Cultivation studies on two commercially important plants; Seasonal variation of *Ceratonia siliqua* and propagation trials on *Athrixia phylicoides*

By

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Abstract

The use of plant extracts or derivatives in the field of medicine, nutraceuticals and cosmeceuticals has occurred for a long time. This is due to the diversity of compounds present in plants. The cosmetic industry has a number of products that include plant actives to combat ageing, pigmentation and blemishes. Extensive studies, such as the seasonal variation of bioactive compounds, are required to be carried out on the plant extracts before they can be included in such products. The present study focused on two commercially important plants: *Ceratonia siliqua* L. (*C. siliqua*) and *Athrixia phylicoides* DC. (*A. phylicoides*).

A. phylicoides is an indigenous plant commonly known as bush tea because an infusion of this plant is a common traditional tea. This plant has a number of medicinal properties and commercialisation of the tea would benefit many. In order to enhance the propagation of the plant, a hormone rooting field trial was carried out on apical cuttings of *A. phylicoides* during spring and autumn. Cuttings were dipped in liquid hormone solutions of 1-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and Gibberellic acid (GA); all of which were tested at 0.1% and 0.3%. During spring, IBA at 0.1% had the highest rooting percentage of 58.33% and highest sprouting percentage of 60%. Following closely behind was NAA at 0.1% with a rooting percentage of 51.67%. IBA and NAA at 0.3% had the highest number of roots for the entire trial: 38.72 and 31.00, respectively. During autumn, NAA at 0.1% was found to have the highest sprouting percentage of 78.33% and at 0.3%, the highest rooting percentage of 83.33%. The highest number of roots formed was 7.88 by NAA at 0.3% with IAA forming 6.67 roots at 0.3%. IBA at 0.1% formed 6.41 roots. IBA was confirmed as an effective hormone for this plant but this study introduced an additional hormone, NAA, to serve the same purpose at a lower cost.

Ethanol extracts of *A. phylicoides* were tested for potential anti-tyrosinase and antimicrobial studies due to the high antioxidant activities of the extracts. At the highest concentration tested (400 µg/ml plant extract) activity was not detected in either assay.

C. siliqua is a popular plant commonly known as carob and is used globally for its bean gum and pods. Previous studies indicated that the leaf extract of this plant has the potential to be used in the cosmetic field. The present study investigated the seasonal variation of five previously isolated bioactive compounds in the leaves of *C. siliqua* (1,2,3,6-tetra-o-galloyl-β-D-glucose, Quercetin-3-o-α-L-rhamnoside, Myricetin-3-o-α-L-rhamnoside, Myricetin-3-o-

glucoside and Gallocatechin-3-o-gallate). Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and the tyrosinase inhibition assay were used in the study. Seasonal samples from three age groups were collected in order to determine the season and age at which optimal levels of the bioactive compounds were present. The leaf extracts prepared from the samples collected from autumn small, autumn medium and summer medium were found to be the most effective at inhibiting the tyrosinase enzyme with inhibitory concentration (IC50) values of 51.77±0.058 µg/ml, 59.49±0.1 µg/ml and 53.62±0.71 µg/ml, respectively. The positive control for the tyrosinase inhibition assay was kojic acid with an IC50 of 6.99±0.14 µg/ml. HPLC detected the highest amount of the key bioactive compounds, based on their previously identified melanin inhibition properties: 1,2,3,6-tetra-o-galloyl-β-D-glucose in winter large (327.92 mg/kg plant material) samples and quercetin-3-o-α-L-rhamnoside in summer medium (2216.84 mg/kg).

A significant amount of variation of bioactive compounds of *C. siliqua* was identified amongst the large plants during the season trial. Follow up variation identification trials were carried out based on plant gender and the drying method of leaf material. TLC and the tyrosinase inhibition assay were used for these trials. Female plants of *C. siliqua* were found to have lower IC50 values overall for the tyrosinase inhibition assay. A female plant growing in the LC area in Hatfield was found to have the highest activity with an IC50 value of 103.70 µg/ml. The drying study found that leaves that were crushed after harvesting, without drying, led to an extract with the highest tyrosinase inhibition activity. The IC50 obtained for the fresh leaf extract was 139.60 µg/ml. The IC50 value of the control averaged to 7.75 µg/ml for the variation identification trials.

The present study concluded that NAA was successful, in addition to IBA, as a rooting hormone for apical cuttings of *A. phylicoides*. Bioactivity of *C. siliqua* was found to be higher in summer and autumn.

List of Abbreviations

3T3-L1	Mouse embryonic fibroblast cell line
A1	(-)-epicatechin -3-o-gallate
A2	1,2,3,6-tetra-o-galloyl-β-D-glucose
A3	Quercetin-3-o-α-L-rhamnoside
A4	Myricetin-3-o-α-L-rhamnoside
A5	Myricetin-3-o-glucoside
A6	Gallocatechin-3-o-gallate
AHA	Alpha Hydroxy Acid
ANOVA	Analysis of Variance
ARC	Agricultural Research Council
B16-F10	Mouse skin cells
C2C12	Mouse myoblast cell line
Chang	Human liver cell line
cm	centimetres
CPSC	Consumer Product Safety Commission
DF/df	Degrees of Freedom
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EtOH	99% Ethanol
FCPC	Fast Centrifugal Partition Chromatography

FCR-3	Strain of *Plasmodium falciparum*
FDA	The Food and Drug Administration
g	gram
GA	Gibberellic Acid
HDL-C	High Density Lipoprotein-Cholesterol
HeLa	Human Uterine Cervical Carcinoma cells
HPLC	High Performance Liquid Chromatography
HTLC	High-Throughput Liquid Chromatography
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
IC_{50}	Median Inhibition Concentration
INCI	International Nomenclature of Cosmetic Ingredients
INT	Tetrazolium Salts
K	Potassium
kg	kilogram
l	Litre
L_DOPA	L-3,4-dihydroxyphenylalanine
LB	Landbou area
LBF	Landbou area Female
LBM	Landbou area Male
LC	L.C de Villers area
LCF	L.C de Villers area Female

LCM	L.C de Villers area Male
LC NMR	Liquid Chromatography Nuclear Magnetic Resonance
LCMS	Liquid Chromatography/Mass Spectrometry
LSD	Least Significant Difference
m	metres
MCF-7	Human breast carcinoma cells
ml	millilitres
MIC	Minimum Inhibitory Concentration
mM	Millimolar
MS	Mean Square
MSDS	Material Safety Data Sheet
N	Nitrogen
NAA	1-Naphthaleneacetic Acid
NIH	National Institute of Health
nm	nanometres
NMR	Nuclear Magnetic Resonance
NW2	Natural Science 2 area
NW2F	Natural Science 2 area Female
NW2M	Natural Science 2 area Male
mm	millimetres
mg	milligrams
OD	Optical Density
P	Phosphorus

P (Statistics)	F-ratio probability
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
R^2	Co-efficient of determination
RCBD	Random Complete Block Design
RSD	Relative Standard Deviation
SA	South Africa
SABIF	South African Biodiversity Information Facility
SAS	Statistical Analysis System
SI	Safety Index
SIBIS	SANBI's Integrated Biodiversity Information System
Theo	Theology building area
TLC	Thin Layer Chromatography
TET	Tetrandrine
TMP	Tetramethylpyrazine
USA	United States of America
UP	University of Pretoria
UV	Ultraviolet
UV/Vis	Ultra Violet Visible
VERO	Monkey kidney epithelial cell line
ZA	South Africa
α	alpha
µl	microlitre

Chapter 1

General Introduction

1.1 The importance of plants

Plants are believed to have made their appearance on land during the Palaeozoic Era which began 542 million years ago (Rafferty, 2011), whereas humankind came into existence approximately 3-5 million years ago during the Cenozoic era (Kim and Weave, 1994) as indicated in figure 1.1. This significant difference in time gave the plants the advantage of having more than 500 million additional years to evolve appropriately for survival.

By taking the time factor into consideration, as well as the fact that plants are sessile and respond to the environment accordingly (Huey, *et al*., 2002), it is most obvious to question as to how their survival was possible. A major part of the evolution of plants involved the synthesis of their secondary compounds (Pichersky and Gang, 2000). The role of many of these compounds was to combat damage from herbivores or as a general defence mechanism against insects (Cotton, 1996).

Many scientists once believed that these secondary compounds were waste products (Taiz and Zieger, 2006; Whittaker and Feeny, 1971); and even now, as these compounds are studied, their roles in the plants are not always clear. The discoveries that have been made up to this point however, indicate that secondary plant compounds are responsible for the beneficial and medicinal value of plants to humans (Cotton, 1996).

These spectacular organisms have developed many methods to deal with environmental factors, herbivores and pests, as they are sessile (APSnet, 2013). These secondary compounds that were produced before humans came into existence are beneficial to current human life. Many secondary compounds derived from plants have been discovered to have medicinal and nutraceutical value (Briskin, 2000).

Many currently used medicines contain components that are derived from higher plants. Despite the actual isolation of some of these compounds becoming less common due to the increasing popularity of their synthetic pathways, there are other plants that have gained therapeutical status in recent years. Another interesting point to note is that a number of novel plant-derived substances have entered the Western drug market (De Smet, 1997).

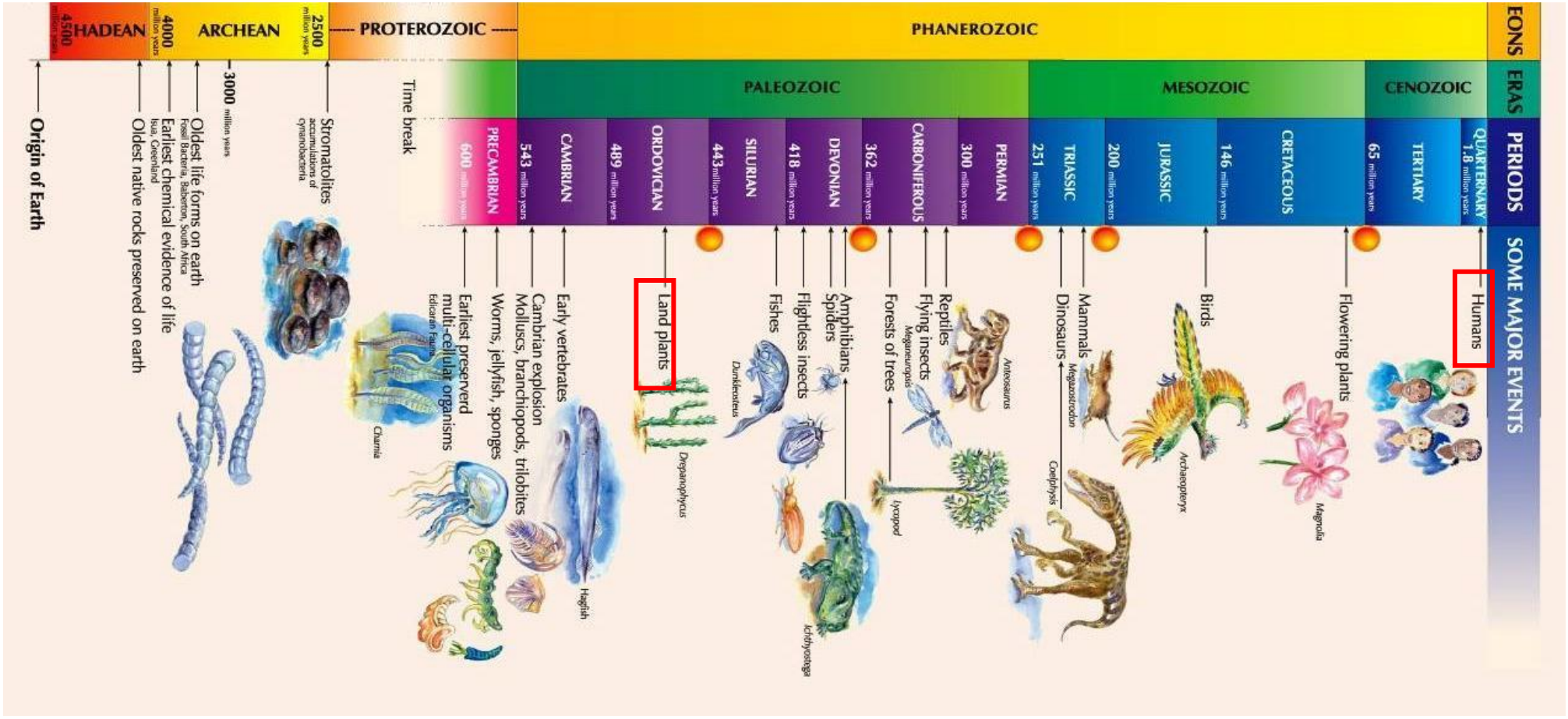

Figure 1.1: Eras of time with the onset of land plants and humans indicated (University of Cape Town, 2013)

Examples of plant derived compounds used for their medicinal properties are shown below in table 1.1. Although purified plant derived compounds are in demand, there is also a market for crude herbal medicines (De Smet, 1997). There are numerous commercially available plant derived medicines, cosmetics and nutritional supplements on the market today (Gellenback, 2012; Gershwin *et al*., 2010; Talalay and Talalay, 2001). The potential to discover novel and more efficient medicinal, cosmeceutical and nutritional compounds from unexplored plant species may seem infinite due to the fact that only a minute fraction of plant life on earth has been explored (Wheeler, 1995). There may even be a plant for every ailment a human being could experience.

Table 1.1: Plant derived drugs valuable for their medicinal potential

Plant derived compounds	Medicinal uses
Taxoids	Anti-cancer properties (Butler and Newman, 2008).
Artemisinin compounds	Anti-malarial properties (Woodrow *et al.*, 2005).
Camptothecins	Anti-cancer properties (Xu *et al.*, 2010)
Tetramethylpyrazine (TMP) and Tetrandrine (TET)	Treatment of cardiovascular disease (Kwan, 1994).
Forskolin	Inhibits platelet aggregation, activates adenylate cyclase (De Souza *et al.*, 2006).
Paclitaxel, vincristine, vinorelbine, teniposide	Anti –tumour properties (Pezzuto**,** 1997).

In addition to these valuable plant derived compounds, there are other imperative functions that plaants play in life on earth. Plants can be said to have made earth liveable for humans. This is indeed the case because life, as it is currently known, would be non-existent without the gas balance provided by plants (The oxygen cycle, 2011). The autotrophic nature of plants allows them to produce food for themselves as well as for all other life forms on the planet. In this process, they emit oxygen and reduce carbon dioxide, thereby making life, as it is currently known, possible. Section 1.2 indicates some general, but imperative functions of plants.

1.2 The general important functions of plants that benefit humans

1.2.1 Food source

The leaves, roots and seeds of many plants serve as foods that are consumable by humans (Better health channel, 2012) as shown in figure1.2 a). A large number of plant products such as maize and cereals serve as food to livestock as seen in figure 1.2 b) (Pimentel and Pimentel, 2003).

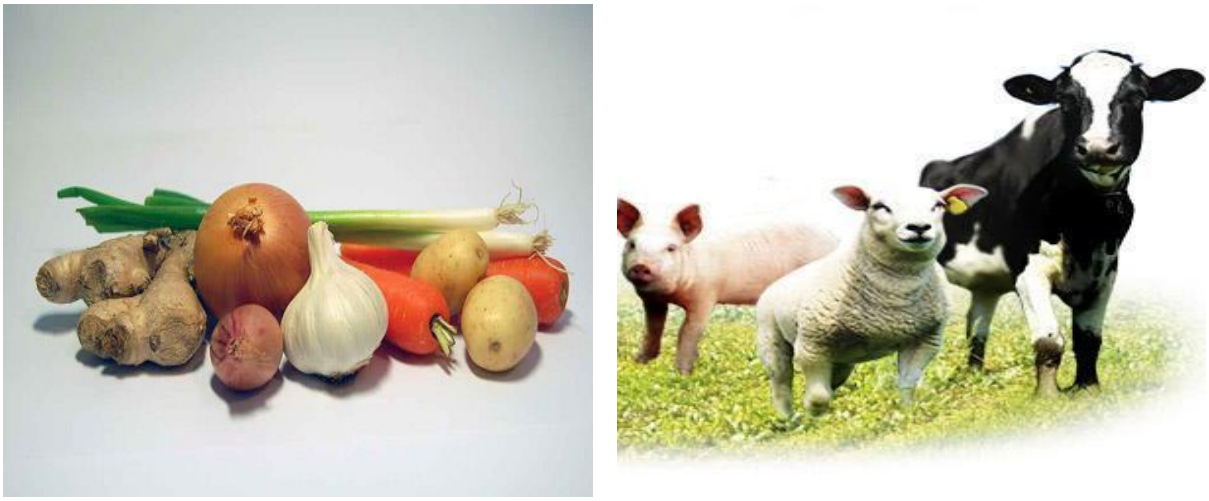

Figure 1.2: a) Roots of plants and livestock that serves as food to humans (Adams county, 2012; No unsacred place, 2011)

1.2.2 Pharmaceuticals and traditional remedies

Many pharmaceuticals are derived from secondary compounds produced by plants. These include: opium, aspirin, cocaine, and atropine (Ghosh, 2000).

1.2.3 Gas balance

Plants provide oxygen globally while reducing carbon dioxide as shown in figure 1.3, thus providing a gas balance for all life on earth (Importance of plants to human life, 2011).

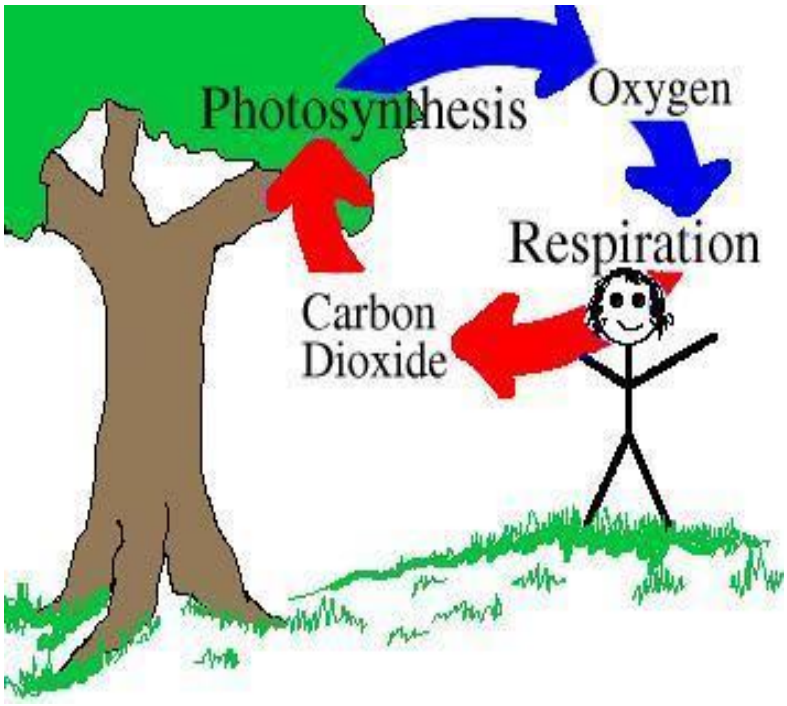

Figure 1.3: Gas balance maintained by plants (The oxygen cycle, 2011)

1.2.4 Provision of materials

Plants provide material that can be used for the building of shelters and structures. Many by-products such as dyes and resins are provided by plants (Importance of plants to human life, 2011).

1.2.5 Environment controllers

Trees and plants can aid as environment adjusters: windbreaks, shade providers, erosion reducers, etc. (Importance of plants to human life, 2011)

Plants have existed and evolved for thousands of years before the onset of human existence and their presence created conditions that allowed humans to flourish as a life form. For the purpose of the present study, the function of plants as cosmeceuticals and teas will be elaborated on.

1.3 Background on cosmetics and the role of plants in this field

From ancient times, women have focused a lot of attention on their appearance. This entailed the use of materials to enhance their natural beauty and to conceal any imperfections. Many of these materials were developed into cosmetics. Although the use of the materials by women of today has not changed, the materials themselves have. Cosmetics have become more varied and technologically advanced in recent time (Rinaldi, 2008).

Philosophically, one of the meanings of the word cosmetic is "to correct defects, especially of the face" or "to make beautiful, with focus on the complexion" (Oumeish, 2001). The Consumer Product Safety Commission (CPSC) has defined a cosmetic product as 'any substance or preparation intended for application to any external surface of the human body 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 diseases' (Aburjai and Natsheh, 2003).

A relatively new concept,"cosmeceuticals" has emerged to describe a hybrid between drugs and cosmetic products. The application of a cosmeceutical is able to enhance both the health and appearance of the skin. The field described by this term fills the gap between skin disorders, cosmetics and pharmaceuticals, as shown in figure 1.4 (Pieroni *et al*., 2004). These cosmeceutical products can include skin moisturisers, lipsticks, hair colours, shampoos, facial make-up and perfumes (Dureja *et al*., 2005).

With reference to plants and cosmetics, it may be surprising to know that at one stage, plants were the main source of all cosmetics. This was before the popularity of synthetic products. In recent times, the popularity of plant derived extracts in cosmeceuticals has increased. This is due to a number of reasons: people are moving in the direction of "going green", the controversy surrounding many currently used actives is instilling fear in individuals and the scientific research on plants is becoming extensive and giving many positive results (Aburjai and Natsheh, 2003; Mahé *et al*., 2003).

Due to the large variation and abundant species of plants, it is simple to apply one plant to a specific skin disorder. This appeals to consumers and also educates them on the different plants and their uses (Aburjai and Natshah, 2003; Mahé *et al*., 2003).

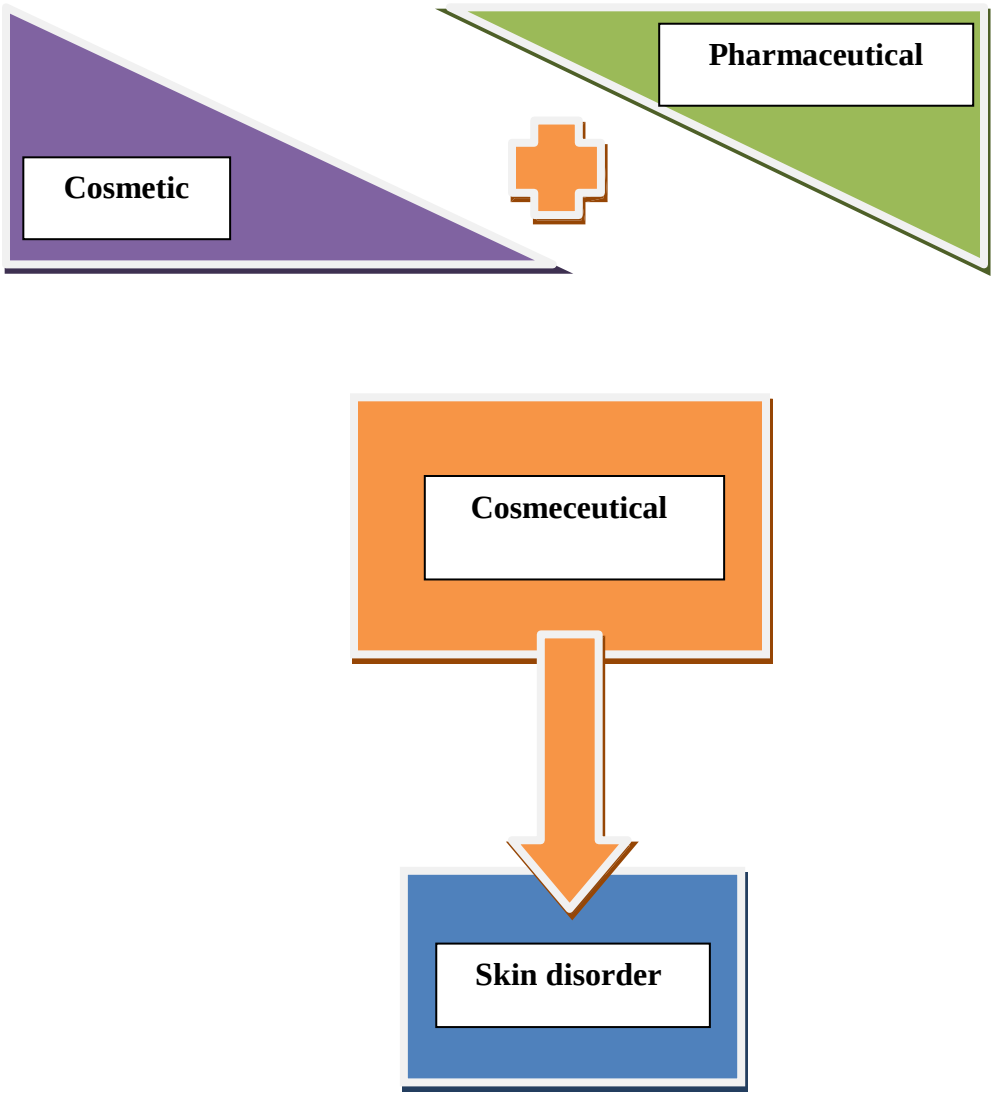

Figure 1.4: The combination of two fields, cosmetics and pharmaceuticals, to alleviate skin disorders more efficiently

Many groups of people have used plants for hundreds of years as a remedy to inflammation, acne and pigmentation (Kapoor and Saraf, 2011; Messele, 2004; Yokota *et al.*, 1998). A number of plants used in cosmetics for skin hyperpigmentation problems are shown in table 1.2.

Table 1.2: Usage of active compounds derived from plants in cosmetics (Yokota *et al.*, 1998)

Plant name	Active compounds	Description
Glycyrrhiza glabra	Hispaglabridin, glabridin and isoliquiritin	Liquorice extract has a mechanism similar to that of kojic acid. This compound can prevent and treat hyper-pigmentation and inflammation by inhibiting the tyrosinase activity in skin cells and has shown no effect on DNA. Liquorice is used in Egypt. (Yokota *et al.*, 1998).
Paeonia suffruticosa	Resacetophenone	Dihydroxyacetophenone (resacetophenone) is present in various peony extracts. Resacetophenone works by inhibiting the formation of tyrosine itself.
Kaempferia pandurata	Isopanduratin	The active compound possesses a related trihydroxybenzene chemical structure.
Alpinia galangal	Galangin	The active compound possesses a related trihydroxybenzene chemical structure.
Arctostaphylos uva ursi	Arbutin and methyl arbutin	The active compounds possess skin-lightening properties

Mitracarpus scaber	Harounoside	The active compound is a hydroquinone derivative with strong anti-tyrosinase activity.
Asparagus officinalis	Glutathione	The reduced form of glutathione has a dual role in the de-pigmentation of coloured skin.

1.4 Plants in the form of teas

Tea is a popular, socially acceptable and economical beverage that is enjoyed throughout the world as illustrated in figure 1.5. It is the second most consumed drink in the world; with water being the first. Tea has been consumed for centuries; however health benefits of this drink has only been researched for past few decades. Tea can be called nature's gift or nature's healthy helper as it contains many actives that are said to affect the pathogenesis of many chronic diseases (Khan and Mukhtar, 2007).

Figure 1.5: Infusion of the tea leaves shown a) green, b) yellow, c) oolong and d) black tea (Katherine's tea pot, 2011)

The polyphenol epigallocatechin-3-gallate found in tea was found to be responsible for disease prevention (Khan and Mukhtar, 2007). In addition to the polyphenols, antioxidants, vitamins and some minerals are also found in tea. The result of the consumption of these compounds may benefit the health of the heart, kidneys and teeth (Trevisanato and Kim, 2000). In addition to the properties of the tea, the method of boiling water as a component also contributes to the safety of a cup of tea (Weisburger, 1997).

Dufresne and Farnworth, (2001) concluded that tea is not merely a drink, but a source of health benefits. Tea is available in the classic sense as well as in capsules and in cool forms in stores. Tea has the potential to be used as a food additive, for diabetes and hypertension (Dufresne and Farnworth, 2001).

Substantial research has indicated the beneficial effects of tea on cardiovascular disease and for the prevention of cancer. These results however were found in animal models and not in humans. One possible reason for the differences in findings is the amount of tea consumed. Researchers believe that higher amounts of tea should be consumed to see positive effects on health; but they are also concerned that too much tea may affect one negatively due to the over consumption of caffeine and polyphenols (Yang, 1999).

Research carried out by Weisburger and Chung, 2002, revealed the possible ways in which tea is beneficial to health. Studies were carried out on black and green tea. The polyphenols found in tea have strong antioxidant effects that lead to a lower risk for heart disease. Polyphenols also play a major role in the excretion of carcinogenic metabolites as well as lowering the rate of cell replication, which reduces the risk of cancer. Tea was also found to promote the good health of the intestine (Weisburger and Chung, 2002).

Extensive research is required to be carried out to confirm and investigate the details of the mechanism of tea compounds on good health (Trevisanato and Kim, 2000). It is evident that the consumption of this drink has positive effects and that research and the introduction of new teas will further benefit consumers.

1.5 Project Focus

The present study investigates the various ways of cultivating two commercially important plants.

1.5.1 *Ceratonia siliqua L.:* Potential in the cosmeceutical field

Ceratonia siliqua (C. siliqua) is indigenous to the Middle East countries. This plant grows abundantly and easily in South Africa. *C.siliqua* is commonly known as carob or the carob tree. This plant has many traditional uses and is well known as a source of food to humans and animals. Previous research completed on extracts prepared from the leaves as well as six bioactive compounds isolated from this plant, indicated significant anti-tyrosinase activity and melanin inhibition properties. Clinical trials established the safety of the extract of *C. siliqua* as well as its efficacy as a de-pigmentation agent (Batlle and Tous, 1997; Momtaz, 2007).

Due to the commercial importance of *C. siliqua*, it is important to know which season is ideal for harvesting the leaves of the plant to ensure its efficacy. Hence, the evaluation of the seasonal variation of bioactive compounds was one of the objectives of the study.

1.5.2 *Athrixia phylicoides* DC.

A. phylicoides is a leafy shrub that is indigenous to South Africa. This plant is commonly known as bush tea and is used in many parts of the country as tea. The infusion of the leaves has been claimed to have many medicinal properties. This plant was found to have a high anti-oxidant and mineral content. *A. phylicoides* is of high commercial value. The products resulting from this plant will benefit many, as discussed above; tea is an additional source of nutrients for humans (McGaw *et al.*, 2007; Mudau *et al*., 2008; Olivier *et al*., 2012; Joubert, *et al.*, 2008).

Hence, the aim of the study was to optimise the propagation of *A. phylicoides*. In addition to this, the plant was also evaluated for its cosmeceutical potential to investigate if there can be additional economic value of this commercially important plant. The motivation for these screening assays was the high anti-oxidant content of the plant.

1.6 Research Questions

Ceratonia siliqua

- At which stage of the plant's growth are the bioactive compounds produced?
- Is there a difference in the presence and concentrations of these actives over the four seasons?
- Is there a difference in the presence and concentrations of these actives between male and female trees?
- For the manufacturing of raw materials, what kind of drying process is ideal for leaves?

Athrixia phylicoides

- What is the effect of four different growth hormones on promoting the rooting of apical cuttings?
- Do the alcoholic extracts prepared from the leaves at each season possess any cosmeceutical activity?

1.7 Objectives of the study

- The determination of the concentration of bioactive compounds from *C. siliqua* amongst three age groups
- The determination of possible seasonal variation of the bioactive compounds in *C. siliqua*
- The determination of the effect of four rooting hormones on growth of apical cuttings of *A. phylicoides*
- The determination of any cosmeceutical activity of alcoholic extracts from the leaves of *A. phylicoides* prepared in four different seasons

1.8 Structure of the thesis

Table 1.3: Structure of thesis

Chapter	Section	Description
1		This chapter provides a brief and concise introduction to the importance of plants. The objectives and motivation for the project are also mentioned.
2	1	The background on *A. phylicoides* is provided.
	2	The hormone field trial carried out on *A. phylicoides* is reported.
	3	The results of different bio-assays carried out on the leaf extracts of *A. phylicoides* prepared over four different seasons are discussed.
3	1	The background on *C. siliqua* and bioactive compounds isolated from this plant are provided.
	2	The seasonal variation of bioactive compounds of *C. siliqua* is presented together with anti-tyrosinase activity over different seasons and at different stages of plant growth.
	3	The difference between male and female trees of *C. siliqua* with regard to the presence of bioactive compounds and anti-tyrosinase activity is discussed.
4		This chapter presents a general discussion and conclusion. General recommendations for future studies are suggested.
5		Acknowledgements
6		Appendices

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Chapter 2

Propagation studies on apical cuttings of *Athrixia phylicoides* DC.

Chapter 2

Section 1

Background Information on *Athrixia phylicoides* DC.

2.1 General background

Athrixia phylicoides DC. (*A. phylicoides)* is also known as bush tea and is indigenous to South Africa. This plant has been used as an infusion for many years by the native people of South Africa and is most popular in rural areas (McGaw *et al.*, 2007). Other common names of this plant are bushman's tea, boesmanstee, Icholocholo, itshelo and umthsanelo (Athrixia phylicoides^{DC}, 2005).

A. phylicoides is a leafy shrub with woolly white stems as seen in figure 2.1. It is an aromatic and perennial plant. The flowers of *A. phylicoides* range from pink to purple with bright yellow centres. The leaves of this plant are simple and alternate. The leaves appear dark green and shiny closer to the base and they progress to a woolly whitish colour as they reach the apical region (Van Wyk and Gericke, 2000). This plant can be grown from cuttings and is a beautiful and relatively hardy garden filler (Mbambezeli, 2013).

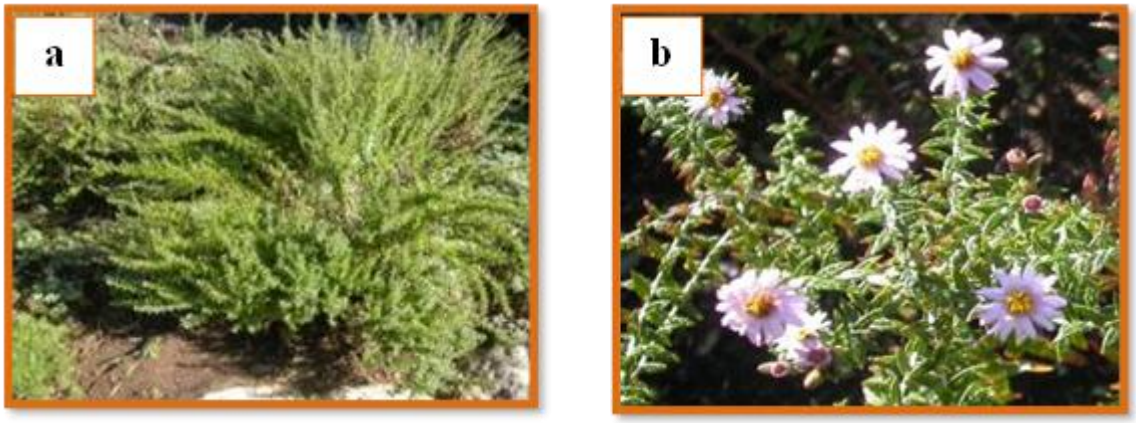


Figure 2.1: *A. phylicoides* a) before flowering and b) with flowers (*Athrixia phylicoides* DC, 2005)

As mentioned in the introductory chapter, the infusion made from bush tea has been claimed to have medicinal value but the tea has not been commercialised. When compared with other teas, bush tea was found to have similar properties in terms of antioxidant activity and therefore, has large commercial potential.

2.1.1 Taxonomy and distribution

Family: Asteraceae

Genus: *Athrixia*

Species: *phylicoides*

The genus name of this plant, *Athrixia*, is derived from the Greek word *thrix* which means hair. This name was given to the plant due to the appearance of the leaves. The species name of the plant, *phylicoides*, is derived from the word Phylica (Athrixia phylicoides^{DC}, 2005).

A. phylicoides belongs to the Asteraceae family also known as the Daisy family (The free dictionary, 2013). The Asteraceae family is amongst the largest families of flowering plants. This family is also predominantly herbaceous (Van Wyk and Van Wyk, 1997). There are 14 species of *Athrixia*, nine of which are endemic to South Africa. *A. phylicoides* is widely distributed throughout South Africa, from the Eastern Cape in the south to the Limpopo Province in the north (Van Wyk and Gericke, 2000). This shrub is found in grasslands, forests, bushvelds and rocky and sloping habitats (Mbambezeli, 2013). The distribution of *A. phylicoides* is indicated in figure 2.2.

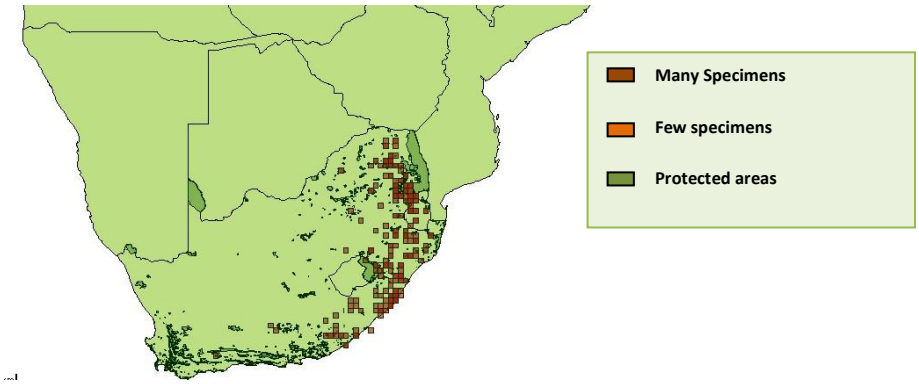


Figure 2.2: Distribution of *A. phylicoides* (SIBIS: SABIF, 2013)

2.1.2 Documented medicinal properties of bush tea

- Potential treatment for diabetes (Chellan, 2012)
- Moderate anti-cancer activity (Fouche *et al.*, 2008)
- Cough remedy and purgative (McGaw *et al.*, 2007)
- Blood cleanser, analgesic, promotes wound healing (Mudau *et al.*, 2008)

- Treatment for acne, infected wounds, infected throats and boils (Joubert, *et al.*, 2008)

2.1.3 General Research completed on *A. phylicoides*

2.1.3.1 Economic uses

Along with the traditional medicinal uses of this plant, it has been reported that it has traditional economic uses as well. The medicinal uses include: the infusion of leaves to prepare a beverage, as anti-helminthics and the chewing of the hard broom material for soothing sore throats (Mbambezeli, 2013).

It has been stated, that to obtain optimum polyphenol content and anti-oxidants, the tea should be brewed at 90ºC for 3 minutes. In an earlier study, it was also found that in the combination of bush tea and black tea (*Camellia sinensis*), the total polyphenol, antioxidant and tannin content decreased when compared to teas brewed individually (Shonisani *et al.*, 2010).

2.1.3.2 Compounds isolated from *A. phylicoides*

The major chemical compounds of the essential oil prepared from the aerial parts of the plant were found to be α-pinene, β-pinene, caryophyllene oxide, β-carophyllene, myrcene and spathulenol. Safety indices (SI) for the methanol extract and the essential oil was reported as 2.28 and 1.77, respectively (Padayachee, 2010).

It was found that phenolic acids were the major phenols in the dry extracts of *A. phylicoides*. 5-hydroxy-6,7,8,3',4',5'- hexamethoxyflavon-3-ol was found to be a unique compound identified in some of the extracts tested. It was found that the most suitable extraction procedure to prepare a dry ethanol extract of *A. phylicoides* with regard to antioxidant activity involved extraction with ethanol (50%) over 20 minutes at 50ºC. The extraction was carried out at volume to mass ratio of 50:3 (Mashimbye *et al*., 2006).

Counter current chromatography was used along with other techniques to isolate 6hydroxyluteolin-7-O-β-glucoside and quercetagetin-7-O-β-glucoside from this plant with the former being the major antioxidant found and the latter, the minor antioxidant (de Beer *et al*., 2011).

The bitterness enhancing principle of *A. phylicoides* was found to be quercetin-3′-Oglucoside. With the use of High Throughput Liquid Chromatography (HTLC), Fast Centrifugal Partition Chromatography (FCPC), High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) and LC Nuclear Magnetic Resonance (LC NMR), three polymethoxylated flavones were isolated (Reichelt *et al*., 2012).

Three flavonoids were isolated from *A. phylicoides* using silica and sephadex column chromatography: 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol(1), 3-0-

demethyldigicitrin (2), and Quecertin (3). The crude extract had an inhibition concentration

(IC_{50}) value of 10.64 ± 0.08 µg/ml for anti-oxidant activity. All flavonoids displayed higher antioxidant activities than the crude extract with compound (3) having the lowest IC_{50}. The cytotoxicity of the extract and flavonoids was tested on Vero cells. The extract was found to be non-toxic. Compound (3) had minimal toxicity while compounds (1) and (2) were found to be highly toxic to Vero cells (Mavundza, *et al*., 2010).

2.1.3.3 Anti-malarial, anti-diabetic, anti-inflammatory, anti-bacterial and anti-oxidant activity

The anti-malarial activity of the essential oil prepared from the aerial parts of *A. phylicoides* was reported as having IC_{50} = 1.006 ± 0.06 µg/ml against the chloroquine-resistant FCR-3 strain. The essential oil and methanol extract of *A. phylicoides* were tested for antiinflammatory activity. The essential oil displayed anti-inflammatory activity with an IC_{50} = 25.68 µg/ml (Padayachee, 2010).

A hot aqueous extract of *A. phylicoides* stimulated *in vitro* glucose uptake and metabolism in the following cell lines: Mouse myoblast cell line (C2C12), Human liver cell line (Chang) and Mouse embryonic fibroblast cell line (3T3-L1), with minimal toxicity. This was due to the polyphenol content of the plant. The most prominent phenolic compounds in the extract were found to be 6-hydroxyluteolin-7-O-glucoside, chlorogenic acid, protocatechuic acid, a di-caffeoylquinic acid and a methoxy-flavonol derivative. It was reported that this plant may have use in the treatment of obesity and Type 2 Diabetes mellitus (Chellan *et al*., 2012).

The methanol extract prepared from the aerial parts of the plant displayed higher activity against microorganisms tested than the essential oil. The extract displayed highest activity against *S. aureus*, *B. cereus* and *B. subtilis* (MIC= 1.0 mg/ml). The methanol extract and

essential oil were found to be more selective against Gram-positive bacteria than Gramnegative bacteria (Padayachee, 2010).

A study was carried out to determine any seasonal variation in the antioxidant activity of wild *A. phylicoides* leaves. The results indicated that the total antioxidant content was lowest in autumn and spring and highest during winter and summer (Mudau *et al*., 2008).

2.1.3.4 Microscopic studies

The essential oil produced by the leaves has anti-inflammatory, antihypertensive, narcotic and analgesic properties. A study explored the structure responsible for producing the oil with light microscopy; it was found that non-glandular and glandular trichomes are present in the leaves (Moller *et al.*, 2013).

2.1.3.5 Toxicity studies

A. phylicoides was found to have slight diuretic properties in rats when an in-depth study was carried out on the toxicity of the plant. After acknowledging that the plant was not toxic to brine shrimp and renal cell lines, toxicity was assayed on Wistar rats. No toxicity was found in any of the tissue tested (Chellan *et al.*, 2008).

The aqueous extracts of *A. phylicoides* showed no toxicity to brine shrimp or Vero kidney cell lines. The ethanolic extract did, however, display some toxicity to brine shrimp (McGaw *et al*., 2007).

2.1.3.6 Cultivation studies

The optimal levels of nutrition for growth of *A. phylicoides* were found to be at 300, 200 and 300kg/ha of nitrogen (N), potassium (K) and phosphorus (P), respectively (Mudau *et al*., 2007). This specific administration of the respective elements was found to increase the dry and fresh shoot mass of the plant, plant height, number of leaves and branches and leaf area as well (Mudau *et al*., 2007). This combination and level of application of these nutritional elements increased the concentration of polyphenols in bush tea regardless of the season (Mudau *et al*., 2006). It was also found that the concentration of tannins increased with the application of N at 300 kg/ha (Mudau et al., 2006). The application of K and P was found to increase the concentration of tannins in bush tea (Mudau *et al*., 2007).

In order to optimise the amount of hydrolysable tannins in autumn and winter and condensed tannins in spring and summer, it is recommended that 300kg ha^{-1} N be applied to the soil (Mudau, *et al*., 2008).

The quality of the plants was determined and it was found that newly cultivated and harvested plants contained a high amount of polyphenols. A high amount of tannins was found in the whole plant. The new and old growths of wild bush tea were found to have a high content of polyphenols and antioxidants, respectively. Pruning of bush tea led mainly to yield loss with a higher amount of polyphenols in un-pruned bush tea. The application of gibberellins to bush tea led to increased growth, however the antioxidant and polyphenol content was found to decrease with the increased application of gibberellins. Tannins were found to peak at the application level of 2% (Maudu, 2010).

A study was carried out to determine the chemical composition of wild bush tea growing at eight locations that differed in altitude, climate and edaphic factors. The polyphenol content, antioxidants and tannins were determined for each sample. The highest polyphenol content was found in Haenertsburg where the altitude was at 1 410 m. The highest tannin content was observed in Levubu. The total antioxidant content of the samples remained the same in all 8 locations (Nchabeleng, *et al*., 2011).

The effect of different amounts of water to fertigate plants was examined in relation to the growth of *A. phylicoides.* The difference between growing *A. phylicoides* in sand and in pine bark was also observed. The results of the study indicate that fertigation with 1 litre (L) of water a day gave optimal growth, even for plants grown in sand. This result contrasted with the results obtained by Araya (2005), who generally found better results with plants grown in pine bark than sand (Rakuambo, 2007).

2.1.4 Research on hormone rooting trial

2.1.4.1 In support of Commercialisation

The infusion produced from *A. phylicoides* was found to have a higher mineral content than other local teas (rooibos, honeybush, green) and non- local black and green teas (Olivier *et al*., 2012).

The antioxidant activity of *A. phylicoides* was found to be comparable to the dry extract of honeybush tea (*Cyclopia spp*.) and lower than that of rooibos (*Aspalathus linearis*). The optimised extraction procedure resulted in an extract with comparable antioxid ant activity to fermented and unfermented commercial rooibos (*Aspalathus linearis*) dry ethanol extract (De Beer and Joubert, 2009).

The potential for the commercialisation of this plant was emphasised by the results of a survey carried out with locals in the Limpopo Province, regarding *A. phylicoides*. The respondents of the survey expressed their familiarity with the plant and their usage of it as material for brooms, as tea and medicinally. The harvesting methods used on these plants, however, may threaten the future survival of the plant (Rampedi and Olivier, 2005).

An informal statement by an individual claimed that the grasslands of Limpopo are at risk of being over harvested. *A. phylicoides* is one of the contributors to this grassland. The leaves and, unfortunately, the roots are harvested to treat boils, wounds, etc. This places a substantial amount of pressure on the survival of the plant. Commercialisation of this plant would therefore, ensure survival of such a valuable plant (Dzerefos, 2011).

The roots and leaves of *A. phylicoides* can be used to treat coughs (McGaw, *et al*., 2008). The usage of the root material is not sustainable. Locals may over harvest this plant and threaten the future survival of it. With respect to this matter, the propagation of this plant on a commercial scale may ensure survival.

2.1.4.2 Propagation trials

The propagation trial in this study aimed to identify whether cuttings could be grown successfully under the outlined parameters.

Araya (2005) investigated the propagation of *A. phylicoides* in which it was found that apical cuttings had a higher number of roots, longer roots, and rooting percentage than basal cuttings. The use of pine bark increased the number of roots per cutting when compared with cuttings grown in sand. The use of Indole-3-butyric acid (IBA) increased the number of roots produced as compared to when no rooting hormone was used. The rooting of cuttings with respect to length was improved during autumn and with respect to number in spring (Araya, 2005).

The beginning of this section clearly indicated that the next obvious step to take with regard to *A. phylicoides* is to commercialise this plant as tea. However, it is necessary for

propagation trials to be carried out so as to determine the viability of producing the tea on a sufficient scale as a widely available product. It is important in commercial propagation systems to initially carry out small propagation trials before propagation is carried out on a large scale (Hartmann, *et al*., 2010). Some examples to support this are:

Burt reported in 2001 that the growth of green tea in Australia would require propagation of plants by cuttings. The mother plant material should be maintained with cuttings taken from the source and re-established. In this way the same product is ensured with the same mineral, antioxidant and tannin content. A variable in this system could be season. Tissue culture is not recommended for the propagation of tea plants as it is laborious and expensive in comparison to simply growing these plants from cuttings (Burt, 2001).

In 2012, Street and Prinsloo, reported the traditional usage, toxicity, biological activity and cultivation and conservation of ten important medicinal plants. These include plants such as *Agathosma betulina, Aloe ferox* and *Aspalathus linearis. Hypoxis hemerocallidea,* a plant used for its anti-cancer and cardiac disease prevention and immune boosting properties, was also mentioned in this list. *H. hemerocallidea* requires a more efficient propagation method in order to provide enough material on a sufficient scale to supply to the market at a reasonable cost. Studies are being carried out on seed propagation of this plant because even though this plant grows easily in a range of environments, growth of large quantities is a challenge (Street and Prinsloo, 2012).

In general shrubs are propagated by cuttings; therefore *A. phylicoides* was propagated in this study by cuttings (Hartmann, *et al*., 2010).

2.1.4.3 Rooting hormones in trials

The growth of plant material from asexual reproduction has been used in horticulture for many centuries. There are many factors that affect the ability of cuttings from a mother plant to re-establish itself as an independent plant (Couvillon, 1988). These include:

Chemical factors--endogenous, exogenous

Plant factors--age, cutting type

Environmental factors--humidity, light, heat (Couvillon, 1988)

Some examples that support the use of hormones for rooting purposes are:

It was found that poor adventitious root formation can be a major hindrance in the propagation of cuttings. The use of an auxin, such as IBA was found to enhance root formation and can be used in combination with other methods (De Klerk, 2002).

A combination of IBA and 1-Naphthaleneacetic acid (NAA) was used along with other components to lead to the successful propagation of *mesquite (Prosopis*) cuttings. Natural and seed propagated plants contained large variations and clonal propagation was required to decrease genetic variability (Felker and Clark, 1981).

A study of the success of rooting of *Chromolaena odorata* that investigated the effect of IBA in combination with growth medium concluded that optimal propagation of stem cuttings was achieved when 0.7% of IBA was combined with different rooting media (Anyasi, 2011).

The studies outlined above contribute to the data required for a plant to be commercialised. In addition to these studies, the present study investigated additional hormones that could be used to enhance rooting. Enhanced rooting could lead to a higher success rate of plants that were propagated through cuttings and not seeds. A large amount of plant material could then be grown and harvested for commercial purposes. The potential of the extract of this plant to be used in the cosmetic field was also studied. This study investigated the effect of exogenous chemical factors on the rooting of *A. phylicoides* cuttings.

2.1.5 Background contributing to this study

It was found, from previous studies that apical cuttings gave rise to a higher root length, number and percentage of roots than basal cuttings. The survival percentage of the apical cuttings with 2-3 leaves was also higher than for the basal cuttings (Araya, 2005). Previous studies also indicated that the cuttings grew best in autumn (longer roots) and spring (number of roots) as compared with growth in winter and summer. It was also discovered that pine bark promoted the growth of a higher number of roots as compared to sand (Araya, 2005).

Previous studies indicated successful propagation of *A. phylicoides* with hormone supplementation. The hormone (IBA at 1 mg/ml and 3 mg/ml) was applied, in previous studies, in powder form after dipping the cut end of a cutting in distilled water (Araya, 2005). In 2005, Araya reported the effect of the application of IBA to bush tea cuttings in powder form. The present study aimed at assessing the difference between a dry and liquid application of IBA as well as the effect of additional rooting hormones. The cuttings were dipped in the hormone solutions for a few seconds and then planted as this is the recommended method of treatment (Gpn, 2014).

Previous studies with honeybush involved dipping the cuttings in liquid hormone solutions for a few seconds. This study concluded that the hormone treatments had no positive effect on rooting for honeybush, and yet the study aimed at determining its impact on *A. phylicoides* (Mbangcolo, 2008). If soaking occurs for too long the plant may be affected negatively, therefore the choice of dipping for a few seconds was decided upon as an intermediate period (Gpn, 2014).

2.1.5.1 Hormones

Auxins

Three of the hormones used in this study were auxins. Auxins are compounds that induce cell growth by inducing cell elongation. The term auxin is derived from a Greek word "auxein'', which means 'to grow' (Plant Hormones, 2013). These compounds are significant for the response of plant to light and gravity. Auxins play an important role in the elongation of lateral shoots and roots, formation of vascular tissue and the development of the embryos (Hobbie, 2013).

Functions of Auxins

A list of some of the functions that auxins are responsible for is given below: (Davies, 1999; Mauseth, 1991; Raven, *et al*., 1992; Salisbury and Ross, 1992).

- Stimulates cell elongation
- Stimulates cell elongation
- Stimulates differentiation of phloem and xylem
- Stimulates root initiation on stem cuttings
- Delays leaf senescence
- Delays fruit ripening
- Stimulates growth of flower parts (Plant Hormones, 2013).

Gibberellins

This class of hormones are derived from gibberellic acid. These hormones promote flowering, elongation of stems and break dormancy of seeds. Gibberellins are natural

hormones. This hormone has vast usage in the field of grain production. In general, they can be used to shorten and therefore strengthen stems for better resistance to weather conditions (Biosynth, 2006). The hormones and their structures that were used in this study are shown in Table 2.1.

2.1.5.2 Justification of hormones used

IAA and IBA were found to aid in the rooting of the cherry rootstock, with IBA being the better hormone in terms of earlier root formations and root numbers (Štefančič, *et al*., 2005). A number of articles reported similar results that agree with the efficacy of IAA and IBA in terms of rooting. IBA was also chosen as a reference point as many studies were carried out with this hormone on *A. phylicoides*. NAA has been reported to have growth regulation properties which may include root formation for the plant in question. GA is normally used for stem growth and is included in this study to observe any positive effect on rooting.

For the plantation of cuttings, seedling trays with deep wells were selected. Figure 2.3 illustrates the length of the well (8 cm). Previous research found deeper wells to support successful rooting of cuttings (Gilman and Harchick, 2008). This finding was outlined in the following study. The use of pines propagated through cuttings has become popular due to their demand in the forestry industry.

These pine cuttings are grown almost exclusively in containers in South Africa. The propagation of cuttings in containers can have a negative effect on plant growth if the cuttings are allowed to grow beyond the constraints of the container. A study was then carried out to investigate the effects of tray types amongst other factors on the field growth of pine cuttings. The study concluded that increased media volumes for the cuttings promoted a higher success rate in the field (Gilman and Harchick, 2008). An increased media volume requires a larger well for the cutting to be rooted in.

Table 2.1: Hormones used in the present study

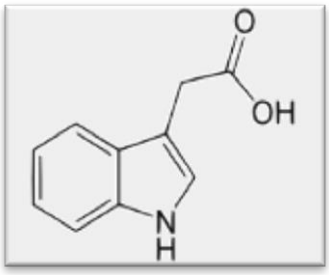
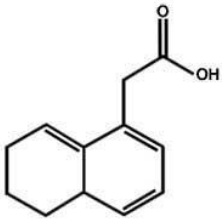
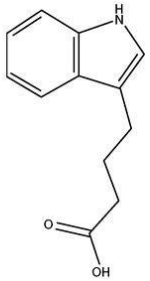
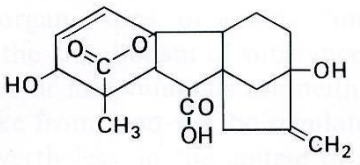
Hormone	Abbreviation	Structure	Information
Indole-3-acetic acid	IAA		Auxin-Induces cell elongation and cell division (USCN, Life Sciences.INC, 2013)
1-Naphthaleneacetic acid	NAA		Auxin-Growth regulator (BioWorld, 2013)
Indole-3-butyric	IBA		Auxin-Synthetic auxin (Bio World, 2013)
Gibberellic acid	GA		Gibberellin-Stimulates cell elongation (Sheilds gardens, 2013)

Figure 2.3: Length of the seedling wells

2.1.6 Background on bioassays

In section 3 of this chapter, the anti-tyrosinase and anti-acne assays were carried out on the ethanol extracts of *A. phylicoides*. Many teas are used in the cosmetic field for their high antioxidant activities and soothing effects. This plant was investigated to identify any potential cosmetic value.

2.1.6.1 *Aspalathus linearis* as a model tea plant

The infusion of rooibos is a popular beverage; however the use of this extract in cosmetics is also becoming common.

Rooibos extract is obtained from the plant *Aspalathus linearis (A. linearis).* This plant belongs to the Fabaceae family and grows in the Western Cape of South Africa in the Cedarburg Mountains. This plant is infused to obtain rooibos tea which is caffeine free and rich in polyphenols. This tea also has antioxidant and anti-mutagenic properties (Iswaldi *et al*., 2011). Figure 2.4 illustrates the bushes of rooibos.

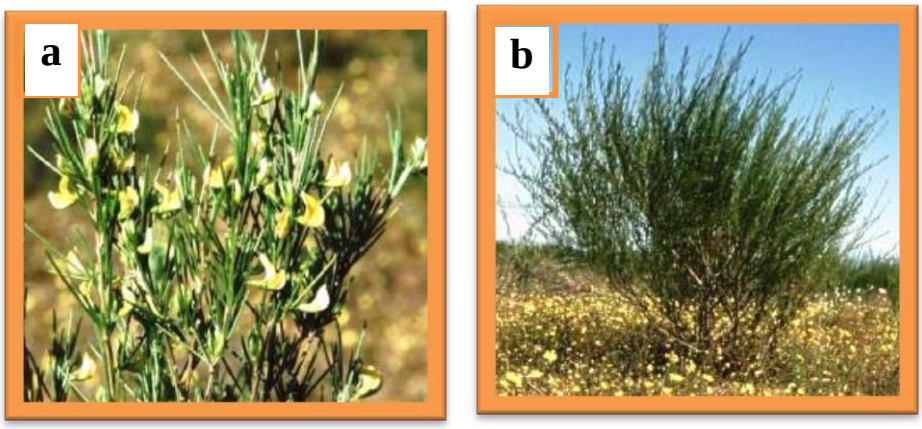


Figure 2.4: a) Flowers and b) bushes of rooibos (*Aspalathus linearis*, 2007)

The antioxidant activity of rooibos was found to be higher than that of black and oolong tea according to the DPPH radical scavenging method. Rooibos extract was found to contain a number of flavonoids such as aspalathin, orientin, vitexin and quercetin. In addition, the phenolic acids found in this include caffeic acid, vanillic acid and syringic acid. These compounds contribute to the antioxidative properties of the rooibos plant extract (Gadow *et al.*, 1997).

According to a global leading company involved with cosmetic products, Symrise, rooibos has substantial potential in the cosmetic field. This company found that a neo-extrapone organic extract of this plant had a similar efficacy as green tea. Despite the bioactive compounds being different in these two plants, their antioxidant activity is comparable. Due to this finding, the extract is used as an active in a number of their current organic products (Cosmetics design.com, 2014). The wide array of uses in the cosmetic field that is fulfilled by rooibos extract has been patented by Nestec. The patent claimed that this extract was able to improve the health of skin and hair (Roger and Crespy, 2010).

The South African Rooibos Council offers extensive information about rooibos. The easy to make rooibos infusion has been recommended to improve the shine of hair, combat acne and revive tired skin and eyes. The alpha hydroxy acids (AHAs), antioxidants and minerals present in the plant are responsible for it's reported soothing, protective and anti-ageing properties (Cosmeticweb, 2009).

There are a number of local companies that include rooibos products in their range such as Annique, African Extracts Rooibos and Clicks Skincare Collection (Cosmeticweb, 2009). Figure 2.5 illustrates products from the Clicks Skincare Collection.

Like the extract of *A. linearis*, *A. phylicoides* was also found to be caffeine free and quercetin was a common compound isolated from both these plants. Two additional flavonoids were isolated from the aerial parts of *A. phylicoides*: 5-hydoxy- 6,7,8,3',4',5-hexamethoxyflavon3-ol and 3-0-demethyldigicitrin (Mavundza *et al.*, 2010; McGaw *et al.*, 2007). The research carried out on *A. phylicoides* is not as extensive as that for *A. linearis*, however, studies like these contribute to the knowledge of the plant as a potential tea and active in cosmetics.

2.1.6.2 Skin care and the role played by plants

Individuals care deeply for the appearance and health of their skin in current times. The stereotype that females have higher concerns is being disproved. This is evident when one notes the number of products aimed at males. These products range from general hygiene to moisture and beauty enhancing products. Skin is an important barrier to the conditions faced by the body and therefore undergoes a number of stresses. On a superficial level, however, the skin is what others initially see when they look at you. For this reason, individuals generally pay a substantial amount of energy, time and money on skin care products (Gediya *et al*., 2011; Oumeish, 2001; Roedel, 2006).

Many products that address general appearance of the skin contain actives that are derived from plants or actives created synthetically to mimic those found in plants. The products that focus on one type of skin problem (acne, pigmentation, un-even tone) generally have one or more actives that were found to combat the specific skin problem at hand (Berkem Products, 2013; Expanscience, 2013; Gediya *et al*., 2011).

Figure 2.5: Rooibos skin care range (Clicks Skincare Collection, 2013)

2.1.6.3 Examples of products relevant to this study

Origa'light-- de-pigmetation (Berkem Products, 2013)
Fresh'ka-- smoothes skin, lightens and brightens complexion (Berkem Products, 2013)

Crema anti-acne-- cleanses skin and reduces acne (Cosmetic Plant, 2013)

Anti-acne gel basil-- anti-inflammatory, diminishes blackheads and spots (Cosmetic Plant, 2013)

The conditions that the skin faces may cause a number of skin related problems. These include acnes, eczema, moles, wrinkles and general pigmentation (NIH Medline Plus, 2008). Two assays were used in the present study: tyrosinase inhibition and anti-microbial assay, to identify potential depigmentation and anti-acne activity of the ethanol extracts of *A. phylicoides.*

2.1.6.4 Tyrosinase inhibition assay

The tyrosinase enzyme, also known as polyphenol oxidase, is widely distributed in nature and contains copper ions. Tyrosinase is involved in the first two steps of melanin biosynthesis: 1) hydroxylation of L-tyrosine and 2) the oxidation L-DOPA to o-quinone. This enzyme is found in fruits and vegetables and is involved in the browning of these foods when they are stored for too long or are bruised. Tyrosinase is responsible for skin pigmentation in mammals and has been linked to neurodegenerative diseases. The inhibition of this enzyme is common practice with many skin-lightening agents. A number of actives used in depigmentation cosmetic products have significant anti-tyrosinase activity (Kim and Uyama, 2005). It is well known that plants are a rich source of bioactive chemicals that are mostly free from harmful side-effects and therefore, the interest in identifying natural tyrosinase inhibitors from plants has increased (Nithitanakool *et al*., 2009). The positive control used in this enzymatic assay is Kojic acid or Arbutin (Cheng *et al.*, 2007; Kim *et al.*, 2005). A number of studies have been carried out on the inhibition of this enzyme by plant extracts.

Many plant extracts were found to have inhibitory activity against this enzyme (Cheng *et al.*, 2007; Momtaz *et al*., 2008).

2.1.6.5 Anti-microbial assay

Propionibacterium acnes (*P. acnes*) is a Gram-positive, anaerobic and rod-shaped bacterium that is present on human skin. Many studies have implicated this bacterium in the progression of acne vulgaris commonly known as acne. Acne is the presence of inflamed lesions that leave scarred areas of skin after the lesions disappear. Acne is one of the diseases that were found to cause psychological stress to patients by the field of psychodermatology. There are also a number of physical discomforts associated with acne, such as pain and itching (Barankin and DeKoven, 2002; Bojar and Holland, 2004; Layton, 2005; Williams *et al*., 2012).

The conventional medication prescribed for this disorder has been shown to be ineffective against certain resistant strains of acne. Many patients are encouraged to take a combination of oral and topical treatment for acne. Due to the inefficacy of current treatments, the search for actives from a natural source has increased. Studies that investigate the potential use of plant actives to treat acne have already been documented. *Garcinia mangostana* was found to be effective in combating acne according to a study carried out on Thai medicinal plants (Chomnawang *et al*., 2007). Additional studies on ayurvedic medicines have also been carried out with positive results (Lalla *et al*., 2001). A tea plant extract, relevant to this study, was found to be active against *P.acnes. Camellia sinensis* (*C. sinensis*) is commonly known as green tea. This tea is well known for its high antioxidant content and positive health effects (Eloff, 1998; Gadow *et al*., 1997; Lee *et al*., 2009).

Chapter 2

Section 2

Hormone rooting trial on apical cuttings of *Athrixia phylicoides* DC.

2.2 Hormone rooting trial

2.2.1 Aim of study

The purpose of the hormone assay was to investigate the effects of four hormones at two concentrations on the rooting of *A. phylicoides* cuttings to contribute to the cultivation data that exists for this plant with application for commercialisation.

2.2.2 Materials and methods

The hormone assay was carried out during autumn and spring. Apical cuttings were prepared by cutting the stem 5-6 cm away from the tip. Figure 2.6 shows the shrubs used in this study. Few leaves were left on the stem. The cuttings were dipped in water in the interval between dipping and planting. A scratch was made on the stem before dipping to aid in hormone uptake. Approximately 4-6 leaves were left on the cutting which was then planted.

Figure 2.6: a) Shrubs from which cuttings were made and b) preparation of the apical cuttings for the trial

Trays with movable seedling wells were filled with compost and watered. Holes were made in the wells, in which the cuttings were placed and firmly potted. The outer seedling wells of the tray contained only compost to prevent drying out of the cuttings as shown in the experimental layout below.

2.2.2.1 Hormone treatments

The following hormone solutions were prepared by dissolving powder hormones in distilled water (dH2O)

- 1-Naphthaleneacetic acid (NAA 0.1%, 0.3%)
- Indole-3-butyric acid (IBA 0.1%, 0.3%)
- Indole-3-acetic acid (IAA 0.1%, 0.3%)
- Gibberellic acid (GA 0.1%, 0.3%)
- Control - no hormone, dH_2O

Hormones were purchased from Sigma- Aldrich® South Africa (Sigma- Aldrich, 2014). A concentration of 3 mg/ml and 1 mg/ml was obtained by dissolving 0.3 g and 0.1 g respectively of hormone powder to 100 ml of dH_2O. Figure 2.7 shows the experimental set up.

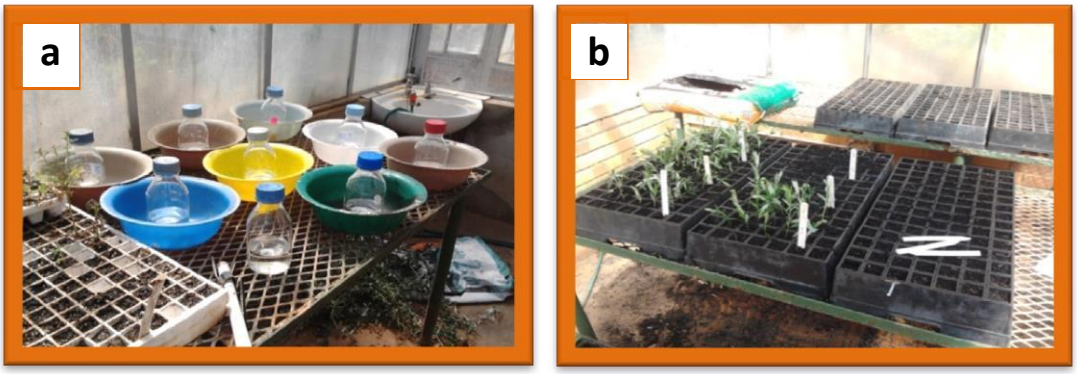


Figure 2.7: a) Hormone solutions and b) planting of treatments in seedling trays.

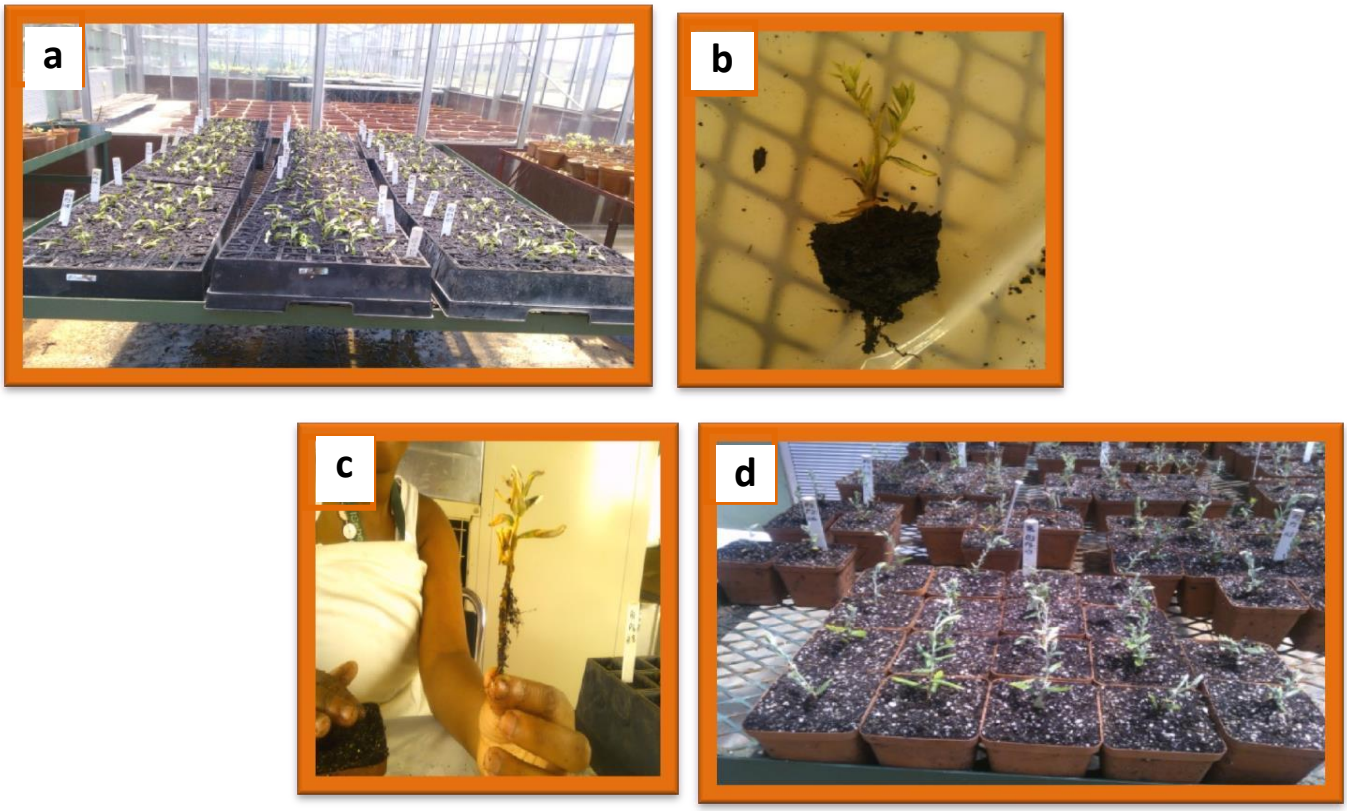
2.2.2.2 Experimental layout

Experimental layout for spring-November 2012

The experiment was carried out on 9 seedling trays, each containing 98 wells in a (14×7) layout as indicated in with two treatments on each tray unlike the previous spring trays that contained three treatments on a tray. This is to accommodate the additional samples per

treatment. Four replicates were planned for the spring trial as shown in appendix B, with the extra trays included for this trial.

Figure 2.8 displays the cuttings during and after the trial. The seedling trays were then placed in a glasshouse. Watering of the cuttings was carried out 3 times a day for a period of 10 minutes. Once rooted, the cuttings were transplanted to 20 litre (L) plastic bags containing pine bark and sand in the ratio of 2:1.



appendix B. Twenty cuttings were included in each of the 9 treatments.

Experimental layout for autumn-March 2013

Based on the results of the spring trial 2012, it was decided to include an extra block (replicate) to the autumn 2013 trial. In addition, 10 extra cuttings were added to each treatment to address the variation and obtain statistical differences. The layout created for the autumn trial 2013 is shown in appendix B

Figure 2.8: a) Planted field trial and cuttings b) after the trial with soil c) without soil and d) planted in individual pots

2.2.2.3 Growth of cuttings after trial

The surviving cuttings were planted in individual pots and maintained in the glasshouse.

Figure 2.9 displays the successful growth of the cuttings.

Figure 2.9: a) Aerial view and b) frontal view of surviving cuttings from spring 2012

2.2.2.4 Statistical analysis

The experiment was designed as a randomized complete block design (RCBC) with 3 replications during spring and four replications during autumn. The treatment design was a 4 x 2 factorial plus a control with factors four hormones (NAA, IBA, IAA and GA) and 2 concentrations (0.1% and 0.3%). The variables measured were sprouting, rooting, the number of roots produced, number of old leaves and number of new leaves. These variables were subjected to a factorial analysis of variance (ANOVA). Means of significant source effects were separated using Fisher's protected t-test with Least Significant Difference (LSD) at a 5% significance level (Snedecor and Cochran, 1980). The Shapiro-Wilk test was performed on residuals to test for non-normality and outliers were identified and removed (Shapiro and Wilk, 1965). All analyses were performed using SAS statistical software (SAS Institute, 1999).

Parameters observed for the hormone cutting trial

The rooting of the surviving cuttings was defined as the most important parameter as the survival of a plant requires the development of roots. The number of roots was then considered as the second most important parameter as the number of roots enhance survival rate by increasing the area that can be reached for nutrients. The sprouting of the cuttings was indicative of the new growth of the cuttings along with observation of the new leaves produced. The number of old leaves observed over both seasons remained relatively constant

over the treatments as this was a controlled parameter when the planting was carried out and it served as a general observation of the health of the plants.

2.2.3 Results and discussion

The means of significant effects from the ANOVA results are outlined below in table 2.2 and 2.3. Significant differences were observed amongst treatments for autumn 2013, whereas trends that support these differences were observed during spring 2012. Table 2.4 follows with the mean values obtained for the spring trial.

2.2.3.1 Spring 2012

Table 2.2: Analysis of variance of spring 2012 hormone trial results

		Sprouted		Rooted		Number of Roots		Number of old leaves		Number of new leaves	
Source	**[1]DF**	**MS**	**P**	**MS**	**P**	**MS**	**P**	**MS**	**P**	**MS**	**P**
Replicate	2	339.8	0.6598	192.59	0.7489	3.64	0.9597	3.52	0.6304	5.24	0.7913
Hormone	4	756.4	0.4609	1073.38	0.2126	591.54	0.0023	6.32	0.5121	64.70	0.0539
Concentration	1	9.4	0.9149	66.67	0.7537	171.63	0.1824	5.55	0.3994	30.17	0.2594
Hormone. Concentration	3	1170.5	0.2602	1069.44	0.2208	241.10	0.0783	6.18	0.4946	12.29	0.6509
Error	16	796.1		654.05		88.33		7.40		22.10	
Total	26										

[1] DF = Degrees of Freedom, MS = Mean Square, P = F-Ratio Probability

A probability greater than 0.05 is considered as not significant (applicable for table 2.2 and 2.3)

Table 2.3: Analysis of variance of autumn 2013 hormone trial results

		Sprouted		Rooted		Number of Roots		Number of old leaves		Number of new leaves	
Source	**[1]DF**	**MS**	**P**	**MS**	**P**	**MS**	**P**	**MS**	**P**	**MS**	**P**
Replicate	3	1638.3	0.0056	2230.45	0.0010	62.61	<.0001	8.29	0.0031	20.86	<.0001
Hormone	4	2826.4	0.0001	2129.67	0.0006	23.54	0.0005	3.51	0.0632	9.86	0.0013
Concentration	1	1250.0	0.0541	642.01	0.1539	0.29	0.7614	3.24	0.1359	0.59	0.5434
Hormone. Concentration	3	253.7	0.4891	492.94	0.2012	2.77	0.4514	0.35	0.8565	1.45	0.4433
Error	24	304.7		296.19		66.96		1.36		1.56	
Total	35										

Table 2.4: Treatment means for spring 2012

Hormone. Percentage(%)	**Sprouted (%)**	**Rooted (%)**	**No. roots**	**Old leaves**	**New leaves**
NAA	30.00 [a]	38.34 [a]	26.29 [a]	6.79 [a]	9.38 [ab]
IBA	51.67 [a]	51.67 [a]	31.67 [a]	7.12 [a]	13.03 [a]
IAA	25.84 [a]	20.84 [a]	21.22 [a]	7.28 [a]	9.45 [ab]
GA	23.33 [a]	19.17 [a]	7.99 [b]	5.65 [a]	4.5 [b]
Control	35.00 [a]	31.61 [a]	7.89 [b]	4.29 [a]	5.29 [b]
[1]$LSD_{p=0.05}$ =	n/a	n/a	12.60	n/a	n/a
[2]Shapiro-Wilk (P>W)	0.83	1.00	0.51	<0.001	0.91

[1] $LSD_{p=0.05}$ = Fisher's Least significant difference at a 5% significance level. Means within columns with the same letter or letters do not differ significantly at the 5% level.

[2] The standardised residuals were considered as normal distributed if the Shapiro-Wilk probability is greater than 0.01.

Statistical discussion of spring 2012 results

A statistical difference was found in terms of the number of roots produced. At the 5% significance level, GA as well as the control, were found to be different from all other treatments. The data was reported to be normal overall according to probability and box plots.

With reference to the control, the efficiency of the rooting hormones on the apical cuttings of *A. phylicoides* was evaluated. It was found that 31.61% of the control treatment rooted. Two treatments were found to have a higher rooting percentage than the control, namely IBA and NAA with rooting as 51.67% to 38.34%, respectively. The number of roots produced by the control averaged at 7.89 roots. IBA, IAA and NAA produced significantly more roots than GA and the control. IBA produced the highest number of roots (31.67), with NAA as a close second (26.29).

The results obtained for the spring trial indicated many trends. Overall, the lower concentrations of hormones led to a higher rooting percentage with the exception of the number of roots (see Appendix C). Approximately 6-8 leaves were left on the cuttings at the time of planting. This is therefore, a variable that is not expected to change much. Many of the old leaves did not survive the trial, however, a trend was observed where the number of roots produced correlated well with the number of old leaves present. There is a possibility that leaves which survived required efficient transport of nutrients and water; and therefore a good rooting system should be considered.

The correlation coefficient between number of old leaves and number of roots was significant with r = 0.40778 (P = 0.0347). This implies a positive correlation between the two variables. The value falls in a range that relates the two variables by a moderate correlation (Choudhury, 2009). The number of new leaves can be accounted for in the same manner as they were produced by obtaining nutrients from the new roots. Table 2.5 below presents the mean values of the autumn trial.

2.2.3.2 Autumn 2013

Table 2.5: Treatment means for autumn 2013

Hormone. percentage (%)	Sprouted (%)	Rooted	No. roots	Old leaves	New leaves
NAA	77.50 [a]	78.75 [a]	6.68 [a]	5.93 ab	4.18 [a]
IBA	63.33 [a]	67.50 [a]	6.11 [a]	5.71 ab	3.57 [a]
IAA	68.33 [a]	67.08 [a]	6.07 [a]	5.05 [b]	4.50 [a]
GA	30.00 [b]	35.42 [b]	2.41 [b]	6.72 [a]	1.71 [b]
Control	76.67 [a]	69.17 [a]	4.88 [a]	6.71 [a]	4.12 [a]
[1]LSDp=0.05	19.63	19.46	3.32	1.32	1.41
[2]Shapiro-Wilk (P>W)	0.81	0.77	0.40	0.50	0.50

[1] $LSD_{p=0.05}$ = Fisher's Least significant difference at a 5% significance level. Means within columns with the same letter or letters do not differ significantly at the 5% level.

[2] The standardised residuals were considered as normal distributed if the Shapiro-Wilk probability is greater than 0.01.

Statistical discussion of autumn 2013 results

With reference to the control, the efficiency of the rooting hormones on the apical cuttings of *A. phylicoides* was evaluated. It was found that 69.17% of the control treatment rooted. NAA produced a higher rooting percentage than the control with a total of 78.75%.

No statistical differences were observed for concentration or for hormone x concentration interaction. However, the hormone in each treatment influenced all the parameters observed. NAA produced the most roots in this trial, (6.68) with IBA following closely behind (6.11). IAA and IBA performed similarly in the trial and did not differ much from the control. GA produced significantly less roots and new leaves than all other hormones. GA also differed significantly from all other hormones in terms of rooting and sprouting. The data was reported to be normal overall according to probability and box plots.

As in the spring trial, a similar number of leaves were left on the cuttings. The correlation coefficient between the number of old leaves and the number of roots produced was found to

be 0.33824 (prob >[r] 0.0504). This implies a positive correlation between the two variables. The value falls in a range that relates the two variables by a moderate correlation (Choudhury, 2009). The best treatments in this experiment were found to be NAA with regard to the most important parameters. IAA was found to perform well in this season. GA was once again found to produce the lowest results.

2.2.3.3 General discussion

With reference to many examples, a general discussion can begin by stating that trials with hormones used for the purpose of enhancing rooting have shown variation and are not easily reproducible. The uptake of hormone in either liquid or dry form will depend on IAA in the plant, the concentration of which will depend on season, weather, plant health, etc. It is then obvious why trials like these include variation. Many cases of trials carried over many years and seasons under controlled environments are indicative of results that are not easily reproduced (Loach, 1988).

With regard to the results obtained in this experiment, IAA performed well in autumn but not in spring; GA was the lowest performer overall, consistently over the two seasons. IBA and NAA performed well over the two seasons. It was found previously that the addition of IBA to growth media increased the rooting of stem cuttings of *Pappea capensis* (jacket plum) by 64% compared to when media alone was used (Mng'omba *et al*., 2007). Another study compared the application of NAA and IBA to shoot cuttings of *Tectona grandis* and found that both were successful with IBA performing better (Husen and Pal, 2007). These lead hormones have performed well in other similar experiments. However, there is not one universally efficient hormone; different hormones will react in a number of ways with different plants. The point of the study is to find the ideal rooting hormone for a plant.

In an earlier study carried out by Araya (2005), the opposite trend was observed in the plants. Araya found an average of approximately 12 roots per cutting in autumn and approximately 6 roots per cutting in spring when IBA was used in dry form (Araya, 2005). The present study that used dissolved hormone solutions found a higher number of roots for spring than autumn averaging at approximately 25 and 6, respectively.

The control performed better in autumn than in spring overall. The temperature in the glasshouse was the same over the two trials, however the temperature of shade net enclosing the source material differed. It is possible that the temperature, at which the mother material was maintained at, over the two seasons, may have affected the success of the trials. Higher

temperature during spring may have affected the stress and survival of the cuttings. This statement is strengthened further by the observation that all treatments performed better in autumn. The same hormones, from the same source, were used over the two seasons. The main differences between the two trials were number of cuttings used, number of blocks, different and more efficient watering system during autumn and finally seasons in terms of the temperature of the source material.

The higher success rate of the cuttings in autumn cannot be attributed to the increased number of cuttings and replicates as the performance of the control treatment also differed between the two seasons. The number of cuttings and replicates may clarify the statistical differences. A larger sample number gives a more accurate representation of the population. The success of the cuttings may be attributed to the season in this experiment. Previous findings by Araya, (2005), indicated high success rates in spring and autumn. The source of the cuttings also plays a role and contributes to variation in the success of cuttings. The environment in which the experiments were carried out also affected the growth and survival of the cuttings.

The key points that are observed in hormone cutting trials are that excessive amount of the hormone is detrimental to the cuttings. This point is observed in this experiment with the majority of the high performers being at the lower concentration (1mg/ml), see Appendix C. This finding is beneficial to the data on cultivation and to the financial provisions. A study compared the application of IAA, IBA and NAA on the root formation of *Malus* 'Jork' plant. IAA performed well over all concentrations while IBA and NAA performed best at lower concentrations (De Klerk *et al*., 1997). These results corresponded with our findings. Plant hormones, if not applied in the correct manner or level may even inhibit root growth (Cao *et al.,* 1993). The second point is that hormones are generally more effective at promoting rooting than the controls. This is observed in the spring trial. However in the autumn trial the control is similar to the treatments. Once again this may be attributed to temperature and the condition of the cuttings.

The general trends observed include that IBA and NAA treated cuttings produced the highest percentage of rooting of the cuttings and that NAA is responsible for the highest number of roots produced overall. IBA treated cuttings produced the most sprouted cuttings. With regard to the number of new leaves produced, NAA and IBA treated cuttings were also found to be the most successful treatments. These results correlate well with the previous findings

by Araya, 2005, with reference to IBA. In addition, these results indicate an additional hormone, NAA, with similar rooting inducing potential.

2.2.3.4 Comparison of cost of hormones

The prices of the hormones per 10g from Sigma Aldrich range from R 80-R 4200.

Approximate costs per 10 g are:

IAA-R 250

IBA-R 700

NAA-R 90

GA-R 4000

NAA was found to induce good root growth and is almost one eighth the price of IBA. The cost of IAA is lower than IBA but still more than twice the cost of NAA. NAA is a good choice based on cost as well as for the optimum root growth of *A. phylicoides*.

2.2.3.5 Structural comparison of hormones

The structures of IAA and IBA are very similar; however, IBA contains a longer hydrocarbon chain. IBA and NAA are similar in structure with the differences being in the nitrogen group that IBA contains as well as the longer hydrocarbon chain. IAA and NAA are strikingly similar with the difference in IAA containing a nitrogen group. The positive effect of these hormones on rooting may lie in their structures. The ring structures may play a role, benzene rings in NAA and indole rings in IAA and IBA. The common group in all the hormones is a carboxyl group which may also be integral to the efficacy of the hormones.

2.2.4 Conclusion

The results indicated that IBA and NAA were successful in inducing rooting that was higher than the control. IBA compared well with previous studies and was found to produce twice the number of roots when dissolved during spring. In general, a higher number of roots were formed during the spring trial but a higher rooting percentage was observed during autumn. During spring, IBA at 0.1 mg/ml had the highest rooting percentage of 58.33% and highest sprouting percentage of 60%. Following closely behind was NAA at 0.1 mg/ml with a rooting percentage of 51.67%. IBA and NAA at 0.3 mg/ml had the highest number of roots for the trial: 38.72 and at 31.00, respectively, See Appendix C.

During autumn, NAA at 0.1 mg/ml had the highest sprouting percentage of 78.33% and at 0.3 mg/ml, the highest rooting percentage of 83.33%. The highest number of roots formed was 7.88 by NAA at 0.3 mg/ml with IAA forming 6.67 roots at 0.3 mg/ml. IBA at 0.1 mg/ml formed 6.41 roots as the third highest value in this category. IAA performed well in autumn along with IBA and NAA. Moderate correlations between new leaves and number of roots formed were also observed over both seasons. IBA was confirmed as an effective hormone for this plant but this study introduced an additional hormone, NAA, to serve the same purpose at a lower cost.

Chapter 2

Section 3

Bioassays on leaf extracts of *Athrixia phylicoides* DC.

2.3 Bioassays

2.3.1 Aim of study

A. phylicoides was found to have comparable anti-oxidant activity to that of *A. linearis*, more commonly known as rooibos. Limited isolation of the active compounds on *A. phylicoides* was carried out compared to that of *A. Linearis*; however, it was found that quercetin is present in both plants. Rooibos extract is commonly used in cosmetic products that claim to revitalise and rejuvenate the skin, as discussed above in 2.1.6. Due to these similarities, extracts of *A. phylicoides* were screened by two bioassays used for potential cosmeceutical extracts. In addition four extracts were prepared over the four seasons to identify whether seasonal variation occurred in terms of the specific bioassays.

2.3.2 Materials and methods

2.3.2.1 Plant collection and extract preparation

Leaves and stems of *A. phylicoides* were collected from a shade house at ARC, Roodeplaat. Collection occurred in the last month of each season from 2012. Material was collected in January, March, June and September. The material to be tested was washed in dH_2O and allowed to dry at room temperature for 10 days away from sunlight. The dried material (60 mg) was then ground and combined with 50 ml of 99.9% ethanol (EtOH). This mixture was placed on a shaker for 48 hours. The resulting solution was filtered and allowed to dry in a fume hood to obtain a dry ethanol extract.

2.3.2.2 Tyrosinase inhibition assay

The anti-tyrosinase assay was carried out in accordance with the methods outlined by Nerya *et al* (2003) and Curto *et al* (1999). Plant extracts and kojic acid (positive control) were dissolved in DMSO to prepare a stock solution of 20 mg/ml. (Lee et al., 1997). The solutions were serially diluted in 50 mM potassium phosphate buffer. In a 96-well microtitre plate, 70 µl of each solution (plant sample, positive and negative control) of different concentrations were added in triplicate. Thereafter, 30 µl of tyrosinase (333 Units/ml in phosphate buffer, pH 6.5) was added. After an incubation period of 5 minutes at room temperature, 110µl of substrate (2 mM L-tyrosine) was added to each well. The final concentrations of the extracts and positive control ranged from 3.13 – 400 µg/ml. The plate was incubated for 30 minutes at

room temperature. Optical densities of the wells were determined at 492 nm with the BIOTEK power Wave XS multi-well plate reader. The median inhibition concentration was calculated using Graph Pad Prism 4.

2.3.2.3 Anti-microbial assay

The anti-microbial activity of the seasonal ethanolic extracts of *A. phylicoides* was determined using the microdilution assay. This assay was carried out in accordance with the method outlined by Eloff (1998). The *Propionibacterium acnes* was cultured on nutrient agar plates under anaerobic conditions at 37°C for 72 hours. The culture of bacteria was dissolved into solution and adjusted by trial and error to 0.5 McFarland standard. Tetracycline was used as a positive control in this assay. The plates were incubated under anaerobic conditions at 37°C for 72 hours in an incubator. The colour changes in the plate were observed after the addition of Presto Blue as an indicator. The MIC was determined visually at the point where no pink colouration occurred, indicative of no reaction.

2.3.3 Results and discussion

2.3.3.1 Tyrosinase inhibition assay

Table 2.6 lists the IC_{50} values of the seasonal samples.

Table 2.6: Tyrosinase inhibition activity of seasonal leaf extracts of *A. phylicoides* in µg/ml

Extract	*IC_{50}(µg/ml)	R^2
Spring	223.65±59.31	0.68
Summer	767.40±111.05	0.85
Autumn	301.40±115.65	0.55
Winter	402.20±203.25	0.19
Positive control (kojic acid)	7.144±0.48	0.87

*Median inhibitory concentration

At the highest concentration of plant extract, 400µg/ml, only moderate tyrosinase inhibition activity was observed for spring and autumn extracts. Two commonly used plant extracts in

the cosmetic field for the purpose of de-pigmentation are, *Glycyrrhiza glabra* and *Morus alba*. The IC_{50} values of these extracts with respect to the anti-tyrosinase assay were 0.91 µg/ml and 11.9 µg/ml, respectively (Moon *et al*., 2010; Nerya *et al*., 2003). These values are for the root and stem extract of *G. glabra* and *M. alba*. These IC_{50} values compared well with the positive control in contrast to the extracts of *A. phylicoides*.

2.3.3.2 Anti-microbial assay

The antimicrobial assay was analysed visually. Figure 2.11 displays the plate in which the assay was carried out at each concentration in triplicate. The positive control indicates a blue to purple colour that represents the lack of living bacteria. The negative control displays a pinkish colour that represents the presence of live bacteria.

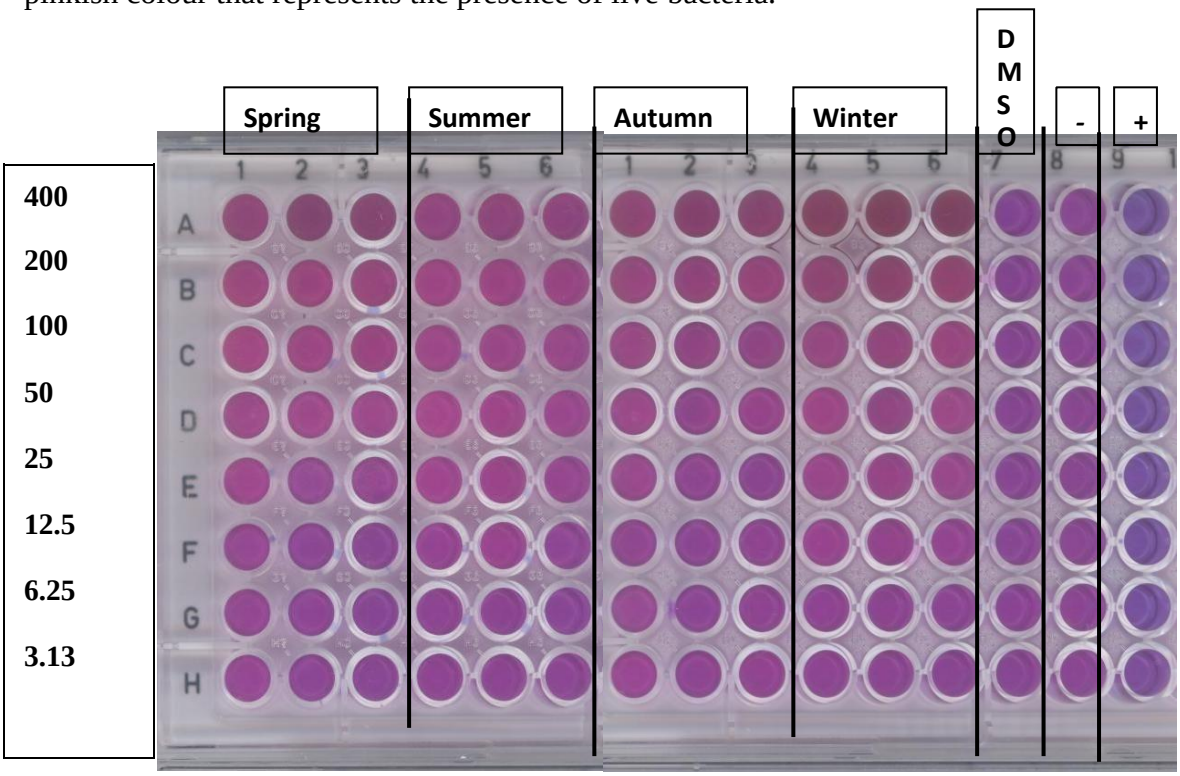


Figure 2.10: Microtitre plate of seasonal samples with presto blue as an indicator

In general, the point where the extract is effective in inhibiting microbial growth will be evident by a colour change. The concentration at such a point will be regarded as the minimum inhibitory concentration (MIC) of the extract. All the test wells resulted in a pink colour similar to that of the negative control. At the highest plant extract concentration tested, 400 µg/ml, no significant antimicrobial activity against *P. acne*s was observed.

2.3.4 Conclusion

The autumn and spring ethanol extract of *A. phylicoides* displayed moderate tyrosinase inhibition activity; IC_{50} = 223.65±59.31µg/ml and IC_{50} = 301.40±115.65µg/ml respectively, compared with the positive control, IC_{50} = 7.144±0.48 µg/ml. The plant parts used to produce the ethanol extracts of *A. phylicoides* were the leaves. The active extracts of the plants mentioned above, *G. glabra* and *M. alba,* were composed of roots and stems, respectively. *A .phylicoides* may have higher tyrosinase inhibition potential in other plant parts. The harvesting of roots and stems is not always sustainable. For this reason, studies on plant leaves are preferred.

At the highest concentration tested, 400 µg/ml, no observable MIC was detected for the seasonal ethanol extracts of *A. phylicoides*. *A. phylicoides* may well have other roles in the cosmetic field that have not been tested for.

2.4 References

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Chapter 3

Seasonal variation studies on the leaf extract C*eratonia siliqua* L.

Chapter 3

Section 1

Background information on C*eratonia siliqua* L.

3.1 General background

Ceratonia siliqua commonly known as the "carob" tree has been of great importance to people of the Mediterranean region, where the tree was used as a food source as well as for agricultural purposes. The carob pod contains a sugary pulp which serves as a nutritious food source for animals and humans. In current times, the uses of the carob tree has expanded to gum and fuel production, landscaping, windbreaks, cosmetic ingredients, food additives, etc. (Batlle and Tous, 1997).

Carob has been referred to in the bible because the pods were once thought to be locusts that were eaten by John the Baptist. Carob is therefore, also known as St. John's bread or locust bean. The word 'carat', that measures the weight of gold and diamonds, comes from 'carob' in the Arabic language due to the uniformity of the carob seeds in weight (Carob, 2011).

3.1.1 General traditional uses of this plant

- Carob pods used as fodder in The Maltese islands and Iberian Peninsula
- Part of diet of people of Maltese islands
- Source of sweetness and sugar before sugarcane and sugar beets
- Dried carob fruit eaten on the Jewish holiday of Tu Bishvat
- Traditional medicine for sore throat and coughs
- Carob juice consumed by Islamic followers during fasts
- Carob syrup known as black gold by people in Cyprus and is widely exported
- In Malta, a sweet is made using carob which is eaten on Good Friday and Lent (Zeta boards, 2013)
- Carob has been used to treat infantile diarrhoea and carob bean gum has been used to control hyperlipidemia. (Natural Standard, 2013)

3.1.2 Appearance and features

C. siliqua is an evergreen tree that can grow up to 15 meters with a thick trunk and a broad crown. The tree flowers from August to October but leaves are found all year round. The flowers are dioecious and generally, one tree will have flowers of only one sex (Batlle and Tous, 1997). The flowers of both sexes are pentamerous and approximately 6-12 mm in length. The flowers comprise mainly of sexual organs and are yellowish-green (Dafni *et al.*, 2011). The leaves of the tree are glossy, deep green and pinnately compound. The fruits of

the tree are edible, prolific brown pods. These fruits are produced in summer and autumn (CalPoly, 2012.) The longevity of the tree is more than 150 years. Figure 3.1 illustrates the appearance of this plant.

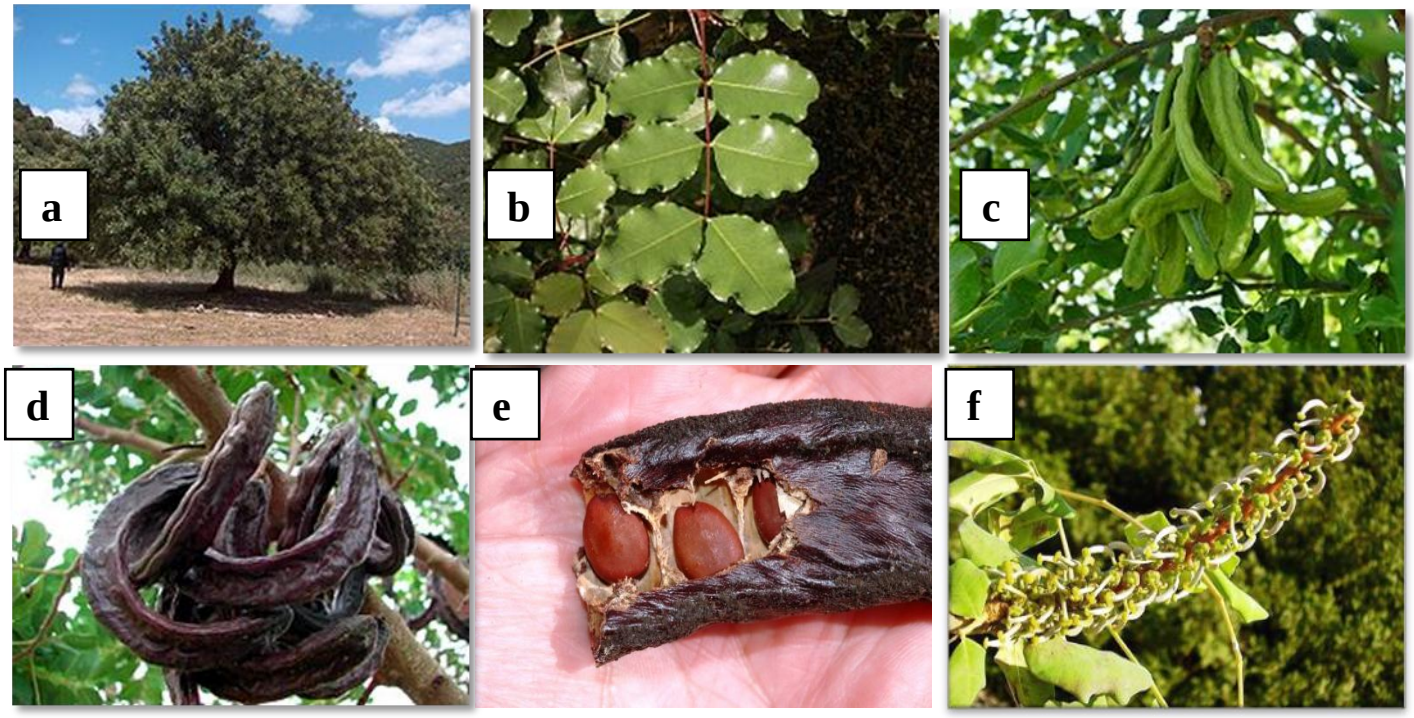


Figure 3.1: *C. siliqua* a) adult tree in native habitat b) leaves c) green fruit pods d) ripe pods e) seeds in the pod and f) female flower (*Ceratonia siliqua*, 2013; *Ceratonia siliqua* L., 2012; Wikimedia, 2011)

These plants have developed interesting features such as the ability to grow in nutritionally poor soil and in most pH ranges of soil. They can also tolerate frost, drought and strong winds (CalPoly, 2012). The *C. siliqua* trees are tolerant to smog and resist oak root fungus. The fragrance of the flowers and pollen is unpleasant. This tree offers high shading capacity as the leaves grow densely (CalPoly, 2012). There are no known health hazards about the plant parts except for the potential allergenic properties of the pollen (Daves Garden, 2014).

3.1.3 Taxonomy and distribution

Table 3.1 outlines the taxonomy of *C. siliqua*.

Table 3.1: The Taxonomy of *C. siliqua* (ITIS report, 2014)

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Genus	*Ceratonia*
Species	*Ceratonia siliqua*

C. siliqua is native to the Middle Eastern countries. These trees were spread to Italy and Greece by the Greeks and to Spain and Portugal by the Arabs. The spread of these trees has continued up to recent times as seen below in Figure 3.2 and 3.3. The carob tree is now cultivated in warm climates all over the world including Florida and California (Batlle and Tous, 1997; Natural Standard, 2013).

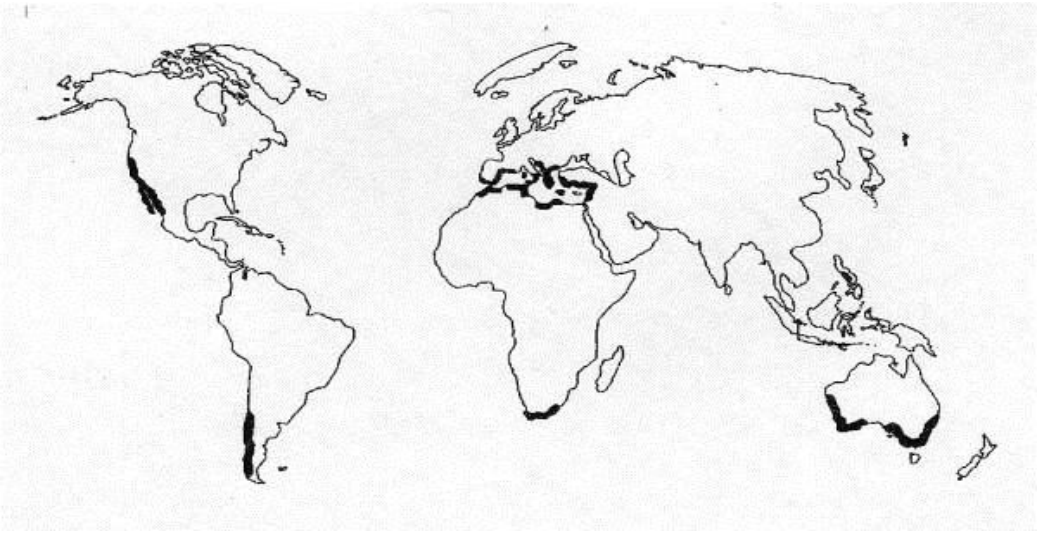


Figure 3.2: Centres of origin and distribution of the carob tree (Batlle and Tous, 1997)

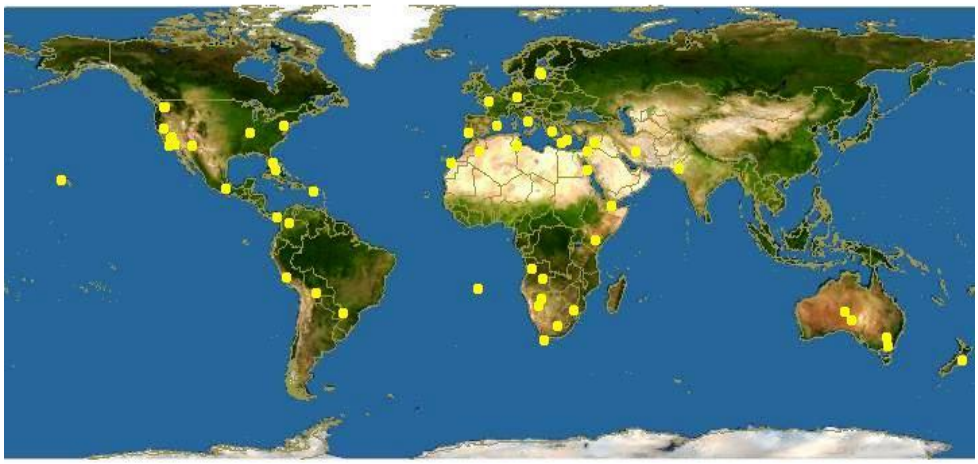


Figure 3.3: Regions where *C. siliqua* grows (Discover life, 2013)

3.1.4 General research completed on *C. siliqua*

3.1.4.1 *C. siliqua* in the field of food and nutrition

The pods of carob have traditionally been used as a source of nutrition. The Food and Drug administration (FDA) has declared that carob is safe as food. The seed pods can be ground to a powder rich in sugar and protein. This can be used as a substitute in cakes, sweets and chocolates. Edible gum can be extracted from the seed which can be used as a thickening agent and as a substitute for egg (Batlle and Tous, 1997; Plants for a future, 2012).

Carob pods have been used as fodder for livestock and added as a flavouring agent for dog biscuits. Research has recently been carried out to investigate the effects of these pods on the health of livestock. The results indicated that the pods can be highly beneficial to livestock (Karabulut *et al*., 2006; Obeidat *et al.*, 2011; Scarecrows garden, 2009).

Parts of this plant have been used as food additives, sweeteners, flavouring and thickening agents. It was recently discovered that the methanolic extract of *C. siliqua* could inhibit the growth of a number of microorganisms and thereby has potential to be used as food preservative. In 2008, work was carried out to identify flower expressed genes in carob. Several agronomically important genes were identified and quantified using polymerase chain reaction (PCR). In addition carob-genic microsatellites were developed (Aissani *et al.*, 2012; Caruso *et al.*, 2008; USDA, 2013).

3.1.4.2 Documented medicinal value of *C. siliqua*

In 2009, a US patent was filed that accounted for the use of an extract of *C. siliqua* leaves and pods for the potential treatment of cancer. Results from studies like these indicated that young leaves of this plant have chemopreventive activity. Polysaccharides from the bean gum extract were found to interrupt virus replication of rubella. The fruit extract of *C. siliqua* was found to be an anti-depressant that mediates action through dopamine and noradrenaline (Avallone *et al.*, 2002; Mastromarino, *et al.*, 1997).

The hydralcoholic seed extract of this plant was found to reduce blood glucose and lipid levels in male rats. The extract was also found to increase high density lipoprotein cholesterol (HDL-C) levels in the blood. The pulp extracted from this plant was found to be nutritious as well as a gut cleanser and a treatment for diarrhoea (Agrawal *et al.*, 2011; Baraldi, 2009; Plants for a future, 2012; Mokhtari *et al.*, 2010).

Carob pulp was found to reduce the glycogen accumulation in the liver of rats. The pulp was suggested to have the potential to be used for weight loss. Studies on the effects of the bean gum on rats, with regard to glucose levels in the blood, have indicated that this extract may have usage in the field of diabetes treatment. The leaves and pods were found to have nephroprotective effects in mice with induced kidney damage by anti-cancer drugs (Ahmed, 2010; Feldman *et al.*, 1995).

3.1.4.3 Economic uses of the plant

Parts of the tree were found to be useful in the production of fuels such as charcoal and fuel wood. Many materials such as gum, resin and alcohol can be extracted from the plant. The tree has also been used as a provision for shelter and has ornamental value as well (Scarecrows garden, 2009; USDA, 2013).

The powder of carob bean was found to be available online, for purchase, priced at around R 50/kg. Carob liquid extract, roasted powder, seeds and carob whole pods are commercially available from amazon.com. *C. siliqua* extract is available at certain chemical stores (Cas Chemnet.com, 2013). The availability of these plant parts are evidence for the economic value of this tree (Algarseeds, 2013; Al-Saadi and Fathi, 2003; Amazon, 2013).

3.1.4.4 Propagation and cultivation studies

A number of propagation studies have been carried out on *C. siliqua* in order to increase the ease of regeneration due to the plant's commercial importance. Perhaps one of the most important studies carried out was that by Osorio *et al.*, in 2012. This team of researchers

found that there were no significant differences amongst the original, micropropagated and seed derived plants. The parameters observed in this study included growth rate, number of leaves, chlorophyll and soluble protein content, amongst others (Osorio *et al*., 2012).

A few examples of these studies are listed below:

Embryogenesis and further regeneration of the *C. siliqua* plant was found to be possible from cotyledonary segments with the addition of auxins and growth enhancers to growth media. Plant regeneration was found to be possible through this method with auxins (Canhoto *et al*., 2006).

Germination of the seeds of *C. siliqua* was found to increase with treatments such as mechanical scarification, soaking in distilled water and application of sulphuric acid. Untreated seeds were found to have a longer dormancy; this was probably due to the impermeability of the seed coat. A large variation was found in seeds in terms of weight, water content and germination potential. Variation was also observed in seeds from the same tree and in the results of the experiment (Perez-Garcia, 2009).

Micrografting of decapitated seedlings used as rootstocks was found to be successful depending on the medium and supplements used (Hsina and Mtili, 2009). In 2005, research carried out by Goncalves *et al.*, found an optimal level of medium in order to obtain optimal amount of macronutrients in the form of micropropagated shoots (Gonçalves *et al.*, 2005).

Many studies involved with the induction of roots in carob have been carried out. This is due to the fact that propagation of this plant has been challenging. Air layering and clonal propagation were found to be successful. Studies were also carried out on the effect of different levels and types of sugars on the rooting of micropropagated shoots. Sucrose at 145mM was found to be most effective with additional recommendations for propagation in this manner (Custodio *et al*., 2004; Gubbuk *et al*., 2011; Hakim *et al*., 2010).

3.1.4.5 Phytochemical constituents of *C. siliqua*

The fibre of carob was found to contain 24 polyphenol compounds. Gallic acid was found to be a major polyphenol in different forms. It was also found that flavonoids represented 26% of the polyphenols with the major compounds being myricetin and quercetin. The purified polyphenols were shown to have antioxidative properties (Owen *et al.*, 2003).

In 2004, the fiber of carob was also investigated. In this study, 41 individual phenolic compounds were isolated. The compounds isolated included gallic acid, tannins, flavonol-

glycosides, and traces of isoflavonoids (Papagiannopoulos *et al.,* 2004). Many studies have been carried out on the compounds isolated from the pods. The phenolic compounds from the green pods were isolated. In addition to this, leucodelphinidin, β-D-glucogallin and β-D-1,6-di-*O*-galloylglucose and other tannins were also isolated (Nishira and Joslyn, 1968).

The flavonoid content of *C. siliqua* leaf extracts (70% ethanol) was determined using reverse phase high performance liquid chromatography (HPLC). Nine flavonoids were identified with myricetin as the major flavonoid (1486 mg/kg extract) (Vaya and Mahmood, 2006). In 2007, Momtaz isolated six compounds from the leaves of *C. siliqua*. Five of these compounds were used as biological markers in the present study. These compounds are discussed in section 3.1.6 and were found to play a role in the potential of this extract to be used in the cosmetic field (Momtaz, 2007).

3.1.4.6 Biological activity and toxicity of *C. siliqua*

The ethanol, methanol, n-hexane and water extract of leaves of *C. siliqua* were found to be toxic in brine shrimp (*Artemia salina*). The ethyl acetate extract of *C. siliqua* was found to be non-toxic against brine shrimp. In the same study, the antimicrobial activity of the same extracts was tested against 10 microorganisms. All extracts displayed good activity against the microorganisms, however the ethyl acetate and n-hexane extracts were found to be most effective (Kivack *et al*., 2001).

The essential oil of carob pods was assayed for its potential antimicrobial activity and cytotoxic effects. A number of compounds were identified in the oil, including Octadecanoic acid, Phenyl ethyl tiglate, Eicosene, *n*-Eicosane, Nonadecane, Heneicosane, Heptadecane, Hexadecanoic acid and Naphthalene. The essential oil was found to have inhibitory effects on a number of bacterial strains with the most important effect being on *Listeria monocytogenes.* The *C. siliqua* essential oil was found to inhibit this food borne pathogen. This extract was also found to be toxic to two cancer cell lines: Human Uterine Cervical Carcinoma cells (HeLa) and Human breast carcinoma cells (MCF-7) (Hsouna *et al.*, 2011).

3.1.4.7 Cosmetic value of *C. siliqua*

The bean gum of *C. siliqua* was found to be valuable in the cosmetic industry. The gum was found to be useful as a thickening agent, stabilizer, gelling agent, sensory agent and in hair care products. Products that contain the carob bean gum extract include AHAVA age control

serum, hydra gel mask and Dermica Switzerland (Cargill, 2013; 6pm.com, 2013; INCI dermica, 2013). Figure 3.4 illustrates one of the above mentioned products.

The incorporation of extracts of *C. siliqua* in cosmetics has occurred for a long time with names like BuyDerm stating that the gum of *C. siliqua* provides a formulation with lubricity and a luxurious feel to the skin. The additive is aesthetically valuable and non-ionic (BuyDerm, 2013).

Figure 3.4: AHAVA age control serum (Elements beauty shop, 2013)

The *C.siliqua* gum was listed under the International Nomenclature of Cosmetics Ingredients (INCI). The gum has been classified as an emollient, film forming, masking and binding agent amongst others. A number of products in the market include this ingredient in their formulation, e.g.: Erno Laszlo, NaturaBisse and Thierry Mugler (Specialchem, 2013).

3.1.5 Background contributing to this study

3.1.5.1 Previous research

Previous research indicated that the methanol leaf extract of *C. siliqua* had significant tyrosinase inhibition activity. The extract was able to inhibit 125% of tyrosinase activity at a concentration of 400 µg/ml. Research on the B16-F10 cell line resulted in 45% melanin reduction at an extract concentration of 12.5µg/ml with a cell viability over 90%. The methanol extract of *C. siliqua* was found to have a high antioxidant activity as well. Six bioactive compounds were isolated which are used in the present study as biological markers for the investigation of seasonal variation. The previous studies indicated that the leaf

material had the potential to be used in the cosmetic against general hyper-pigmentation (Momtaz, 2007).

Due to the safety issues surrounding methanol in skin care products, ethanol was used to produce extracts of *C. siliqua* for further *in vitro* and clinical trials. The *in vitro* studies carried out are listed below:

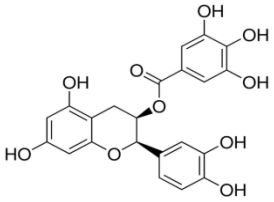
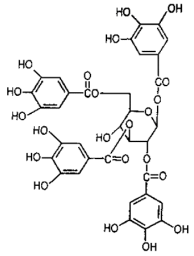
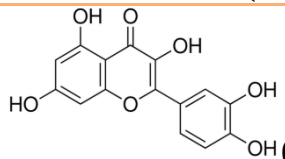
- **Mutagenicity**
- **Stability**
- **Microbial count**
- **Preservative challenge**
- **Heavy metal detection**
- **Physiochemical data**

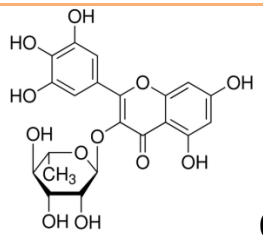
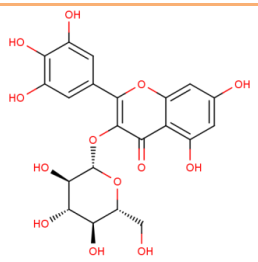
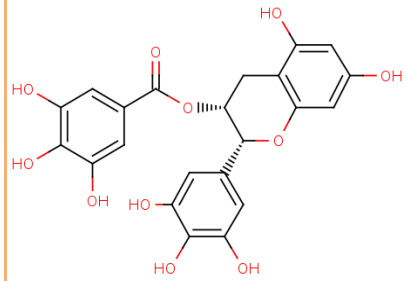
Three clinical trials were carried out on the extracts after the *in vitro* studies were successful. These included irritancy study, skin even tone study and anti-blemish trial, all of which, were successful. The present study was therefore undertaken to evaluate the seasonal variation of the leaf extract of *C. siliqua*.

3.1.5.2 Biological markers used in this study

Table 3.2 outlines six bioactive compounds referred to as standards in HPLC and assigns each of them a code from A1-A6. The name, structure and *in vitro* results with regard to the tyrosinase inhibition and melanin inhibition assay are provided. Standard A1 was not accessible during the research phase and was excluded from the study. All standards were identified as flavonoids with the exception of A2, which was categorised as tannin.

Table 3.2: Anti-tyrosinase, melanin inhibition and cytotoxivity of bioactive compounds isolated from *C. siliqua* (Momtaz, 2007)

Standards	Names of compounds	Structures of compounds	Monophenolase activity IC_{50} (µg/ml)	Melanin Inhibition %	Cell viability %
A1	**(-)-epicatechin -3-o-gallate**	 ([a]Sigma-aldrich, 2014)	27.52	-	-
A2	**1,2,3,6-tetra-o-galloyl-β-D-glucose**	 (Plant expert, 2010)	83.30	30 (at 6.25 µg/ml)	50 (at 6.25 µg/ml)
A3	**Quercetin-3-o-α-L-rhamnoside**	 ([b]Sigma-aldrich, 2014)	>200	28 (at 6.25 µg/ml)	100 (at 6.25 µg/ml)

A4	**Myricetin-3-o-α-L-rhamnoside**	 (cSigma-aldrich, 2014)	>200	60 (at 12.5µg/ml)	70 (at 12.5 µg/ml)
A5	**Myricetin-3-o-glucoside**	 (YMDB, 2011)	>200	0(at 100µg/ml)	100 (at 100µg/ml)
A6	Gallocatechin-3-o-gallate	 (BRENDA , 2013)	28.3	25(at 200µg/ml)	100 (at 200µg/ml)

Chapter 3

Section 2

Seasonal variation studies on Ceratonia siliqua L. using tyrosinase inhibition assay, TLC and HPLC

3.2 Seasonal variation studies

The seasonal variation of the ethanol leaf extracts of *C. siliqua* were evaluated using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and the tyrosinase inhibition assay (as mentioned in chapter 2). TLC was selected as a simple and inexpensive screening technique to identify the compounds. HPLC was used as a quantitative analysis of the concentration and presence of the biological markers.

3.2.1 Aim of Study

The purpose of the present study was to identify variation in the anti-tyrosinase activity of extracts obtained from each season as well as from three age groups based on the heights of the plants. The study also focused on the detection of isolated bioactive compounds over the four seasons using TLC and HPLC.

3.2.2 Materials and methods

3.2.2.1 Plant collection and extract preparation

Leaves of *C. siliqua* were collected from ARC (Roodeplaat), University of Pretoria and LC de Villiers (Hatfield) during June and September in 2012 and December and March in 2013. Voucher specimens of the material used were deposited into the H.G.W.J. Schweikerdt Herbarium. Voucher specimen numbers for the representative small and medium plants were 119649 and 119648. The specimen numbers for adult plants collected from different areas were submitted under the numbers 119644, 119645, 119646 and 119647.

The study included plants from three age groups with reference to their height: adult/large (5-10 m), adolescent/medium (0.8-1.2 m) and juvenile/small (12-15 cm) over the four seasons of the year. The material to be tested was washed in distilled water (dH_2O) and allowed to dry for 10 days away from sunlight at room temperature. The dried material (60 mg) was then ground and combined with 99.9% EtOH (50 ml). This mixture was placed on a shaker for 48 hours. The resulting solution was filtered and allowed to dry in a fume hood to obtain a dry ethanol extract.

3.2.2.2 Statistical collection of samples

The experimental design was a completely randomised design with 5 replicates. The treatment design was a two-factor factorial, with factors, season (spring, summer, autumn and winter) and age (small, medium and large). The study included plants from three age groups: adult, adolescent and juvenile over the four seasons of the year. In order to obtain samples that would be representative of the population, 1 leaf was removed from the lowest branch of the plant. These were the youngest leaves observed at the site. The leaves of 10 plants composed one sample per season per age for this study. Five replicates were collected for each age group per season. Therefore, a total of 50 trees were sampled per age group and 150 per season. Figure 3.5 illustrates the number of plants required in the present study.

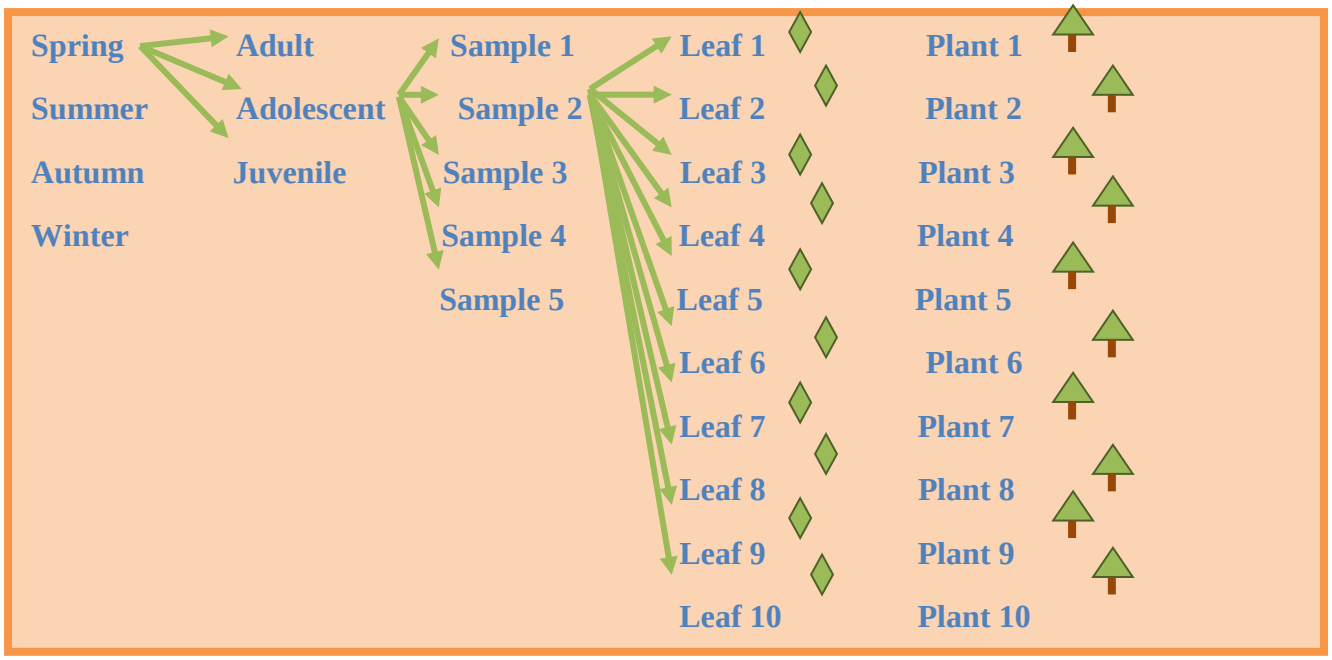


Figure: 3.5: The number of plants used in this study

Figure 3.6 illustrates the plants required for the seasonal study. Additional samples were added for the HPLC analysis during autumn and summer for large samples to account for the variation observed in previous seasons. Codes (lc[1], theo[2], nw2[2] and land[2]) were provided to differentiate samples.

[1] lc- L. C de Villiers

[2] theo- Theology, nw2- Natural sciences,land- Landbou- areas outside buildings on main campus, University of Pretoria

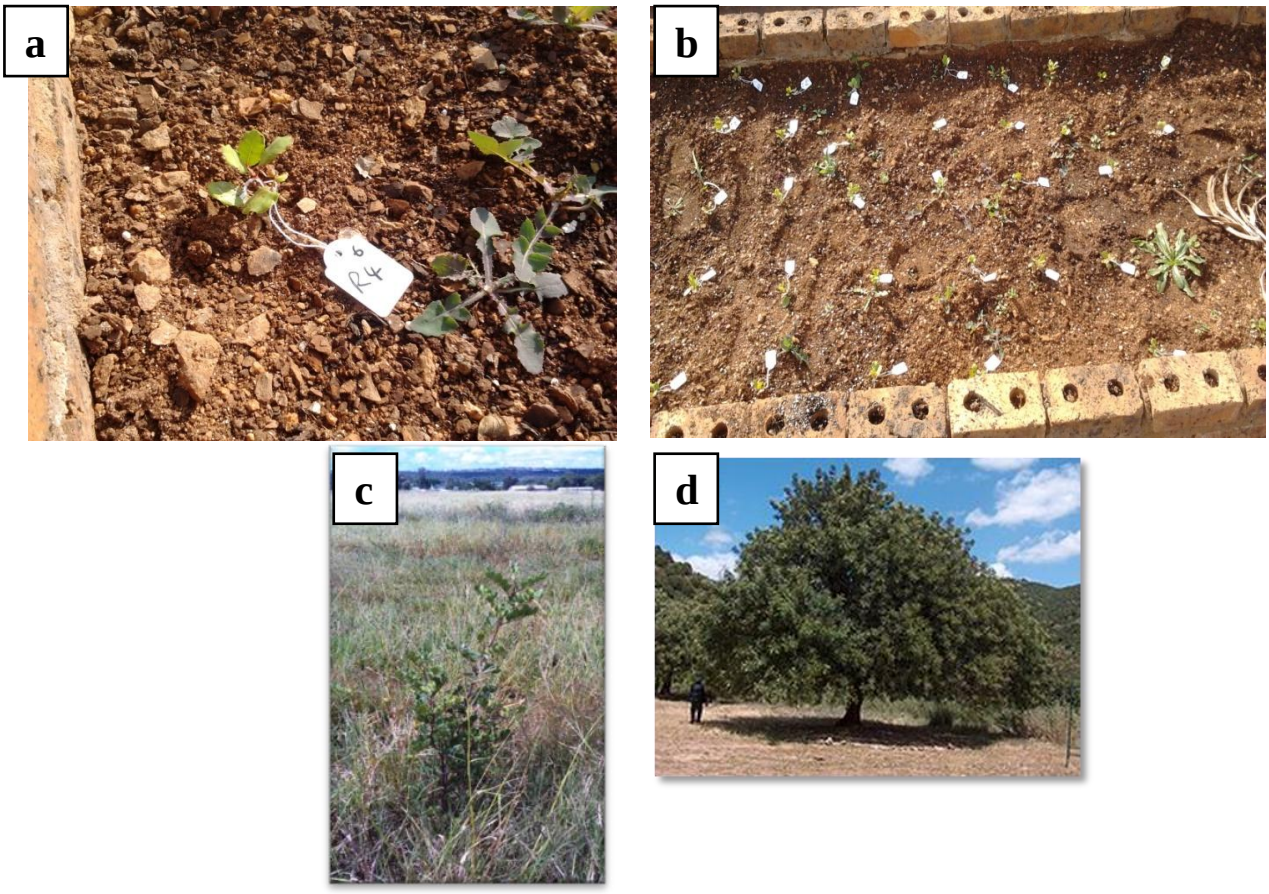


Figure 3.6: Sample plants a) juveniles with label b) juveniles/small c) adolescent/medium plant and d) adult/large plant (*Ceratonia siliqua*, 2013)

Figure 3.7: Ethanol extracts of leaves collected during the season of winter

Figure 3.7 illustrates the extracts prepared for each season of the present study.

3.2.2.3 Tyrosinase inhibition assay

Refer to chapter 2 section 2.3.2.2

3.2.2.4 Thin layer chromatography

In order to obtain solutions of the test samples and standards, 2 mg of each standard (A2-A6) was dissolved in 500 µl EtOH and 2 mg of dry ethanol extract was dissolved in 1 ml EtOH. Approximately 10 µl of each sample was spotted on silica gel 60 F_{254} aluminium sheets alongside each other. The plates were allowed to develop in a mobile phase solvent system composed of ethyl acetate::methanol (8:2) in a chamber. The plates were removed from the chamber before the solvent reached the edge of the plate. Plates were allowed to dry and were sprayed with vanillin. The separated components were observed under ultraviolet and visible light at 366 and 254 nm respectively. The appropriate markings were made on the plates so it could be analysed at a later stage. Figure 3.8 illustrates the method of TLC.

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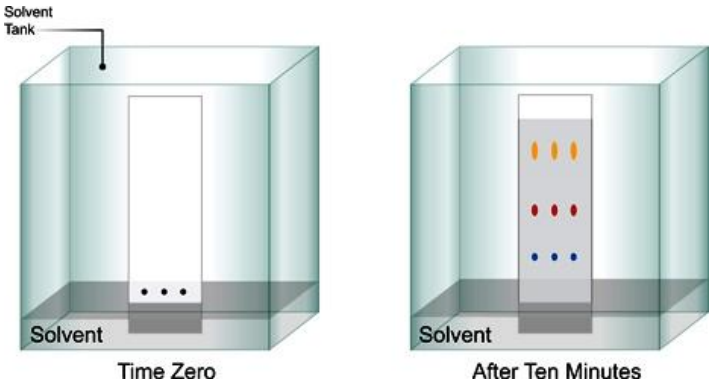


Figure 3.8: TLC as a chromatographic technique (Waters, 2013)

3.2.2.5 High Performance Liquid Chromatography

HPLC is a separation technique based on the affinity of dissolved components in a mixture to react with a column. The detection and amount of a certain compound can be determined from the resulting peaks and equations (Cziczo, 2004). Figure 3.9 displays the general apparatus required for this technique.

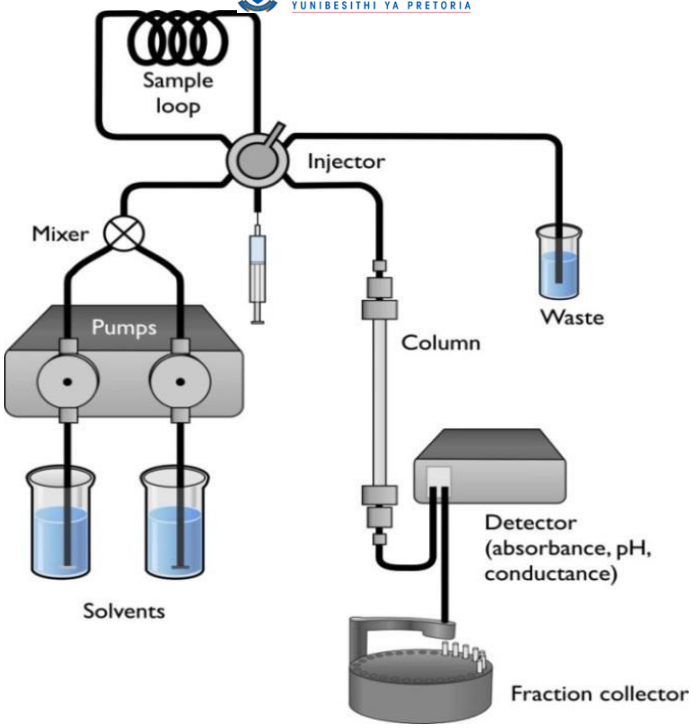


Figure 3.9: Simplified layout of HPLC method (HPLC, 2013)

In order to separate and detect the isolated bioactive markers (A2-A6) a PerkinElmer series 200 HPLC system was used. The system is composed of an analytical pump system part no. N2910100, auto-sampler system part no. 09938912, column oven, UV/Vis detector system part no. 09936504, refractive index system part no. 02711465 and fluorimeter. All the components were from the series 200 and manufactured in the US. An illustration of the system used is displayed below in figure 3.10. The HPLC method was automated with an auto-sampler illustrated in figure 3.11.

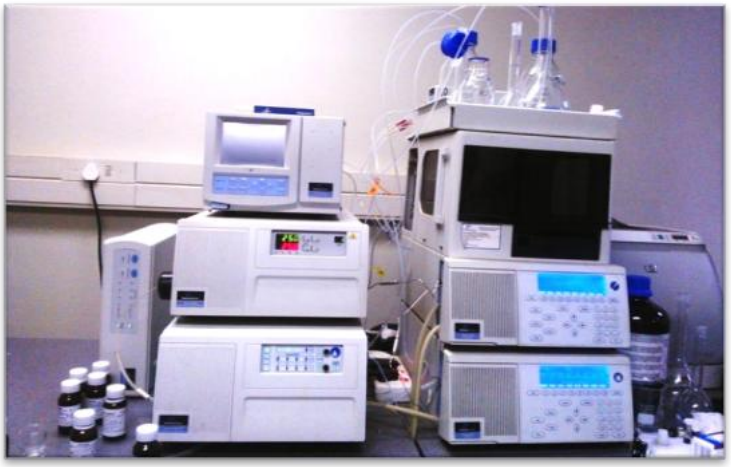


Figure 3.10: PerkinElmer HPLC system used in the present study

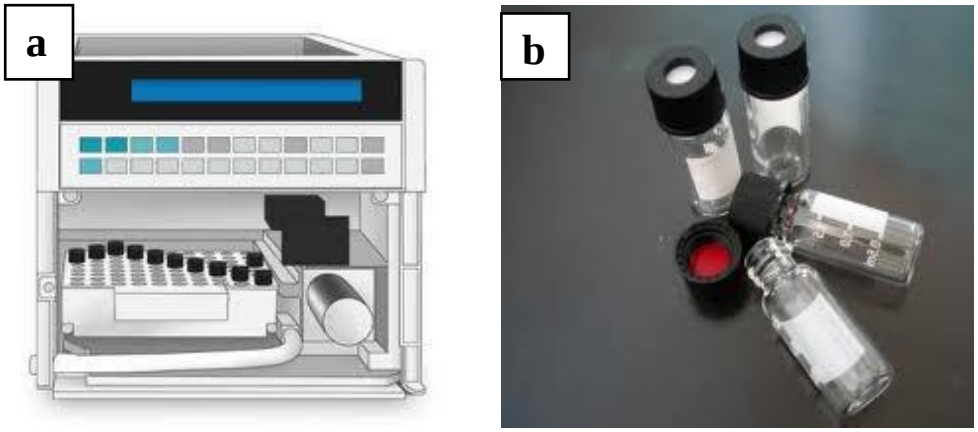


Figure 3.11: a) Auto-sampler b) vials used in the auto-sampler (Esslab, 2013; Weiku, 2011)

Standardisation

Ten microliters (10 µl) of each standard was injected into the system. The concentration of each standard is listed below in table 3.3 as the final dilution value. Dilutions were made and injected until optimal standard peaks were found. The standards were detected at 254 nm.

Table 3.3: Dilution of standards for injection into the system

Samples	Amount(mg)	Volume dissolved in(ml)	Concentration (mg/ml)	Dilution1 (mg/ml)	Final Dilution (mg/ml)
A2	5.2	2	2.6	0.26	0.13
A3	5.4	2	2.7	0.27	0.135
A4	5.3	2	2.65	0.265	0.135
A5	5.4	2	0.27	0.27	0.135
A6	2	2	1	0.2	0.1
Plant extracts	30	2	15	-	15

Calibration curves for each standard were created from the resulting peak areas using the linear least-square regression as in appendix E. Standard curves are illustrated in figure 3.12 below. Equations for these curves are provided in table 3.4.

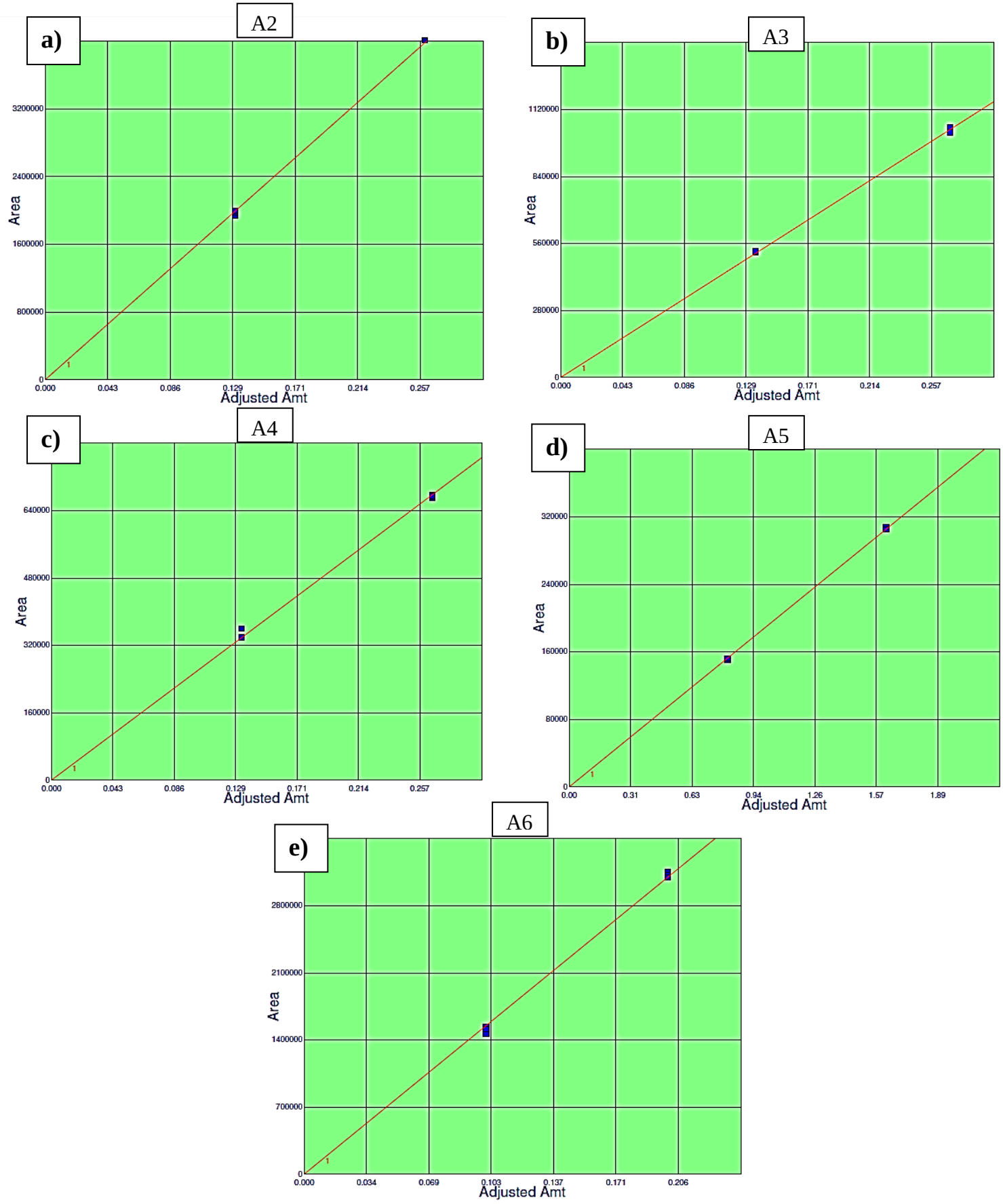


Figure 3.12: Standard curves of a) A2 b) A3 c) A4 d) A5 and e) A6

Table 3.4: Equations from standard curves of each compound	
Standard	Equation
A2	Y=(15303927.14)X
A3	Y=(3841628.85)X
A4	Y=(2551344.48)X
A5	Y=(188363.04)X
A6	Y=(15495497.46)X

Injection of samples

Ten microlitres (10 µl) of the crude extracts (final dilution) were injected three times into the column to obtain peaks of separated compounds. The stationary phase comprised of a Synergi Fusion RP (150mm×4.6mm) 4 um column at a constant temperature of 25°C. The mobile phase consisted of 20% acetic acid in water (A) and acetonitrile (B) used in isocratic mode with a flow rate of 1.2 ml/minute. The gradient elusion used for this study was: 0-2.5 minutes (A:B 9:1), 2.5-17.5 minutes (A:B 6:4) and 17.5-19.5 minutes (A:B 8:2). The total run time was 20 minutes.

The crude extracts were injected into the system in triplicate. Peaks were identified based on the expected retention times of standards. Detection of standards occurred at a wavelength of 254 nm. The analysis time was 10 minutes for both standards and samples. The resulting areas of the integrated peaks were used to determine the concentration of standards in crude extracts.

Statistical analysis

All contributing factors were considered in order to calculate the amount of each standard per kg of plant material as outlined in appendix E. The Relative Standard Deviation (RSD) was calculated from the three values for each standard per sample analysed. The RSD was < 5 overall for standards A3-A5. For standards A2 and A6 the RSD value fluctuated substantially.

3.2.3 Results and discussion

3.2.3.1 Tyrosinase inhibition assay

3.2.3.1.1 Statistical analysis

Table 3.5 and 3.6 below represent the Analysis of variance (ANOVA) and interactions of the tyrosinase inhibition values respectively. The factors were the 4 seasons (autumn, spring, summer and winter) and 3 ages (large, medium and small).

Table 3.5: Analysis of variance of tyrosinase inhibition data

Source of variation	d.f. (m.v.)	Sum of squares	Mean square	Variance ratio	F statistic
Season	3	3.01981	1.00660	55.05	<.001
Age	2	0.26960	0.13480	7.37	0.002
Season.Age	6	1.13575	0.18929	10.35	<.001
Residual	41 (7)	0.74970	0.01829		
Total	52 (7)	4.80884			

There was strong evidence of a significant Season×Age interaction therefore these means were compared using Fischer's protected t-LSD (Least Significant Difference) at a 5% significance level as evident in table 3.6 below.

Table 3.6: Log transformed (and untransformed) interaction means for tyrosinase inhibition data

Season	Size					
	Large		Medium		Small	
Autumn	2.256 [b]	(197.9)	1.773 [d]	(59.5)	1.707 [d]	(51.8)
Spring	2.395 [ab]	(248.2)	2.287 [b]	(194.4)	2.366 [ab]	(237.9)
Summer	2.048 [c]	(114.8)	1.731 [d]	(53.6)	1.974 [c]	(94.1)
Winter	2.227 [b]	(170.8)	2.509 [a]	(369.7)	2.394 [ab]	(255.6)

Means with the same letter or letters do not differ significantly based on the transformed means at the 5% significance level.

According to the data above, significant differences were found between values from autumn small, autumn medium and summer medium collectively and the rest of the samples. These

data points also had the lowest Inhibitory concentration (IC_{50}) values and therefore the highest activity.

Table 3.7 reports the IC_{50} values obtained for the anti-tyrosinase assay. Each extract was tested in triplicate with each experiment repeated thrice. Values could not be obtained for some samples as indicated by a"–" sign. In these instances, the IC_{50} value was estimated to be too high for the program to report. These samples were therefore, assumed to be ineffective at inhibiting the tyrosinase enzyme.

Kojic acid was the positive control used in this assay. As outlined in table 3.7, the IC_{50} of the control averaged at 6.99±0.14 µg/ml. Samples with the highest activity with regard to the tyrosinase inhibition assay were autumn small, autumn medium and summer medium with IC_{50} values of 51.77±0.058 µg/ml, 59.49±0.1 µg/ml and 53.62±0.71 µg/ml, respectively.

With reference to harvesting, a substantial amount of leaves may be required in order to produce extracts commercially. It is therefore impractical to harvest these leaves from very small plants as this may hinder their survival. It is possible that these plants require a high level of these secondary compounds for their defence. While it would be most convenient to harvest leaves from adult trees which grow leaves in abundance, the anti-tyrosinase activity was found to be low in these age groups over all the seasons. Leaves can be harvested from medium, during summer and autumn trees according to the results obtained in this study.

The values obtained for medium and small trees had a low amount of variation, however, activity varied substantially between different large trees. A follow up trial was carried out and is presented in section 3 of this chapter that deals with an attempt to identify the source of variation.

Table 3.7: Anti-tyrosinase activities of samples tested from each age group and season

Sample	Season								
	Spring		Summer		Autumn		Winter		
Extract	*IC_{50} (µg/ml)	R^2	*IC_{50} (µg/ml)	R^2	*IC_{50} (µg/ml)	R^2	*IC_{50} (µg/ml)	R^2	**Average[1]**
Small 1	219.6±3.10	0.87	76.14±0.26	0.98	46.85±0.04	0.99	377.9±6.80	0.86	
Small 2	177.7±6.10	0.85	81.70±0.31	0.97	62.62±0.09	0.99	196.9±3.30	0.90	
Small 3	-	-	110.8±0.50	0.98	71.74±0.05	0.99	197.7±1.30	0.96	
Small 4	-	-	96.89±0.47	0.96	44.78±0.03	0.99	212.5±3.45	0.91	
Small 5	316.3±17.85	0.71	104.8±0.39	0.98	32.88±0.08	0.99	292.9±18.40	0.63	
Average[2]	**237.87±9.02**		**94.07±0.39**		**51.77±0.06**		**255.58±6.65**		**159.82**
Medium 1	209.5±4.25	0.90	44.17±2.97	0.72	47.97±0.07	0.98	615.3±18.30	0.74	
Medium 2	206.5±3.00	0.92	47.54±0.16	0.98	45.94±0.13	0.98	596.0±26.80	0.65	
Medium 3	226.6±10.45	0.80	46.32±0.24	0.98	67.86±0.34	0.97	246.7±13.45	0.72	
Medium 4	161.8±1.30	0.94	69.59±0.04	0.99	78.14±0.10	0.99	191.1±20.55	0.46	
Medium 5	167.5±1.90	0.93	60.50±0.13	0.99	57.55±0.14	0.98	199.6±7.65	0.78	

Average[2]	**194.38±4.18**		**53.62±0.71**		**59.49±0.16**		**369.74±17.35**		**169.31**
Large 1	266.6±15.65	0.63	168.1±0.4	0.96	227.9±1.15	0.89	204.8±5.10	0.88	
Large 2	-	-	128.4±0.2	0.99	-	-	129.4±2.85	0.90	
Large 3	229.9±3.85	0.80	87.48±0.27	0.98	92.28±0.51	0.95	135.8±6.60	0.83	
Large 4	-	-	79.59±0.40	0.97	273.6±5.15	0.85	195.5±5.50	0.86	
Large 5	-	-	-	-	-	-	-	-	
Average[2]	**248.25±9.75**		**115.89±0.32**		**197.93±2.27**		**166.38±5.01**		**182.11**
Average[3]	**226.83**		**87.86**		**103.06**		**263.90**		
Kojic Acid	6.78±0.03	0.98	7.21±0.01	0.99	6.82±0.001	0.99	7.15±0.51	0.87	

- Median inhibitory concentration

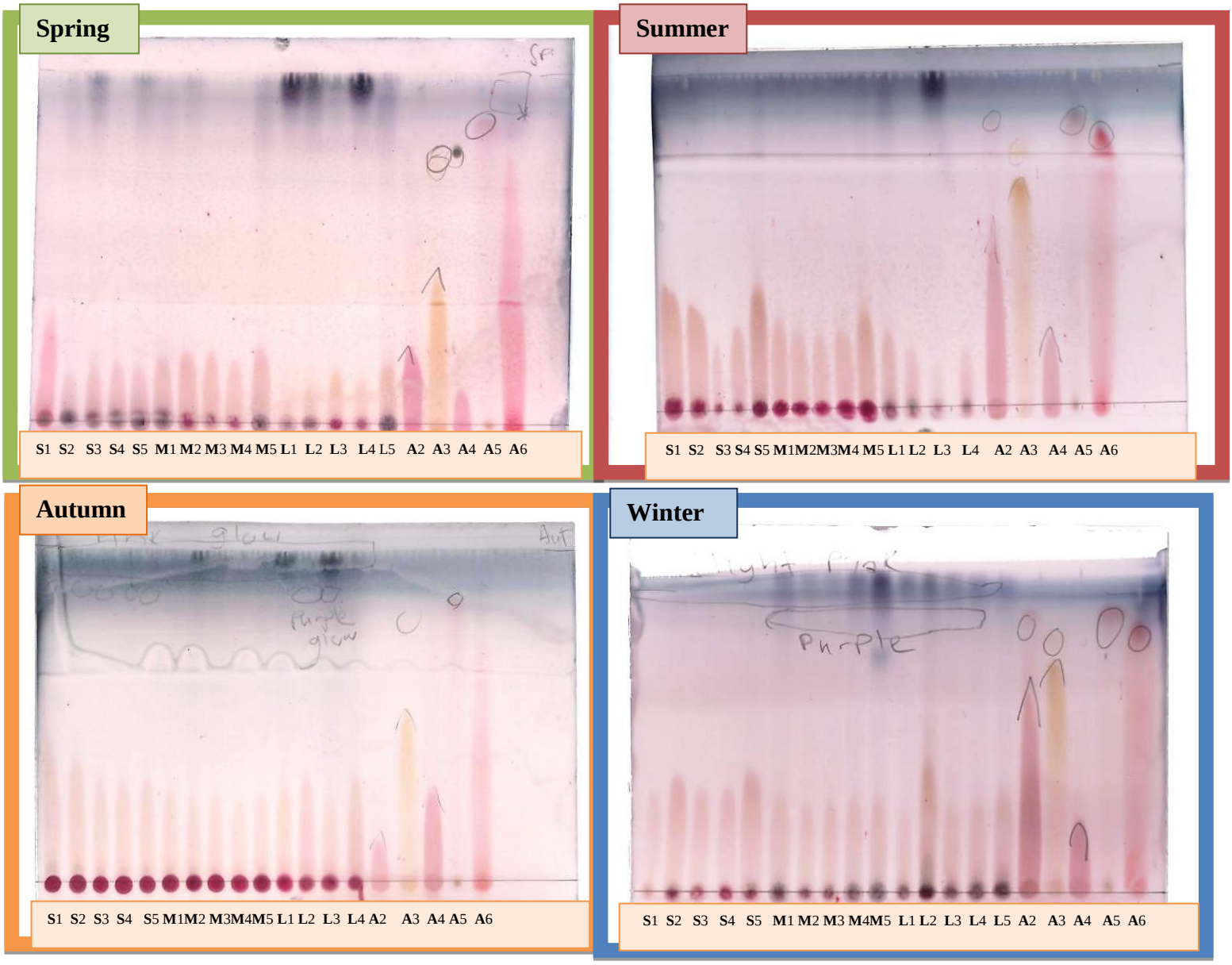
[1]per age group

[2]per season per age group

[3]per season

3.2.3.2 Thin Layer Chromatography

Figure 3.13 illustrates the TLC chromatograms labelled per season. The standards were spotted next to the extracts in order to identify them.



S1-small 1, M1-medium 1, L1-large 1, A2-A6-standards

Figure 3.13: Thin layer chromatogram of small, medium and large plant extracts per season

With reference to the spring plate, the spots observed for the large samples were lighter than the small and the medium. The large sample spots seemed to correspond with A4. The

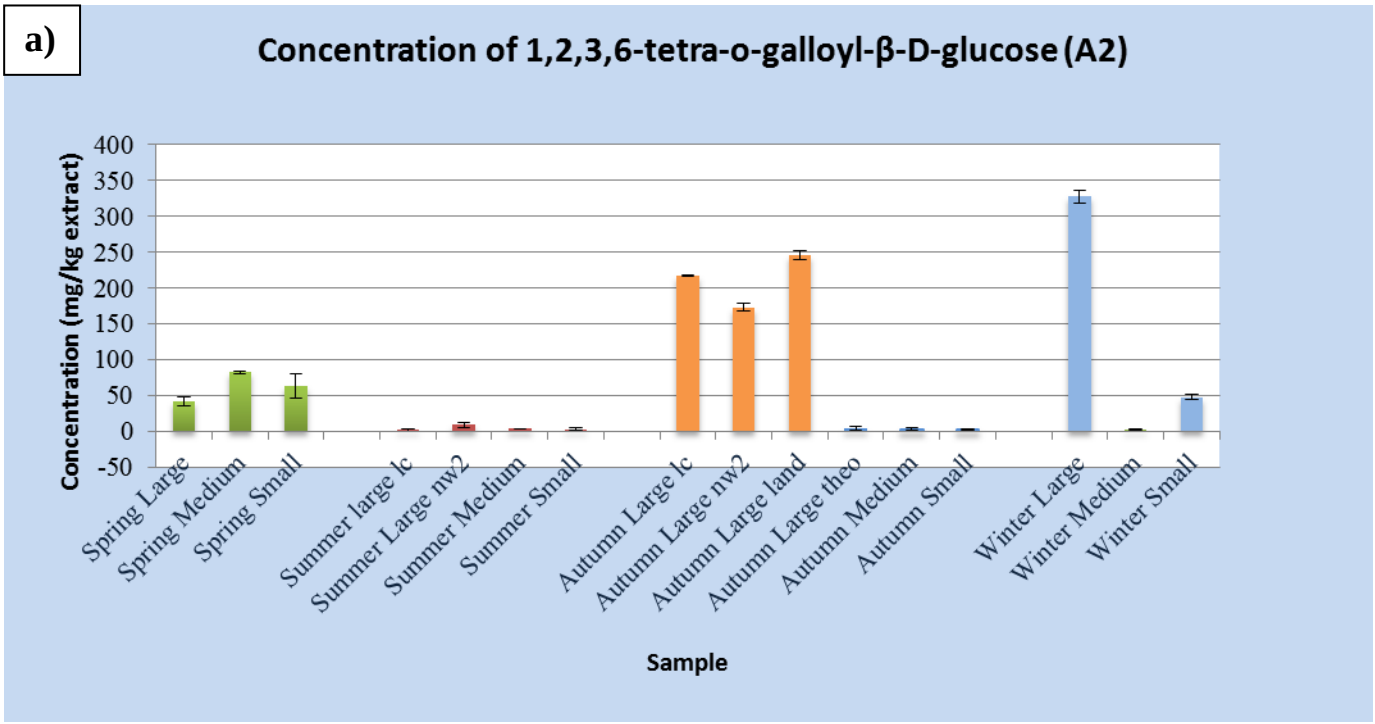
medium and possibly the small samples corresponded with A2 or A4 or both. The rest of the standards (A3, A5, and A6) were not visible in the extracts. The plate for summer was indicative that all samples may include A4; however the observation of any other standard in the extracts was not apparent.

The plate for autumn indicated well defined spots for all extracts. The spots observed were similar to that of A4. The presence of A2, if any, was not well defined. The same was seen for A3 and A6. Pencil outlines that were made under UV showed that A5 seemed to be present in S1-S5 as well as in two of the large extracts. For the plate that represented winter samples, spots of extracts were observed which may have corresponded with A4. The standards may not have been present or may not have been present in detectable amounts.

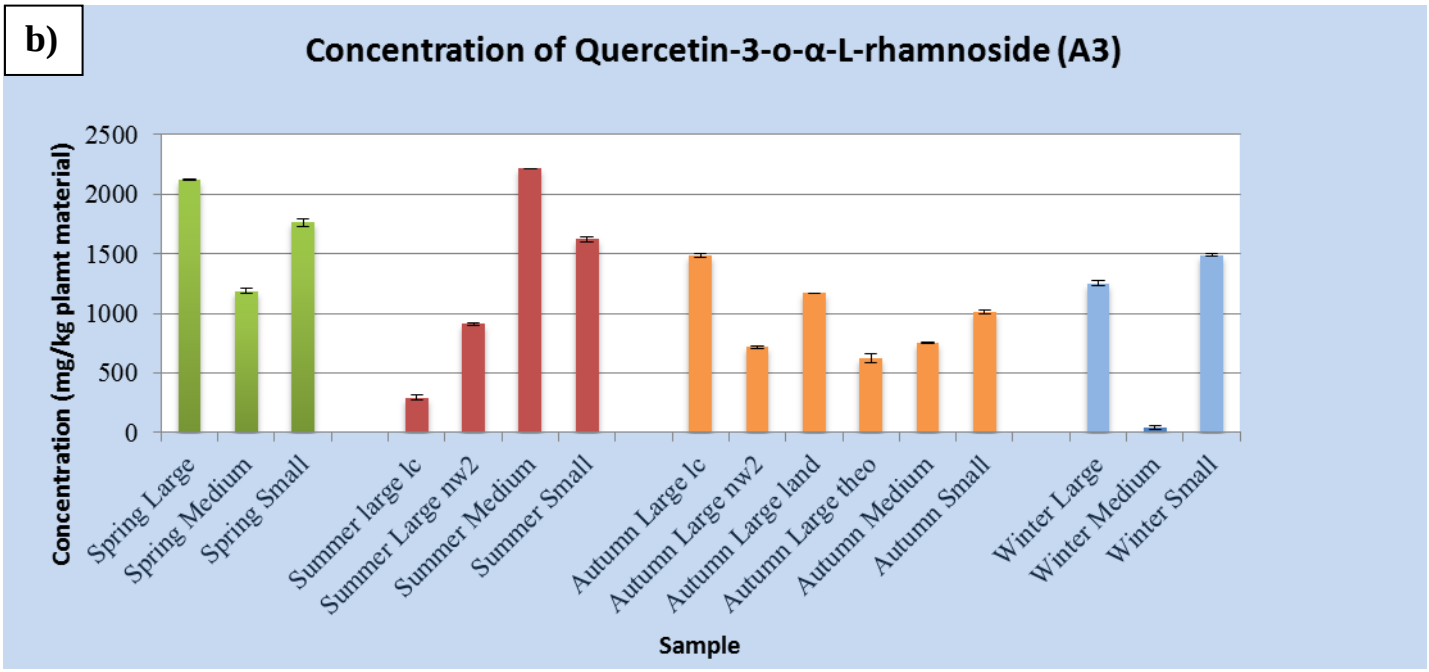
3.2.3.3 High Performance Liquid Chromatography

The calculated concentration of each compound in each sample identified with HPLC is illustrated below in figure 3.14 a) - e). The standard curve equations were used to calculate the concentrations of standards. An overview of the amount of each standard in a sample can be easily observed in the figures below, after which table 3.8 provides their actual concentrations in samples.

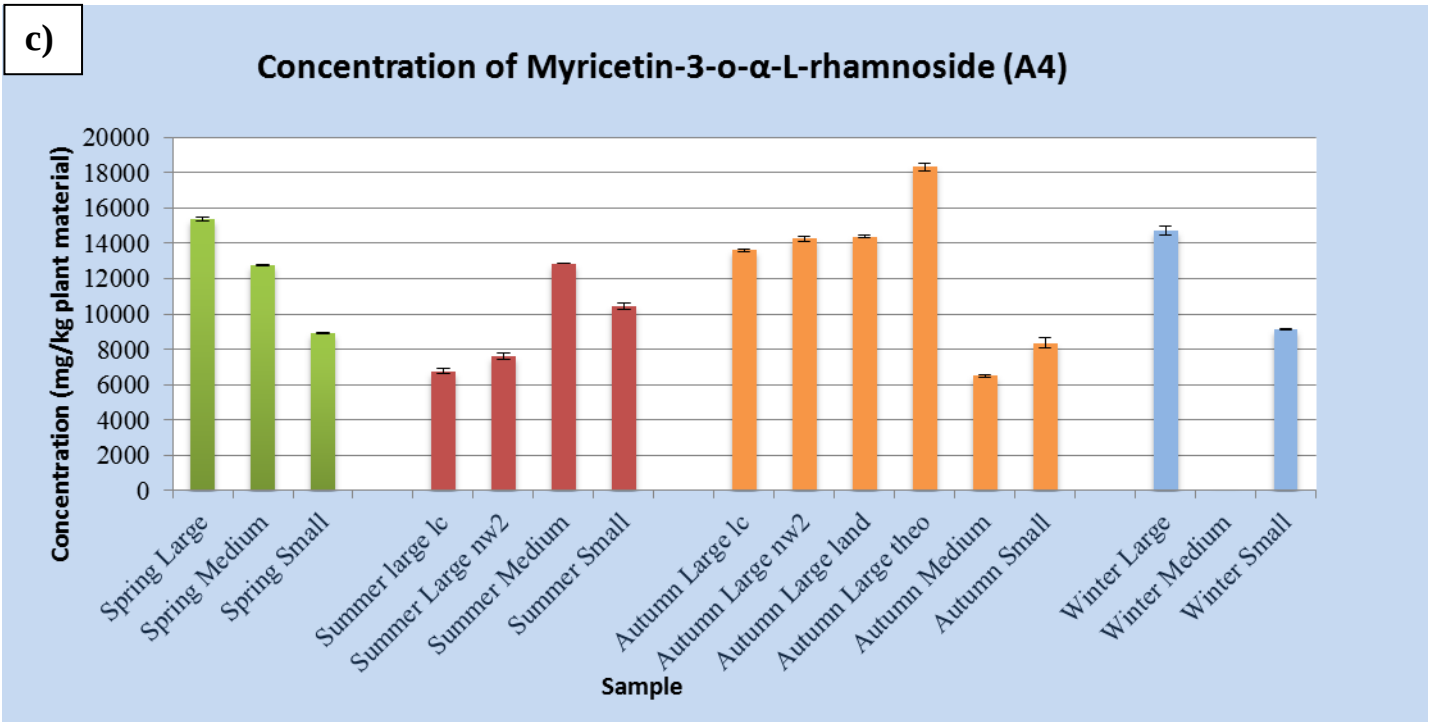
a)

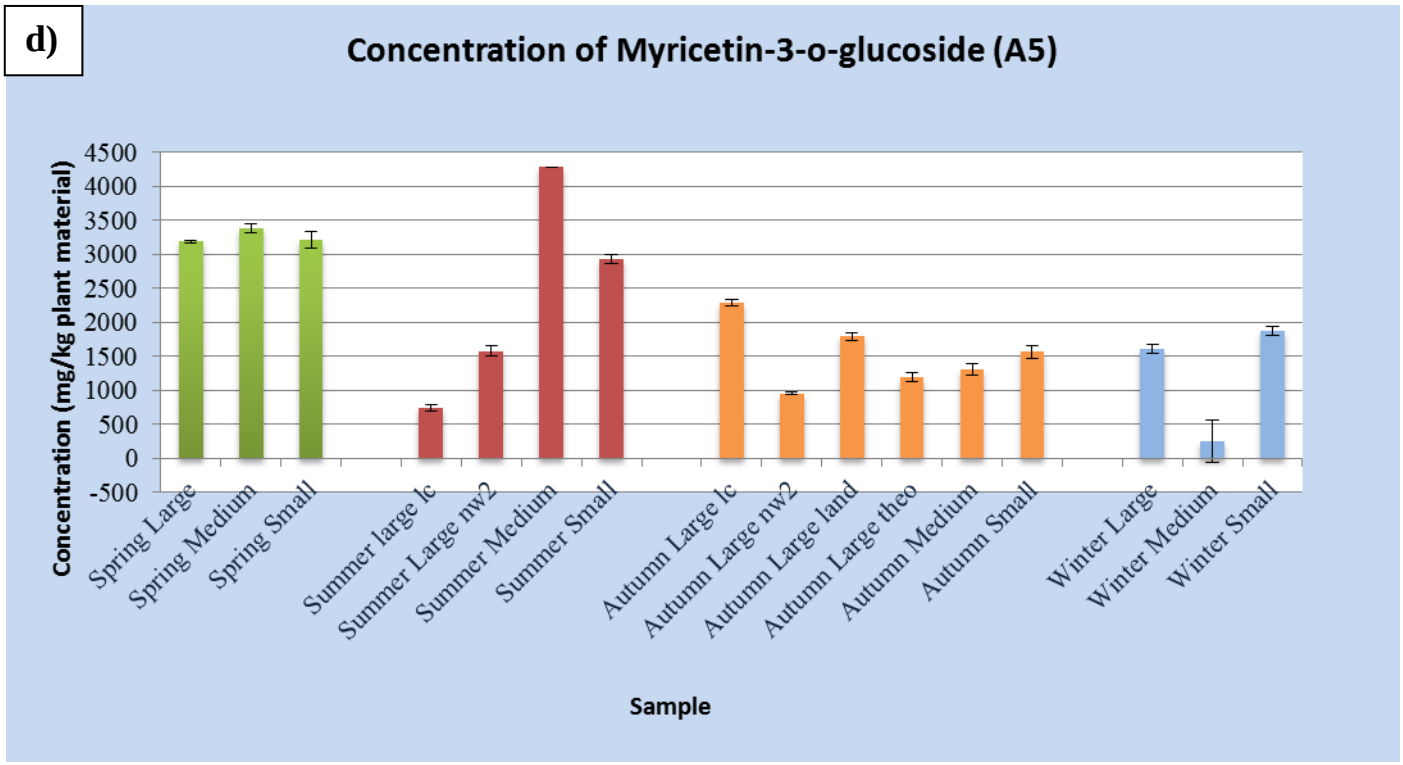


b)



c)





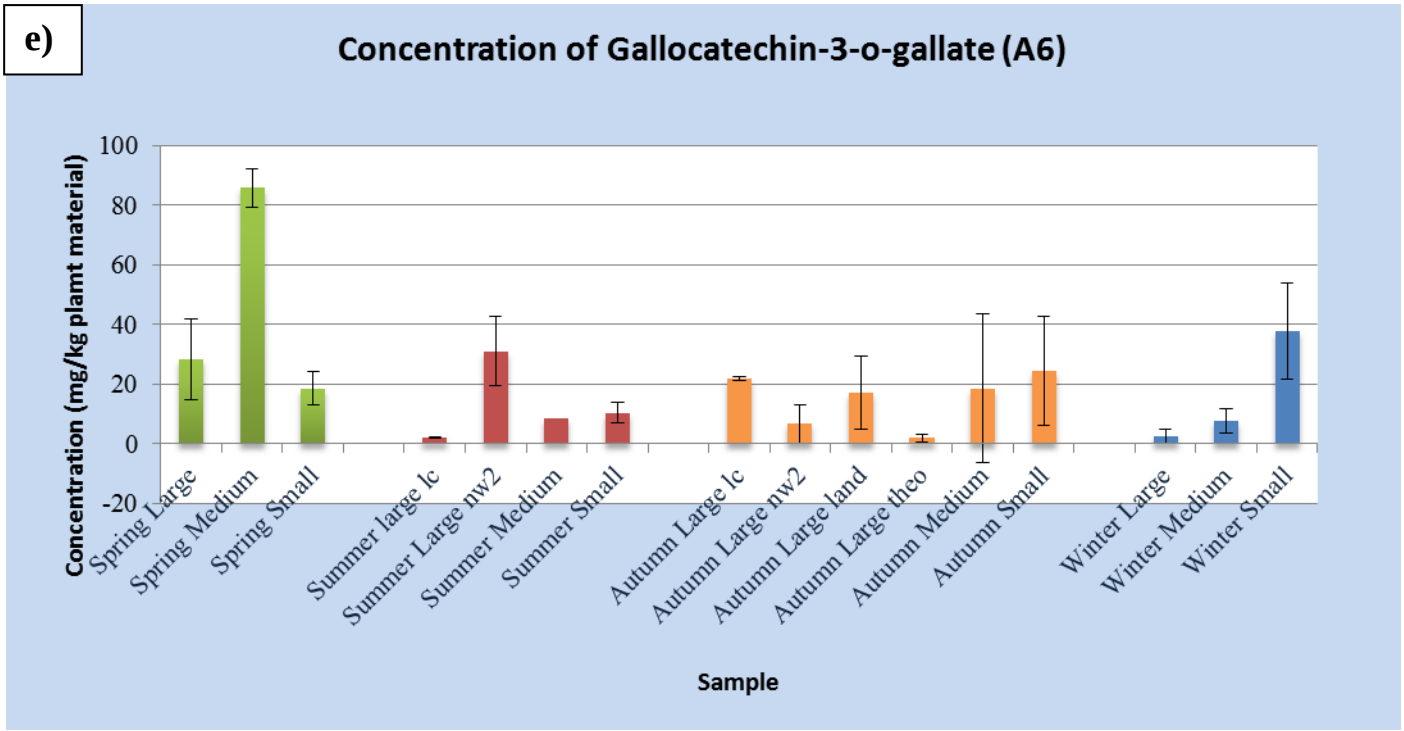


Figure 3.14: Concentrations of compounds in the leaves of *C. siliqua* during the four seasons a) 1,2,3,6-tetra-o-galloyl-β-D-glucose b) Quercetin-3-o-α-L-rhamnoside c) Myricetin-3-o-α-L-rhamnoside d) Myricetin-3-o-glucoside and e) Gallocatechin-3-o-gallate

Table 3.8: Concentrations of standards in samples

Sample	Concentration (mg/kg plant material)				
	A2	**A3**	**A4**	**A5**	**A6**
Spring Large	41.77±6.37	2117.93±5.41	15360.96±123.46	1274.98±10.28	28.31±13.53
Spring Medium	81.98±1.33	1187.49±20.03	12778.20±49.49	1354.11±29.58	85.71±6.45
Spring Small	63.43±16.75	1761.22±30.89	8908.29±59.40	1288.42±48.51	18.53±5.59
Summer Large lc[1]	0.92±1.43	464.55±3.89	6764.00±145.55	295.51±19.44	2.15±0.36
Summer Large nw2[2]	2.48±1.95	910.79±12.82	7596.44±185.99	630.31±30.83	31.02±11.54
Summer Medium	3.18	2216.84	12852.30211	1712.53	8.651975649
Summer Small	2.48±1.95	1623.01±21.14	10451.16±164.58	1173.15±26.78	10.41±3.47
Autumn Large lc	216.86±1.37	1487.44±17.64	13607.39±60.29	718.44±19.83	21.83±0.60
Autumn Large nw2	172.54±5.35	717.15±9.50	14258.84±125.66	381.73±9.98	6.64±6.38
Autumn Large land[3]	245.48±6.80	1170.09±0.94	14390.82±46.64	859.83±107.00	17.19±12.32
Autumn Large theo[4]	4.13±2.74	623.71±33.43	18323.32±236.0	1186.15±67.35	1.93±1.39

			5		
Autumn Medium	3.01±1.62	755.85±7.39	6482.85±96.46	523.27±34.31	18.60±24.81
Autumn Small	2.28±1.14	1009.99±14.09	8367.57±283.21	626.29±36.04	24.41±18.30
Winter Large	327.92±8.69	1250.64±23.05	14710.92±249.62	642.79±27.52	2.48±2.36
Winter Medium	1.89±1.12	40.65±14.60	408.95±174.70	98.78±123.79	7.52±4.12
Winter Small	47.74±4.21	1487.57±11.54	9107.23±48.76	750.82±27.88	37.85±16.26

[1] Large plants at L.C de Villiers, Pretoria

[2] Large plants on main campus (UP) Natural sciences building

[3] Large plants main campus (UP) Landbou building

[4] Large plants main campus (UP) Theology building

Standards A2 and A3 were identified as key compounds due to their anti-tyrosinase and melanin inhibition properties as provided in table 3.2. A2 was detected irregularly over winter in higher amounts than other seasons. A2 was also found in autumn samples at lower concentrations than winter. A3 was found to be highest in summer medium plants at a concentration of 2216.84 mg/kg plant material. Standard A4 was detected at higher levels than all other standards peaking in spring and autumn adult trees within a range of 13000-18000 mg/kg plant material. Standard A5 was found at its highest concentration in summer medium plants at 1712.53 mg/kg plant material. A5 was found at slightly lower concentrations in spring samples averaging at 1305.84 mg/kg for all ages. Standard A6 was found in the lowest levels compared with other standards. A6 was found at its highest concentration in spring medium plants at 85.71 mg/kg plant material.

HPLC has been used in a number of seasonal studies. The seasonal variation of the presence of 13 tannins in the leaves of *Betula pubescens* was assayed using HPLC. Many trends were observed over the seasons in this study and certain compounds were found in higher levels in young plants (Salminen *et al*., 2001). A similar trend was found in the present study with younger plants were found to have higher activities.

An interesting study that did not include plants, also involved the use of HPLC to analyse the seasonal content of certain pharmaceutical drugs in a sewage system plant. In this study, it was found that there was a high amount of certain compounds during winter. This study has important implications as a drinking water treatment plant is affected by the contents of the sewage system plan (Vieno *et al*., 2005).

3.2.4 Conclusion

Autumn small, autumn medium and summer medium samples were most effective at inhibiting the tyrosinase enzyme with IC_{50} values of 51.77±0.058 µg/ml, 59.49±0.1 µg/ml and 53.62±0.71 µg/ml respectively. The positive control for the tyrosinase inhibition assay was kojic acid with an averaged IC_{50} of 6.99±0.14 µg/ml. Due to the limitation of TLC, the clear presence of compounds in samples were not observed, however A4 seemed to be abundant from this technique. HPLC analysis confirmed that A4 was present in higher amounts than the remainder of the standards. HPLC detected the highest amount of the key compounds, based on their melanin inhibition properties, 1,2,3,6-tetra-o-galloyl-β-D-glucose in winter large (327.92 mg/kg plant material) samples and quercetin-3-o-α-L-rhamnoside in summer medium (2216.84 mg/kg). For the purpose of harvesting, based on this study, leaves can be collected during autumn and summer from

medium plants. Small plants may be excluded as harvesting could affect their survival. However, investigations on the link between bioactivity and juveniles can be carried out by pruning adult trees for new growth. The lack of correlation between the presence of compounds (HPLC) and activity (tyrosinase study) may be due to the interaction of all the compounds despite their individual properties.

Chapter 3

Section 3

Variation of bioactive compounds due to the gender and method of preparation of the leaf extract of *Ceratonia siliqua* L.

3.3 Variation identification trials

The seasonal studies were initiated in the season of winter with spring as the second season. During both these seasons, the tyrosinase inhibition activity was found to be lower than expected as compared with previous studies by Momtaz, (2007). A substantial amount of variation was observed in the large samples. Small scale trials were proposed to identify the source/s of variation.

3.3.1 Aim of study

The variation identification study aimed to identify if gender and the method of drying leaf material affected tyrosinase inhibition activity and the presence of biological markers with TLC. In addition an aqueous extract, preferred by consumers, of *C. siliqua* leaf material was prepared to observe the activity of such an extract.

3.3.2 Materials and methods

3.3.2.1 Plant collection and extract preparation

Gender study

Leaves of *C. siliqua* were collected from six different trees from main campus and L.C. de Villiers, Lynnwood, University of Pretoria, Hatfield during early winter. Three of the trees were female and three were male. Collection was carried out in May 2013. A male and female pair were identified in a certain area and used for collection. The areas collected from included the Landbou (LB) area (main campus), NW2 (NW2) area (main campus) and L.C de Villiers (LC). Voucher specimens of the representative material used were deposited into the H.G.W.J. Schweikerdt Herbarium under the numbers 119650 and 119651. The material to be tested was washed in dH_2O and placed to dry away from sunlight at room temperature. Dry plant material (60 mg) was ground and placed in 99.9% EtOH (50 ml). This mixture was placed on a shaker for 48 hours. The resulting solution was filtered and allowed to dry in a fume hood to obtain dry ethanol extracts.

Drying study

Leaves of *C. siliqua* were collected from one tree on main campus, Lynnwood, University of Pretoria, Hatfield in early winter. Collection occurred in May 2013. The material to be tested was separated into five equal portions (60 mg each). The material was washed in dH_2O. For the fresh sample,

material was immediately ground. Leaves that belonged to the freeze dried samples were placed in a -70°C freezer overnight. The frozen leaves were then freeze dried with a Virtis freeze drier (model #2KBTXL-75). The sun dried sample leaves were placed in direct sunlight until they were dry. Shade dried samples were prepared by allowing material to dry away from sunlight. The oven dried sample leaves were placed in an oven maintained at 50°C until dry. Dry plant material of each sample was ground and placed in 99.9% EtOH (50 ml). This mixture was placed on a shaker for 48 hours. The resulting solutions were filtered and allowed to dry in a fume hood to obtain dry ethanol extracts.

Aqueous extract study (liquid and dry)

Leaves of *C. siliqua* were harvested from a few adult trees growing in the same region. They were washed in dH_2O and allowed to dry away from sunlight at room temperature. When dry, the material was ground. For every 6.25 g of plant material, 25 ml of dH_2O was added to a jar. The mixture was placed on a shaker for 48 hours. Thereafter, half the mixture was filtered to obtain a clear liquid. Preservative was added to the liquid extract. The other half was placed in an evaporator (Genevac EZ-2 plus) to obtain a dry aqueous extract.

3.3.2.2 Tyrosinase inhibition assay

The anti-tyrosinase activity of the extracts at concentrations ranging from 3.13 – 400 µg/ml was carried out as mentioned in chapter 2 section 2.3.2.2.

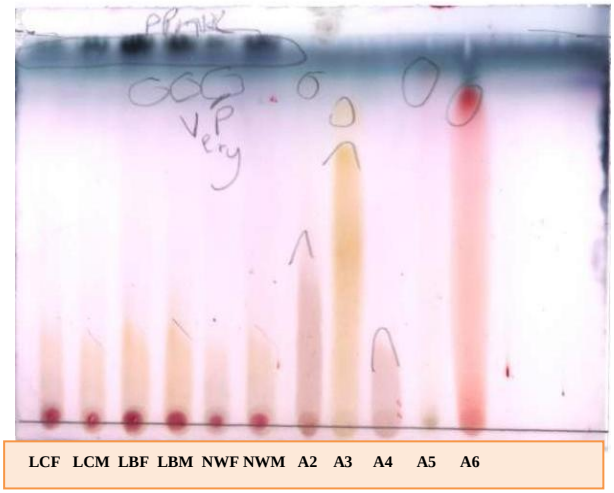
3.3.2.3 Thin layer chromatography (gender and drying study)

A chromatogram was prepared for the gender and drying study in the same manner as mentioned in section 3.2.2.4.

3.3.3 Results and discussion

3.3.3.1 Gender study

Figure 3.15 below displays the TLC plate obtained for the gender study.



LCF/M-L.C de VilliersFemale/Male, LBF/M-Landbou Female/Male, NWF/M-NW2 Female/Male.

Figure 3.15: Thin layer chromatogram of male and female plants

Standard A4 appears to be present in all samples; however under UV light no other compounds were clearly detectable. If present, they may have occurred at low concentrations. Table 3.9 below outlines the IC_{50} values obtained for the six samples that differ in gender.

Table 3.9: Anti-tyrosinase activities of male and female plants

Extract	*IC_{50}	R^2
Female NW2	117.70±0.20	0.98
Male NW2	128.60±0.20	0.98
Female LC	103.70±0.24	0.97
Male LC	139.60±0.45	0.95
Female LB	119.80±0.10	0.99
Male LB	174.70±0.55	0.94
Control (Kojic acid)	7.85±0.004	0.99

*Median inhibitory concentration

The female plant in the NW2 area was found to have the lowest IC_{50} value of 103.70 µg/ml. The male plant in the Landbou was found to have the highest IC_{50} in the study with a value of 174.7 µg/ml. Female plants were found to have lower values in each pair. The control averaged to an

IC_{50} value of 7.85 µg/ml. Pairs in each area were chosen in order to minimise the differences in an area such as climate, shade, watering, etc.

Variation could be observed between pairs. This may indicate that the area in which the plant grows may affect the activity. In addition there are other factors (genetic, stress tolerance, etc) that may affect the activity of a plant. However from this trial, a difference in activity between male and female plants can be observed. This is a basis for future studies on a larger scale. These results also added value to the commercial information about the plant.

In support of the above observation, a previous study found a general trend in willows (*Salix* spp.), where males grew faster than females, but were less protected against herbivory (Ahman, 1997). Studies like these indicated that gender can affect a plant in many ways. Another study was carried out to determine the effect of a number of parameters on the lateral shoot formation and adventitious root formation in *Salix planifola.* In this study it was found that female cuttings were superior in terms of root number and shoot biomass (Houle and Babeux, 1998).

3.3.3.2 Drying study

Figure 3.16 below displays the TLC plate obtained for the drying study.

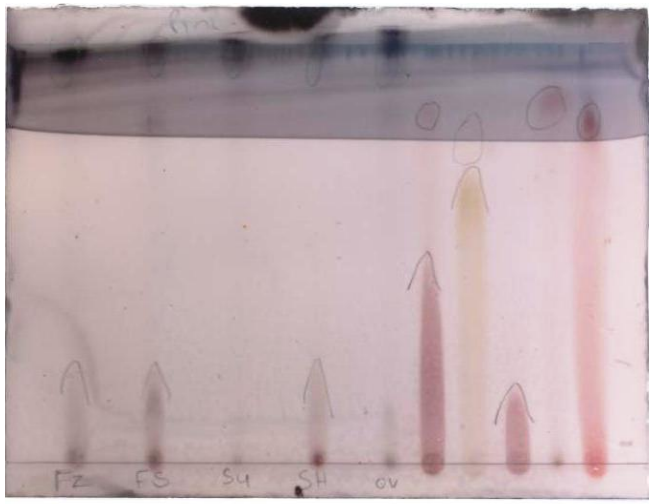


Figure 3.16: Thin layer chromatogram of different drying methods

Standard A4 seems to be present in all the samples above. In a similar observation to that of the gender study, the detection of other compounds did not occur. If present, the compounds may have

been present at concentrations that were below a detectable level. Table 3.10 below outlines the IC_{50} values obtained for the drying study.

Table 3.10: Anti-tyrosinase activities of different drying methods in µg/ml

Extract	IC_{50} (µg/ml)	R^2
Sun	99699±361.02	0.09
Shade	168.30±19.90	0.51
Freeze dried	941±15.80	0.14
Fresh	139.60±2.05	0.91
Oven	2643±96.02	0.09
Control	7.64±0.002	0.99

The methods of drying the leaf material, which resulted in the lowest IC_{50} values, were found to be the fresh and shade dried sample at 139.6 µg/ml and 168.30 µg/ml, respectively. The IC_{50} values of the control averaged at 7.64 µg/ml. The other methods produced extracts with low activities. In addition, the use of equipment to dry the leaves appear to be unnecessary. This minimises the costs involved in preparing extracts. Leaves that were not dried and crushed immediately after harvesting produced the highest tyrosinase inhibition activity. If verified and safe to use in large scale extract preparation, this method could save the time and space required for drying the material.

In support of these findings, a study was carried out on the effect of six different drying methods on the level and composition of the essential oil of *Laurus nobilis.* The results indicated that one method, of air drying at ambient temperature, was appropriate in order to obtain the highest level of bioactive components. The other methods included oven and microwave drying (Sellami *et al*., 2011).

A similar experiment was carried out on the essential oils of *Juniperus phoenicia.* The effect of the drying method relative to the chemical composition and bioactivities was observed. It was found that drying in the sun was beneficial for essential oil yield and drying in the shade was more suitable for concentrating specific compounds (Ennajar *et al*., 2010).

3.3.3.3 Aqueous extract study

Figure 3.17 below provides two inhibition graphs to compare and observe any tyrosinase inhibition of the aqueous extract of *C. siliqua*. The dry extract shows no inhibition whereas the liquid aqueous extract was found to be active against the tyrosinase enzyme.

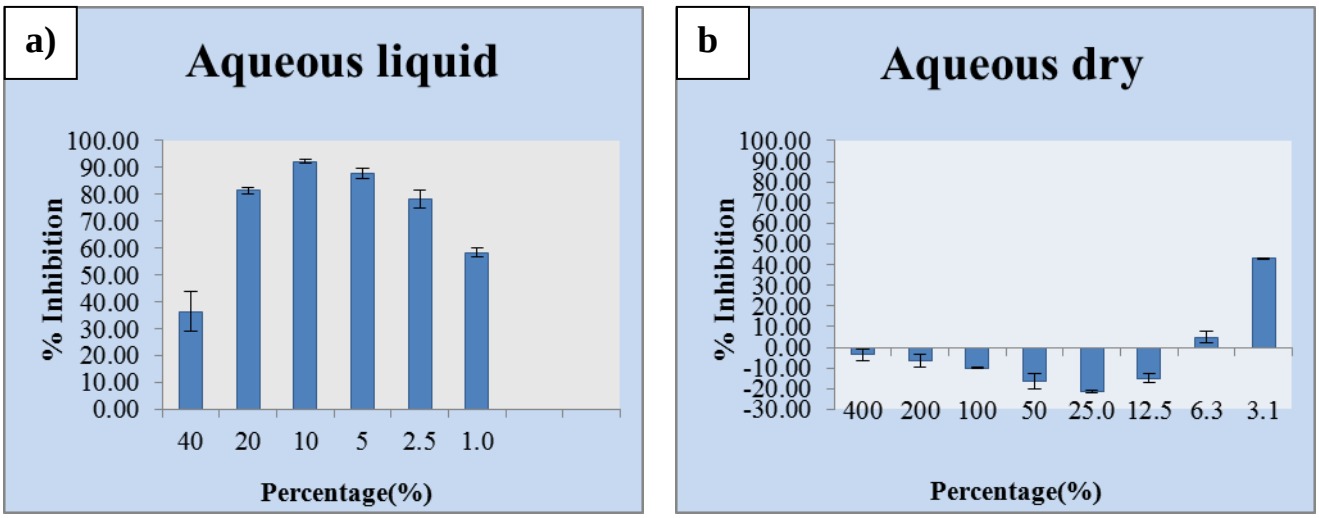


Figure 3.17: Tyrosinase inhibition graphs of aqueous *C. siliqua* leaf extracts a) liquid and b) dry

However, since this extract is in liquid form, the quantification is challenging. The extract cannot be reported in weight but in volume. Active aqueous actives are becoming popular in cosmetics. The more natural a product, the more the consumers are likely to trust a brand. In addition, the elimination of other commonly used solvents for extraction may be welcomed as many of these solvents are potential irritants. This observation trial indicates that the aqueous extracts of this plant can be further studied for application in the cosmetic field.

3.3.4 Conclusion

Results from the gender study indicated a trend where female plants of *C. siliqua* had lower IC_{50} values for the tyrosinase inhibition assay. A female plant growing in the LC area in Hatfield was found to have the highest activity with an IC_{50} value of 103.70 µg/ml. Even though variation between the pairs of trees was observed, a number of factors contributed to the activity of this plant, the gender may be one factor. During the drying study, it was found that leaves that were crushed after harvesting, without drying, led to an extract with the highest tyrosinase inhibition activity. This method of extract preparation could save time and resources that would normally be required for drying leaves. The IC_{50} obtained for the fresh leaf extract was 139.60 µg/ml. The TLC

analysis was unclear with regard to the appearance of spots. As this was just a small scale observation trial, more elaborate and analytical techniques such HPLC were not used. Methods such as HPLC/LCMS can be used at a large scale and well-designed experiments to further investigate the presence of the bioactive compounds in different extracts. The results from the aqueous study indicated that these extracts inhibited the tyrosinase enzyme. Aqueous extracts are becoming popular in cosmetic products.

The small scale studies described here can be expanded to obtain a clearer trend that is also statistically significant. These variation identification trials have laid the foundation for future projects. Results from such projects can give insight to the correct season, age, gender, extraction method and manner of preparing the extract to obtain the most active extract.

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Chapter 4

General Conclusion

4.1 General Conclusion

Athrixia phylicoides

- In the spring trial, IBA (0.1%) had the highest rooting percentage of 58.33% and highest sprouting percentage of 60%. NAA (0.1%) had a rooting percentage of 51.67%. IBA and NAA at 0.3% had the highest number of roots for the trial: 38.72 and 31.00, respectively.
- In the autumn trial, NAA (0.1%) was found to have the highest sprouting percentage of 78.33% and at 0.3%, the highest rooting percentage of 83.33%. The highest number of roots formed was 7.88 by NAA at 0.3% with IAA forming 6.67 roots at 0.3%. IBA at 0.1% formed 6.41 roots.
- NAA and IBA were identified as potential hormones to be used for the propagation of cuttings with NAA having more advantage in terms of cost per 10 g, NAA-R 90 vs. IBA-R 700.
- Ethanol extracts of *A. phylicoides* did not show anti-tyrosinase and antimicrobial activity at the highest concentration tested (400 µg/ml extract).

Ceratonia siliqua

- Autumn small, autumn medium and summer medium samples were found to be most effective at inhibiting the tyrosinase enzyme, with IC50 values of 51.77±0.058 µg/ml, 59.49±0.1 µg/ml and 53.62±0.71 µg/ml, respectively as compared with kojic acid (IC50 = 6.99±0.14 µg/ml).
- Based on their melanin inhibition properties, the concentration of key compounds 1,2,3,6-tetra-o-galloyl-β-D-glucose and quercetin-3-o-α-L-rhamnoside, were found at the highest concentrations in winter large (327.92 mg/kg plant material) and summer medium (2216.84 mg/kg) samples, respectively as quantified by HPLC.
- TLC produced unclear results with regard to the presence of bioactive compounds. However standard A4 or Myricetin-3-o-α-L-rhamnoside was detected in noticeable amounts on the plates for all samples.
- Leaves can be collected during autumn and spring from medium sized trees in order to obtain the highest tyrosinase inhibition activity.

- Variation identification trials indicated that female trees were found to have lower IC50 values than males in the tyrosinase inhibition assay. The lowest value obtained was IC50 = 103.70 µg/ml. The drying study found that leaves that were crushed after harvesting, without drying, led to an extract with the highest tyrosinase inhibition activity. The IC50 obtained for the fresh leaf extract was 139.60 µg/ml. The IC50 value of the control averaged to 7.75 µg/ml for the variation identification trials.

4.2 Publications, conferences and works from study

- Propagation studies on apical cuttings of *Athrixia phylicoides* DC. South African Journal of Botany (under submission)
- Seasonal variation studies on leaf extracts of *Ceratonia siliqua* L. South African Journal of Botany (under submission)
- Presented at departmental conference, Department of Plant Science, University of Pretoria
- Lall, N. and Singh , S., University of Pretoria S.A. , 2014. Skin Lightening. South Africa. Pat ZA2012/05718.

4.3 Recommendations for future work

Athrixia phylicoides

- An increase in the number of cuttings for hormone rooting trial to obtain a clearer idea of trends.
- Strict control of the factors affecting the growth of cuttings (watering, temperature control, etc.).

Ceratonia siliqua

- Collection of seasonal samples per month to obtain a clear trend of variations.
- Isolation of standard A1 or (-)-epicatechin -3-o-gallate to obtain an idea on the seasonal variation of this compound.
- Replicates of seasonal HPLC samples can be prepared to obtain statistically significant results.

- Variation identification trials can be carried out on a large scale to investigate variation and compare with the findings of the current study.
- Variation of large trees can be investigated according to factors such as stress, area, and genetic variation.
- Pruning trials on adult trees to investigate the apparent link between bioactivity and juvenile plants.

Chapter 5

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Chapter 6

Appendices

Appendix A

Hormone Rooting Trial Raw Data

Spring 2012

Block	position	hormone	Overall comments	Survival
1	1	6	High mortality	5/20
1	2	5	High mortality	1/20
1	3	2	High mortality	3/20
1	4	8	Good appearance	14/20
1	5	7	No survivors	0/20
1	6	4	Good appearance	13/30
1	7	1	High mortality	2/20
1	8	9	Good appearance	9/20
1	9	3	Good appearance	18/20

Block	position	hormone	Overall comments	Survival
2	1	9	No survivors	0/20
2	2	5	High mortality	4/20
2	3	2	High mortality	3/20
2	4	1	Good appearance	18/20
2	5	7	Good appearance	10/20
2	6	8	Good appearance	14/20
2	7	4	High mortality	7/20
2	8	6	High mortality	5/20
2	9	3	High mortality	4/20

Block	position	hormone	Overall comments	Survival
3	1	1	High mortality	7/20
3	2	6	High mortality	2/20
3	3	9	Good appearance	1/20
3	4	3	Good appearance	18/20
3	5	5	Good appearance	15/20
3	6	9	Good appearance	16/20
3	7	4	High mortality	8/20
3	8	7	High mortality	0/20
3	9	2	Good appearance	10/20

Autumn 2013

Block	position	hormone	Overall comments	Survival
1	1	9	60% leaves had red tips, good appearance and survival	28/30
1	2	2	Green and healthy, moderate	24/30
1	3	1	Good healthy looking	30/30
1	4	7	Light green with red tips	27/30
1	5	4	Green firm stems, some red tips	28/30
1	6	3	Green healthy looking	30/30
1	7	6	Good, green with some redness	27/30
1	8	8	Deep green, dry, moderate,	23/30

			red tips	
1	9	5	Green healthy	28/30

Block	position	hormone	Overall comments	Survival
2	1	2	Very light green and healthy	29/30
2	2	7	Light green , dry, slight redness, white masses on stems and roots	29/30
2	3	5	Green healthy looking	29/30
2	4	1	Most green healthy, some redness	25/30
2	5	9	Deep green healthy, red tips	26/30
2	6	8	Long stems, deep green , healthy	21/30
2	7	3	Very healthy, slight red	26/30
2	8	6	Green healthy, slight red	18/30
2	9	4	Tall, green healthy, red tips	13/30

Block	position	hormone	Overall comments	Survival
3	1	9	Firm stems, red tips, deep green	28/30
3	2	4	Very slight red, light healthy	29/30

			green, firm stems	
3	3	3	Green , no redness	27/30
3	4	2	Green, dry	24/30
3	5	8	Dry and red, not healthy	27/30
3	6	6	Very light green, no redness, healthy	25/30
3	7	7	Tall, light green, some red	26/30
3	8	1	Green healthy, slight redness	30/30
3	9	5	Deep green with red tips, firm	30/30

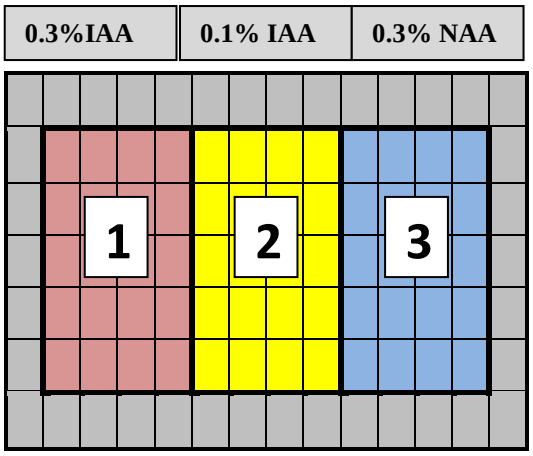
Block	position	hormone	Overall comments	Survival
4	1	7	Slight redness, quite green	23/30
4	2	8	Tall, with red tips, moderate	25/30
4	3	6	Deep green, firm slight redness, moderate	23/30
4	4	4	Dry, light green, red tips	20/30
4	5	9	Moderate green with red tips	24/30
4	6	1	Good, firm, deep green	30/30
4	7	5	Dry and green	29/30
4	8	3	Dry and green with red tips	25/30
4	9	2	Very good looking, green with very slight red	30/30

Appendix B

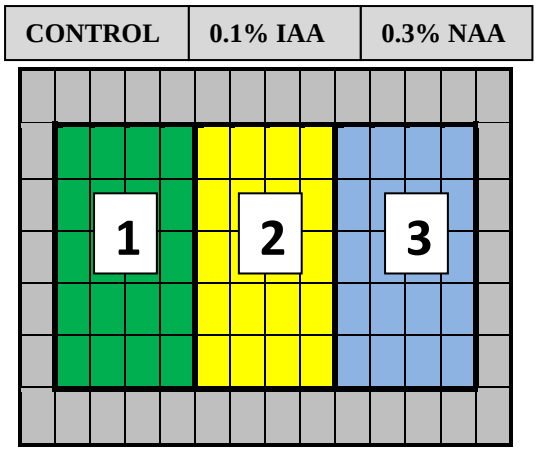
Hormone Rooting Trial Layout

Treatment	
1) 1-Naphthaleneacetic acid 0.1%	
2) 1-Naphthaleneacetic acid 0.3%	
3) Indole-3-butyric acid 0.1%	
4) Indole-3-butyric acid 0.3%	
5) Indole-3-acetic acid 0.1%	
6) Indole-3-acetic acid 0.3%	
7) Gibberellic acid 0.1%	
8) Gibberellic acid 0.3%	
9) No hormone	

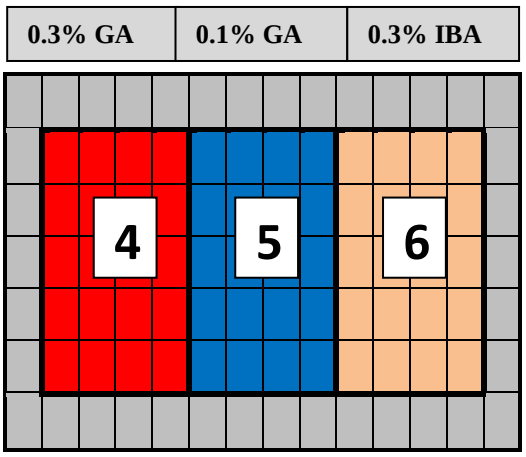
Replicate 1 - Tray 1



Replicate 2 - Tray 4



Replicate 1 - Tray 2



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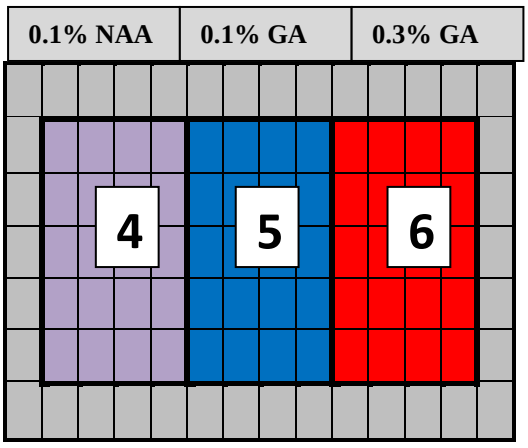

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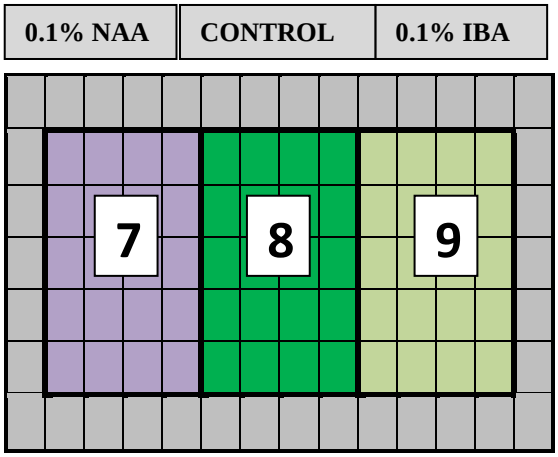

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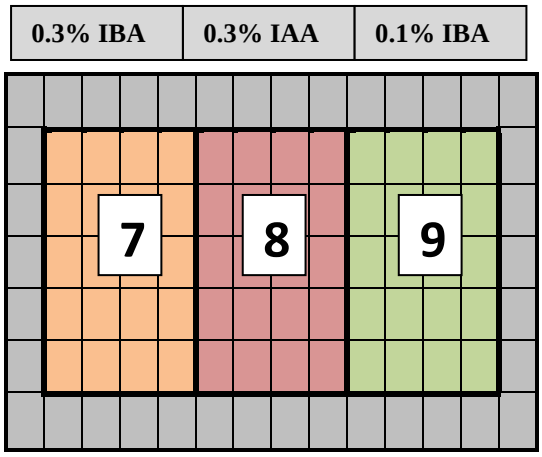

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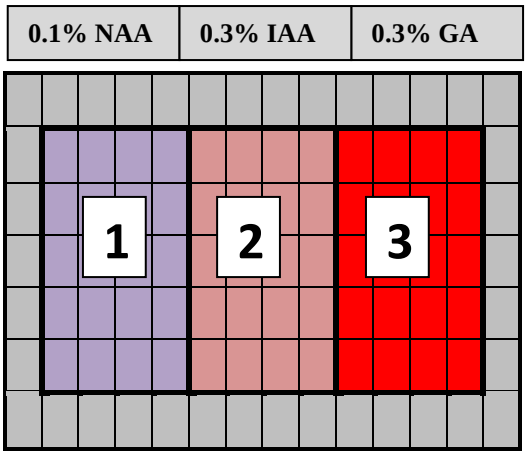

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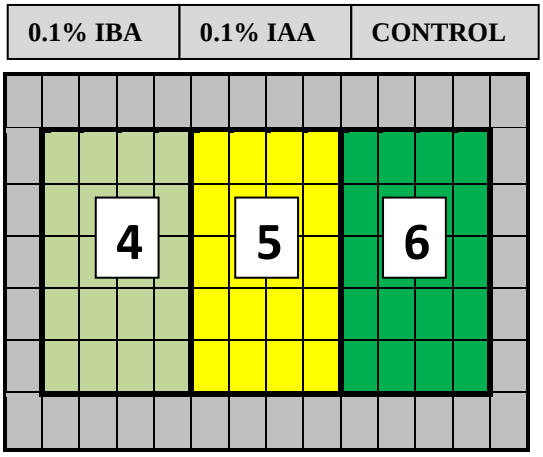

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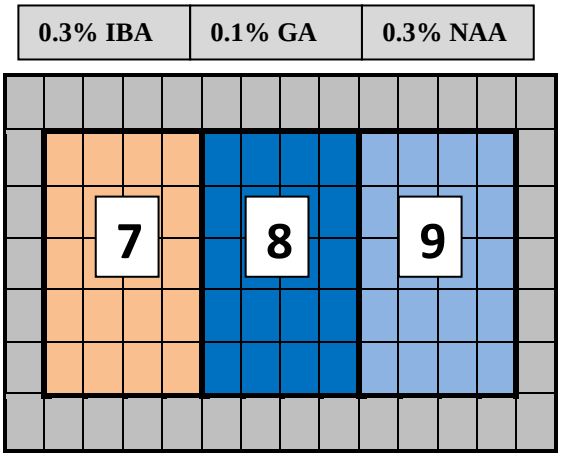
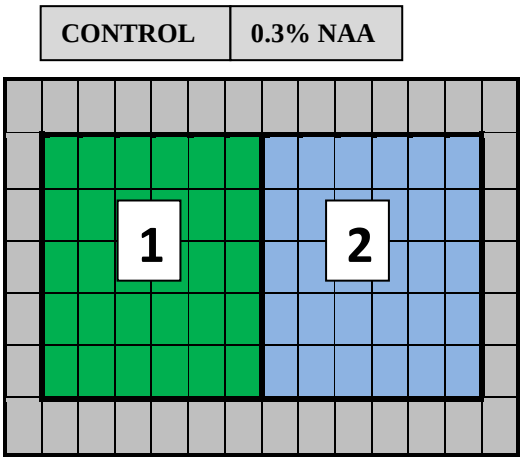


Autumn 2013

Replicate 1- Tray 1



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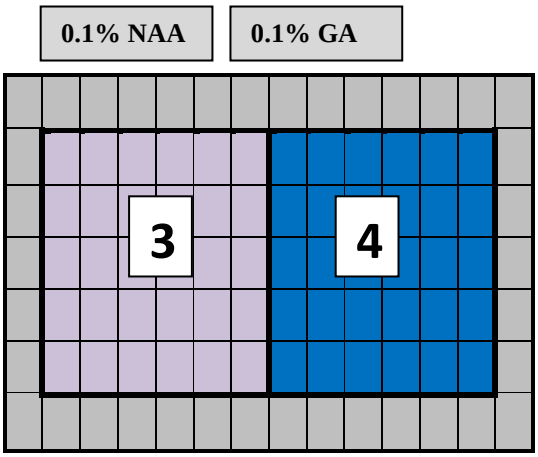

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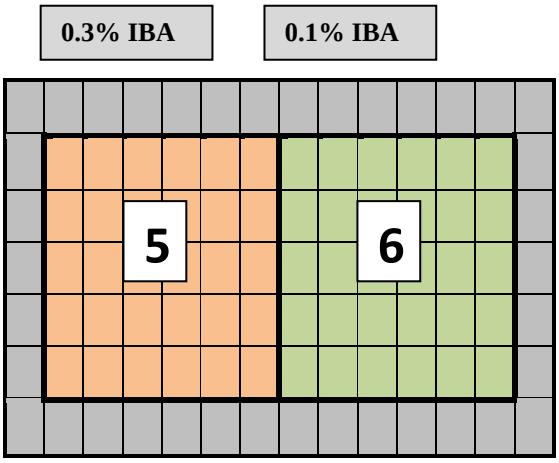

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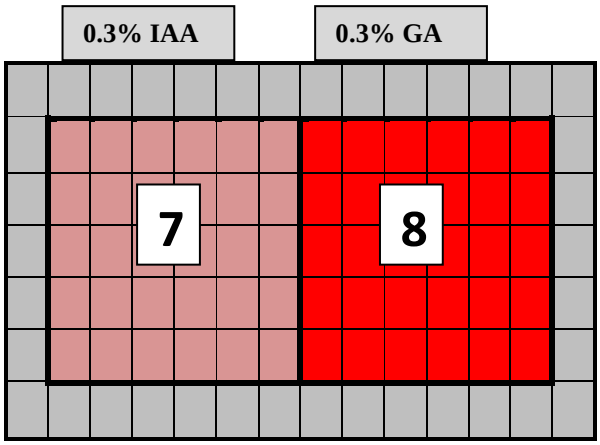

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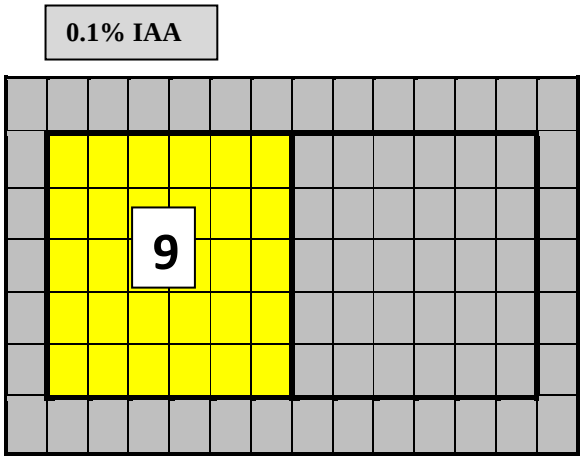

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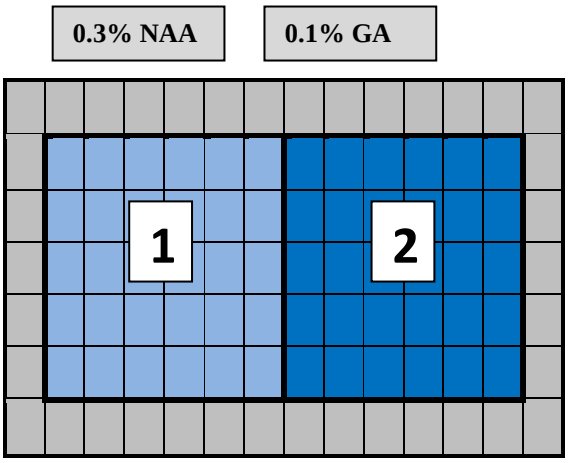

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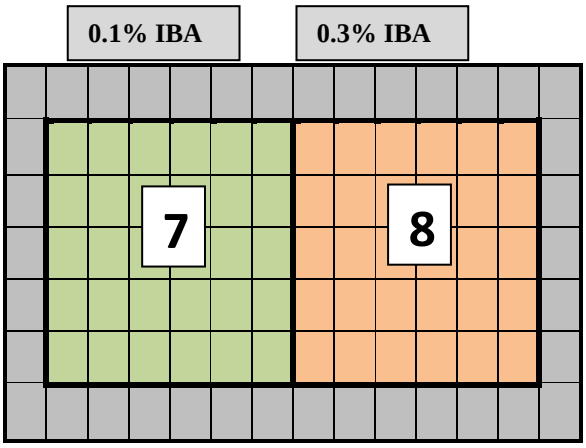

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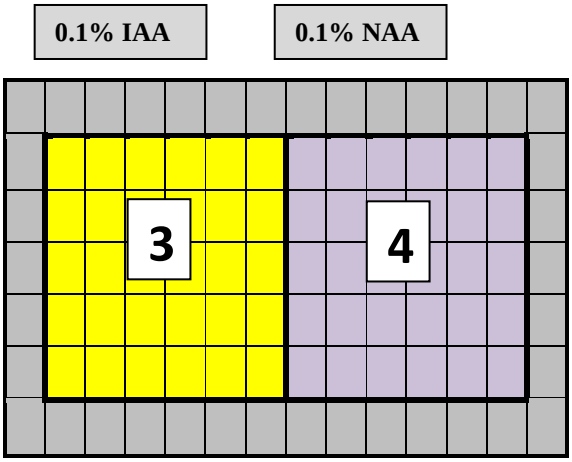

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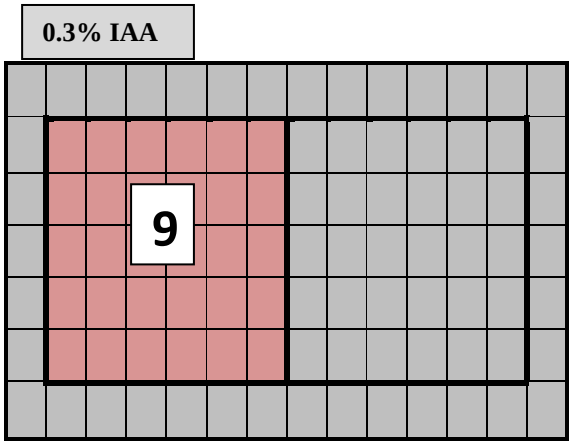

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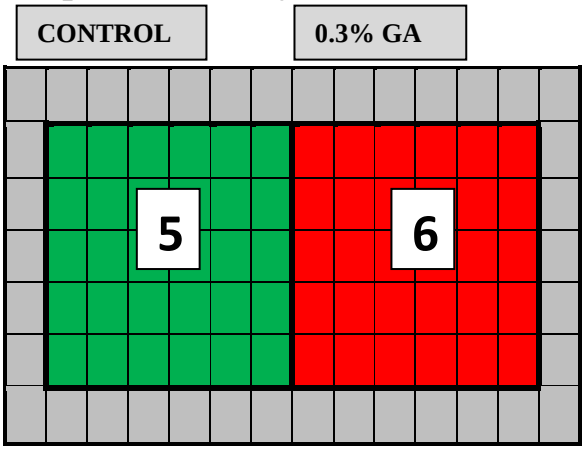

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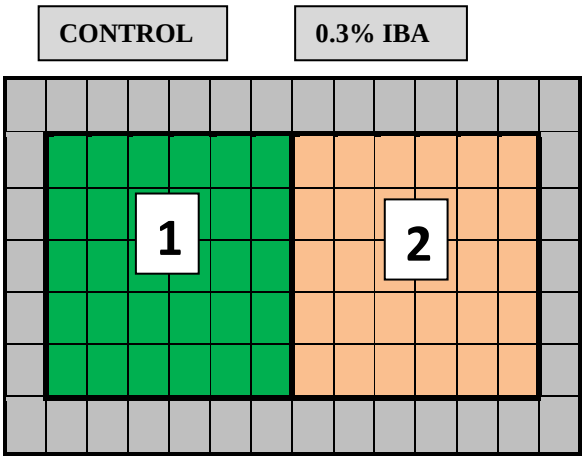

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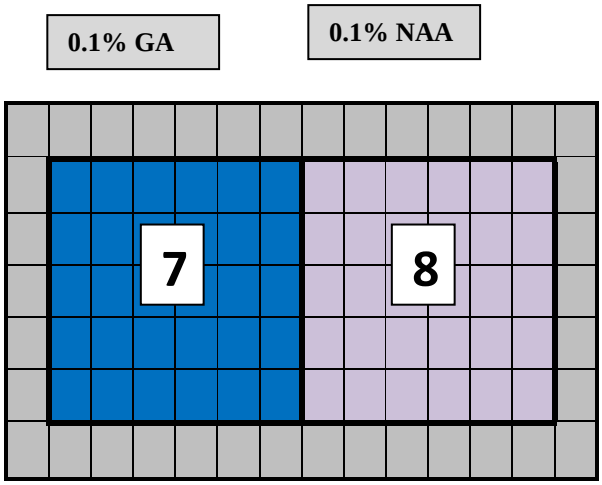

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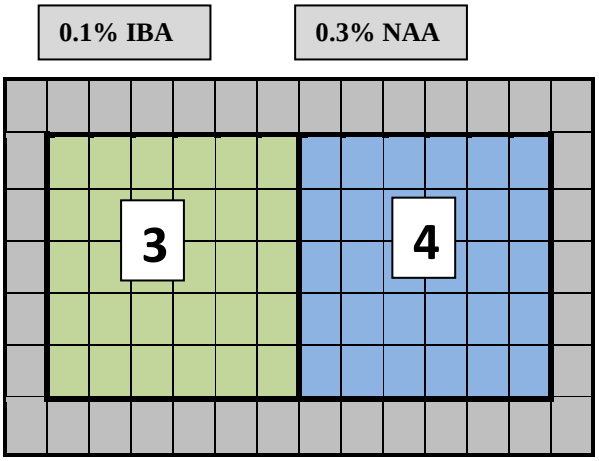

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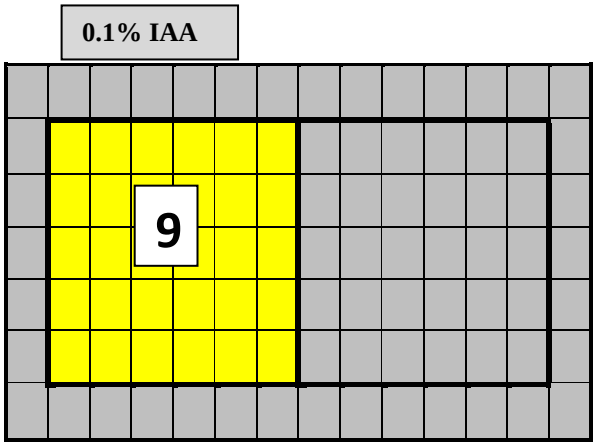

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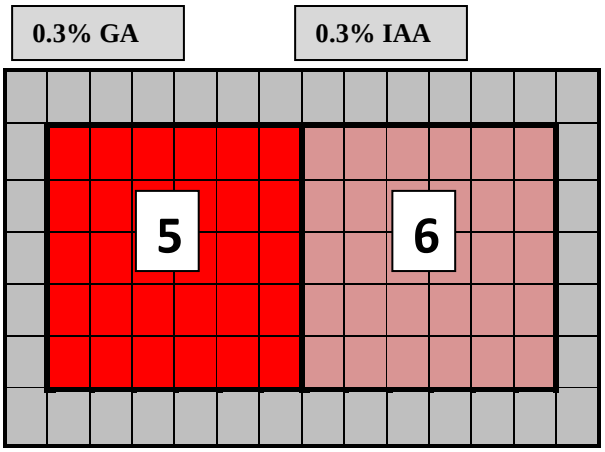

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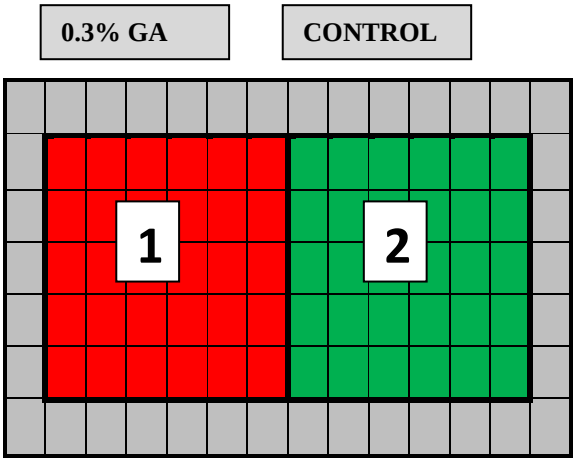

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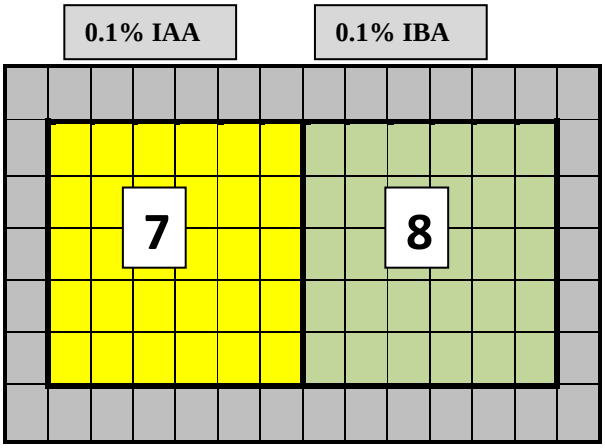

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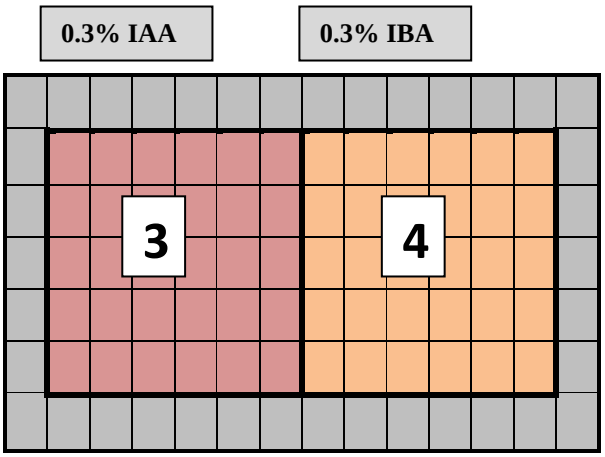

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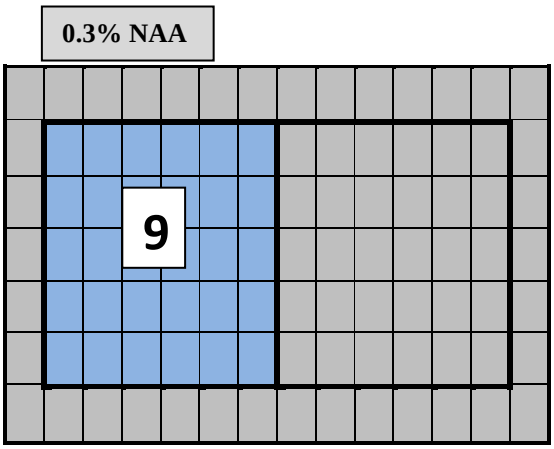

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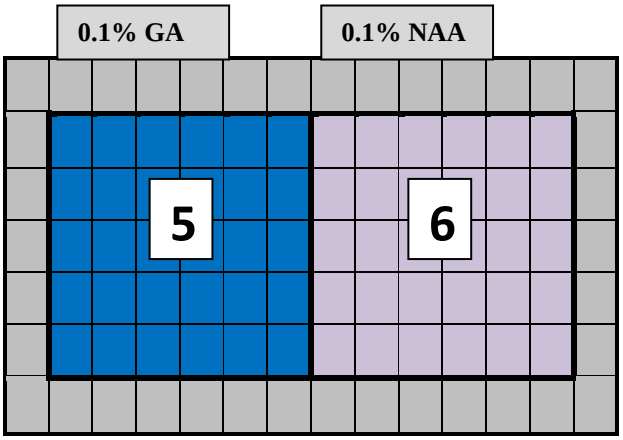

Appendix C

Hormone Rooting Trial Means

Treatment means for spring 2012

Hormone. Concentration(mg/ml)	Sprouted (%)	Rooted (%)	No. roots	Old leaves	New leaves
NAA 0.1	40.00	51.67	21.57	6.60	7.31
NAA 0.3	20.00	25.00	31.00	6.98	11.44
IBA 0.1	60.00	58.33	24.62	7.08	14.00
IBA 0.3	43.33	45.00	38.72	7.15	12.05
IAA 0.1	30.00	25.00	27.94	7.55	8.06
IAA 0.3	21.67	16.67	14.49	7.00	10.83
GA 0.1	3.33	1.67	2.33	3.67	2.50
GA 0.3	43.33	36.67	13.65	7.62	6.51
Control	35.00	31.61	7.89	4.29	5.29

Treatment means for autumn 2013

Hormone. Concentration(mg/ml)	Sprouted (%)	Rooted	No. roots	Old leaves	New leaves
NAA 0.1	78.33[a]	74.17[a]	5.47[a]	6.55[a]	4.41[a]
NAA 0.3	76.67	83.33[a]	7.88	5.31	3.94
IBA 0.1	68.33	71.67[a]	6.41	5.96	3.74
IBA 0.3	58.33	63.33[a]	5.80	5.46	3.40
IAA 0.1	73.33	70.83	5.47	5.32	4.10
IAA 0.3	63.33	63.33	6.67	4.77	4.90
GA 0.1	44.17	50.00	3.18	6.85	2.29
GA 0.3	15.83	20.83	1.63	6.58	1.13
Control	76.67	69.17	4.88	6.71	4.12

Appendix D
HPLC Standard Curves

(Attached in folder)

Appendix E
HPLC Sample Areas

(Attached in folder)