Tyrosinase inhibitors isolated from *Ceratonia siliqua* (L.) and *Sideroxylon inerme* (L.)

by

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

(Plant Science)

In the Department of Plant Science, Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

April 2007

Promoter

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List of Abbreviations

ACTH	**Adrenocorticotrophic hormone**
AKT	**Serine-threonine kinase**
ANOVA	**Analysis of variance**
AsA	**Ascorbic acid**
ATRA	**Tretinoin, all-trans retinoic acid**
BC	**Before Christ**
BSE	**Bovine spongiform encephalitis**
Ca^{2+}	**Calcium ion**
CAF	**Caffeine**
^{13}C-NMR	**Carbon nuclear magnetic resonance**
CPSC	**Consumer Products Safety Commissions**
DHI	**Dihydroxyindole**
DHICA	**Dihydroxyindole-carboxylic acid**
DH_2O	**Distilled water**
DMEM	**Dulbeccos modified eagle's medium**
DMSO	**Dimethyl sulphoxide**
DNA	**Deoxyribonucleic acid**
2D-NMR	**2-dimentional nuclear magnetic resonance**
DOPA	**l-3,4-dihydroxyphenylalanine**
DPPH	**1,2-diphenyl-2-picrylhydrazyl**
EC	**Epicatechin**
EGC	**Epigallocatechin**
EGCG	**Epigallocatechin-3-O-gallate**
EPR	**Electron paramagnetic resonance**
ERK	**Extracellular signal-regulated kinase**
EtoH	**Ethanol**
FCS	**Fetal calf serum**
GCG	**Gallocatechingallate**
HA	**Hydroxyanisol**

HEPES	**N-(2—hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) sodium salt**
^{1}H-NMR	**Proton nuclear magnetic resonance**
H_2O_2	**Hydrogen peroxide**
HPLC	**High performance liquid chromatography**
HQ	**Hydroquinone**
Mg^{2+}	**Magnesium ion**
MITF	**Microphetalmia transcription factor**
MSH	**Melanocyte stimulating hormone**
$NaHCO_3$	**Sodium hydrogen carbonate**
NH_4OH	**Ammonia solution**
NMR	**Nuclear magnetic resonance spectroscopy**
.OH	**Hydroxyl radical**
$^{3}O_2$	**Oxygen triplet bi-radical**
$^{1}O_2$	**Single oxygen**
O_2^-	**Superoxide radical**
PBS	**Phosphate buffer saline**
PKB	**Protein kinase B**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
4-SCAP	**4-S-cystaminylphenol**
TLC	**Thin layer chromatography**
α-Toc	**α-tocopherol**
TRP-1	**Tyrosinase related protein-1**
TRP-2	**Tyrosinase related protein-2**
UV	**Ultra violet**
VG-PMG	**Magnesium-l-ascorbyl-2-phosphate**
WHO	**World Health Organization**
XTT	**sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro benzene sulfonic acid hydrate), labelling reagent**

Tyrosinase inhibitors isolated from *Ceratonia siliqua* L. and *Sideroxylon inerme* L.

Abstract

Tyrosinase is known to be a key enzyme in melanin biosynthesis, which is involved in determining the color of mammalian skin and hair. Various dermatological disorders, such as melasma, age spot and sites of actinic damage arise from the accumulation of an excessive level of epidermal pigmentation. The inadequacy of current therapies to treat these conditions as well as high toxicity and mutagenicity, poor skin penetration and low stability of existing depigmenting agents led us to seek new whitening/anti-pigmentation agents from plants.

Acetone, dichloromethane and methanol extracts of three plant species namely; *Cassipourea malosana* L. (stem-bark), *Ceratonia siliqua* L. (leaves) and *Sideroxylon inerme* L. (stem-bark) were evaluated for tyrosinase inhibitory activity using mushroom tyrosinase (monophenolase and diphenolase used as substrates) at concentration ranging from 3.125 to 400 µg/ml.

Acetone, dichloromethane and methanol extracts of *C. malosana* did not exhibit any significant inhibition of monophenolase activity at the concentrations tested but a significant ($P<0.01$) inhibitory activity was achieved by its methanol extract at 12.5 µg/ml when L-DOPA was used as a substrate.

Methanol extract of *C. siliqua* showed significant ($P<0.01$) inhibition of tyrosinase activity (125%) at 400 µg/ml whilst L-tyrosine was used. No significant reduction ($P<0.01$) of tyrosinase activity was observed by its acetone and dichloromethane extracts. Its methanolic extract exhibited 43% and 23% inhibition of diphenolase activity at 400 and 200 µg/ml ($p<0.01$) respectively. Following the results obtained from tyrosinase assay, the methanol extract of *C. siliqua* was selected for

further investigation to examine its ability on melanin production in an *in vitro* environment. A significant reduction in melanin production (45%) was observed by this extract at 12.5 µg/ml while the cells' viability was more than 90%. Consequently, the methanol extract was selected for isolation of its bioactive compounds.

The methanol extract was fractionated using phase-partitioning with n-hexane, ethyl acetate and n-butanol, subsequently. Anti-tyrosinase activities of these fractions were determined, *in vitro* and *in vivo*. The ethyl acetate fraction exhibited the highest tyrosinase inhibitory activity. Therefore, this fraction was subjected to further separation by chromatographic methods. Two flavonoids (quercetin-3-*O*-α-L-rhamnoside and myricetin-3-*O*-α-L-rhamnoside) and a gallotannin compound (1,2,3,6-tetra-*O*-galloyl-ß-D-glucose) were isolated and their structures were determined by NMR spectroscopic methods. Quercetin-3-O-α-L-rhamnoside showed significant ($p<0.01$) anti-tyrosinase activity (50%) at 50 µg/ml while L-DOPA was used. Myricetin-3-O-α-L-rhamnoside exhibited significant reduction ($p<0.01$) of monophenolase (30%) and diphenolase (43%) activity at 200 µg/ml. Also a significant ($p<0.01$) tyrosinase inhibitory activity (90%) was observed by 1,2,3,6-tetra-O-galloyl-ß-D-glucose at 200 µg/ml using L-tyrosine as a substrate. This compound showed 65% anti-DOPA activity at 200 µg/ml ($p<0.01$). Quercetin-3-O-α-L-rhamnoside showed 28% inhibition of melanin production at 6.2 µg/ml significantly ($p<0.01$). Myricetin-3-O-α-L-rhamnoside exhibited a significant ($p<0.01$) reduction of melanin content (60%) in B16 melanocytes at 12.5 µg/ml without being toxic to the cells at this concentration. 1,2,3,6-tetra-O-galloyl-ß-D-glucose decreased the cell viability/proliferation at all the concentrations examined in this study. Compounds "1,2,3,6-tetra-O-galloyl-ß-D-glucose, quercetin-3-O-α-L-rhamnoside and myricetin-3-O-α-L-rhamnoside" have been isolated before from *C. siliqua*. 'Quercetin-3-O-α-L-rhamnoside' has not been explored before for its inhibitory activity for monophenolase, diphenolase activity and for melanogenesis.

The antioxidant activity of *C. siliqua* (methanol extract) and its purified compounds was determined by the DPPH radical scavenging method. The methanol extract of *C. siliqua* showed the highest antioxidant activity which was followed by the purified compounds, 1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside. EC_{50} value of extract was found to be 19.33 µg/ml. 1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside exhibited EC_{50} values of 42.55 µg/ml and 76.59 µg/ml respectively. Vitamin C and quercetin were used as

standard controls which showed EC_{50} values of 3.339 µg/ml and 51.06 µg/ml respectively.

Results of monophenolase-inhibitory activity by the methanol and acetone extracts of *S. inerme* were found to be similar, (70% of tyrosinase activity) at 200 µg/ml ($P<0.01$). No significant ($P<0.01$) anti-tyrosinase activity was obtained by its dichloromethane extract at any concentration tested. The methanolic extract of this plant did not exhibit significant ($P<0.01$) anti-DOPA activity at any concentration tested in this study. The methanol extract of *S. inerme* was subjected to a toxicity assay in conjunction with the melanin production assay. The sample showed significant inhibition (37%) of melanin production at 6.2 µg/ml while 80% of cells were viable. The methanolic extract of *S. inerme* was further subjected to bioassay guided fractionation. The extract was partitioned with n-hexane, ethyl acetate and n-butanol. Monophenolase inhibitory activities of these fractions were determined, *in vitro* and *in vivo*. The ethyl acetate fraction exhibited the highest anti-tyrosinase inhibition. Bioassay guided phytochemical investigation of this extract led to the isolation of two pure compounds namely; 'epicatechin gallate' and 'procyanidin B2'. Epicatechin gallate exhibited 58% anti-tyrosinase activity at 200 µg/ml significantly ($p<0.01$) whereas procyanidin B2 showed 55% reduction of monophenolase activity at the same concentration. Diphenolase activity and inhibition of melanin formation were not observed by these compounds at any concentration tested in this study ($p<0.01$).

Epicatechin gallate and procyanidin B2 exhibited DPPH discolouration by 72% and 67% at 3.9 µg/ml respectively. Antioxidant activities of these two compounds were found to be higher than those observed for the standards (vitamin C and quercetin). These compounds are likely to be responsible for the high antioxidant activity of the extract of *S. inerme* (EC_{50}= 1.56 µg/ml).

C. siliqua (methanol extract), *S. inerme* (methanol extract) and quercetin-3-O-α-L-rhamnoside (isolated from the methanol extract of *C. siliqua*) have not been explored before for their inhibitory activities for monophenolase, diphenolase and melanogenesis. In addition, the present study reports for the first time the isolation of compounds, 'epicatechin gallate' and 'procyanidin B2' from the methanol extract of *S. inerme*. The DPPH scavenging capacity of *S. inerme* (methanol extract), purified compound '1,2,3,6-tetra O-galloyl-ß-D-glucose' has not been reported previously.

Considering the positive anti-tyrosinase activity of pure compounds isolated from *C. siliqua* and *S. inerme* found in this study, it can be concluded that these compounds can be worth considering to be used as skin-lightening agents. A significant activity for melanogenesis observed by the methanol extract of *C. siliqua* and acetone and methanol extracts of *S. inerme*, make these extracts good candidates for hyperpigmentation-problems.

Chapter 1

General introduction

1.1. Background and motivation of the study

It has been assumed that "there is a plant for every need on every continent". Remarkably, this statement appears to be true. Finding healing powers in plants is an ancient idea (Cowan, 1999). The World Health Organization (WHO) estimates that 80% of the people living in developing countries almost exclusively use traditional medicine. Medicinal plants used in traditional medicine should therefore be studied for safety and efficacy. It has been estimated that 14-28% of higher plant species are used medicinally, which are only 15% of all angiosperms that have been investigated chemically. According to the database, 74% of pharmacologically-active plant derived drugs have been discovered after following up on ethnomedical use of the plant (Eloff, 1998). Natural molecules derived from plant extracts offer a particularly exciting avenue for further research (Aburjai & Natsheh, 2003).

The traditional use of plants against skin disease and especially for cosmeceutical purposes is a common practice in the domestic medicine of many cultures. Most of the plant ingredients (oat, walnuts, chamomile, carrot, almonds, cucumber, lavender, mint, rose and sweet violet petals) are used also today in the modern phytocosmetics as shampoos, creams, lotions and sun care products (Pieroni *et al*. 2004). It is interesting how plants produce a compound for one purpose and humans use this compound for an unrelated purpose. Phytotherapeuticals are a group of natural constituents from plants used to heal well-defined afflictions of the skin apparatus. A number of the well known medicinal plants are widely used in modern phytotheraphy to heal skin diseases. External or topical applications of some of these plants were never recorded before in modern ethnobotanical studies (Pieroni *et al*. 2004). These medicinal plants can be employed either for dermatological or cosmeceutical purposes.

Along human history, people have been struggling with numerous skin diseases, specially skin pigmentation (hyper/hypo-pigmentation). It is well documented that tyrosinase (EC 1.14.18.1) is an essential enzyme in pigment formation in mammalian's body as well as plants, microorganisms and fungi. Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin-whitening effects. Scientists and herbalist have investigated the effect of different plant extracts (oat, walnuts, chamomile, carrot, almonds, cucumber, lavender, mint, rose and sweet violet petals) on skin for the treatment of hyper/hypo-pigmentation disorders. A number of tyrosinase inhibitors are reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. Representative of tyrosinase-inhibiting agents from plants include: vitamin C, hydroquinones, aloesin, arbutin, azelaic acid, licorice and *Morus alba* extracts.

For many years, tyrosinase has been studied for its use in cosmetics as well as in food industries. Browning in fruits and vegetables is of great concern because of tyrosinase to growers and the food industry. Various recent papers have exposed some previously unexplored aspects of tyrosinase in clinical studies. Contradictory results are available regarding the role of tyrosinase in cancer as some papers suggest a tumor-suppressing effect of mushroom tyrosinase, whereas others predict a possible role in mutagenicity (Seo *et al*. 2003). Nowadays the importance of tyrosinase inhibitors is well known and as plants are naturally gifted as a source of bioactive and mostly safe chemicals (without harmful side effects) therefore, there is an ongoing effort to search for tyrosinase inhibitors from them.

1.2. Philosophical concepts of cosmetics

The word *cosmetic* (originated from Greek), *kosmétikos*, means "skilled in adornment." *Kosmein* means "arrange," or "adorn." *Kosmos* means "order:" It also means "to make for beauty, especially of the complexion, or beautifying", it also means "done or made for the sake of appearance," or "correcting defects especially of the face." More than that it is "decorative," or "ornamental" (Oumeish, 2001).

According to the Consumer Products Safety Commission (CPSC, USA) 1989; a **cosmetic** product refers to 'any substance or preparation intended for application to any external surface of the human body (epidermis, hair system, nails, lips and external genital organs), or to the teeth or buccal mucosa wholly or mainly for the purpose of cleaning, perfuming or protecting them, or keeping them in good condition or changing their appearance or combating body odour or perspiration except where such cleaning, perfuming, protecting, keeping, changing or combating is wholly for the purpose of treating or preventing disease' (Aburjai & Natsheh, 2003).

For many centuries, cosmetics were made to serve beauty, elaborate it, or promote it. Beauty was only one aspect of the Greek word “komes”, which means harmony. It is an aspect of the truth and close to the perfection of human beings. Over time, the meaning changed into masking, concealing, covering up and camouflaging. After all, real beauty could never be created only from the outside; it had to originate mainly from the inner being.

Since the dawn of history, humans have searched for materials and developed many products for women to enhance their beauty, especially in the eyes of men. Women also embellished themselves and tried always to be aesthetically attractive to men and in particular to their lovers. They also meant to intimidate their enemies and compensate for any physical defect, whether real or imagined. Women used every possible means, including cosmetics, to excite the keenest of pleasures and stir the emotions of men through their senses (Oumeish, 1999).

1.3. Cosmetics through history

1.3.1. The Pharaohic period

The ancient Egyptians were, in fact, masters of the cosmetics art. Many artifacts discovered in royal tombs are vessels filled with rare species and precious oils, and portraits show feminine faces enhanced by beauty products. Cleopatra, queen of Egypt (51 BC), was known to bathe in goat’s milk, almond and honey to soften her

skin. Milk contains lactic acid and so acts as a mild exfoliant, and honey is a natural humectant.

Aromatherapy began in ancient Egypt, as mentioned in the medical papyri that are believed to date back to 1600 BC. Women used oils of sweet and delicate odor and scent extracted from flowers and pine trees. In addition, they used different types of salts and alabaster to aesthetically improve the skin shape. They also produced creams that contain fruit acids (glycolic acid) from sugar cane, mangos and apples. Fruits with peels and were used mainly by women of the royal family. Women also used sour milk, which contains lactic acid, to exfoliate and smooth the skin.

To prevent or diminish wrinkles, the Egyptians used a mixture of incense, wax, freshly squeezed olive oil and cypress, combined with fresh milk, which was applied to the face for 6 days. Ancient Egyptian women used clay and herbal masks as far back as 69 BC. They believed that clay draw out toxins from deep within the skin and masks are useful for dry skin. The "Papyrus Ebers" records the use of 'Aloe' in many different cosmetics, as well as a medication for burns, cut wounds and skin rashes (Oumeish, 2001).

1.3.2. Ancient Europe period

The Romans collected rich herbs from the empire and used them in public hot and cold baths, steam rooms and massages. The Greeks worshiped the body and believed that cold baths improved fitness. They scrubbed with goat's fat soap and bathed with olive oil plus oil massages. Roman women used walnut extracts to darken their hair, antimony as eye shadow and cold creams for their face. Romans took much of their medical knowledge from the Greeks. They improved the effect of aromatics. They also used steam, sauna and vapour baths for recreation and beauty purposes and eventually Rome became the bathing capital of the world. Using oils and massage was a continuous practice of the Romans. They imported herbs and aromatic products from India and Arabia through the opening of trade routes.

Figs became a popular fruit in Rome with the conquest of Carthage and were used as a face mask when mixed with banana, oatmeal and rose water. Olive oil was also

used in Italy and Mediterranean countries for dry wrinkled skin. It was used warm and rinsed off at the end of bath.

The Greeks used herbal medicine. The eminent physician 'Pedarios Discurides' wrote his herbal medicine book that has continued for 1200 years to be a standard Western medical reference for herbs. The Greeks were the first Europeans to use aromatic oils of Eastern origin and also ointments as a kind of make-up. They used white lead carbonate as a face whitener. They also used charcoal crocodile excrement to darken the colour of the face. They used incense from the extracts of aromatic Indian plants and rose leaves. A bread-and-milk poultice as a cosmetic ritual to prevent aging was applied at night and removed in the morning with a mixture of beans cooked in butter. A cosmetic mixture was prepared by grinding the wood of cypresses, cedars, incense trees and water and applied to the whole body for one night. The face foundation paints were often based on lead and mercury products that caused skin erosen. Asparagus roots, wild anise, wild lily bulbs in goat's milk and manure were filtered and applied with soft bread to smooth and beautify the skin of the face (Oumeish, 2001).

With the demise of the Western Roman Empire, city life in Europe collapsed into a landscape dominated by castles and cathedrals, with literate men and women confined to monastic cloisters. Throughout those Dark Ages, sciences and medical manuscripts were preserved, copied, and studied within the sanctuaries provided by abbeys and cathedral schools. Medical knowledge declined until around 1100, when medicine regained its strength first in Salerno in southern Italy. The medical encyclopedia during that era contains some information concerning cosmetics.

Italian women used heavy make up, but German lower class, Spanish, English prostitutes and upper class Spanish women used the pink make up. French women whitened their faces with wheat powder (Blunchet). During the Crusades between the eleventh and thirteenth centuries, many women accompanied their husbands to the Middle East and knew a large number of cosmetics. One of the most famous cosmetics was '**Arabic kohl**', which was used as black liquid or powder eyeliners. The eyeshadow was also used in brown, grey, blue-green or violet. French women preferred azure eyeshadow and English women grey (Oumeish, 2001).

1.3.3. The Elizabethan Period

During the seventeenth century Elizabethan Period, the queen of England was held up as the ideal beauty. English women wore red wigs and reflected a pale complexion to resemble and copy closely their monarch's style. Ladies used a number of different techniques to keep their skin white, including washing their faces in their own urine. Other methods involved mixing rosewater with wine, as well as extracting the flower of lemon rinds by boiling and mixing it with water in which beans had been boiled.

Cosmetics were also used to cover scars, in particular those of smallpox, which spread all over Europe for centuries. White lead paint was commonly used to paint the face. It was used in the form of lead carbonate in a powder or as a paste in seed oil, which was later called "white lightening" or "moonshine." It was also mixed with a dye as well as ochre; an impure iron ore of red or yellow and with mercuric sulphide and used in rouge and lip adhesive cover. This was believed to be the cause of the gradual destruction of Queen Elizabeth's skin and the decay of her teeth. Women in the Elizabethan period used a number of methods to make their skin appear pale—a feature associated with "blue blood" (Oumeish, 2001).

1.4. Cosmeceuticals: an emerging concept

If "drugs" have been defined as compounds used in the treatment and prevention of diseases, or are intended to affect a physiological function, "cosmetics" have been labelled as substances that clean or enhance the appearance of the skin without therapeutic benefits. There is also a grey area bordering these two fields, for which the term "*cosmeceuticals*" has been defined. "Cosmeceuticals" are described as hybrids between drugs and cosmetic products and are able to enhance both health and beauty of the skin by external application. Cosmeceuticals, cover the gap between pharmaceuticals, skin diseases and cosmetics (Fig. 1.1) (Pieroni *et al*. 2004).

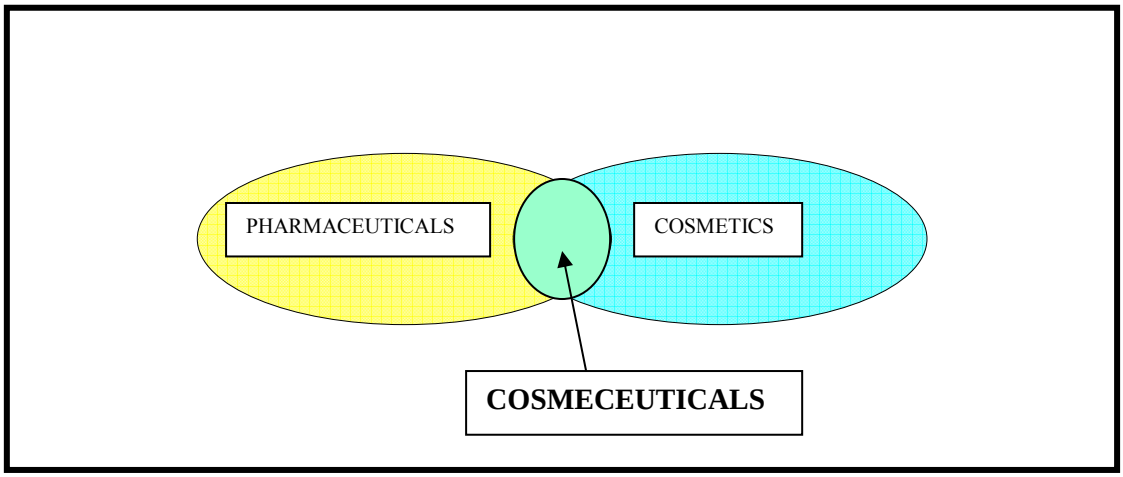

Figure 1.1. Cosmeceuticals (Pieroni *et al*. 2004)

According to Dureja *et al*. (2005), 'Cosmeceuticals' are topical cosmetic-pharmaceutical hybrids intended to enhance the beauty through ingredients that provide additional health-related function or benefits. Among the products included in this definition are skin moisturizers, perfumes, lipsticks, fingernail polishes, eye and facial makeup preparations, shampoos, permanent waves, hair colours, toothpastes, and deodorants, as well as any material intended for use as a component of a cosmetic product.

1.5. Skin cosmeceuticals

Cosmetics and skin care products are part of everyday grooming. Our skin, the largest organ in the body, separates and protects the internal environment from the external one. Environmental elements, air pollution, exposure to solar radiation as well as normal aging process cause cumulative damage to building blocks of skin-DNA, collagen and cell membranes. Use of cosmetics will not cause the skin to change or heal but cosmeceuticals have medicinal or drug-like benefits that are able to affect the biological functioning of skin owing to type of functional ingredients they contain (Dureja *et al*. 2005). Some of the skin cosmeceutical categories are as follows:

1. ***Skin-care products***; that go beyond colouring and adorning the skin. They improve the functioning/texture of the skin by encouraging collagen growth by

combating harmful effects of free radicals, thus maintaining keratin structure in good condition and making the skin healthier.

2. ***Sunscreens***; regular use of sunscreens is the most important step to keep healthy, youthful-looking skin. Traditional chemical sunscreens act primarily by binding to skin protein and absorbing UVB photons (280-320 nm).
3. ***Moisturizers***; their function is to smooth out the age lines, help brighten and tone the delicate skin.
4. ***Bleaching agents***; are used for bleaching/fading the various marks and act to block the formation of the skin pigment melanin.

1.6. Preparation of herbs in cosmeceuticals

Fresh plant materials undergo various chemical processing before getting converted into a cosmeceutical and this process affects the biological activity of the botanical. The most important factor for the biological activity of a herb to be incorporated into a cosmeceutical product is the source of the plant material because each plant part may contain hundreds of different individual chemicals and molecules.

Growing conditions, including soil composition, amount of available water, climate variations, plant stress, harvesting conditions, care of plant materials during shipping, storage conditions prior to manufacture and preparation of the herb and final product, as well as mixing with other herbs, are other factors that may substantially alter solubility, stability, biological availability, pharmacokinetics, pharmacologic activity and toxicity (Thornfeldt, 2005).

For herbal-cosmetic preparation usually, the plant material is extracted using various solvents, heated or processed to obtain essential oils or other distillates that can be easily added to a cosmetic formulation. This processing may destroy or unfavourably modify some of the physiologically active molecules. The results can be oil, wax, juice, tincture, decoction, tea, infusion and/or powders, which are then formulated into topical applications, including solutions, gels, lotions, creams, ointments and pastes.

The concentration of the herb, its extract and the active molecules affects therapeutic activity. Usually in cosmeceuticals, the medicinal botanicals are added in very small, subtherapeutic amounts for marketing. Few herbs, unlike most synthetic pharmaceuticals, are required only in a very low concentration to provide the desired effect because of their high potency. Herbal efficacy is first challenged by the 'stratum corneum' permeability barrier. Delivery across the mucocutaneous surface is difficult owing to the botanical's multiple active compounds with different solubility, polarity and therapeutic concentration, as well as the reactivity of different mucoctaneous targets (Thornfeldt, 2005).

These complex biological science and formulation issues indicate that the only validation of herbal activity in a cosmeceutical formulation is a human clinical trial conducted by a reputable researcher.

1.7. Plants used in cosmetics

According to Aburjai & Natsheh (2003), novel "bioactive" ingredients are derived from the sea, the earth and the plant kingdom. Popular ingredients include Chinese herbs, vitamins, minerals, antioxidants, enzymes, hormones and a multitude of 'naturals'. Plants were once the main source and foundation of all cosmetics, before methods were discovered of synthesizing substances with similar properties. The use of plant extracts in cosmetic formulation is increasing, mostly because of the poor image of animal-derived extracts. This is due, in part, to news reports regarding bovine spongiform encephalitis (BSE). Herbal "total extracts" as well as "selective extracts" are used in cosmetics. Total extracts are applied mainly according to the historical tradition of their use. On the other hand selective extracts are employed more by reason of investigation into their specific activity. Some selective extracts are introduced for different areas of use: licorice for skin irritations; ginkgo as a free radical scavenger; bearberry for skin lightening (complexion); walnut for skin tanning and wheat germ for stimulating cell proliferation.

A cosmetic formulation, including active principles of strictly natural origin, is designed to protect the skin against exogenous or endogenous harmful agents as well

as to balance again the dermal homeostasis lipids altered by dermatosis and ageing. Emollient natural remedies are often found to contain mucilage, consisting of polysaccharides, complex sugars and starch derivatives that relieve dryness and provide a soothing membrane that covers the skin. Protection of the skin hydration and producing softening effects to skin and hair preparations is achieved using seed oils rich in fatty acids and triglycerides that reduce transepidermal water loss. Those plants with anti-inflammatory properties often have a high level of the flavonoids; those that are used to firm and tone the skin are rich in tannins, which have an astringent effect and for skin healing in the case of infections the use of plants with number of antimicrobial and antifungal biocides are beneficial (Aburjai & Natsheh, 2003).

It has been reported that castor oil from castor bean (*Ricinus communis*), cocoa butter from *Theobroma cacao*, coconut oil from *Cocos nucifera*, sunflower oils from the seeds of *Helianthus annuus* and olive oil from *Olea europaea* are frequently used for treatment of skin dryness. Turmeric the rhizome of *Curcuma longa* is employed as a remedy for eczema (Aburjai & Natsheh, 2003; Athar & Nasir, 2005; Dweck; Fisher & Agner, 2004).

For the treatment of acne, spots and pimples; *Artemisia vulgaris*, *Artemisia absinthum*, *Ocimum sanctum* (basil), *Pisum sativum* (pea), *Cucurbita pepo* (pumpkin) and *Allium cepa* (onion) are commonly used by people from different cultures (Dweck, http://www.dweckdata.co.uk/Published_papers/Indians.pdf). Korean ginseng (*Panax ginseng*) is known as an antiaging skin treatment (Kim & Lee, 2004). There are several herbal drugs that are used for their anti-inflammatory properties such as red clover (*Trifolium pretense*), chamomile (*Matricaria recutita*), fenugreek (*Trigonela foenum-graeceum*) (Abebe, 2002), jojoba (*Buxus chinensis*) (Habashy *et al*. 2005) and Licorice root (*Glycyrrhiza glabra*). *Aloe* species (*Aloe vera*, *Aloe barbadensis, Aloe ferox*, etc.) (Dagne *et al*. 2000), oats (*Avena sativa*) and cucumber (*Cucumis sativa)* are widely used as skin protectives for various skin conditions.

1.8. African plants used in cosmetics

Africa, the cradle of mankind, bears a long history of people-plant interaction. It is the second largest of the earth's seven continents and is characterised by rich ethic and biotic mosaics that represent 13% of the earth's human population and the largest of the continental floras, estimates of which range between 50 000 and 70 000 plant taxa. The African flora is remarkable not only for its diversity but its distinctiveness: as many as 88% of its species are endemic (Nigro ***et al***. 2004).

It has been extensively reported that many people in African continent are struggling with various type of skin diseases. According to an investigation in Nigeria, allergic skin diseases (24.9%) were the commonest skin disorders. Within the allergic disorders; eczemas/dermatitis were found to be the most prevalent followed by follicular (13.7%) and pigmentary disorders (11.1%) (Nnoruka, 2005). Herbal folklore in practice for centuries, for the purpose of skin fairing, is one such nature's gift, which in recent years has been noticed by the scientific fraternity. There are numerous South African plants that are used in the cosmetic industry. They are being used as additives to shampoo, shaving and skin care creams, for skin disorders, treatment of burns and as skin lightening agents.

The indigenous flora of South Africa is notable for its species richness and high degree of endemicity (Scott ***et al***. 2004). It is estimated by the World Health Organization (WHO) that between 60-90% of Africa's population relies on medicinal plants totally or partially to meet their health care needs. This is true also for South Africa where up to 60% of the population consult of an estimated 200 000 traditional healers, especially in rural areas where traditional healers are numerous and more accessible than Western doctors (Taylor ***et al***. 2001).

The South African flora consists of over 25 000 species of higher plants (1/10 of world's plant diversity), of which 3 000 of these are used as medicines. Approximately 350 species are the most commonly used medicinal plants (Taylor ***et al***. 2001). Detailed analysis of the pharmacological properties of medicine used traditionally has brought to light the presence of acids, alkaloids, flavonoids, terpenoids, oils, gums, resins and fats in medicinal plants. Some of these ingredients

with a specific pharmacological action have been identified by pharmacologists. Many South African medicinal plants have been known to possess potentially valuable therapeutic agents (Lall, 2000).

For instance, *Cinchona succiruba*, ginger (*Zingiber officinale*), *Aloe vera* and Rooibos tea (*Aspalathus linearis)* have been used to stimulate hair growth. Baobab (*Adansonia digitata*)**,** coconut (*Cocos nucifera*) and mango (*Mangifera indica*) contain edible fruits and valuable emollient butter (rich in oleic and stearic acids) that are frequently employed as skin moisturisers. The leaves of *Asparagus africanus* are used by the women in South Africa as an ointment. False Daisy (*Eclipta prostrata*) is used in Egypt as the fresh juice of the plant and applied directly to the scalp. Taken internally, it is believed to blacken the hair and beard. Shea butter (*Butyrospermum parkii*) and Cocoa butter (*Theobroma cacao*) are also said to be beneficial to the skin. The juice from the aerial parts of *Borreria verticillata* is used on the west coast of Africa to treat skin diseases and is also used against eczema (Dweck, http://www.dweckdata.co.uk/Published_papers/Indians.pdf).

Anogeissus leiocarpus, *Anogeissus schimperi* and *Conocarpus leiocarpus* are used externally as a decoction of the leaves in Nigeria for washing in the treatment of skin diseases and the itch of 'Psoriasis'. *Plumbago zeylan*ica (Ceylon leadwort), *Artemisia absinthium*, *Artemisia herba-alba* and *Artemisia afra* are extremely popular throughout Africa and Asia as a remedy for parasitic skin diseases, especially leprosy, scabies, acne vulgaris, sores and leg ulcers (Dweak, 1996). *Aloe* species, *Cassipourea malosana*, *Sideroxylon inerme* are traditionally identified and used for their anti-hyperpigmentation properties (Fig 1.2) (Van Wyk & Gerick, 2000).

A number of South African plants are known to have phenolic compounds such as catechin, chalcone, flavones, quercetin etc. Most of the existing skin lightening agents belong to the phenolic group of secondary metabolites. Phenolic compounds afford protection against (UV) radiation and pathogens (Kim & Uyama, 2005). Melanin formation beneath the skin proceeds through free-radical mechanism. UV-radiation facilitates this chain reaction and it could be disrupted by selective use of antioxidants, potent enough to inhibit this reaction. Plants selected for the present study are known to be rich in the phenolic group of compounds.

Figure 1.2. Phyto-cosmetic products

1.9. Selected plants

Our goal was to identify bleaching agents from plants, which can contribute towards skin-pigmentation problems. A preliminary study conducted during my Honours studies indicated very good anti-tyrosinase activity of plants namely; Onionwood (*Cassipourea malosana* L.) from the Rhizorphoraceae, Carob tree (*Ceratonia siliqua* L.) from the Fagaceae and White milkwood (*Sideroxylon inerme* L.) from the Sapotaceae (Momtaz, 2006). A detailed phytochemical investigation of these plants was undertaken in the present study. A brief distribution, description and usage of selected plants are as follows:

1.9.1. *Cassipourea malosana* (L.)

(a)

(b)

Figure 1.3. *Cassipourea malosana*, (a) tree (b) stem-bark (www.vumba-nature.com/habitat-forest.htm)

Cassipourea malosana belongs to the Rhizophoraceae family and its common names are Onionwood; umemezi-obomvu (Zulu) and Tshamulevhu (Venda). It is a shrub or small forest tree with dark green leathery leaves, small greenish flowers and thin fleshy orange capsules. Freshly cut timber has a distinctive smell like onions. Trees grow up to 25m high, with a trunk of 1 to 2 meter in diameter and occur in the forest. It produces simple opposite leaves, a smooth grey bark when young, lamina of leaf is small to medium and flowers are yellowish or greenish (Van Wyk[a] *et al*.1997).

1.9.1.1. Distribution of *Cassipourea malosana*

C. malosana occurs across a wide range including three provinces of South Africa namely; KwaZulu-Natal, Mpumalanga and Limpopo. It is also found in Mozambique. It extends northwards into Zimbabwe and tropical Africa. It occurs naturally in forests, wooded hillsides, reverie, forest fringes as well as in open dry woodland and bushfields. The wide range suggests that it would be ideally suited to cultivation in summer rainfall areas of medium to lower altitude areas where frost is minimal or absent (Van Wyk[a] *et al*.1997).

1.9.1.2. Uses of *Cassipourea malosana*

The bark of *C. mlosana* has many medicinal and magical properties, but it is widely used as a skin lightener, particularly in KwaZulu–Natal. Skin-lightener

mixture is prepared by grinding dry stem-bark into powder which is mixed with sodium carbonate and milk/water to make a paste. This mixture is applied on the face for the whole night. Results can be seen after three weeks. It is also applied on the face for sunburn. This mixture is also used for treating pimples in post adolescent years (Van Wyk[a] ***et al.*** 1997). Wood of these plants has been used for floors turnery and tool handles. Because of their heavy wood, they are always applicable for timbers, building poles and sleepers. (Van Wyk[a] ***et al.*** 1997).

1.9.2. *Ceratonia siliqua* (L.)

(a)

(b)

Figure 1.4. *Ceratonia siliqua*, (a) fruits and leaves (b) tree (www.hort.purdue.edu)

Ceratonia siliqua, *Johannesbroodboom* (Afrikaans), is a highly drought resistance tree which reaches 50 to 55 ft (15-17m) in height and at an age of 18 years may have a trunk of 33 inches (85cm) in circumference. The evergreen leaves are pinnate with 6 to 10 opposite leaflets, oval, rounded at the apex, dark green and leathery. The tiny red flowers are in short, slender racernes borne in clusters along the branches–male, female or hermaphrodite on separate trees. The pod is light to dark-brown, oblong, flattened, straight or slightly curved with a thickened margin (Morton, 1987).

1.9.2.1. Distribution of *Ceratonia siliqua*

C. siliqua originates from the Mediterranean region. Throughout the Mediterranean region, it is grown only in the warmest areas near the coast and

neighboring islands. In Spain and Portugal it survives only on their Atlantic coasts. It has been introduced to South Africa where it sometimes occurs as an escape species (Morton, 1987).

1.9.2.2. Uses of *Ceratonia siliqua*

The seed gum is employed in the manufacture of cosmetics, pharmaceutical products, detergents and insecticides. Fruits are used as food, for both humans and livestock. Tannins from its pod have been reported for bowel diseases like cancer. Polysaccharide from its seeds is used for skin infection caused by some kinds of fungi and bacteria. Root-bark is used for skin diseases (Morton, 1987).

From its fruit pulp, products such as sugar syrups or molasses, unroasted and roasted carob powder are used as cocoa substitutes or especially tannin-rich preparations as antidiarrhoeals obtained. Its pulp product especially rich in insoluble dietary fiber (carob fiber) shows promising cholesterol-lowering properties in animal, as well as in human trials. Additionally, water extract from its pods shows a high antioxidative activity in different *in vitro* tests. Carob pods contain condensed tannins (proanthocyanidins), gallic acid, (+)-catechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate and quercetin glycosides (Papagiannopoulos *et al*. 2004).

1.9.3. *Sideroxylon inerme* (L.)

(a) **(b)**

Figure 1.5. *Sideroxylon inerme,* (a) aerial part of the plant (b) stem-bark

Sideroxylon inerme, is a large, evergreen tree belonging to the Sapotaceae family. It is commonly known as white milkwood, amasethole (Zulu), witmelkhout (Afrikaans) and Mububulu (Venda). It has a rounded crown which may reach up to 15m under ideal conditions. It is a shrub or small tree in its natural range and only reaches its full potential in valleys and forest margins where moisture is more available (Van Wyk ***et al.*** 1997).

Leaves are glossy, dark green and carried on the spreading crown. It has the potential of making an excellent evergreen garden specimen for larger gardens, parks and golf courses. Small, white and sweetly scented flowers are borne in October to February. Fruit are oval with a pointed tip ripening yellow or orange from April to September (Van Wyk ***et al***. 1997).

1.9.3.1. Distribution of *Sideroxylon inerme*

S. inerme occurs across a wide range including the four northern provinces of South Africa as well as KwaZulu-Natal, Swaziland and Mozambique. It also extends northwards into Zimbabwe and tropical Africa. It occurs naturally on the rocky outcrops, wooded hillsides and reverie and forest fringes as well as in open, dry woodland and bushfields. The natural wide range occurrence suggests that it would be ideally suited to cultivation in summer rainfall areas of medium to lower altitudes areas where frost is minimal or absent (www.plantzafrica.com/plantklm/mimusopzey.htm).

1.9.3.2. Uses of *Sideroxylon inerme*

The bark is mainly used in traditional medicine by Zulus. The Zulus take an infusion of the bark to dispel bad dreams. It is emetic. They administer the powdered bark to calves and goats as a tonic. The Xhosas (a South African tribe) use a decoction of the bark for gall-sickness in stock. Medley Wood states that the bark is used as an astringent (Watt & Breyer-Brandwijk, 1962). Its bark has many medicinal and magical properties, but it is widely used as a skin lightener, particularly in

KwaZulu –Natal. (Van Wyk & Gerick, 2000). The skin-lightener mixture is prepared as described earlier in section 1.9.3.

1.10. Objectives of the study

- To discover and characterize new tyrosinase inhibitors from plants using bioassay guided fractionation which can help in preventing pigmentation disorders and other melanin-related problems in human beings.
- To understand tyrosinase activators and inhibitors towards regulation of the discoloration process.
- To determine the chemical structure and activities of possible pure compounds isolated by bioassay-guided fractionation.
- To evaluate the efficacy of isolated compounds from plants for skin-hyperpigmentation.
- To investigate oxidant scavenging activity of plant extracts and pure compounds.

1.11. Structure of the thesis

Chapter 1 This chapter provides a concise review of the concept of cosmetics, cosmeceuticals, preparation of herbs for cosmecuticals and the usage of plants is cosmetics. Selected plants, their extraction, description, distribution and uses have also been briefly discussed.

Chapter 2 The structure and function of skin, pigmentation associated with hyper and hypo-pigmentation; their cause and treatments, tyrosinase (its structure and function) and melangenesis pathway have been argued in detail.

Chapter 3 Inhibitory effect of plant extracts on tyrosinase activity and melanin production in mouse melanocyte cells has been discussed.

Chapter 4 Isolation and purification of active compounds from *C. sliqua* using various isolation methods such as solvent-solvent separation, column chromatography, thin layer chromatography, paper chromatography, high performance liquid chromatography and various spectral analysis such as nuclear magnetic resonance, mass spectra and etc. have been discussed. In addition, this chapter documents the inhibitory activity on tyrosinase and melanin production by all the purified compounds from *C. siliqua*.

Chapter 5 Isolation and purification of active compounds from *S. inerme* using various isolation procedures such as solvent-solvent separation, column chromatography, thin layer chromatography and various spectral analysis such as nuclear magnetic resonance, mass spectra and etc. have been discussed. In addition, this chapter demonstrates the inhibitory activity on tyrosinase and melanin production by all the purified compounds from *S. inerme*.

Chapter 6 The antioxidant and radical scavenging capacity of plant extracts and purified compounds using both quantitative and qualitative methods have been discussed.

Chapter 7 In the general discussion and conclusion, an attempt is made to synthesize a more coherent picture of the results of this study.

Chapter 8 Acknowledgements

Chapter 9 Appendices

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Chapter 2

Skin pigmentation; cause and treatments

2.1. Introduction

Pigmentation has become an important phenotypical characteristic, in the pharmaceutical, medicinal as well as in the cosmetic field. Natural and synthetic chemical agents can frequently modulate the metabolism of pigmentation produced. Alterations in skin pigmentation may often have a dramatic expression in individuals with a dark skin complexion. Unwanted skin pigmentation can be a source of significant emotional distress in individuals (Stratigos & Katsambas, 2004).

Tyrosinase is known as a key enzyme in melanin biosynthesis. Melanin is an effective pigment in mammalian skin and hair colour. Melanin formation beneath the skin proceeds via a free-radical mechanism. UV-radiation facilitates this chain reaction and it could be disrupted by selective use of antioxidants, potent enough to poison this reaction. Melanin biosynthesis can be inhibited by avoiding UV exposure, by inhibition of melanocyte metabolism and proliferation, by inhibition of tyrosinase, and by removal of melanin by corneal ablation (Wang *et al*. 2006).

In this chapter; skin's structure and function, skin pigmentation; causes and treatments, tyrosinase, its structure and function, melanogenesis and tyrosinase inhibitors have been discussed.

2.2. Skin's structure and function

Skin forms a remarkable protective barrier against the external environment, helping to regulate temperature and fluid balance, keeping out harmful microorganisms and chemicals and offering some protection against sunlight. Figure 2.1 represents the structure of normal human skin

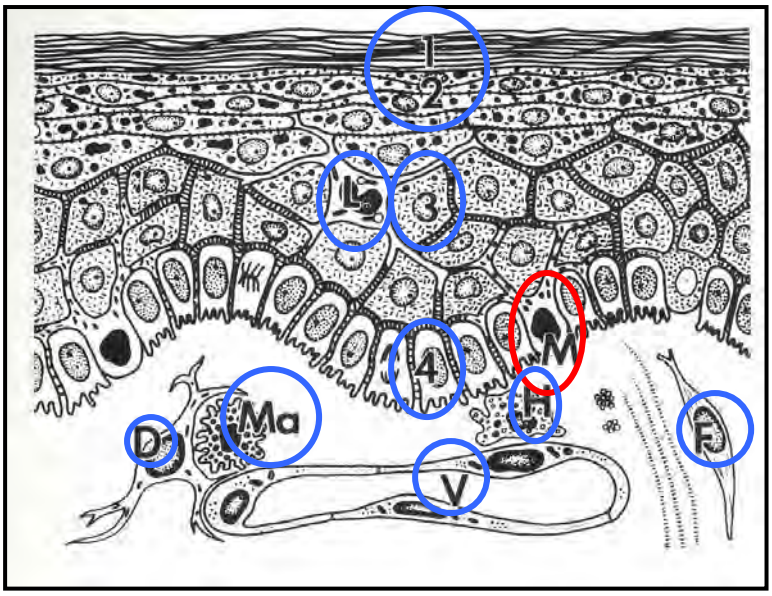


Figure 2.1. Normal human skin. The epidermis is composed of four layers of keratinocytes: (1) the stratum corneum, (2) the stratum granulosum, (3) the stratum spinosum and (4) the stratum basalis. Within the midepidermis are dendritic Langerhans cells (L) and admixed within the stratum basalis are pigment-producing melanocytes (M). The underlying collagenous dermis contains microvessels (V), fusiform fibroblasts (F), histiocytes (H), dendrocytes (D) and granule-containing mast cells (Ma) (Murphy, 1995)

Skin is a living organ that consists of epidermis, dermis and subcutaneous fat layers;

i. ***Epidermis***; consist of two main parts: the 'Stratum germinativum' and the 'Stratum corneum'. Epidermis also contains; keratinocytes, Langerand cells, melanocytes and Merkel cells.

ii. ***Dermis***; is a connective tissue layer and separated from the epidermis by a basement membrane. Dermis contains many elastin and collagen fibers, as well as abundance of blood vessels, mast cells, endothelial cells and specialized nerve endings.

iii. ***Horny layer***; the outermost layer; which comes in direct contact with environment. This layer is not simply a collection of dead cells, but a complex organism that is part of a homeostatic system and all the phenomena occurring at this layer, including the use of cosmetics, are

transmitted to the epidermis and the inner skin. The disturbance of the homeostatic balance, which accompanies aging and rapid changes in the external environment, clearly affects the epidermis and dermis (Murphy, 1995; Tranggono, 2000).

An equally important component of skin is adnexal epithelium, which consists of pilar and related sebaceous structures as well as apocrine and eccrine glands (Murphy, 1995).

2.3. Skin pigmentation

The dominant component of normal skin colour is provided by melanin (Launey & Land, 1984). Nevertheless, it is not a simple concept; there are actually four chromophores that contribute to skin colour; haemoglobin, oxyhaemoglobin, carotenoids and melanin (s) (Summers, 2006). Among them melanin plays the root role in skin colour and pigmentation.

Melanin is one of the most widely distributed pigments and is found in bacteria, fungi, plants and animals. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis (Fig. 3) (Kim & Uyama, 2005). Melanocytes are neural crest-derived cells that migrate to the epidermis during embryogenesis and subsequently synthesize and distribute melanin to surrounding keratinocytes (Yaar *et al*. 2006). Melanocytes have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin (Tiedtke *et al*. 2004). Melanin plays an important role in protecting human skin from the harmful effects of ultra violet (UV) radiations by absorbing UV sunlight, removing reactive oxygen species (ROS) and scavengering toxic drugs and chemicals. Melanin also induces the expression and synthesis of a variety of cytokines, primarily of keratinocyte origin, that act in a paracrine fashion to further induce melanogenesis (Yaar *et al*. 2006).

The accumulation of an abnormal melanin amount in specific parts of the skin as more pigmented patches (melasma, freckles, ephelide, senile lentigines etc.) might

become an aesthetic problem (Solano *et al*. 2006). The type and amount of melanin synthesized by the melanocytes and its distribution in the surrounding keratinocytes determine the actual colour of the skin (Kim & Uyama, 2005).

Melanins are polymers of 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) or dihydroxyindole (DHI) and have a range of forms. They fall into three main groups:

- Phaeomelanins; which are yellow or red-brown in colour and alkali soluble;
- DHICA-enriched eumelanins; which are light brown; and
- DHI-enriched eumelanins; which are blue, black, insoluble and very stable (only able to be degraded under drastic chemical conditions (e.g. alkali fusion at over 200 ºC)) (Summers, 2006).

Switching between eumelanin and phaeomelanin indicates a temporary shift from eumelanogenesis to phaeomelanogenesis. Eumelanogensis consists of the enzymatic oxidation of tyrosine or L-DOPA to its corresponding *o*-dopaquinone catalyzed by tyrosinase. The amino group of an *o*-dopaquinone side chain first undergoes an intramolecular 1, 4-addition to the benzene ring, which causes its cyclization into leucodopachrome. This intermediate is quickly oxidized to dopachrome. A series of chemical and enzymetic reaction occur after dopachrome formation, which lead to the synthesis of eumelanin. During phaeomelanogenesis; tyrosinase enzymatically attacks *o*-dopaquinone to produce cysteinyldopa. Subsequent cyclization and polymerization of cysteinyldopa in an uncharacterized series of reaction result in the production of phaeomelanin. The interaction between the eumelanin and phaeomelanin gives rise to mixed-type melanins (Fig. 2.2) (Kim & Uyama, 2005).

Summers (2006), illustrated that the relative ratios of phaeomelanins and eumelanins vary depending on skin colour. Celtic skins have phaeomelanins but virtually no eumelanins. Dark negroid skins have a predominant proportion of eumelanins.

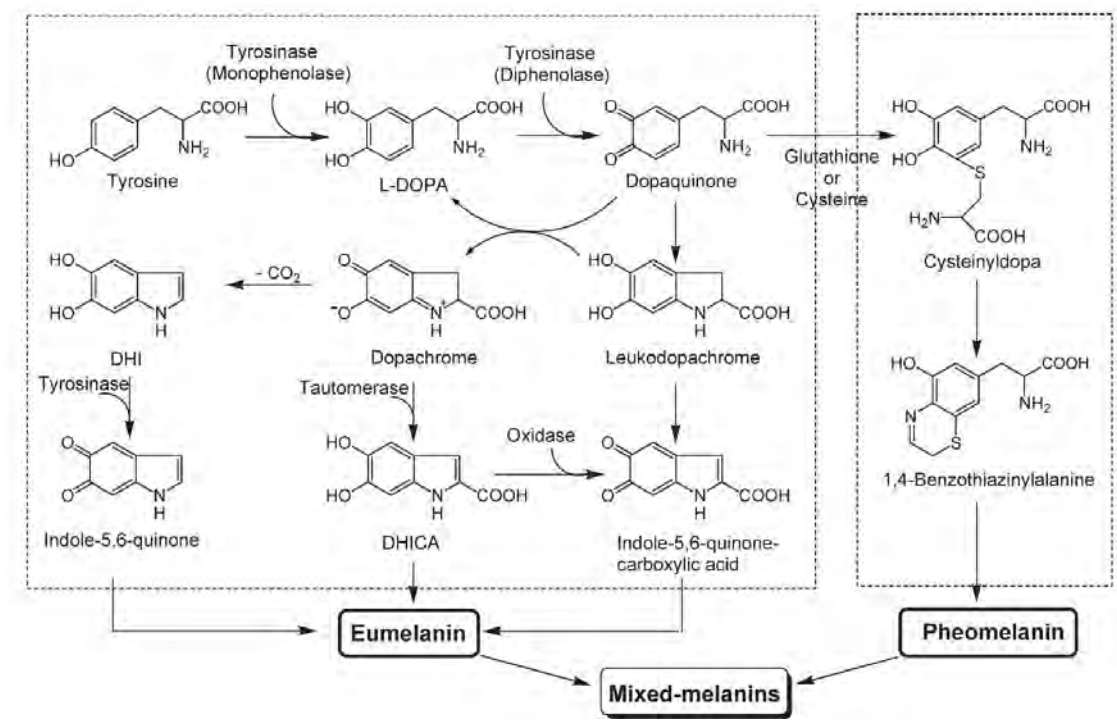


Figure 2.2. Biosynthetic pathway of melanin (Kim & Uyama, 2005)

2.4. Tyrosinase

2.4.1. The properties of tyrosinase

Tyrosinase is a multifunctional copper containing enzyme. Tyrosinase plays diverse physiological roles in different organisms. In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine. In plants, the physiological substrates are a variety of phenolics. Tyrosinase oxidizes them in the browning pathway observed when tissues are injured. One possible role is protection of the wound from pathogens or insects. Tyrosinase is thought to be involved in wound healing and possibly sclerotization of the cuticle (Kim & Uyama, 2005). In addition, tyrosinase is known to be involved in the molting process of insects and adhesion of marine organisms (Haq *et al*. 2006; Khan *et al*. 2003; Kim *et al*. 2006; Bernard & Berthon, 2000; Lee *et al*. 2006).

2.4.2. Classification of tyrosinase

In higher plants and fungi, tyrosinases exist in immature, mature latent and active isoforms. Tyrosinase contains a coupled binuclear copper active site. Tyrosinase catalyzes both the *o*-hydroxylation of monophenols and the two-electron oxidation of *o*-diphenols to *o*-quinones. The later is more rapid than the former; thus, the hydroxylation of tyrosine to L-DOPA is considered the rate-determining step.

The best characterized tyrosinases are derived from *Streptomyces glaucescens*, the fungi *Neurospora crassa* and *Agaricus bisporus*, as shown in Table 2.1.

Table 2.1. Properties of various tyrosinases (Kim & Uyama, 2005)

Source	Number of subunits	Molecular weight of subunits (kDa)
Streptomyces glaucescens (Eubacteria)	1	30.9
Neurospora crassa (Fungi)	1	46
Agaricus bisporus (Fungi; mushroom)	2	13.4 43
Beta vulgaris (Plant; spinach-beet)	1	40
Human melanocyte (Animal)	1	66.7

The first two are monomeric proteins, while the last is a tetramer with two different subunits; light and heavy. Tyrosinase has been isolated from numerous plant and animal sources but few of them have been characterized. The fungal and human tyrosinases are membrane-bound glycoproteins (13% carbohydrate). A variety of mutations in the human tyrosinase gene has been correlated with pigment deficiency of oculocutaneous albinism (Kim & Uyama, 2005).

2.4.3. Structure of the active center of tyrosinase

During the process of melanin-pigments formation, three types of tyrosinase (met, oxy and deoxytyrosinases) (Fig. 2.3) with different binuclear copper structure of the active site are involved:

- ***Mettyrosinase***; The resting form of tyrosinase, contains two tetragonal Cu(II) ions antiferromagnetically coupled through and endogenous bridge, although hydroxide exogenous ligands other than peroxide are bound to the copper site. The antiferromagnetically coupling between the Cu(II) ions of mettyrosinase triggers the lack of an electron paramagnetic resonance (EPR) signal, which requires a super-exchange pathway associated with a bridging ligand. The resting form of tyrosinase is found to be a mixture of 85% met and 15% oxy forms (Kim & Uyama, 2005).

- ***Oxytyrosinase***; can be produced by the two-electron reduction of deoxytyrosinase, followed by reversible binding of dioxygen, which react with monophenol as well as *o*-diphenol substrate. Thus, its geometric and electronic structures are keys to understanding the hydroxylation chemistry of tyrosinase. Oxytyrosinase consists of two tetragonal Cu(II) atoms, each coordinated by two strong equatorial and one weaker axial N_{His} ligands. The exogenous oxygen molecule is bound as peroxide and bridges the two copper centers. Peroxide bound in this mode confers a distinct $O^{2-} \rightarrow Cu(II)$ charge transfer spectrum, which can be correlated to the optical features of the oxy form of tyrosinase and includes an extremely intense absorption band at 350 nm (Kim & Uyama, 2005).

- ***Deoxytyrosinase;*** An analogue of deoxyhemocyanin, has a bicuprous structure [(Cu(I)-Cu(I)]. The bridging ligand must be hydroxide from water and a similar situation is likely the case for mettyrosinase.

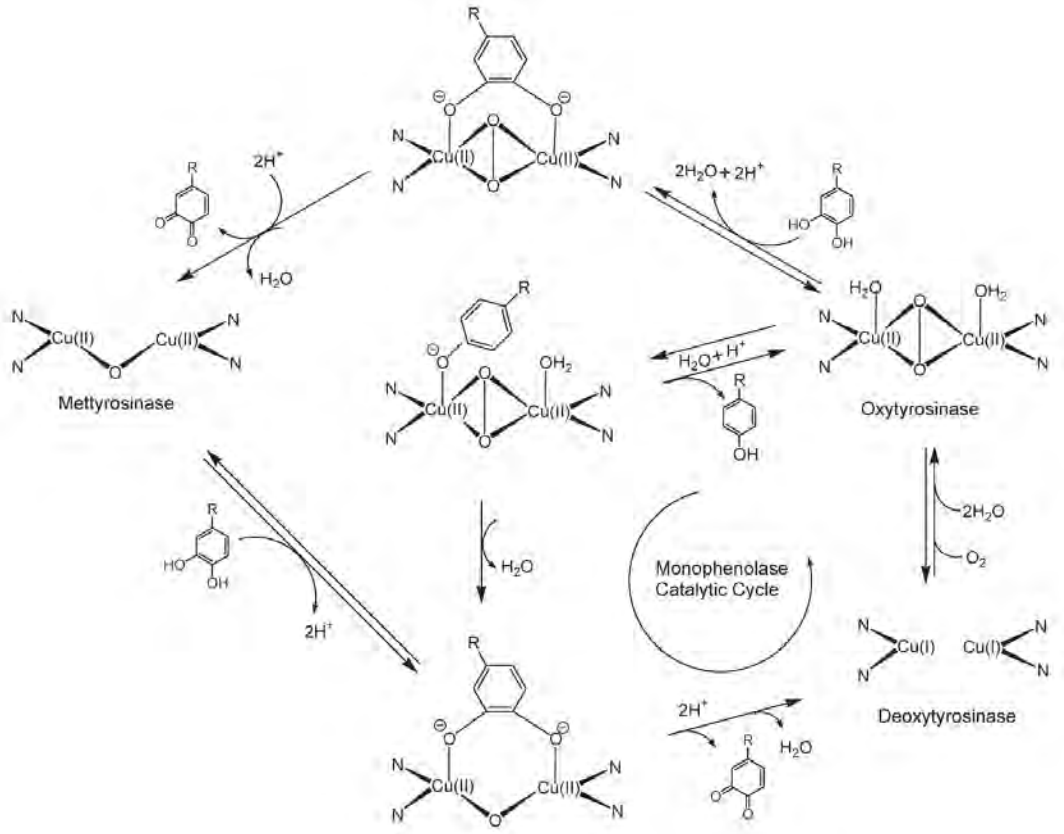


Figure 2.3. Structure of tyrosinases and catalytic cycles of the hydroxylation of monophenol and oxidation of *o*-diphenol to *o*-quinone by tyrosinase (Kim & Uyama, 2005)

Tyrosinase has three domains, of which the central domain contains two copper binding sites. Six histidine residues bind a pair of copper ions in the active site of tyrosinase, which interact with both molecular oxygen and its phenolic substrate. The location of cysteine also plays an important role in the formation of disulfide linkages; which stabilizes protein structure. The number of cysteine residues varies from one organism to another, as long the N-terminal and central part of protein, human and mouse tyrosinases have 17 cystein residues and plants have 11, whereas the C-terminal domain contains 1 cysteine residue. Interestingly, *Neurospora crassa, Agaricus bisporus* and prokaryotic tyrosinases contain 0 or 1 cysteine in mature protein. In the mushroom tyrosinase sequence, only two cysteine residues are found in the C-terminal domain. It was found that large-size ligands have a higher affinity for the active site compared with smaller ones (Kim & Uyama, 2005).

2.4.4. Mechanism of tyrosinase action

According to Kim & Uyama (2005), the mechanism of monophenolase activity of tyrosinase has been widely studied based on the three forms of the enzyme. In the monophenolase cycle, the monophenol can react only with the oxy form and binds to the axial position of one of the coppers of this oxy form. Rearrangement through a trigonal bipyramidal intermediate leads to *o*-hydroxylation of monophenol by the bound peroxide (Fig. 2.3). This generates a coordinated *o*-diphenol, which is oxidized to *o*-quinone, resulting in a deoxy form ready for further dioxygen binding. The *o*-diphenol can react with the met form present to give the coordinated *o*-diphenol in the monophenolase cycle.

In the diphenolase cycle, both the oxy and met forms react with o-diphenol, oxidizing it to the *o*-quinone. However, monophenol can compete with *o*-diphenol for binding to the met form site, inhibiting its reduction. While monophenolic substrates require the axial to equatorial arrangement for *o*-hydroxylation, *o*-diphenolic substrates need not undergo rearrangement at the copper site for simple electron transfer (Kim & Uyama, 2005).

Kinetic studies of the steady state of the pathway show the lower catalytic efficiency of tyrosinase on monophenols than on *o*-diphenols. Monophenolase activity is typically characterized by a lag time, which is dependent on factors such as substrate, the enzyme concentrations and presence of hydrogen donar. Lag time; is the time required for the resting met form to be drawn into the active deoxy form by reducing agent, arising via action of the small amounts of the oxy form that usually accompany the met form. In the presence of reducing agents known as cofactors, especially *o*-diphenol derivatives such as L-DOPA and (+)-catechin, tyrosinase is activated and the lag time is shortened or abolished. L-DOPA at a very low concentration is the most effective reducing agent for eliminating lag time (Kim & Uyama, 2005).

2.4.5. Enzymatic browning of plant-derived foods by tyrosinase

The Enzymatic browning of plant-derived foods and beverages takes place in the presence of oxygen when tyrosinase and their polyphenolic substrates are mixed after brushing, peeling and crushing operations, which leads to rupture of cell structure (Kim & Uyama, 2005). The fundamental step in enzymatic browning is the transformation of an *o*-diphenol such as L-DOPA to the corresponding *o*-quinone, which can undergo further oxidation to brown melanin pigment (Martinez & Whitaker, 1995). According to Kim & Uyama (2005), *o*-quinones are powerful electrophiles that can suffer nucleophilic attack by water, other polyphenols, amino acids, peptides and proteins, leading to Michael-type addition products. This enzymatic browning can be prevented by trapping the *o*-dopaquinone intermediate with cysteine or ascorbic acid (Fig. 2.4).

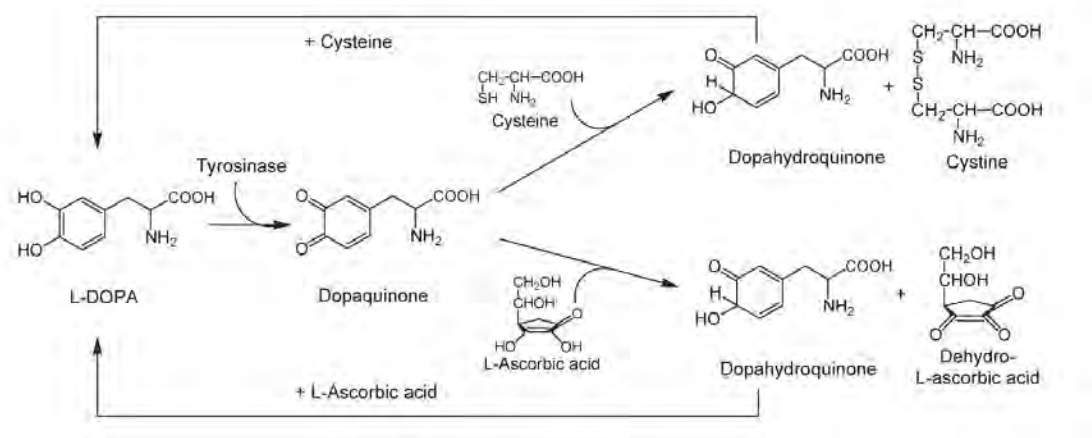


Figure 2.4. Inhibition of tyrosinase-catalyzed enzymatic browning by trapping the dopaquinone intermediate with cysteine or ascorbic acid (Kim & Uyama, 2005)

2.4.6. Regulation of melanogenic enzymes

Depigmentation can be achieved by regulating (i) the transcription and activity of tyrosinase, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2), and/or peroxidase; (ii) the uptake and distribution of melanosomes in recipient keratinocytes and (iii) melanin and melanosome degradation and turnover of

'pigmented' keratinocytes (Fig. 2.5 & Table 2.2). In mammalian melanocytes, melanogenesis is known to be regulated by many biologically relevant natural agents, including interleukins, vitamins, prostaglandins, interferons, UV light and melanocyte stimulating hormone (MSH) (Aroca *et al*. 1993). As a result of the key role played by tyrosinase in melanin biosynthesis, most of the lightening agents act specifically to reduce the function of this enzyme by means of several mechanisms: (i) interference with its transcription and/or glycosylation, (ii) inhibition by different modalities, (iii) reduction of by-products and (iv) post-transcriptional control (Briganti *et al*. 2003).

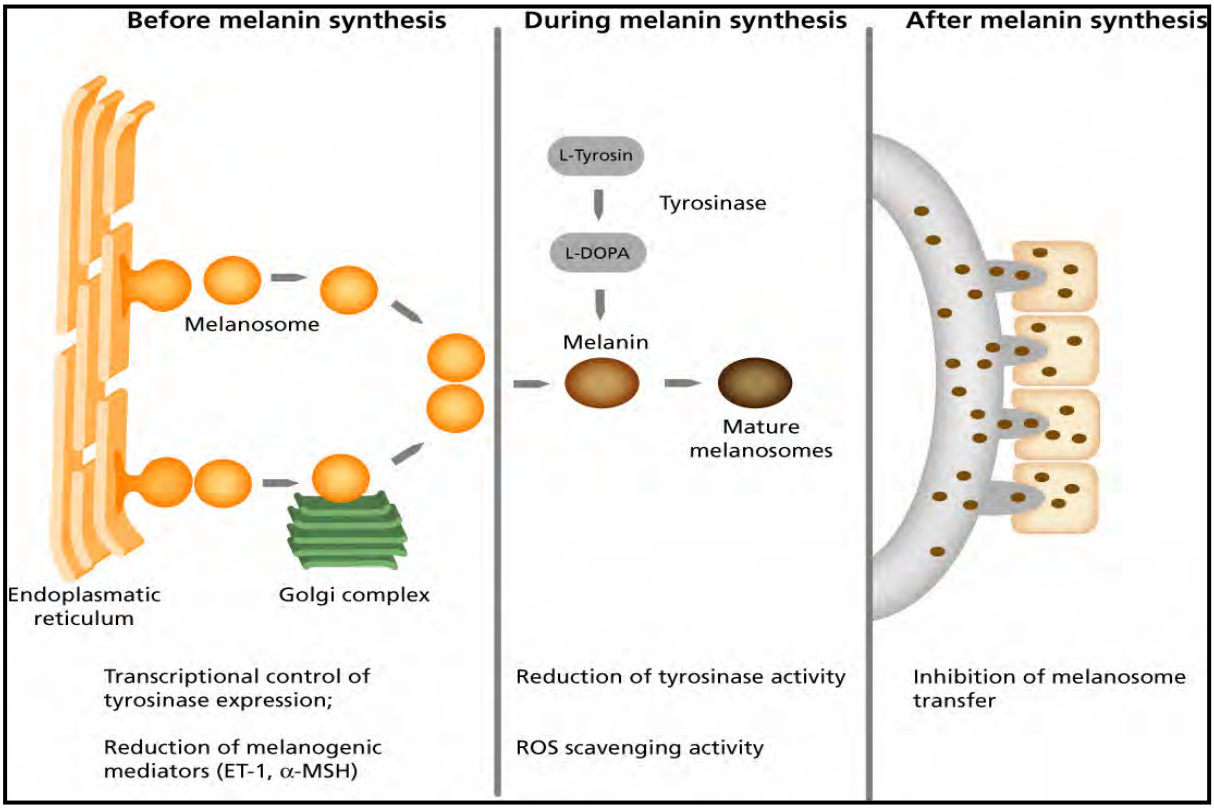


Figure 2.5. Melanogenesis pathway. Tyr: tyrosinase, M: melanosomes, ROS: reactive oxygen species (Briganti *et al*. 2003)

2.4.6.1. Transcriptional control of tyrosinase expression

Transcription of genes encoding tyrosinase and TRP-1 is under transcriptional control of the microphethalmia transcription factor (MITF). Substances able to inhibit MITF expression and activity, as well as the extracellular signal-regulated kinase

Table 2.2. Classification of depigmenting agents. Compounds have been divided in categories on the basis of the reported mechanisms of interference with melanin synthesis and deposition (Briganti *et al*. 2003)

Before melanin synthesis	
Tyrosinase transcription	
C_2-ceramide	Tretinoin
Tyrosinase glycosylation	
$PaSSO_3Ca$	
During melanin synthesis	
Tyrosinase inhibition	
Hydroquinone	Kojic acid
4-hydroxy-anisole	Methyl Gentisate
4-S-CAP & derivatives	Ellagic Acid
Arbutin	Resveratrol
Aloesin	Oxyresveratrol
Azelaic acid	
Peroxidase inhibition	
Methimazole	Phenols/catechols
Product reduction and ROS scavengers	
Ascorbic acid	α-Toc
Ascorbic Acid Palmitate	D, L-α TF
VC-PMG	Hydrocumarins
Thioctic acid	
After melanin synthesis	
Tyrosinase degradation	
Linoleic acid	α-Linolenic acid
Inhibition of melanosome transfer	
Serine protease inhibitors	Niacinamide
Lecthins and Neoglycoproteins	RW-50353
Soybean/milk extracts	
Skin turnover acceleration	
Lactic acid	Retinoic acid
Glycolic acid	Linoleic acid
Liquiritin	

PaSSO3Ca, Calcium D-panthethine-S-sulphonate; 4-SCAP, 4-S-cystaminylphenol; VC-PMG, magnesium-L-ascorbyl-2-phosphate; α-Toc, α-tocopherol; α-Toc-F, α-Tocopherol ferulate.

(ERK) and serine-threonine kinase (AKT)/protein kinase B (PKB) pathways could represent depigmenting agents (Briganti *et al*. 2003). For instance tretinoin (all-trans retinoic acid; ATRA), acting on retinoid-activated transcription factors, interferes with melanocyte development and melanogenesis.

2.4.6.2. Control of tyrosinase activity

The classification of tyrosinase inhibitors (Table 2.2, p.49) is difficult because of the capability of several compounds to work in different ways, interacting with both catalytic and regulatory sites of the enzyme or being metabolized to a product that, in turn, can act as either a non-competitive or a competitive inhibitor. Most of the inhibitors are phenol/catechol derivatives, structurally similar to tyrosinase or DOPA, which act as alternative substrates of tyrosinase without producing pigment (Briganti *et al*. 2003).

Compounds with redox properties can have depigmenting effects by interacting with o-quinones, thus avoiding the oxidative polymerization of melanin intermediates, or with the copper at the active site. Moreover, redox agents, by scavenging reactive oxygen species (ROS) generated in the skin following UV exposure can inhibit possible second messengers which are able to stimulate epidermal melanogenesis either directly or indirectly (Briganti *et al*. 2003). Ascorbic acid, magnesium-l-ascorbyl-2-phosphate (VG-PMG), and α-tocopherol (α-Toc) belong to this group of melanogenic inhibitors.

2.4.6.3. Post-transcriptional control of tyrosinase

Substances, which are able to regulate melanin synthesis without affecting the expression of melanogenic proteins, are likely to exert a post-transcriptional control of melanogenic enzymes. Unsaturated fatty acids, such as oleic acid (C18:1), linoleic acid (C18:2), or α-linoleic acid (C18:3), suppress pigmentation, *in vitro*, correlated with the degree of unsaturation, whereas saturated fatty acids, such as palmitic acid, increase the rate of melanogenesis. Linoleic acid *in vivo* showed the greatest lightening effect in UVB induced pigmentation, without toxic effects on melanocytes.

Tyrosinase is selectively targeted by fatty acids, which seems to act on the degradation of the enzyme during the physiologic proteasome-dependent mechanism, leading to the alteration of tyrosinase protein content in hyperactive melanocytes. Linoleic acid accelerates the process whereas palmitic acid works in an antagonistic manner mimicking protease inhibitors (Briganti *et al*. 2003).

2.5. Disorders of pigmentation

Disorders of pigmentation are mainly disorders of melanin synthesis or distribution (hyperpigmentation and hypopigmentation) (Fig. 2.6). There may be:

1. Localized hyperpigmentation
2. Generalized hyperpigmentation
3. Localized hypopigmentation
4. Generalized hypopigmentation
5. Poikiloderma
6. Abnormal pigmentation not due to melanin

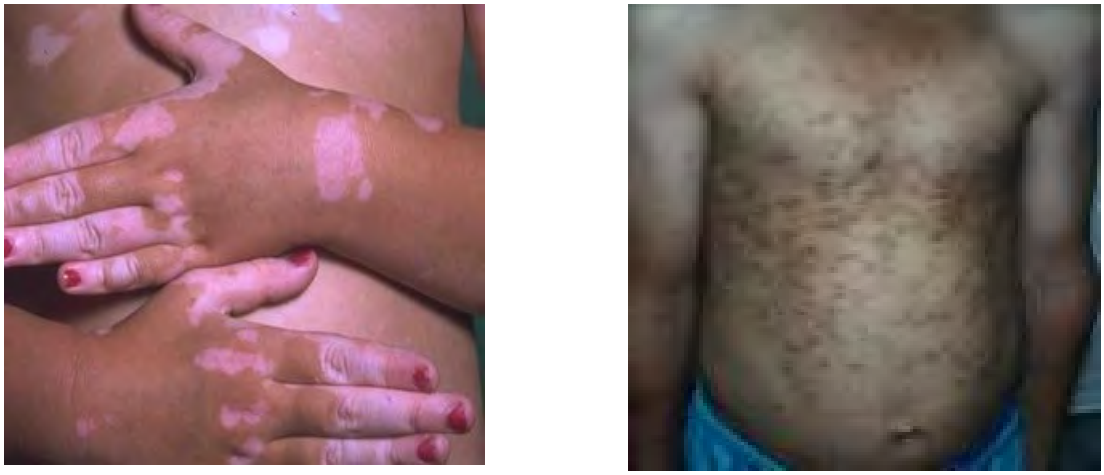


Figure 2.6. Patients with hypopigmentation (left) and hyperpigmentation (right) (www.answers.com)

2.5.1. Localized hyperpigmentation

2.5.1.1. Melasma (chloasma)

Melasma is a sepia to brown, blotchy melanosis, which is usually confined to symmetrical areas of the cheeks and forehead, but sometimes extends onto the upper lip, chin and neck (Launey & Land, 1984). Melasma is found most often on the facial areas of women who either are taking anovulatory drugs or are pregnant and has been called the 'mask of pregnancy'. It also may be found in men or women with no apparent endocrinologic abnormalities. Its causes remain unknown, but exposure to the sun is necessary for its development. It is present more frequently in dark-skinned whites (Arndt & Bowers, 2002).

2.5.1.2. Freckles

A freckle is a small brown patch on the skin, coloured by abundant epidermal melanin without any increase in the number of melanocytes. Freckles occur only on light-exposed skin and tend to darken in summer and fade in winter. The brown to black colour of lentigines is also produced by epidermal melanin, but in the lentigo there is an increased number of melanocytes scattered along the basal layer.

2.5.1.3. Lentigines

Lentigines are small, rounded and brown to black areas, which may coalesce to form patches or slightly elevated lesions, a centimeter or more across (Launey & Land, 1984). Unlike freckles, lentigines do not darken on exposure to sunlight, but elderly people often develop large lentigines on light-exposed areas (Burton, 1979).

2.5.1.4. Peri-orificial lentiginosis (Peutz-Jegher's disease)

Peri-orificial lentiginosis is a rare autosomal dominant disease characterized by multiple small deep brown macules around the mouth with multiple polyps throughout the intestinal tract. These tend to cause recurrent intussusceptions and

house-surgeons who examine the skin can make the diagnosis pre-operatively (Burton, 1979).

2.5.1.5. Postinflammatory hyperpigmentation

Postinflammatory hyperpigmentation may be a sequel to any pattern of dermatitis, but is characteristic of the phototoxic type. Continued handling of such phototoxic agents as creosote and tar may darken skin on the back of the hands and forearms (Launey & Land, 1984). It is common in darkly pigmented persons and is more related to the nature of the insult than to the degree of previous inflammation. Epidermal changes leading to hyperpigmentation may be secondary to pathological changes in the dermis, as in mastocytosis and the cutaneous lesions of porphyria or may follow some conditions such as thermal, mechanical or other forms of injury by physical agents. There is an increase in epidermal melanin, but there also may be melanin granules present in dermal macrophages. Postinflammatory hyperpigmentaion may persist for months to years (Arndt & Bowers, 2002).

2.5.1.6. Hereditary syndromes with hyperpigmentation

Neurofibromatosis; in this condition there are usually several large molecules of a characteristic 'café-au-lait' colour, in addition to the soft pedunculated Schwann cell tumours. These coffee-colourd patches can be shown on biopsy to contain bizarre giant melanosomes (Burton, 1979). Pigmentary changes are a relatively minor component of these diseases, but are the outstanding feature of another inherited disorder, incontinentia pigmenti (Launey & Land, 1984).

2.5.2. Generalized hyperpigmentation

2.5.2.1. Endocrine

Endocrine is the prototype of the diffuse melanoses. Elevated levels of MSH (Melanocyte-Stimulating hormone) and ACTH (Adrenocorticotrophic hormone) lead to a generalized increase of melanogenesis. The increase is more apparent in areas

where melanin synthesis is normally greater, as in light-exposed skin and areas of fraction. The skin may darken to brown or black but in fair-skinned patients, there may be no more than a moderate tan of a colour, which is normal to healthy people of darker complexion (Launey & Land, 1984).

2.5.2.2. Metabolic and nutritional

Starvation and malabsorption may lead to patchy or diffuse hyperpigmentation, and melanosis complicates the jaundice of biliary cirrhosis. The pigmentation of haemochromatosis, renal and hepatic failure is at least partly due to increased melanin synthesis (Launey & Land, 1984).

2.5.2.3. Connective tissue diseases

Hupus erythematosus, scleroderma and dermatomyositis may all be accompanied by hyperpigmentation, often diffuse Addisonian in distribution.

2.5.2.4. Drugs

Pigmentation may be a non-specific sequel to drug eruption, or may develop without a preceding rash. Cytotoxic agents, especially ‘Busulphan’ and less often ‘Cyclophosphamide’, ‘Bleomycin’ and ‘Procarbazine’ may cause diffuse melanosis. High doses of chlorpromazine, chloroquine and hydroxychloroquine may slowly produce a diffuse bluish grey colour, more marked on light-exposed areas. Chronic intoxication with inorganic arsenic may cause diffuse pigmentation, some times dotted with small pale macules producing a characteristic ‘rain drop’ pattern. Silver salts are now a very rare cause, but large cumulative doses of gold salts lead to diffuse melanosis, more marked on light-exposed areas with relative sparing of the flexures (Launey & Land, 1984).

2.5.2.5. Lymphoma and carcinoma

Lymphoma and carcinoma may be accompanied by generalized darkening of the skin, usually in the late stages of the disease (Launey & Land, 1984).

2.5.3. Localized hypopigmentation

2.5.3.1. Vitiligo

Vitiligo is a common, acquired diseases affecting 1% to 2% of the population with women being more affected than men. Fifty percent develop their lesions before age of 20 and 25%, before the age of 10 (Arndt & Bowers, 2002). It is likely that predisposition to Vitiligo is inherited as an autosomal dominant characteristic (Launey & Land, 1984). Localized or generalized areas of the skin completely lack melanin pigmentation. Pigment cells (melanocytes) cannot be detected in depigmented areas, even on inspection by electron microscopy. This finding is in contrast to albinism, in which melanocytes are present but there is little or no pigmentation because of faulty or absent melanin synthesis. The cause of Viltiligo is unknown; an abnormal neurogenic stimulus, intrinsic genetic defect of melanocytes as an enzymatic self-destruction mechanism involving a deficiency of a melanocytic growth (Arndt & Bowers, 2002).

2.5.3.2. Postinflammatory or posttraumatic hypopigmentatin

Postinflammatory hyperpigmentation may be profound enough to mimic Vitiligo but frequently appears as slightly scaly, slightly lighter areas of skin. It is often seen following cutaneous disease such as Psorisis and atopic dermatitis and in those instances may be related to the inability of altered epidermal cells to accept melanin granules rather than to the decreased production of pigment in melanocytes (Arndt & Bowers, 2002).

2.5.4. Generalized hypopigmentation

Some inherited disorders of amino acid metabolism are associated with diminished pigmentation of skin and hair, and poor ability to tan.

2.5.4.1. Albinism

Albinism is a heterogeneous cluster of disorders linked by an impaired capacity for melanin synthesis. The skin and hair are pigmented normally but affected males have translucency of the iris, defective pigmentation of the retina, nystagmus and photophobia. Female heterozygotes are less severely affected (Launey & Land, 1984).

2.5.5. Abnormal pigmentation not due to melanin

There are several causes of abnormal pigmentation that are created not due to melanin, such as the yellow or yellowish green colour of jaundice is to some extent mimicked by the yellow skin of hypercarotenaemia. A melanin-like pigment is deposited in the connective tissue and cartilage of patients with ochronosis and may be visible over ears, eyelids and sclera.

2.5.5.1. Exogenous pigment

Exogenous pigment may be impregnated in the skin (tattoos, coal dust, grit etc.) or may be merely staining the surface. Many dermatological remedies stain the skin (e.g., dithranol, potassium permanganate, iodine) and general practitioners are occasionally consulted by patients worried by exogenous pigmentation (Burton, 1979).

2.5.5.2. Drugs

Drugs such as mepacrine (yellow), silver (slate-blue), and gold (blue-grey) can cause hyperpigmentation (Burton, 1979).

2.6. Depigmenting agents (Tyrosinase inhibitors)

There are numerous types of depigmenting agents that basically are divided in to two main categories; natural and synthetic agents. Natural agents include; plant polyphenols, aldehydes and other compounds from higher plants, fungal metabolites, and derivatives of natural compounds. A number of synthetic depigmenting agents from drugs and simple chemicals (e.g. captopril, tropolone and hydroxylamines) have been reported to date (Kim & Uyama, 2005). The most common depigmenting agents from natural sources are as follows:

2.6.1. Hydroquinone [a]

Hydroquinone (HQ) is an effective and widely used skin-lightening agent for the treatment of melasma, postinflammatory hyperpigmentation and other hyperpigmentation disorders. HQ seems to exert its effect mainly in melanocytes with active tyrosinase activity. The oxidation products are quinones and ROS (reactive oxygen species), which lead to oxidative damage of membrane lipids and proteins, including tyrosinase and depletion of glutathione contributes to the lightning action. Several other phenolic compounds have been screened as depigmeting agents such as 4-hydroxyanisole [b], 4-SCAP [c] and monobenzone [d]. HQ is available over the counter in the United States in strengths up to 2% and by prescription in strengths of 3 to 4%. Higher concentrations are available through compounding pharmacies. Four to 6 weeks of monotherapy with hydroquinone is generally required before depigmenting effects are seen. Various products containing hydroquinone in combination with tretinoin, glycolic acid, vitamin C, retinol and fluorinated steroids are now available. The length of time necessary for these products to take effect varies. The most common side effects associated with hydroquinone are skin irritation and contact dermatitis, which can be treated with topical steroids. A rare side effect is the development of exogenous ochronosis, a sooty hyperpigmentation in the treatment area, which can be extremely difficult to reverse. Although this adverse event is uncommon with normal use, it may result from hydroquinone use for extended periods of time. Dark-complexioned individuals living in sunny climates are most at risk. Alternating the use of hydroquinone in 4-month cycles with one of the natural

depigmenting agents listed below can prevent or reduce side effects, such as irritation or even exogenous ochronosis (Briganti *et al*. 2003; Katsambas & Stratigos, 2001; Nerya *et al*. 2003; Rendon & Gaviria, 2005; James, 2006).

2.6.2. Aloesin [e]

Aloesin is a natural derivative of *Aloe vera* that inhibits tyrosinase at noncytotoxic concentrations. Aloesin acts as a competitor inhibitor of DOPA oxidation and as a noncompetitor on tyrosine hydroxylase activity. Aloesin is an experimental product and is not available clinically (Rendon & Gaviria, 2005).

2.6.3. Arbutin [f]

Arbutin (hydroquinone-α-D-glucopyranoside) and methylarbutin are skin-lightening glucosides found in bearberry (*Arctostaphylos uva ursi*), a fruit enjoyed by bears but not by humans. Arbutin is also present in certain herbs and pear trees. Its effect occurs by inhibiting melanosomal tyrosinase activity. A dose-dependent reduction in tyrosinase activity and melanin content in melanocytes has been demonstrated. The manufacturers of products containing arbutin cite a concentration of 1% as effective for depigmentation. Nevertheless, studies have shown the efficacy of arbutin to be less than that of Kojic acid. Arbutin (3%) is also available, although controlled studies have not been done in the United States (Rendon & Gaviria, 2005; Nerya *et al*. 2003).

2.6.4. Azelaic acid [g]

Azelaic acid is a naturally occurring dicarboxylic acid derived from *Pityrosporum ovale*. Compounds able to bind either amino or carboxyl groups may block the access of tyrosine to the active site, behaving as competitive inhibitors. Among these, azelaic acid inhibits tyrosinase activity *in vitro* and may also interfere with DNA synthesis and mitochondria activity in hyperactive and abnormal melanocytes. Azelaic acid has a minimal effect on normal pigment and the greatest effect on heavily pigmented melanocytes. It has been used successfully in the treatment of facial lentigo maligna,

rosacea, solar keratosis and hyperpigmentation associated with burns and herpes labialis. Some studies have reported a superior beneficial effect on melasma compared with hydroquinone, whereas other studies have found no significant difference between these two agents for this condition. Topical azelaic acid in strengths of 15 and 20% is available in the United States by prescription. It is applied twice daily for 3 to 12 months. Azelaic acid is generally well tolerated and can be used for extended periods. It is effective on dark skin. The most common side effects are transient erythema and skin irritation, which normally resolve after 2 to 4 weeks of use (Briganti *et al*. 2003; Katsambas & Stratigos, 2001; Nerya *et al*. 2003).

2.6.5. Glycolic acid

Glycolic acid is an α-hydroxy acid derived from sugarcane. In low concentrations, glycolic acid produces rapid desquamation of pigmented keratinocytes. In higher concentrations, glycolic acid results in epidermolysis. Removal of superficial layers of epidermis with glycolic acid peels using glycolic acid in concentrations of 30 to 70% can enhance the penetration of other topical skin lighteners, such as hydroquinone. The addition of glycolic acid to hydroquinone enhances efficacy. When used for postinflammatory hyperpigmentation, glycolic acid treatment should commence at low concentrations to avoid skin irritation. The risk of exaggerated hyperpigmentation can be reduced with the use of hydroquinone prior to and following a glycolic acid peel (Rendon & Gaviria, 2005).

2.6.6. Kojic acid [h]

Kojic acid (5-hydroxy-2-hydroxymethyl-4-H-pyran-4-one) is a fungal metabolic product that is structurally related to maltol. It is a potent tyrosinase inhibitor and functions by chelating copper at the active site of the enzyme. It also acts as an antioxidant and prevents the conversion of the o-quinones of L-DOPA, norepinephrine and dopamine to their corresponding melanin. Kojic acid is used in a 1–4% cream base, alone or in combination with tretinoin, hydroquinone and/or a corticosteroid. It appears to act synergistically with glycolic acid. Compared with 2% HQ, kojic acid alone appears to be less effective. There are scant data from the

literature with regard to its long-term side effects, although some investigators have reported a high frequency of contact sensitivity to this product (Briganti *et al*. 2003; Katsambas & Stratigos, 2001; Nerya *et al*. 2003).

2.6.7. Licorice extract (glabridin)

The principal active compound of licorice root extract is glabridin, which is used in concentrations of 10 to 40% in skin-lightening products. The depigmenting effect of glabridin has been shown to be 16 times greater than that of hydroquinone. It has been shown that UV-B-induced pigmentation and erythema were inhibited by topical application of 0.5% glabridin. The main commercial problems of glabridin are its poor skin-penetrating ability and its instability in formulations (Nerya *et al*. 2003).

2.6.8. Mequinol (4-hydroxyanisol) [b]

Mequinol is widely used in Europe in concentrations ranging from 5 to 20% and is approved in the United States and Canada for the treatment of solar lentigines. Mequinol is a substrate to the enzyme tyrosinase and acts as a competitive inhibitor of melanogenesis. Its exact mechanism is unknown. The therapeutic effect of 4-hydroxyanisol (4HA-mequinol) is based on the structural analogy between 4HA and tyrosine. The combination of mequinol 2% and retinoic acid 0.01% has also proven effective in the treatment of solar lentigines. The use of pigment-specific lasers in combination with mequinol appears to potentiate the depigmenting action and could potentially reduce the number of laser treatments required, as well as prevent recurrences. In darkly pigmented patients, mequinol can also prevent the development of postprocedural postinflammatory hyperpigmentation (James, 2006; Rendon & Gaviria, 2005).

2.6.9. Melatonin

Melatonin is a hormone secreted by the pineal gland in response to sunlight. It has been shown to inhibit melanogenesis in a dose-related manner. However, the concentration needed for depigmentation in human skin has not been established.

Anti-inflammatory activity has been shown at a dosage of 0.6 mg/cm^2 and one company is marketing a topical melatonin cream as an antioxidant (Rendon & Gaviria, 2005).

2.6.10. Niacinamide

Niacinamide (also known as nicotinamide, 3-pyridinecarboxamide) is the physiologically active amide of niacin (vitamin B3). Niacinamide inhibits the transfer of melanosomes to the epidermal keratinocytes. Several reports suggest that niacinamide may have various effects on the skin: it acts as an anti-inflammatory agent in acne, as an antioxidant, prevents photoimmunosuppression and photocarcinogenesis, and increases intercellular lipid synthesis. Topical niacinamide has been used to treat cutaneous lesions of dermatitis but has not previously been demonstrated to affect pigmentation (Hakozaki *et al*. 2002).

2.6.11. Paper mulberry

Paper mulberry extract, a popular skin-lightening agent in Europe and South America is prepared from the roots of the *Broussonetia papyrifera* tree.

2.6.12. Retinoids and retinoid combination therapy

Retinoids such as tretinoin and retinol are derived from vitamin A. These products are used successfully to treat melasma, postinflammatory hyperpigmentation and other pigmentation disorders. Retinol is found in a variety of moisturizers and antiaging products sold over the counter. Retinol is less effective and less irritating than tretinoin. A prescription product containing 0.3% plus 4% hydroquinone and sunscreens has been shown effective for the treatment of hyperpigmentary disorders. Retinol is less effective and less irritating than tretinoin. The actual mechanism of depigmentation has not been established. In animal studies, retinol has been shown to inhibit tyrosinase induction. Retinoids may also interfere with pigment transfer to keratinocytes and accelerate pigment loss by causing the epidermis to be shed more quickly. Triple combinations of corticosteroids, hydroquinone and retinoic acid have a

synergistic effect and studies have demonstrated 79% clearing of melasma (Griffiths, 2001; Rendon & Gaviria, 2005).

2.6.13. Soy

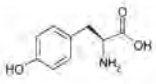
Natural soybeans contain Bowman-Birk inhibitor and soybean trypsin inhibitor, two serine protease inhibitors that interfere with the protease-activated receptor 2 pathway, thereby reducing melanin transfer and inducing depigmentation. The effect can be obtained only with unpasteurized soymilk. Soy has proven to be both efficacious and safe. Several skin care products containing total soy are available to improve mottled hyperpigmentation and solar lentigines. Skin-lightening benefit is seen after 12 weeks of twice daily application (Rendon & Gaviria, 2005).

2.6.14. Vitamin C (L-ascorbic acid)

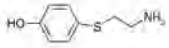
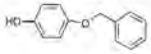
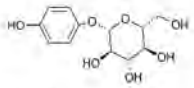
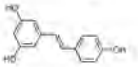
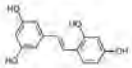
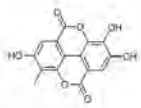
Vitamin C or L-ascorbic acid (AsA), interferes with pigment production at various oxidative steps of melanin synthesis, for example 5,6 dihydroxyindole oxidation. The involvement of peroxidase in the polymerization of melanogenic intermediates has been suggested by the high efficiency of peroxidase in the oxidation of 5,6-dihydroxyindole (DHI) with generation of hydrogen peroxide (H_2O_2) as a by-product. Interacellular H_2O_2 is generated after UV irradiation or in response to cytokines. The inhibition of peroxidase, however, results in depigmentation, reducing the polymerization rate of eumelanin in different experimental models. Several melanocytotoxic phenol/catechol derivatives, are suitable substrates of either tyrosinase or peroxidase and the inhibition of peroxidase could play a role in their mechanism of action as well as of depigmenting agents, such as glucocorticoids or ascorbic acid, that do not exhibit any effect against the melanogenic enzymes. Methimazole, an antithyroid agent belonging to the thionamide group, exerts inhibitory action towards both tyrosinase and peroxidase. AsA has a reducing effect on o-quinones and oxidized melanin and it can alter melanin from jet black to light tan. A disadvantage of AsA is its chemical instability in aqueous solution where it becomes quickly oxidized and denatures. AsA esters have been tested in an effort to overcome this problem. A stable derivative of AsA, magnesium L-ascorbyl-2-

phosphate (VC-PMG), was used in a 10% cream base and produced a significant lightening effect in 19 of 34 patients with melasma after 3 months of twice daily application (Briganti *et al*. 2003; Katsambas & Stratigos, 2001). Chemical structures and the mechanism of action of a few depigmenting agents are depicted in table 2.3.

Table 2.3. Chemical structure of modulators melanogenic activity. Most of the compounds show chemical analogy with L-tyrosine (natural substrate of tyrosinase) (Briganti *et al*. 2003)



L -Tyrosine.

Chemical structure	Name	Mechanism of action	Inhibition constants	Other effects
	Hydroquinone [a]	Alternative substrate	IC_{50}= 75 μM	Oxidative stress-mediated melanocytotoxic effect
	4-hydroxyanisole [b]	Alternative substrate		Oxidative stress-mediated melanocytotoxic effect
	4-SCA [c]	Alternative substrate		Melanocytotoxic effect mediated by alkylation of cell proteins
	Monobenzone [d]	Alternative substrate		Oxidative stress-mediated melanocytotoxic effect
	Arbutin [f]	Hydroquinine pro-drug	IC_{50}= 17 mM	Inhibition DHICA polymerase activity
	Aloesin [e]	Competitive inhibition DOPa oxidation	IC_{50}= 0.167 mM	Non-Competitive inhibition tyrosine hydroxilation
	Azelaic acid [g]	Block of tyrosinase access to active site?	K_i= 2.73*10^{-3} M	
	Resveratrol	Alternative substrate (product ortho-hydroxylated)	IC_{50}= 54 μM [a] IC_{50}= 250 μM [b]	ROS scavenger, COX-2 inhibitor anti-cancer activity
	Oxyresveratrol	Non-competitive inhibition	IC_{50}= 1.2 μM [a] IC_{50}= 52.7 μM [b]	Same effects described for resveratrol
	Kojic acid [h]	Copper chelation	IC_{50}= 6.2 μM [a] IC_{50}= 250 μM [b]	Antioxidant activity, iron chelator Inhbibition NF-kB activation
	Methyl gentisate	Copper chelation	IC_{50}= 11.2 μM [a] IC_{50}= 30.9 μM [b]	
	Ellagic acid	Copper chelation		Scavenger activity

a, b, c & d (p. 43 & 46); e, f & g (p. 44); h (p. 46)

2.7. Application and importance of tyrosinase inhibitors

2.7.1. Agricultural and food fields

Browning of plant-derived foods and beverages occurs due to enzymatic oxidation of phenols by tyrosinases, which can cause the destruction of essential amino acids, the impairment of digestibility and nutritional quality, the formation of toxic compounds. This unfavorable browning has been of great concern, and it is necessary to search for potent tyrosinase inhibitors (Kim & Uyama, 2005).

2.7.2. Cosmetic and medicinal fields

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic and medicinal industries due to their preventive effect on pigmentation disorders. Tyrosinase inhibitors may result in a reduction in melanin biosynthesis and are used in cosmetic products for hyperpigmentation-related concerns, including the formation of freckles. Tyrosinase may also be targets for developing medicines to treat hypopigmentation-related problems, such as albinism and piebaldism (Kim & Uyama, 2005). Various results are available regarding the role of tyrosinase in cancer as some papers suggest a tumor-suppressing effect of mushroom tyrosinase, whereas others predict a possible role in mutagenicity (Seo *et al*. 2003).

2.8. Discussion and conclusions

Skin depigmentation can be achieved by interfering with the different steps of melanogenesis pathway. Thus, association of depigmenting agents with different mechanisms of action is a useful strategy to improve clinical efficacy, reducing the duration of therapy and the risk of adverse effects. In addition to tyrosinase inhibition, other parameters related to cytotoxicity, solubility, cutaneous absorption, penetration and stability of the agents should be considered (Solano ***et al.*** 2006).

Current therapies are inadequate in terms of high toxicity, poor skin penetration, instability in formula, low effects and etc. According to Nerya ***et al***. (2003), among the present depigmenting agents, 1,4-dihydroquinone is one of the most widely prescribed. It causes reversible inhibition of cellular metabolism by affecting both DNA and RNA synthesis. Hence, 1,4-dihydroquinone can be considered to be a potent melanocyte cytotoxic agent and has also been reported to induce mutations. As a result of these and other side effects, such as chronosis in African nations, there has been increasing impetus to find alternative herbal and pharmaceutical depigmenting agents.

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Chapter 3

Anti-tyrosinase activity of *Cassipourea malosana*, *Ceratonia siliqua* and *Sideroxylon inerme*

3.1. Introduction

Tyrosinase inhibition has so far been the most common approach for depigmentation, but only a few of tyrosinase inhibitors have practical application for several reasons (Solano ***et al***. 2006). Therefore, the research of natural chemical agents able to modulate the metabolism of pigmentation is of great interest.

Our goal was to identify bleaching agents from plants that can contribute towards skin-pigmentation problems. A preliminary study conducted during my Honours studies indicated very good anti-tyrosinase activity of plants namely; *Cassipourea malosana* L. (stem-bark), *Ceratonia siliqua* L. (leaves) and *Sideroxylon inerme* L. (stem-bark) (Momtaz, 2006). Therefore, they have been selected for further investigation for their efficacy of tyrosinase inhibition. The bark of *C. malosana* and *S. inerme* are traditionally used in Southern African for skin lightening purposes. The leaves of *Ceratonia siliqua* was selected based on its chemical components; i.e., polyphenols that are well known as tyrosinase inhibitors. An investigation of *in vivo* tyrosinase inhibitory and *in vitro* anti-melanogenesis activities of these plants were pursued.

3.2. Material and methods

3.2.1. Materials

3.2.1.1. Collection of plant materials

The *Ceratonia siliqua* leave material was collected from the Botanical Garden of the University of Pretoria during September 2005. The bark of C*assipourea malosana* and *Sideroxylon inerme* were collected in Holly Thathe Forest next to Thohoyandou in Limpopo province (South Africa) in June 2006. The plants were identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria. Herbarium voucher specimens have been submitted in the herbarium of the University of Pretoria (Table 3.1).

Table 3.1. Plant samples collected for the present study:

Name of Plant	Plant Parts	Voucher Herbarium Specimen Number
***Cassipourea malosan* L. (Rhizorphoraceae)**	**Stem-bark**	**PRU 09484**
***Ceratonia siliqua* L. (Fagaceae)**	**Leaves**	**S.M. 95502**
***Sideroxylon inerme* L. (Sapotaceae)**	**Stem-bark**	**PRU 96216**

3.2.1.2. Chemicals and reagents

Tyrosinase with an activity of 6680 units/mg, Kojic acid (100%) and Arbutin (98%) (positive controls) were purchased from Sigma-Aldrich (South Africa). Fetal calf serum (FCS), trypsin, EDTA, L-glutamine, HEPES buffer, penicillin/streptomycin/fungizone and sodium pyruvate were purchased from Highveld Biological. The Cell Proliferation Kit II (XTT) (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro benzene sulfonic acid

hydrate) labelling reagent) was purchased from Roche Diagnostics (Nerya *et al*. 2003).

3.2.2. Methods

3.2.2.1. Extraction of plant materials

Selection of an appropriate extractant (when the chemical nature of the potentially active constituents is unknown) can be a daunting task. The first step in the process of obtaining unknown metabolites from biogenic materials is to release them from the matrix by means of extraction. Because of their complex composition of the material and the minute amounts of some of the constituents present, the choice of extraction method is of great importance (Yrjonen, 2004). Physical properties of solvents (availability, detector compatibility, solvent reactivity, boiling point, viscosity, miscibility and safety) to be used for extractions and fractionations need to be carefully considered to make sure the desired compounds are extracted or separated (Rabie, 2005). Many studies employ Soxhlet extraction of dried plant material using solvents with increasing polarity.

For the present study, 40 g of each plant material (shade dried) were ground to a fine powder using a Junke & Kunkel grinder. In order to find an extractant that would be optimally useful in both the bioassay and isolation of anti-tyrosinase components from plants, it was decided to compare a number of extractants. Dichloromethane, acetone and methanol were used as non-polar, medium-polar and polar solvents respectively to extract all kind of compounds from plant materials.

Each plant material was extracted with (200 ml) of acetone, dichloromethane and methanol separately, constant stirring for 5 hours at 50 ºC temperature. The extracts (solvent and plant material) were kept at room temperature for 24 hours. The solvent was then removed and replaced with an equal volume of the solvent. This procedure was repeated two times. Extracts were then filtered through filter paper (Whatman filter paper 15.0 cm) and the solvent was removed under vacuum (BUCHI, Rotavapor, R-200) to yield dry extracts.

3.2.2.2. Tyrosinase enzyme assay

This assay was performed using the method described by Curto *et al*. (1999) and Nerya *et al*. (2003). Extracts were dissolved in DMSO (dimethyl sulphoxide) to a final concentration of 20 mg/ml. This extract stock solution was then diluted to 600 µg/ml in 50 mM potassium phosphate buffer (pH 6.5). 'Kojic acid' and 'Arbutin' were used as control drugs (Lee *et al*. 1997; Kim *et al*, 2006). In the wells of a 96-well plate, 70 µl of each extract dilution was combined with 30 µl of tyrosinase (333 Units/ml in phosphate buffer) in triplicate. After incubation at room temperature for 5 minutes, 110 µl of substrate (2 mM L-tyrosine or 12 mM L-DOPA) was added to each well. Final concentrations of the extract samples and positive controls ranged from 1.5 to 400 µg/ml. Incubation commenced for 30 minutes at room temperature. Optical densities of the wells were then determined at 492 nm with the BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa). All the experiments were repeated thrice. Plant extracts with good anti-tyrosinase activity, were further investigated for their effect on melanin production by melanocyte cells.

3.2.2.3. Melanocyte cell culture for the investigation of inhibition of melanin formation by plant extracts

The mouse melanocyte cell line, 'B16-F10', was cultured in complete basal medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM *L*-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone at 37°C with 5% CO_2 in a humidified atmosphere. Cells were sub-cultured in a ratio of 1:3 on every third or fourth day. For *in vitro* experiments, B16-F10 cells, were resuspended in complete DMEM medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM *L*-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone.

3.2.2.3.1. Transferring cells to 96-well plates

Ethanol (70%) was sprayed on to the tissue culture flask (250 ml, 75 cm^2, Lasec, South Africa) with the confluent cells before it was placed upright in the flow hood. The spent medium was removed from the flask and the confluent culture was washed

five times with 5ml of PBS (without Ca^{2+} or Mg^{2+}). The PBS was later removed from the culture flask and trypsin/versene (1.5 ml) was added to the cells. The flask was rotated to cover the cell layer with trypsin/versene and then incubated at 37 °C for 15 minutes. The flask was tapped every 5 minutes to help loosen the cells. Once the cells were detached, complete medium (3 ml) was added to the flask to inactivate the trypsin/versene. The cell medium mixture was then transferred to a 10 ml conical tube to which some more complete media was added. By centrifuging at 970 rpm (1619 rotor) for five minutes the cells were pelleted. The supernatant was removed and by tapping the tube gently the pellet was loosened. Thereafter, the cells were re-suspended in 2 ml complete medium after which they were counted with trypan blue and a hemacytometer.

3.2.2.3.2. Counting cells with a hemacytometer

A 1:10 dilution of the cell suspension in trypan blue solution (e.g. 10 µl cells in 90 µl trypan blue) in an eppendorf tube was prepared and mixed. Ten microlitres of this dilution was transferred to 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.

The concentration of cells was determined by using the following formula:

Cell concentration (cells/ml) = Number of cells per square x 10 x 10000

A cell suspension of the given concentration was determined using the following formula:

$$\text{Volume (cell suspension)} = \frac{\text{Cell concentration wanted x volume wanted}}{\text{Concentration of cells in suspension}}$$

3.2.2.4. Toxicity and effect of plant extracts on melanin biosynthesis *in vitro*

Mouse melanocyte cells (B16F10) were obtained from the American Type Culture Collection (Rockville, MD, USA). B16F10 cells were maintained in culture flasks in complete DMEM (Dulbeccos Modified Eagle's Medium), supplemented with 10% FCS, and (10 ml) antibiotics (penicillin/streptomycin/fungizone).

Subculture was done every 2-3 day after it had formed a confluent monolayer. During subculture, cells that attached to the culture flask were trypsinized (0.25% trypsin containing 0.01% EDTA) for 10 min at 37 $^{\circ}$C then stopped by the addition of complete medium. About 10^5 of the viable cells were then re-suspended in complete medium.

The B16F10 cells were seeded into the wells of a 96-well plate (100 µl/10^4 cells per well) for toxicity experiment and 24-well plate (1 ml/10^5 cells per well) for melanin inhibition investigation. The plates (24-well and 96-well) were incubated for 24 hours to allow the cells to attach to the bottom of the plate. A DMSO control was prepared by adding 80 µl of DMSO to 8 ml complete medium as well as a medium control (the final percentage of DMSO was 0.018%). A dilution series of samples and positive control (Kojic acid) (final concentration 100 µg/ml – 1.563 µg/ml) were made and then added to the microtiter plates. The 96-well and 24-well plates were incubated for 48 hours at 37 ºC in 5% CO_2 and a humidified atmosphere (Fig 3.1).

Cytotoxicity was measured by the XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) method using the Cell Proliferation Kit II (Zheng *et al*. 2001). The XTT reagent was added to the cells in 96-well plate at the final concentration of 0.3 mg/ml incubated for 1-2 hrs. A toxicity reference plate (96-well) was prepared by adding 100 µl of medium, 100 µl of samples and 50 µl of XTT. The plate was incubated for 1-2 hrs. After incubation, the absorbance of the color complex was spectrophotometrically quantified at 490 nm using an ELISA plate reader with a reference wavelength set at 690 nm. By referring to the control (medium with DMSO), cell survival was assessed. The effect of the extracts on melanin synthesis was determined by washing the cells in the 24-well

plate with PBS and lysing with 200 µl of sterile distilled water. Optical densities were determined at 405 nm. The effect on melanin production was determined by referring to the control sample (medium with DMSO). All the experiments were repeated three times.

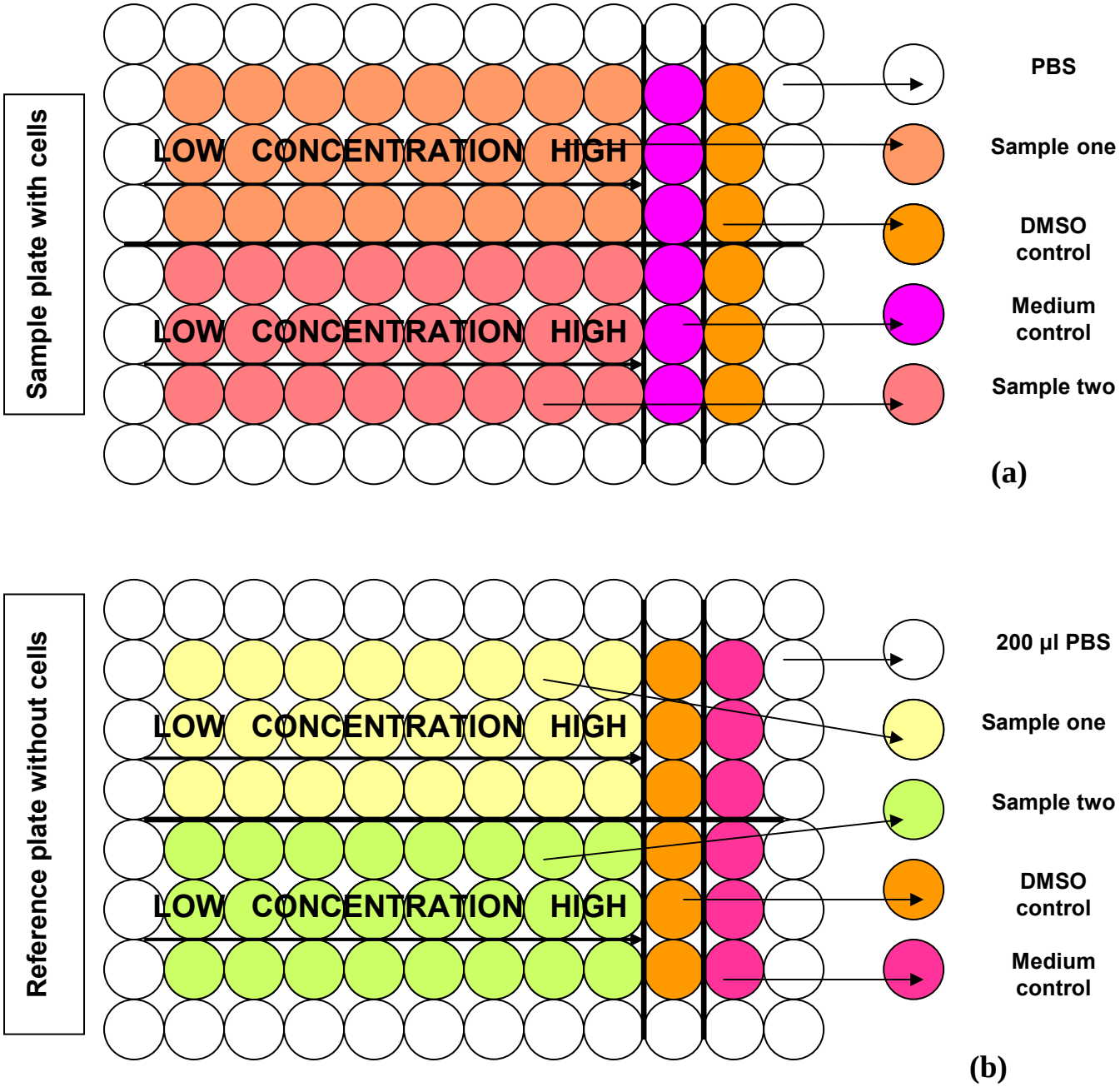

Figure 3.1. Microtiter plate for the investigation of effect of plant extracts on melanocyte cells; (a) sample plate with melanocyte cells and (b) reference plate without cells for comparing the intensity of XTT

3.3. Statistical analysis

The final results are expressed as the mean (standard deviation, ±SE.S). The group means were compared using the ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple Range Test was applied to compare the means. Values were determined to be significant when *p* was less than 0.01 ($p<0.01$).

3.4. Results

3.4.1. Inhibition of tyrosinase activity by positive controls (arbutin and kojic acid) and plant extracts (*C. malosana*, *C. siliqua* and *S. inerme*) using L-tyrosine as substrate

Kojic acid showed a complete inhibition of tyrosinase activity at all the concentrations tested ($p<0.01$ at 3.1 µg/ml) while arbutin exhibited 35 % anti-tyrosinase activity at 200 µg/ml (Fig. 3.2).

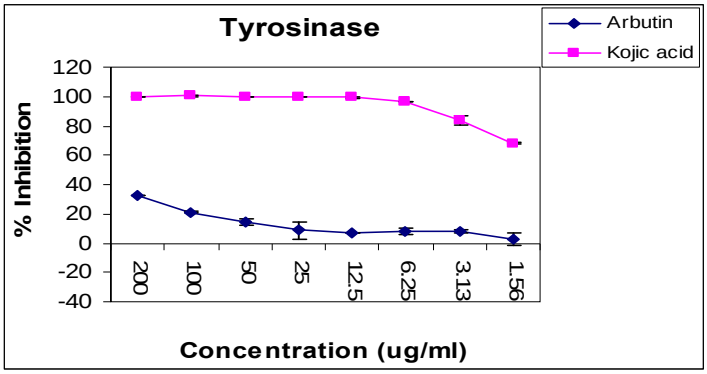

Figure 3.2. Tyrosinase inhibitory activity by Arbutin and Kojic acid (positive controls), using L-tyrosine as a substrate

Results of tyrosinase inhibitory activity by the methanol and acetone extracts of *S. inerme* were found to be similar. *S. inerme* (acetone and methanol extracts) showed significant inhibition ($P<0.01$) (70%) of tyrosinase activity at 200 µg/ml. No

significant reduction (P<0.01) of tyrosinase activity was obtained by dichloromethane extract at any concentration tested (Fig. 3.3).

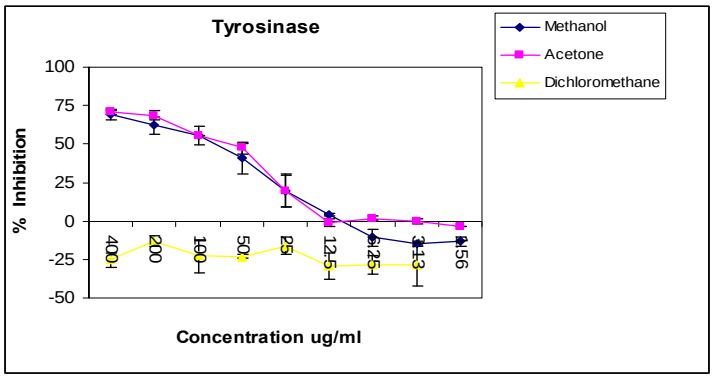

Figure 3.3. Tyrosinase inhibitory activity by acetone, dichloromethane and methanol extracts of *Sideroxylon inerme*, using L-tyrosine as a substrate

Methanol extract of *C. siliqua* showed significant inhibition (P<0.01) of tyrosinase activity at 400, 200 and 100 µg/ml. No significant reduction (P<0.01) of tyrosinase activity was observed by its acetone and dichloromethane extracts (Fig. 3.4).

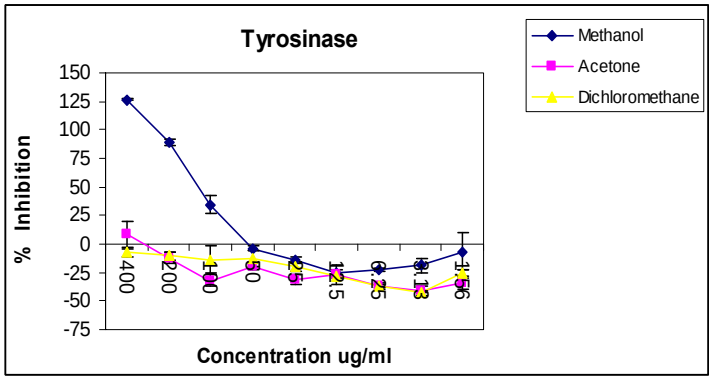

Figure 3.4. Tyrosinase inhibitory activity by acetone, dichloromethane and methanol extracts of *Ceratonia siliqua*, using L-tyrosine as a substrate

Acetone, dichloromethane and methanol extracts of *C. malosana* did not show significant inhibition (P<0.01) of tyrosinase action at any concentration tested (Fig. 3.5).

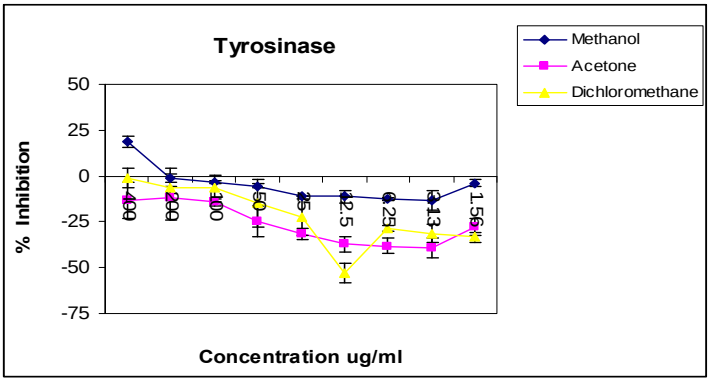

Figure 3.5. Tyrosinase inhibitory activity by acetone, dichloromethane and methanol extracts of *Cassipourea malosana*, using L-tyrosine as a substrate

3.4.2. Inhibition of tyrosinase activity by positive controls (arbutin and kojic acid) and plant extracts (*C. malosana*, *C. siliqua* and *S. inerme*) using L-DOPA as substrate

Kojic acid showed 62% inhibition of tyrosinase activity at 50 µg/ml significantly ($p<0.01$) while arbutin showed 32 % anti-tyrosinase activity at 12.5 µg/ml using L-DOPA as substrate ($p<0.01$) (Fig. 3.6).

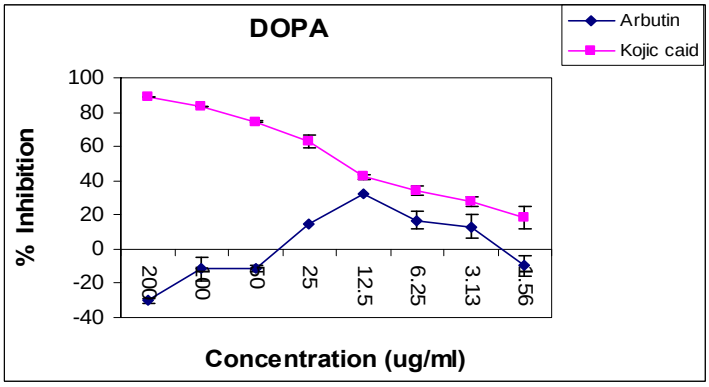

Figure 3.6. Tyrosinase inhibitory activity by Arbutin and Kojic acid (positive controls), using L-DOPA as substrate

Methanol extract of *C. siliqua* showed 43% and 23% inhibition of tyrosinase activity at 400 and 200 µg/ml ($p<0.01$) respectively when L-DOPA was used as a

substrate. A significant ($P<0.01$) tyrosinase inhibitory activity was achieved by the methanol extracts of *C. malosana* at 12.5 µg/ml whereas, *S. inerme* (methanol extract) did not exhibit significant activity at any concentration tested in this study (Fig. 3.7). Table 3.2 depicts the IC_{50} values of plant extracts and positive controls (arbutin and kojic acid).

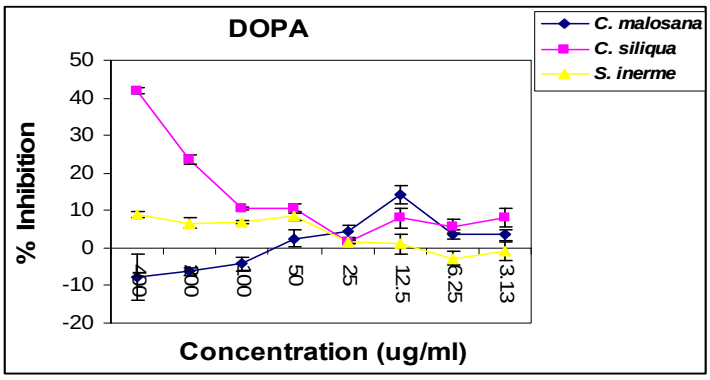

Figure 3.7. Tyrosinase inhibitory activity by the methanol extracts of *Cassipourea malosana*, *Ceratonia siliqua* and *Sideroxylon inerme*, using L-DOPA as a substrate

Table 3.2. IC_{50} values of plant extracts for mushroom tyrosinase

Material	IC_{50} monophenolase (µg/ml)	IC_{50} diphenolase (µg/ml)
Arbutin	**>200**	**>200**
Kojic acid	**1.138±0.05**	**50.51±1.7**
C. malosana* (A)	**>200**	**-**
C. malosana* (D)	**>200**	**-**
C. malosana* (M)	**>200**	**>200**
***C. siliqua* (A)**	**>200**	**-**
***C. siliqua* (D)**	**>200**	**-**
***C. siliqua* (M)**	**112.1±0.7**	**>200**
***S. inerme* (A)**	**63±2.1**	**-**
***S. inerme* (D)**	**>200**	**-**
***S. inerme* (M)**	**82.1±2.7**	**>200**

***A (acetone), D (dichloromethane) and M (methanol) extracts**

3.4.3. The effect of plant extracts on melanin biosynthesis by mouse melanocytes

Following the results obtained from tyrosinase assay, methanol extracts of *Ceratonia siliqua* and *Sideroxylon inerme* were selected for further investigation to examine their ability on melanin production in an *in vitro* environment. To correlate whether a reduction in melanin observation is caused by enzyme inhibition or a decrease in cell number and/or viability, it was imperative to perform a toxicity assay in conjunction with the melanin production assay.

Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and exhibited 60% reduction in melanin content at 3.1 µg/ml and 25 µg/ml (Fig. 3.8). *S. inerme* showed significant inhibition (37%) of melanin production at 6.2 µg/ml while 80% of cells were viable. The sample showed some toxicity to the cells when exposed to concentrations greater than 50 µg/ml. A significant reduction in melanin amount (45%) was obtained by *C. siliqua* at 12.5 µg/ml when the cells viability was found to be more than 90%.

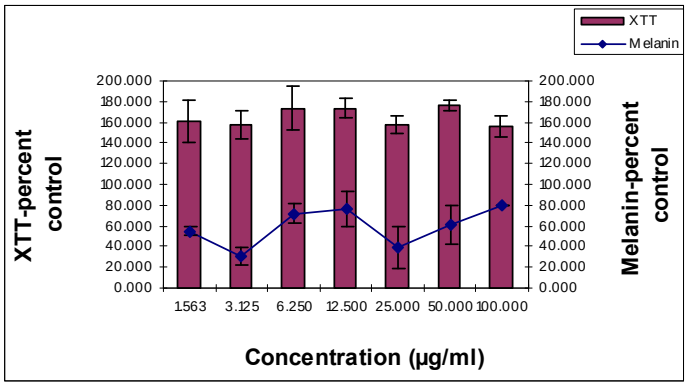

Figure 3.8. The effect of kojic acid on cell viability/proliferation and melanin production

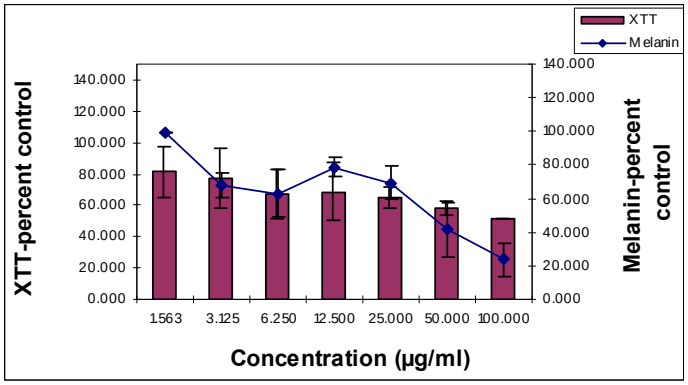

Figure 3.9. The effect of *Sideroxylon inerme* on cell viability/proliferation and melanin production

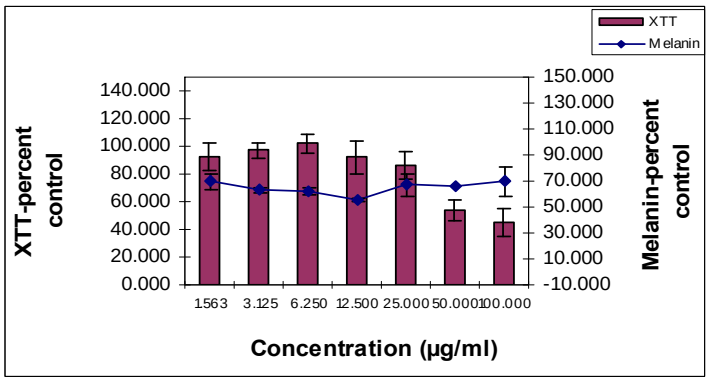

Figure 3.10. The effect of *Ceratonia siliqua* on cell viability/proliferation and melanin production

3.5. Discussion and conclusions

Acetone and methanol extracts of *S. inerme* showed significant inhibition ($P<0.01$) of tyrosinase activity (70%) at 200 µg/ml. Methanol extract of *C. siliqua* exhibited very good inhibition ($P<0.01$) of tyrosinase activity at 400, 200 and 100 µg/ml when L-tyrosine was used as substrate. Arbutin and kojic acid demonstrated 32% and 100% inhibition of monophenolase activity at 200 µg/ml respectively. Methanol extract of *C. siliqua* showed significant inhibition ($P<0.01$) (45%) of tyrosinase activity at 200 µg/ml when L-DOPA was used as substrate. *C. siliqua* and *S. inerme* (methanol extract) showed more anti-tyrosinase activity than arbutin but less than that of kojic acid.

Our results compare well with other reported results of the effect of plant extracts for anti-tyrosinase activity. It has been reported that *Glycyrrhiza glabra*, *Morus alba* and *Gastrodia ellata* (80% ethanol extract) showed 65%, 68% and 85% tyrosinase inhibition at the concentration of 333 µg/ml respectively (Lee *et al*. 1997). *Entada africana* (water extract), *Portulaca pilosa* (water extract), *Prosopis africana* (water extract) and *Cariniana brasiliensis* (water extract) exhibited inhibition of tyrosinase activity of 94%, 93%, 91% and 90% respectively *in vitro* (Baurin *et al*. 2002).

Methanol extracts of *C. siliqua* and *S. inerme* were selected to investigate their ability to inhibit melanin production in mouse melanocyte cells (B16F10). Methanol extracts of *C. siliqua* and *S. inerme* exhibited 45% and 37% reduction of melanin content at 12.5 and 6.2 µg/ml in melanocytes, respectively, without being significantly ($p<0.01$) toxic to the cells. Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and gave 60% reduction in melanin content at 3.1 µg/ml. According to the earlier reports on the other plants, it has been stated that ethanolic extract of *Areca catechu* demonstrated 49% inhibition of melanogenesis at 500 µg/ml (Lee & Choi, 1999). Kim *et al*. (2006) reported *Morus alba* (70% ethanol extract) showed 32% reduction of melanin production in murine melanoma cells at 50 µg/ml. Based on our results, methanol extracts of *C. siliqua* and *S. inerme* can be considered for the development of skin-depigmentation agents.

In a Thai study in which 0.4% licorice extract was combined with 0.05% betamethasone and 0.05% retinoic acid, 70% of patients reported the effects of their treatment to be "excellent" (Katsambas & Stratigos, 2001). It has been reported earlier in one study that in patients with epidermal melasma, a product containing 0.05% hydroquinone, 10% glycolic acid and 2% kojic acid proved superior to a product containing 2% hydroquinone and 10% glycolic acid and no Kojic acid. In another study of patients given 4% kojic acid in combination with 0.05% tretinoin and valerate betamethasone, 12% reported an excellent response, 48% reported a satisfactory response, 27% had some response, and 13% had no response. One percent of patients reported mild facial erythema (Rendon & Gaviria, 2005). It is speculated that the extracts may be more effective in combination with existing skin-depigmentation agents.

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Chapter 4

Isolation and purification of multifunctional actives from *Ceratonia siliqua*

4.1. Introduction

The skilful application of fractionation methods exploits the fact that an extract can be separated into groups of compounds sharing similar physico-chemical characteristics. The process is called fractionation and can be carried out in various ways, each of, which group compounds according to one or more particular features. Solubility, size, shape, electrical charge and several other features may influence grouping (Houghton & Raman, 1998). The suitable application of fractionation methods can be employed to obtain many fractions, each containing only one or two components. The initial fractionation may be based on solubility and polarity differences (e.g. solvent-solvent separation) while the second may utilise molecular size (e.g. Sephadex column chromatography).

After a specific plant's activity has been established, the plant extract is fractionised by solvent-solvent separation and the activity of different fractions determined with bioassay-guided fractionation. Utilising this method in initial purification, the isolation of an active fraction to improve the probability to find pure compounds with better activity than that of crude extract increases (Gailliot, 1998; Houghton & Raman, 1998).

Sample preparation is the crucial first step in the analysis of herbs, because it is necessary to extract the desired chemical components from the herbal materials for further separation and characterization (Williams & Fleming, 19991). There are usually four main steps followed for the isolation of a compound:

a. The first step in the process of obtaining secondary metabolites from biogenic materials is to release them from the matrix by means of extraction. Because of their complex composition of the material and the minute amounts of some of the constituents present, the choice of extraction method is of great importance.

b. The second step in the isolation process is to separate the target compounds from the crude extract. It can be done by liquid-liquid partition or by some low-resolution chromatographic isolation.

c. The third step in the isolation process usually involves some type of high resolution method such as silica gel column chromatography, sephadex column chromatography etc. to separate the compounds of interest from the other compounds still remaining in the extract.

d. Often the final isolation step is liquid chromatography, especially HPLC, also other separation methods have been applied (Yrjonen, 2004).

Various compounds especially polyphenols (one of the most numerous and ubiquitous groups of plant metabolites, ranging from simple phenolic molecules to highly polymerized compounds with molecular weights of greater than 30,000 Da, such as tannins, quercetins, myricetines and etc.) have been isolated from *C. siliqua* before (Papagiannopoulos *et al*. 2004, Owen *et al*. 2003). Antibacterial, antifungal, antiviral and antithelmintic activities of different extracts of *C. siliqua* have been reported earlier (Papagiannopoulos *et al*. 2004, Van Wyk & Grick, 2000). There is no information till date about the efficacy of *C. siliqua* to treat hyperpigmentation.

Having obtained good inhibitory activity of the methanol extracts of *C. siliqua*, this sample was further selected for solvent-solvent separation (chapter 3, sections 3.4.1 & 3.4.3). The aim of this experiment was to first simplify the extracts of *C. siliqua* by fractionation. Anti-tyrosinase activity and inhibition of melanin production by different fractions and purified compounds have been discussed in this chapter.

4.2. Material and methods

4.2.1. Materials

Methanol extract of the leaves of *C. siliqua* was prepared as mentioned in chapter 3 (section 3.1.2.1).

4.2.2. Methods

Various techniques are available for the purification and identification of natural constituents of plant species. In this study the compounds have been isolated, purified and identified by means of 'solvent-solvent separation', 'Paper partition chromatography' method, 'High performance liquid chromatography' (HPLC) and 'Nuclear magnetic resonance spectroscopy' (^{1}H-NMR ,^{13}C-NMR and 2D-NMR).

4.2.2.1. Solvent-Solvent separation

Dried methanol extract of *C. siliqua* (95 g) was re-dissolved in 80% methanol (methanol/distilled water; 80: 20) and partitioned with n-hexane, ethyl acetate, and n-butanol in separating funnel (PYREX®, 29/32) (Fig. 4.1). Funnels were shaken gently for few minutes and left steady to obtain two separate layers. The organic layers (top layers) were evaporated to dryness at 40 °C to give 11 g, 32.5 g, 19 g and 5g of n-hexane, ethyl acetate, n-butanol and water fractions respectively.

Figure 4.1. Phase-partition of methanol extract of *Ceratonia siliqua* using n-hexane and ethyl acetate

4.2.2.2. Thin layer chromatography (TLC) of separated fractions

Four fractions (n-hexane, ethyl acetate, n-butanol and water) were spotted on thin layer chromatography (TLC) plate, developed using ethyl acetate: ethyl acetate/ formic acid/ acetic acid/ distilled water; (13.5:10:1:1:1.5) as an eluent system (Fig. 4.2). The plate was sprayed with 0.34% vanillin in 3.5% sulphuric acid in methanol.

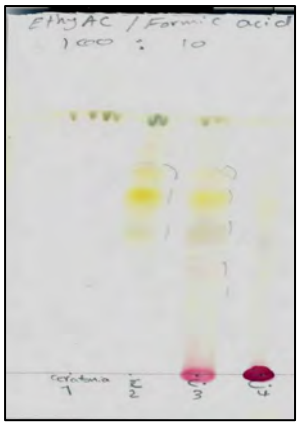


Figure 4.2. Thin layer chromatogram of (1) n-hexane, (2) ethyl acetate, (3) n-butanol and (4) water fractions of solvent-solvent separation of the methanol extract of *Ceratonia siliqua*

4.2.2.3. Isolation of active compounds from ethyl acetate fraction of *C. siliqua* using 'column chromatography'

The ethyl acetate fraction of *C. siliqua*, which showed the highest anti-tyrosinase activity (section 4.3.5.1) as compared to n-hexane, n-butanol and water fractions was dried and quantified (32.5 g). Dry Sephadex powder (LH × 20, Sigma-Aldrich, South Africa) was added to a suitable glass column. The sample size dictates the column size. This mixture was re-dissolved in a minimum amount of methanol and kept at room temperature until the methanol evaporated. Later, the mixture was subjected to a Sephadex column and eluted with ethanol/distilled water mixture (1:1) followed by 100% ethanol, without disturbing the extract alternatively. As the column's elution started, compounds started to form bands and moved down the column at different rates. Fifteen fractions were collected (200 ml each) and concentrated separately.

A series of Sephadex columns were developed with 100% ethanol as eluent (Fig. 4.3). The fractions were applied to TLC plates using ethyl acetate: ethyl acetate/ formic acid/ acetic acid/ distilled water; (13.5:10:1:1:1.5) as an eluent system and those with similar TLC-profile were combined. The plates were sprayed with vanillin (7.5 g vanillin, 250 ml acetone, 5 ml H_2SO_4). Figure 4.4 shows the schematic representation of the column purification steps for the isolation of different compounds from *C. siliqua*.

Figure 4.3. Chromatographic fractionation of ethyl acetate fraction of *Ceratonia siliqua* using Sephadex chromatography

4.2.2.4. Isolation of compounds from *C. siliqua* by 'paper partition chromatography'

Fractions 5 and 6 of column (VII) were mixed, dried, and quantified (0.051 g). Then, the mixture was re-dissolved in a minimum amount of ethanol and was applied on the paper sheets (Whatman No.3 (46× 57 cm)) by a 230 mm Pasture Pipette. The diameters of lines at the origin were approximately 1 cm. The quantity of the sample is important for sensitivity, resolution and purification, for known mixtures as well as unknown ones (Buch, 1952). Thereafter, papers were kept under a cool dryer (hair dryer) until ethanol evaporated. Paper sheets were placed in a glass cylinder and hung vertically from the trays at the top of the tank and held in place by small glass bars (0.25× 0.125× 4 inches). Then, solvent system (acetic acid/distilled water; 15: 85) was placed in the trays. The tank cover was clamped in position and the chromatograms were developed over night until the solvent front had moved 35-40 cm (Buch, 1952; Bush, 1951).

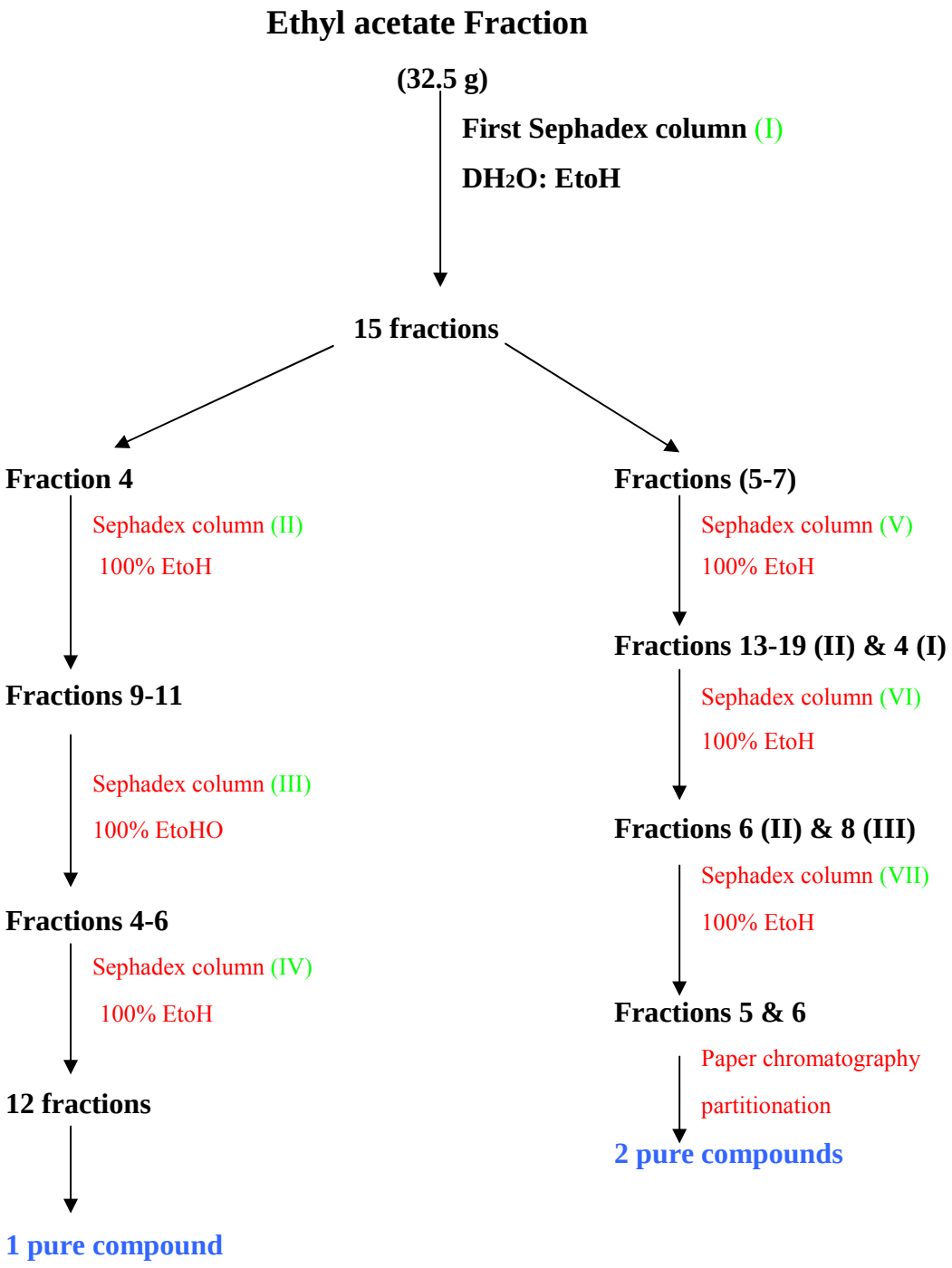


Figure 4.4. Schematic representation of the column purification for the isolation of different compounds from *Ceratonia siliqua*

After development of the chromatograms, the sheets were removed from the tank and hung vertically at room temperature for few hours to evaporate solvents in a stream of air in a

laboratory hood. The lines locations were determined by spraying 'ammonia solution (NH_4OH)' (Chemical Suppliers (PYT) LTD, South Africa).

Four lines were separated and named as A, B, C, and D (Fig 4.5). The lines were examined under UV light at 254 and 366 nm, to confirm the presence of compounds and marked. Each line was cut with a scissor in small pieces. The paper pieces (of each line) were dissolved in 80% methanol separately and kept for 3 hours at 50 ºC while shacking occasionally. Subsequently, each mixture was filtered off and the filtrate was dried using a rotary evaporator (BUCHI Rotavapor, R-200). These procedures were repeated three times until the solvent became colourless. A few mg of each filtrate was dissolved in ethanol and spotted on a TLC plate to determine the purity of obtained compounds.

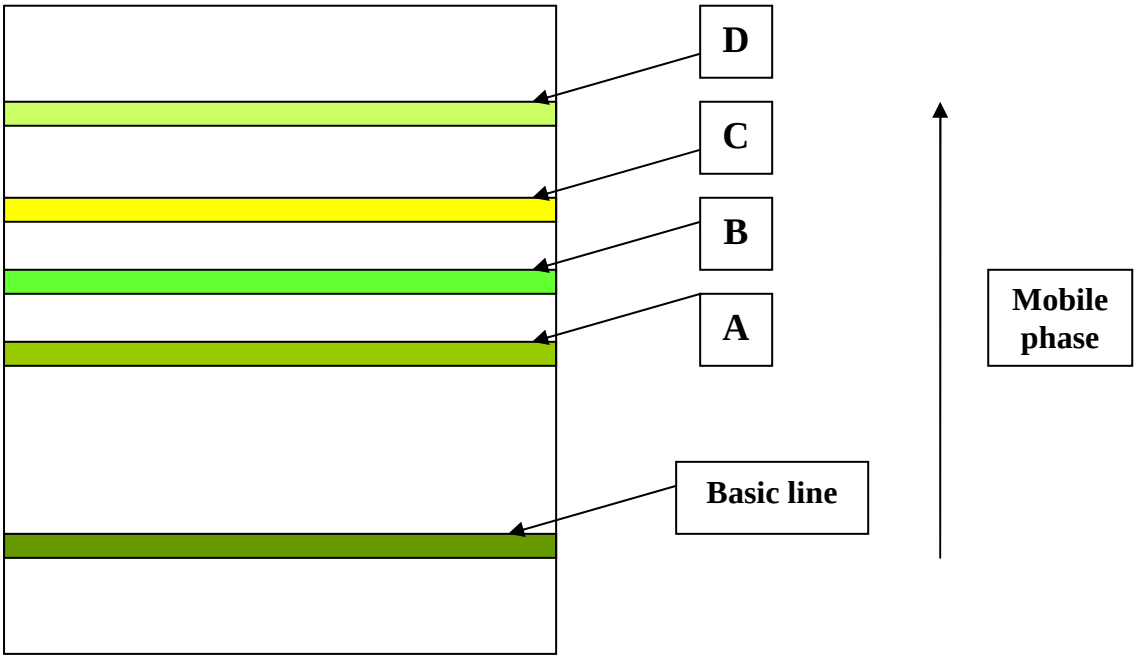


Figure 4.5. Paper chromatography; A, B, C, and D represent the separated lines from the basic (mixture) line

4.2.2.5. Determining anti-tyrosinase activity

The tyrosinase activity of different fractions (n-hexane, ethyl acetate, n-butanol and water) of methanol extract of *C. siliqua* and purified compounds were determined as described in chapter 3 (section 3.1.2.2). The pure compounds were tested at the final

concentrations 1.5 to 200 µg/ml. The effect of separated fractions and purified compounds on cell viability/proliferation and melanin production was examined as described in chapter 3 (section 3.1.2.3). The samples were tested at the final concentration ranging from 1.5 to100 µg/ml.

4.2.2.6. Statistical analysis

The final results are expressed as the mean (standard deviation, ±SE.S). The group means were compared using the ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple Range Test was applied to compare the means. Values were determined to be significant when *p* was less than 0.01 ($p<0.01$).

4.3. Results

4.3.1. Fractions of solvent-solvent separation

Four fractions namely; n-hexane, ethyl acetate, n-butanol and water were obtained from the methanol extract of *C. siliqua*. The ethyl acetate fraction showed the highest activity, therefore was selected for further purification and isolation (sections 4.3.5.1 & 4.3.6.1).

4.3.2. Purification of active compounds from ethyl acetate fraction of *C. siliqua* using 'column chromatography'

One pure compound was isolated from column (IV) namely; ‘myricetin-3-O-α-L-rhamnoside’ (yellow powder, yield: 0.0022%).

4.3.3. Purification of active compounds from ethyl acetate fraction of *C. siliqua* using 'paper partition chromatography'

Two pure compounds were isolated from paper partitionation method namely; ‘quercetin-3-O-α-L-rhamnoside’ (greenish powder, yield: 0.003%) and ‘1,2,3,6-tetra-O-galloyl-ß-D-

glucose' (red powder, yield: 0.003%). The structure of compounds were identified by comparison of their ^{1}H-NMR and ^{13}C-NMR spectral data with published articles (Papagiannopoulos *et al*. 2004, Owen *et al*. 2003). The NMR spectral of compounds are shown in appendix A (Page 126-131).

4.3.4. Purified compounds from the methanol extract of *C. siliqua*

NMR spectra indicated that 'myricetin-3-O-α-L-rhamnoside' and 'quercetin-3-O-α-L-rhamnoside' belong to 'flavonol glycosides' group of compounds while '1,2,3,6-tetra-O-galloyl-ß-D-glucose' belongs to 'gallotannins' (Fig. 4.6).

4.3.5. Anti-tyrosinase activity

4.3.5.1. Anti-tyrosinase activity of various fractions from *C. siliqua*

The tyrosinase inhibitory activity of fractions (n-hexane, ethyl acetate, n-butanol and water) was measured using L-tyrosine as substrate. Ethyl acetate fraction showed 50% inhibition of tyrosinase activity at 200 µg/ml. Water and n-butanol fractions exhibited almost similar tyrosinase inhibitory activity. Figure 4.7 shows the percent inhibition of tyrosinase activity by the fractions of *C. siliqua*.

4.3.5.2. Anti-tyrosinase activity of pure compounds isolated from *C. siliqua* using L-tyrosine as substrate

Quercetin-3-O-α-L-rhamnoside did not show any significant anti-tyrosinase activity at the concentrations tested. Myricetin-3-O-α-L-rhamnoside exhibited significant reduction ($p<0.01$) of tyrosinase activity at 200 µg/ml. Also a significant ($p<0.01$) tyrosinase inhibitory activity (90%) was observed by 1,2,3,6-tetra-O-galloyl-ß-D-glucose at 200 µg/ml (Fig 4.8). Kojic acid showed a complete inhibition of tyrosinase activity at all the concentrations tested ($p<0.01$ at 3.1 µg/ml) while arbutin exhibited 35 % anti-tyrosinase activity at 200 µg/ml.

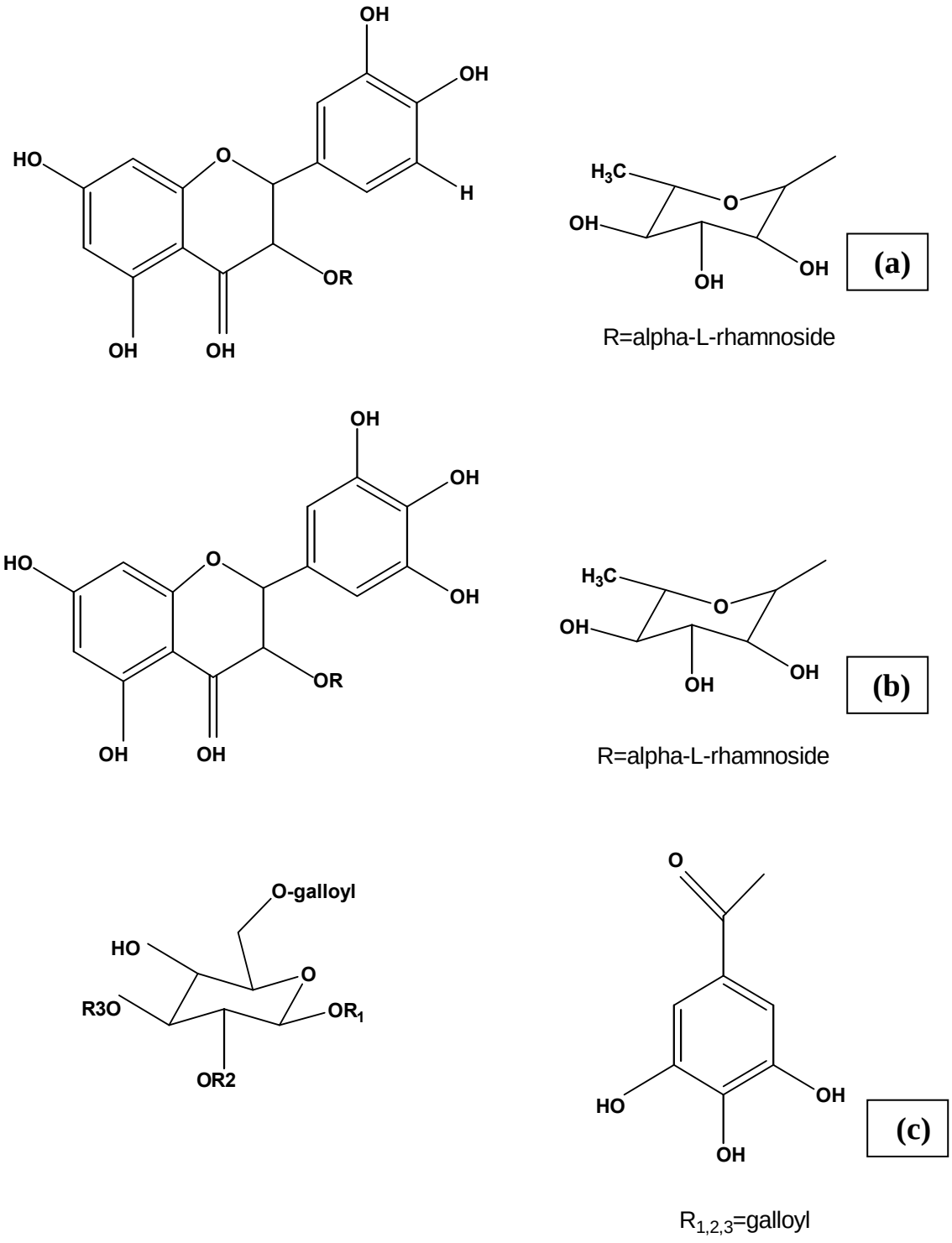


Figure 4.6. Isolated compounds from the methanol extract of *Ceratonia siliqua*; (a) quercetin-3-O-α-L-rhamnoside, (b) myricetin-3-O-α-L-rhamnoside and (c) 1,2,3,6-tetra-O-galloyl-ß-D-glucose

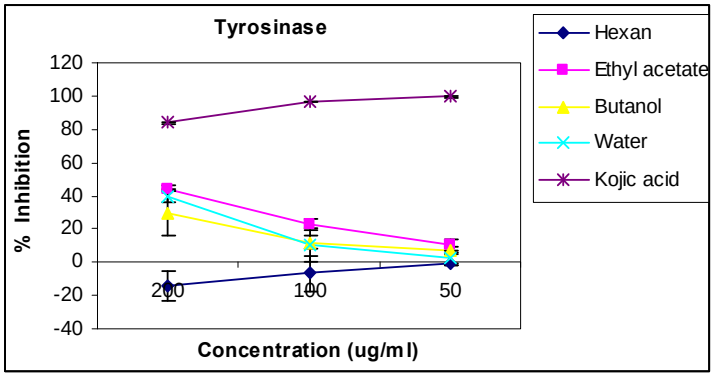


Figure 4.7. Tyrosinase inhibitory activity by n-hexane, ethyl acetate, n-butanol and water fractions of *Ceratonia siliqua* and kojic acid (positive control), using L-tyrosine as a substrate

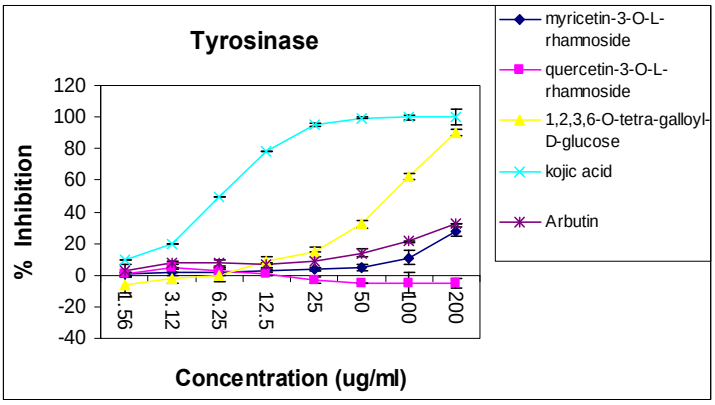


Figure 4.8. Dose-dependent inhibition of mushroom tyrosinase by 'myricetin-3-O-α-L-rhamnoside', 'quercetin-3-O-α-L-rhamnoside' and '1,2,3,6-tetra-O-galloyl-ß-D-glucose' isolated from *Ceratonia siliqua*, Arbutin and Kojic acid (positive controls), using L-tyrosine as a substrate

4.3.5.3. Anti-tyrosinase activity of pure compounds isolated from *C. siliqua* using L-DOPA as substrate

Quercetin-3-O-α-L-rhamnoside showed significant ($p<0.01$) tyrosinase inhibitory activity (50%) at 100 and 50 µg/ml. Myricetin-3-O-α-L-rhamnoside exhibited significant ($p<0.01$) reduction of tyrosinase activity (43%) at 200 µg/ml. 1,2,3,6-tetra-O-galloyl-ß-D-glucose showed 65% anti-tyrosinase activity at the highest concentration tested in this study ($p<0.01$)

(Fig. 4.9). Kojic acid showed 62% inhibition of tyrosinase activity at 50 µg/ml significantly ($p<0.01$) while arbutin showed 32 % anti-tyrosinase activity at 12.5 µg/ml using L-DOPA as substrate ($p<0.01$). The IC_{50} values of the tyrosinase inhibitory activity of isolated compounds from *C. siliqua* (methanol extract) are depicted in table 4.1.

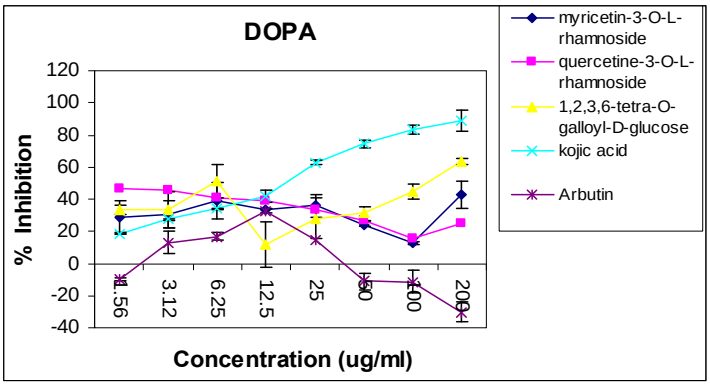


Figure 4.9. Dose-dependent inhibition of mushroom tyrosinase by 'myricetin-3-O-α-L-rhamnoside', 'quercetin-3-O-α-L-rhamnoside' and '1,2,3,6-tetra-O-galloyl-ß-D-glucose' isolated from *Ceratonia siliqua*, Arbutin and Kojic acid (positive controls), using L-DOPA as substrate

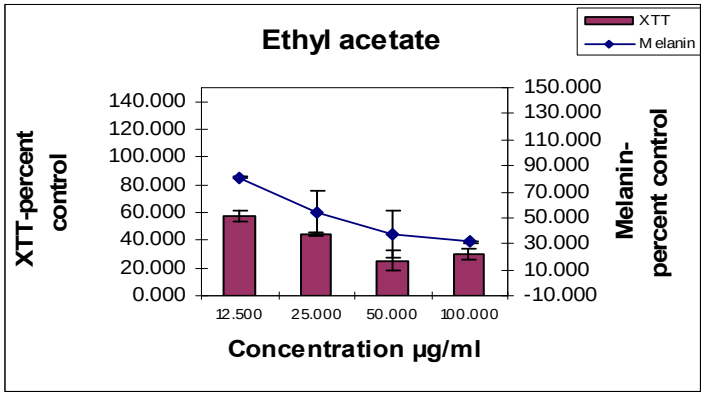
Table 4.1. IC_{50} values of pure compounds isolated from *Ceratonia siliqua* (methanol extract) for mushroom tyrosinase

Sample	IC_{50} monophenolase (µg/ml)	IC_{50} diphenolase (µg/ml)
Arbutin	>200	>200
Kojic acid	1.138±0.05	50.51±1.7
Myricetin-3-O-α-L-rhamnoside	>200	>200
Quercetin-3-O-α-L-rhamnoside	>200	>200
Tetra-O-galloyl-ß-D-glucose	83.3±2.0	129.3±2.4

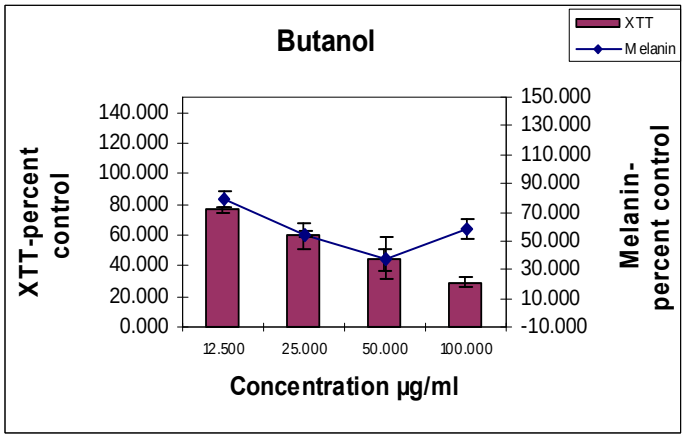
4.3.6. Inhibition of melanin production in melanocyte cells

4.3.6.1. Inhibition of melanin production in melanocyte cells by separated fractions from *C. siliqua*

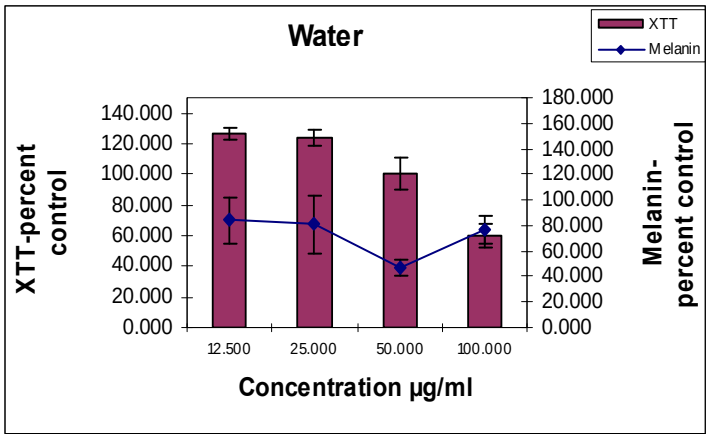
Ethyl acetate, n-butanol and water fractions of the methanol extract of *C. siliqua* exhibited potential anti-tyrosinase activity. Therefore, these fractions were selected to examine their ability on melanin production in an *in vitro* environment. Water fraction showed 50% inhibition of melanin production at 50 µg/ml without being toxic to the cells. Ethyl acetate and n-butanol fractions showed similar inhibitory activity and were toxic to the cells at the highest concentrations tested. The results are shown in figure 4.10.



(a)



(b)



(c)

Figure 4.10. The effect of (a) ethyl acetate, (b) n-butanol and (c) water fractions of *C. siliqua* on cell viability/proliferation and melanin production

4.3.6.2. Inhibition of melanin production in melanocyte cells by pure compounds isolated from *C. siliqua*

Quercetin-3-O-α-L-rhamnoside became toxic to the melanoma cells at the highest concentrations but this compound showed 28% inhibition of melanin production at 6.2 µg/ml significantly ($p<0.01$). 1,2,3,6-tetra-O-galloyl-ß-D-glucose decreased the cell viability/proliferation at all the concentrations examined in this study. Myricetin-3-O-α-L-rhamnoside exhibited a significant ($p<0.01$) reduction in melanin content (60%) in B16 melanocytes at 12.5 µg/ml without being toxic to the cells at this concentration. Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and exhibited similar reduction in melanin content at 3.1 and 25 µg/ml (60%). The results are shown in figures 4.11-4.14.

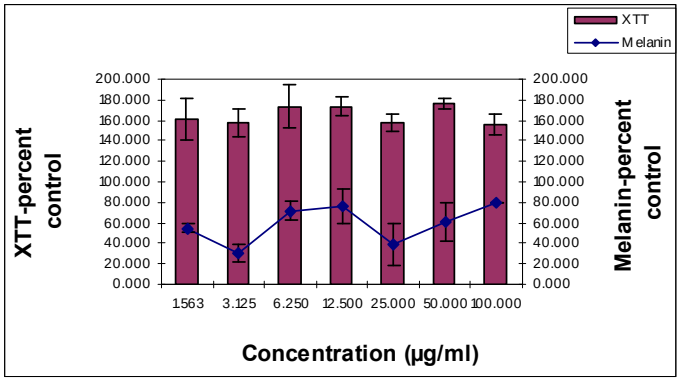


Figure 4.11. The effect of Kojic acid (positive control) on cell viability/proliferation and melanin production

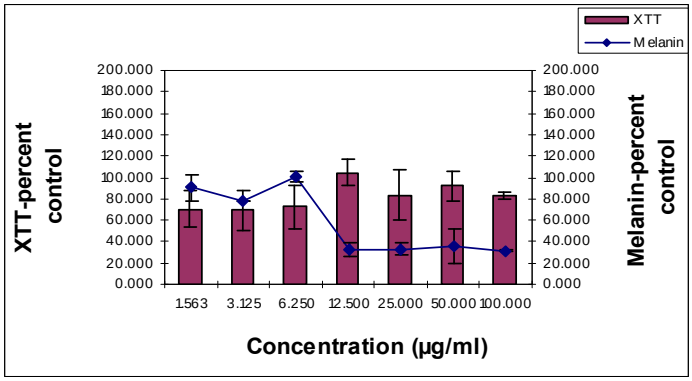


Figure 4.12. The effect of 'myricetin-3-O-α-L-rhamnoside' on cell viability/proliferation and melanin production

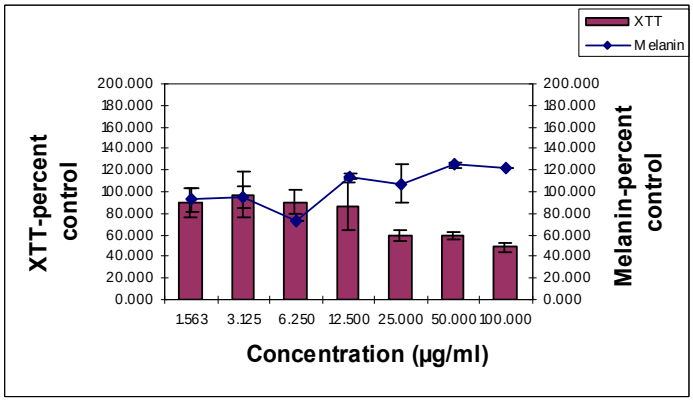


Figure 4.13. The effect of 'quercetin-3-O-α-L-rhamnoside' on cell viability/proliferation and melanin production

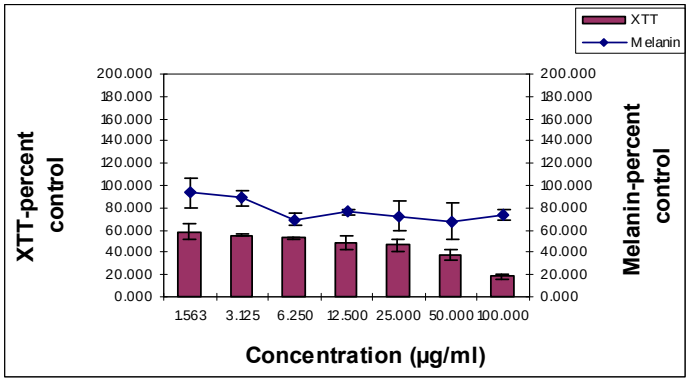


Figure 4.14. The effect of '1,2,3,6-tetra-O-galloyl-ß-D-glucose' on cell viability/proliferation and melanin production

4.4. Discussion and conclusions

Ethyl acetate and water fractions of *C. siliqua* showed anti-tyrosinase activity at three concentrations 50, 100 and 200 µg/ml. Ethyl acetate fraction of *C. siliqua* showed the highest anti-tyrosinase activity but in melanocyte cells non of the fractions showed inhibition of melanin production except the water fraction of *C. siliqua*, which showed 50% reduction of melanin production at 50 µg/ml. The ethyl acetate fraction of *C. siliqua* was selected for isolation and purification of compounds.

Quercetin-3-*O*-α-L-rhamnoside, myricetin-3-*O*-α-L-rhamnoside and 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose have been isolated from the fruits of *C. siliqua* previously (Papagiannopoulos *et al*. 2004, Owen *et al*. 2003). Quercetin-3-*O*-α-L-rhamnoside did not show any anti-tyrosinase activity when L-tyrosine was used as a substrate. This compound demonstrated 50% inhibition of DOPA auto-oxidation at 100 and 50 µg/ml. Myricetin-3-*O*-α-L-rhamnoside exhibited 20% and 43% ($p<0.01$) inhibition of monophenolase and diphenolase activities at 200 µg/ml respectively. A significant ($p<0.01$) tyrosinase inhibitory activity (90%) was observed by 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose at 200 µg/ml using L-tyrosine. This compound exhibited 65% anti-DOPA activity at the highest concentration tested ($p<0.01$). Kojic acid demonstrated a complete inhibition of monophenolase activity at all the concentrations tested ($p<0.01$ at 3.1 µg/ml). Kojic acid showed 62% inhibition of diphenolase activity at 50 µg/ml significantly ($p<0.01$). Arbutin exhibited 35 % anti-

tyrosinase activity at 200 µg/ml and showed 32 % anti-DOPA activity at 12.5 µg/ml ($p<0.01$).

A number of natural products have been reported by other researchers for their anti-tyrosinase activity. Kim *et al*. (2006) reported that mulberroside A and oxyresveratol isolated from the ethanolic extract of *Morus alba* exhibited more than 80% inhibition of mushroom tyrosinase activity at 60 µM. Butin and liquiritigenin isolated from *Spathololobus suberectus* (95% ethanol extract) showed 80% and 36% inhibition of monophenolase activity at 100 µM respectively (Lee *et al*. 2006). It also has been documented that (-)-epigallocatechin-3-O-gallate (EGCG), (-)-epigallocatechin (EGC) and caffeine (CAF) isolated from green tea (methanol extract) showed 55%, 40% and less than 5% anti-tyrosinase activity at 40 µM (No *et al*. 1999). Among the *C. siliqua* constituents, 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose exhibited stronger activity than myricetin-3-*O*-α-L-rhamnoside or quercetin-3-*O*-α-L-rhamnoside of L-tyrosinase.

Myricetin-3-O-α-L-rhamnoside exhibited a significant reduction in melanin content (60%) in B16 melanocytes at 12.5 µg/ml and it was not toxic to the cells at this concentration. Quercetin-3-O-α-L-rhamnoside was found to be toxic to the melanoma cells at the highest concentrations but the compound showed 28% inhibition of melanin production at 6.2 µg/ml significantly ($p<0.01$). Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and exhibited 60% reduction in melanin content at 3.1 µg/ml. Purified compounds from plant extracts have been reported earlier for anti-melanogenesis activity. Lee *et al* (2006) stated that 'butin' isolated from *Spathololobus suberectus* (95% ethanol extract) exhibited 29.8% decrease of melanin production at 100 µM (cell viability > 90%). According to him, arbutin showed 7% anti-melanogenesis activity at 2.5 mM (608.7 µg/ml). Glabridin, glabrene and isoliquiritigenin isolated from licorice extract demonstrated 55%, 27% and 13 % inhibition of melanin production at 10 µM respectively (Nerya *et al*. 2003).

Quercetin-3-*O*-α-L-rhamnoside, myricetin-3-*O*-α-L-rhamnoside and 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose have been isolated from *C. siliqua* before. There is no report till date on the ability of quercetin-3-*O*-α-L-rhamnoside for the inhibition of tyrosinase activity or melanin formation in mouse melanocyte cells.

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Chapter 5

Isolation and purification of multifunctional actives from *Sideroxylon inerme*

5.1. Introduction

Sideroxylon inerme is being used traditionally by people in South Africa for the treatment of hyperpigmentation. As mentioned earlier in chapter 1 (section 1.9.9) a decoction of this plant is used for its lightener property. No medicinal research has been conducted on *S. inerme* before. People have focused on the conservation and secure vegetation of this plant due to its over exploitation (www.york.ac.uk/projects). In this chapter, the isolation and purification of bioactive compounds from this plant, their chemical structure and inhibitory activity of tyrosinase and melanin production described.

5.2. Material and methods

5.2.1. Materials

Methanol extract of the leaves of *S. inerme* was prepared as mentioned in chapter 3 (section 3.1.2.1).

5.2.2. Methods

In order to isolate the compound from the crude methanol extract of *S. inerme*, a solvent-solvent separation method was employed to simplify the extract as described in section 4.2.2.1. The bioactive anti-tyrosinase fraction was then subjected to a series of Sephadex columns using different eluents (Fig. 5.1).

The 'Nuclear magnetic resonance spectroscopy' (^{1}H-NMR and ^{13}C-NMR) have been employed to elucidate and identify the structure of purified compounds.

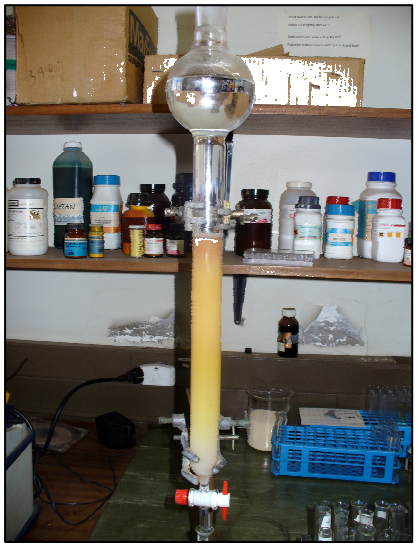


Figure 5.1. Chromatographic fractionation of ethyl acetate fraction of *Sideroxylon inerme* using Sephadex chromatography

5.2.2.1. Solvent-Solvent separation

Dried methanol extract of *S. inerme* (105 g) was fractionated using solvent-solvent separation method as explained earlier in chapter 4 (section 4.2.2.1).

5.2.2.2. Thin layer chromatography (TLC)

Four fractions (n-hexane, ethyl acetate, n-butanol and water) were spotted on thin layer chromatography (TLC) plate, developed using ethyl acetate: ethyl acetate/ formic acid/ acetic acid/ distilled water; (13.5:10:1:1:1.5) as an eluent system (Fig. 5.2). The plate was sprayed with 0.34% vanillin in 3.5% sulphuric acid in methanol.

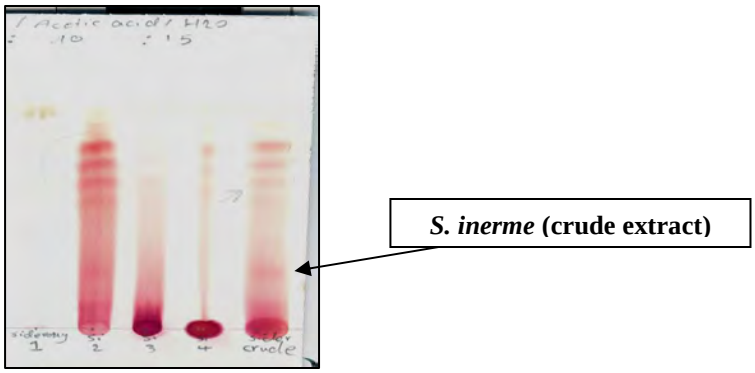


Figure 5.2. Thin layer chromatogram of the solvent-solvent separation of the methanol extract of *Sideroxylon inerme*, (1) n-hexane, (2) ethyl acetate, (3) n-butanol and (4) water fractions

5.2.2.3. Isolation of active compounds from ethyl acetate fraction of *S. inerme*

The ethyl acetate fraction of *S. inerme*, which showed the highest anti-tyrosinase activity (section, 5.3.4.1) as compared to n-hexane, n-butanol and water fractions was dried and quantified (30.05 g). This fraction was applied to a Sephadex column chromatography and eluted with 100% distilled water followed by 25%, 50%, 75% and 100% of ethanol and later 100% ethyl acetate. Forty-four fractions (500 ml each) were collected and concentrated. A series of sephadex columns were prepared and developed by 100% ethanol. The fractions were spotted on TLC plates using ethyl acetate: ethyl acetate/ formic acid/ acetic acid/ distilled water; 13.5:10:1:1:1.5 were used as eluents.

5.2.2.4. Determining anti-tyrosinase activity

The tyrosinase activity of different fractions (n-hexane, ethyl acetate, n-butanol and water) of the methanol extract of *S. inerme* and purified compounds were determined as described in chapter 3 (section 3.1.2.2). The pure compounds were tested at the final concentrations; 1.5-200 µg/ml. The effect of separated fractions and purified compounds on cell viability/proliferation and melanin production was examined as described in chapter 3 (section 3.1.2.3).

5.2.2.5. Statistical analysis

The final results are expressed as the mean (standard deviation, ±SE.S). The group means were compared using the ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple Range Test was applied to compare the means. Values were determined to be significant when *p* was less than 0.01 ($p<0.01$).

5.3. Results

5.3.1. Fractions of solvent-solvent separation

Four fractions namely; n-hexane, ethyl acetate, n-butanol and water were obtained from the methanol extract of *S. inerme*. The ethyl acetate fraction showed the highest activity, therefore was selected for further purification and isolation (sections 5.3.4.1 & 5.3.5.1).

5.3.2. Purification of active compounds from ethyl acetate fraction of *S. inerme*

Two pure compounds were isolated namely; 'epicatechin gallate' from column II (orangish powder, yield: 0.035%) and 'procyanidin B2' from column V (orangish powder, yield: 0.084%). Figure 5.3 shows the pure compounds on TLC plates.

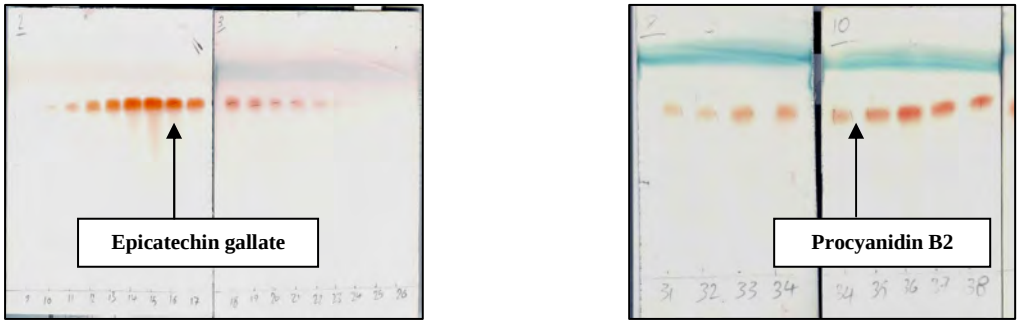
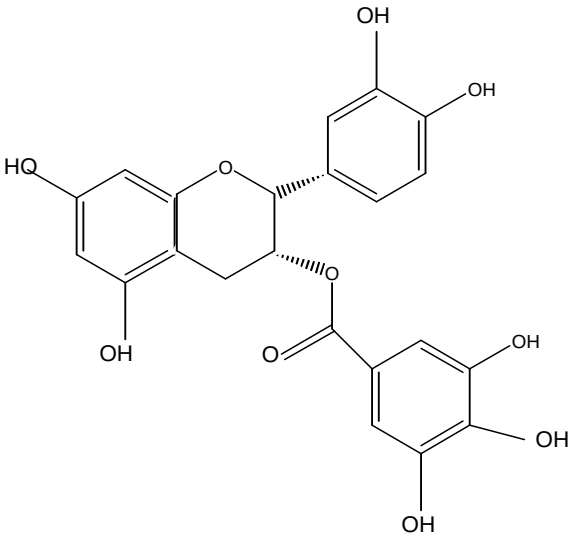


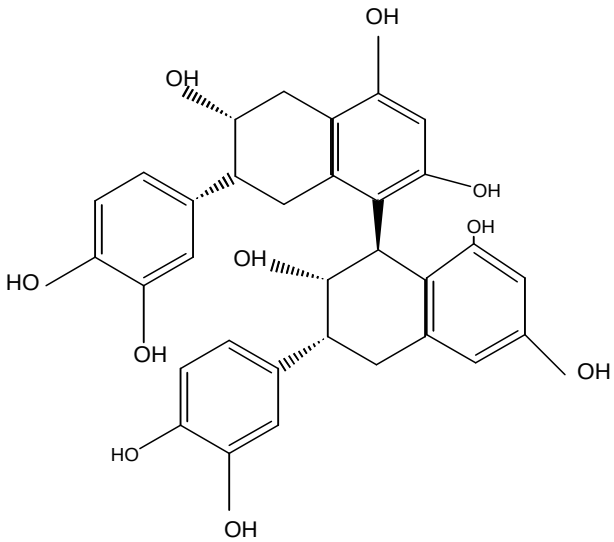
Figure 5.3. Thin layer chromatogram of pure compounds epicatechin gallate and procyanidin B2, developed in (ethyl acetate: ethyl acetate/ formic acid/ acetic acid/ distilled water; 13.5:10:1:1:1.5)

5.3.3. Purified compounds from the methanol extract of *S. inerme*

The chemical structure of compounds was elucidated using their ^{1}H-NMR and ^{13}C-NMR spectral data. The compounds belong to 'flavonoids' and have not been isolated from the bark of S. *inerme* before. Figure 5.4 shows the chemical structure of epicatechin gallate and procyanidin B2. The ^{13}C-NMR and ^{1}H-NMR spectrums of these compounds are shown in appendix B (Page 133-136).



(a)



(b)

Figure 5.4. Isolated compounds from the methanol extract of *Sideroxylon inerme*; (a) epicatechin gallate and (b) procyanidin B2

5.3.4. Anti-tyrosinase activity

5.3.4.1. Anti-tyrosinase activity of various fractions from *S. inerme*

Inhibition of tyrosinase activity by n-hexane, ethyl acetate, n-butanol and water fractions of the methanol extract of *S. inerme* was measured using L-tyrosine. Ethyl acetate fraction exhibited 75% tyrosinase inhibitory activity at 200 µg/ml. It showed the highest anti-tyrosinase activity as compared to n-butanol, n-hexane and water fractions.

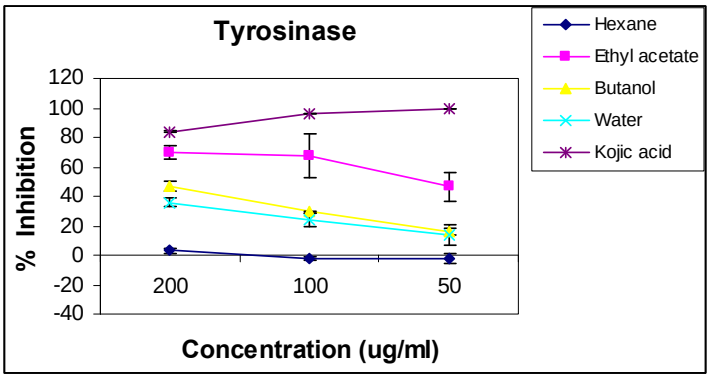


Figure 5.5. Tyrosinase inhibitory activity by n-hexane, ethyl acetate, n-butanol and water fractions of *Sideroxylon inerme*, Kojic acid (positive control), using L-tyrosine as a substrate

5.3.4.2. Anti-tyrosinase activity of pure compounds isolated from *S. inerme* using L-tyrosine as substrate

Epicatechin gallate exhibited 58% anti-tyrosinase activity at 200 µg/ml significantly ($p<0.01$) whereas procyanidin B2 showed 55% reduction of tyrosinase activity at the same concentration ($p<0.01$) (Fig. 5.6). Kojic acid showed a complete inhibition of tyrosinase activity at all the concentrations tested ($p<0.01$ at 3.1 µg/ml) while arbutin exhibited 35 % anti-tyrosinase activity at 200 µg/ml.

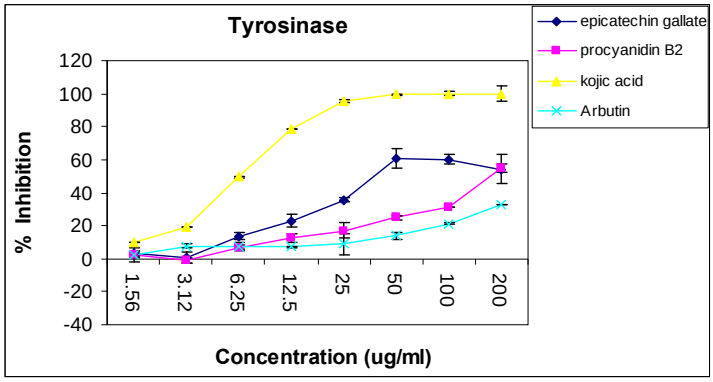


Figure 5.6. Dose-dependent inhibition of mushroom tyrosinase by 'epicatechin gallate' and 'procyanidin B2' isolated from *Sideroxylon inerme*, Arbutin and Kojic acid (positive controls), using L-tyrosine as a substrate

5.3.4.3. Anti-tyrosinase activity of pure compounds isolated from *S. inerme* using L-DOPA as substrate

No significant ($p<0.01$) inhibition of tyrosinase activity by epicatechin gallate and procyanidin B2 was observed when L-DOPA was used as substrate at any concentration tested in this experiment (Fig. 5.7). Kojic acid showed 62% inhibition of tyrosinase activity at 50 µg/ml significantly ($p<0.01$) while arbutin showed 32 % anti-tyrosinase activity at 12.5 µg/ml using L-DOPA as substrate ($p<0.01$). Table 5.1 depicts the IC_{50} values of isolated compounds from methanolic extract of *Sideroxylon inerme* and positive controls (arbutin and kojic acid).

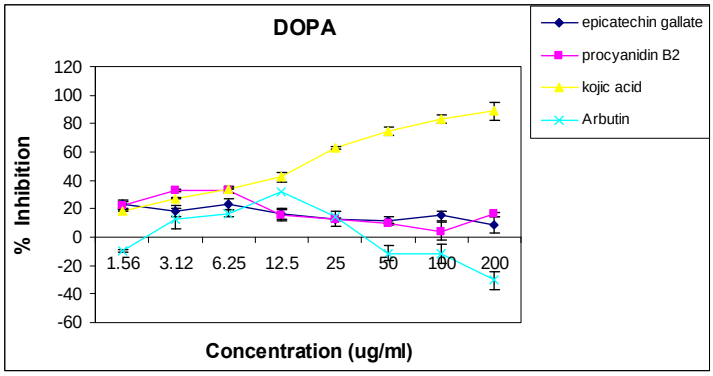


Figure 5.7. Dose-dependent inhibition of mushroom tyrosinase by 'epicatechin gallate' and 'procyanidin B2' isolated from *Sideroxylon inerme*, Arbutin and Kojic acid (positive controls), using L-DOPA as a substrate

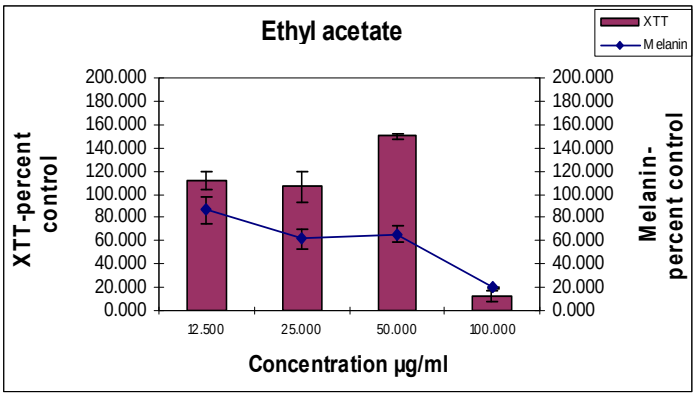
Table 5.1. IC_{50} values of pure compounds isolated from *Sideroxylon inerme* for mushroom tyrosinase

Sample	IC_{50} monophenolase (µg/ml)	IC_{50} diphenolase (µg/ml)
Arbutin	**>200**	**>200**
Kojic acid	**1.138±0.05**	**50.51±1.7**
Epicatechin gallate	**30**	**>200**
Procyanidin B2	**200**	**>200**

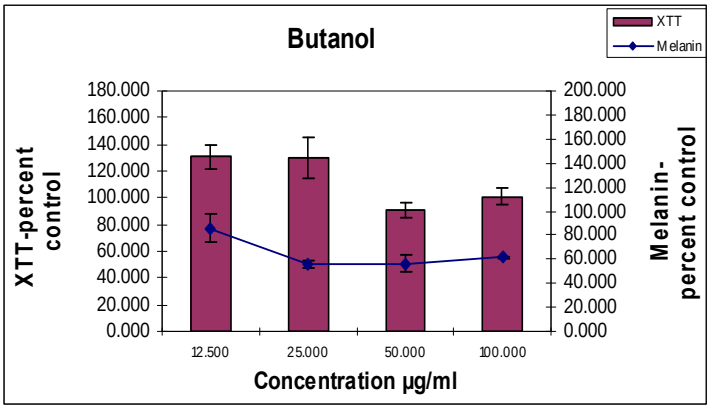
5.3.5. Inhibition of melanin production in melanocyte cells

5.3.5.1. Inhibition of melanin production in melanocyte cells by separated fractions from *S. inerme*

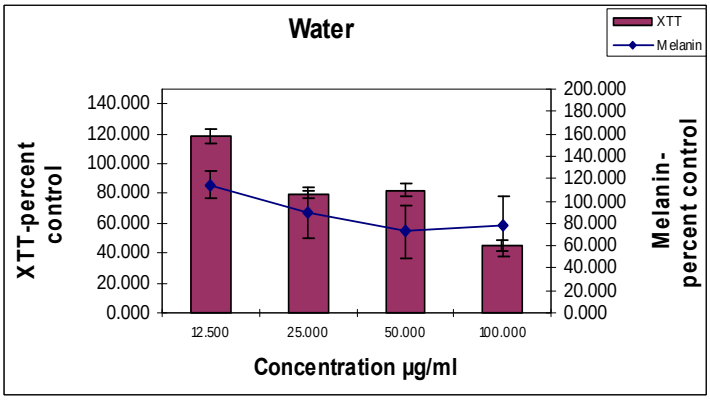
Ethyl acetate and butanol fractions of methanol extract of *S. inerme* showed 40% inhibition of melanin production in melanocytes at 25 and 50 µg/ml. Water fraction showed some toxicity to the cells at the highest concentrations tested (Fig. 5.8).



(a)



(b)



(c)

Figure 5.8. The effect of (a) ethyl acetate, (b) n-butanol and (c) water fractions of *Sideroxylon inerme* on cell viability/proliferation and melanin production

5.3.5.2. Inhibition of melanin production in melanocyte cells by pure compounds isolated from *S. inerme*

Figures 5.9 and 5.10 show the effect of 'epicatechin gallate' and Kojic acid on cell viability/proliferation and melanin production in B16F10 cells respectively. Epicatechin gallate did not show any significant ($p<0.01$) inhibition of melanogenesis at the concentrations tested in this study. Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and exhibited similar reduction in melanin content at 3.1 and 25 µg/ml (60%).

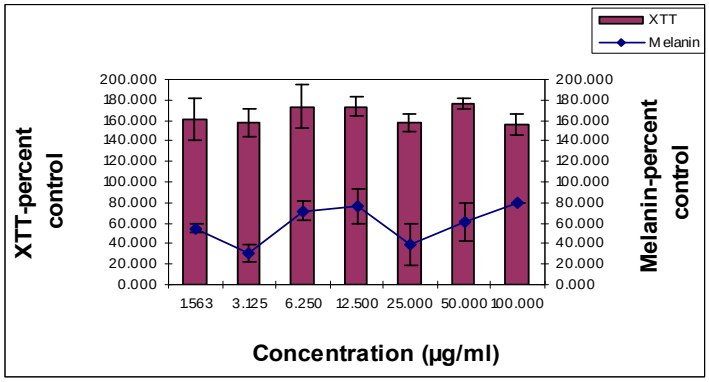


Figure 5.9. The effect of Kojic acid (positive control) on cell viability/proliferation and melanin production

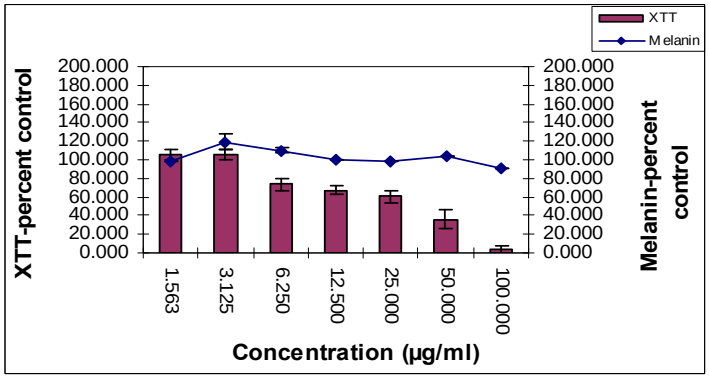


Figure 5.10. The effect of 'epicatechin gallate' on cell viability/proliferation and melanin production

5.4. Discussion and conclusions

Ethyl acetate fraction of *S. inerme* showed the highest anti-tyrosinase activity at the concentrations tested in this study rather than water and n-butanol fractions. This fraction showed 40% inhibition of melanin production in melanocytes at 25 and 50 µg/ml. Based on the results obtained from tyrosinase assay, the ethyl acetate fraction of *S. inerme* was selected for isolation and purification of compounds.

Among the *S. inerme* constitutes, epicatechin gallate exhibited 58% inhibition of monophenolase activity at 200 µg/ml whilst procyanidin B2 showed 55% anti-tyrosinase activity at the same concentration ($p<0.01$). These compounds did not

show any inhibition of DOPA auto-oxidation at the concentrations tested in this study. Kojic acid demonstrated a complete inhibition of monophenolase activity at all the concentrations tested ($p<0.01$ at 3.1 µg/ml). Kojic acid showed 62% inhibition of diphenolase activity at 50 µg/ml significantly ($p<0.01$). Arbutin exhibited 35 % anti-tyrosinase activity at 200 µg/ml and showed 32 % anti-DOPA activity at 12.5 µg/ml ($p<0.01$). Plant extracts and their bioactive constituents have been explored frequently for tyrosinase inhibitory activity before. According to Hara *et al*. (1997), epicatechin gallate, epigallocatechin and epicatechin isolated from tea leaves exhibited 95%, 17% and 11% inhibition of monophenolase activity at 0.15 mg/ml (150 µg/ml). No *et al*. (1999) reported, - (-)epicatechin (EC), (-)-epigallocatechin (EGC) and (+)-catechin purified from the methanolic extract of green tea showed < 10%, 40% and < 10% inhibitory effects of mushroom tyrosinase at 40 µM respectively. He also demonstrated gallocatechingallate (GCG) (isolated from same extract) exhibited 40% anti-DOPA activity at 50 µM. According to Yamakoshi *et al* (2003), little is known of the lightening effect of proanthocyanidin on UV-induced pigmentation of the skin. He also reported, grape seed extract (rich of proanthocyanidin) inhibited the activity of mushroom tyrosinase and inhibited melanogenesis without inhibiting the growth of cultured B16 mouse melanoma cells. 'Quebracho' (quebracho compound similar to proanthocyanidins) isolated from the heartwood of *Schinopsis lorentzii* (70% aqueous acetone) showed 47% tyrosinase inhibition (Takagi & Mitsunaga, 2003). As compared with positive controls (arbutin and kojic acid), epicatechin gallate isolated from the stem-bark of *S. inerme* exhibited more anti-tyrosinase activity than arbutin but less than kojic acid. This compound also showed stronger tyrosinase inhibitory activity than procyanidin B2.

No significant ($p<0.01$) inhibition of melanin content was observed by epicatechin gallate at the concentrations tested in this study and the sample exhibited some toxicity on cells viability at tested concentrations. Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and exhibited 60% reduction in melanin content at 3.1 µg/ml.

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Chapter 6

Antioxidant activity of *Cassipourea malosana, Ceratonia siliqua* and *Sideroxylon inerme*

6.1. Introduction

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Phytomedicene Practical Guide, 2006). Oxidative stress, an imbalance of reactive radicals and antioxidants, is associated with numerous human disorders, including: lipid peroxidation and secretion of inflammatory cytokines, ischemia/reperfusion syndrome, multiple sclerosis, senile dementia, autoimmune disorders, Parkinson's diseases, Alzheimer, aging, cancer and coronary heart diseases (Briganti & Picardo, 2003; Phuwapraisirisan *et al*. 2006; Khan *et al*. 2003; Yang *et al*. 2001; Ng *et al*. 2000) . Free radicals can react with biochemicals such as nucleic acids, proteins and cell membranes to cause structural damages and resulting malfunction of vital molecules. To protect from damaging effect of free radicals, all aerobic organisms possess a natural antioxidant defense system (Khan *et al*. 2003).

Oxygen is present in the atmosphere as a stable triplet bi-radical ($^3O_2$), in the ground state. Once inside the human body it can be transformed by a four-electron reduction process to water, producing a superoxide radical (O_2^-), a hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2) as the reactive intermediates. Single oxygen ($^1O_2$) is formed from the excited state of various sensitizers (such as chlorophyll, acridine and other pigments) which primarily targets and damages cellular and extra cellular components, proteins, enzymes, lipids, DNA and RNA. It can also oxidize unsaturated fatty acid components of cell membranes (Ramarathnam *et al*. 1995).

Free radicals are described as unstable molecules with unpaired electrons that float freely through the body, seeking out completely healthy cells from with they can 'steal' electrons to rebalance themselves. This can cause a chain reaction in body cells and if left unchecked, can cause cellular damage. Free radical molecules are formed naturally in the body, through normal metabolic processes like oxidation, or they can be generated by car fumes, smoking, alcohol, pesticides, solvents and even illness or infection. Antioxidant nutrients have the ability to scavenge free radicals in the system and neutralize them before they can damage the body cells.

Most plants have protective functions of naturally occurring antioxidants in the cells (Larson, 1988). Naturally occurring antioxidants in plant cells are:

- enzymatic and peptide defence mechanisms (catalases, peroxidises, superoxide dismutases, glutathione and other proteins),
- phenolic defence compounds (vitamin E, flavonoids, phenols and phenolic acids)
- nitrogen compounds (alkaloids, amino acids and amines),
- carotenoids, chlorophyll derivatives,
- vitamin C, ketones and;
- synergism between more than one type of compound (for example; phenolic compounds and proteins) (Larson, 1988).

Two basic categories of antioxidants are known; synthetic and natural. In general synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitutions. Natural antioxidants can be phenolic compounds, nitrogen compounds, carotenoids as well as ascorbic acid (Phytomedicine Practical Guide, 2006). Several studies have also suggested that balanced intake of dietary antioxidants may also help in prevention of diseases (Khan ***et al***. 2003).

The protective role of antioxidants has promoted investigators to search for compounds with potential antioxidant activity. Skin is one of the most important antioxidant defense systems in animal's body. Skin is constantly exposed to oxidative stress induced by reactive oxygen species (ROS), that are generated both from endogenous sources, such as enzyme activity or activated neutrophils and external

pro-oxidant stimuli, such as UV. To deal with the harmful effect of oxidative stress, mammalian skin is equipped with antioxidant defense mechanisms. The skin antioxidant system consists of a network of enzymatic and non-enzymatic antioxidants. Among enzymatic antioxidants; gluthatione peroxidase, catalase and superoxide dismutase play a pivotal role. Non-enzymatic antioxidants present in cells are α-tocopherol, ubiquinone, ß-carotene, ascorbate and glutathione. A number of skin diseases such as; Psoriasis, acne, irritant contact dermatitis and pigmentary system skin diseases are related to imbalance function of its antioxidant system (Briganti & Picardo, 2003).

It is well known that melanin plays an important role in protecting human skin from the harmful effects of UV radiations by absorbing UV sunlight and removing ROS and in scavenging toxic drugs and chemicals. Khan *et al*. (2006) demonstrated that three phenolic compounds isolated from *Salsola foetida* exhibit tyrosinase inhibition with moderate antioxidant activity. It has been investigated that most of the benefits of herbal, black, green and Rooibos tea, also fruits are attributed to their antioxidant content (Toit *et al*. 2001; Speisky *et al*. 2006) while a few researches have reported on their anti-tyrosinase activities (Tiedtke & Marks, 2002; Tiedtke *et al*. 2004; No *et al*. 1999). A study showed that 'resveratol' (known as antioxidant agent) as an original substrate for tyrosinase and can be used as an additive compound in whitening cosmetics, particularly with a *Morus alba* extract (Bernard & Berthon, 2000). Therefore, compounds with antioxidant properties may have anti-tyrosinase activity as well.

In this chapter, the antioxidant activities of methanol extract of *C. malosana, C. siliqua*, *S. inerme* and their purified compounds have been investigated using both quantitative and qualitative assays. The DPPH method has been performed to quantify the total radical scavenging capacity (RSC) of samples.

6.2. Material and methods

6.2.1. Materials

Plant materials were collected as described in chapter 3 (section 3.1.1.1).

6.2.2. Methods

6.2.2.1. Preparation of plant extracts

Methanolic extract of samples (methanol extract of *C. malosana*, *C. siliqua*, *S. inerme* and their isolated compounds) were selected to investigate their antioxidant potential. The extraction method is described in chapter 3 (section 3.2.1.1). Final concentrations of 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml were prepared for the extracts for 1,2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, South Africa) antioxidant assays. Pure compounds were tested at the concentration ranging from 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 µg/ml by re-dissolving the dried extracts in 80% methanol. 'Myricetin-3-O-α-L-rhamnoside' was not tested due to unavailability of enough quantity needed for this experiment. For each sample, a dilution series was prepared in a 96 well ELISA plate by adding distilled water as a dilution medium. All the samples were prepared in triplicate. 'Vitamin C' and 'Quercetin' were used as controls and were tested at the concentrations ranging from 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml. Their stock solutions were prepared using distilled water.

6.2.2.2. DPPH qualitative antioxidant assay

Methanolic extracts of *C. malosana*, *C. siliqua*, *S. inerme* and their purified compounds (80%) (10µl of stock solution) were applied to a Merck Silica gel F_{254} plate. The plate was developed with a solution containing ethyl acetate/formic acid/acetic acid/distilled water (10:1:1:1.5). Thereafter, the plate was sprayed with a 90 µM methanolic DPPH solution and was incubated for 20 min at room temperature to determine the presence of antioxidant compounds in the extracts.

6.2.2.3. DPPH quantitative antioxidant assay

This assay was performed using the method described by Toit ***et al***. (2001) with slight modifications. Distilled water (100 µl) was added as medium to the wells of 96 well ELISA plates. In plate (I), 10 µl of each extract (stock concentration 29 mg/ml) was added into the wells in triplicate. For each extract, 8 dilution series (doubling dilutions) were prepared separately. Vitamin C and quercetin were prepared in the same way as extracts and used as standard controls. Later, 90 µl (90 µM) of methanolic DPPH was added to each well. In plate (II) (reference plate), 10 µl of each extract was added in to the wells in triplicate. A doubling dilution series was prepared for each sample and 90 µl of distilled water was added in all wells. The plates were covered with aluminium foil and incubated at room temperature for 1 h before spectrophotometry.

6.2.2.4. Spectrophotometric assays

The radical scavenging capacities of the samples were determined by using a BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa) to measure the disappearance of DPPH at 550 nm. The radical scavenging activity was mesured in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Toit ***et al***. 2001). The EC_{50} value for each sample was determined graphically by plotting the absorbance of DPPH as a function of the sample concentration in µg/ml for the standard and samples. The EC_{50} is the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50%. The results are expressed as the mg vitamin C equivalents/g dry weight and are calculated as follows:

EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = × mg vitamin C equivalents/ g dry weight
Zero mg/ml was taken as 100%.

6.2.2.5. Statistical analysis

The final results are expressed as the mean (standard deviation, ±SE.S). The group means were compared using the ANOVA test (MSTATC software, East Lansing, MI,

USA) and the Duncan's Multiple Range Test was applied to compare the means. Values were determined to be significant when *p* was less than 0.01 ($p<0.01$).

6.3. Results

6.3.1. Qualitative antioxidant activity

The Merck silica gel TLC plate were sprayed with a DPPH solution in methanol, the regions where substances with antioxidant capacity occurred stained white to yellowish and the remainder of the plate stained purple. Figure 6.1 represents chromatogram of a TLC plate sprayed with DPPH and the antioxidant chromatogram of the plant extracts and their isolated compounds.

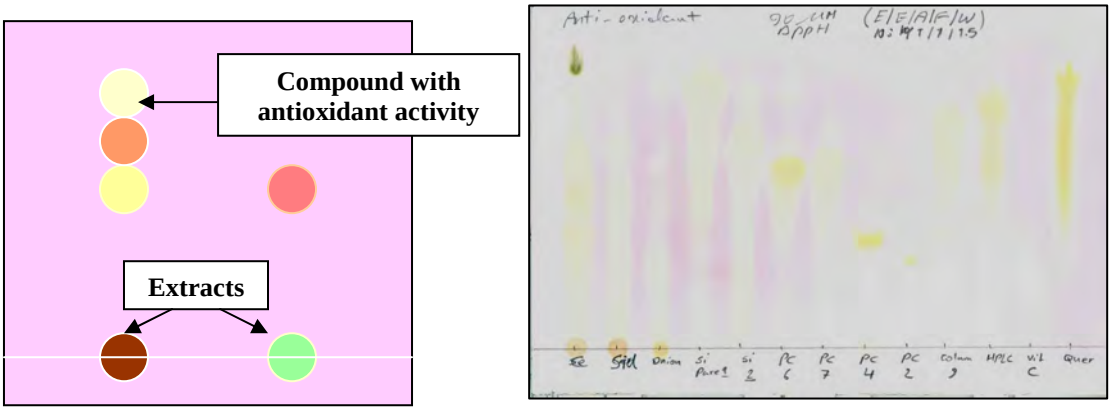


Figure 6.1. Qualitative antioxidant activity of plant extracts

6.3.2. Quantitative antioxidant activity

The intensities of extracts with DPPH were measured by a multiwell plate reader (Figure 6.2).

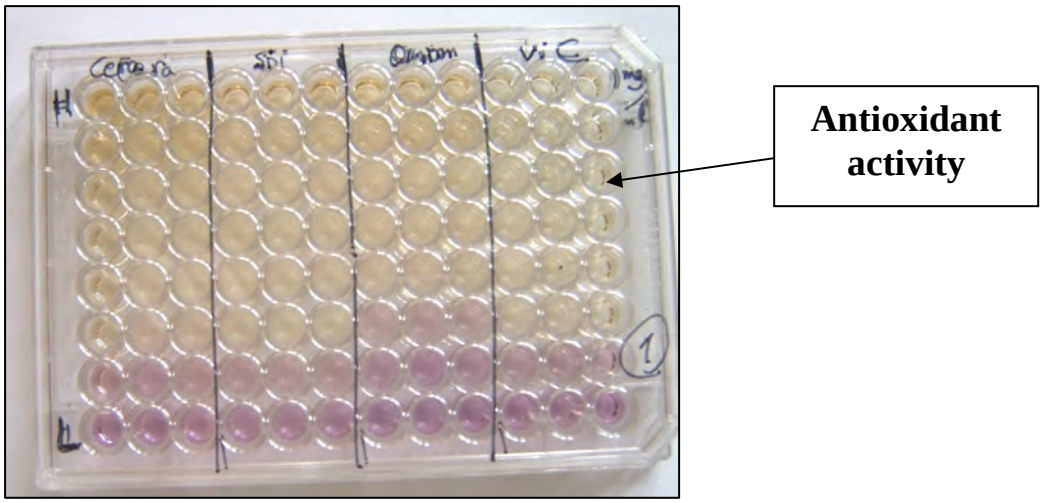
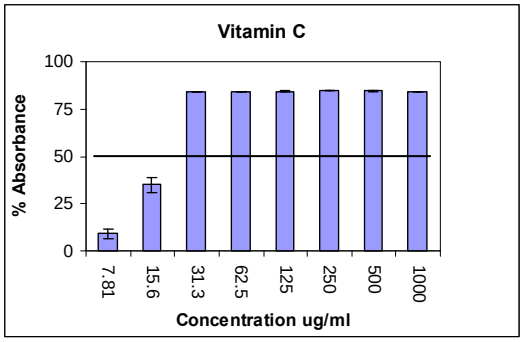
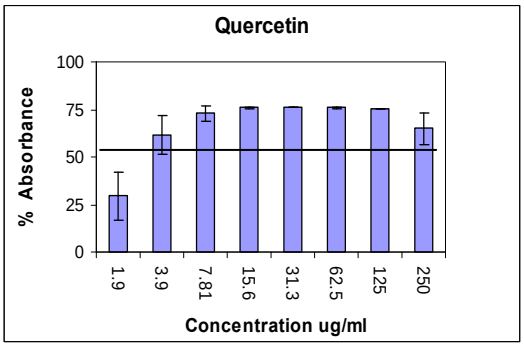


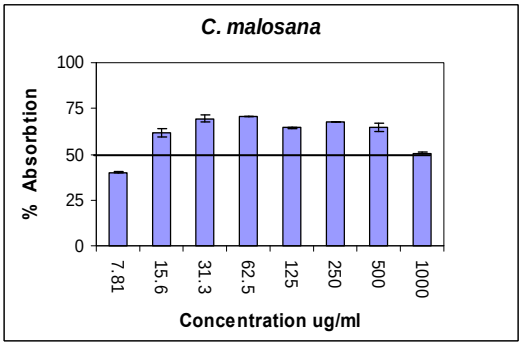
Figure 6.2. Antioxidant activity of *Cassipourea malosana, Ceratonia siliqua, Sideroxylon inerme* and vitamin C

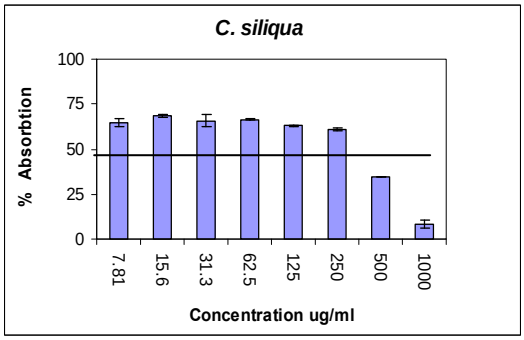
Colour formation with DPPH is indicative of a balance between antioxidants and free radicals. Yellow represents antioxidants in excess where the concentration of plant extracts is high (1000 µg/ml) and pink is indicative of free radicals in excess (shows the lower concentrations). Therefore, a concentration range is sought where the yellow colour just disappears or become translucent before pink appears. The colour reaction shows a gradual changing from yellow to pink and indicates that the optimum concentration range has been reached. Vitamin C (standard control) represented complete antioxidant activity at all the concentrations tested ($p<0.01$). Quercetin (standard control) demonstrated 61% radical scavenging capacity at 3.9 µg/ml significantly ($p<0.01$). *S. inerme* (methanol extract) showed almost 75% DPPH discolouration at all the concentrations tested in this experiment. *C. siliqua* (methanol extract) exhibited 65% DPPH scavenging activity at all the concentrations tested except at 500 µg/ml and 1000 µg/ml. Methanolic extract of *C. malosana* led to 70% DPPH discolouration at 62.5 µg/ml significantly ($p<0.01$). Methanol extract of *C. siliqua* showed the highest antioxidant activity which was followed by the purified compounds; 1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside. EC_{50} value of extract was found to be 19.33 µg/ml. 1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside exhibited an EC_{50} values of 42.55 µg/ml and 76.59 µg/ml respectively. Vitamin C and quercetin were used as standard controls, which showed EC_{50} values of 3.339 µg/ml and 51.06 µg/ml respectively. Epicatechin gallate and procianidin B2 exhibited DPPH discolouration of 72% and

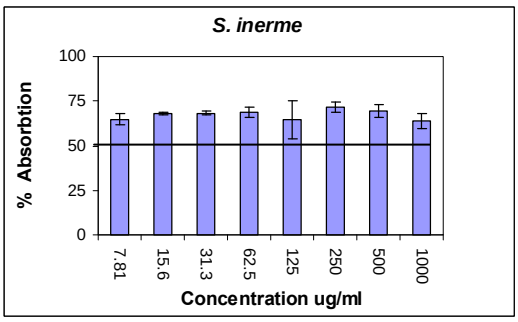
67% at 3.9 µg/ml respectively. Antioxidant activities of these two compounds were found to be higher than those observed for the standards (vitamin C and quercetin). These compounds are likely to be responsible for the high antioxidant activity of the extract of *S. inerme* (EC_{50}= 1.56 µg/ml). Dose-response antioxidant activity of vitamin C, quercetin, the extracts and their purified compounds are shown in figure 6.3.

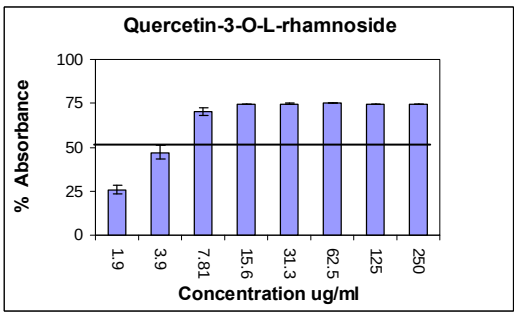












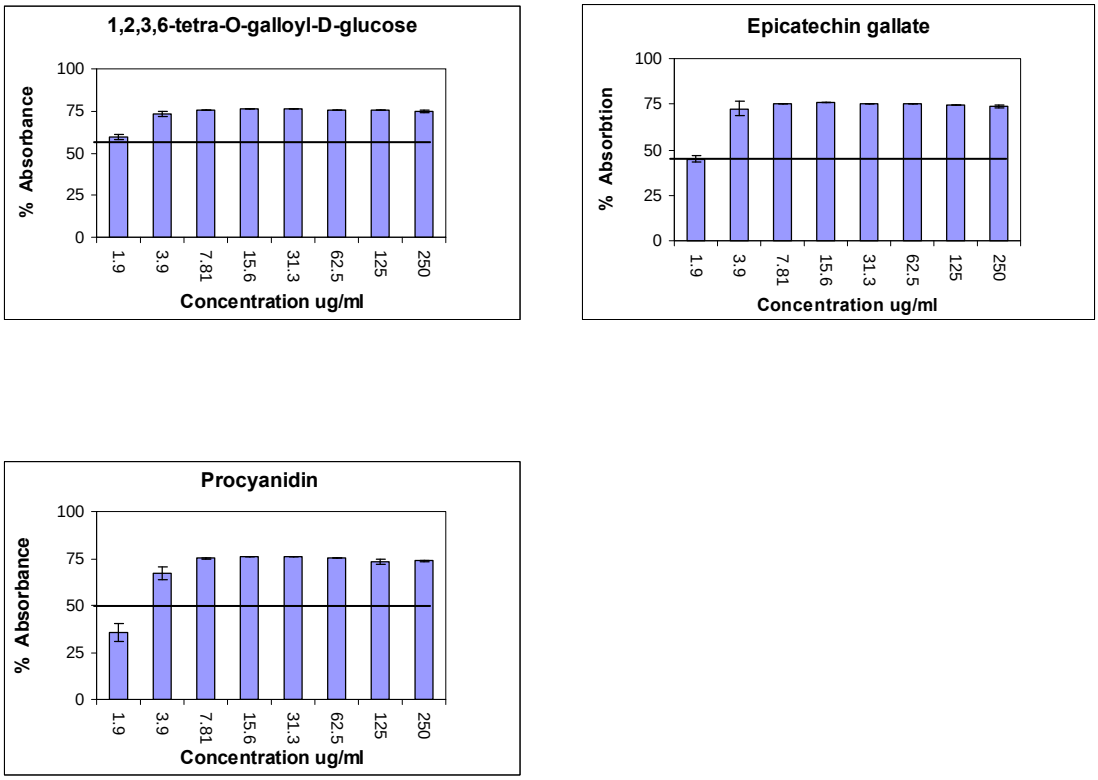


Figure 6.3. The percentage absorbance of the antioxidant activities of the methanol extracts of *Cassipourea malosana, Ceratonia siliqua, Sideroxylon inerme* and their purified compounds, vitamin C and quercetin (standard controls)

The expression of the antioxidant activity of plant extracts in mg vitamin C equivalent has the benefits that the antioxidant activity is quantified and different plant extracts are compared. The EC_{50} values (the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%) were calculated (EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = × mg vitamin C equivalents/g dry weight) and are listed in Table 6.1.

Table 6.1. EC_{50} values for the methanol extract of *Cassipourea malosana, Ceratonia siliqua, Sideroxylon inerme* and their isolated compounds (Vitamin C, EC_{50} = 3.339 µg/ml)

Extracts	EC_{50} (µg/ml)	mg vitamin C equivalents/g dry weight (EC_{50} value)
C. malosana	22.00	151.7
C. siliqua	19.33	172.7
S. inerme	1.54	2168.1
Epicatechin gallate	1.33	2510.6
Procyanidin B2	1.68	1987.6
Quercetin-3-O-α-L-rhamnoside	76.59	43.59
1,2,3,6-tetra-O-galloyl-ß-D-glucose	42.55	78.47
Quercetin	51.06	65.39

Standard deviation values (present as error bars on graphs) were all ≤ a 1.3% confidence interval.

6.4. Discussion and conclusions

The results showed that *S. inerme* exhibited the least EC_{50} value (1.54 µg/ml) as compared to *C. siliqua* (EC_{50}=19.33 µg/ml) and *C. malosana* (EC_{50}= 22.0 µg/ml). This demonstrates that the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%, is less when *S. inerme* is used. Compared to *C. malosana* where one gram of dry weight had a vitamin C equivalent of 151.7 mg, *C. siliqua* and *S. inerme* had a vitamin C equivalent of 172.7 and 2168.1 mg respectively. There are numerous reports on the radical scavenging capacity of plant extracts. According to Kumazawa *et al* (2002), water extract of carob pods (*C. siliqua*) exhibited 60% DPPH radical scavenging activity (125 µM) at 25 µg/ml. Mensor *et al* (2001) reported ethanolic extract of *Hyptis elegans* and *Vitex cyamosa* exhibited EC_{50} values of 89.91 µg/ml and 98.27 µg/ml of DPPH (ethanolic solution, 0.3 mM) discolouration respectively.

Vitamin C and quercetin were used as standard controls, which showed EC_{50} values of 3.339 µg/ml and 51.06 µg/ml respectively. Methanol extract of ***C. siliqua*** showed the highest antioxidant activity which was followed by the purified compounds; 1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside. EC_{50} value of extract was found to be 19.33 µg/ml. 1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside exhibited an EC_{50} values of 42.55 µg/ml and 76.59 µg/ml respectively. Our results differ from the one obtained by other scientists. Quercetin-3-O-rhamnoside isolated from the methanolic extract of ***Sanicula graveolens*** exhibited 29% and 25% DPPH discolouration at 100 µg/ml and 10 µg/ml respectively (Viturro ***et al***. 1999). Yan ***et al***. (2002) demonstrated that quercetin-3-rhamnoside and quercetin-3-arabinoside isolated from the methanolic extract of Cranberry fruit (***Vaccinium macrocarpon***) showed DPPH radical scavenging activities with EC_{50} values of 12 µg/ml and 15 µg/ml respectively. The difference in results could be due to different methods used. It has been reported that quercetin, quercetin-3-O-α-L-arabinofuranoside, quercetin-3-O-β-D-galactopyranoside, quercetin-3-O-β-D-galactopyranoside-7-O-β-D-glucopyranoside (1 µg/ml), isolated from the ethyl acetate fraction of the methanol extract of ***Hypericum hyssopifolium*** showed 70%, 80%, 60% and 55% DPPH (1mM/ml, methanolic solution) radical scavenging activity (Cakir ***et al***. 2003).

Epicatechin gallate and procyanidin B2 isolated from ***S. inerme*** showed the lowest EC_{50} values of 1.33 µg/ml and 1.68 µg/ml respectively. With these results, it can be concluded that epicatechin gallate and procyanidin B2 were found to have DPPH radical scavenging activities higher than those observed for the standards (vitamin C and quercetin). These compounds are likely to be responsible for the antioxidant activity of the extract of ***S. inerme***. Our results are in agreement with the reports of other researchers. Lu & Foo (2000) stated that EC_{50} values of - (-) epicatechin , epicatechin gallate and procyanidinB2 isolated from apple pomace for free radical scavenging activity (0.1 mM of methanolic solution of DPPH) were 0.135 µg/ml , 0.051 µg/ml and 0.012 µg/ml respectively. According to Kumazawa ***et al*** (2002), epicatechin gallate and procyanidin B2 isolated from the water extract of carob pods (***C. siliqua***) exhibited 80% and 70% DPPH (125 µM) discolouration at 5 µg/ml respectively. Aspalathin, caffeic acid, (+)-catechin and isoquercitrin isolated from

Rooibos tea exhibited 91%, 93%, 92% and 91% antioxidant activity (at either 0.25 or 0.5 mol of antioxidant/mol of DPPH) respectively (Gadow *et al* .1997).

Melanin is the root cause for blackening of the skin. Its formation beneath the skin proceeds through free radical mechanism. UV-radiations facilitate this chain reaction, and it could be disrupted by selective use of antioxidants, potent enough to poison this reaction. Therefore, *S. inerme* can be considered as either anti-tyrosinase or antioxidant agent. Based on our results, all the compounds showed strong antioxidant activities. The DPPH-scavenging activities of *S. inerme* (methanol extract) and its isolated compounds were 2 times better than those of the antioxidant vitamins C and quercetin. These results display that the *in vitro* radical scavengering potential of an extract is related to its chemical properties and reflects the *in vivo* activity (in this case tyrosinase inhibition). The DPPH scavenging capacity of *S. inerme* (methanol extract) and 1,2,3,6-tetra O-galloyl-ß-D-glucose has not been reported previously.

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Chapter 7

General conclusion and discussion

7.1. Motivation for this study

The lack of an efficient whitening agent which does not suffer from low activity (kojic acid), high cytotoxicity and mutagenicity (hydroquinone), poor skin penetration (arbutin) or low stability in formulation (glabridin) encouraged us for continuation of research on new anti-pigmentation/skin-lightening agents.

7.2. Tyrosinase inhibitory activity of active constituents isolated from *Ceratonia siliqua*

Ceratonia siliqua (methanol extract) is a well known species based on its polyphenol components and these compounds exhibited various valuable biological and medicinal properties. This point led us to examine the constituents present in its extract. *C. siliqua* exhibited a great inhibitory effect on tyrosinase activity as was expected based on its richness of polyphenols, known as tyrosinase inhibitors. Various chromatographic methods were used to isolate some of these polyphenol components. By using different assay methods, we have demonstrated that 1,2,3,6-tetra-O-galloyl-ß-D-glucose, myricetin-3-O-α-L-rhamnoside and quercetin-3-O-α-L-rhamnoside can inhibit the mono- and diphenolase activities of mushroom tyrosinase. Furthermore, myricetin-3-O-α-L-rhamnoside inhibited the biosynthesis of melanin in melanocytes. A review of the literature reveals that numerous depigmenting or skin-lightening agents are in use or under investigation. Some of these agents, such as hydroquinones, kojic acid and azelaic acid are well known to most dermatologists. Several other natural compounds, such as quercetin, myricetin and a glycoside of myricetin, have been reported to have varying degrees of inhibitory activity toward tyrosinase.

Structure-related activity studies of flavonoids, stilbenes, and related 4-substituted resorcinols, obtained from *Artocarpus incisus* and other plants, suggest that compounds with the 4-substituted resorcinol skeleton have a potent tyrosinase inhibitory ability (Shimizu *et al*. 2000). However, the effective topical concentration of these compounds in disorders of hyperpigmentation is not yet known.

Among the *C. siliqua* constituents that we have isolated, 1,2,3,6-tetra-O-galloyl-ß-D-glucose which exhibited stronger activity than myricetin-3-O-α-L-rhamnosid or quercetin-3-O-α-L-rhamnoside. All the *C. siliqua*-inhibitors tested were more effective on the monophenolase than on the diphenolase activity except quercetin-3-O-α-L-rhamnoside that underlines their role in the first step of oxidation, the rate-limiting reaction. We originally expected that the gallic acid moiety in *C. siliqua* components that is similar to tyrosine or L-DOPA may be the active inhibitory structure. However, our preliminary study on the gallic acid produced a strong inhibitory effect therefore, 1,2,3,6-tetra-O-galloyl-ß-D-glucose was the most active of the *C. siliqua* constituents with a gallotanin structure; a group of compounds, which exert a high inhibitory activity of tyrosinase.

1,2,3,6-tetra-O-galloyl-ß-D-glucose and quercetin-3-O-α-L-rhamnoside exhibited significant antioxidant activity which is again related to their polyphenolic structures. Polyphenols, including flavonoids, can exert their antioxidant activity most importantly by scavenging free radicals. Generally, polyphenols are potent free radical scavengers because phenolic groups are excellent nucleophiles. According to Zhao *et al*. (2005), two gallotannins; Pistafolin A and Pistafolin B isolated from the leaves of *Pistacia weinmannifolia* showed potent antioxidant activities. By donating hydrogen in the active hydroxyl groups to form resonance-stabilized phenoxyl radicals, polyphenols are usually called hydrogen-donating antioxidants. In the present investigation, 1,2,3,6-tetra-O-galloyl-ß-D-glucose was potent hydrogen donor, which eliminated the stable DPPH-free radicals and blocked the oxidative chain reactions of bio-macromolecules by acting as chain-break antioxidants. As compared with quercetin-3-O-α-L-rhamnoside, 1,2,3,6-tetra-O-galloyl-ß-D-glucose was a more effective antioxidant. Their antioxidant efficiencies are closely related to their molecular structure. 1,2,3,6-tetra-O-galloyl-ß-D-glucose contains four galloyl groups whilst quercetin-3-O-α-L-rhamnoside contains an α-L-rhamnopyranose group. The

galloyl groups containing active phenolic hydroxy groups were major contributors to the antioxidant capacity, so 1,2,3,6-tetra-O-galloyl-ß-D-glucose with four galloyl groups showed stronger antioxidant ability. It is interesting and worthy noting that in our study this four galloyl moiety compound containing is also the most effective tyrosinase inhibitor (Fig. 7.1).

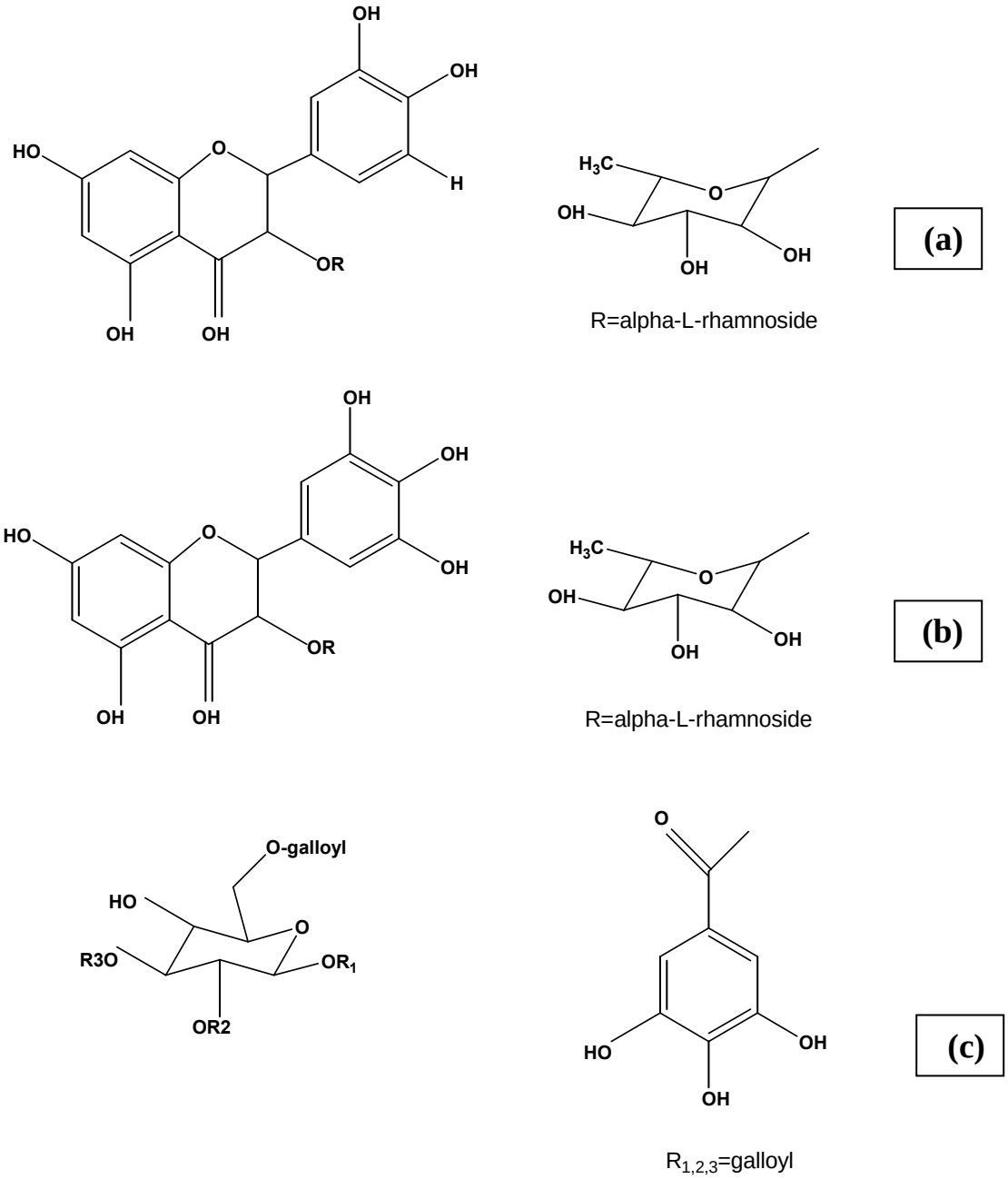


Figure 7.1. Chemical structures of (a) quercetin-3-O-α-L-rhamnoside, (b) myricetin-3-O-α-L-rhamnoside and (c) 1,2,3,6-tetra-O-galloyl-ß-D-glucose, isolated from the methanol extract of *Ceratonia siliqua*

7.3. Tyrosinase inhibitory activity of active constituents isolated from *Sideroxylon inerme*

The bark of *Sideroxylon inerme* is the one of the most commonly used skin-lightener in South Africa. Two major components of *S. inerme*, epicatechin gallate and procyanidin B2 showed potent inhibition of monoplenolase activity but not diphenolase *in vitro*. Our data imply that the depigmenting effect of *S. inerme* components likely work through an inhibition of the tyrosinase activity.

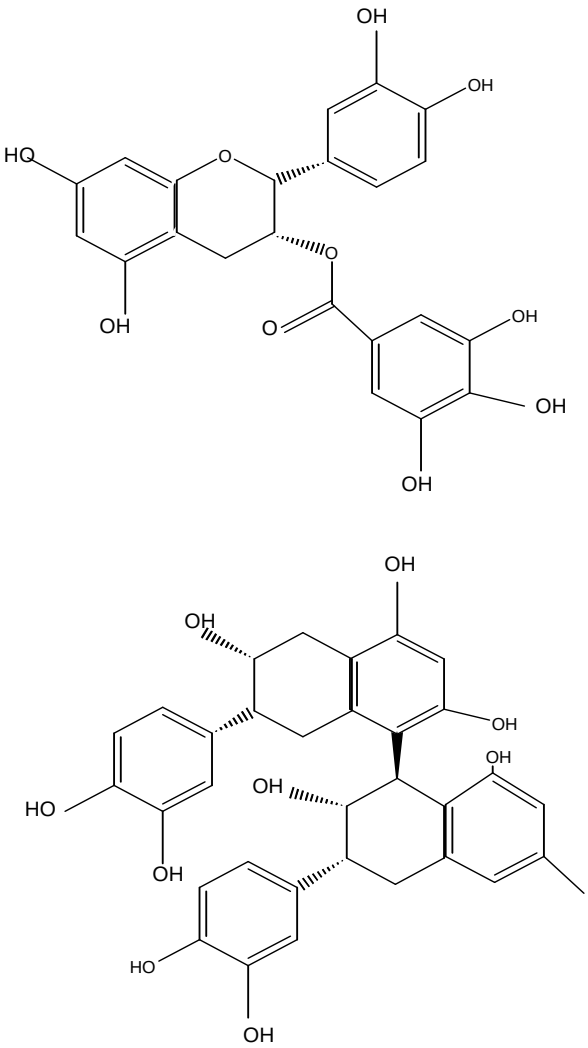
Catechins, a predominant group of polyphenols present in various plant species have been reported to inhibit tyrosinase activity. Considering the relation of free radicals and activation of tyrosinase, tyrosinase which biosynthesizes melanin could be activated by UV generating several free radicals. These compounds exhibited stronger antioxidant activities than those obtained with standards (vitamin C and quercetin) at the concentrations tested in our studies. Epicatechin gallate represented stronger DPPH scavenging activity than procianidin B2 and as explained earlier this can be related to its chemical structure (gallate group) (Fig 7.2). In addition, *S. inerme*, the extract contains these compounds showed very high antioxidant ability, which is likely related to its potent antioxidant components.

7.4. Recommendations for future work

Further studies are required to understand the effect of different functional group substitutions and the mode of inhibition of tyrosinase activity by purified compounds. The effect of the active compound (s) on the kinetic parameters of mushroom tyrosinase should be performed. The regulation of tyrosinase-related proteins and mRNA in epidermal melanocyt cells should be investigated.

Despite the large number of molecules exhibiting anti-pigmentation properties *in vitro*, only some of them are able to induce an effective hyperpigmenting effect measurable in clinical trials. This gap between *in vitro* and *in vivo* studies suggests that new strategies are needed for discovering new depigmenting agents and validating their efficacy and safety. The combination of two or more agents acting on

different mechanisms to produce a synergistic hyperpigmenting effect should be considered. These approaches can be achieved in account of the following steps: (i) synergistic effects of combined therapies; (ii) stability of whitening formulation; (iii) toxicity and skin penetration and (iv) definition of markers and targets for evaluating depigmenting properties *in vitro* and *in vivo*.



(a)

(b)

Figure 7.2.Chemical structures of (a) epicatechin gallate and (b) procyanidin B2, isolated from the methanol extract of *Sideroxylon inerme*

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Chapter 8

Acknowledgments

8.1. Acknowledgments

A project of this magnitude involves many more people than just the writer. The completion of this work would not have been possible without the help of many people. I would like to express special gratitude to:

- Prof. N. Lall for being my supervisor and for her consistent guidance, without which the completion of the project would not have been possible. I am indebted to her for her support and for always being ready to assist. Special thanks for her critical comments during the write up of this dissertation.

- Prof. J.J.M. Meyer, Head of Botany department for giving me the opportunity to do my Master and for being always approachable.

- The entire project would have come to a halt had it not been for the extremely support by Prof. Ahmed Hussein. I wish to express my utmost and sincere thanks for his assistance rendered to me and giving me opportunity to get familiar with different purification techniques in University of Pretoria. I am grateful to him for his help for the collection of plant materials used in this study.

- Dr. Adrian Basson, for helpful discussions and valuable advice in the various enzymatic and cell cultural techniques. He deserves an extraordinary mention for giving me the opportunity to learn valuable information about cell culture laboratory and being always accountable.

- Dr. Seyed Mahdi Ziaratnia, for his camaraderie and motivation for analyzing the data (statistics) of the thesis and for being always approachable, responsible and patience.

- Mr. Eric Palmer, for kindly providing his technical assistance with NMR spectroscopy.

- Mrs. Rene Swart, for her friendship and support through complicated times.

- To those unknown and unnamed people whose efforts over the years have dedicated the breakneck increase in traditional knowledge since the beginning of the 20th century.

- To my colleagues in the Department of Botany for their inspirations and supports.

- Finally, I express my particular gratitude to my husband Ramin and my daughter Kimia for their support, patience and understanding throughout the period of the study and to my family; Kazem Momtaz, Khadijeh Khonveh, M.R. Momtaz and H.R. Momtaz for their encouragement during this study.

Chapter 9

Appendices

Appendix A

9.1. ^{1}H-NMR and ^{13}C-NMR of purified compounds from *C. siliqua*

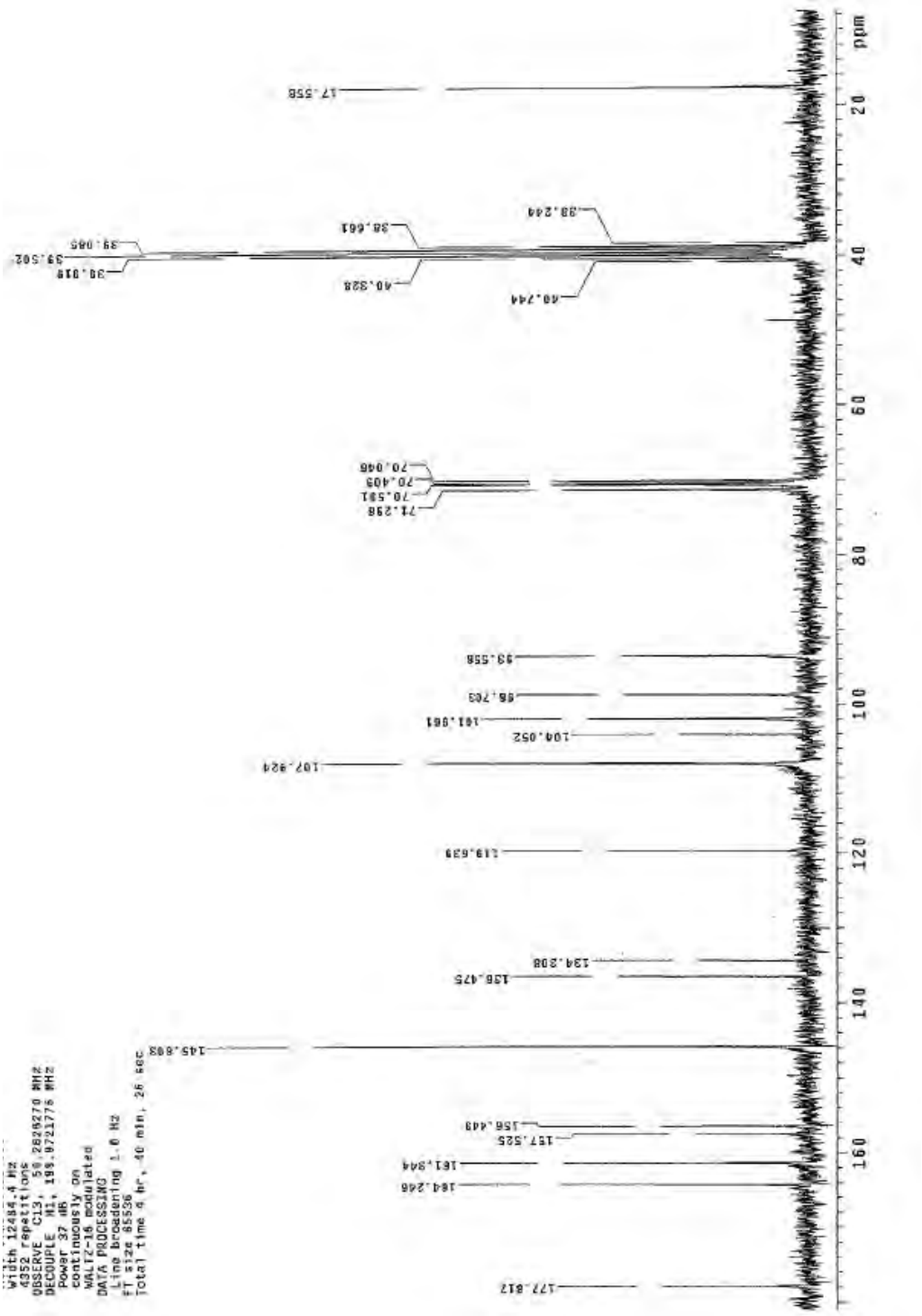

Figure 9.1.1. ^{13}C-NMR spectrum of 'myricetin-3-O-α-L-rhamnoside' isolated from the methanol extract of *Ceratonia siliqua*

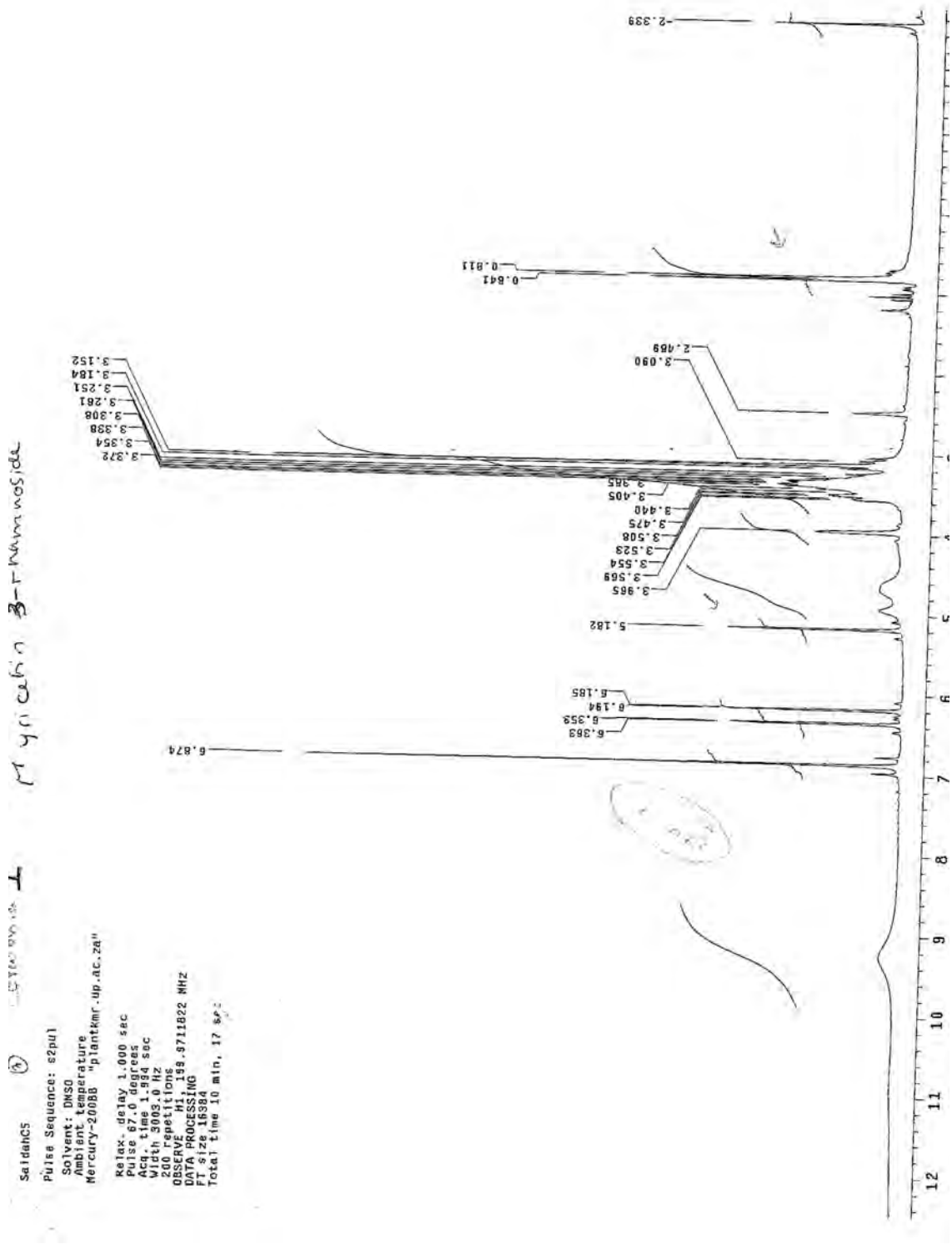

Figure 9.1.2. ^{1}H-NMR spectrum of 'myricetin-3-O-α-L-rhamnoside' isolated from the methanol extract of *Ceratonia siliqua*

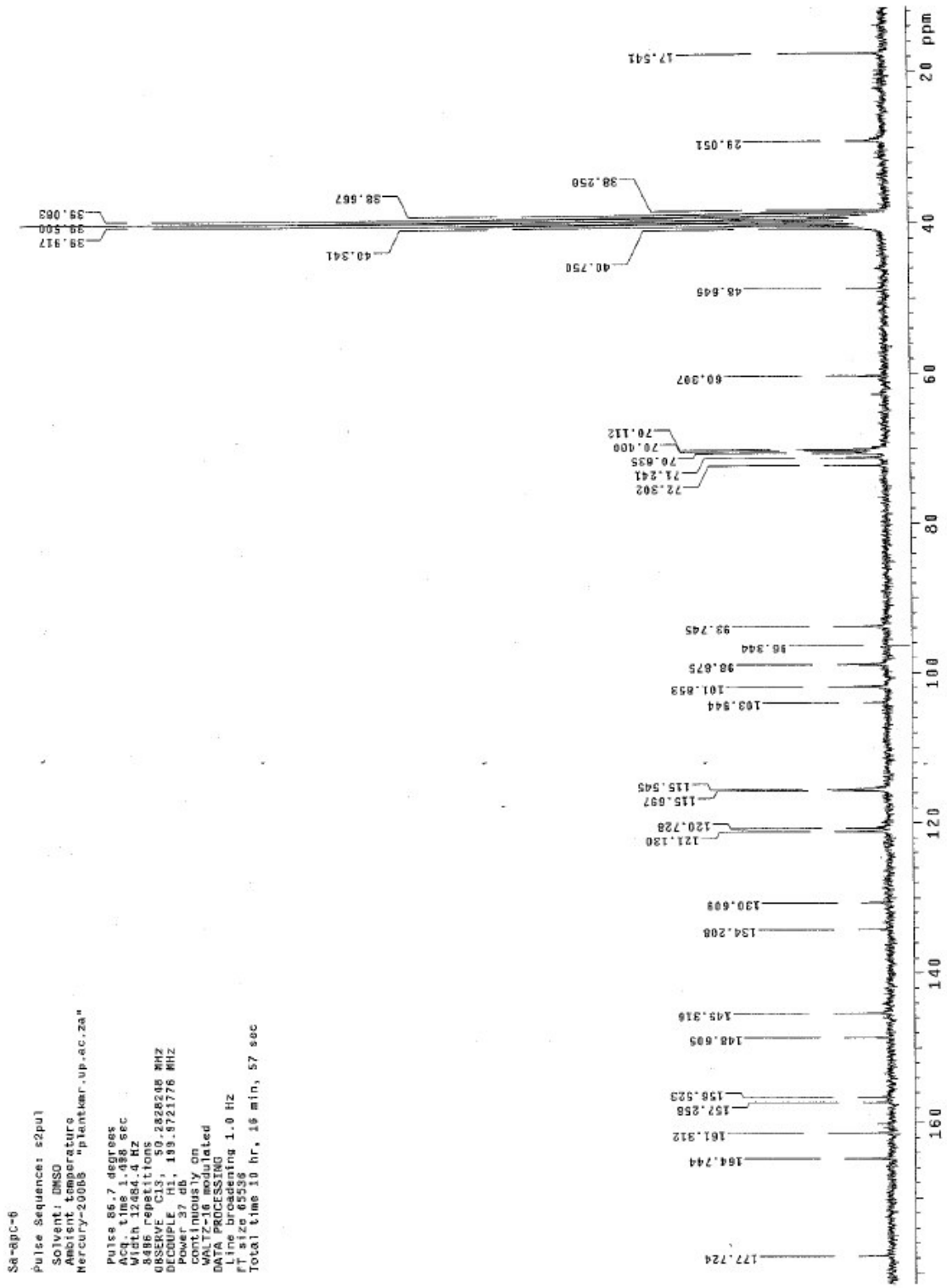

Figure 9.1.3. ^{13}C-NMR spectrum of 'quercetin-3-O-α-L-rhamnoside' isolated from the methanol extract of *Ceratonia siliqua*

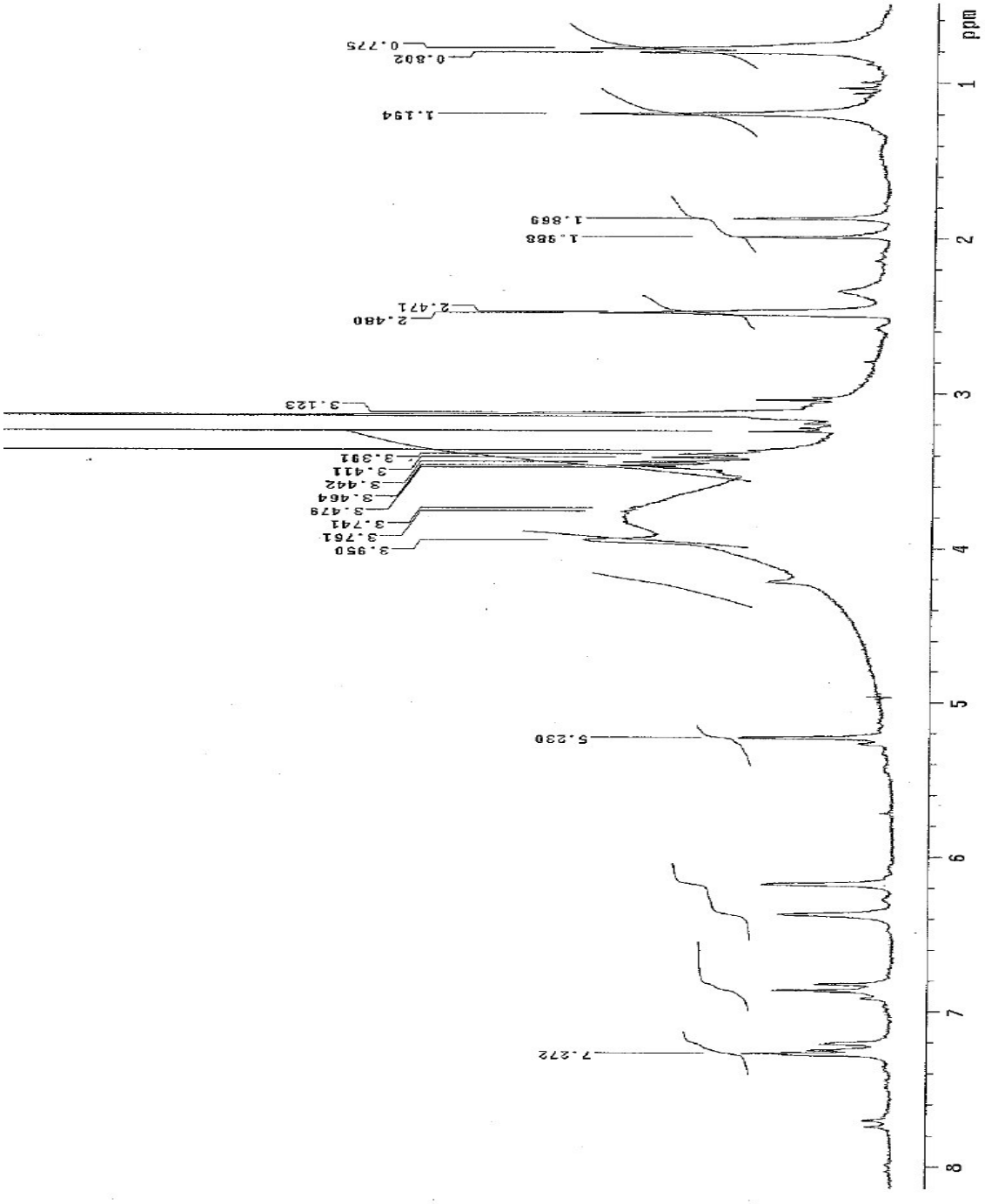

Figure 9.1.4. ^{1}H-NMR spectrum of 'quercetin-3-O-α-L-rhamnoside' isolated from the methanol extract of *Ceratonia siliqua*

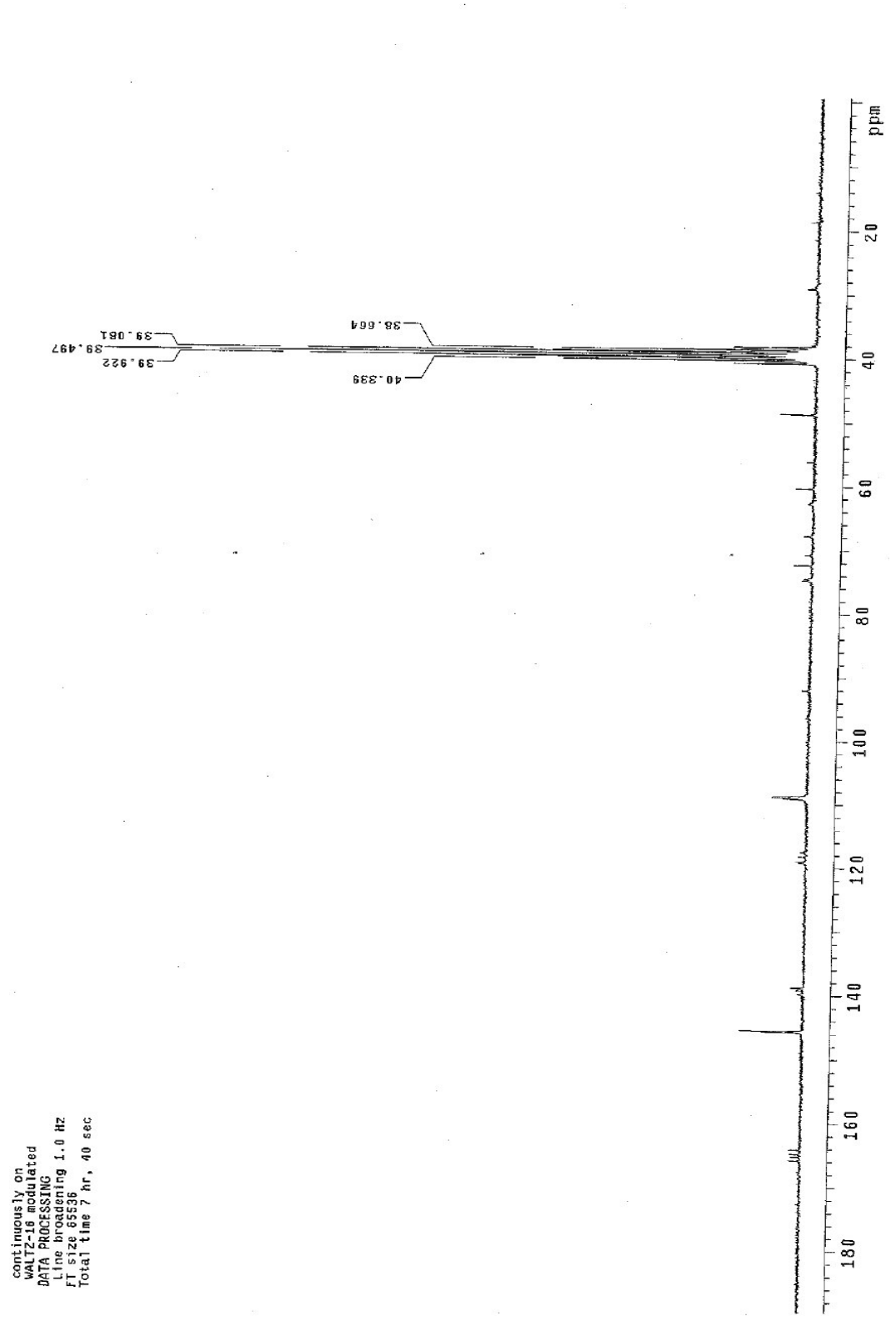

Figure 9.1.5. ^{13}C-NMR spectrum of '1,2,3,6-tetra-O-galloyl-ß-D-glucose' isolated from the methanol extract of *Ceratonia siliqua*

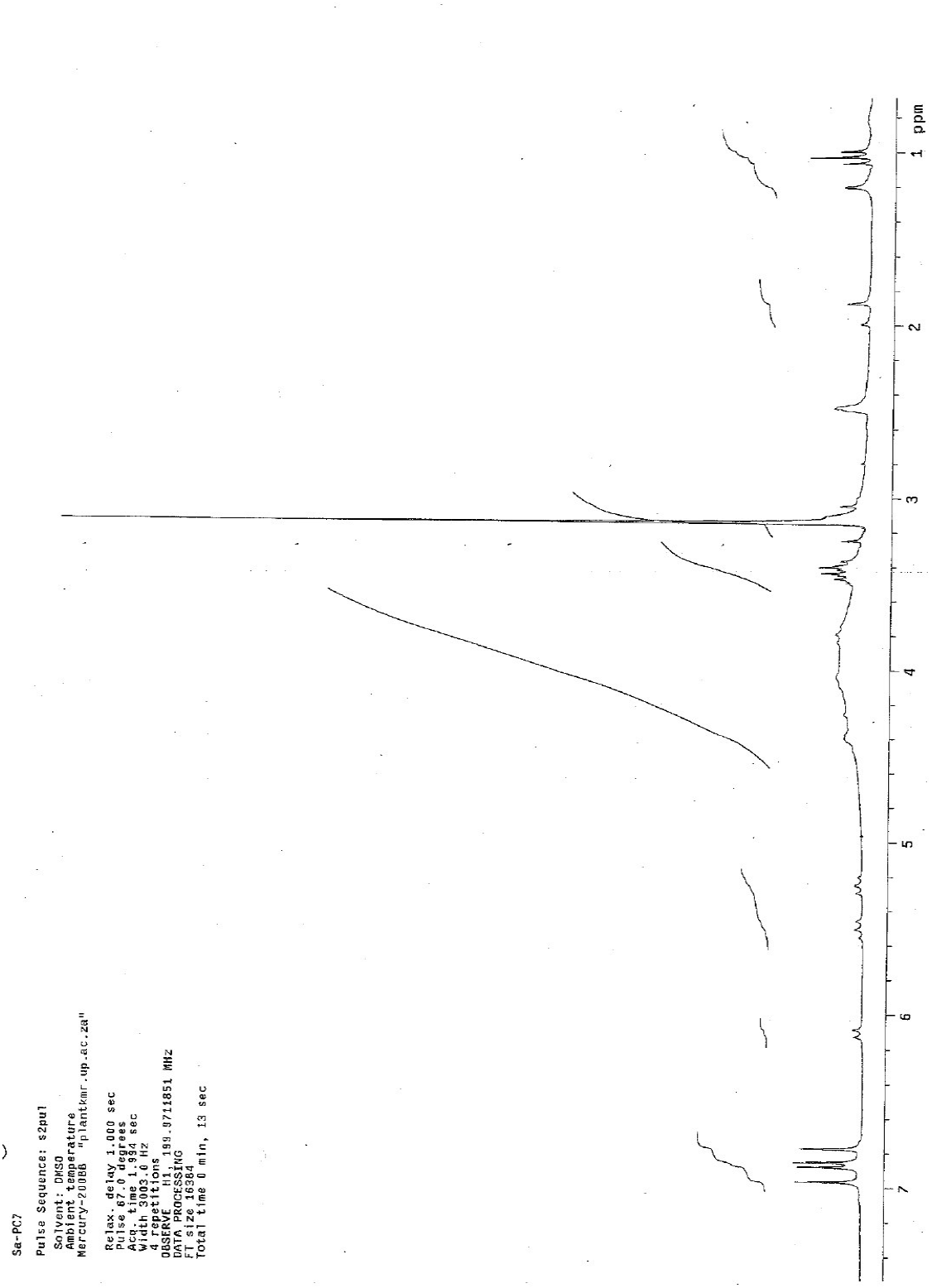

Figure 9.1.6. ^{1}H-NMR spectrum of '1,2,3,6-tetra-O-galloyl-ß-D-glucose' isolated from the methanol extract of *Ceratonia siliqua*

Appendix B

9.2. ^{1}H-NMR and ^{13}C-NMR of purified compounds from *S. inerme*

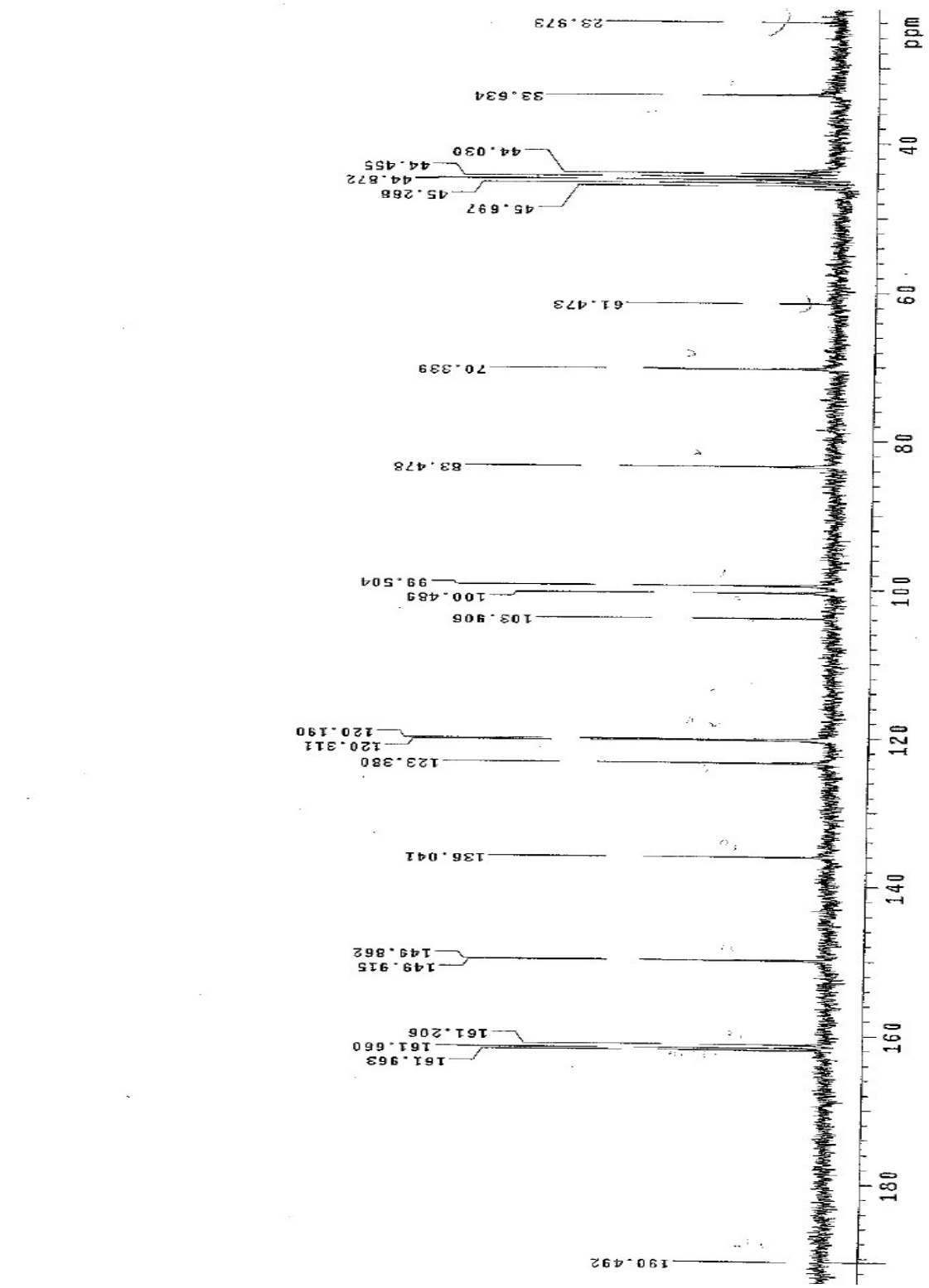

Figure 9.2.1. ^{13}C-NMR spectrum of 'epicatechin gallate' isolated from the methanol extract of *Sideroxylon inerme*

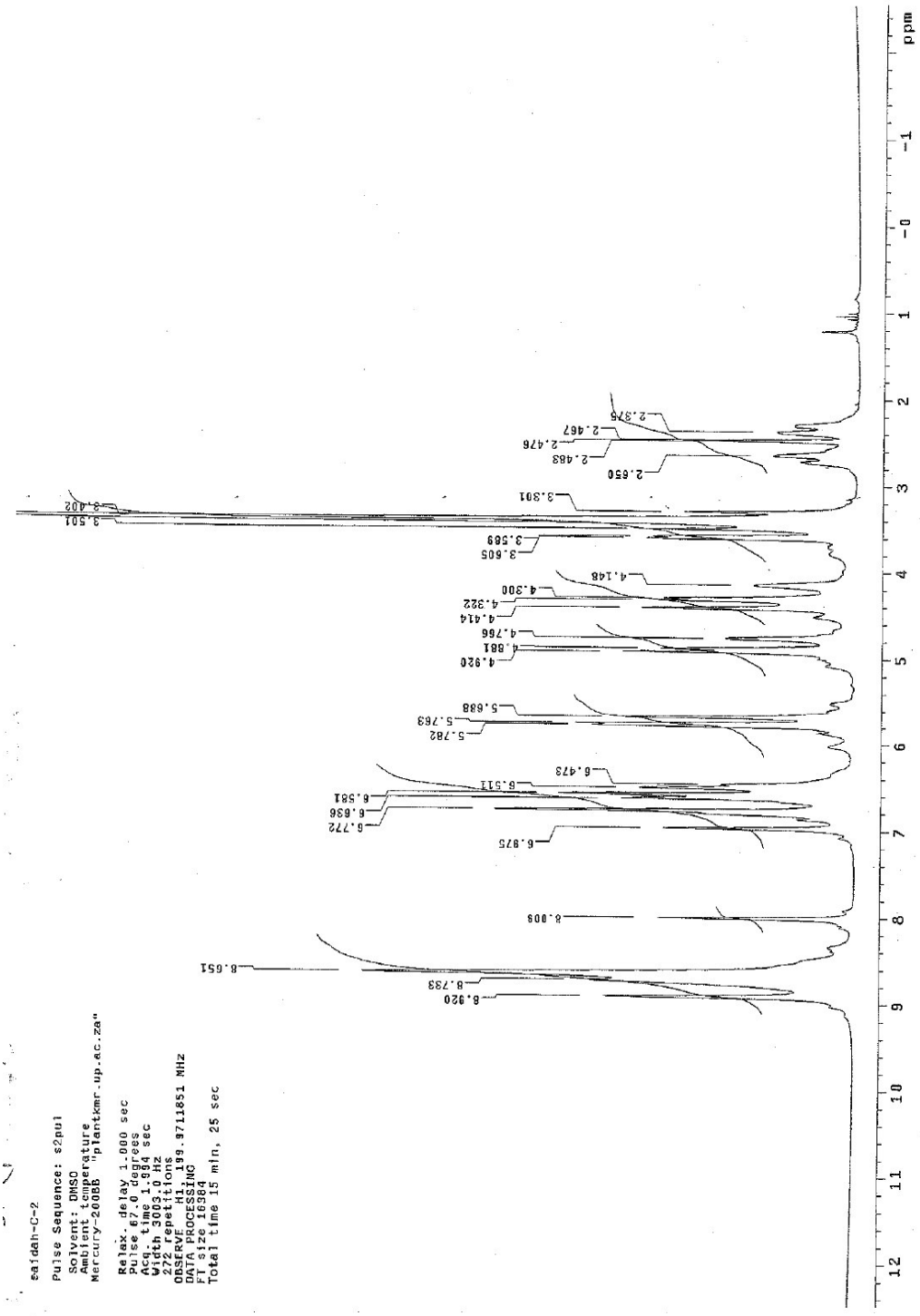

Figure 9.2.2. ^{1}H-NMR spectrum of 'epicatechin gallate' isolated from the methanol extract of *Sideroxylon inerme*

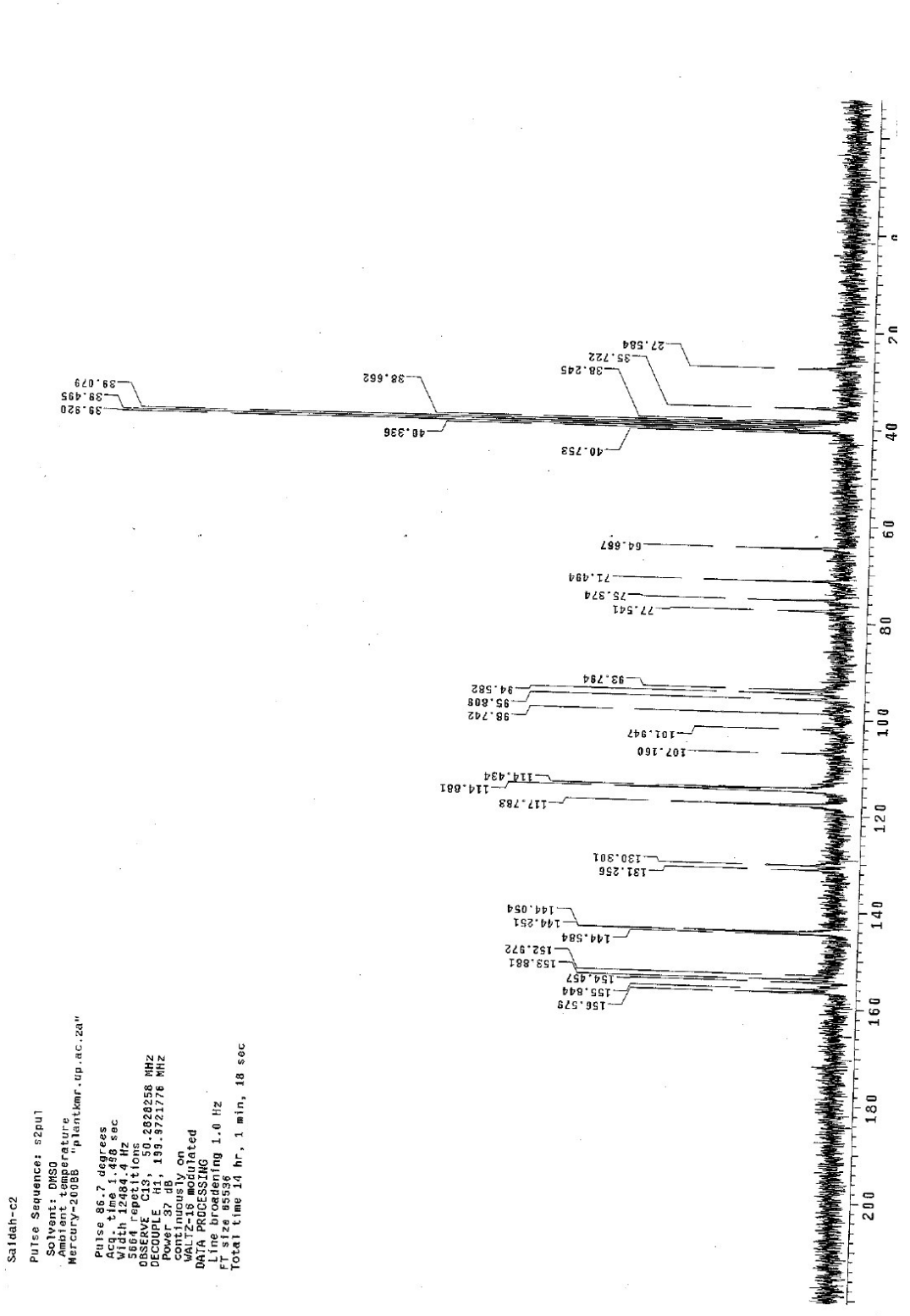

Figure 9.2.3. ^{13}C-NMR spectrum of 'procyanidin B2' isolated from *Sideroxylon inerme*

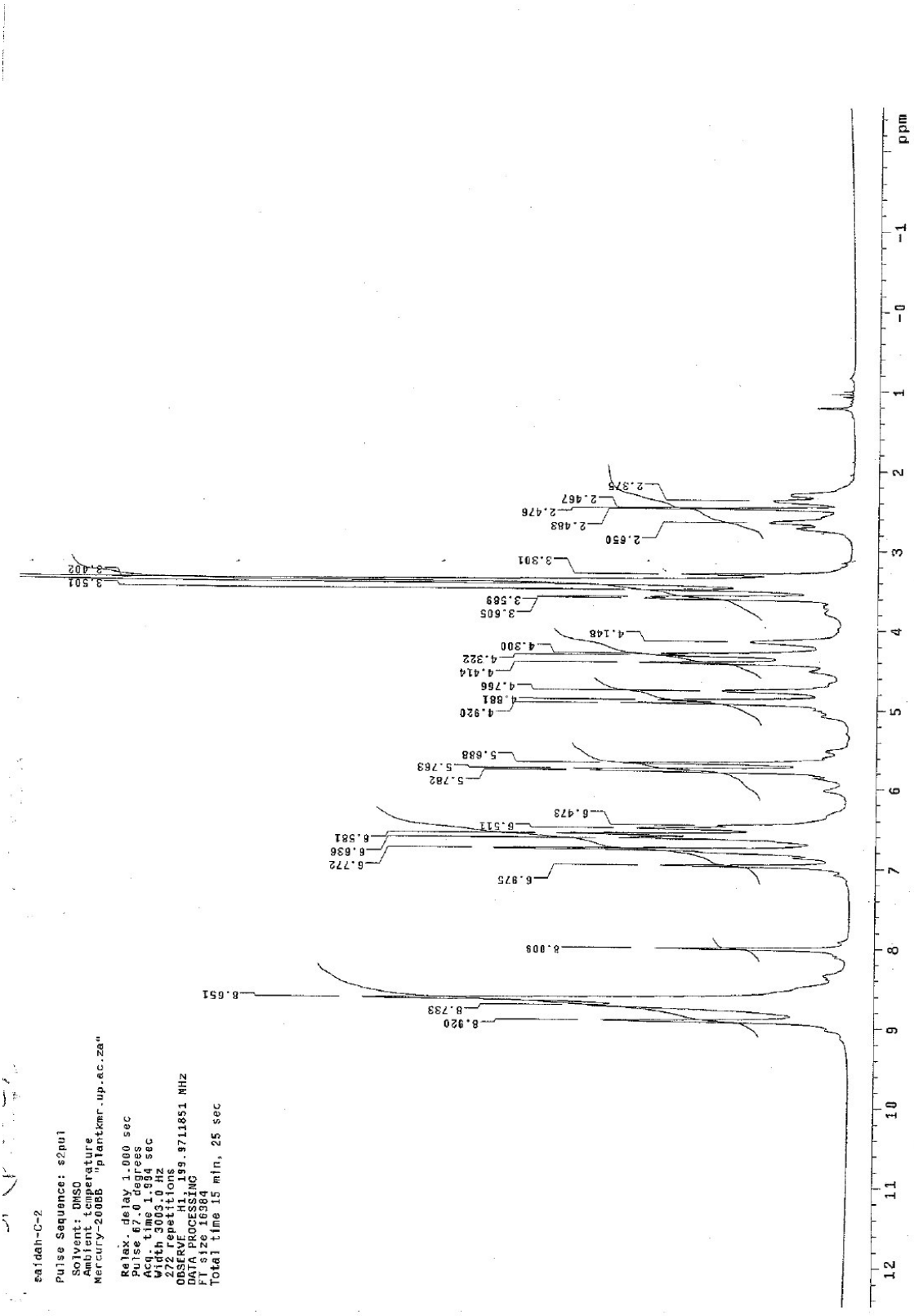

Figure 9.2.4. ^{1}H-NMR spectrum of 'procyanidin B2' isolated from *Sideroxylon inerme*

Appendix C

9.3. Patent/publication/Conference presentations resulting from this thesis

9.3.1. Publications

Manuscripts under publication:

Momtaz, S., Lall, N., Hussein, A., & Basson, A.E (April 2007). Plant-derived multifunctional actives for skin hyperpigmentation from *Ceratonia siliqua.*

Momtaz, S., Lall, N., Hussein, A & Basson, A.E (April 2007). Plant-derived multifunctional actives for skin hyperpigmentation from *Sideroxylon inerme.*

9.3.2. Conference presentations

International Conference:

Momtaz, S & Lall, N., (13-14 September 2006). Anti-tyrosinase activity of South African plants. *International Society of Cosmetic Chemists; Coschem* ***(Oral presentation).*** *Johannesburg, South Africa.*

9.3.3. Patent

Momtaz, S., Lall, N., Hussein, A., Basson, A & Mashamba, A (April 2007). Anti-tyrosinase activity of plants from South Africa. Patent document. *Department of Botany, University of Pretoria, Pretoria, 0002, South Africa.*

Anti-tyrosinase activity of plants from South Africa

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ABSTRACT

Tyrosinase is known to be a key enzyme in melanin biosynthesis, which is involved in determining the color of mammalian skin and hair. Various dermatological disorders, such as melasma, age spot and sites of actinic damage arise from the accumulation of an excessive level of epidermal pigmentation. The inadequacy of current therapies to treat these conditions as well as high toxicity and mutagenicity, poor skin penetration and low stability of existing depigmenting agents led us to seek new whitening/anti-pigmenting agents from plants.

Extracts of four plant species namely; *Hyaenache globosa* L. (aerial parts), *Myrsine africana* L. (aerial parts), *Ceratonia siliqua* L. (leaves) and *Sideroxylon inerme* L. (stem-bark) were evaluated for tyrosinase inhibitory activity using mushroom tyrosinase (monophenolase and diphenolase used as substrates) at concentrations ranging from 3.1 to 400.0 µg/ml. Extracts and purified compounds were also evaluated for their inhibitory activity on melanin production by melanocyte cells at concentrations ranging from 1.5 to 100.0 µg/ml.

Methanol extract of *Hyaenanche globosa* exhibited 82% inhibition ($p<0.01$) of tyrosinase activity at 200.0 µg/ml when L-tyrosine was used as substrate. It also demonstrated 30% inhibition of diphenolase activity at 400.0 µg/ml ($p<0.01$). Methanol extract of *Hyaenanche globosa* did not show any significant ($p<0.01$) decrease in melanin production at the highest concentration tested (100.0 µg/ml) in mouse melanoma cells but the extract was not found to be toxic to the cells at any concentration tested.

Methanol extract of *Myrsine africana* showed 93% inhibition of monophenolase activity at 200.0 µg/ml ($p<0.01$). *Myrsine africana* (methanol extract) exhibited 57% inhibition of tyrosinase activity at 400.0 µg/ml ($p<0.01$) when L-DOPA was used as substrate. *Myrsine africana* (methanol extract) showed 18% reduction in melanin content in melanocytes at 100.0 µg/ml.

Results of monophenolase inhibitory activity by the methanol and acetone extracts of *S. inerme* were found to be similar, (70%) of tyrosinase activity at 200.0 µg/ml ($P<0.01$). Methanolic extract of this plant did not exhibit significant ($P<0.01$) anti-DOPA activity at any concentration tested in this study. Based on the results achieved by tyrosinase assay, methanol extract of *S. inerme* was subjected to a toxicity assay in conjunction with the melanin production assay. The sample showed significant inhibition (37%) of melanin production at 6.2 µg/ml while 80% of cells were found to be viable.

Methanol extract of *C. siliqua* showed significant ($P<0.01$) inhibition of tyrosinase activity (125%) at 400.0 µg/ml whilst L-tyrosine was used. No significant reduction ($P<0.01$) of tyrosinase activity was observed by its acetone extract. Its methanolic extract revealed 43% and 23% inhibition of diphenolase activity at 400.0 and 200.0 µg/ml ($p<0.01$) respectively. A significant reduction in melanin amount (45%) was obtained by *C. siliqua* at 12.5 µg/ml while the cells viability was more than 90%. In total 3 pure compounds (quercetin-3-*O*-α-L-rhamnoside, myricetin-3-*O*-α-L-rhamnoside and 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose) have been purified of which anti-tyrosinase activity of 2 compounds have been reported earlier. Quercetin-3-*O*-α-L-rhamnoside is a flavonoid, this is the first report of its anti-tyrosinase activity. Quercetin-3-O-α-L-rhamnoside showed significant ($p<0.01$) anti-tyrosinse activity (50%) at 50.0 µg/ml while L-DOPA was used. Quercetin-3-O-α-L-rhamnoside showed 28% inhibition of melanin production at 6.2 µg/ml significantly ($p<0.01$).

C. siliqua (methanol extract), *S. inerme* (methanol extract), *H. globosa* (methanol extract), *M. africana* (methanol extract) and quercetin-3-O-α-L-rhamnoside (isolated from the methanol extract of *C. siliqua*) have not been explored before for their inhibitory activities for monophenolase, diphenolase activity and for melanogenesis. These samples can therefore, be considered for use as tyrosinase inhibitors.

1. Introduction

Melanin is a pigment in mammals that determine the colour of their skin and hair (Césarini & I.N.S.E.R.M., 1996; Riley, 1997). It is produced through a series of oxidative reactions that involves tyrosine and the key-enzyme, tyrosinase (monophenol monooxygenase, E.C. 1.14.18.1) (Fenoll et al., 2004). Tyrosinase catalyses two reactions: the first involves the hydroxylation of monophenols to *o*-diphenols; the second reaction involves the oxidation of the *o*-diphenols to *o*-quinones. The enzyme therefore, has two activities, namely a monophenolase activity and a diphenolase activity. The quinones that result from the second activity, can spontaneously polymerise to form melanins (Riley, 1997). Melanin, secreted by melanocytes in the basal layer of the epidermis, protects the skin from ultraviolet (UV) irradiation by absorbing UV light (Jablonski & Chaplin, 2000; Rouzaud et al., 2005). Various dermatological disorders result in the accumulation of excessive or inadequately levels of epidermal pigmentation of the skin (Taylor, 2002; Halder & Nootheni, 2003; Passeron, et al., 2005). Tyrosinase inhibitors are important for their use as skin lightening agents to treat hyperpigmentation (Hearing, 2005).

The bark of *Sideroxylon inerme* is traditionally used in Southern African for skin lightening purposes. Flavonoids are known to be with high anti-tyrosinase activity. The leaves of *Ceratonia siliqua* are rich in flavonoids and were therefore, selected for this study. The aerial parts of *Myrsine africana* and *Hyaenache globosa* were selected randomly. An investigation of the tyrosinase inhibitory activity and *in vitro* anti-melanogenesis activities of these plants were pursued.

2. Materials and Methods

2.1. Materials

L-Tyrosine, L-DOPA, tyrosinase, and isoliquirtigenin were obtained from Sigma-Aldrich (Kempton Park, South Africa). Cell culture reagents and equipment were purchased from Highveld Biological (Sandringham, South Africa), LASEC (Randburg, South Africa), and The Scientific Group (Midrand, South Africa). The B16-F10 mouse melanocyte cell line was obtained from Highveld Biological (Sandringham, South Africa).

2.2. Collection of plant material

The bark of *Sideroxylon inerme* was collected during July 2004 in Venda. The leaves of *Ceratonia siliqua*, *Myrsine africana* (aerial part) and *Hyaenanche globosa* (aerial part) were collected during July 2005 from the botanical garden of the University of Pretoria, South Africa. The South African National Biodiversity Institute (SANBI) and the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria confirmed the identities of the collected plants (Table 1).

2.3. Extraction

The dried bark of *Sideroxylon inerme, Ceratonia siliqua* (leaves), *Myrsine africana* (aerial part) and *Hyaenanche globosa* (aerial part) ware ground to a fine powder. Forty grams of the bark of *Sideroxylon inerme* was extracted with 200 ml of acetone and the same amount of the bark of *Sideroxylon inerme*, the leaves of *Ceratonia siliqua,* the aerial part of *Myrsine africana* and *Hyaenanche globosa* were extracted separately with 200 ml of methanol each with constant stirring for 3 hours at room temperature. The solvent was then removed and replaced with an equal volume of respective solvents. This procedure was continued until the solvent remained clear. Extracts were then filtered through filter paper and the solvent was removed under vacuum to yield dry extracts.

2.4. Isolation of compounds from Ceratonia siliqua

Various compounds especially polyphenols have been isolated from *C. siliqua* before (Papagiannopoulos et al. 2004, Owen et al. 2003). Antibacterial, antifungal, antiviral and antithelmintic activities of different extracts of *C. siliqua* have been reported earlier (Papagiannopoulos et al. 2004, Van Wyk and Grick, 2000). There is no information till date about the efficacy of *C. siliqua* to treat hyperpigmentation. Having obtained good inhibitory activity of the methanol extracts of *C. siliqua*, this sample was further selected for isolation of its bioactive constituents.

2.4.1. Solvent-Solvent separation

Dried methanol extract of *C. siliqua* (95 g) was re-dissolved in 80% methanol (methanol/distilled water; 80: 20) and partitioned with n-hexane, ethyl acetate and n-butanol in separating funnel (PYREX®, 29/32). Funnels were shaked gently for few minutes and left steady to obtain two separate layers. The organic layers (top layers) were evaporated to dryness at 40 °C to give 11 g, 32.5 g, 19 g and 5g of n-hexane, ethyl acetate, n-butanol and water fractions respectively.

2.4.2. Isolation of compounds from C. siliqua by 'Paper partition chromatography'

A series of Sephadex columns (9 columns) were developed with 100% ethanol as eluent. The obtained fractions were applied on TLC plates using ethyl acetate: ethyl acetate/ formic acid/ acetic acid/ distilled water; (13.5:10:1:1:1.5) as an eluent system and those with similar TLC-profile were combined.

Fractions 5 and 6 of the Sephadex column were mixed, dried and quantified (0.051 g). Then, the mixture was re-dissolved in a minimum amount of ethanol and was applied on the paper sheets [Whatman No.3 (46× 57 cm)] by a 230 mm Pasture Pipette. The diameters of lines at the origin were approximately 1 cm. Thereafter, papers were kept under a cool dryer (hair dryer) until ethanol evaporated. Paper sheets were placed in a glass cylinder and hung vertically from the trays at the top of the tank and held in place

by small glass bars (0.25× 0.125× 4 inches). Solvent system (acetic acid/distilled water; 15: 85) was used in the tanks.

The tank cover was clamped in position and the chromatograms were developed over night until the solvent front had moved 35-40 cm (Buch, 1952; Bush, 1951). After development of the chromatograms, the sheets were removed from the tank and hung vertically at room temperature for few hours to evaporate solvents using a stream of air in a laboratory hood. The lines' locations were determined by spraying 'ammonia solution (NH_4OH)' (Chemical Suppliers (PYT) LTD, South Africa).

The lines were examined under UV light at 254 and 366 nm, to confirm the presence of compounds and marked. Each line was cut with a scissor in small pieces. The paper pieces (of each line) were dissolved in 80% methanol separately and kept for 3 hours at 50 ºC while shaking occasionally. Subsequently, each mixture was filtered off and the filtrate was dried using a rotary evaporator (BUCHI Rotavapor, R-200). These procedures were repeated three times until the solvent became colourless. A few mg of each filtrate was dissolved in ethanol and spotted on a TLC plate to determine the purity of obtained compounds.

2.4.3. Chemical structure of purified compound

A pure compound 'quercetin-3-O-α-L-rhamnoside' (greenish powder, yield: 0.003%) was isolated using paper partitionation method. The structure of the compound was identified by comparing its ^{1}H-NMR, ^{13}C-NMR, 2D-NMR (COSY and NOESY) spectral data with that of the published articles (Papagiannopoulos et al. 2004, Owen et al. 2003).

2.5. Tyrosinase enzyme assay

This assay was performed using Curto et al. (1999) and Nerya et al. (2003) as references. Extracts were dissolved in DMSO to 20 mg/ml. This extract stock solution was then diluted to 600 µg/ml in 50 mM potassium phosphate buffer (pH 6.5).

Isoliquirtigenin was used as a control drug (Nerya et al., 2003). In the wells of a 96-well plate, 70 µl of each extract dilution was combined with 30 µl of tyrosinase (333 Units/ml in phosphate buffer) in triplicate. After incubation at room temperature for 5 minutes, 110 µl of substrate (2 mM L-tyrosine or 12 mM L-DOPA) was added to each well. Final concentrations of the extract samples were 1.5, 3.1, 6.2, 12.5, 25.0, 25.0, 50.0, 100.0, 200.0 and 400.0 µg/ml (for pure compound final concentrations were 0.7, 1.5, 3.1, 6.2, 12.5, 25.0, 25.0, 50.0, 100.0 and 200.0 µg/ml). Incubation commenced for 30 minutes at room temperature. Optical densities of the wells were then determined at 492 nm with the BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa).

2.6. Cell culture

The mouse melanocyte cell line, B16-F10, was cultured in complete Basal medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM L-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone at 37^{O}C with 5% CO_2 in a humidified atmosphere. Cells were sub-cultured in a ratio of 1:3 on every third or fourth day.

For *in vitro* experiments, B16-F10 cells were resuspended in complete DMEM medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM L-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone.

2.7. Determination of extract toxicity and melanin content in melanocytes

On day 0, B16-F10 cells in complete DMEM 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^{O}C in 5% CO_2 and a humidified atmosphere, extract samples were added to the cells to final concentrations of 1.5, 3.1, 6.2, 12.5, 25.0, 50.0 and 100.0 µg/ml. Isoliquirtigenin was used as a control drug (Nerya et al., 2003). Incubation at 37 ^{O}C in 5% CO_2 and a humidified atmosphere followed for 3 days.

The toxicity of the extracts on the B16-F10 cells was assayed using XTT cytotoxicity assay (Zheng et al., 2001). Fifty microlitres of XTT reagent (1 mg/ml XTT with 0.383 mg/ml PMS) was added to the wells and incubation commenced for 1-4 hrs. The optical densities of the wells were then measured at 450nm (690nm reference wavelength). By referring to the control (medium with DMSO), cell survival was assessed.

The effect of the extracts on melanin synthesis was determined by washing the cells in the 24-well plate with PBS, and lysing with 200.0 µl of sterile distilled water. Optical densities were determined a 405 nm. The effect on melanin production was determined by referring to the control sample (medium with DMSO).

3. Results

3.1. Inhibition of tyrosinase

The ability of the extract samples to inhibit the *o*-hydroxylation of tyrosine could be determined by using L-tyrosine as substrate. Acetone extract of *Sideroxylon inerme* showed a significant inhibition ($p<0.01$) of the monophenolase-activated form of tyrosinase of 30% at a low concentration of 25.0 µg/ml (Fig. 1). A significant inhibition ($p<0.01$) of monophenolase activity was seen by *Ceratonia siliqua* (128%) at the highest concentration (400.0 µg/ml). Methanol extract of *Hyaenanche globosa* exhibited 82% inhibition ($p<0.01$) of tyrosinase activity at 200.0 µg/ml when L-tyrosine was used as substrate. It also demonstrated 30% inhibition of diphenolase activity at 400.0 µg/ml ($p<0.01$). Methanol extract of *Myrsine africana* showed 93% inhibition of monophenolase activity at 200.0 µg/ml ($p<0.01$). *Myrsine africana* (methanol extract) exhibited 57% inhibition of tyrosinase activity at 400.0 µg/ml ($p<0.01$) when L-DOPA was used as substrate. 'Quercetin-3-O-α-L-rhamnoside' showed 46% inhibition of diphenolase activity at 200.0 µg/ml. A successful inhibition ($p<0.01$) of the *o*-hydroxylation of tyrosine was obtained at all concentrations of the control drug, isoliquirtigenin (Fig. 2).

3.2. Cytotoxicity and melanin content

Following the results obtained from the tyrosinase assay, *Sideroxylon inerme* was selected for further investigation to examine its inhibitory activity on melanin production in an *in vitro* environment. To correlate whether a reduction in melanin observation is caused by enzyme inhibition or a decrease in cell number and/or viability, it was imperative to perform a toxicity assay in conjunction with the melanin production assay.

Sideroxylon inerme demonstrated a significant reduction melanin (37 %) *in vitro* at 6.2 µg/ml without being significantly ($P>0.01$) toxic to B16-F10 cells at this concentration (Fig. 3). At 3.1 µg/ml there was no significant inhibition ($P>0.01$) of melanin

production, whilst at concentrations of ≥25.0 µg/ml melanin content abridged as a result of a decrease in cell number/viability due to toxic effects of the extract on the cells. A significant reduction in melanin amount (45%) was obtained by *C. siliqua* at 12.5 µg/ml when the cells viability was found to be more than 90%. (Fig. 4).

Methanol extract of *Hyaenanche globosa* did not show any significant ($p<0.01$) decrease in melanin production in mouse melanoma cells but the extract was not toxic to the cells at any concentration tested (Fig. 5). *Myrsine africana* (methanol extract) showed 18% reduction in melanin content in melanocytes at 100.0 µg/ml (Fig. 6). Bioassay guided fractionation of the methanol extract of *C. siliqua* led to the isolation of 'quercetin-3-O-α-L-rhamnoside'. 'Quercetin-3-O-α-L-rhamnoside' showed 32% inhibition of melanin production at 6.250 µg/ml without being toxic to B16F10 melanoma cells (Fig.7). Isoliquirtigenin showed no significant toxicity to B16-F10 cells over any of the concentrations tested, and showed a low but significant ($P<0.01$) reduction in melanin content at 0.897 µg/ml (Fig. 8).

4. Discussion and Conclusion

The exploration of plants traditionally used to treat certain ailments is a good source for the discovery of novel active compounds. Extracts from the bark of ***Sideroxylon inerme***, the leaves of ***Ceratonia siliqua*** and the aerial part of ***Hyaenanche globosa*** and ***Myrsine africana*** showed significant ($p<0.01$) inhibition of the monophenolase activity of mushroom tyrosinase. They also proved to reduce the melanin content of mouse melanocytes without being significantly toxic at that concentration. The tyrosinase inhibitory activity of ***S. inerme***, ***C. siliqua***, ***H. globosa***, ***M. africana*** and 'quercetin-3-O-α-L-rhamnoside' has not previously been reported.

Three compounds namely; - (-) epicatechin-3-O-gallte, 1,2,3,6-tetra-O-galloyl-ß-D-glucose and myricetin-3-O-L-rhamnoside were isolated also in this study from the leaves of ***C. siliqua***. These compounds showed 93% (- (-) epicatechin-3-O-gallate), 90% (1,2,3,6, tetra-O-galloyl-ß-D-glucose) and 28% (myricetin-3-O-L-rhamnoside) inhibition of tyrosinase activity at 200.0 µg/ml. It has been reported earlier by No et al., (1999) that – (-) epicatechin 3-O-gallate (ECG) isolated from green tea (methanol extract) exhibited more than 60% tyrosinase inhibition at 40 µM. Lee et al., (1999) reported the anti-tyrosinase activity of myricetin-3-O-L-rhamnoside isolated from the leaves of ***Cercis chinensis***. Lee et al., (1998) described the anti-tyrosinase activity of 1,2,3,6-tetra-O-galloyl-ß-D-glucose isolated from the bark of ***Paeonia moutan.*** Methanol extract of ***C. siliqua*** showed good inhibition of tyrosinase activity and melanin production which further clarify the potential of this plant to be used as skin-lightening agents.

Two pure compounds namely 'epicatechin gallate' and 'procyanidin B2' purified from the bark of ***S. inerme*** showed 60% and 54% inhibition of tyrosinase respectively. According to Hara ***et al***. (1997), epicatechin gallate, epigallocatechin and epicatechin isolated from tea leaves exhibited 95%, 17% and 11% inhibition of monophenolase activity at 0.15 mg/ml (150 µg/ml). It has been reported earlier by No et al., (1999) that – (-) epicatechin purified from green tea (methanol extract) showed 10% inhibition of tyrosinase activity at 40.0 µM. Quebracho (a compound similar to procyanidin) isolated from the heartwood of ***Schinopsis lorentzii*** (70% aqueous acetone) showed 47% tyrosinase inhibition (Takagi and Mitsunaga, 2003). It can therefore be concluded

that the methanol and acetone extract of *S. inerme* is worth considering to be used as skin-lightening agents.

Main Results:

1. The following extracts can be considered for use as tyrosinase inhibitors:

1.1. Acetone and methanol extracts of *Sideroxylon inerme*

1.2. Methanol extract of *Ceratonia siliqua*

1.3. Methanol extract of *Hyaenanche globosa*

1.4. Methanol extract of *Myrsine africana*

2. The following pure compound can be considered as tyrosinase inhibitor:

2.1. 'Quercetin-3-O-α-L-rhamnoside' isolated from the leaves of *Ceratonia siliqua*

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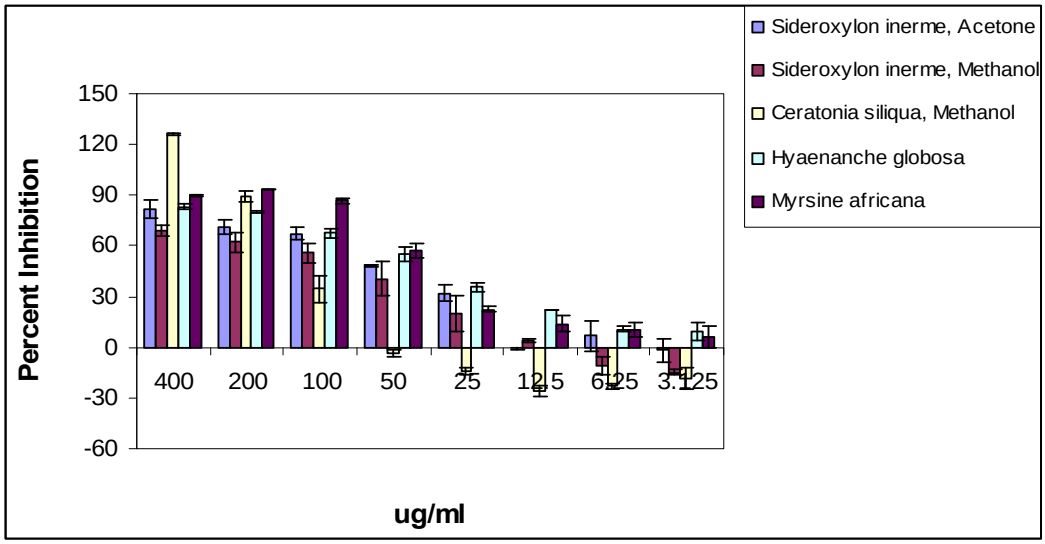

Figure 1. Inhibition of tyrosinase activity, using L-tyrosine as a substrate, by different concentration of extracts

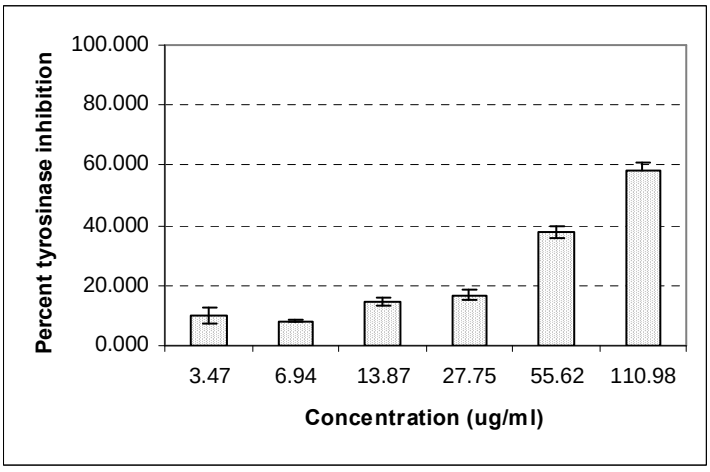

Figure 2. Inhibition of tyrosinase, using L-tyrosine as a substrate, by different concentrations of isoliquirtigenin (positive control)

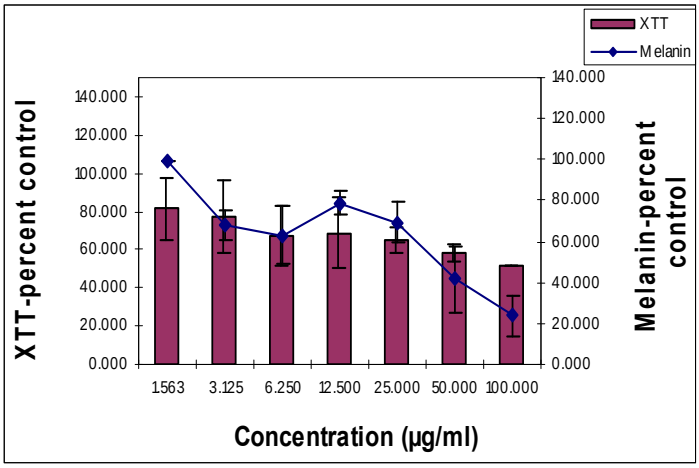

Figure 3. The effect of the acetone extract of *Sideroxylon inerme* on cell viability/proliferation and melanin production. ■ XTT — Melanin

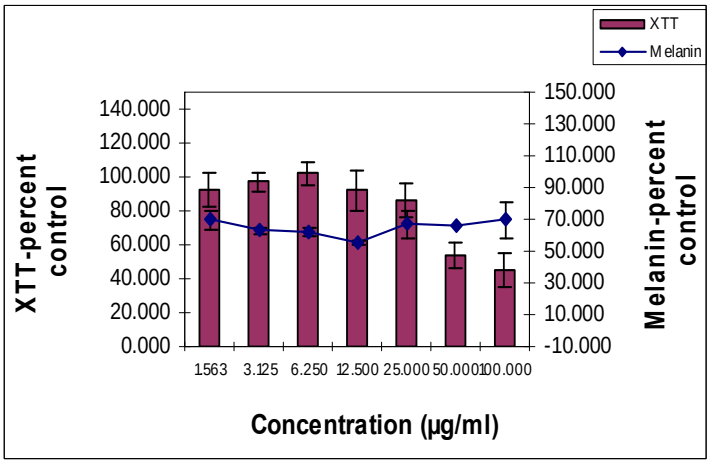

Figure 4. The effect of the methanol extract of *Ceratonia siliqua* on cell viability/proliferation and melanin production. ■ XTT — Melanin

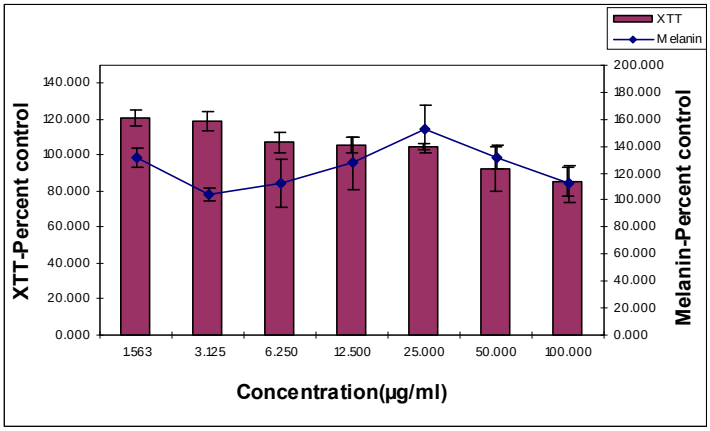

Figure 5. The effect of the methanol extract of *Hyaenanche globosa* on cell viability/proliferation and melanin production. ■ XTT — Melanin

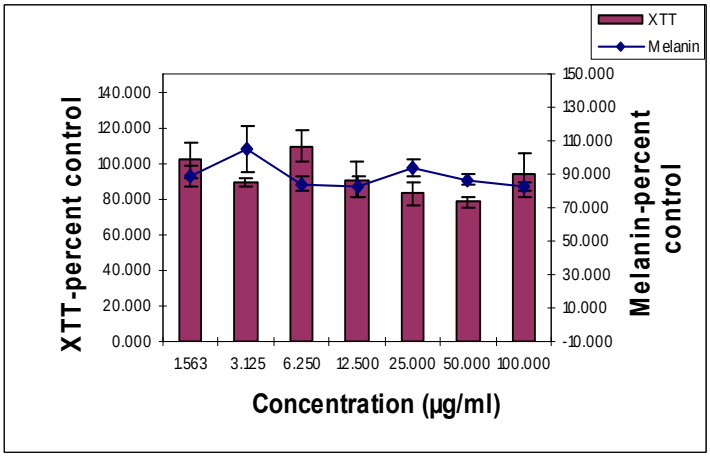

Figure 6. The effect of the methanol extract of *Myrsine africana* on cell viability/proliferation and melanin production. ■ XTT — Melanin

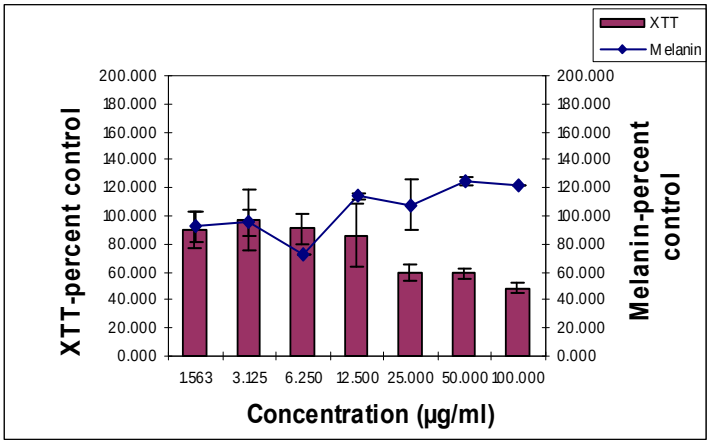

Figure 7. The effect of 'quercetin-3-O-α-L-rhamnoside' on cell viability/proliferation and melanin production. ■ XTT — Melanin

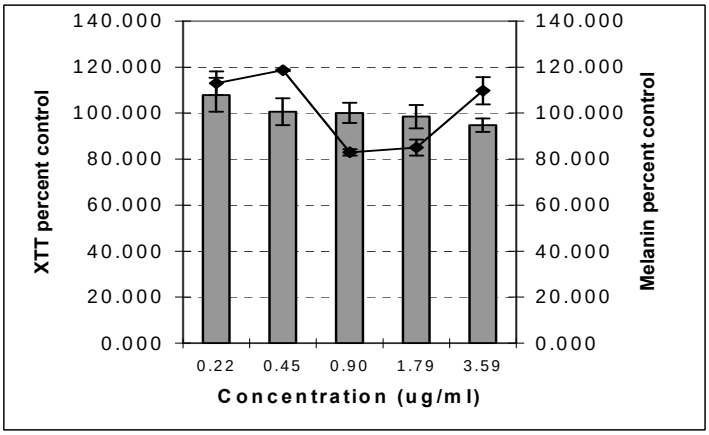

Figure 8. The effect of isoliquiritigenin (positive control) on cell viability/proliferation and melanin production. ■ XTT — Melanin

Table 1. Plant samples collected for the present study:

Name of Plant	Plant Parts	Voucher Herbarium Specimen Number
***Hyaenanche globosa* L.**	**aerial part**	**S.M. 95499**
***Myrsine africana* L.**	**aerial parts**	**S.M. 95503**
***Ceratonia siliqua* L.**	**leaves**	**S.M. 95502**
***Siderxylon inerme* L.**	**stem-bark**	**PRU 96216**