atmosphere, extract sample and positive controls were added to the cells to the final concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.13 μ l/ml. Plates were incubated at 37 °C in 5% CO₂, humidified atmosphere for three days. Toxicity effect of the extracts on the B16-F10 cells was assayed using XTT (sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitrobenzene sulfonic acid hydrate) cytotoxicity assay. Fifty microlitres of

XTT reagent (1 mg/ml XTT with 0.383 mg/ml PMS) was added to the wells and incubated for 1 h. The optical densities of the wells were measured at 450 nm (690 nm reference wavelength). By referring to the control (medium with DMSO), cell survival was assessed. The effect of the plant extracts on melanin synthesis was determined by washing the melanocytes cells in the 24-well plate with potassium phosphate buffered saline (PBS), and lysing with 200 μ l of sterile distilled water (dH_2O). Optical densities were determined at a wavelength of 405 nm. The effect on melanin production was determined by referring to the control sample (medium with DMSO). The absorbances of a series of known concentrations of pure melanin were used to construct a calibration curve to determine the amount of melanin produced by the cells. The IC_{50} values were determined by the use of MSTATC software (East Lansing, MI, US).

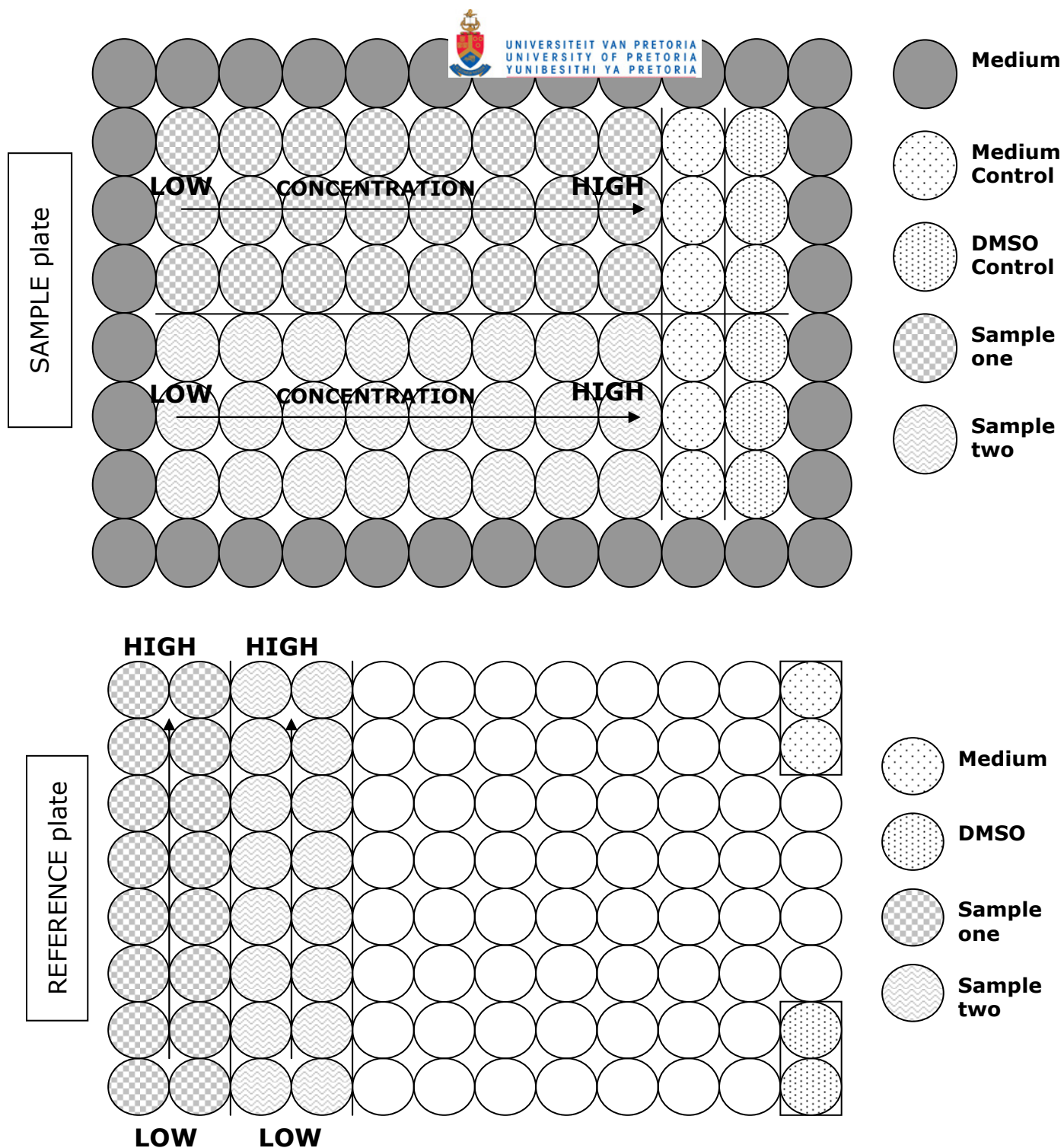


Figure 5.1 Plates layout for the investigation of cytotoxicity of the extract on melanocytes cells

5.2.5 Antioxidant activity

Since melanin formation beneath the skin proceeds through a free radical mechanism, it could be disrupted by selective use of antioxidants, potent enough to poison this reaction (Momtaz et al 2008). Antioxidant activity of *Greyia flanaganii* (Bulus) was investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, South Africa) antioxidant assay. Following the procedures as described by Toit et al (2001) for each sample, a dilution series (8 dilutions) was prepared in a 96-well ELISA plate by adding distilled water as a dilution medium. Final concentrations of the ethanolic extract of *Greyia flanaganii* ranged from 2.0 to 250 µg/ml. Each concentration was tested in triplicates. Vitamin C was used as the positive control and was tested in concentrations ranging from 0.78 to 100 µg/ml. Ninety microlitres (90 µM) of ethanolic DPPH was added to each well. The plates were covered with aluminium foil and incubated at room temperature for 30 minutes before being read by BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa). The radical scavenging capacities of the samples were determined using a BIOTEK plate reader to measure the disappearance of DPPH at 550 nm. The radical scavenging activity was measured in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC₅₀) (Toit et al, 2001). The EC₅₀ value for each was determined graphically by plotting the absorbance of DPPH as a function of the sample concentration in µg/ml. The EC₅₀ is the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50%. The results are expressed as the mg Vit C equivalents/g dry weight and are calculated as follows:

$$\text{VitEAC (mg AA/100g)} = (\text{EC}_{50} (\text{vit c}) / \text{EC}_{50} (\text{sample})) \times 1000$$

5.2.4 Antibacterial activity of ethanol extract of *Greyia flanaganii* against *Propionibacterium acnes*

5.2.4.1 Bacteria

The antibacterial activity of *G. flanaganii* was investigated against *Propionibacterium acnes* (ATCC 11827) which was purchased from ANATECH Company South Africa. The bacteria was cultured from a Kwik-Stick on nutrient agar and incubated at 37°C for 72 h under anaerobic conditions before the assay.

5.2.4.2 Microdilution assay

The minimal inhibitory concentration (MIC) of the ethanolic leaf extract of *G. flanaganii* was determined by microdilution assay. This assay was done using methods as described by McGaw and Eloff (2005) and Sahin et al (2003) with slight modifications. The bacteria was cultured from a Kwik-Stick on nutrient agar and incubated at 37°C for 72 h under anaerobic conditions before the assay. The extract and positive control (erythromycin) were dissolved in 10% DMSO to a stock solution of 1mg/ml. The 72 h culture of the bacteria was dissolved in ringer's solution and the suspensions were adjusted to 0.5 McFarland standard turbidity. This resulted in 10^7 - 10^8 CFU/ml. The 96 well plates were prepared by dispensing into each well 100 µl of nutrient broth. A 100 µl of the samples initially prepared at 1 mg/ml was added to the first wells. Then 100 µl of their serial dilutions were transferred into eight consecutive wells. Erythromycin was used as the positive control (Lim et al, 2007). About 100 µl of bacterial inoculum was then added to all the wells. The plates were then covered with parafilm and incubated at 37°C for 72 h in an anaerobic condition. The minimum inhibitory concentration (MIC)

value was determined by observing the colour change in the wells after addition of *p*-iodonitrotetrazolium violet salt (INT) (defined as the lowest concentration that showed no bacterial growth).

5.3. Statistical analysis

The results were analyzed statistically using one-way analysis of variance (ANOVA) and the least significant differences ($P < 0.05$) were determined according to Duncan's t-test. Values were determined to be significant were P (probability) was less than 0.05 ($P < 0.05$).

5.4 Results and discussions

5.4.1 Antityrosinase activity of plant extracts using L-tyrosine and L-DOPA as the substrates

Ethanollic leaf extract of *Greyia flanaganii* and two positive controls (hydroquinone and kojic acid) were tested on their activity for tyrosinase activity using L-tyrosine as the substrate. *Greyia flanaganii* showed high % inhibition of the enzyme with the IC_{50} of 32.68 ± 0.5 $\mu\text{g/ml}$ as compared to the two controls used (kojic acid and hydroquinone) exhibiting IC_{50} of 7.02 ± 0.03 $\mu\text{g/ml}$ and 13.47 ± 0.1 $\mu\text{g/ml}$ respectively (monophenolase activity) (figures 5.3 A, B and C). Samples were further tested for their effect on tyrosinase using L-DOPA as the substrate. *G. flanaganii* exhibited 24% inhibition of the enzyme at 200 $\mu\text{g/ml}$. Hydroquinone also exhibited no significant inhibition of the enzyme. Kojic acid showed an IC_{50} value of 19.46 $\mu\text{g/ml}$ when L-DOPA was used as the substrate (figure 5.4 A, B and C). Inhibition of the enzyme is represented

by the clear wells in the microtitre plate as compared to the wells which shows orange to reddish colour due to melanin production (figure 5.2).

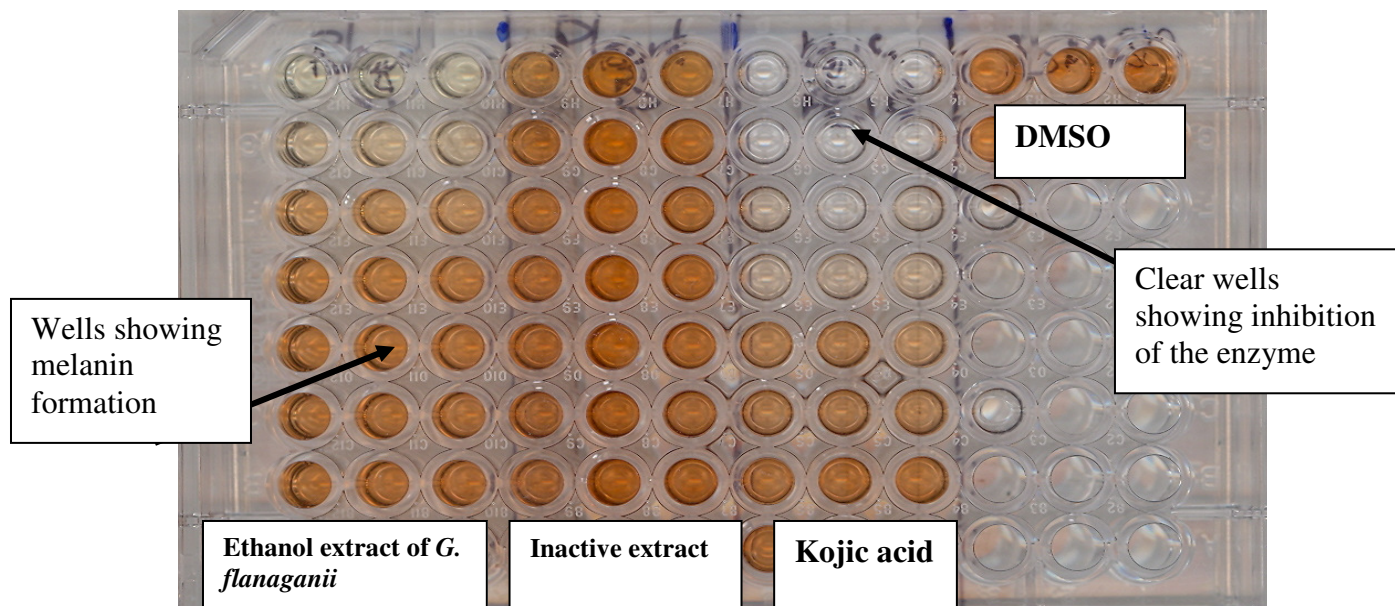
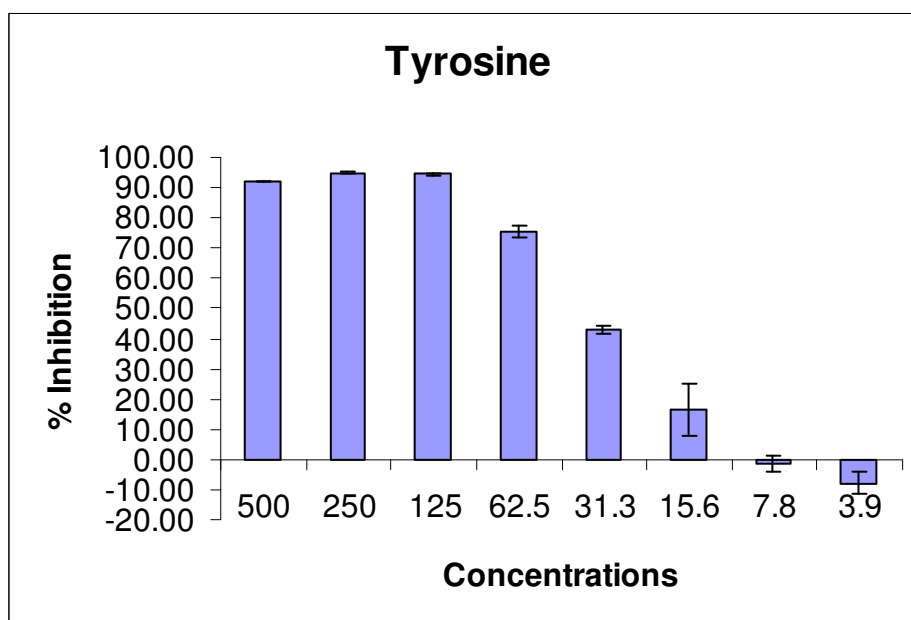
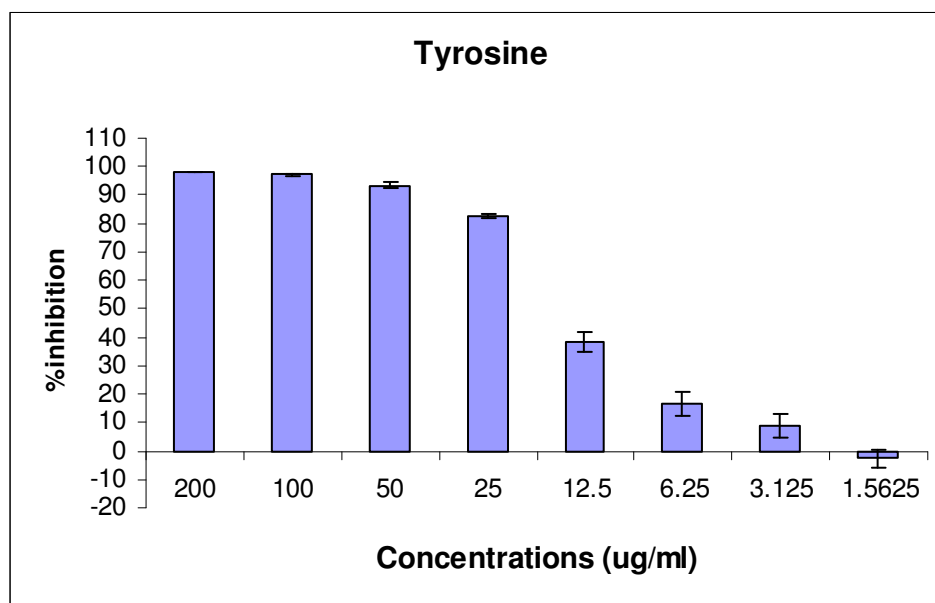


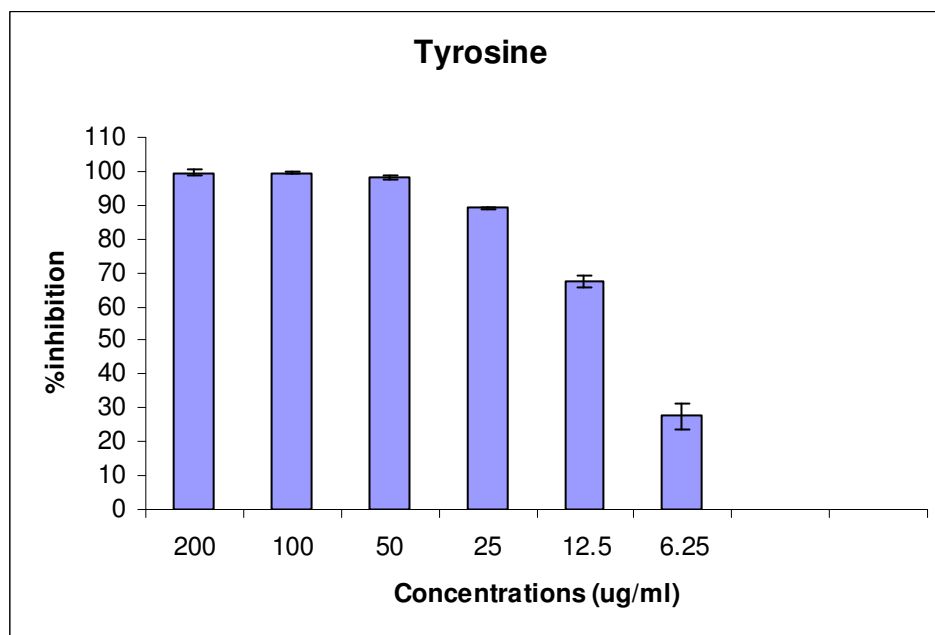
Figure 5.2 Antityrosinase activity of plant extracts and positive control



(A)

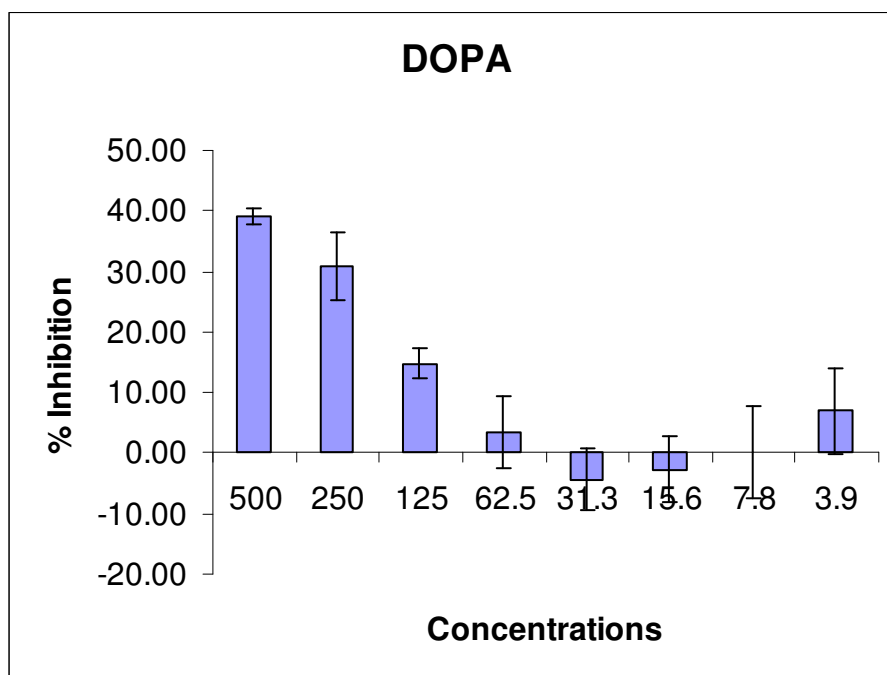


(B)

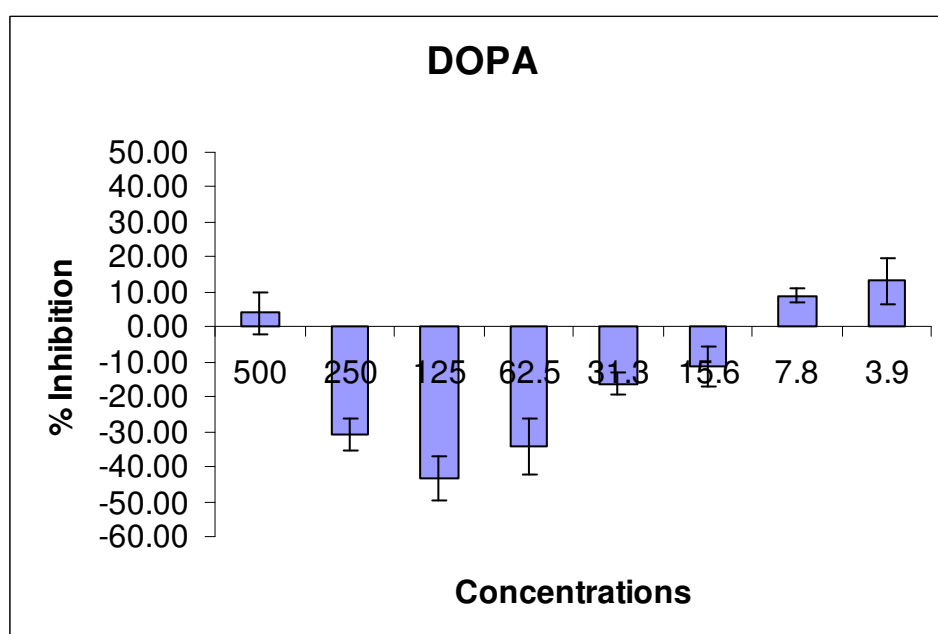


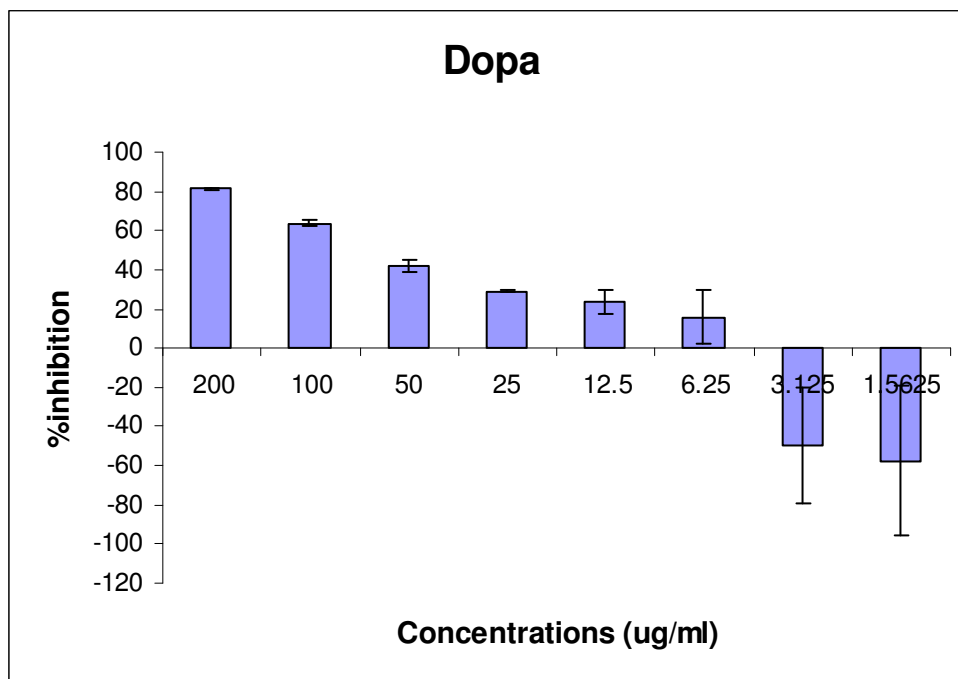
(C)

Figure 5.3 Antityrosinase activity of ethanolic leaf extract of *Greyia* *flanaganii* (A), Hydroquinone (B), and kojic acid (C) when tyrosine was used as a substrate



(A)





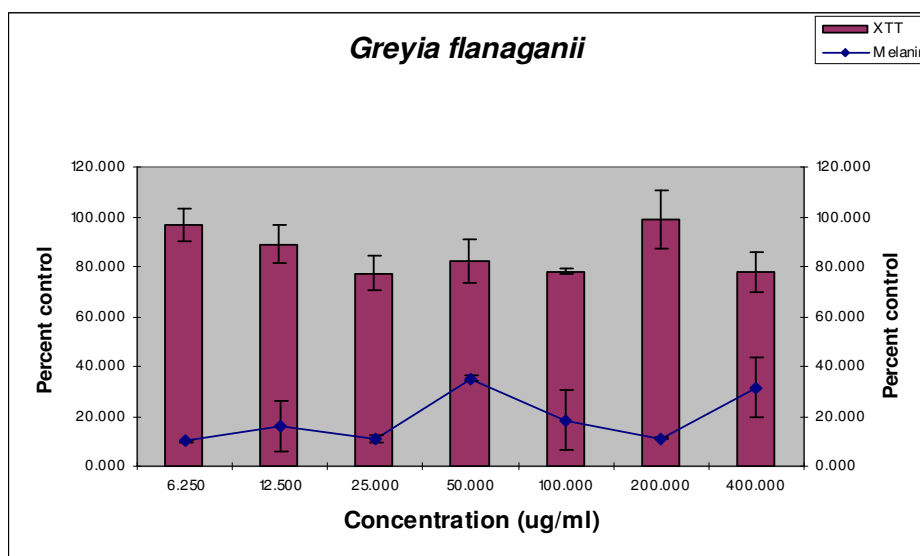
(C)

Figure 5.4 Antityrosinase activity of ethanolic leaf extract of *Greyia flanaganii* (A), Hydroquinone (B), and kojic acid (C) when L-DOPA was used as a substrate

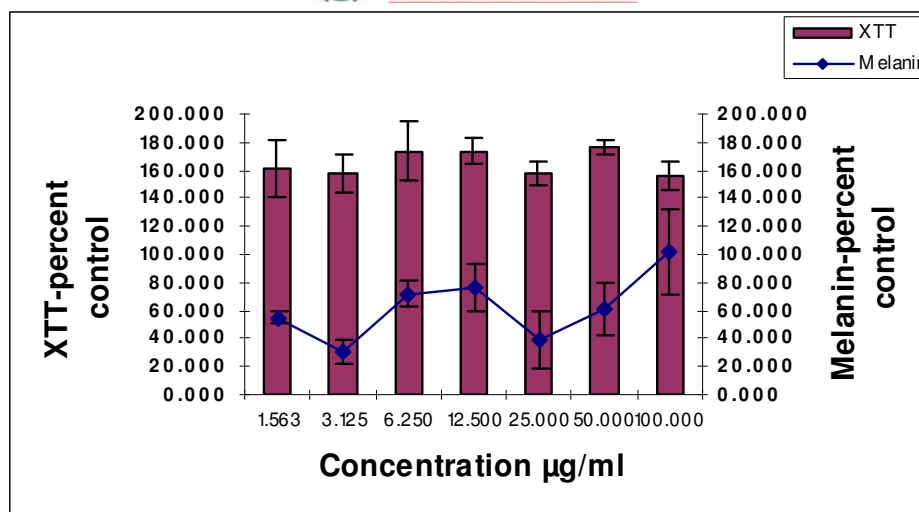
5.4.2 Cytotoxicity of plant extracts and their effect on melanogenesis

It was decided to test the effect of the extract of *G. flanaganii* on melanocytes cells for investigating its cytotoxicity as well as melanogenesis effect *in-vitro*. To relate whether the reduction in melanin observation is caused by enzyme inhibition or a decrease in cell number, it was imperative to perform toxicity assay in conjunction with melanin biosynthesis or production assay. The concentrations of Kojic acid ranged from 100-1.56

$\mu\text{g/ml}$ while for *G. flanaganii* concentrations ranged from 400-6.25 $\mu\text{g/ml}$. Figure 5.5 shows the effect of *G. flanaganii* (ethanolic extract) and kojic acid on cell viability/proliferation and melanin production in B16F10 cells. *G. flanaganii* showed significant inhibition ($P < 0.05$) (20%) of melanin production at 6.25 $\mu\text{g/ml}$ while 80% of cells were found to be viable, thus indicating low levels of cytotoxicity ($\text{IC}_{50} < 400 \mu\text{g/ml}$). Kojic acid showed no toxicity effect on the cells at the highest concentrations tested and exhibited about 60% reduction in melanin content of the cells at 3.1 $\mu\text{g/ml}$ (figure 5.5b).



(A)



(B)

Figure 5.5 The effect of *Greyia flanaganii* (A) and Kojic acid (B) on cell viability and melanin production

5.4.3 Antioxidant activity of plant extract

Ethanollic leaf extract of *G. flanaganii* was tested for its scavenging capacity using the DPPH method with Vitamin C as the positive drug. The colour formation of the extract and DPPH shows the balance between antioxidants and free radicals, this reaction is measured with the multiwell plate reader. Yellowish to clear wells represents antioxidants in excess where the sample or extract concentration is high and purple to pinkish represents free radicals in excess (figure 5.6). *G. flanaganii* exhibited above 90% radical scavenging activity at 200 µg/ml (figure 5.7a). Vitamin C showed better antioxidant activity than the extract of *G. flanaganii*. Table 5.1 represents the EC₅₀ (concentration of the extract which decreases the initial DPPH absorbance by 50%) values for the ethanollic extract of *Greyia flanaganii*. Results are also represented in mg vitamin C equivalents.

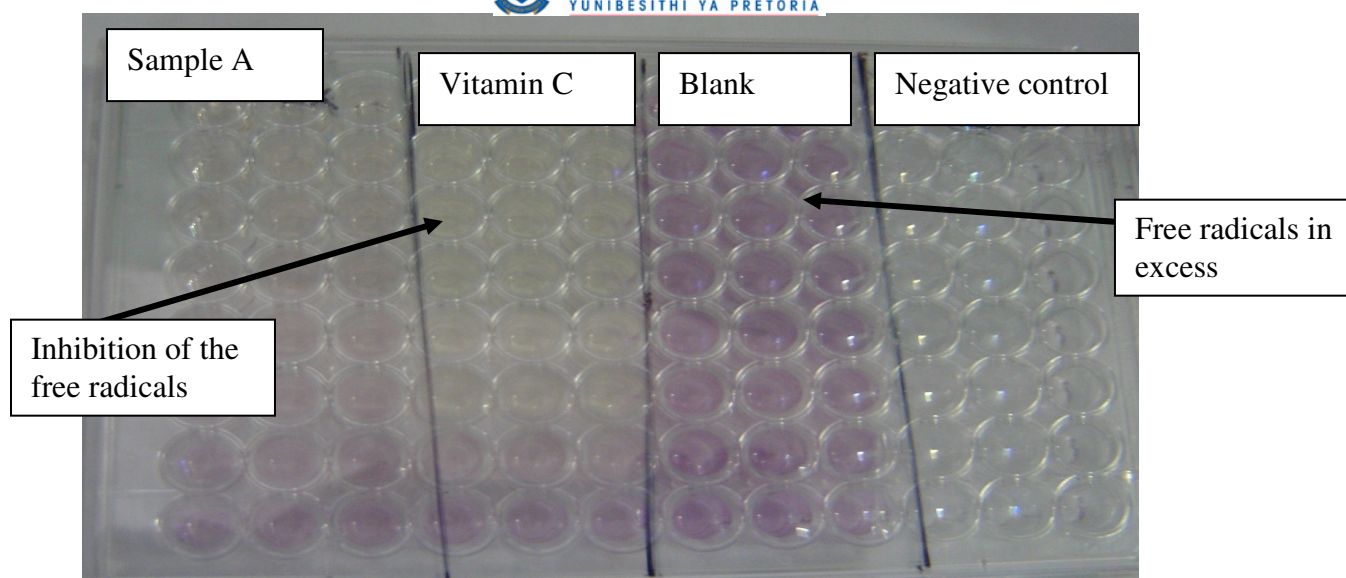
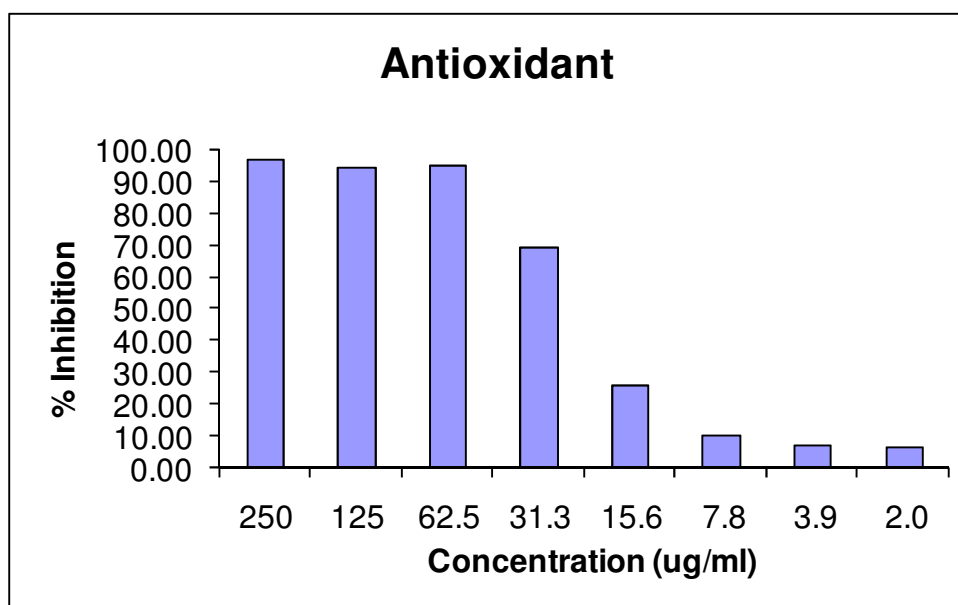
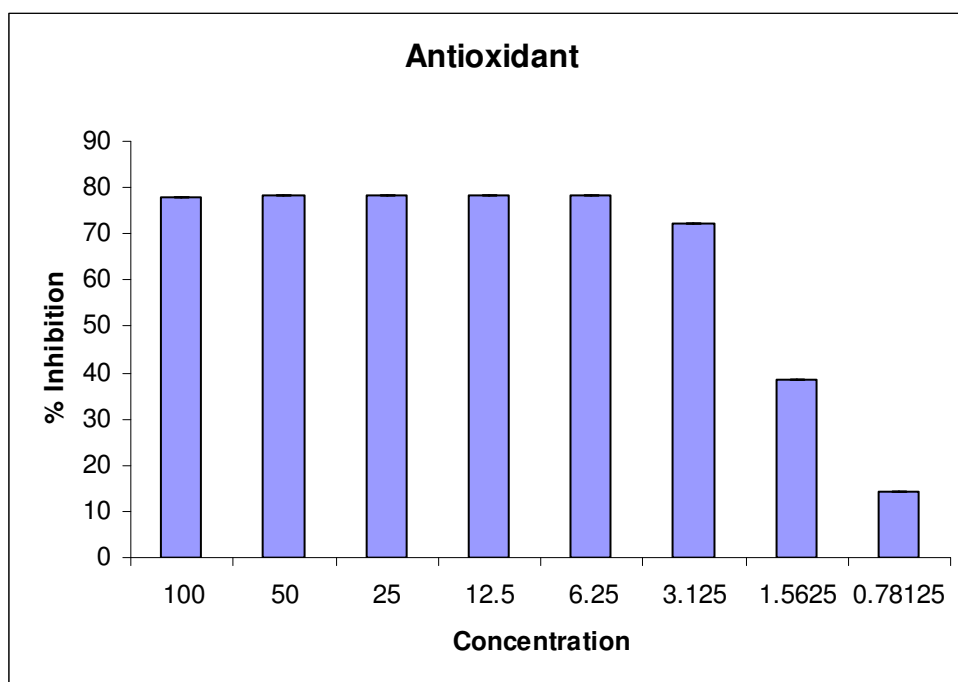


Figure 5.6 DPPH radical scavenging activity of *Greyia flanagani*



(A)



(B)

Figure 5.7 Antioxidant activities of *Greyia flanaganii* (A) and Vitamin C

(B)

Table 5.1 EC₅₀ (concentration of extract which decreases the initial DPPH absorbance by 50%) values for the ethanolic extract of *Greyia flanaganii*

Sample	EC ₅₀ (µg/ml) (µM)	Mg Vitamin C equivalents/dry weight (EC ₅₀ value)
Vitamin C	2.0 ± 0.3	
<i>G. flanaganii</i>	22.01± 0.02	90.87

5.4.4 Antibacterial activity of plant extract

Ethanollic leaf extract of *Greyia flanaganii* was tested for its activity against *P. acnes*. *G. flanaganii* was tested at the final concentrations ranging from 3.9 µg/ml to 500 µg/ml. The wells that showed no colour (pink) of the reagent (INT) indicates the concentration at which the bacterial growth was inhibited and the lowest concentration showing no colour was recorded as the MIC value (figure 7.1). *G. flanaganii* exhibited the MIC value of 250 µg/ml against *P.acnes* as compared to erythromycin which indicated the MIC value of 100 µg/ml.

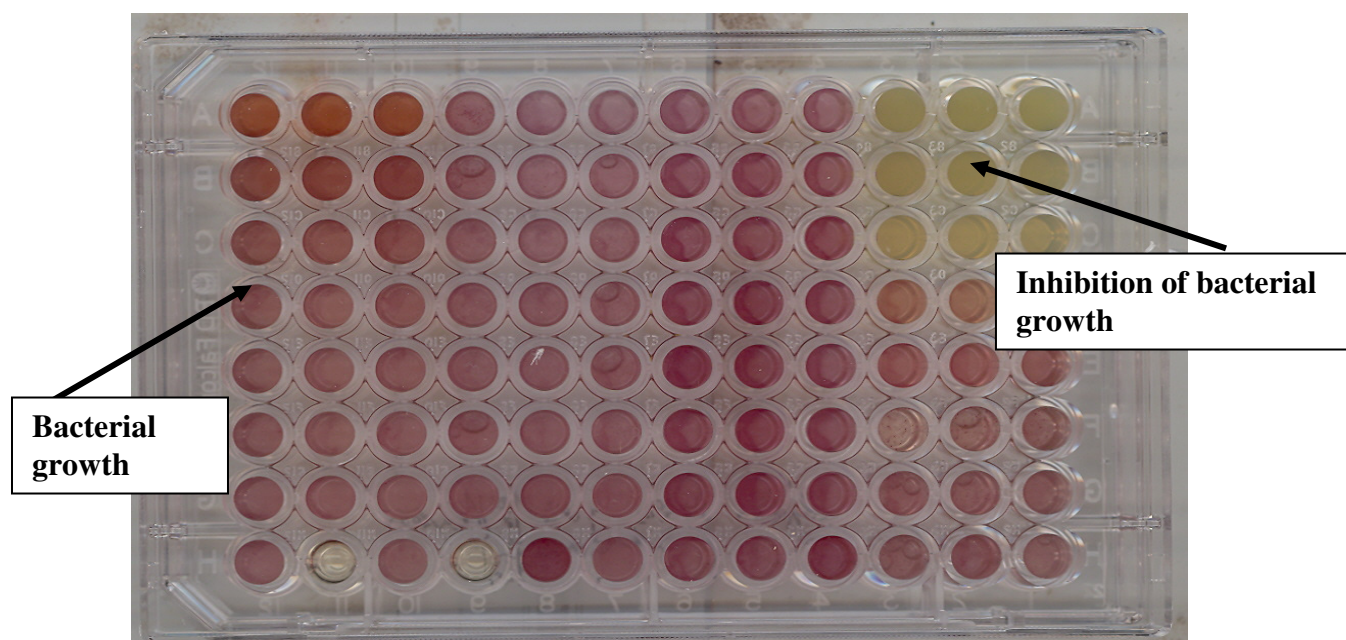


Figure 5.8 Antibacterial activity of leaf extracts of *Greyia flanaganii* against *Propionibacterium acnes*



Table 5.2 The minimum inhibitory concentration (MIC) of the leaf extract of *Greyia flanaganii* against *Propionibacterium acnes*

Samples	MIC (µg/ml)
Extract	250
Erythromycin	100

Table 5.3 Cytotoxicity, Antityrosinase, antioxidant and antibacterial activities of ethanolic leaf extract of *Greyia flanaganii* (Bolus)

Test	<i>G. flanaganii</i>	Kojic acid	Vitamin C	
Tyrosine (IC ₅₀)	32.68 ± 0.5 µg/ml	7.02 ± 0.03 µg/ml	-	
L-DOPA (IC ₅₀)	N/A	19.46 µg/ml	-	
Cytotoxicity (IC ₅₀)	>400 µg/ml	>100 µg/ml	-	
Antioxidant (EC ₅₀)	22.01± 0.02 µg/ml	-	2.0 ± 0.3 µg/ml	
Antibacterial (MIC)	250 µg/ml	-	-	100 µg/ml

5.5 Discussions and conclusions

Greyia flanaganii inhibited tyrosinase enzyme exhibiting an IC₅₀ value of 32 µg/ml when tyrosine was used as the substrate, however it did not show significant inhibitory activity of the enzyme when L-DOPA was used as the substrate. Antityrosinase activity of this plant has never been reported before. Our results are in agreement with the findings of previous studies where plant extracts have been reported to inhibit tyrosinase enzyme either through the monophenolase activity or diphenolase mechanism. According to Lee et al (1997) the extracts of *Morus alba*, *Glycyrrhiza glabra* showed 68 and 65 % inhibition of the tyrosinase enzyme respectively when tyrosine was used as a substrate at 1mg/ml. In another study done by Momtaz et al (2008) both acetone and methanol extracts of *Sideroxylon enerve* exhibited 70% inhibition of the enzyme at 200 µg/ml.

The antioxidant activity of this plant has not been explored earlier. From this study it be concluded that *G. flanaganii* has a remarkable antityrosinase activity. The extract is not toxic to melanocytes cells and it does prevent marginal melanin production *in-vitro*. The antioxidant and antibacterial activity were found to be significant. It was therefore, decided to identify the bioactive compounds of *Greyia flanaganii*.

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CHAPTER 6

**Isolation and identification of active
compounds from *Greyia flavaganii*
(Bolus)**

6.1 Introduction

Biologically active compounds isolated from plant materials represent a constant challenge to researchers all over the world. More and more extensive investigations as a consequence of development of new technologies and involvement of much more human potentials in research area than ever in the history, especially in economically progressive countries, lead to numerous scientific discoveries in this field like in many others. The final results of these investigations are often new products that enhance the quality of life, considering that they are mostly drugs and dietary supplements. Some other uses also exist, for example in cosmetic industry.

Greyia flanaganii (Bolus) is a shrub belonging to the family Greyiaceae. It is used by the Xhosa people whereby they place the roots of this Eastern Cape tree on either side of their kraals (enclosures) as it is thought to ward off sickness and prevent theft of livestock (Mbambezeli G., 2002). The family has been investigated before and found to contain different types of flavonoids. Flavonoids are a type of phenolic compounds that have shown to possess antityrosinase activities. Ethanolic leaf extract of *G. flanaganii* showed the best inhibitory activity of the tyrosinase enzyme when L-tyrosine was used as the substrate. The main aim of this study was to isolate the active compounds from this extract, and to check if tyrosinase inhibition of this plant is due to flavonoids or any other type of compounds.

6.2 Materials and methods

6.2.1 Plant material

Leaves of *Greyia flanaganii* Bolus were collected from the botanical garden of the University of Pretoria (Manie Van der Schijff Botanical Garden).

6.2.2 Extraction, isolation and purification of active compounds

Isolation of active compounds from *G. flanaganii* was carried out using bioassay guided fractionation. Ethanol extract was prepared as described in chapter 4 (section 4.41) but using 800 g of fresh material yielding 71.192 g of dry powdered material. About 71.192 g of the extract was dissolved in a minimal amount of acetone solvent and mixed with silica gel. This mixture was left to dry to fine powder and was chromatographed on a silica gel column using hexane (HEX): ethylacetate (EtOAc) mixtures of increasing polarity (100:0 to 0:100 EtOAc) to yield 64 primary fractions of about 500 ml each. The column was then washed with 2L of 100% methanol (MEOH). Fractions which contained the same compounds as evident on the TLC plate were combined together to 8 secondary fractions and concentrated to dryness under reduced pressure. The TLC plate of the combined fractions (labeled as AC1 – AC8) was developed with dichloromethane (DCM): MEOH 1% (figure 6.1). All 8 fractions were tested at a concentration of 200 and 20 µg/ml for antityrosinase activity using tyrosine as a substrate. Fractions AC1, AC4 and AC6 showed stronger inhibitory activity of the enzyme at 200 µg/ml (75, 63 and 59 % respectively) while other fractions did not show any inhibition of the enzyme hence these

fractions were further chromatographed for the isolation and identification of bioactive compounds. Fraction AC6 (1.52g) was subjected to a series of sephadex column LH-20 (Sigma-Aldrich, South Africa) eluted with 100% ETOH and then washed with 100% MEOH to yield compounds **C1** (253 mg), **C2** (208 mg) and **C7** (114 mg). Fraction AC4 was subjected to a preparative TLC eluted with DCM: MEOH 9:1 from which compounds **C3** (10 mg) and **C4** (10 mg) were isolated. Fraction AC1 was also subjected to preparative TLC (DCM: MEOH 9:1) which led to the isolation of compounds **C5** (24 mg) and **C6** (24 mg). A flow chart representing the stages followed to isolate these compounds is included (figure 6.3). Figure 6.1 represents the 8 combined fractions of *G. flanaganii*.

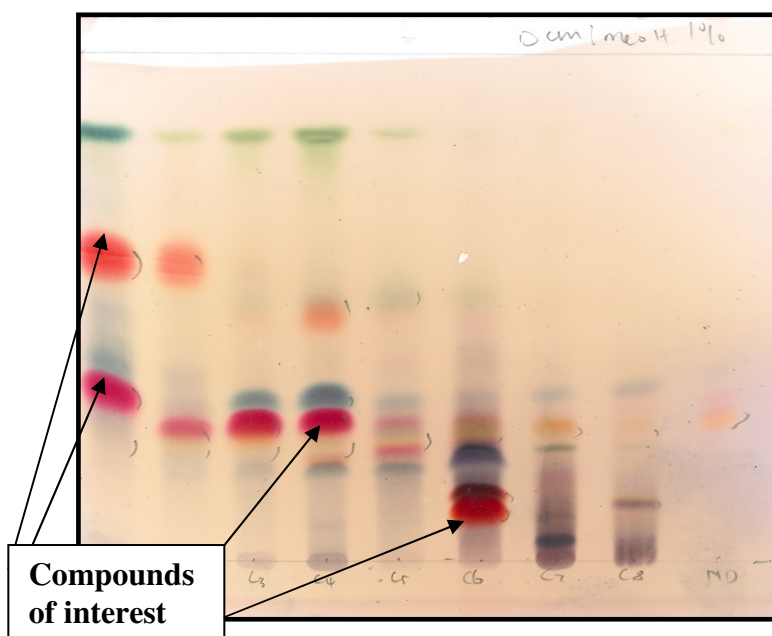


Figure 6.1 Fractions obtained from column chromatographic purification of ethanol leaf extract of *Greyia flanaganii* (bolus) (Eluent DCM: MeOH 1%)

6.2.3 Antityrosinase activity of isolated compounds

Antityrosinase of the isolated compounds was determined as described in chapter 5 (section 5.2.3). These compounds were tested at final concentrations of 1.5 to 200 µg/ml. They were further tested on cell viability and melanin biosynthesis and method followed are same as described in chapter 5 (section 5.2.4) at final concentrations ranging from 1.5-100 µg/ml.

6.2.4 Antioxidant activity of isolated compounds

Radical scavenging activity of the isolated compounds was determined as described in chapter 5 (section 5.2.5), tested at the final concentrations ranging from 0.78-100 µg/ml.

6.2.5 Antibacterial activity of isolated compounds against

Propionibacterium acnes

Antibacterial activity of isolated compounds against *P. acnes* was determined as described in chapter 5 (section 5.2.4). Compounds were tested at the final concentrations ranging from 3.9 to 500 µg/ml.

6.3 Statistical analysis

The results were analyzed statistically using one-way analysis of variance (ANOVA) and the least significant differences ($P < 0.05$) were determined according to Duncan's t-test. Results were significant where $P < 0.05$.



Figure 6.2 Silica gel column chromatography

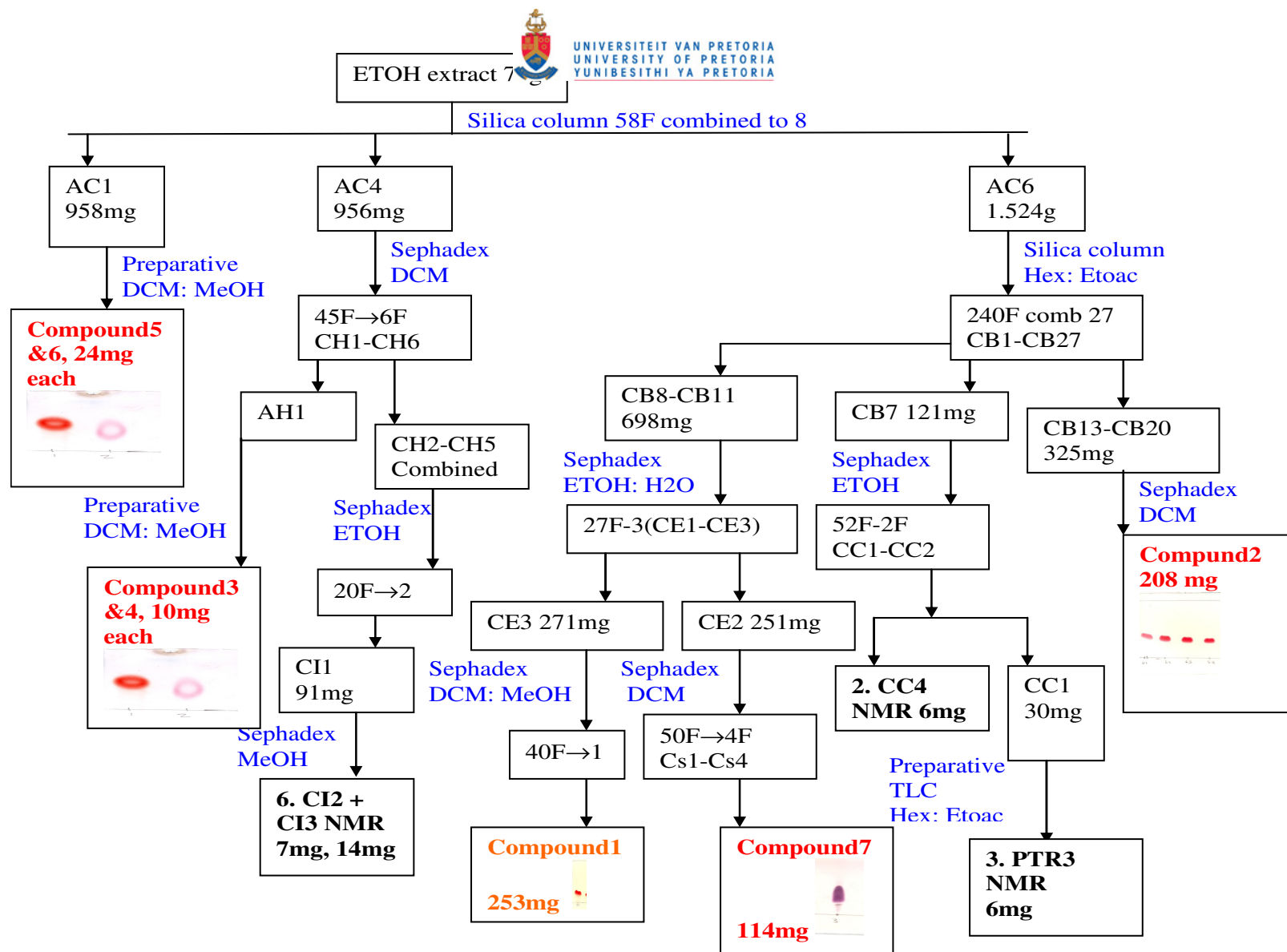


Figure 6.3 Schematic diagram representing the isolation of active compounds from ethanolic leaf extract of *Greyia flanaganii*

6.4 Results and discussion

The column chromatography (figure 6.2) using silica gel as the stationary phase yielded 65 fractions (figure 6.1) which were pooled together according to their TLC profile. This led to 8 combined fractions. From these combined fractions seven compounds were isolated. The isolated compounds were different types of phenolic compounds (six already known compounds and one novel compound).

Fraction AC6 yielded pure compounds 1 (253mg), 2 (208 mg) and 7 (114 mg) respectively (table 6.1). Fraction AC1 yielded pure compounds 3 (24 mg) and 4 (24 mg) and AC4 yielded compounds 5 (7 mg) and 6 (7 mg) respectively (table 6.4). TLC plates which showed these compounds were examined under UV light after development and also dipped in vanillin (7.5g vanillin, 250ml ETOH and 5ml concentrated 98% sulphuric acid (H_2SO_4)) and heated to detect compounds not absorbing under UV. The compounds were identified by comparing their ^1H -NMR and ^{13}C -NMR spectral data with published articles. The ^1H -NMR and ^{13}C -NMR of the isolated compounds done from this study are also attached



Table 6.1 Chemical structures of the isolated compounds from *Greyia* *flanaganii*

Structures of isolated compounds	
C1 2',6',4-trihydroxy-4'-ethoxydihydrochalcone (asebogenin/lyonogenin)	C2 2',4',6'-trihydroxydihydrochalcone
C3 2',6'-dihydroxy-4',4-dimethoxydihydrochalcone	C4 (2<i>R</i>,3<i>R</i>)-3,5,7-trihydroxy-3-<i>O</i>-acetylflavanone (pinobanksin-3-acetate/dihydrogalangin-3-acetate)
C5 2',6'-dihydroxy-4'-methoxydihydrochalcone	C6 5,7-dihydroxyflavanone [(2<i>S</i>)-pinocembrin]
C7 4-hydroxyphenethyl 3-hydroxy-5-phenylpentanoate	

Compound **2** and compound **5** showed similar ^1H and ^{13}C pattern (Table 6.2 and 6.3), compound **2** showed a signals of $\text{CH}_2\text{-CH}_2$ (3.32, 2.94, 2H, t, $J = 7.9$ Hz/each) close to a carbonyl group from one side and a phenyl group from the other side, in addition a signals of two aromatic protons at 5.81, (2H, s, H-3', 5') identical to 1,2,4,6-tetrasubstituted benzene ring. The only difference between both compounds is the existence of a methoxyl group in compound **5** at 3.77. The compounds were identified as 2',4',6'-trihydroxydihydrochalcone,^{1,2} (**2**) and 2',6'-dihydroxy-4'-methoxydihydrochalcone^{3,4} (**5**), previously known compounds. Compounds **1** and **3** showed similar NMR pattern to those of **1** and **5** except the splitting of the signals of ring B to AA BB system (compounds **1**, **3**) instead of the signals of a phenyl group (compounds **3**, **5**), in addition, compound **3** contains extra methoxyl group at position 4 (NMR data in Tables 6.2 and 6.3, which are identical with published data). Both Compounds **1** and **3** were identified as 2',6',4-trihydroxy-4'-methoxydihydrochalcone (**1**, asebogenin/lyonogenin),⁵⁻¹⁰ and 2',6'-dihydroxy-4',4-dimethoxydihydrochalcone (**3**)^{11,12}.

^1H and ^{13}C NMR, (Tables 6.4 and 6.5, respectively) for compounds **4**, **6** were similar and identified as 2*R*,3*R*)-3,5,7-trihydroxy-3-*O*-acetylflavanone (**4**, pinobanksin-3-acetate/dihydrogalangin-3-acetate)^{13,14}, and 5,7-dihydroxyflavanone [(2*S*)-pinocembrin]^{15,16}, the optical rotation showed 2*R*,3*R* of compound **4** and 2*S* for compound **6**, by comprising the values obtained with those in literature $[\alpha]_{\text{D}}^{20} +15.1$ (c 0.61, MeOH), **4**, -46.3 (c 0.35, acetone). For **4**, **6** respectively]

For the assignments of the HMBC spectra, please note that in aromatic compounds the ^2J values are close to zero, or $^2J \ll ^3J$. Moreover, in compounds **1-3** and **5**, the observed ^4J

HMBC correlation between the C=O carbon and the H-3' and H-5' protons is expected for acetophenone derivatives. In any case, the substitution pattern of ring A in compounds **1-3** and **5** is **strongly supported by biogenetic reasons** and also by NOE experiments. When the 4'-methoxyl protons signal of compounds **1** and **4** was irradiated, a clear NOE enhancement was observed in the signal of the H-3' and H-5' protons. Thus, ring A is 1',2',4',6'- and not 1',3',4',5'- tetrasubstituted.

High-resolution mass spectrometry and combustion analysis indicated a molecular formula of C₁₉H₂₂O₄ for **C7**, and its IR spectrum showed hydroxyl (3446, 3312 cm⁻¹) and ester (1721, 1183 cm⁻¹) absorptions. The ¹H and ¹³C NMR spectra of **C7**, together with 2D-NMR COSY and HSQC experiments, revealed that it possessed a 3-hydroxy-5-phenylpentanoic acid ester structural moiety, because the chemical shifts of the C-1 – C-11 protons and carbons (Table 6.6) were almost identical to those reported for the methyl ester of 3-hydroxy-5-phenylvaleric acid.¹ Moreover, the remaining proton and carbon signals of **C7** (C-1' – C-8', Table 1) were in complete agreement with those of esterified tyrosol.² The observed HMBC correlations between the carbonyl carbon of the acyloxy part (C-1, δ 173.4) and the C-1' methylene protons of tyrosol (δ 4.22 and 4.19) further supported that this new substance is 4-hydroxyphenethyl 3-hydroxy-5-phenylpentanoate (**C7**).

The absolute stereochemistry of the C-3 asymmetric center of **C7** was not ascertained. However, we assume that it possesses a 3*S* absolute configuration because the specific optical rotation of **C7** {[α]_D²⁴ -4.7 (*c* 0.738, CHCl₃)}, is almost identical to that reported³ for methyl (3*S*)-3-hydroxy-5-phenylpentanoate {[α]_D²⁵ -3.35 (*c* 1, CHCl₃)}.

All the ¹H and ¹³C NMR assignments (Tables 6.2 to 6.6) were in agreement with 2D COSY, HSQC and HMBC spectra.



Table 6.2 ¹H NMR data for compounds 1, 2, 3 and 5^a

H	1	2	3	5
2,6	7.03, 2H, d, <i>J</i> = 8.2 Hz	7.23 m ^b	7.13, 2H, d, <i>J</i> = 8.7 Hz	7.24 m ^b
3,5	6.70, 2H, d, <i>J</i> = 8.2 Hz	7.23 m ^b	6.81, 2H, d, <i>J</i> = 8.7 Hz	7.24 m ^b
4	-	7.14, 1H, tt, <i>J</i> =6.7, 2.0 Hz	-	7.15, 1H, tt, <i>J</i> =6.7, 2.0 Hz
3',5'	5.89, 2H, s	5.81, 2H, s	5.92, 2H, s	5.93, 2H, s
□	3.30, 2H, t, <i>J</i> = 7.7 Hz	3.32, 2H, t, <i>J</i> = 7.9 Hz	3.30, 2H, t, <i>J</i> = 7.8 Hz	3.35, 2H, t, <i>J</i> = 7.8 Hz
□	2.86, 2H, t, <i>J</i> = 7.7 Hz	2.94, 2H, t, <i>J</i> = 7.9 Hz	2.87, 2H, t, <i>J</i> = 7.8 Hz	2.95, 2H, t, <i>J</i> = 7.8 Hz
4-OMe	-	-	3.75, 3H, s	-
4'-OMe	3.74, 3H, s	-	3.77, 3H, s	3.77, 3H, s

^aAt 500 MHz, CD₃OD solution, referenced to the solvent at □ 3.30.

^bOverlapped signal, 4H.

Table 6.3 ¹³C NMR data for compounds 1, 2, 3 and 5^a

C	1	2	3	5
1	133.1 (C)	143.2 (C)	135.1 (C)	143.1 (C)
2,6	129.7 (CH) [2C]	129.35 (CH)* [2C]	130.3 (CH) [2C]	129.36 (CH)* [2C]
3,5	115.4 (CH) [2C]	129.43 (CH)* [2C]	114.8 (CH) [2C]	129.43(CH)* [2C]
4	155.1 (C)	126.9 (CH)	159.4 (C)	126.9 (CH)
1'	105.4 (C)	105.3 (C)	106.1 (C)	106.0 (C)
2',6'	164.5 (C) [2C]	165.8 (C) [2C]	165.6 (C) [2C]	165.6 (C) [2C]
3',5'	93.7 (CH) [2C]	95.8 (CH) [2C]	94.3 (CH) [2C]	94.4 (CH) [2C]
4'	166.2 (C)	166.2 (C)	167.5 (C)	167.5 (C)
CO	206.0 (C)	206.1 (C)	206.6 (C)	206.4 (C)
□	46.4 (CH ₂)	46.9 (CH ₂)	47.2 (CH ₂)	47.0 (CH ₂)
□	30.3 (CH ₂)	32.2 (CH ₂)	31.3 (CH ₂)	32.1 (CH ₂)
4-OMe	-	-	55.7 CH ₃)	-
4'-OMe	55.5 (CH ₃)	-	55.8 (CH ₃)	55.8 (CH ₃)

^aAt 125 MHz, in CD₃OD solution, referenced to the solvent signals (□49.00).

*Interchangeable assignments.



Table 6.4 ^1H NMR data for compounds 4 and 6^a

H	4	6
2	5.41, 1H, d, $J = 11.7$ Hz	5.44, 1H, dd, $J = 12.7, 3.0$ Hz
3	5.82, 1H, d, $J = 11.7$ Hz	3.07, 1H, dd, $J = 17.1, 12.7$ Hz, axial H-3 ($\square$) 2.76, 1H, dd, $J = 17.1, 3.0$ Hz, equatorial H-3 ($\square$)
6	5.95, 1H, d, $J = 2.2$ Hz	5.89, 1H, d, $J = 2.2$ Hz
8	5.97, 1H, d, $J = 2.2$ Hz	5.93, 1H, d, $J = 2.2$ Hz
2'(6')	7.51, 2H, dd, $J = 7.8, 2.0$ Hz	7.48, 2H, dd, $J = 7.1, 1.8$ Hz
3'(5')	7.41, 2H, m ^b	7.40, 2H, tt, $J = 7.2, 1.8$ Hz
4'	7.41, 1H, m ^b	7.35, 1H, tt, $J = 7.2, 1.8$ Hz
3-O-Ac	1.95, 3H, s	-

^aAt 500 MHz, CD_3OD solution, referenced to the solvent at $\square$ 3.30.

^bOverlapped signal, 3H.

Table 6.5 ^{13}C NMR data for compounds 4 and 6^a

C	4	6
2	82.5 (CH)	80.4 (CH)
3	73.8 (CH)	44.2 (CH_2)
4	193.0 (C)	197.3 (C)
5	164.1 (C)	164.7 (C)
6	96.7 (CH)	97.2 (CH)
7	169.2 (C)	168.4 (C)
8	97.8 (CH)	96.2 (CH)
9	165.5 (C)	165.5 (C)
10	102.1 (C)	103.4 (C)
1'	137.2 (C)	140.4 (C)
2'(6')	128.7 (CH) [2C]	127.3 (CH) [2C]
3'(5')	129.6 (CH) [2C]	129.7 (CH) [2C]
4'	130.4 (CH)	129.6 (CH)
3-O $\underline{\text{C}}$ COCH ₃	170.8 (C)	-
3-OCO $\underline{\text{C}}$ H ₃	20.1 (CH_3)	-

^aAt 125 MHz, in CD_3OD solution, referenced to the solvent signals ($\square$ 49.00).



Table 6.6 NMR Spectroscopic Data [500 (^1H) and 125 (^{13}C) MHz, CD_3OD] for Compound **C7^a**

Position(s)	δ_{C} , mult.	δ_{H} (<i>J</i> in Hz)
1	173.4, qC	
2	43.5, CH_2	2.47, dd (15.0, 5.0) 2.41, dd (15.0, 8.0)
3	68.6, CH	3.96, tt (8.0, 5.0)
4	40.0, CH_2	1.74, 2H, m
5	32.8, CH_2	2.74, ddd (13.5, 9.2, 5.9) 2.62, ddd (13.5, 9.3, 7.0)
6	143.3, qC	
7, 11	129.5, CH	7.17, 2H, br d (7.6)
8, 10	129.4, CH	7.24, 2H, br t (7.5)
9	126.8, CH	7.14, tt (7.7, 1.2)
1'	66.6, CH_2	4.22, dt (10.8, 6.9) 4.19, dt (10.8, 6.9)
2'	35.2, CH_2	2.80, 2H, t (6.9)
3'	130.0, qC	
4', 8'	130.9, CH	7.02, 2H, dt (8.6, 2.4)
5', 7'	116.2, CH	6.69, 2H, dt (8.6, 2.4)
6'	157.1 qC	

^aAssignments were in agreement with COSY, HSQC, and HMBC spectra. The hydroxyl protons at C-3 and C-6' positions were not observed in CD_3OD solution, but they appeared at δ 3.30, br s and 6.56, br s, respectively, when the ^1H NMR spectrum of **C7** was registered in CDCl_3 solution.

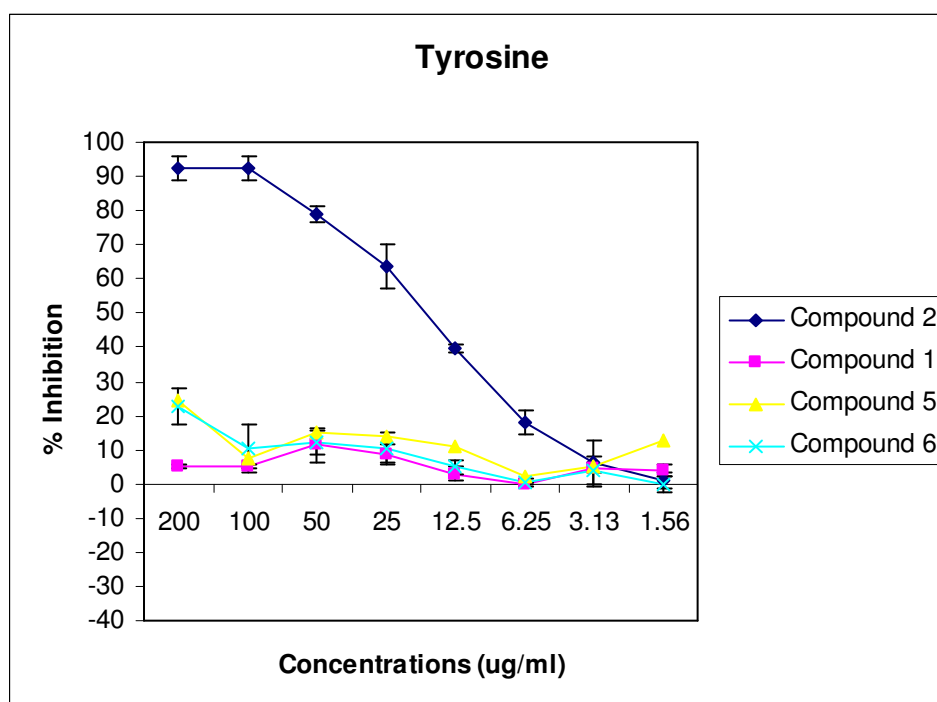
^1H and ^{13}C NMRs spectra of **C1**, **C2**, **C3**, **C4**, **C5**, **C6** and **C7** are represented in chapter 9

Appendix A

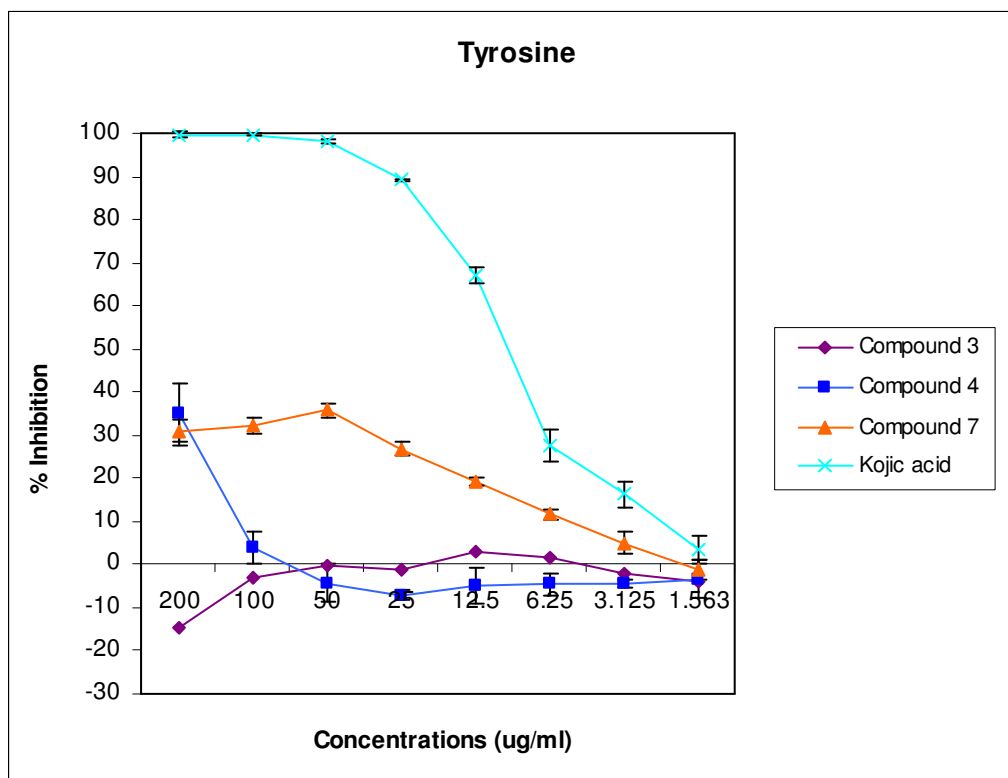
6.4.1 Antityrosinase activity of isolated compounds

Seven isolated compounds from *G. flanaganii* were tested for their activity on tyrosinase.

C2 exhibited the best inhibitory activity of the enzyme as compared to the other compounds with the IC_{50} value of 69.1 μ M. **C3** did not show any significant ($P>0.05$) inhibition of the enzyme even at the highest concentrations tested. **C1**, **C4**, **C5**, **C6** and **C7** exhibited 11.3, 35, 24, 22, and 31% inhibition of tyrosinase enzyme at 200 μ g/ml. The results were compared with those of kojic acid (positive drug) with the IC_{50} of 49.3 μ M. IC_{50} values of all the compounds are given in table 6.2



(A)



(B)

Figure 6.4 Dose-response curves representing inhibitory activity of isolated compounds (1, 2, 5 and 6) (A) and (3, 4, 7 and kojic acid) (B) from *G. flanaganii* on tyrosinase enzyme

Table 6.7 The IC₅₀ (concentration of the samples at which half the original tyrosinase is inhibited) values of the isolated compounds for mushroom tyrosinase

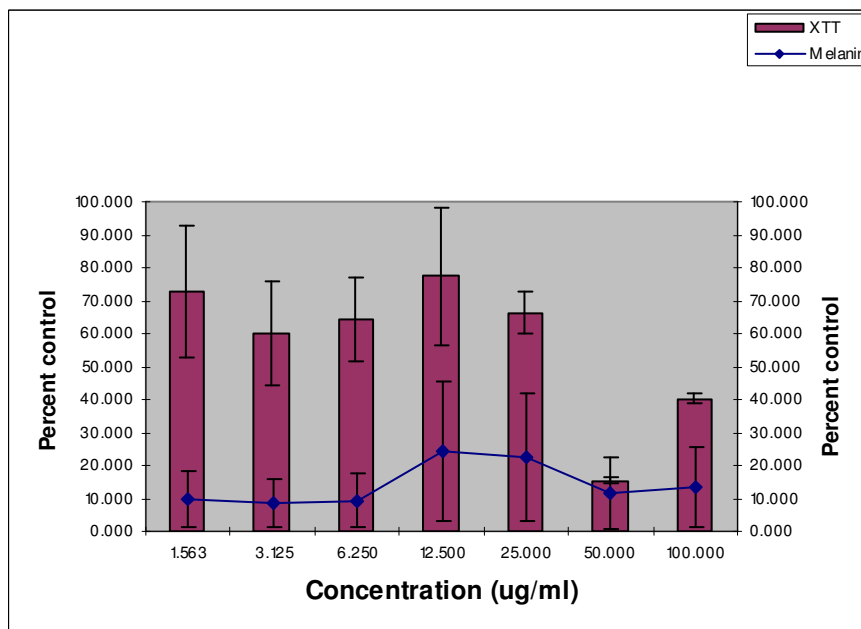
Sample	IC ₅₀ monophenolase activity µg/ml/ (µM)
Kojic acid	7.020 ± 0.03/(49.3)
2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (C1)	>200
2',4',6'-trihydroxydihydrochalcone (C2)	17.86 ± 0.02/(69.15)
2',6'-dihydroxy-4',4-dimethoxydihydrochalcone (C3)	N/A
(2 <i>R</i> ,3 <i>R</i>)-3,,5,7-trihydroxy-3- <i>O</i> -acetylflavanone (pinobanksin-3-acetate/dihydrogalangin-3-acetate) (C4)	> 200
2',6'-dihydroxy-4'-methoxydihydrochalcone (C5)	> 200
5,7-dihydroxyflavanone [(2 <i>S</i>)-pinocembrin] (C6)	>200
4-hydroxyphenethyl 3-hydroxy-5-phenylpentanoate (C7)	> 200

6.4.2 Cytotoxicity of isolated compounds on melanocytes and melanogenesis

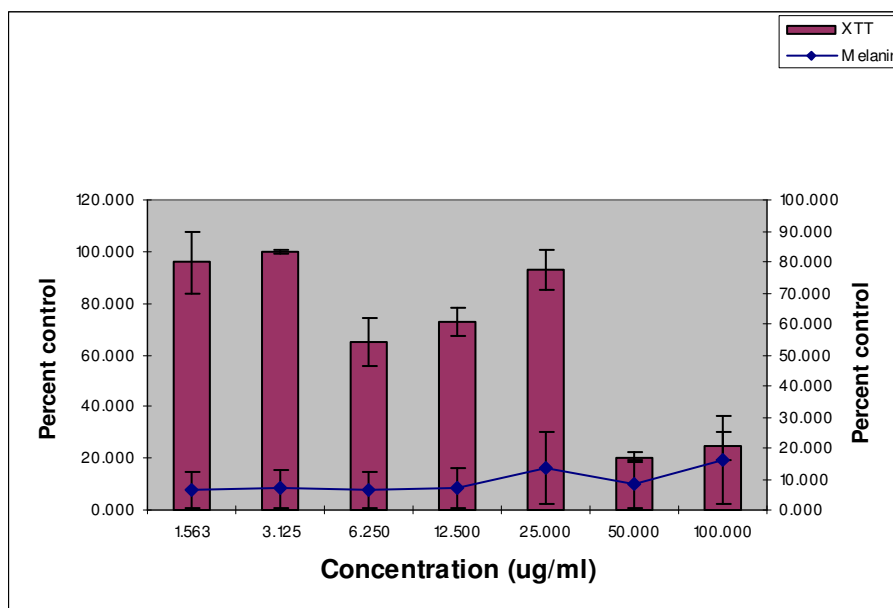
Melanin biosynthesis pathway involves enzymatic and nonenzymatic reactions. Tyrosinase is known to be the key enzyme as it catalysis two reactions hence it is targeted by researchers in the development of new skin lighteners. Some skin lighteners do not inhibit melanin production at the enzyme level but rather they target other molecules that

are involved in the production of melanin. Compounds such as nobiletin, naringin and neohesperidin isolated in a study done by Chang (2009) showed poor inhibition of the enzyme but showed inhibition of melanin *in-vitro* on melanoma cells. Another example of a compound that inhibits melanin production at post translational level is deoxyArbutin. This indicates that some compounds might not inhibit tyrosinase but rather they can inhibit melanin production by the cells *in-vitro*. Therefore in the present study, it was decided to test all the isolated compounds (even if they did not inhibit tyrosinase) for their melanin inhibitory effect by melanocytes cells. Due to low yield of compounds **3** and **4** no further tests were done on these compounds. Five compounds (**C1**, **C2**, **C5**, **C6** and **C7**) were further tested for their effect *in-vitro* on melanocytes cells to check if the compounds can inhibit melanin production through other mode of action rather than enzyme inhibition. Compound 5,7-dihydroxyflavanone [(2*S*)-pinocembrin] (**C6**) showed less cytotoxicity effect on the cells exhibiting IC_{50} of 373 μ M and 10% reduction in melanin content of the cells at 1.5 μ g/ml (figure 6.5D). 2',6'-dihydroxy-4'-methoxydihydrochalcone (**C5**) has shown toxicity effect of the cells giving low cell viability (less than 30%) at most concentrations tested with the IC_{50} of 30.15 μ M (figure 6.5C). 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (**C1**) was less toxic to the cells at lower concentrations with the IC_{50} of 120 μ M (figure 6.5B) and 2',4',6'-trihydroxydihydrochalcone (**C2**) was also less toxic with the IC_{50} value of 219 μ M (figure 6.5A). Compound (**C7**) showed no toxicity effect of the cells even at the highest concentrations tested with no reduction of melanin content of the cells at all concentrations (figure 6.5E). From the present study it shows that **C2** which did not show any tyrosinase inhibition showed less toxicity of the cells with some reduction of melanin content which clearly shows that different compounds/ extracts have different mode of

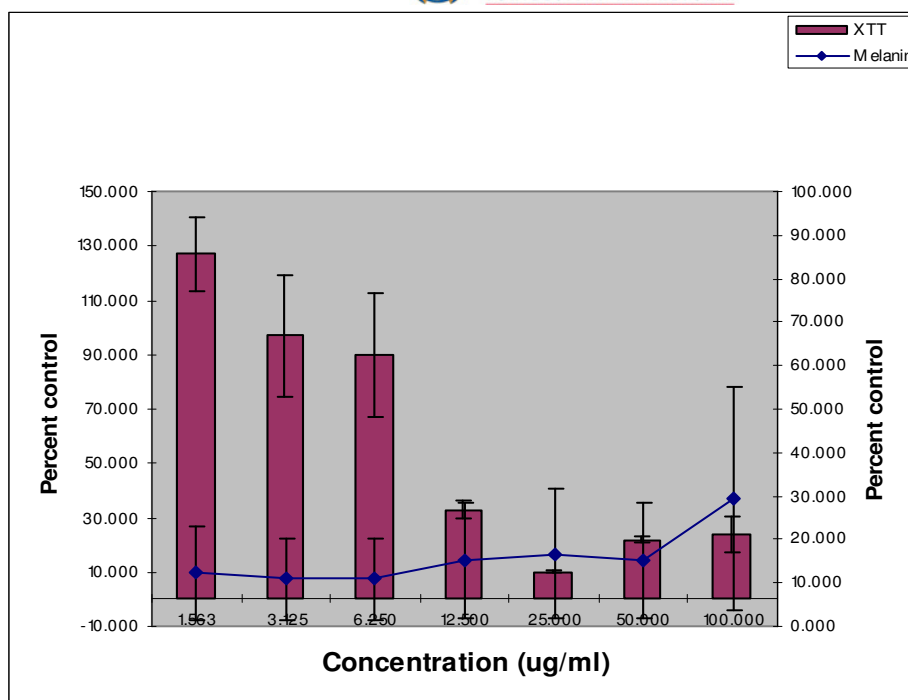
action for the inhibition of melanin production. These results were analyzed in comparison with the positive drug kojic acid (figure 6.5F).



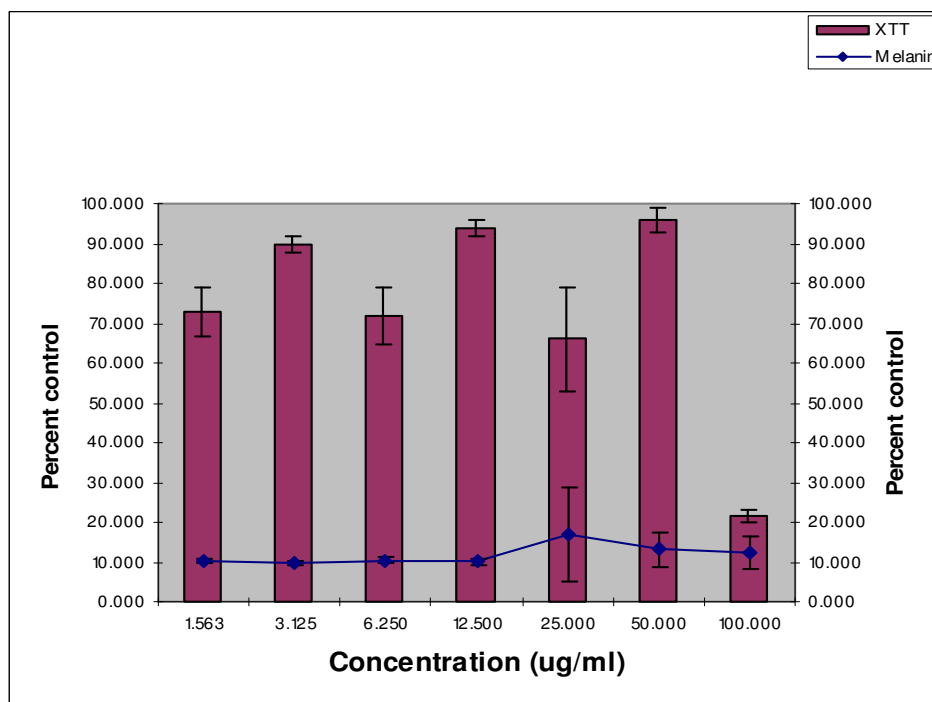
(A)



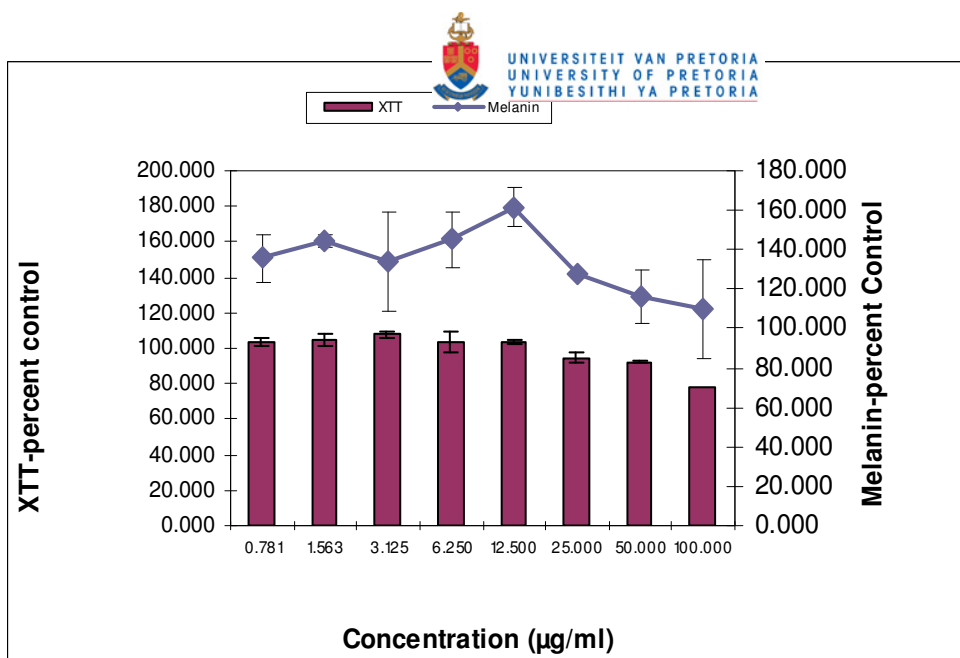
(B)



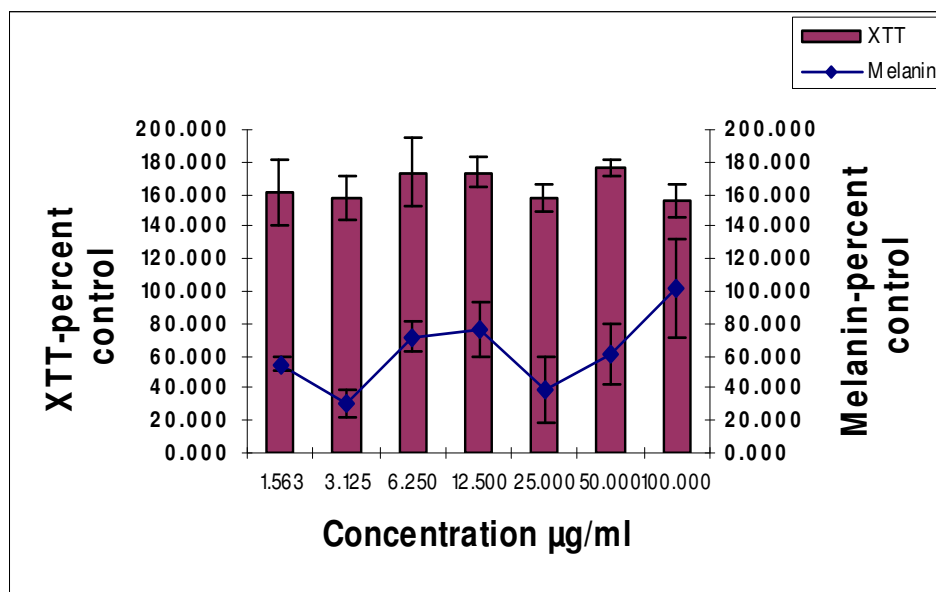
(C)



(D)



(E)



(F)

Figure 6.5 The effect of purified compounds on cell viability and melanin production 2',4',6'-trihydroxydihydrochalcone (A), 2',6',4'-trihydroxy-4'- methoxydihydrochalcone (asebogenin/lyonogenin) (B), 2',6'-dihydroxy-4'-methoxydihydrochalcone (C), 5,7-dihydroxyflavanone [(2*S*)-pinocembrin] (D), Compound 7 (E), Kojic acid (positive control) (F)

6.4.3 Antioxidant activity of isolated compounds

The radical scavenging activity of five compounds was carried out using the DPPH method. All five compounds showed good inhibitory activity of the radical formation in almost all concentrations tested. The EC₅₀ values of all the compounds are represented in table 6.2.

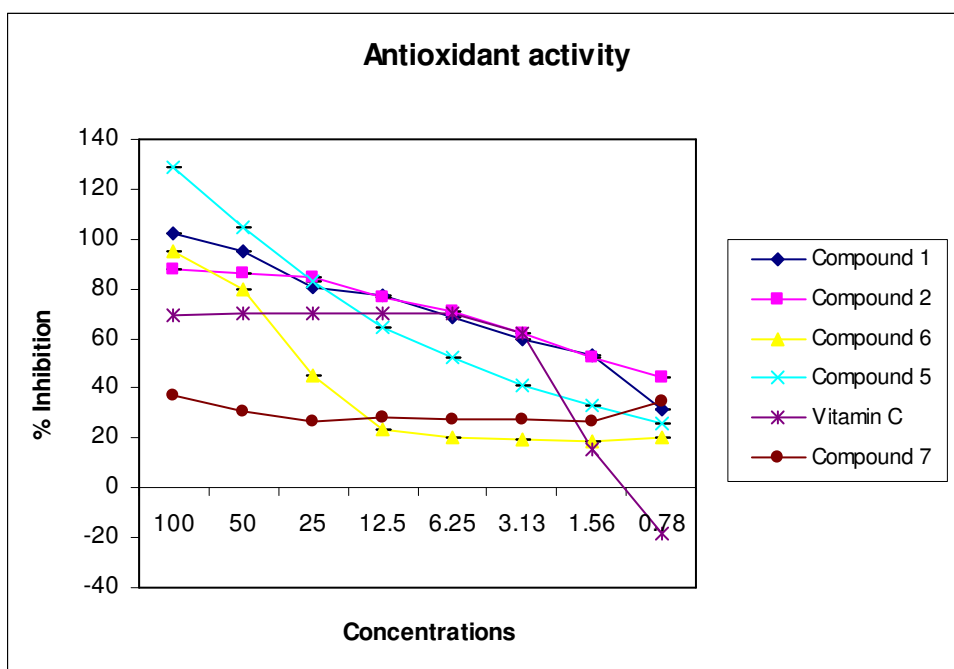


Figure 6.6 Dose-response curves representing antioxidant activity of the isolated compounds from *G. flanaganii*

Table 6.8 The EC₅₀ (concentration of extract which decreases the initial DPPH absorbance by 50%) values for the isolated compounds from ethanolic leaf extract of *Greyia flanaganii*

Sample	EC ₅₀ µg/ml (µM)	Mg Vit C equivalents/g dry weight
Vitamin C	2.0 (11.3)	
2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (C1)	2.00 (6.69)	1000
2',4',6'-trihydroxydihydrochalcone (C2)	0.895 (3.46)	2234
2',6'-dihydroxy-4'-methoxydihydrochalcone (C5)	8.72 (32.0)	229.4
5,7-dihydroxyflavanone [(2S)-pinocembrin] (C6)	19.5 (76.1)	102.56
Compound (C7)	> 100	-

6.5 Antibacterial activity of isolated compounds from *Greyia flanaganii*

Five compounds were tested for their activity against *P. acnes* with erythromycin used as the positive drug (McGaw and Eloff, 2005, Sahin et al, 2003). Compounds **3** and **4** were not tested due their unavailability (low yield during isolation). Samples were tested at the final concentrations ranging from 3.9 µg/ml to 500 µg/ml. Compound **7** showed inhibition of the growth of bacteria exhibiting the MIC value of 1590 µM as compared to erythromycin which exhibited the MIC value of 136 µM. **C1** exhibited a lower MIC value of 418 µM. **C5** and **C6** did not show any inhibitory activity of the bacteria at 500 µg/ml.

Table 6.9 The minimum inhibitory concentration (MIC) of the leaf extract of *Greyia flanaganii* and its isolated compounds against *Propionibacterium acnes*

Samples	MIC µg/ml (µM)
2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (C1)	125 (418)
2',4',6'-trihydroxydihydrochalcone (C2)	250 (968)
2',6'-dihydroxy-4'-methoxydihydrochalcone (C5)	-
5,7-dihydroxyflavanone [(2S)-pinocembrin] (C6)	-
Erythromycin (Positive drug)	100 (136)
Compound (C7)	500 (1590)

-=not active even at the highest concentration tested (500 µg/ml)

Table 6.10 Antityrosinase, toxicity, antioxidant and antibacterial activity of isolated compounds from the leaf extract of *Greyia flanaganii* (Bolus)

Test	Compounds							Positive controls		
µg/ml/(µM)	C1	C2	C3	C4	C5	C6	C7	Kojic acid	Vit C	Erythromycin
Tyrosinase	>200	17.86/ (69.1)	-	>200	>200	>200	>200	7.020/ (49.3)		
Cytotoxicity	36.0/ (120)	56.59/ (219)	*	*	8.21/ (30.1)	95.56/ (373)	>100			
Antioxidant	2.0/ (6.69)	0.895/ (3.46)	*	*	8.72/ (32.0)	19.5/ (76.1)	>100		2.0 /(11.3)	
Antibacterial	125/ (418)	250/ (968)	*	*	-	-	500/ (1590)			100/ (136)

*Not tested, -Not active

6.6 Conclusion

Seven phenolic compounds were isolated from *Greyia flanaganii* (Bolos). For the isolated compounds six (compounds **1**, **2**, **3**, **4**, **5** and **6**) were known compounds and have been isolated before from other plants such as *Piper mollicomum*, *Lindera umbellata* var. *lancea* and *Lindera umbellata* etc. However this compounds are being reported for the first time in *Greyia flanaganii* (Bolos) though other flavonoids such as pinostrobin, chrysin, apigenin, tectochrysin, galangin and kaempferol 3-methyl ether have been isolated from this species including from *G. sutherlandii* and *G. radlkoferi* (Bohn and Chan, 1992). Compound **7** was found to be a novel compound. The isolated compounds have shown to have antityrosinase, antioxidant activities and some showed low toxicity effects on the melanocytes cells in the present study. Antibacterial activity against *P. acnes* of this compounds is been reported for the first time. It was also proved that the inhibitory activity of *G. flanaganii* was due to the presence of different phenolic compounds, because the isolated compounds were chalcones (compounds **1**, **2**, **3** and **5**) and flavones (Compound **4** and **6**). Some of the six known compounds isolated in the present study have been tested before for different biological activities. The two groups of compounds (chalcones and flavonoids) have been tested before for their activity on tyrosinase for example in study done by Tanaka et al (1985) and Ichino et al (1987). 2',4',6'-trihydroxydihydrochalcone (**C2**) was synthesized in a study by Jun et al (2007) and was evaluated for its tyrosinase activity where it exhibited the IC₅₀ of 120 µM. In a study by Lago et al (2007) 2',6'-dihydroxy-4'-methoxydihydrochalcone (**C5**) was isolated from *Piper mollicomum* and tested for antifungal activity. It was tested against *Cladosporium cladosporoides* and *Cladosporium sphaerospermum* on a TLC plate (MIC 5.0 and 10µg/ml) respectively. **C1**, **C3**, **C4** and **C6** have been isolated from *Lindera*

umbellata var. *lancea* and synthesized before but their tyrosinase activity has not been reported. Antityrosinase, antioxidant and antibacterial activities of **C7** are reported for the first time. The antityrosinase results in literature compares to the one we obtained from this study. Table 6.9 gives a summary of the activity exhibited by the isolated compounds for each assay studied. The five compounds were also tested for antibacterial activity against acne causing bacteria *Propionibacterium acnes*. Compound 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (**C1**) showed to be good inhibitor of *P. acnes* exhibiting the MIC value (418 μ M) comparing to that of the positive drug (erythromycin) used (136 μ M). Therefore this compound shows some potential to be employed in the cosmetical industries for the treatment of acne; however for maximum results in depigmentation it might need combination of other compounds. Our results compares with that in literature wherein plants extracts have been tested for their activity against *P. acnes*. In a study done by Chomnawang et al (2005) leaf extracts of *Andrographis paniculata* showed inhibitory activity of the bacteria exhibiting a MIC value of 625 μ g/ml. in another study done by Park et al (2004) the isolated compounds honikiol and magnolol isolated from stem bark of *Magnolia* species also showed inhibitory activity against *P. acnes* with the MIC values of 3 and 9 μ g/ml respectively.

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CHAPTER 7

General discussions and conclusions

7.1 Motivation of the study.

Plants have been used for centuries for the treatment of different disorders/ ailments. The use of plants in cosmetics used as skin lighteners also has recently gained momentum. Preferences over chemical and fungal derived compounds is due to the fact that they have shown to have mutagenic, cytotoxic effects, poor skin penetration and low stability in formulation, therefore there is a need for new herbal derived compounds for the treatment of skin hyper-pigmentation.

7.2 Antityrosinase activity of extract of *Greyia flanaganii* and its isolated compounds

Greyia flanaganii (Bolus) is a shrub belonging to the Greyiaceae family. This family is known to be rich in flavonoids compounds in a study done by Bohm and Chan (1992). As flavonoids are also examples of compounds that have shown to have antityrosinase activity therefore this let us to investigate the effect of this extract on tyrosinase. Using tyrosine as the substrate ethanol leaf extract of *G. flanaganii* showed a significant inhibition of the enzyme with the IC_{50} of 32 μ g/ml comparing it to the positive control kojic acid. These results led us to further investigate the kind of compounds that maybe involved in this activity. Different isolation methods were followed to isolate the active compounds from this plant. Seven compounds were isolated namely: 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (C1), 2',4',6'-trihydroxydihydrochalcone (C2), 2',6'-dihydroxy-4',4-dimethoxydihydrochalcone (C3), (2*R*,3*R*)-3,5,7-trihydroxy-3-*O*-acetylflavanone (pinobanksin-3-acetate/dihydrogalangin-

3-acetate) (**C4**) 2',6'-dihydroxy-4'-methoxydihydrochalcone (**C5**), 5,7-dihydroxyflavanone [(2*S*)-pinocembrin] (**C6**), and 4-hydroxyphenethyl-3-hydroxy-5-phenylpentanoate (**C7**). Tyrosinase activity of these compounds was also done using tyrosine as the substrate. All compounds were tested for their antityrosinase activity. **C1**, **C2**, **C4**, **C5**, **C6**, and **C7** showed to inhibit the monophenolase activity of mushroom tyrosinase. Many plant derived compounds are being investigated for their activities on tyrosinase. However their effective concentrations used alone or in combination with other constituents to treat hyper-pigmentation disorders is still unknown.

7.3 Melanogenesis and cytotoxicity

Synthetic or chemical derived compounds have previously shown to have toxicity effect on people therefore it was important to test if this extract and compounds have any toxicity effect. Low melanin synthesis is at times due to decreasing number of melanocytes, we are interested in a compound that will decrease melanin biosynthesis and not kill the cells themselves. The extract and compounds were further investigated for their effect on melanogenesis. Only five compounds were tested. Compounds **3** and **4** were not tested due to their low yield during isolation. *G. flanaganii* showed minimal toxicity effect on the cells with the IC₅₀ value of >400 µg/ml, and at least 20% reduction of melanin content. 5,7-dihydroxyflavanone [(2*S*)-pinocembrin] (**C6**) showed minimal toxicity effect on the cells with 10% in melanin content reduction. 2',4',6'-trihydroxydihydrochalcone (**C2**) and 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) (**C1**), 4-hydroxyphenethyl-3-hydroxy-5-phenylpentanoate (**C7**) were not toxic at lower concentrations. 2',6'-dihydroxy-4'-methoxydihydrochalcone (**C5**)

was toxic at most concentrations tested. Therefore from this results shows that this compounds work at the enzyme level to reduce melanin synthesis as they showed less toxicity effect on the cells. Not all tyrosinase inhibitors are toxic to human beings. Plant derived compounds such as Arbutin have been proven to aid in removal of age spots but its draw down is that it causes paradoxical hyper-pigmentation if its used for three months (Mashhood, 2006).

7.4 Antioxidant activity

Increased melanin synthesis is also caused by availability of free radicals. A compound that can have the ability to be a free radical scavenger will give some advantage as it will protect the skin against UV damage (a factor generating free radicals). Flavonoids isolated from plants have been previously reported to possess this activity. Compounds isolated from *G. flanaganii* and the extract exhibited strong antioxidant activities. 2',4',6'-trihydroxydihydrochalcone showed good radical scavenging activity as compared to all the compounds. Antioxidant activity observed in this study could be due to their chemical structures of the isolated compounds as they posses the flavonoid backbone.

7.5 Antibacterial activity against *P. acnes*

Actinic damage of the skin is also a problem leading to hyper-pigmentation. This mostly occurs during puberty. A product that can posses all the above mentioned activities as well as anti-acne activity will have an added advantage to be considered as a skin

lightener. *G. flanaganii* leaf extract showed inhibition of the growth of the *P. acnes*. 2',4',6'-trihydroxydihydrochalcone, 4-hydroxyphenethyl-3-hydroxy-5-phenylpentanoate and 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) showed inhibition of the growth of the bacteria with the MIC of 968, 1590 and 418 μ M respectively. This shows that even if one compound cannot reduce melanin formation at the enzyme level then it can be used for other factors to help in reaching one goal .i.e. inhibition of increased melanin biosynthesis. Overall it was found that *G. flanaganii* has antityrosinase, antioxidant and antibacterial activities, showing low cytotoxic effects on melanocytes cells. Therefore it can be considered as a possible cosmeticeutical agent.

7.6 Future work

Further studies are required to effectively understand the mode of action of each compound on tyrosinase inhibition. Kinetic studies of each compound (s) on tyrosinase enzyme should also be studied in detail. The effect of antityrosinase agents on the regulation of tyrosinase-related proteins and mRNA in epidermal melanocytes cells should also be investigated. *In-vivo* studies are also recommended as at times *in-vitro* studies only, does not validate a compound to be effective *in-vivo* due to many mechanisms of the body system involved. Therefore, new strategies should be formed to combine the two systems (*in-vivo* and *in-vitro*) for discovering of new skin depigmentation agents.

7.7 References

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CHAPTER 8

Acknowledgements

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- Lastly I dedicate this thesis in memory of the Late Mr. Kenoven Cheung, for we struggled together in some of the assays in this study but through dedication and patience the experiments aimed for the present study were completed



CHAPTER 9

Appendices

Appendix A

9.1 ^1H -NMR and ^{13}C -NMR of isolated compounds from ethanol leaf extract of *Greyia flanagani* (Bolus)

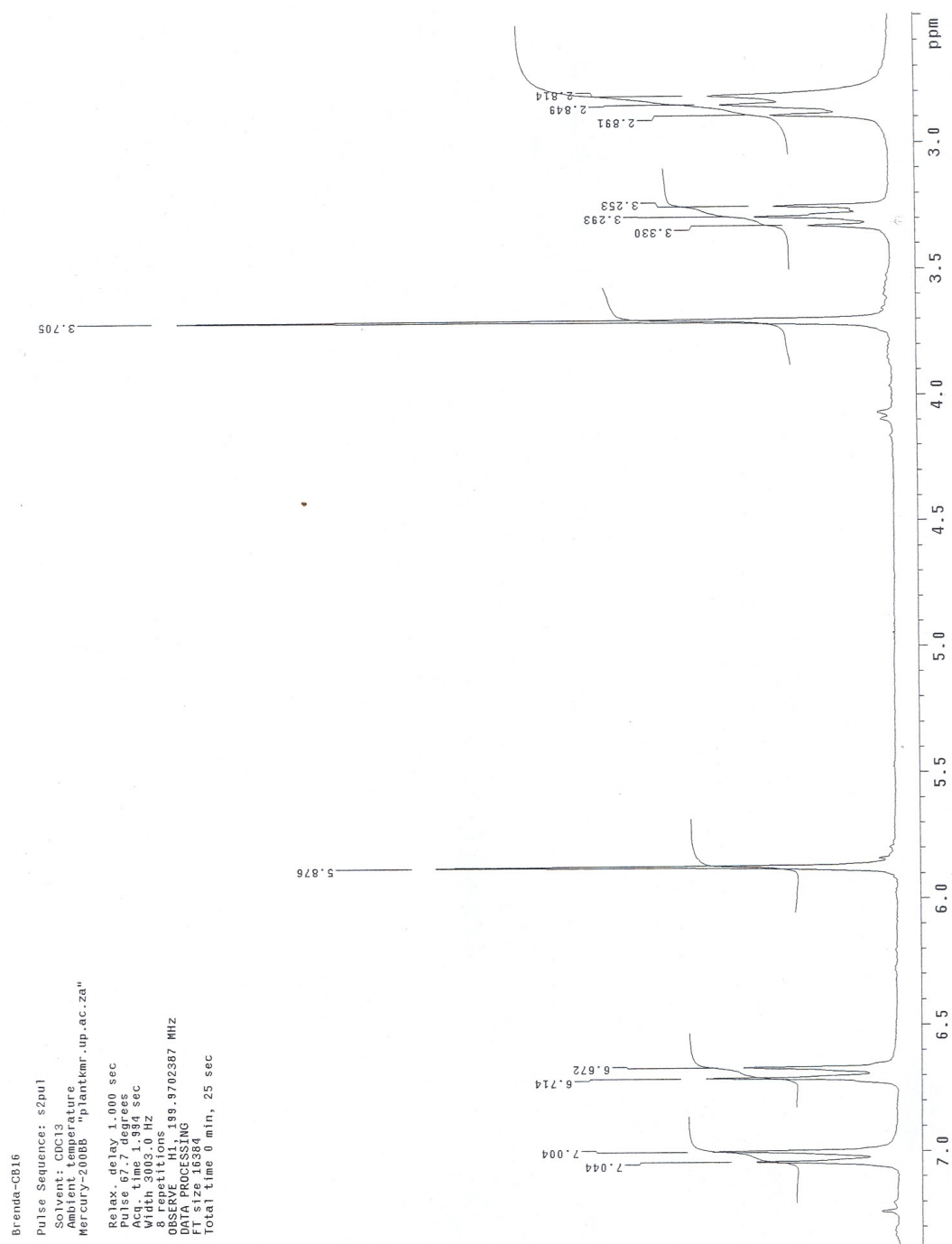


Figure 9.1 ^1H -NMR spectra of 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) isolated from *G. flanaganii*

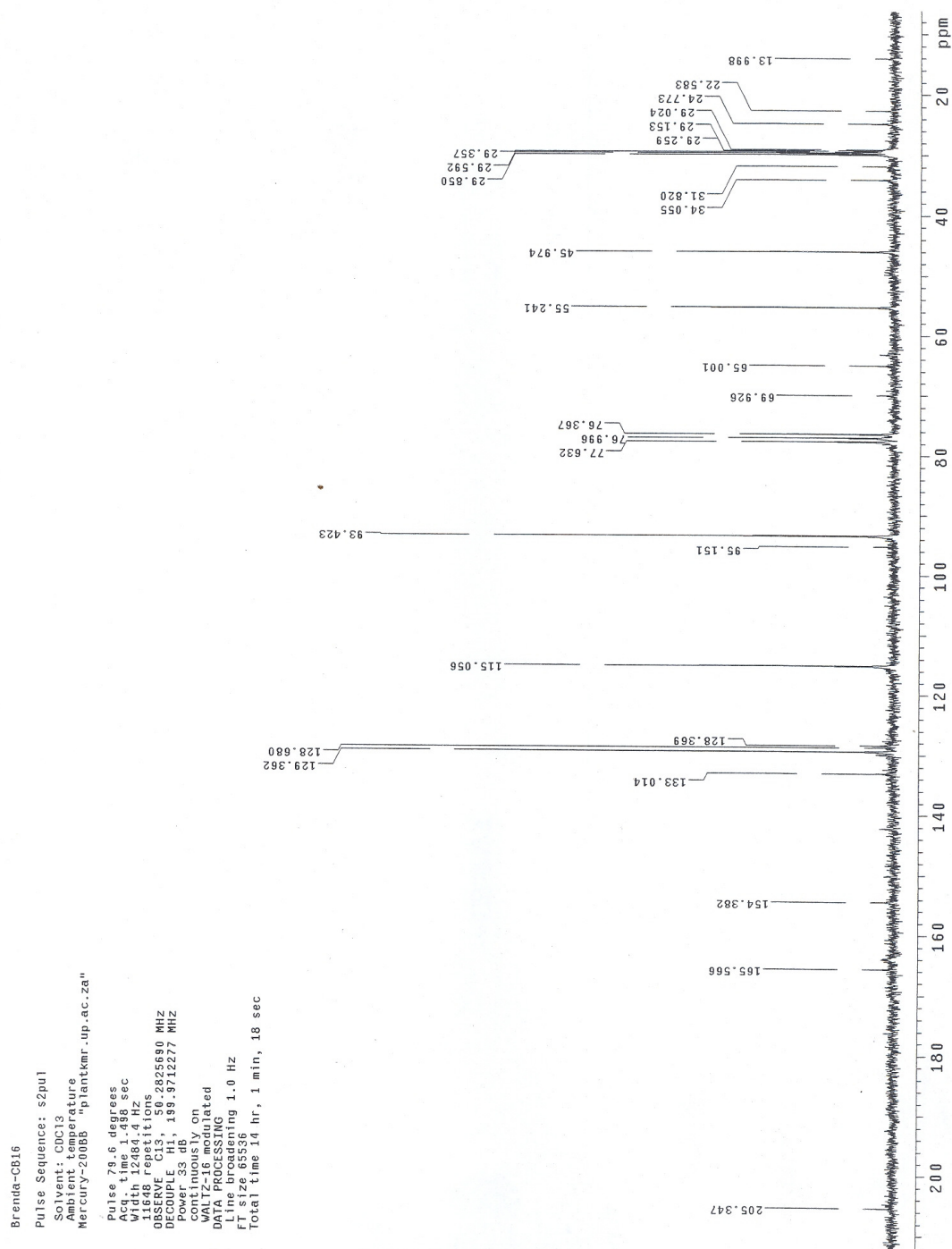


Figure 9.2 ^{13}C -NMR spectra of 2',6',4'-trihydroxy-4'-methoxydihydrochalcone (asebogenin/lyonogenin) isolated from *G. flanaganii*

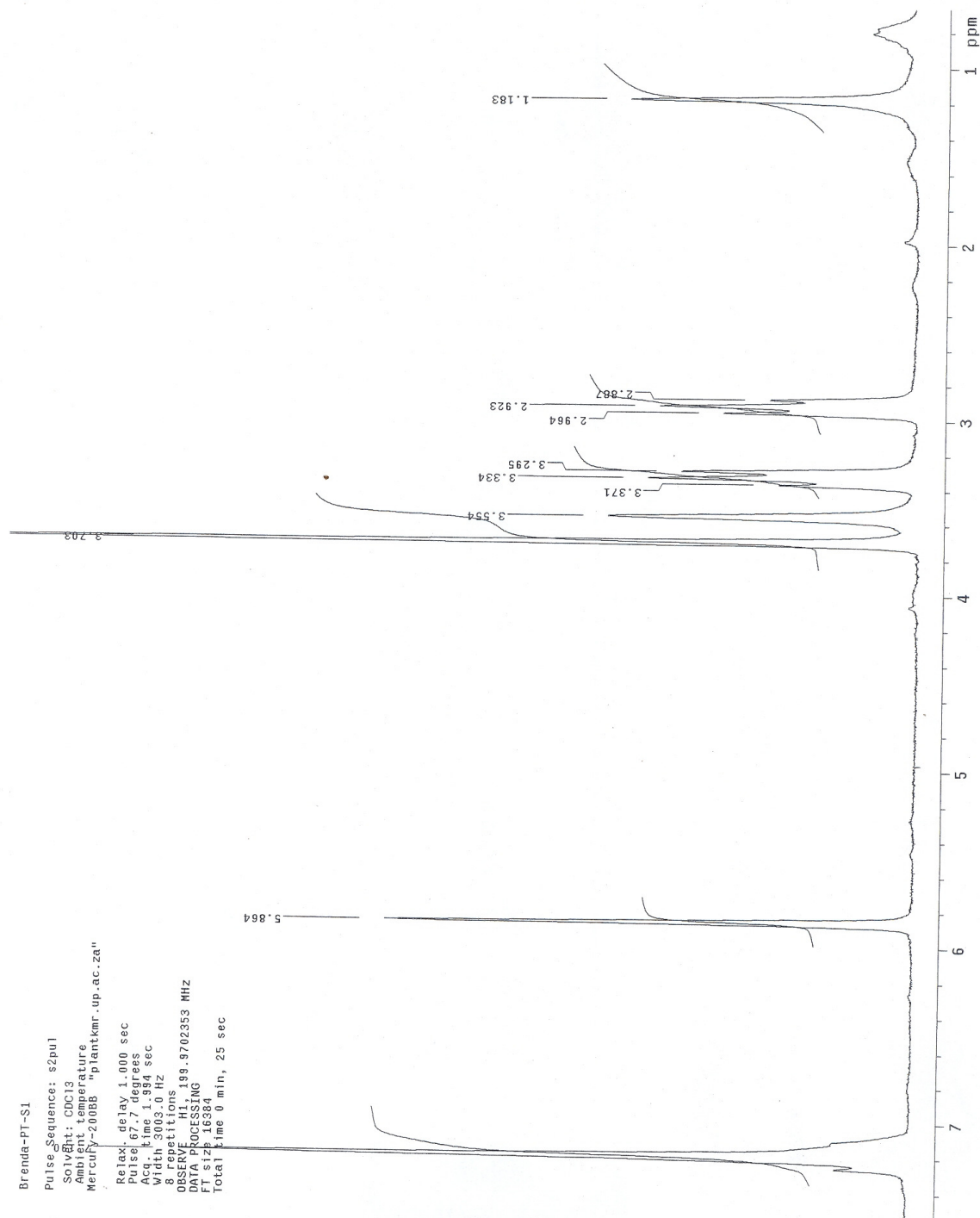


Figure 9.3 ^1H -NMR spectra of 2',6'-dihydroxy-4'-methoxydihydrochalcone isolated from *G. flanaganii*

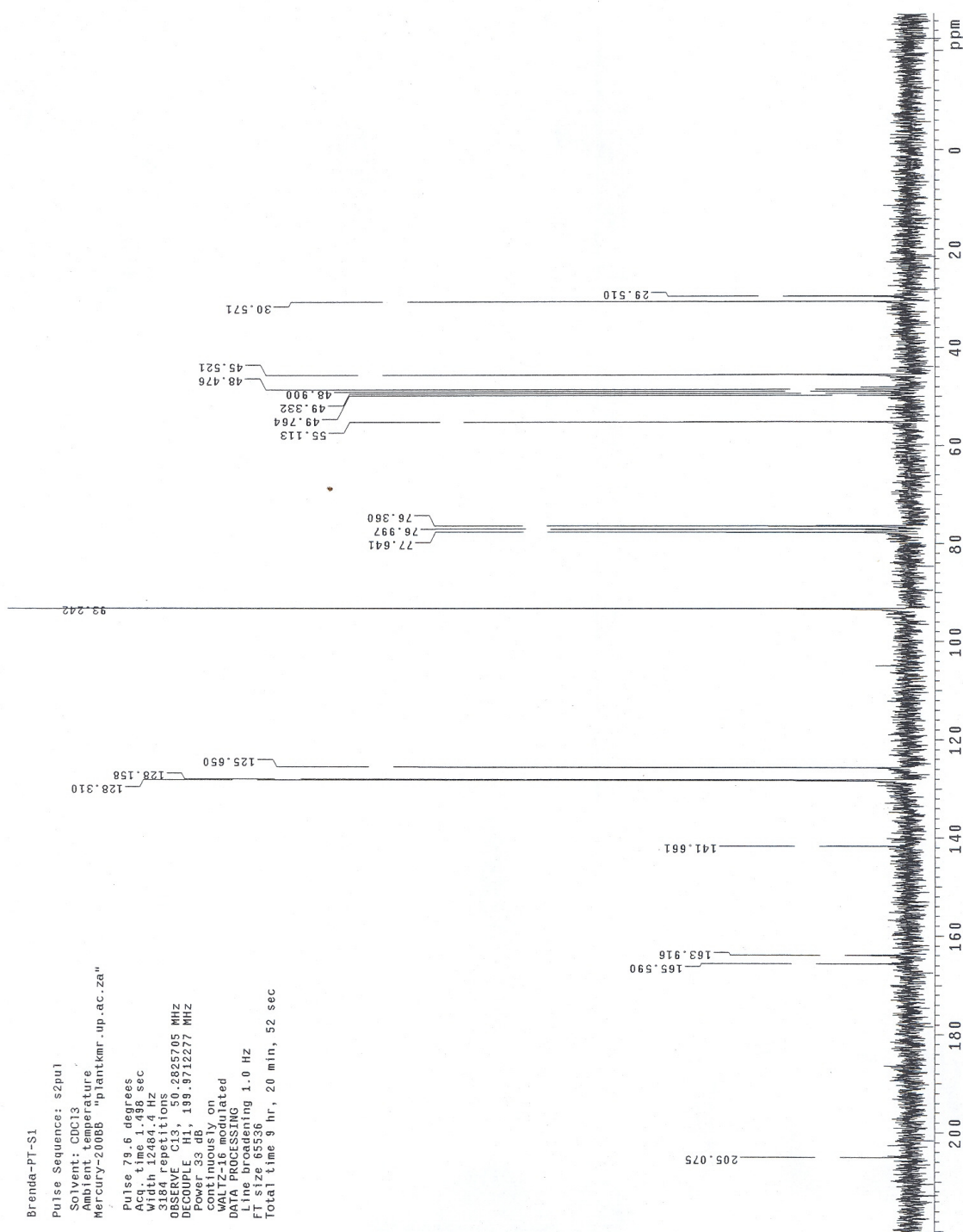


Figure 9.4 ^{13}C -NMR spectra of 2',6'-dihydroxy-4'-methoxydihydrochalcone isolated from *G. flanaganii*

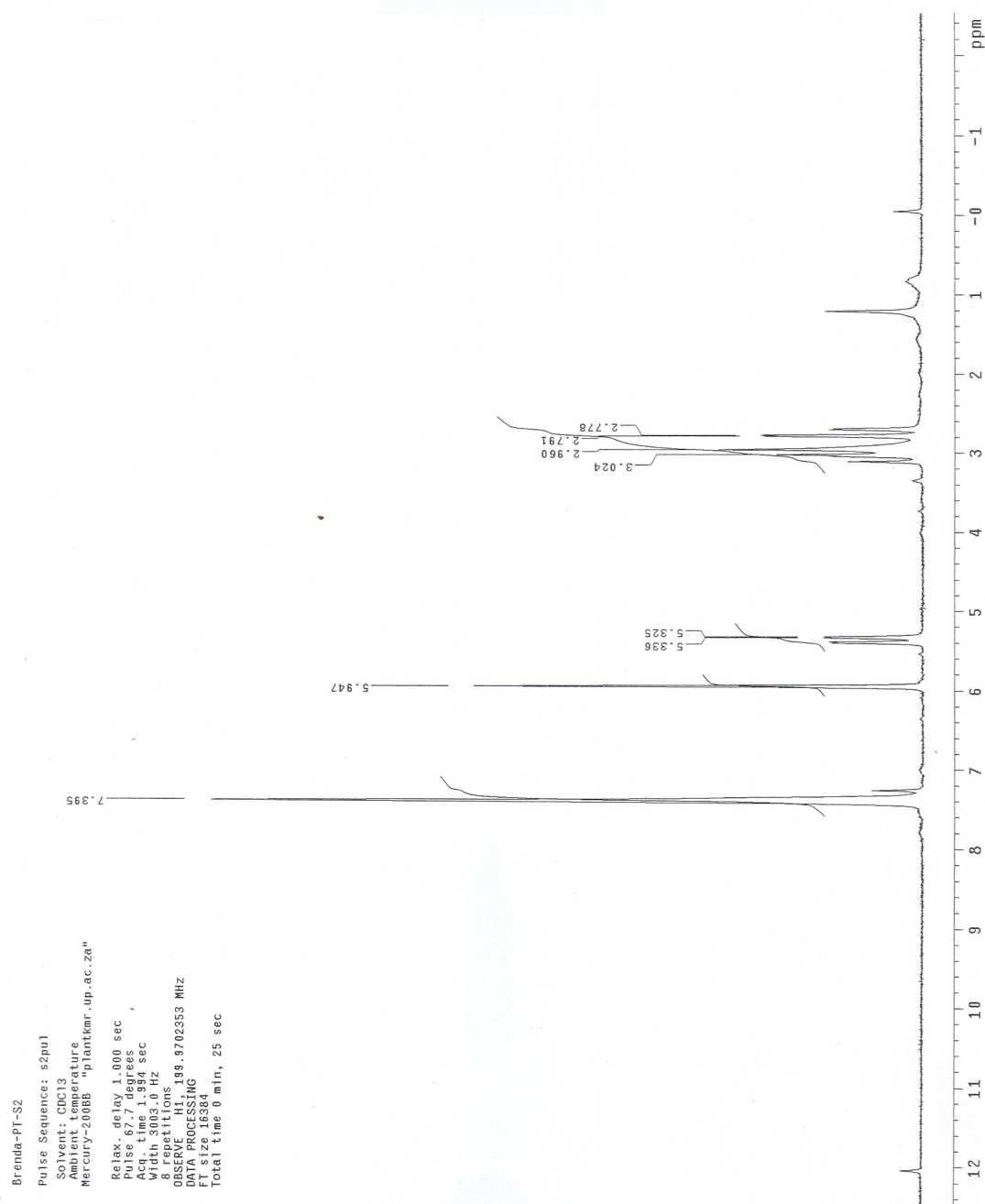


Figure 9.5 ^1H -NMR spectra of 5,7-dihydroxyflavanone [(2S)-pinocembrin] isolated from *G. flanaganii*

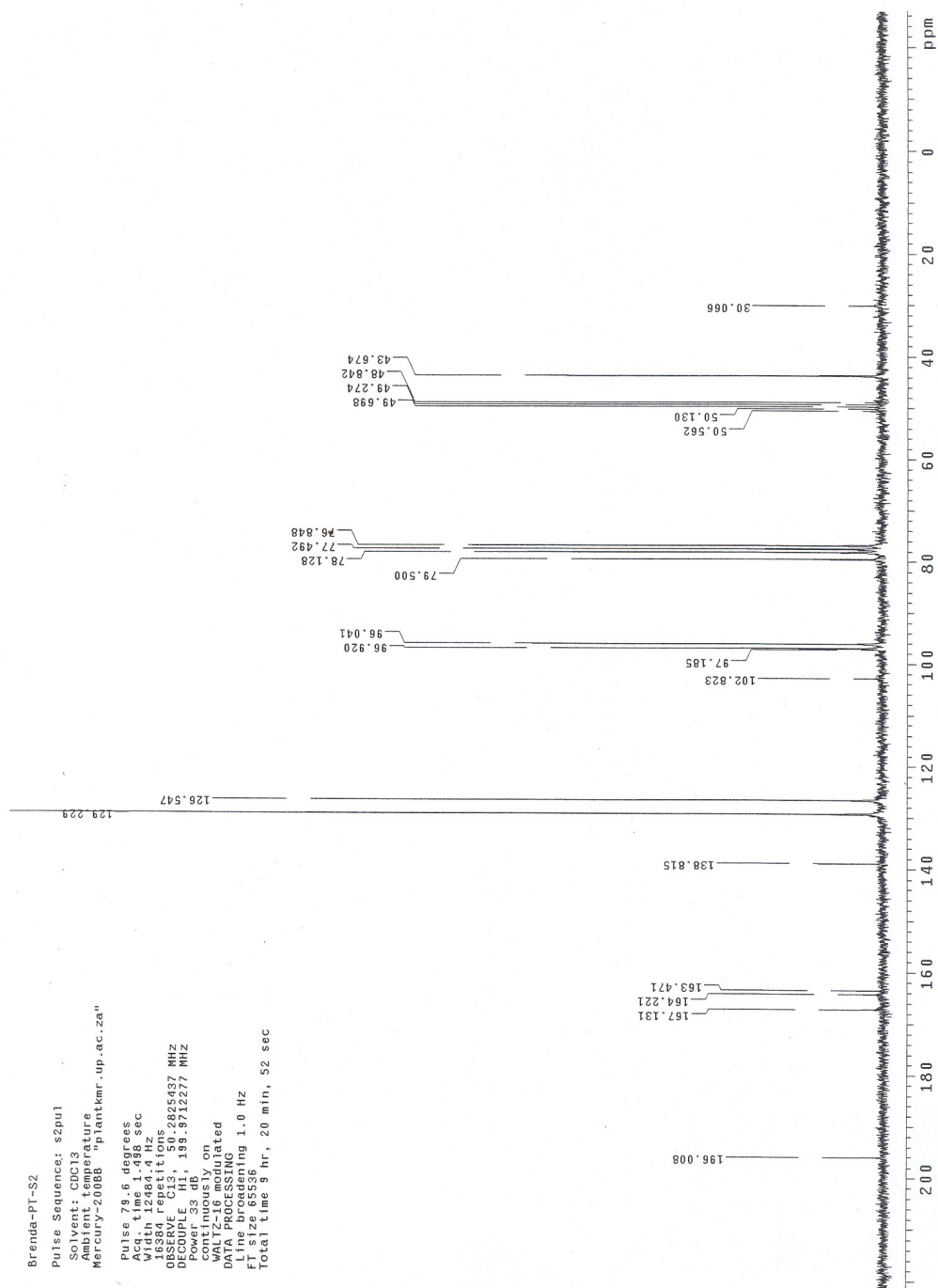


Figure 9.6 ¹³C-NMR spectra of 5,7-dihydroxyflavanone [(2*S*)-pinocembrin] isolated from *G. flanaganii*

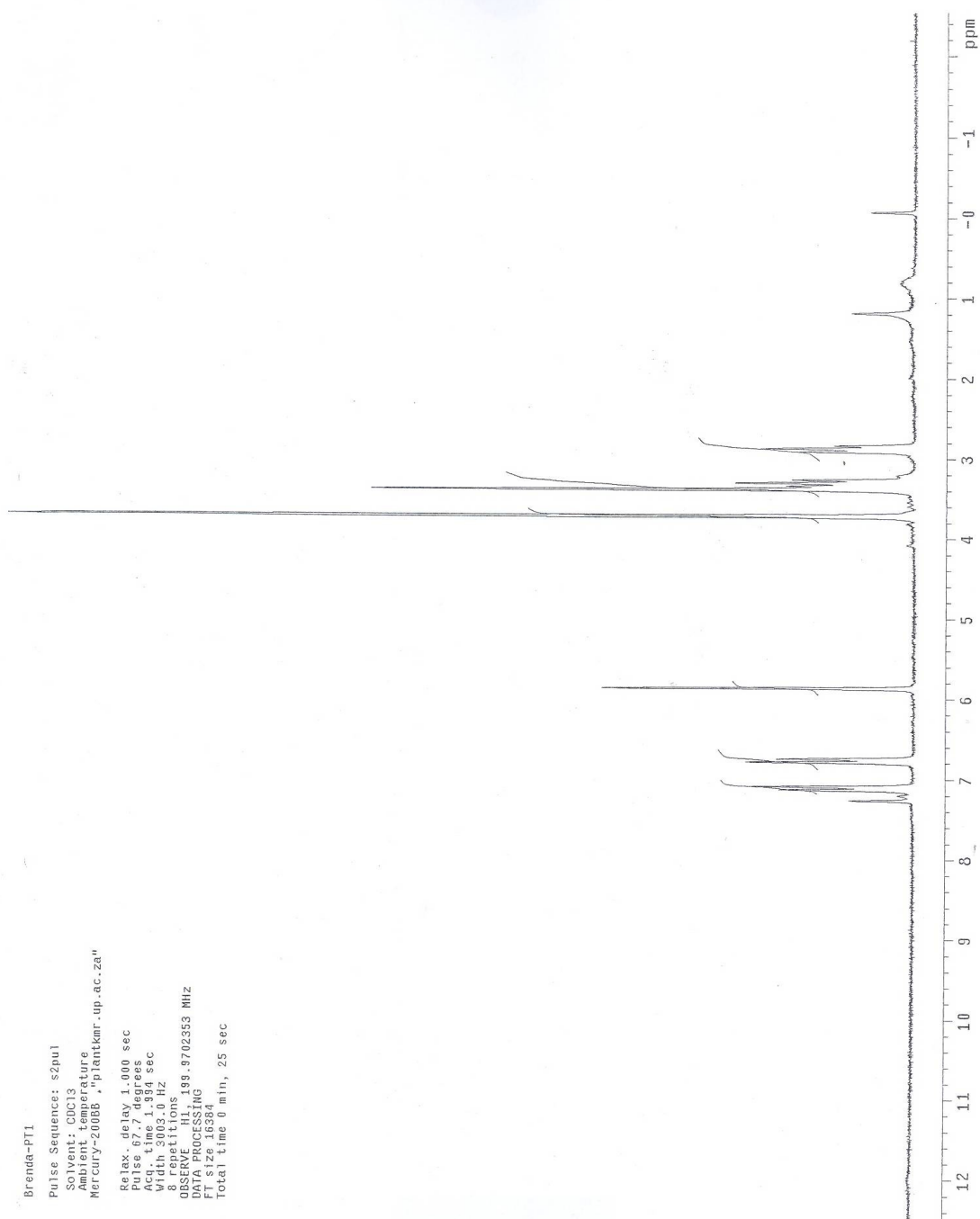


Figure 9.7 ^1H -NMR spectra of 2',6'-dihydroxy-4',4-dimethoxydihydrochalcone isolated from *G. flanaganii*

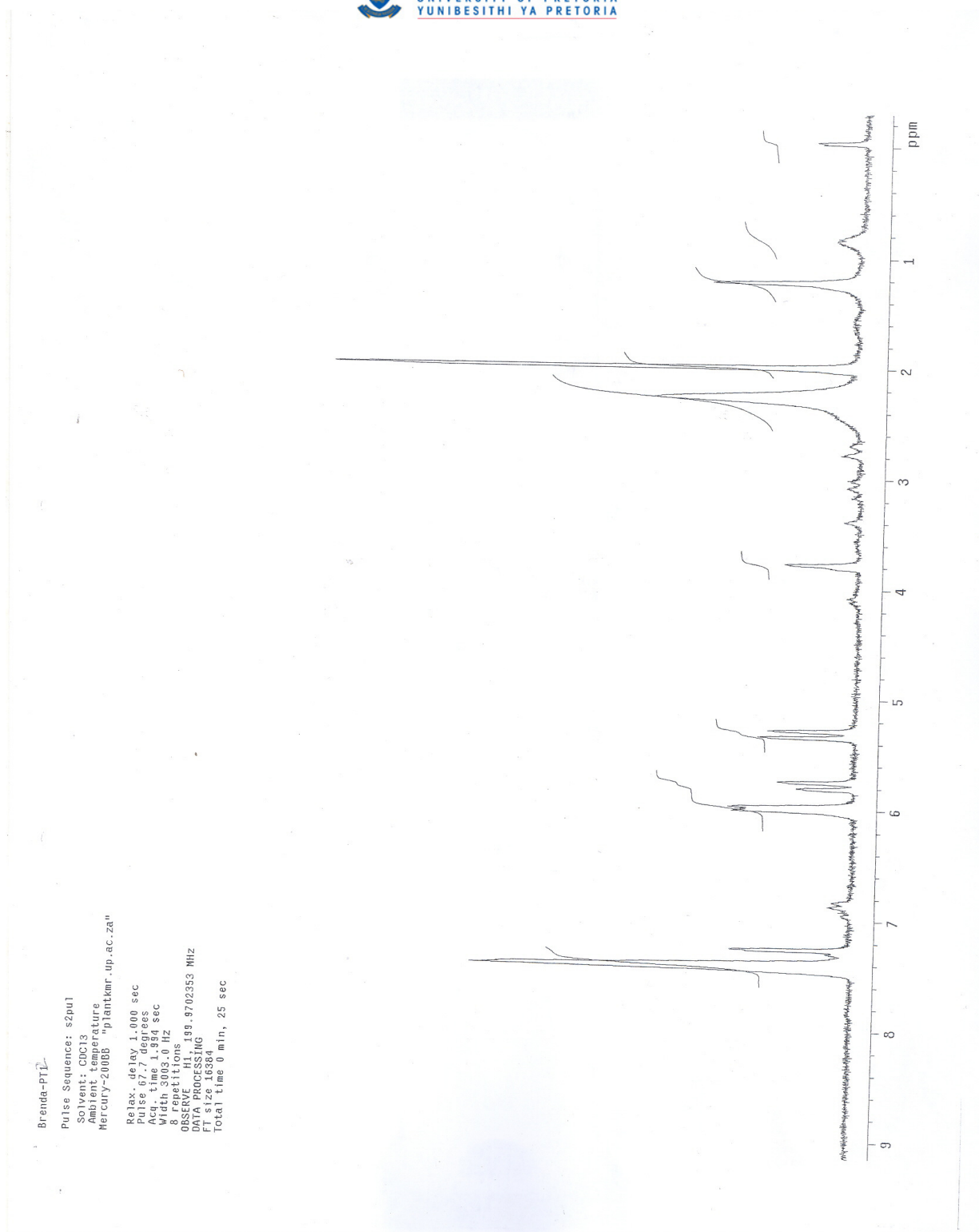


Figure 9.8 ^1H -NMR spectra of (2*R*,3*R*)-3,5,7-trihydroxy-3-*O*-acetylflavanone (pinobanksin-3-acetate/dihydrogalangin-3-acetate) isolated from *G. flanaganii*

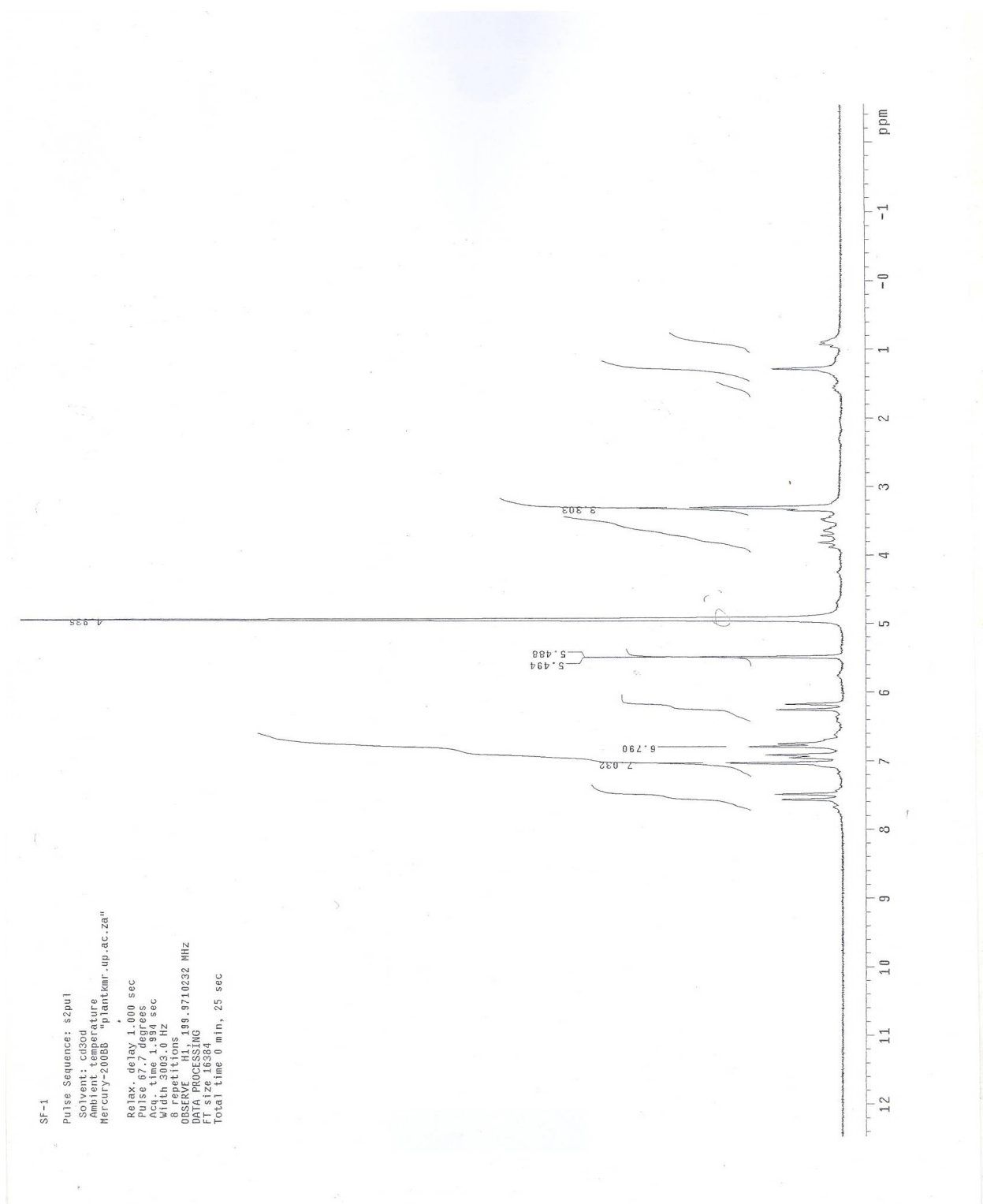


Figure 9.10 ^1H -NMR spectra of 2',4',6'-trihydroxydihydrochalcone isolated from *G. flanaganii*

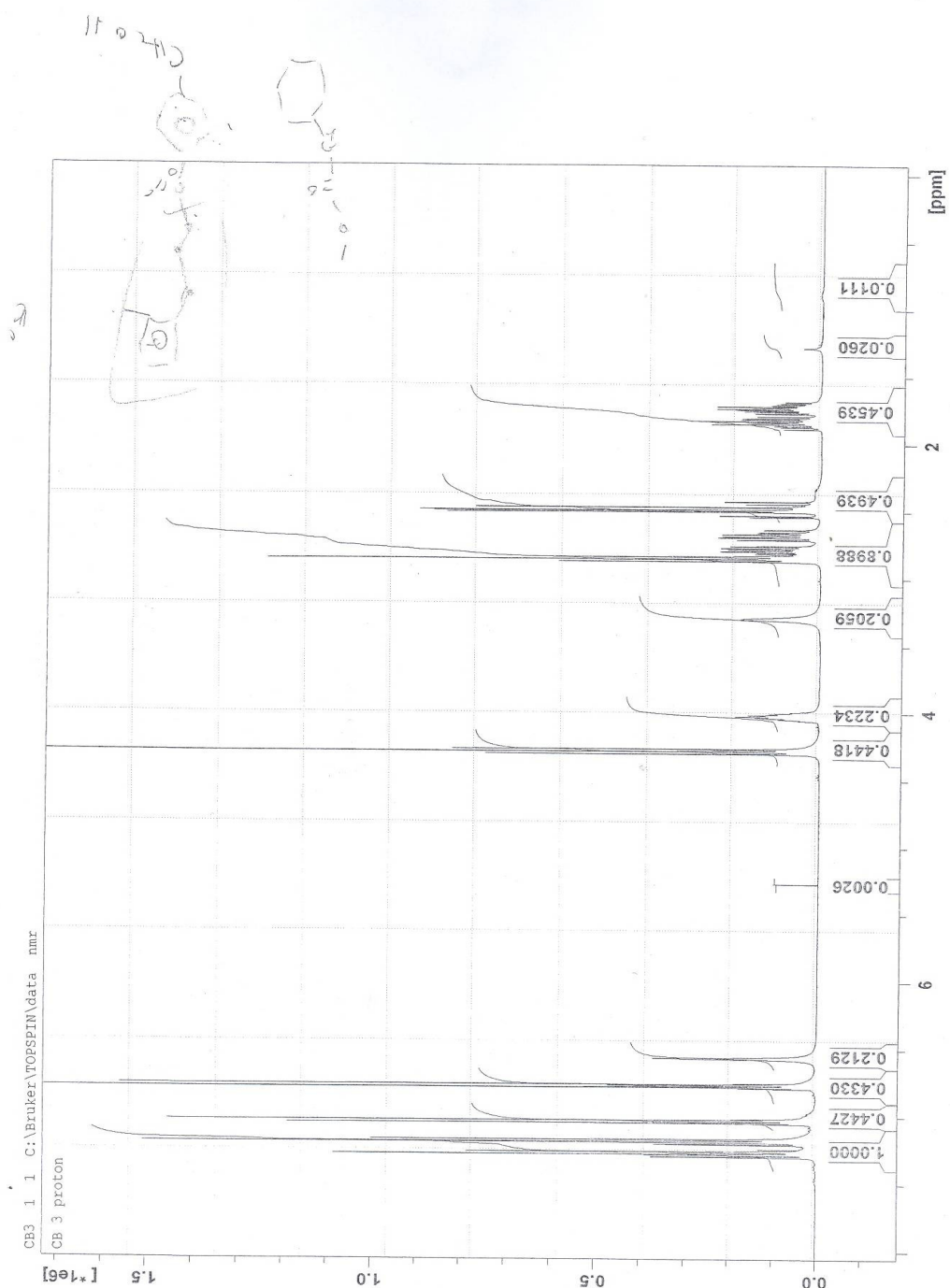


Figure 9.10 ¹H-NMR spectra of 4-hydroxyphenethyl 3-hydroxy-5-phenylpentanoate isolated from *G. flanaganii*

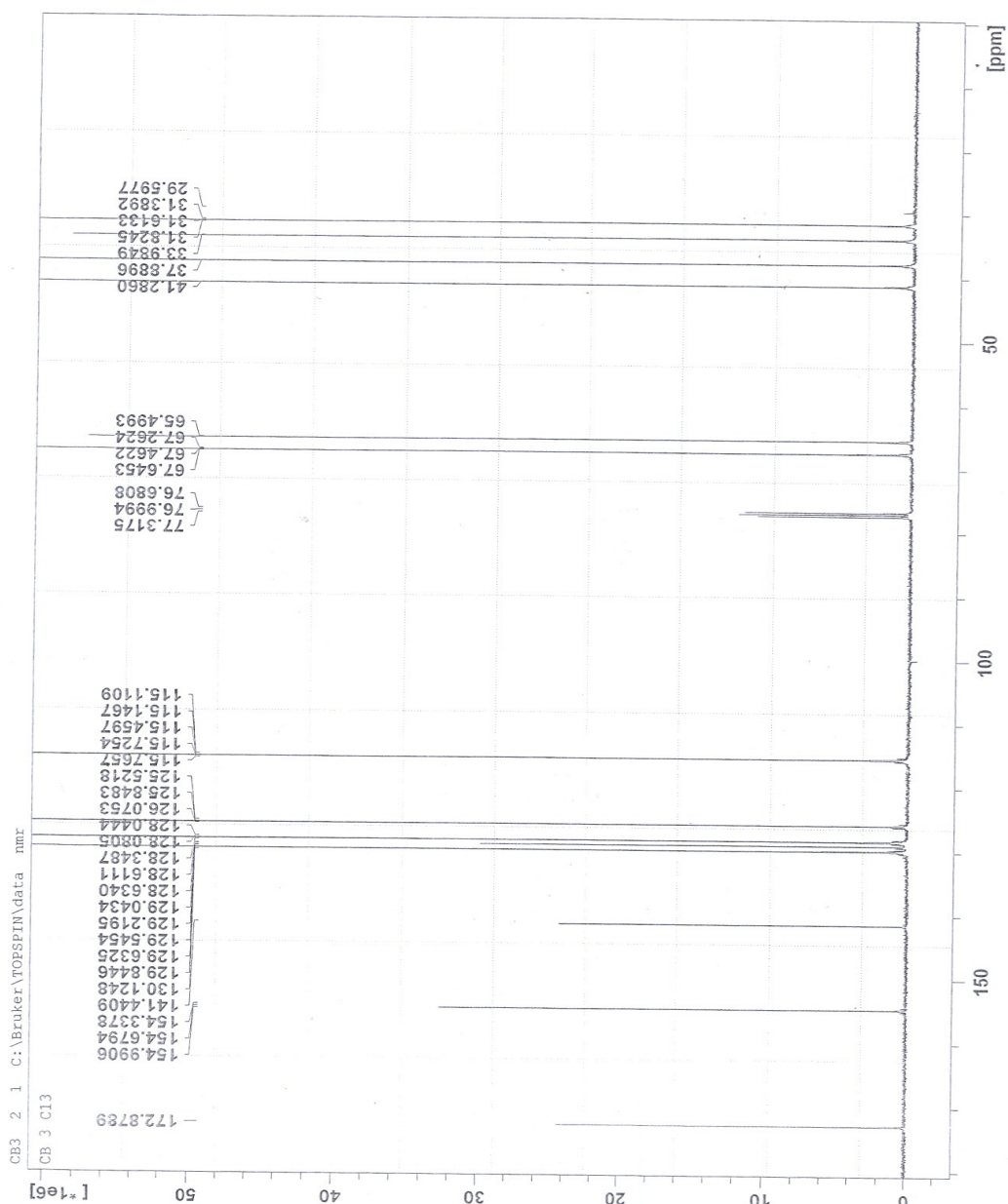


Figure 9.10 ^{13}C -NMR spectra of 4-hydroxyphenethyl 3-hydroxy-5-phenylpentanoate isolated from *G. flanaganii*

Appendix B

9.2 Conference presentations resulting from this thesis

- SAAB conference (2009) (Oral presentation): Mapunya MB, Lall N, Hussein AA, 2009. Multifunctional actives from *Greyia flanaganii* (Bolus) with anti-acne and skin-depigmentation activities.
- University of Pretoria post graduate symposium (2008) (Oral presentation): Mapunya MB, Lall N, Hussein AA, 2009. Multifunctional actives from *Greyia flanaganii* (Bolus) with anti-acne and skin-depigmentation activities.

9.3 Article in preparation:

Mapunya MB, Lall N, Hussein AA, 2009. Mushroom tyrosinase activity of novel and known phenolic compounds isolated from *Greyia flanaganii* (Bolus)