Phytochemical, Elemental and Biotechnological Study of *Cryptocarya latifolia*, an Indigenous Medicinal Plant of South Africa

by

Mohammed Falalu Hamza

Submitted in fulfillment of the academic requirements for the degree of Master of Science in Chemistry in the School of Chemistry and Physics, University of KwaZulu-Natal, Durban

2013

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As the candidate's supervisor, I have approved this thesis for submission.

Signed________________________Name___________________________Date_________

DECLARATION

I Mohammed Falalu Hamza declare that

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DECLARATION 2 - PUBLICATIONS

Publication 1

Title: Phytochemical and elemental investigations of the leaves and edible fruits of *Cryptocarya latifolia.*

Authors: *Mohammed Falalu Hamza*, Shakira Shaik and Roshila Moodley

(Manuscript in preparation)

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Authors: *Mohammed Falalu Hamza*, Shakira Shaik and Roshila Moodley

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Authors: *Mohammed Falalu Hamza*, Shakira Shaik and Roshila Moodley

Presenter: *Mohammed Falalu Hamza*

Conference: Postgraduate Research Day, UKZN, Durban, 2013.

In all of the publications I have performed all the experimental work and written the manuscripts. The co-authors were involved in discussion of the results and were responsible for verifying the scientific content and accuracy of the results as well as editing the manuscripts.

Signed…………………………………………

DEDICATION

This work is dedicated to my loving brother; Murtala Hamza Mohammed Turabu

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ABSTRACT

Cryptocarya latifolia (Lauraceae) is an indigenous South African plant used in ethnomedicinal practices. Traditional healers in KwaZulu-Natal, South Africa are extensively using the bark of *C. latifolia* as a substitute for *Ocotea bullata,* resulting in the decline of existing populations and also causing its conservation status to come under scrutiny. If no conservation efforts are instigated, the depletion and extinction of this species in the natural habitat is highly probable. Therefore, in this study, a phytochemical investigation of the leaves and fruits of *C. latifolia* was performed to determine their suitability, as a replacement for the bark, for medicinal use. From the leaves, a new compound, 5-hexyltetrahydro-2*H*-pyran-2-one (**4**) was isolated together with known compounds, quercetin-3-*O*-rhamnoside (**1**), β-sitosterol (**2**), copaene (**3**) and nerolidol (**5**). In addition, some important monoterpenoids, diterpenoids, sesquiterpenoids and essential oils were isolated and identified using gas chromatography-mass spectrometry (GC-MS). Quercetin-3-*O*-rhamnoside (**1**) and β-sitosterol (**2**) were also isolated from the edible fruits. Anti-oxidant activity of the isolated compounds using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay indicated good anti-oxidant activity of all compounds, except β-sitosterol (**2**), compared to ascorbic acid. The results reveal that the leaves and fruits can be used, instead of the bark, as a source of antioxidants. The analytical study conducted on the fruits of *C. latifolia* indicated that they are a good source of important dietary elements and may contribute significantly to the diet. The elements were found to be in increasing order of Pb <Cr < Se < Ni < Cu < Zn < Mn < Fe< Mg< Ca. Consumption of the edible fruits of *C. latifolia* could be beneficial to the rural populace and other vulnerable communities. In this study, an evaluation of decontamination, bud break, *in vitro* and *ex vitro* rooting in *C. latifolia* was also performed. Different types, combinations and concentrations of sterilants, fungicides and antibiotics were tested for the establishment of contaminant-free cultures. The most effective decontamination

regime which resulted in 94% contaminant-free explants included the use of 70% ethanol, 1.75% sodium hypochlorite, Tween20®, Ampicillin®, Celest®, Heritage® and autoclaved distilled water at varying combinations, concentrations and time exposures. Thereafter, explants were grown in culture tubes containing Murashige and Skoog (MS) nutrient medium supplemented with various concentrations and combinations of cytokinin (benzylaminopurine - BAP) and auxin (naphthaleneacetic acid - NAA) to stimulate bud break. The treatment containing BAP:NAA at 1.0:0.01 mg L^{-1} resulted in the highest percentage of explants forming shoots (96%). This treatment also resulted in the longest shoot length (8.06 mm). The bud break protocol established can be used to produce *C. latifolia* shoots for medicinal or conservation purposes.

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ABBREVIATIONS

ANOVA	Analysis of variance
ABU	Ahmadu Bello University
ABA	Abscisic acid
ATP	Adenosine triphosphate
BAP	Benzulaminopurine
BUK	Bayero University, Kano
CC	Column chromatography
CCPs	Critical control points
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
^{13}C NMR	C-13 nuclear magnetic resonance spectroscopy
COSY	Correlated spectroscopy
COMTRADE	Commercial trade
CRMs	Certified reference materials
d	Doublet
dd	Double doublet
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer

DPPH	2,2-diphenyl-1-picrylhydrazyl
DRI	Dietary reference intake
EXSY	Exchange Spectroscopy
FTIR	Fourier-transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
LC-ESI-MS	Liquid chromatography-electrospray ionization-mass spectrometry
HACCP	Hazard Analysis Critical Control Point
MHz	Megahertz
HMBC	Heteronuclear multiple bond coherence
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
HSQC	Heteronuclear single quantum coherence
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
IR	Infrared
ISO	International organisation for standardisation
KIN	Kinetin
m	Multiplet
MS	Murashige and Skoog

NAA	Naphthalene acetic acid
ND	Not determinable
NOESY	Nuclear overhauser effect spectroscopy
nm	Nano metre
OS	Oxidative stress
ppm	Part per million
RDA	Recommended dietary allowance
RF	Radio frequency
ROS	Reactive oxygen species
s	Singlet
SADC	Southern African development community
t	Triplet
TLC	Thin layer chromatography
TMPs	Traditional medical practitioners
TMS	Tetramethyl silane
TOCSY	Total correlation spectroscopy
WHO	World health organisations
UKZN	University of KwaZulu-Natal
UL	Tolerable upper intake level

UNICEF	United nations international children's emergency fund
UNCTAD	United nations conference on trade and development
UV	Ultra violet radiation
ZEA	Zeatin

CHAPTER ONE

Introduction

Medicinal plants contain both primary and secondary metabolites, of which the secondary metabolites impart medicinal value (Wink, 2013). Primary metabolites include sugars, amino acids and nucleic acids, while secondary metabolites includes flavonoids, terpenoids, alkaloids and polyphenolics (Mahlangeni, 2012; Eid *et al*., 2012; Lee *et al*., 2013). Plants synthesize secondary metabolites as a defense mechanism, for ecological purposes and physiological functions. Therefore, they are essential for the plant's survival in its ecosystem. The medicinal value of plants has been recognized by traditional healers for centuries. Extracts from medicinal plants have been used for their anti-inflammatory, anti-oxidant, anti-bacterial and anti-fungal properties and they are used to treat various diseases like cancer, hypertension, diabetes and cardiovascular disease (Khan, 2010).

The World Health Organization (WHO) has reported that 65% of the world's population and 70-95% of developing countries depend on traditional medicine for their primary healthcare needs (Verschaeve *et al*., 2004; Moyo *et al*., 2011; Nielsen *et al*., 2012; Mabona and Van Vuuren, 2013). It is also reported that traditional medicine is the only system of medicine in some rural communities (Mabona and Van Vuuren, 2013). Consequently, WHO has sanctioned that traditional medicine, like conventional medicine, be represented in national drug policies as part of the healthcare system (Cunningham, 1993).

Natural product chemists are interested in systematically investigating plant species for their secondary metabolites. These researchers are interested in the scientific justification for the plant's usage and, more importantly, they seek to discover novel compounds that have pharmaceutical significance. The traditional knowledge on medicinal plants assists scientists to focus on and target specific species that may be of medicinal value instead of randomly

selecting and screening plants (Fennell *et al*., 2004). The study of natural products cannot be overemphasized as it has led to the discovery of a variety of drugs that are useful in the treatment of various ailments (Khan, 2010).

A range of assays are used to test for the biological activity of isolated and characterized compounds (Ibelgaufts, 2012). Firstly, the assays have to start *in vitro* where a standard drug is used to ensure the effectiveness of the assay and, secondly, *in vivo* to ascertain the activity of the natural product. Fractionated extracts and pure phytocompounds are screened for anti-inflammatory, anti-fungal, anti-bacterial, anti-amoebic, anti-oxidant and anti-malarial activity. Sometimes, psychotropic and neurotropic properties are also studied (Fennell *et al*., 2004). Besides the medicinal advantages of plants, they are also the elementary source of biologically active substances such as minerals and vitamins (Onibon *et al*., 2012). The plant's mineral elements are inorganic substances found in all its tissues and fluids. In the plant, their presence is essential for metabolic processes, growth and development. Plants are intermediates through which mineral elements enter the human body (Moodley *et al*., 2012). These nutrients are categorized into macronutrients and micronutrients as required by the human body (Soetan *et al*., 2010). Macronutrients are those nutrients needed in larger quantities and include Na, C, N, O, K, P, S, Ca and Mg. Micronutrients are required in smaller quantities and include Co, Mn, Cu, Fe, Mo, Cr, Ni, Se, Zn and I (Abdulla, *et al*., 1996). Inadequate consumption of nutrients will result in the disturbance of metabolic processes and may result in poor health and sickness and, in children, it can lead to impaired development (Welch and Graham, 2004).

People from developing countries continue to harvest medicinal plants from wild sources including forests in order to meet their demands (Mander *et al*., 2007). Excessive, uncontrolled, and unsustainable harvesting techniques has led to the depletion of wild

populations causing many plants to become extinct, threatened and endangered (Oladele *et al*., 2011). In an effort to conserve and ensure the existence of representative vulnerable medicinal plant species in wild populations, alternative cultivation techniques have to be established (Cunningham, 1993). Plant tissue culture is defined as the aseptic culture of cells, tissues, organs and their components under chemical and physical conditions *in vitro* (Thorpe, 2007; George *et al*., 2008). In plant tissue culture, any part of the plant can be cultured on a nutrient medium, under sterile conditions, with the purpose of obtaining growth. Leaf explants, shoot tips, root explants, lateral buds or stem explants can regenerate whole plants i.e. clones, within a short period of time (George and Sherrington, 1984; Rout *et al*., 2006; Žiauka *et al*., 2013). *Lauraceae* is a large family of tropical evergreen trees and shrubs that comprises about 55 genera and 2500-3000 species that are distributed worldwide (Nishida, 1998; Yang, 1998; Rohwer, 2000; Yen, 2010; Bannister *et al*., 2012; Moraes, 2012). In Africa, the genus *Cryptocarya* is commonly found in South Africa, Mozambique and Swaziland (Renner, 2005). In South Africa, six species of *Cryptocarya* are commonly distributed in the coastal regions of KwaZulu-Natal and Eastern Cape. These are tall evergreen plants mostly found along rivers and streams. They include *Cryptocarya latifolia, C. myrtifolia* and *C. woodii*.

C. latifolia extracts are used for the treatment of headaches, morning sickness, pulmonary diseases, bacterial and fungal infections as well as for magical purposes (Kumar and Meshram, 2011). Due to the scarcity of the highly endangered *Ocotea bullata*, an important medicinal plant among the Zulu people of South Africa, traditional healers in KwaZulu-Natal often use *Cryptocarya* species as an alternative to this plant (Zschocke and van Staden, 2000). The conservation status of *C. latifolia* has recently come under scrutiny. Existing populations of this species are rapidly declining probably as a consequence of their substitutive use for *Ocotea bullata* (Drewes *et al*., 1995; Zschocke and Van Staden, 2000).

Therefore, there is a need for alternative cultivation techniques to promote the conservation of this indigenous medicinal plant. Tissue culture technology can be used to develop micropropagation protocols to produce large sustainable populations of *C. latifolia*.

1.1 Aims

The aim of the study was to phytochemically and analytically investigate various extracts of *Cryptocarya latifolia*. The phytochemical investigation was done on the leaves and fruits of the plant to determine if they contain any secondary metabolites that would validate their ethnomedicinal use; this could help to reduce the enormous removal of bark. The analytical investigation was done to determine the nutritional value of the fruits. Plant tissue culture techniques were investigated to ascertain effective protocols for sustainable mass propagation of this species to eliminate the enormous strain on natural resources and to conserve biodiversity.

1.2 Objectives

1. To extract and isolate the phytocompounds from the leaves and fruits of the plant.
2. To identify and characterize the isolated compounds using spectroscopic techniques (NMR, IR, UV and GC-MS).
3. To determine the anti-oxidant activities of the isolated compounds.
4. To determine the elemental concentrations in the fruits and to assess for nutritional value by comparing elemental concentrations to recommended dietary allowances (RDAs).
5. To establish suitable explants for micropropagation by developing appropriate decontamination protocols.

6. To develop an efficient shoot multiplication protocol for sustainable mass propagation of the plant.
7. To assess *in vitro* and *ex vitro* rooting.

CHAPTER TWO

Literature Review

2.1 History of traditional medicine

"*Traditional medicine is defined as a body of knowledge, skills and practices indigenous to different cultures based on theories, beliefs and experiences utilized to maintain good health*" (WHO, 2002).

Plants have been utilized by human beings for thousands of years to treat diseases and disorders, and sometimes for spiritual purposes as part of their health care needs (Shahidullah, 2007). From historical records, the use and properties of these plants for medicinal use were known by the Babylonians, Egyptians, Assyrians and ancient Hebrews (Ghani, 2003). The use of plants became popular during the Greek civilization. Hippocrates (born 460 BC) and Theophrastus (born 370 BC) also used herbs for medicine. The *Materia Medica* written by Hippocrates listed about 400 medicinal plants and later the encyclopaedic work of Dioscorides, *De Materia Medica* (in 78 AD), captured approximately 600 medicinal plants (Parker, 1915). Hence Dioscorides is regarded as the ancestor of all modern pharmacopoeias and authoritative texts on botanical medicine. Galen (131-200 AD) was the first pharmacist-physician to come up with many different formulations and guidelines that explained the use of plants as medicine in the Middle Age. He wrote nearly 500 volumes describing such formulations and guidelines (Shahidullah, 2007). Pen Tsao, the earliest famous Chinese pharmacopoeia, attributed to the legendary emperor of Shen Nung, appeared around 1122 BC; this authoritative work documented the medicinal use of many plants. The use of medicinal plants in Europe in the 13th and 14th centuries was based on the doctrine developed by Paracelsus (1490-1541 AD), a Swiss alchemist and physician. According to this doctrine, "*all plants possessed some sign, given by the Creator, which indicated the illness,*

symptom or diseased organ for which they were intended". A common example of this doctrine includes Ginseng - *Panax ginseng* (Al-Achi, 2008). Some Arabian physicians (9th to 12th century AD) like Al-Razi and Ibn Sina, contributed to the history of medicine; they introduced new drugs of plant and mineral origin into general use. Al-Kanun of Ibn Sina laid down the groundwork of modern western medicine to improve the innovative Greek system. Hundreds of years ago, Australian aborigines used medicinal plants for their health care needs which add to the increase in global medicinal plant stock (Shahidullah, 2007). Plants that were grown naturally in the forests of South America were carefully planted in medicinal plant gardens by herbalists for ease of access as well as for conservation, and these provided the world with many useful medicinal plants all year round. Rig Veda (4500-1600 BC), the oldest book in the library of mankind mentioned the use of medicinal plants in the Indian subcontinent (Doniger, 1981).

2.2 Medicinal plants

Medicinal plants contain both primary and secondary metabolites, of which, the secondary metabolites impart medicinal value (Thakare, 2004; Shahidullah, 2007; Wink, 2013). Nature has provided us with numerous varieties of plants that form an important source of materials for traditional medicine. They are the natural source of drugs worldwide (Al-Daihan *et al*., 2013).

2.2.1 Medicinal plant trade

About 80% of the world's population engages in medicinal plant trade for ethnomedicinal practices (Cunningham, 1993; Steenkamp, 2003). This trade also exists in developed countries where about 25% of prescribed medicines contain many active ingredients commonly derived from plants (Cunningham, 1993). Since 1962, the statistical information

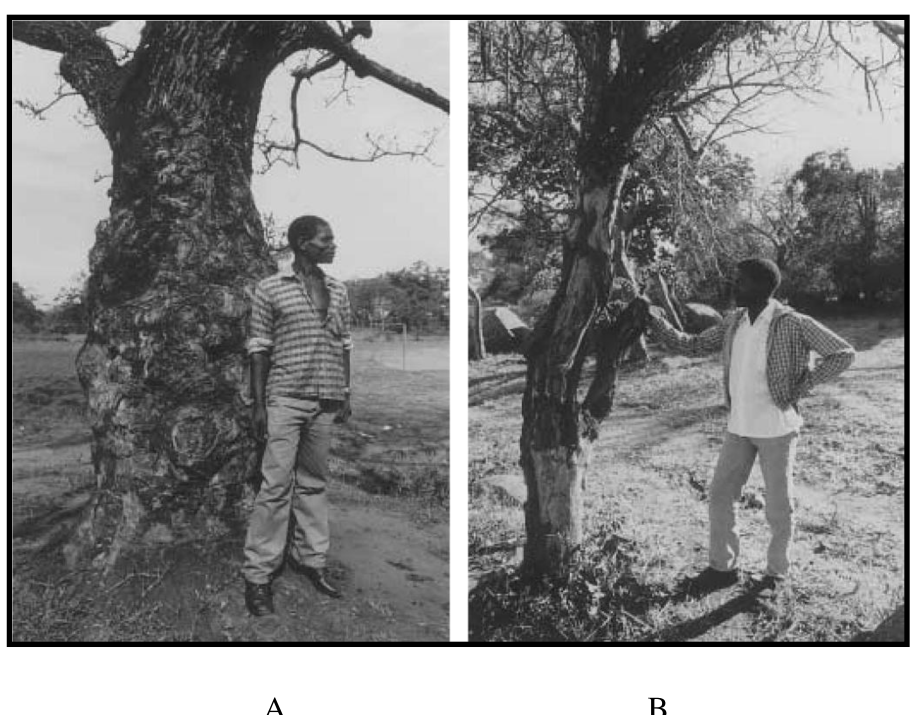
on the import and export of medicinal plants traded across the world are contained in the United Nations Conference on Trade and Development (UNCTAD) database. The largest importer of pharmaceutical plants is Hong Kong (Wiersum *et al.*, 2006). Approximately 25% is traded internationally by Europe (Mander, 2003). In Africa, the markets for medicinal plants stretch out across the indigenous cultures where traditional medicines remain an important health service. Local trade is basically informal (Figure 1) (Mander, 1998; Mander, 2003).

Figure 1: Raw or semi- processed medicinal plants traded in Durban street markets (Mander, 2003).

There are an estimated 28 million users of medicinal plants including 255 000 traditional healers in southern Africa (Mander, 2003). The demand for medicinal plants is increasing, with a growing consumer population and with no suitable alternatives or substitutes available. The bulk trade in medicinal plant products takes place at informal street markets, and involves the sale of relatively large quantities of unprocessed or semi-processed products (Figure 1). In KwaZulu-Natal, an estimated 4500 tones of plants are traded annually, of which 1200 tones are traded in the Durban street markets. Over 400 species are traded in the markets, both wholesale and retail (Mander, 2003).

2.2.2 Conservation and management of medicinal plants

Medicinal plants play an important role as a source of medicinally useful drugs in traditional medicine (Tang, 2005; Al-Daihan *et al*., 2013). These plants are still collected in present day from wild populations. Because of the high demand of medicinal plants for health care needs, the selling of these plants has become highly commercialized. Considerable numbers of important medicinal plants are destroyed as a result of excessive use by traditional medical practitioners (TMPs) (Figure 2).



A B

Figure 2: *Erythrina abyssinica* (A) and *Cassia abbreviata* (B) after extensive debarking (Cunningham, 1993).

This uncontrolled removal of medicinal plants from wild populations has led to the enforcement of conservation legislation which prohibits plant gatherers from uncontrolled collecting of plant species from their natural habitat (Mahlangeni, 2012). It has become

necessary for healthcare systems depending on them to ensure sustainability of these plant resources. Coordinated efforts are necessary in order to change unsustainable practices of collecting medicinal plants to more ecologically sustainable and socially acceptable practices (Parotta, 2002).

2.2.3 The use of medicinal plants in South Africa

South Africa has an enormous diversity of tribes. Traditional healers within these tribes are referred to as *inyangas* and herbalists as *isangomas* by the Zulu people; however, the difference between the two is unclear. Practitioners in other groups are known as *ixwele* and *amaquira* (Xhosa), *nqaka* (Sotho) and *nanga, mungome* or *maine* (Vhavenda) (Steenkamp, 2003). Aged people and faith healers in villages practice their herbal wisdom in traditional health care systems (Steenkamp, 2003). In metropolitan areas, remedies are obtained at various markets or shops. The TMPs pay particular attention to the use of herbs in treating various diseases and rely on symptomatic diagnosis of diseases (Eloff and McGaw, 2006). The majority of medicinal plants in South Africa are used to enhance fertility (Veale *et al.*, 1992). A considerable number of South African women look for treatment from traditional healers for a variety of complications and disorders associated with the reproductive and genital organs (Steenkamp, 2003). Many plants are also used in the treatment of wounds (Mabona and Van Vuuren, 2013) and tuberculosis (Arya, 2011). The part of the plant used varies from one species to another, from practitioner to practitioner and depends on the nature and state of the disease.

2.3 Secondary metabolites

Secondary metabolites are chemical compounds obtained from higher plants. They are stored in plants as a complex mixture in relatively high concentrations (Croteau *et al*., 2000). They are sometimes stored in organs that do not produce them, as inactive substances, but in the case of danger, they are activated by certain enzymes. They are known as phytochemicals and occur naturally in the plant's body and are used as a defense mechanism against herbivores and pathogens. Secondary metabolites are also used by plants to attract insects or animals for pollination (Tesso, 2005). Today, they are used for the treatment of diseases like cancer, hypertension, diabetes, cardiovascular disease, fungal and bacterial infections. They are known to have biological activities such as anti-microbial, anti-oxidant and anti-inflammatory activities (Khan, 2010). About 200 000 secondary metabolites have been discovered and reported by phytochemists (Bino *et al*., 2004). Secondary metabolites include alkaloids, chalcones, flavonoids, terpenoids, sterols and polyphenolic compounds (Tesso, 2005).

2.3.1 Types of secondary metabolites

Some secondary metabolites contain nitrogen while some are nitrogen-free compounds (Table 1).

Table 1: Types of secondary metabolites and approximate number isolated (Tesso, 2005).

Type of secondary metabolites	Approximate number
Secondary metabolites containing Nitrogen	
Alkaloids	21000
Non-protein amino acids	700
Lectins, peptides, polypeptides	2000
Alkamides	150
Amines	100
Glucosinolates	100
Cyanogenic glycosides	60
Secondary metabolites without Nitrogen	
Monoterpenes including iridoids	2500
Sesquiterpenes	5000
Diterpenes	2500
Triterpenes, steroids, saponins	5000
Tetraterpenes	500
Flavonoids, tannins	5000
Phenylpropanoids, lignin, coumarin	2000
Polyacytelene, wax, fatty acids	1500
Anthraquinones and other polyketides	750

2.3.2 Classification of secondary metabolites

2.3.2.1 Flavonoids

These are the earliest natural compounds studied and are the most widely distributed in plants. About 2000 flavonoids were discovered and reported. They are mostly found in all kinds of terrestrial plants. Flavonoids consist of a three ring system, and these rings can be substituted with methoxyl groups, hydroxy groups or other substituents. This possibility of substitution has led to the formation of large structures by flavonoids. Generally, flavonoids are categorized into flavones (**1**), flavonols (**2**), isoflavones (**3**), flavanones (**4**) and chalcones (**5**) (Figure 3) (Tang, 2005).

Flavone (**1**)

Flavonol (**2**)

Isoflavone (**3**)

Flavonone (**4**)

Chalcones (**5**)

Figure 3: Structures of five major flavonoids.

Flavonoids are known to anti-fungal, anti-malarial, anti-bacterial as well as anti-oxidant and anti-inflammatory activities. As a result, they have been used to supplement medicines or vitamins, like catechin, which is found in green tea and helps to fight against viral hepatitis, oxidative heart damage and other diseases associated with the kidneys, lungs, and spleen. Large numbers of these flavonoids play a crucial role in medicine. For instance, nevadensin is

used in anti-inflammatory and antioxidant drugs, rutin is used in blood pressure reducing drugs and quercetin is used in anti-tumor drugs (Tang, 2005).

2.3.2.2 Polyphenolic compounds

These are aromatic hydrocarbons containing the -OH group (Figure 4) directly attached to the ring. The simplest class of this compound is phenol which bears a single OH group directly attached to the ring. Other examples are polyphenolic compounds and gallic acid.

OH

OH

OH

HO

Phenol (**6**) Resveraterol (**7**)

Figure 4: Examples of polyphenolic compounds

2.3.2.3 Terpenoids:

Terpenoids are made up of isoprene units containing five carbon atoms and are mostly found in higher plants in considerable amounts (Harrewijn *et al*., 2001). Wallach (1887), was the first to formulate the structural relationship among terpenes (McGarvey and Croteau, 1995) after rigorous research on them. He then came up with the theory that states that terpenes contain one or more isoprene units connected in a head tail manner. Later Ruzicka (1953), proved the theory with the biogenetic isoprene rule. Terpenes are classified into many groups based on the number of isoprene units.

Hemiterpenes

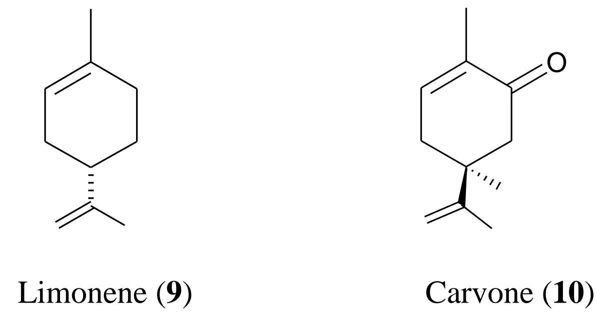
This is the group of terpenes that consists of one isoprene unit (Figure 5). Isoprene itself is the best known example of hemiterpenes (Köksal *et al.*, 2010).

Isoprene (**8**)

Figure 5: An example of a hemiterpene.

Monoterpenes

Contain two isoprene units (Figure 6). They have a pleasant scent which make them an important ingredient in flavour industries (Singh, 2007; Hyatt *et al.*, 2007).



Limonene (**9**) Carvone (**10**)

Figure 6: Examples of monoterpenes.

Sesquiterpenes

Sesquiterpenes are a class of terpenes bearing three isoprene units (Figure 7). They are mostly found in higher plants (Merfort, 2002; Shchepin *et al.*, 2003).

Fernesol (**11**)

Figure 7: An example of sesquiterpene.

Diterpenes

This class contains four isoprene units (Figure 8) (Matsui *et al*., 2004).

H
H
COOH

Abietic Acid (**12**)

Figure 8: An example of a diterpene.

Triterpenes

This class contains six isoprene units (Figure 9) (Singh, 2007).

H_3C
H_3C
H
H_3C
H
H
H
HO

β-sitosterol (**13**)

Figure 9: An example of a triterpene.

Tetraterpenes

Tetraterpenes (Figure 10) consists of eight isoprene units (Matsui *et al*., 2004; Baliga *et al*., 2011).

β-Carotene (**14**)

Figure 10: An example of a tetraterpene.

Polyterpenes

Polyterpenes (Figure 11) contain n-multiples of isoprene units in the structure (Lange, 2003; Khan, 2010).

MeO MeO O O H n

n= >9

Ubiquinone (**15**)

Figure 11: An example of a polyterpene.

Sterols

Sterols are naturally occurring compounds produced by plants and animals; they are found in almost all parts of the plant especially the fat soluble fractions. Plant and animal cell membranes are made up of sterols (Figure 12) as one of the major constituents (Baliga *et al.*, 2011).

β- Sitosterol (**16**) Stigmasterol (**17**)

Figure 12: Examples of sterols.

2.4 The Lauraceae family

Lauraceae is a family of many species found in tropical forests. Their taxonomy at genus or species levels is not well understood (Nishida, 1998). The family consists of about 50 genera and 2500-3000 species (Nishida, 1998; Hu *et al*., 2007; Bannister *et al*., 2012; Cuca *et al*., 2013; Miller and Tuck, 2013). They are aromatic evergreen trees and shrubs with the exception of *Cassytha* (Nishida, 1998). This tropical family can be recognized by their distinct morphology; the flowers consist of alternating trimerous whorls: four whorls of three stamens and the gynaecium, two whorls of three petals. Stamens from the third whorl have a pair of glands in the androecium. Sometimes stamens from the four whorls are reduced or absent. Anthers have two or four locules and dehisce by flap-like valves. The pistil has one carpel with a single pendulous anatropous ovule. The ovary is superior, the seeds are hard, brown and covered by fleshy like cupuls (fruits), the fruits are leathery to the touch, green in colour and turns black when dry (Yang, 1998). The family has great economic importance as a source of wood, seasoning, food and some essential oils. They also contain important secondary metabolites with biological activity (Cuca *et al*., 2013).

2.4.1 Geographical distribution of Lauraceae

The *Lauraceae* family is distributed worldwide with many species found commonly in southeast Asia and the tropics of America (Bannister *et al*., 2012). They grow mostly in low land and montane rainforests. Their widespread diversity indicates their fossil history (Bannister *et al*., 2012). Their leaves are simple with entire margin and venation (camptodromous or acrodromous), however their cuticles are very resistant to decay, and this is the reason why the family has fossil history. They can be preserved for a long period of time (Hu *et al*., 2007). The species in northern Asia is distributed across China and Japan. The family is also found in Chile and Argentina. Many species are found in Madagascar and

the family is not well distributed across Africa but some genera such as *Cryptocarya* are found in South Africa, Mozambique and Swaziland. The family is also found in the rain forests of Australia (Bannister *et al*., 2012).

2.4.2 Classification of Lauraceae

Large numbers of the species belonging to the family *Lauraceae* are widely distributed across the world, and the distribution is not properly understood (Rohwer, 2000). This results in difficulty in classifying the *Lauraceae* family. However, many classification systems of this family based on their morphology, have been reported (Van der Werff and Richter, 1996). But the most recent classification, Van der werff and Richter's classification system, is outlined below (Yang 1998).

Subfamily *Laureae*

Tribe *Perseas*

Aiouea	*Aniba*
Alseodaphne	*Apollonias*
Cinnamomum	*Dehaasia*
Endlichera	*Licaria*
Mezilaurus	*Neocinnamomum*
Nectandra	***Ocotea****
Persea (machilus)	*Phoebe*
Pleurothyrium	*Aspidostemon*
Chlorocardum.	

Tribe *Cryptocaryeae*

Beilschmedia	*Caryodaphnopsis*
Cryptocarya**	*Endiadra*
Eusideroxylon	*Hypodaphnis*
Potoxylon	*Triadodaphne*

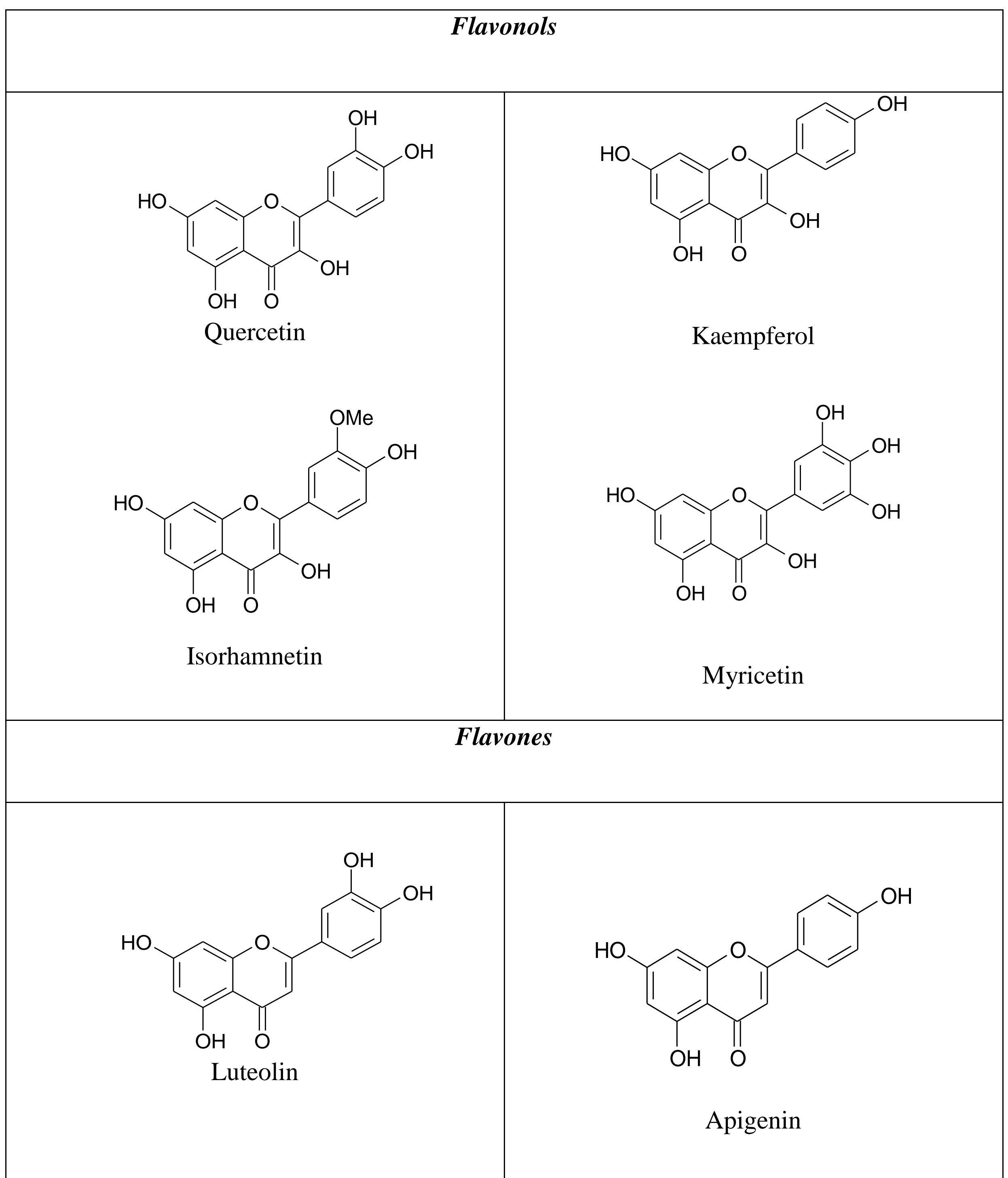
*Genus of the species claimed to have similar medicinal properties as *C. latifolia*

**Genus of the species in this study.

2.4.3 Common phytocompounds found in Lauraceae

The *Lauraceae* family contains many important phytochemical compounds (Table 2) (Yang, 1998).

Table 2: Some common phytocompounds found in *Lauraceae*



Flavanones	
Naringenin	Pinocembrin
C-Glycosine flavones	
Vitexin	Orientin

2.4.4 The genus Cryptocarya

The genus *Cryptocarya* belongs to the plant family *Lauraceae* (Nishida, 1998). In southern Africa, *Cryptocarya* species are distributed in KwaZulu-Natal and Eastern Cape (Bannister *et al*., 2012). This genus includes three species in South Africa viz. *Cryptocarya latifolia*, *Cryptocarya myrtifolia* and *Cryptocarya woodii*. The species are mostly found along rivers and streams. They are tall evergreen plants with smooth bark (Figure 13-A). Their leaves are leathery with alternate venation (Figure 13-B). The fruits are green before ripening and turn green-brown after ripening (Figure 13-C). The fruits are shed around October. The seeds are hard and brown in colour (Figure 13-D). The fruits are eaten by humans, birds and antelopes (Thomas, 2004).

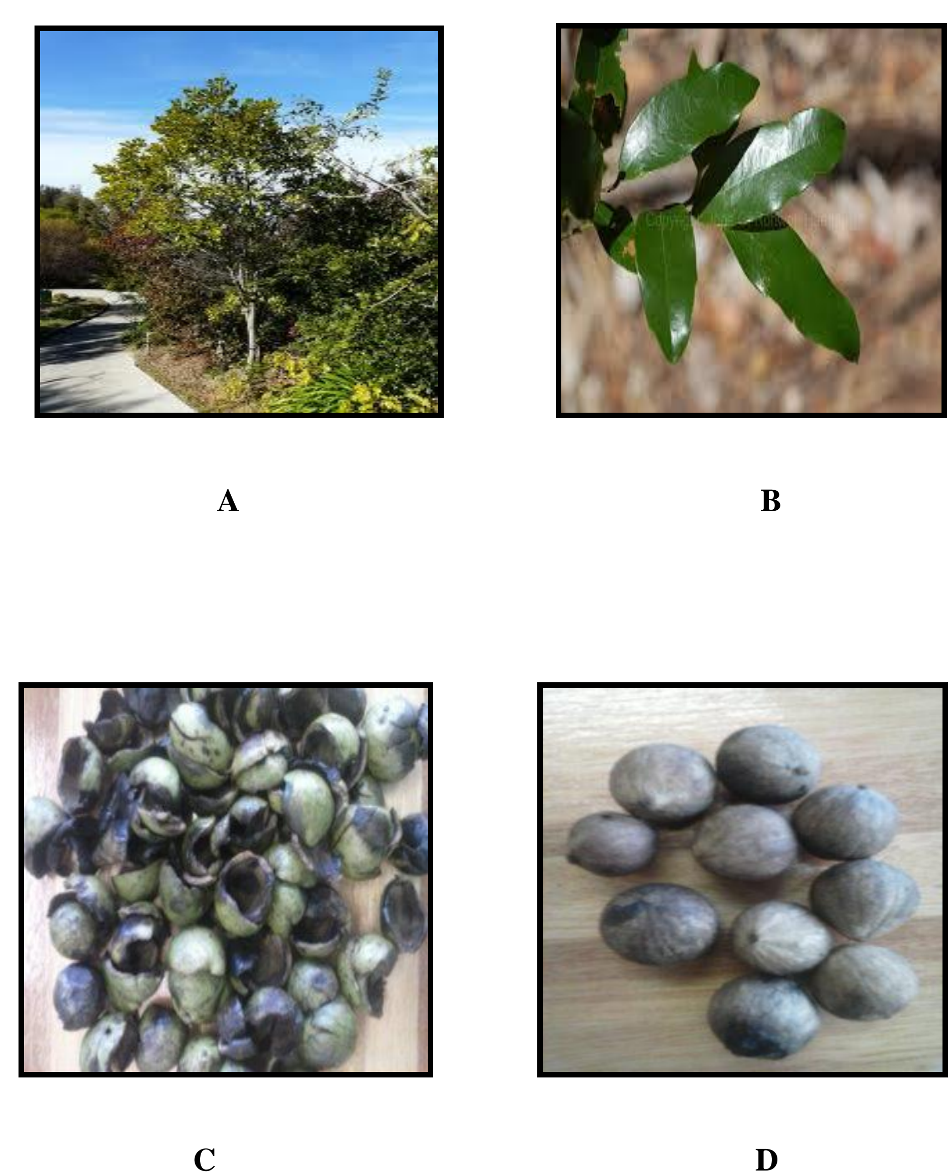


Figure 13: *C. latifolia* A- tree, B- leaves C- fruits and D- seeds.

2.4.4.1 Compounds previously isolated from some Cryptocarya species

Many compounds have been isolated from *Cryptocarya species*. These are highlighted in Table 3 and their structures are shown in the appendix.

Table 3: Compounds isolated from common *Cryptocarya* species.

Plant species	***Isolated compounds***	***Reference***
Cryptocarya alba (fruits)	Cryptofolione (**1**), Cryptofoliondiacetate (**2**)	Schmeda *et al*., 2001
C. chartacea (bark)	Pinocembrin (**3**), Chartaceones (**4**)	Allard *et al*., 2011
C. chinensis (leaves)	Cryptochinone (**5**), Cryptocaryanone (**6**), infecticaryone (**7**)	Chou *et al*., 2010
C. liebertiana (bark)	Cryptocaryalactone (**8**), Cryptofolione (**1**)	Drewes *et al*., 1995 & 1996
*C. latifolia** (bark)	Cryptocaryadiacetate (**9**), Cryptocaryatriacetate (**10**), Cryptocaryolonediacetate (**11**), Cryptofolione (**1**)	Drewes *et al*., 1996; Sehlapelo, 1994
C. myrtifolia (bark)	Cryptocaryadiacetate (**9**), Cryptocaryolone (**12**), Cryptofolione (**1**), Cryptocaryalactone (**8**)	Drewes *et al*., 1995; Sehlapelo, 1994
C. nigra (leaves)	Scoparone (**13**), , β-sitosterol (**14**), Stigmasterol (**15**)	Yen, 2010
C. strictifolia (bark)	Strictifolione (**16**)	Juliawaty *et al*., 2000
C. woodii (bark)	Cryptofolion (**1**)	Drewes *et al*., 1995

*species investigated in this study.

2.4.4.1.1 Taxonomy of Cryptocarya latifolia

Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Laurales*

Family: *Lauraceae*

Genus: *Cryptocarya*

Species: *Cryptocarya latifolia*

2.4.4.1.2 Distribution of Cryptocarya latifolia in Africa

Cryptocarya latifolia is found in South Africa, Mozambique and Swaziland. The map below (Figure 14) represents these areas (highlighted in green).

Figure 14: Distribution of *C. latifolia* in Africa (adopted from BOLD systems database 2013)

2.4.4.1.3 Medicinal uses of C. latifolia

C. latifolia has been used in the treatment of various ailments including headache, morning sickness, pulmonary disease, inflammation, tuberculosis, bacterial and fungal infections, as well as for magical purposes (Drewes *et al*., 1995; Wang *et al*., 2009; Sabitha *et al*., 2009; Arya, 2011; Kumar and Meshram, 2011; Yen, 2010). The compounds previously isolated from the plant are given in Table 3.

2.5 Applications of micropropagation and macropropagation for plant conservation

The conservation status of *C. latifolia* has recently come under scrutiny. Existing populations of this species are rapidly declining probably as a consequence of their substitutive use for *Ocotea bullata* (Drewes *et al*., 1995; Zschocke and van Staden, 2000). As a result of this substitutive use, *C. latifolia* is massively exploited and is listed among the declining indigenous medicinal plants in South Africa (Cunningham, 1993). The applications of micropropagation and macropropagation procedures in the conservation of declining medicinal plant species have gained momentum. These techniques have also gained international recognition in the Convention on Biological Diversity (CBD) (Sarasan *et al*., 2006). Considerable numbers of declining/threatened/endangered medicinal plants have been conserved through the use of *in vitro* micropropagation methods (plant tissue culture) (Fay, 1992) in which plants can be rapidly propagated (Rout *et al*., 2006). Macropropagation is the traditional vegetative propagation of plants from their growing parts. This can be done through stem cuttings, grafting, budding, layering etc. Important plants such as *Shorea guios* (guijo) and the critically endangered *Centaurea tchihatcheffii* have been successfully conserved using macropropagation techniques (Patricio *et al*., 2006; Ozel, 2006). These propagation techniques are also commonly used to study biomass production of biochemical secondary metabolites, germ-plasm preservation and plant pathology (Hudson, 2002).

2.6 Plant tissue culture and micropropagation

Plant tissue culture refers to the *in vitro* cultivation of plants, seeds and plant parts (cells, tissues, organs and embryos) (George and Sherrington, 1984; Bhojwani and Razdan, 1986; Rout *et al*., 2006; Smith, 2012) on nutrient media under aseptic conditions. The *in vitro* clonal propagation of plants, from axillary buds, embryos, shoot tips and leaves is called micropropagation. The term 'clonal' is derived from the Greek word 'clon' referring to a group of genetically identical cells derived from a single cell. Plants that grow from vegetative parts are not individuals in the common sense, but instead they are transplanted parts of the same individual and are identical. Thus, clonal propagation is the multiplication of genetically identical individuals by asexual reproduction (Shokri *et al*., 2012). The word 'micro' is often used to refer to the small size of explant used to produce large numbers of identical plants under certain physical and chemical conditions *in vitro*. The biggest advantage of micropropagation over conventional breeding is that, within a short span of time and space, large populations of identical plants can be produced from a single individual (Chawla, 2002). The major stages in micropropagation are:

1. Selection of suitable and healthy explants from the mother plant.
2. Sterilization/decontamination of the selected explants.
3. Culture of explants in nutrient media.
4. Multiplication (proliferation) of shoots from explants in nutrient media.
5. Transfer of shoots to rooting media.
6. Planting of rooted explants in soil (acclimatization).

2.6.1 Brief history of plant tissue culture

Plant tissue culture can be defined as the aseptic culture of cells, tissues and organs under certain defined chemical and physical conditions *in vitro* (Thorpe, 2007). Gottliebs Haberlandt (1902) attempted to culture single cells into mature plants, however, he was unsuccessful because he worked on monocotyledons (Chawla, 2002). And thus, he established the concept of totipotency. Presently, it is not easy to culture monocotyledons, but dicotyledonous tissue can be cultured relatively easily (Bhojwani and Razdan, 1986). Two of Haberlandt's students were able to culture small root systems from excised root tips but because of the contamination their culture did not grow and the tissues died after few days. It was concluded that tissue culture must be carried out under sterile conditions (Thorpe, 2007).

The first true plant tissue cultures were carried out successfully by Gautheret from cambial tissue of *Acer pseudoplatanus*. He also achieved success with similar explants of *Ulmus campestre, Robinia pseudoacacia,* and *Salix capraea* using agar-solidified medium of Knop's solution, glucose and cysteine hydrochloride (Smith, 2012).

2.6.2 Specific in vitro culture systems

Almost any part of a plant can be used as a starting material to grow a full plant *in vitro* (Bhojwani and Razdan, 1986). Figure 15 describes various types of *in vitro* culture systems (Chawla, 2002).

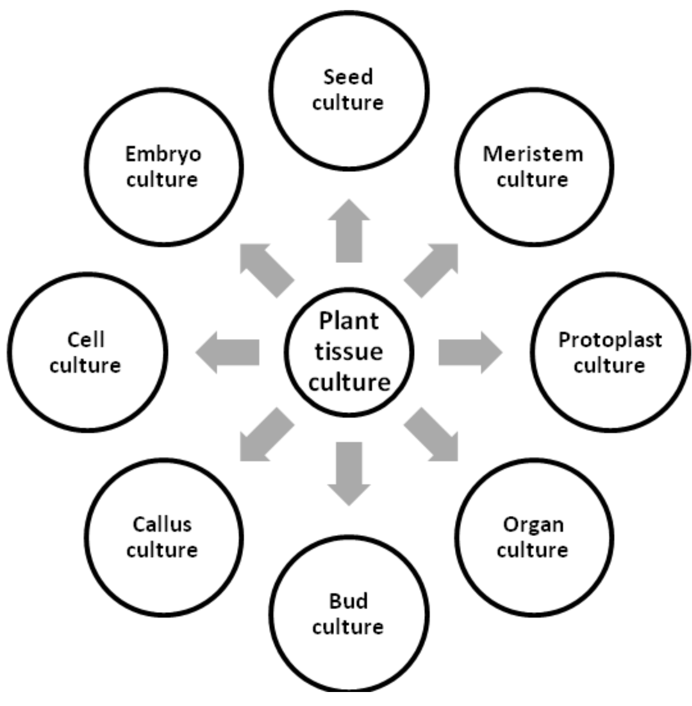


Figure 15: Specific types of *in vitro* culture systems (Chawla, 2002)

2.6.3 Plant tissue culture nutrient media

Nutrient media is the artificial liquid or solid "soil" prepared or designed for the growth of plants. Nutrient media may be defined with respect to their nutrient composition (mineral salts, vitamins, source of carbon and phytohormones) (Gamborg *et al*., 1976). A number of varieties of basic media (Table 4) have been formulated based on the composition and concentrations of their ingredients (George and George., 1988). The most common and widely used media (Figure 16) in various culture systems is the one formulated by Murashige and Skoog (1962).

Figure 16: *C. latifolia* explants producing shoots in Murashige and Skoog nutrient media in this study.

The nutritional composition of common nutrient media (Table 4) is made up of the following ingredients:

Inorganic nutrients (K, Ca, S and Mg)

Carbon and energy source (sucrose)

Vitamins (vitamin B_1 and B_6)

Organic supplement (organic nitrogen and acids)

Nutrient media contains different concentrations of inorganic and organic salts, carbon source and vitamins. The effect of concentrations of carbon source (carbohydrates), organic and inorganic salts have been explained in the review of Thorpe (1980) and Kirby *et al*. (1987).

Table 4: Composition of various plant tissue culture media (expressed in mg L^{-1}) (Chawla, 2002).

Constituent	**Nitsch (1956)**	**White (1963)**	**Murashige & Skoog (1962)**	**Gautheret (1942)**	**Gamborg's (1968)**	**Chu (1978)**
KCl	1500	65	-	-	-	-
$MgSO_4.7H_2O$	250	720	370	125	250	185
$NaH_2PO_4.H_2O$	250	16.5	-	-	150	-
$CaCl_2.2H_2O$	-	-	440	-	150	166
KNO_3	2000	80	1900	125	2500	2830
$CaCl_2$	25	-	-	-	-	-
Na_2SO_4	-	200	-	-	-	-
NH_4NO_4	-	-	1650	-	-	-
KH_2PO_4	-	-	170	125	-	400
$Ca(NO3)_2.4H_2O$	-	300	-	500	-	-
$(NH_4)_2SO_4$	-	-	-	-	134	463
$NiSO_4$	-	-	-	0.05	-	-
$FeSO_4.7H_2O$	-	-	27.8	0.05	-	27.8
$MnSO_4.4H_2O$	3	700	22.3	3	-	-
$MnSO_4.2H_2O$	-	-	-	-	10	3.3
KI	-	0.75	0.83	0.5	0.75	0.8
$CoCl_2.6H_2O$	-	-	0.025	-	0.025	-
$Ti(SO_4)_3$	-	-	-	0.2	-	-
$ZnSO_4.7H_2O$	0.5	3	8.6	0.18	2	1.5

$CuSO_4.5H_2O$	0.025	-	0.025	0.5	0.025	-
$BeSO_4$	-	-	-	0.1	-	-
H_3BO_3	0.5	1.5	6.2	0.05	3	1.6
H_2SO_4	-	-	-	1	-	-
$Na_2MoO_4.2H_2O$	0.025	-	0.25	-	0.25	-
$Fe_2(SO4)_3$	-	2.5	-	-	-	-
EDTA disodium salt	-	-	37.3	-	-	37.3
EDTA-Na ferric salt	-	-	-	-	43	-
m-inositol	-	-	100	-	100	-
Thiamine	-	0.1	0.1	0.1	1.0	1.0
Pyridoxine	-	0.1	0.5	0.1	1.0	0.5
Nicotinic acid	-	0.5	0.5	0.5	1.0	0.5
Glycine	-	3	2	3	-	-
Cysteine	-	1.0	-	-	10	-
Sucrose	34,000	20,000	30,000	30,000	20,000	30,000

2.6.4 Plant growth regulators (PGRs)

Plant growth regulators (PGRs) are natural or synthetic substances that are used to regulate plant growth and development. PGRs alone do not usually bring about a growth response, it is usually necessary to have a specific balance between PGRs that are used (Hannweg, 1995). The two broad categories of PGRs are auxins and cytokinins. Generally, high auxin concentrations encourage the development of unsystematic growth such as callus. Callus can

be defined as undifferentiated plant tissue developed as a result of wound, stress or insect attack (Taylor and Van Staden, 2001). By manipulating the ratios between cytokinin and auxin the balance between shoot and root growth can be controlled. Cytokinin at relatively high concentration does not promote rooting, whilst auxin at high concentration does not promote shoot formation. The molecular mechanism of rooting by auxins has been explained in the review of Han and Zhang (2009). Some examples of PGRs are Kinetin (KIN), Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Abscisic acid (ABA), Benzylaminopurine (BAP) and Zeatin (ZEA).

2.6.5 Sterilization

Sterilization is the process of eliminating contaminants (microorganisms) from culture systems. It is very important to maintain culture vessels, media, explants, handling instruments and culture environments free from all contaminants for successful culture systems. A culture environment is suitable when there is flow of air currents which carry microbial spores away during tissue culture operation. A small room or laminar airflow cabinets are usually used (Figure 17). These cabinets are equipped with germicidal lamps emitting ultra violet irradiation and designed to direct a flow of filtered sterile air across the working environment (Bhojwani and Razdan, 1986). The wall and surface of the cabinet is preferably washable in order to keep the cabinet clean. It is important to regularly check the condition of the cabinets to ensure that air and lamp units are working properly.

Figure 17: Laminar flow cabinet at the School of Life Sciences, UKZN.

In general, sterilization techniques are grouped into:

Maintenance of aseptic environment

It is important that operators' hands and laminar cabinets be sprayed regularly with 70% alcohol (ethanol) or sanitizing solution before, during and after working in the culture environment. Instruments that are continuously used such as forceps and blades should also be sprayed and placed in a heated bead sterilizer repeatedly during work sessions. All culture containers should preferably be covered with foil and kept at one side of the cabinet. Talking should be avoided while work is in progress to prevent re-introduction of contaminants into the culture (Chawla, 2002).

Sterilization of media, vessels (containers) and handling instruments

An autoclave (Figure 18) is the most widely employed device for sterilizing culture media, vessels, water and handling instruments. The autoclave provides steam for sterilization and is only effective when the autoclave has reached the proper temperature and air in the chamber has been replaced by steam. The effectiveness of the sterilization (Table 5) depends on temperature, pressure, time and volume of the object to be sterilized. It is also important to note that prolonged sterilization

decomposes the chemical constituents of nutrient media and also affects the pH by decreasing it to about 3-4.5. All autoclaved materials should be kept immediately in the laminar flow.

Figure 18: Image of an autoclave at the School of Life Sciences, UKZN.

Table 5: Conditions of autoclaving for effective sterilization (Chawla, 2002).

Material	***Volume (cm^3)***	***Temperature (^oC)***	***Pressure (psi)***	***Time (min)***
Test tube	20-50	121	15	20
Flask	50-500	121	15	25
Flask	500-5000	121	15	35

Sterilization of explant material

Plant materials are sterilized by the use of various chemicals. The effectiveness of the type, combination, concentration and exposure time of these chemicals usually depends on the physiology of the plant. Table 6 shows the type, concentration and time of treatment of commonly used chemical sterilants. As mentioned above, the effectiveness of these chemicals depends upon the kind of plant species and therefore, the concentration and time should be decided empirically.

Table 6: Common surface sterilants and their recommended concentration and treatment time (Chawla, 2002).

Chemical sterilants	*Concentration*	*Treatment time (min)*
Sodium hypochlorite	1-1.4%[a]	5-30
Calcium hypochlorite	9-10%	5-30
Hydrogen peroxide	10-12%	5-15
Bromine water	1-2%	2-10
Silver nitrate	1%	5-30
Mercuric chloride	0.01-1%	2-10
Alcohol	70-80%[b]	30sec-1
Tween20®	1-2 drops	20-30
Antibiotics	4-50mg/l	30-60

[a]Common usage rate is 20% v/v

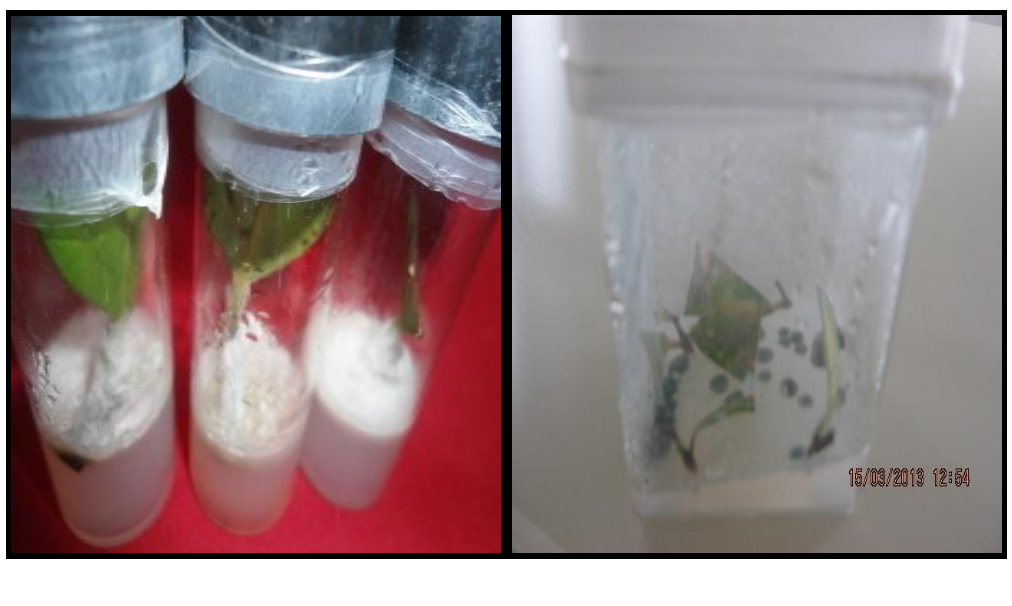
[b]Common usage rate is 70% v/v

2.6.6 Tissue culture contamination

Contamination is a serious problem in plant cell and tissue culture systems. It is caused by pathogens and microbial contaminants such as multicellular fungi, yeasts and bacteria (Figure 19). Plant growth media often provides a favourable environment needed by these microorganisms (Cassells *et al*., 2000; Leifert and Cassells, 2001; Ryan, 2009) in which they inhibit the growth of *in vitro* plants. Contaminants may sometimes express themselves without delay after their introduction into the culture or they may take a long period of time before showing any kind of visible symptoms on *in vitro* plants or media (Reed and Tanprasert, 1995; Cassells, 2010).

According to Leifert and Cassells (2001), the following are the major causes of contaminant introduction:

1. Contamination may be introduced into the culture as a result of micro-arthropod vectors which carry microbial organisms into the culture during work sessions.
2. Presence of thrip vectors in a plant growth room due to lack of proper and regular maintenance may also result in the appearance of specific microbial contaminants.
3. Contamination may appear in the culture if the disinfection process is not performed appropriately. For example, the presence of Gram-negative bacteria such as *P. fluorescens* at the initial stage of culture indicates that the disinfection procedure is not sufficient, while Gram-positive bacteria such as *Bacillus spp.* indicates the media is not well sterilized.
4. Lack of good training of culture operators may also contribute towards contaminant introduction.



A B

Figure 19: *C. latifolia* explants suffering from the attack of fungi (A) and bacteria (B) in this study.

2.7 Nutrients in humans

Nutrients are chemical or mineral elements needed in the body of living organisms for metabolism (Moodley *et al*., 2012). These nutrients are required in the correct proportion (Soetan *et al*., 2010). They are categorised as macronutrients (those needed in large amounts) and micronutrients (needed in small amounts). Carbohydrates, proteins, fats and oils, and the elements Na, C, N, O, K, P, S, Ca and Mg are regarded as macronutrients (Abdulla, *et al*., 1996; Duruibe *et al*., 2007; Soetan *et al*., 2010). Micronutrients include Co, Mn, Cu, Mo, Cr, Ni, Se, Zn and I (Abdulla, *et al*., 1996). Minerals have been found to show significant activity against viral, bacterial and fungal infections (Dembitsky *et al*., 2011; Dahech *et al*., 2013). For metabolic processes to be sustained in the human body, it requires approximately 49 nutrients. Deficiency of a single nutrient may result in the disturbance of metabolic processes

resulting in poor health, sickness and impaired development among children (Welch and Graham, 2004). Fruits, herbs and vegetables are the basic sources of biologically active substances such as minerals and vitamins (Onibon *et al*., 2012; Gebrekidan *et al*., 2013). Consumption of these foods is recommended because of the beneficial role they play in the human body; the values usually tend to vary from one country to another regarding the recommended level of intake. Individuals who consume adequate fruits and vegetables stand a lower chance of developing wide varieties of cancer (Dembitsky *et al*., 2011). Metals have been found to help in regulating osmotic body fluid pressure, and to enhance growth as well as increase the overall well-being of the human body. They are part of supplementary nutrients and are internationally recommended to be used as food additives (Onibon *et al*., 2012). Tables 7 and 8 show the Dietary Reference Intakes (DRIs) for most individuals and Tolerable Upper Intake levels (ULs) that are likely to pose no risk of adverse effects.

Table 7: Dietary Reference Intakes (DRIs)-Recommended intake for Individual - Food and Nutrition Board, Institute of Medicine.

Life stage (y)	Ca	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Se	Zn
Children	(mg d^{-1})	(µg d^{-1})	(µg d^{-1})	(mg d^{-1})	(mg d^{-1})	(mg d^{-1})			(µg d^{-1})	(mg d^{-1})
4-8	800	15	440	10	130	1.5	ND*	ND	ND	5.0
Males										
14-30	1000-1300	35	890-900	8-11	240-410	1.9-2.2	ND	ND	55	11
31-70	1000-1200	30-35	900	8.0	420	2.3	ND	ND	55	11
> 70	1200	30	900	8.0	420	2.3	ND	ND	55	11
Females										
14-30	1000-1300	24-25	890-900	15-18	310-360	1.6-1.8	ND	ND	55	8-9
31-70	1000-1200	20-25	900	8-18	320	1.8	ND	ND	55	8
> 70	1200	20	900	8.0	320	1.8	ND	ND	55	8

*ND not determined.

Table 8: Dietary Reference Intakes (DRIs)-Tolerable Upper Intake Levels (ULs) - Food and Nutrition Board, Institute of Medicine.

Life stage	Ca	Cr	Cu	Fe	Mg[a]	Mn	Ni	Pb	Se	Zn
Children	(g d^{-1})		(µg d^{-1})	(mg d^{-1})	(mg d^{-1})	(mg d^{-1})	(µg d^{-1})		(µg d^{-1})	(mg d^{-1})
4-8 y	2.5	ND*	3 000	40	110	3.0	0.3	ND	150	12
Males/females										
14-18	2.5	ND	800	45	350	9.0	1.0	ND	400	11
19-70	2.5	ND	10 000	45	350	11	1.0	ND	400	11
> 70	2.5	ND	10 000	45	350	11	1.0	ND	400	11

*ND not determined, [a]UL from Mg do not include intake from foot and water but represent pharmacological agent.

2.8 Nutrients in plants

Besides the secondary metabolites in plants that are beneficial to human health, plants also contain nutrients that are equally important. Nutrients in plants are inorganic mineral substances found in all tissues of the plant. Their presence in the plant is essential for metabolic processes that leads to growth and development (Malhotra, 1998). Mineral elements are categorized into macro elements such as Ca, Na, Mg, P and Cl and micro elements such as Ni, Fe, Co, Cu, Zn and Be. The former are required in large amounts (≥100 mg dL^{-1}) and the latter in small amounts (≤100 mg dL^{-1}) by the plant's body for normal metabolism (Murray 2000; Tapiero *et al*., 2003; Soetan *et al*., 2010).

2.9 Phytochemical and analytical techniques

The following techniques were employed to achieve the objectives of this research.

2.9.1 Chromatography

Chromatography is a Greek word which means chroma (colour). Chromatography is a generic term that is used for the set of laboratory techniques to separate mixtures of compounds. The mixture is dissolved in a solvent called a "mobile phase" which passes through a stationary phase that allows the constituents of the mixture to be separated and isolated. Chromatography could either be preparative (used for purification) or analytical (for measurement).

2.9.1.1 Column Chromatography

Column chromatography (CC) is used to purify mixtures of different compounds. In column chromatography, a glass tube (of varying size) with a tap at the bottom is used. A slurry of the stationary phase (usually silica gel or alumina) with the mobile phase (solvents) is

prepared and carefully poured into the column. Care has to be taken to prevent air bubbles in the packed column. A small layer of sand is usually placed on top of the stationary phase to provide a bed for the crude extract. The prepared extract is then carefully transferred into the column. As soon as the column tap is opened, the components of a mixture are passed through the stationary phase with the help of the mobile phase (Figure 20). This process is known as elution (Skoog *et al*., 2004), and compounds are eluted separately based on their polarity. Fractions that elute (Figure 21) are collected and analysed using thin layer chromatography (TLC).

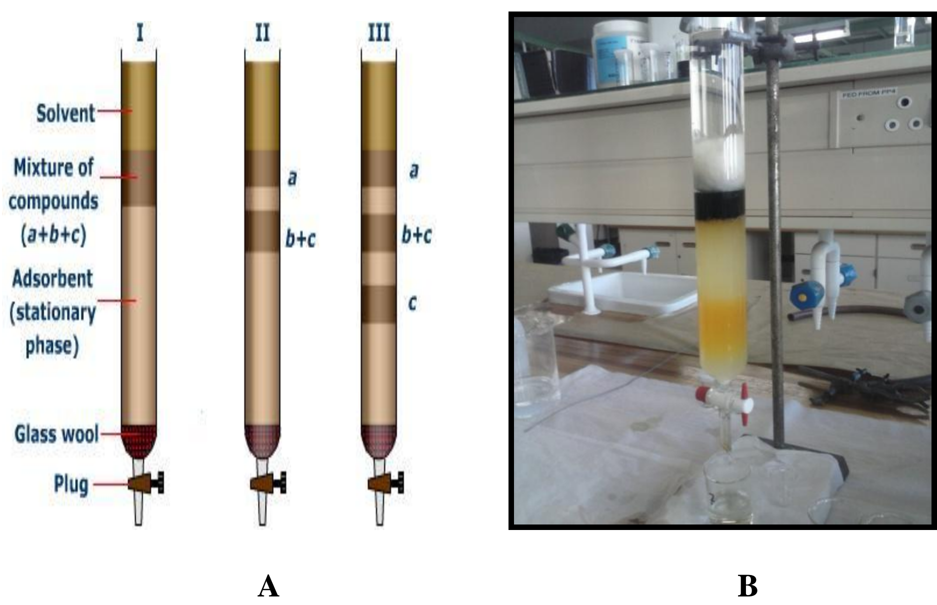
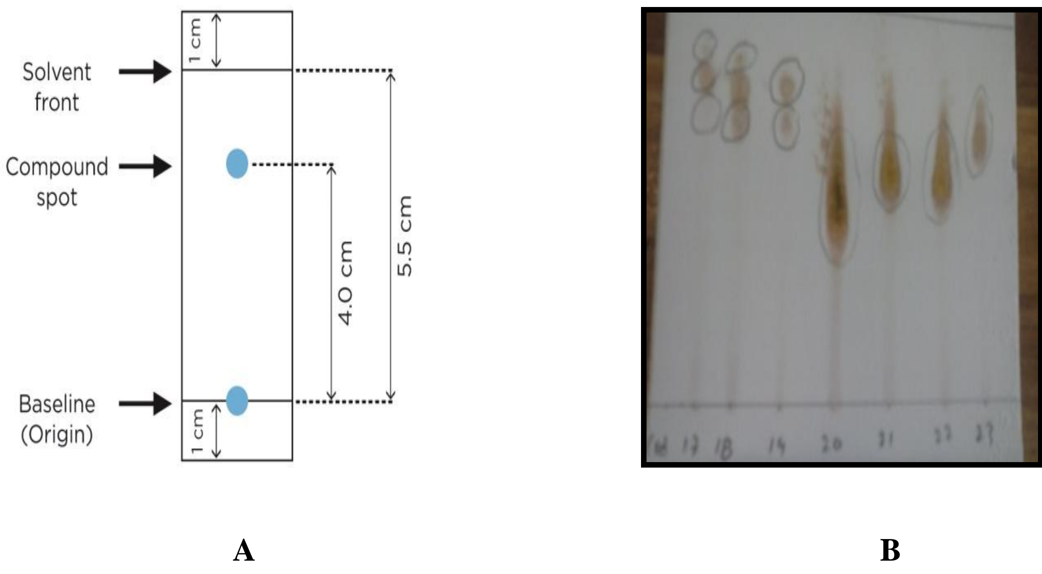


Figure 20: (A) Schematic diagram of column chromatography describing the separation of components (a, b and c) of a mixture (Chimicamo 1999). (B) A column that was used for the separation of compounds in this study.

Figure 21: A series of fractions collected in this study.

2.9.1.2 Thin layer chromatography

TLC (Figure 22) is used for qualitative study. The TLC plate is usually aluminium, glass or rubber coated with a stationary phase like silica gel or alumina. TLC has advantage of a faster run rate and better separations of the components than paper chromatography in which paper serves as the stationary phase.



A **B**

Figure 22: Schematic diagram of TLC plate (A) (adopted from Silicycle 2013) and TLC plate that was used to isolate a compound in this study (B)

2.9.2 Spectroscopic Techniques

The principles behind spectroscopic methods depend on the interaction of matter and radiation and the amount of quantum of radiation absorbed or produced by atomic or molecular species.

2.9.2.1 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy (Figure 23) uses magnetic properties of nuclei. The technique is dependent on the ability of atomic nuclei to act like a small magnet and how they arrange themselves with an external magnetic field. An unpaired atomic nuclei spins upon interacting with radio frequency (RF) in an external field (B_0). The spinning nuclei generate tiny magnetic fields. Some nuclear spins are aligned parallel and anti-parallel to the external field. Nuclei that are aligned in a parallel position to the external magnetic field are irradiated with the right frequency of electromagnetic radiation. A quantum of energy is absorbed and the spin of the nuclei flips to a higher energy anti-parallel alignment. The nuclei that flip as a result of applied radiation are said to be in resonance with the applied radiation. The energy frequency can be measured and displayed as an NMR spectrum. This spectrum is a series of vertical peaks/signals distributed along the x-axis of the spectrum, and therefore, each atom within the molecule corresponds with the peaks/signals. The positions of the peaks provide useful information with regards to the local structural environment of the atom. Detailed structural information of the compounds can be established using one dimensional NMR (1D-NMR) spectroscopy. Proton (^{1}H) and Carbon-13 (^{13}C) NMR are the most important nuclear spins to chemists and their spectrum show signals against corresponding chemical shifts. The chemical shifts for most organic molecules in ^{13}C-NMR rages from 0-230 ppm whilst those in ^{1}H-NMR range from 0-13 ppm. The ^{13}C-NMR signals appear as singlets due to decoupling of protons attached.

Figure 23: The 600 MHz and 400 MHz ultra-shield NMR spectrometry at the School of Chemistry, (UKZN).

Distortionless Enhancement by Polarization Transfer (DEPT 90, 135) is also a 1D-NMR technique that provides useful information on signals of methyl, methylene, methine and quaternary carbon atoms.

Two-Dimensional (2D) NMR provides supporting information for structure elucidation of compounds, particularly when the structure of a molecule is too complicated and cannot be fully elucidated by 1D-NMR.

Some examples of 2D-NMR are as follows:

Correlation Spectroscopy (COSY)

Nuclear Overhauser Effect Spectroscopy (NOESY)

Heteronuclear Singlet Quantum Coherence (HSQC)

Heteronuclear Multiple Bond Quantum Coherence (HMQC)

2.9.2.2 Infrared spectroscopy (IR)

Infrared Spectroscopy (IR) (Figure 24) is another spectroscopic technique which provides information on the functional groups of a molecule or a compound. It is used to identify and examine both organic and inorganic compounds. IR spectroscopy uses the infrared region of the electromagnetic spectrum; the energy courses the molecule to vibrate or rotate at a certain positions and is not sufficient for electron transition.

Fourier-Transform Infrared Spectroscopy (FTIR); is a high speed, sensitive and high resolution technique. It does not record the intensity of energy absorbed when IR light is not constant, but instead measures and collects IR spectra. FTIR performs mathematical Fourier transformations on the signals of the investigated sample and displays the result on a spectrum.

Figure 24: IR Spectrometry at the School of Chemistry, (UKZN).

2.9.2.3 Ultraviolet-visible spectroscopy (UV-Vis)

Ultraviolet-visible spectroscopy (UV-Vis) (Figure 25) is another of the spectroscopic techniques used to identify organic compounds bearing unsaturated heteroatoms or organic chromophores. It also measures the absorptions for transition from ground state to excited state (Skoog, *et al*., 2004).

Figure 25: UV-Vis spectrometry at the School of Chemistry, (UKZN).

2.9.2.4 Gas chromatography-mass spectrometry (GC-MS)

The combination of gas chromatography (GC) and mass spectrometry (MS) to form a unit system (Figure 26) is to identify different substances within a test sample. GC separates the components of a mixture based on the adsorption between a mobile phase (carrier gas) and stationary phase (solid or liquid) while the mass spectrometer determines the mass-to-charge ratio (*m/z*) of ions. The molecules are bombarded with a strong energy of beams of electrons and results in the fragmentation of the molecule into positive and negative ions and neutral species.

Figure 26: Image of GC-MS spectrometry at School of Chemistry (UKZN).

2.9.3 Instrumentation

Microwave digestion and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) were used in this study to determine the elemental concentrations in the fruits.

2.9.3.1 Microwave digestion

Microwave digestion is a technique used to dissolve metals in organic matter before their analysis. A sample under digestion is exposed to a strong acid such as (HNO_3) in a closed vessel under high temperature and pressure. This increases the solubility of heavy metals in a solution which makes it easier to quantify through elemental techniques. Microwave vessels are made up of liners and caps. These materials can withstand high temperatures and pressures as well as resistance to chemical attack.

2.9.3.2 Inductively coupled plasma-optical emission spectrometer

The Inductively Coupled Plasma (ICP) (Figure 27) is an analytical instrument used to determine the concentration of elements in a sample under investigation. The ICP produces excited atoms and ions using inductively coupled plasma. The excited atoms or ions produced would then emit electromagnetic radiation at the characteristic wavelength of the particular element. The concentration level can be identified or measured by the intensity of the emitted

radiation. The sample is fed into the capillary tube by a peristaltic pump and decomposed into charged ions after colliding with electrons and charged ions in the plasma and emit characteristic wavelengths (Table 9) of the element engaged.

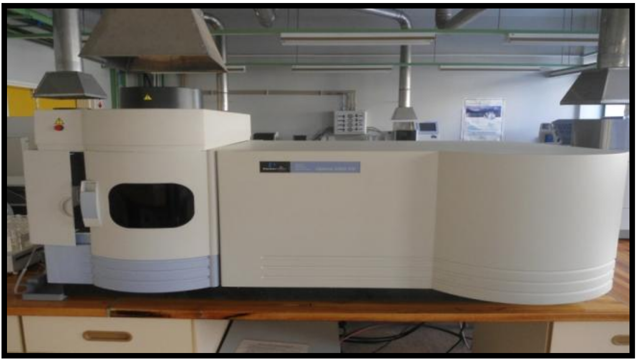


Figure 27: Image of an ICP-OES spectrometry at the School of Chemistry, (UKZN).

Table 9: ICP-OES detection limits.

Element	*Wavelength (nm)*	*Detection limit (ppb)*
Ac	193.75	0.90
Cd	228.80	0.07
Co	228.61	0.25
Cr	267.71	0.25
Cu	324.75	0.90
Fe	259.94	0.20
Mn	257.61	0.03
Ni	221.64	0.37
Pb	220.35	1.40
Se	196.09	4.00
Zn	206.19	0.20

A certified reference material (CRM) is a control or standard reference material used to confirm the quality of an analyte. Samples of known analytical composition (reference materials) are used to compare analytical instrumentation for accuracy. The accuracy of the trace element measurements in this study were accomplished using a standard reference material, *lyophilized brown bread* (BCR 191), from the community Bureau of Reference of the Commission of the European Communities (attached in the Appendix).

2.10 Reactive oxygen species (ROS)

The presence of high amounts of reactive molecules containing oxygen species in the human body causes chemical stress known as oxidative stress (OS). This oxidative stress may cause age-related illness. Due to the increased harmful effects of free radicals in the human body and deterioration of the components of foodstuff, interest in anti-oxidants that would prevent the occurrence of such processes has increased (Molyneux, 2004). Several methods are used today to estimate the effectiveness of a substance as an anti-oxidant. The most common method is the use of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is a deep coloured crystalline solid powder that is made up of stable free radical molecules (Sharma and Bhat, 2009). It has the approximate melting point of 130 ^{o}C depending upon its lattice symmetry. It is known as a radical scavenger due its ability to trap other radicals. Therefore, it is used as an indicator to determine the rate reduction of a chemical reaction. DPPH has a deep violet colour in solution and changes to light yellow or sometimes to colourless when it is neutralized. It absorbs at about 517 nm in its radical form ($DPPH^{\bullet}$) and the absorption disappears when reduced by an anti-oxidant agent (Figure 28) (Brand-Williams *et al*., 1995).

Figure 28: Reaction of DPPH and radical species.

CHAPTER THREE

Phytochemical and Elemental Investigations of the Leaves and Edible Fruits of Cryptocarya latifolia

Abstract

Cryptocarya latifolia (*Lauraceae*) is an indigenous South African plant used in ethnomedicinal practices. Traditional healers in KwaZulu-Natal, South Africa continue to use the bark of *C. latifolia* as a substitute for *Ocotea bullata (Lauraceae)* for medicinal purposes. The conservation status of *C. latifolia* has recently come under scrutiny. Existing populations of this species are rapidly declining probably as a consequence of their substitutive use for *O. bullata.* Uncontrolled and excessive removal of bark of the species has led to the death of many of these plants and may eventually result in the depletion and extinction of this plant species in the natural habitat. In this study, a phytochemical investigation on the leaves and fruits of *C. latifolia* was done to determine their medicinal value. From the leaves, a new compound, 5-hexyltetrahydro-2*H*-pyran-2-one (**4**) was isolated together with known compounds, quercetin-3-*O*-rhamnoside (**1**), β-sitosterol (**2**), copaene (**3**) and nerolidol (**5**). However, some important monoterpenoids, diterpenoids, sesquiterpenoids and essential oils were identified with the aid of gas chromatography-mass spectrometry (GC-MS). Quercetin-3-*O*-rhamnoside (**1**) and β-sitosterol (**2**) was also isolated from the edible fruits of the plant. Anti-oxidant activity of the isolated compounds using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay indicated that all the compounds, except β-sitosterol (**2**), exhibited good anti-oxidant activity compared to ascorbic acid. This study shows that excessive removal of the plant's bark can be minimized by utilizing the leaves and fruits for ethnomedicinal practices and this can help in the management and conservation of this declining plant. The analytical study conducted on the fruits of *C. latifolia* indicates that they are a good source of important dietary elements and may contribute significantly to the recommended dietary intake for most individuals. These elements were found to be in increasing order of Pb <Cr <

Se < Ni < Cu < Zn < Mn < Fe< Mg< Ca. This study confirms that consumption of the indigenous edible fruits of *C. latifolia* could be useful to the rural populace and other vulnerable communities and may be as alternative source of important dietary elements.

Keywords: *Lauraceae, Cryptocarya latifolia*, phytochemical, analytical, fruits, leaves, anti-oxidant, conservation.

3.1 Introduction

The loss of valuable indigenous medicinal plant species is a growing environmental concern in South Africa. About 3000 species are used for medicinal purposes by approximately 200 000 traditional healers established within a large informal business system (Mander, 1998). Many plants are collected illegally, via unsustainable harvesting techniques, resulting in the depletion of wild populations (Cunningham, 1993). If sustainable quantities of raw medicinal plant materials can be made available, then the use of indigenous plants in primary health care can be maintained. *Lauraceae* is a large family consisting of about 50 genera and 2500-3000 species (Nishida, 1998; Hu *et al*., 2007; Bannister *et al*., 2012; Cuca *et al*., 2013; Miller and Tuck, 2013). They are aromatic evergreen trees and shrubs with the exception of *Cassytha* (Nishida, 1998). The family is distributed across the world (Rohwer, 2000) with many species found commonly in the Southeast of Asia and tropics of America (Bannister *et al*., 2012). In the region of Europe, a single species (*Laurus nobilis*) is found (Yang, 1998). The species found in northern Asia is distributed across China and Japan. The family is also found in Chile and Argentina. Many species are found in Madagascar but the family is not well distributed in Africa. However, the genus *Cryptocarya* is found in South Africa, Mozambique and Swaziland. In southern Africa, some species of *Cryptocarya* (*Cryptocarya latifolia*, *Cryptocarya myrtifolia* and *Cryptocarya woodii*) are commonly distributed in the coastal regions of KwaZulu-Natal and Eastern Cape (Bannister *et al*., 2012).

Plants are basic sources of biologically active substances such as minerals and vitamins (Onibon *et al*., 2012; Gebrekidan *et al*., 2013). Nutrients are chemical or mineral elements needed in the body of living organisms for metabolism (Moodley *et al*., 2012). They are required in the correct proportions for effective functioning of the organs (Soetan *et al*., 2010). *C. latifolia* has great economic importance as a source of food and medicine. The fruits are eaten by humans, birds and antelopes. In South Africa, *C. latifolia* has been used in

the treatment of various ailments and diseases including headaches, morning sickness, pulmonary disease, tuberculosis, bacterial and fungal infections, as well as for magical purposes (Drewes *et al*., 1995; Wang *et al*., 2009; Sabitha *et al*., 2009; Yen, 2010; Arya, 2011; Kumar and Meshram, 2011). Consequently, this important indigenous South African plant faces serious ecological threat due to uncontrolled and excessive removal of the plant parts used in ethnomedicinal practices. This study is aimed at investigating the leaves and fruits of *C. latifolia* to determine their medicinal value to provide a means through which this endangered medicinal plant could be conserved. Apart from medicinal significance, a nutritional investigation was also conducted to assess the nutritional benefits of consuming the indigenous edible fruits which could be useful to the rural populace and vulnerable communities. No previous studies on the phytochemistry of the leaves, fruits and elemental concentrations in the edible fruits of *C. latifolia* have been reported.

3.2 Materials and methods

The experimental section of this chapter explains the various methods employed in the collection, preparation of samples, isolation, identification and characterisation of the isolated compounds, the protocols used for the bioassays and the analytical techniques used for elemental analysis of the fruits of *C. latifolia*.

3.2.1 General experimental procedure

The compounds were successfully isolated with the aid of column chromatography and characterized using different spectroscopic techniques (1D and 2D-NMR, GC-MS, IR, and UV). The antioxidant activities of the isolated compounds were determined using the DPPH free radical scavenging assay.

NMR spectra (1D and 2D) were recorded in deuterated methanol (CD_3OD) and deuterated chloroform ($CDCl_3$) at room temperature using the Brucker Avance^{III} 400 MHz and 600 MHz spectrometer with tetramethylsilane (TMS) as an internal standard. IR spectra were recorded using the Perkin Elmer Universal ATR Spectrometer. UV spectra were recorded using the UV-Vis-NIR Shimadzu UV-3600 spectrometer with methanol as a solvent. GC-MS data were recorded on an automated GC-MS (split-less mode) equipped with a DB-5SIL MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Helium (0.70 mL min^{-1}) was used as a carrier gas and acetonitrile (ACN) was used to dissolve the sample. 1 µL of each sample solution was injected into the GC-MS. The injector was kept at 250 °C whilst the transfer line was at 280 °C. The column temperature was held at 60 °C for 2 min, and then ramped to 280 °C at 20 °C min^{-1} where it was held for 10 min.

The MS was operated in the EI mode at 70 eV. Compounds were identified based on their fragmentation pattern and molecular mass, and the mass spectra were compared to the National Institute of Standard and Technology (NIST 05, 2005) database and literature.

Melting points (uncorrected) were recorded on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus. All chemicals used were supplied by Merck and Sigma Chemical Companies and were of analytical-reagent grade. All spectral data obtained were compared with those reported in literature for identification of known compounds.

3.2.2 Collection, preparation and extraction

The leaves and fruits of *C. latifolia* were collected in December 2012 from the province of Kwazulu Natal, South Africa, and identified by herbarium technician, Mr Edward Khathi, from the Ward Herbarium, School of Life Sciences, UKZN (Westville).

The plant material was taken to the natural products laboratory where the leaves and fruits were separated from the stems. These were then air dried at room temperature to constant weight in the drying room. Thereafter, they were crushed using a domestic blender (Russell Hobbs) and kept in plastic containers in the refrigerator for further analysis.

Ground leaves (560.6 g) and fruits (50.35 g) were sequentially extracted with hexane, dichloromethane (DCM) and methanol (MeOH) for 72 hr each on an orbital shaker set at 120 rpm. Crude extracts were filtered using Whatman No. 1 filter paper then concentrated under reduced pressure using a rotary evaporator. The crude extracts were transferred into beakers and were placed open in the drying room for 48 hr. The beakers were then sealed with plastic paraffin film (Parafilm) and stored in a refrigerator at 4 ^{o}C for further analysis.

3.2.3 Column separation

Crude extracts were subjected to column chromatography using suitably sized columns and silica gel (kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM). Fractions collected were monitored by TLC (Merck silica gel 60, 20 x 20 cm F254 aluminium sheets), were visualized using anisaldehyde spray reagent (97: 2: 1; MeOH: conc. H_2SO_4: anisaldehyde) and analyzed under UV (254 nm). For the crude methanol extract from leaves, partitioning was performed in a separating funnel before isolation. The crude methanol extract (main) was dissolved in a small volume of MeOH then transferred to a separating funnel, to which, 200 mL each of DCM and water was added, shaken vigorously and left to stand for approximately 48 hr. The DCM fraction (bottom layer) was collected and concentrated using a rotary evaporator. This process was repeated twice. The collected fractions were combined and allowed to dry. The aqueous mixture remaining in the separating funnel was then extracted with 200 mL ethyl acetate, similar to DCM. This was also done in triplicate and the collected fractions were combined and concentrated before being subjected to column chromatography.

The ethyl acetate fraction (main) from the methanol extract of the leaves was subjected to column chromatography using a hexane: ethyl acetate solvent system starting with 100% hexane which was gradually increased by 10% to 100% ethyl acetate in hexane. Ten fractions containing 50 mL each were collected at every solvent system. The fractions collected at 100% ethyl acetate, which had the same TLC profiles, were combined to give fraction **A**. This fraction was rechromatographed using an ethyl acetate: methanol (80:20) solvent system and purified to yield compound **1** (72 mg).

The same solvent system (hexane: ethyl acetate) was used to separate the DCM fraction (main) from the methanol extract of the leaves using column chromatography. Fractions with the same TLC profiles were combined and purified. Compound **2** (28 mg), a white crystalline solid, was isolated in fraction 16 (90:10, hexane: ethyl acetate) and compound **3** (158 mg) was isolated in fractions 35-49.

The crude hexane and DCM extracts from the leaves showed similar spots on the TLC plate therefore these extracts were combined then subjected to column chromatography. The mass of the combined extracts was 21.04 g. Initially elution was effected using 100% hexane which was increased gradually by 5% with ethyl acetate until 100% ethyl acetate was reached. Ten fractions of 50 mL were collected for each solvent system. Two fractions (33 and 34) yielded compound **4** (17.8 mg). Fractions 31-32 were combined and rechromatographed to give an oily compound, compound **5** (450 mg), which eluted with a hexane: ethyl acetate (95:5) solvent system.

The methanol extract (4.56 g) from the fruits was subjected to column chromatography using a hexane: ethyl acetate solvent system which was increased gradually by 10% to 100% ethyl acetate. Ten fractions containing 50 mL each were collected at every solvent system. The fractions collected at 100% ethyl acetate yielded compound **1** (38.8 mg).

For the crude hexane and DCM extracts from the fruits (main), the extracts were combined as the TLC profiles for both extracts were similar. The column was eluted with 100% hexane which was stepped by 5% to 100% ethyl acetate. Compound **2** (12 mg) was eluted with a hexane: ethyl acetate (85:5) solvent system.

3.2.4 Anti-oxidant activity

The antioxidant activity of the isolated compounds was determined using the DPPH radical scavenging assay. The radical scavenging activity using the DPPH assay was evaluated as described by Murthy (2012) with little modification. Methanolic stock solutions of 600 µg mL^{-1} for each compound were prepared. A range of different concentrations (240, 120, 60, 30 and 15 µg mL^{-1}) from respective stock solutions of the compounds were prepared and each of these were mixed with 500 µL of methanolic DPPH solution (0.1 mM). The prepared solutions were then incubated in the dark for 30 min at room temperature. The absorbance was recorded at 517 nm against the blank (methanol) with the aid of a UV spectrophotometer. A standard solution of ascorbic acid was also prepared for comparison. The methanolic DPPH solution without phytocompounds was used as a control. The percentage scavenging activity was calculated according to the equation below:

$$\%\ \text{scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

Where Ac = Absorbance of Control, As = Absorbance of Sample.

3.2.5 Elemental Analysis

3.2.5.1 Reagents and standards

The analytical reagents used were of analytical reagent grade and were supplied by Merck and Sigma Chemical Companies. Double distilled water was used throughout the experiments. All the glassware used were washed with 6.0 M HNO_3 and rinsed with double distilled water to remove contaminants.

Digestion was achieved with the aid of a CEM MARS microwave digester (Model No. Mars6) by means of Teflon (TFM) lined vessels (HF 50). In order to get accurate results, five replicate digestions were done. Approximately 0.5 g of the fruit samples were accurately weighed and transferred into the vessels, to which, 10 mL of 70% HNO_3 was added and sealed for digestion. The power was set at 1600 W and the temperature was held at 210 ^{o}C for 30 min. After the digestion was completed, the power was then reduced gradually until cooled. The digests were then transferred to 50 mL volumetric flasks and diluted with double distilled water to the graduation mark. These were then analysed for As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). The accuracy of the elemental analysis was achieved by the use of the Certified Reference Material (CRM), *lyophilized brown bread* (BCR 191), from the Community Bureau of Reference of the Commission of the European Communities.

The emission lines were selected according to minimal spectral interference. Table 10 highlights the wavelengths that were selected for the elements analysed.

Table 10: **Emission lines (wavelengths) selected for the studied elements.**

Element	Emission line (nm)
Ca	317.9
Cr	267.7
Cu	324.8
Fe	259.9
Mg	285.2
Mn	294.9
Ni	341.5
Pb	217.0
Se	196.0
Zn	213.8

3.3 Results and Discussion

3.3.1 Isolation of Compound 1

Compound **1** (Figure 29) was isolated as a yellow, amorphous solid with a mass of 72 mg and 38.8 mg from the leaves and fruits, respectively. The NMR data is given in Table 11 and corresponding spectra are attached in the appendices section.

Figure 29: Structure of compound **1**.

The ^{1}H-NMR spectrum of compound **1** showed a resonance at δ_H 7.33 (1H, dd, J = 8.40, 2.00 Hz, H-6’) that appeared as a double-doublet due to coupling with the proton at δ_H 6.93 (1H, d, J = 8.40 Hz, H-5’) and meta-coupling with the proton at δ_H 7.35 (1H, d, J = 2.00 Hz, H-2’); the coupling of protons was confirmed by COSY correlations. The resonances at δ_H 6.21 (1H, d, J = 2.0 Hz, H-6) and δ_H 6.38 (1H, d, J = 2.0 Hz, H-8) were due to the methine protons in the A-ring as confirmed by 2-D NMR spectra. The presence of a sugar moiety was indicated by a proton resonance at δ_H 5.36 (1H, d, J = 1.2 Hz) typical of an anomeric proton and confirmed by resonances between δ_H 3.32-4.24. For rhamnose, the 6-deoxy sugar may be identified by a doublet methyl signal upfield with a coupling constant of about 6-7 Hz. The prominent peak at δ_H 0.96 (3H, d, J = 6.08 Hz, H-6’’) confirmed the presence of the rhamnose unit. A weak singlet resonating at δ_H 12.56 was due to the free OH groups in the structure.

The ^{13}C-NMR spectrum of compound **1** showed a resonance at δ_C 103.6 (C-1'') which correlated to the anomeric proton in the HSQC experiment. The two peaks at δ_C 99.8 (C-6) and δ_C 94.7 (C-8) correlated with the protons at positions 6 and 8, respectively in the HSQC experiment. The resonances at δ_C 165.9 (C-7), 163.2 (C-5), 149.8 (C-4'), and 146.4 (C-3') were assigned according to ^{13}C-NMR and DEPT (90 and 135) experiment. Similarly, ^{13}C-NMR and DEPT (90 and 135) spectra confirmed C-2, C-3, C-3', C-4, C-4', C-5, C-7, C-9 and C-10 to be quaternary carbons (Table 11).

The IR spectrum of compound **1** showed a broad absorption stretching band at 3231 cm^{-1} due to the presence of the hydroxyl (-OH) group, an absorption at 2933 cm^{-1} (stretch) due to –CH, an absorption band at 1651 cm^{-1} (stretch) due to the carbonyl group (C=O), a sharp absorption band at about 1600 cm^{-1} (stretch) indicating the presence of an aromatic ring (-C=C) and an absorption band at 1499cm^{-1}, 1355 cm^{-1} and 1267 cm^{-1} due to C-H, C-OH and C-O bonds, respectively.

The UV absorption spectrum of compound **1** showed two bands, a maximum peak at 250 nm (0.1510 A) and the other at 340 nm (0.1150 A) which is consistent with the literature. The melting point of compound **1** was recorded as 184-186 ^{o}C. The GC-MS spectrum of compound **1** showed *m/z*: 448 (M^{+}) and 303 (Figure 30). The data obtained is in agreement with that reported in literature (Lawrence *et al*., 2004; Ma *et al*., 2005; Amani *et al*., 2006; Thomas, 2007; Liu *et al*., 2009; Park *et al*., 2011, Mediani *et al*., 2012). Therefore, compound **1** was identified as quercetin-3-*O*-rhamnoside.

$- C_6H_{11}O_4^{\bullet}$ 147.066

m/z; 448.4 *m/z;* 301.0

Figure 30: Fragmentation pattern of compound **1** by GC-MS.

Table 11: ^{1}H-NMR, ^{13}C-NMR, DEPT (90, 135) and HMBC data for compound **1** (CD_3OD, 400 MHz).

Position	$\delta_{C\ (ppm)}$	DEPT ppm	δ_{H} ppm	HMBC Correlation
2	158.5	C	-	-
3	136.3	C	-	1’’
4	179.7	C	-	-
5	163.2	C	-	-
6	99.8	CH	6.21 (1H, d, *J* = 2.0 Hz)	8
7	165.9	C	-	-
8	94.7	CH	6.38 (1H, d, *J* = 1.96 Hz)	6
9	159.3	C	-	-
10	105.9	C	-	-
1’	122.9	C	-	-
2’	116.4	CH	7.35 (1H, d, *J* = 2.0 Hz)	6’
3’	146.4	C	-	2’

4’	149.8	C	-	5’, 3’
5’	116.9	CH	6.93 (1H, d, *J* = 8.40 Hz)	6’
6’	122.9	CH	7.33 (1H, dd, *J* = 8.40, 2.0 Hz)	5’, 2’
1’’	103.6	CH	5.36 (1H, d, *J* = 1.2 Hz)	
2’’	72.0	CH	4.24 (m, rhamnose-H)	1’’, 3’’
3’’	72.1	CH	3.78 (m, rhamnose-H)	2’’
4’’	73.3	CH	3.36 (m, rhamnose-H)	-
5’’	71.9	CH	3.31 (m, rhamnose-H)	6’’
6’’	17.8	CH_3	0.96 (3H, d, *J*=6.08 Hz)	5’’

3.3.1.1 Medicinal significance of Compound 1 (quercetin-3-O-rhamnoside)

Recently, a survey on seasonal influenza conducted by the National Institute for Communicable Diseases (NICD) in South Africa revealed that there was an increase rate of 4% in the number of people infected by the influenza virus in two months (NICD Report, 2013). In September 2013, it was also reported that an outbreak of diarrhea killed 30 people across some provinces of South Africa (Global Times News, 2013). It is well known that vulnerable communities are more prone to these diseases because of a lack of access to medication and unsanitary conditions. Utilization of medicinal plants could help as a preventive measure against these viral infections. For instance, the leaves and fruits of *C. latifolia* contain quercetin-3-*O*-rhamnoside which is known to have anti-influenza and anti-diarrheal activity (Choi *et al*., 2009a; 2009b). Quercetin is also used for the treatment of stage I hypertension; a person's blood pressure can be reduced if 730 mg of quercetin is taken in two divided doses in a month (Edwards *et al*., 2007). There is a need for South Africans suffering from such problems to know which plants to utilize or consume for maximum benefit especially those that have closer access to these indigenous medicinal plant species.

3.3.2 Isolation of Compound 2

Compound **2** (Figure 31) was isolated as a white crystalline solid with a mass of 28.7 mg and 19.5 mg from the leaves and fruits of the plants, respectively. The NMR data is given in Table 12 and corresponding spectra are attached in the appendices section.

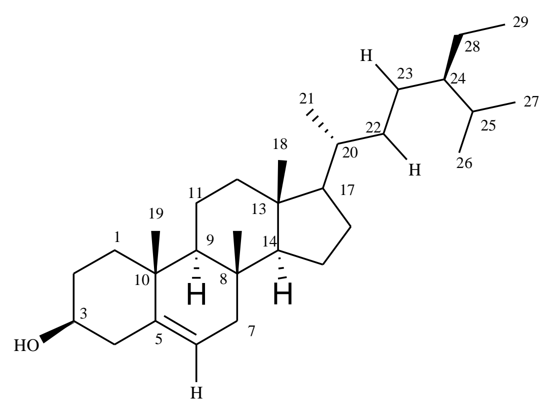


Figure 31: Structure of compound **2**.

The ^{1}H-NMR spectrum of compound **2** showed six methyl resonances; a triplet at δ_H 0.84 (3H, t, *J* = 7.5 Hz, H-29), two singlets at δ_H 0.68 (3H, s, H-18) and 1.01 (3H, s, H-19), and three doublets at δ_H 0.81 (3H, d, *J* = 6.5 Hz, H-26), 0.83 (3H, d, *J* = 6.5 Hz, H-27) and 0.93 (3H, d, *J* = 6.4 Hz, H-21). The ^{1}H-NMR spectrum of compound **2** also showed a resonance for a single olefinic proton at δ_H 5.36 (1H, d, *J* = 5.3 Hz, H-6) and another resonance at δ_H 3.53 (m, *J* = 12.0, 11.4, 4.9 Hz, H-3) that appeared as a multiplet corresponding to the proton attached at δ_C 71.8 (C-3) in the ^{13}C-NMR spectrum. The ^{13}C-NMR spectrum of compound **2** showed 29 carbon resonances. Resonances at δ_C 140.7 and 121.7 were assigned to C-5 and C-6, respectively. The structure of compound **2** (Figure 32) was further supported by DEPT (90 & 135) and from the literature (Chaturvedula and Prakash, 2012) (Figure 32).

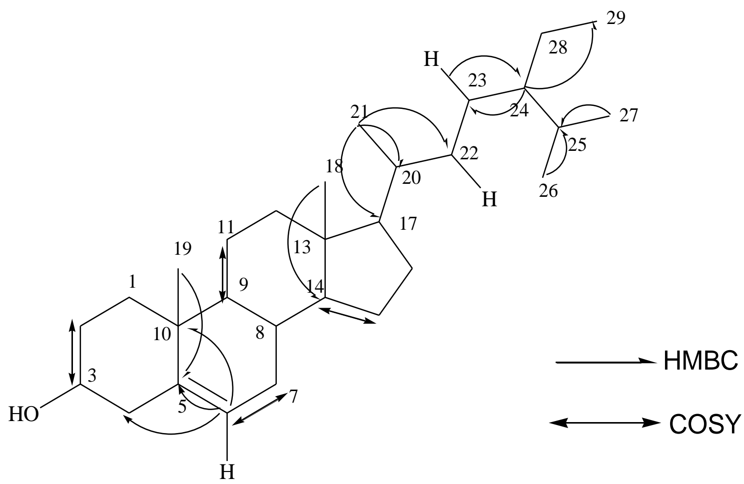


Figure 32: HMBC and COSY correlations of compound **2**

Table 12: ^{1}H-NMR, ^{13}C-NMR and DEPT (90, 135) data for compound **2** (CD_3OD, 400 MHz).

Position	δ_{C} (ppm)	DEPT (ppm)	δ_{H} (ppm)
1	37.3	CH_2	
2	29.7	CH_2	
3	71.8	CH	3.53 (m, J = 12.0, 11.4, 4.9 Hz)
4	42.3	CH_2	
5	140.8	C	-
6	121.7	CH	5.36 (d, J = 5.3 Hz)
7	31.9	CH_2	
8	31.7	CH	
9	50.1	CH	
10	36.5	C	-
11	21.1	CH_2	
12	39.8	CH_2	

13	42.3	C	-
14	56.8	CH	
15	24.3	CH_2	
16	28.3	CH_2	
17	56.1	CH	
18	36.1	CH_3	0.68 (s)
19	19.4	CH_3	1.01 (s)
20	34.0	CH	
21	26.1	CH_3	0.93 (d, *J* = 6.4 Hz)
22	45.8	CH_2	
23	23.1	CH_2	
24	12.0	CH	
25	29.2	CH	
26	19.8	CH_3	0.81 (d, *J* = 6.5 Hz)
27	19.0	CH_3	0.83 (d, *J* = 6.5 Hz)
28	18.8	CH_2	
29	11.9	CH_3	0.84 (t, *J* = 7.5 Hz)

The IR spectrum of compound **2** showed a broad band at 3414 cm^{-1} and an intense band at 1052 cm^{-1} due to O-H and C-O stretching vibrations, respectively. Another band at 1643 cm^{-1} was due to the presence of a double bond (-C=C-) and absorptions at 1464 cm^{-1} and 1376 cm^{-1} were due to C-H and C-OH, respectively. The melting point of compound **2** was recorded to be 134-135 ^{o}C. The UV (hexane) spectrum of compound **2** contains two peaks at 234.9 nm and 222.2 nm. The GC-MS spectrum showed fragment peaks at *m/z*: 414(M+), 329, 301and 273. The data obtained is in agreement with that reported in literature (De-Eknamkul and

Potduang, 2003; Chaturvedula and Prakash, 2012, Sosińska *et al*., 2013) therefore compound **2** was identified as β-sitosterol.

3.3.2.1 Medicinal significance of Compound 2 (β-sitosterol)

β-sitosterol is a useful phytocompound in cosmetics, pharmaceutical and food industries because of its biological activity (Xu *et al*., 2005). It is also capable of modifying the level of cholesterol concentration in cancer membranes, thereby inhibiting the viability of cancer-cell growth in the stomach, lungs and breast (Hąc-Wydro, 2013a; Hąc-Wydro 2013b; Sosińska *et al*., 2013).

3.3.3 Isolation of Compounds 3 and 5

Compounds **3** and **5** (Figure 33) were isolated with a mass of 158 mg and 450 mg, respectively. These compounds were the major components of the essential oil from *C. latifolia* leaves. Their structural identification was by GC-MS (Figure 34) according to the fragmentation patterns (attached in the appendices) and by comparison with the National Institute of Standard and Technology (NIST 05, 2005) database and literature (Donath and Boland, 1995; Klopell *et al*., 2007; Xie *et al*., 2012). Compound **3** was identified as copaene while compound **5** was identified as nerolidol.

OH

compound **3** compound **5**

Figure 33: Structures of compounds **3** and **5**.

- $C_3H_7^{\bullet}$ 43.05

- $C_5H_9^{\bullet}$ 69.07

$C_{15}H_{24}$ 204.2 | $C_{12}H_{17}$ 161.1 | $C_{14}H_{23}O$ 222.0 | $C_{10}H_{17}O$ 153.1

compound **3** compound **5**

Figure 34: GC-MS fragmentation pattern of compounds **3** and **5**.

In general, sesquiterpenes in plants are localized and tend to catalyze the formation of acyclic to tricyclic products through the formation of monocyclic and bicyclic intermediates (Xie *et al.*, 2012). Figure 35 describes the cyclization pathway of compound **5** to form compound **3**.

-OH
1,10 Cyclization
1,6-Closure
2,7 Closure
Compound **5**
Compound **3**

Figure 35: Cyclization pathway of compound **5** to form compound **3**.

3.3.3.1 Medicinal significance of Compounds 3 and 5 (copaene and nerolidol)

Nerolidol is a sesquiterpene present in the essential oil of *C. latifolia.* The United States Food and Drug Administration (FDA) approved the use of this compound to enhance the flavour and aroma of foods. It is used as a topical skin penetration enhancer for the delivery

of therapeutic drugs. Nerolidol also possesses anti-fungal, anti-malarial, anti-leishmanial and anti-ulcer activity and is cytotoxic on renal cell adenocarcinoma (Klopell *et al.*, 2007; Sperotto *et al.*, 2013). Similarly, copaene was shown to possess anti-hepatotoxic activity (Vinholes *et al.*, 2013).

3.3.4 Isolation of Compound 4

Compound **4** (17.8 mg) was isolated as a yellowish amorphous solid (Figure 36). The NMR data is given in Table 13 and the corresponding spectra attached in the appendices section.

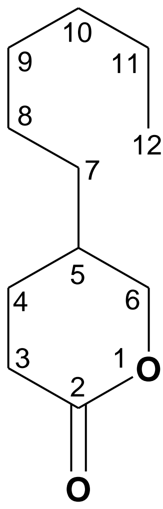


Figure 36: Structure of compound **4**.

The ^{1}H-NMR spectrum of compound **4** showed a resonance at δ_H 3.99 (2H, dd, J = 2.42, 5.86 Hz, H-6) and a multiplet resonating at δ_H 1.59 (1H, H-5). The proximity and position of these protons was confirmed by the COSY experiment. The triplet at δ_H 2.33 (2H, t, J = 0.68 Hz, H-3) correlated with the carbon resonance at δ_C 33.8 as confirmed by the HSQC experiment and with the carbon at δ_C 24.3 (C-4) in the HMBC experiment (Figure 37). The resonance at δ_H 1.69 (2H, m, H-4) was assigned to this position due to HSQC, COSY and HMBC correlation attached in the appendices. A single methyl resonance at δ_H 0.91 (3H, t, J = 7.42 Hz, H-12) and five methylene resonances between δ_H 1.35 to 1.25 (H-7, H-8, H-9, H-10 and

H-11) indicated the presence of a hydrocarbon side chain. The ^{13}C-NMR spectrum of compound **4** showed 11 carbon signals. The quaternary carbon resonance at δ_C 173 (C-2) as indicated by the DEPT (90 & 135) spectra was due to the carbonyl functional group. The DEPT (90 & 135) spectra also confirmed the presence of a methine group at δ_C 38.6 (C-5).

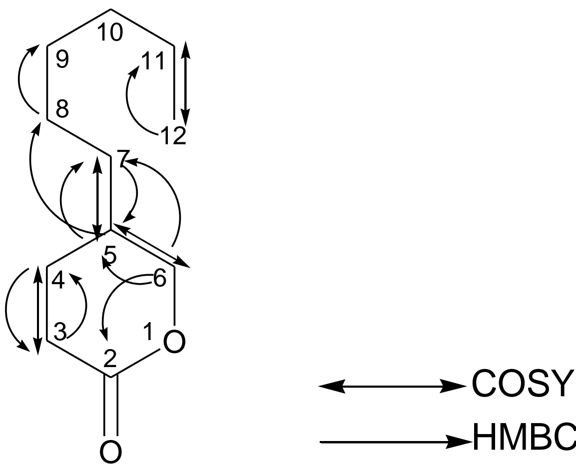


Figure 37: HMBC and COSY correlation of compound **4**.

The IR spectrum of compound **4** showed a band at 2923 cm^{-1} due to the C-H stretching vibration. A sharp band at 1714 cm^{-1} indicated the presence of the carbonyl group (-C=O), a band at 1167 cm^{-1} confirmed the presence of a C-O bond. The UV spectrum showed a peak at λ_{max} 223 nm characteristic of lactone absorption. The GC-MS spectrum showed molecular ion peaks at; *m/z* 185 (M^+ + 1), 129 and 99.0 (Figure 38). Therefore, compound **4** was identified as 5-hexyltetrahydro-2*H*-pyran-2-one with molecular formula of $C_{11}H_{20}O_2$.

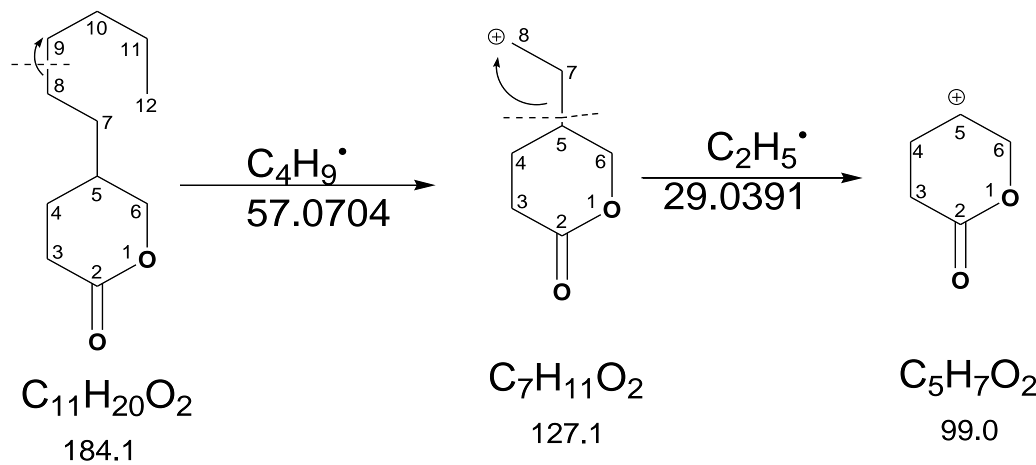


Figure 38: Proposed fragmentation pattern of compound **4** by GC-MS.

Table 13: ^{1}H-NMR, ^{13}C-NMR and DEPT (90, 135) data for compound 4 ($CDCl_3$, 400 MHz).

Position	**δ_{C} (ppm)**	**DEPT (ppm)**	**δ_{H} (ppm)**
1	-	-	-
2	173	C	-
3	33.8	CH_2	2.33 (t, *J*= 0.68 Hz)
4	24.3	CH_2	1.69 (m)
5	38.6	CH	1.59 (m)
6	66.7	CH_2	3.99 (dd, *J* = 2.42, 5.86 Hz)
7	29.6	CH_2	1.35 to 1.26 (m)
8	23.7	CH_2	1.35 to 1.26 (m)
9	28.7	CH_2	1.35 to 1.26 (m)
10	30.3	CH_2	1.35 to 1.26 (m)
11	22.8	CH_2	1.35 to 1.26 (m)
12	10.8	CH_3	0.91 (t, *J* = 7.42 Hz)

3.3.5 Other compounds profiled by GC-MS in C. latifolia leaves.

The chemical investigation revealed the presence of sesquiterpenes, monoterpenes, diterpenes, alcohols and organic acids from the fractionated extracts. The mass spectra (attached in the appendices) of the compounds were compared with the NIST (2005) library and literature. Table 14 shows the chemical structures of the compounds identified with their retention time and molecular ion/base ion peaks. This is the first report of these compounds from the leaves of *C. latifolia*.

Table 14: Compounds identified by GC-MS analysis from the extract of the leaves of *C. latifolia*.

Compounds	Molecular ion/Base ion peak	T_R (min)	Chemical structure
tau-Cadinol	204.2/161.2	8.967	OH
Phytol	278.3/71.2	11.279	OH
Naphthalene	204.2/119.2	8.894	

Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	279/149	13.598	
4-methoxybenzenmethanol	138/93	9.003	
Diisooctyl adipate	370/129	12.647	
Benzyl butyl phthalate	311.9/149		
Methyl hexadecanoate	270.2/74	10.372	
Pentadecanone	250/58.1	9.933	
(-)-Globulol	222/109	8.791	

α-Cubebene	204.2/161.2	8.982	
Vitamin E	430.4/164.1	21.820	HO, O
Azulene	222.2/109.1	8.821	
Dodecatrien-3-ol	189.2/69.1	8.367	OH

Cadinol and globulol were shown to have good anti-oxidant potential and potential as anti-bacterial agents against both gram positive and gram negative bacteria (Hammami *et al.*, 2011). The leaves were found to contain vitamin E which is known for lowering the risk of coronary heart diseases (Rimm *et al.*, 1993). Azulene is an anti-fungal agent (Iwu *et al.*, 1990) and cubebene is known to reduce the risk of atherosclerotic disease (Choi *et al.*, 2009c). This investigation has led to the isolation and identification of various medically important phytochemicals from the leaves of *C. latifolia*.

3.3.6 Antioxidant Activity

The DPPH radical scavenging assay most widely used for testing the ability of plant extracts to scavenge free radicals generated from the DPPH reagent (Dahech *et al.*, 2013). Figure 39 shows the anti-oxidant capacity of the various compounds isolated from *C. latifolia* using the DPPH free radical scavenging assay. The results show that the anti-oxidant capacity of the compounds is dependent on concentration similar to the standard (ascorbic acid).

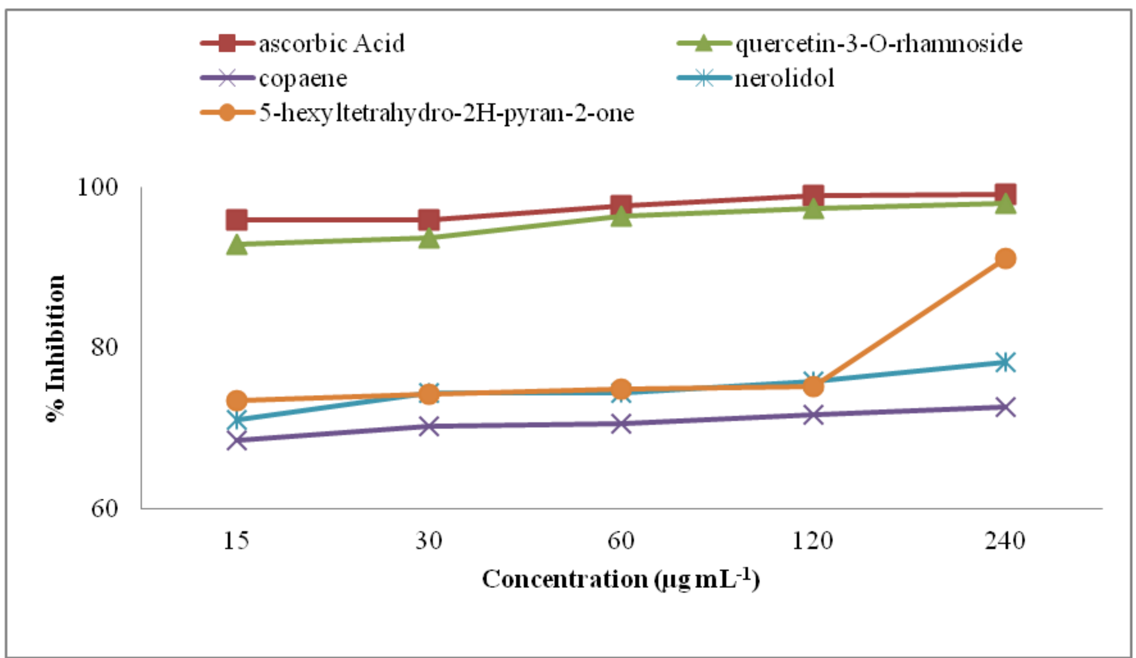


Figure 39: Anti-oxidant activity of compounds.

All the compounds except β-sitosterol (**2**) exhibited good anti-oxidant activity. Therefore, β-sitosterol was omitted from the figure and further discussions. Quercetin-3-*O*-rhamnoside (**1**) showed percentage scavenging inhibition between 92-98% at all concentrations tested. Similarly, 5-hexyltetrahydro-2*H*-pyran-2-one (**4**) was also found to have 91% inhibition at a higher concentration (240 μg mL^{-1}), however, at a lower concentration (15 μg mL^{-1}) it showed 73% inhibition, indicating good oxidative effect at high concentrations. Copaene (**3**) was also found to exhibit anti-oxidant activity, though it was lower than the other compounds

tested. Similarly, nerolidol (**5**) has free radical scavenging activity with highest inhibition at 240 µg mL^{-1}. This assay shows that extracts from *C. latifolia* fruits or leaves possess radical scavenging abilities, therefore it can help reduce oxidative stress in the body.

3.3.7 Elemental Analysis

The accuracy of the method for elemental analysis was measured by comparing results obtained with certified results (Table 15). The values for Fe, Mn, Cu and Zn are certified whilst those for As, Mg, and Ca are suggestive therefore no uncertainties are provided for these elements. All the values were found to be in agreement with the CRM values thereby validating the method. Elements that were not detected were As, Cd, and Co because their concentrations were below the detection limit of the instrument.

Table 15: Comparison of measured and certified values in the CRM (*lyophilized brown bread* (BCR 191).

	Concentration*	
Elements	**Measured****	**Certified****
Fe	40. 6± 1.2 µg g^{-1}	40.7 ± 2.3 µg g^{-1}
Mn	20.3 ± 0.6 µg g^{-1}	20.3± 0.7 µg g^{-1}
Cu	2.6 ± 0.1 µg g^{-1}	2.6 ± 0.1 µg g^{-1}
Zn	19.5± 0.4 µg g^{-1}	19.5 ± 0.5 µg g^{-1}
As	23.1 mg g^{-1}	23.0 mg g^{-1}
Mg	0.50 mg g^{-1}	0.51 mg g^{-1}
Ca	0.41 mg g^{-1}	0.40 mg g^{-1}

*Based on dry mass, **Mean ± S.D, at 95% confidence interval, n = 4.

The elemental distribution in the fruit was compared to Dietary Reference Intakes (DRIs) (Table 16). The table shows the contribution of 50 g dry mass (DM) of *C. latifolia* fruits to the nutritional requirements of most individuals for most elements.

Table 16: Dietary Reference Intake (DRI) – Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Levels (UL) of elements for most individuals – compared to average concentration of elements (n = 4) in the fruits.

Element	Average Concentration (mg/50 g, DM)	DRI (mg/day)		Estimated Contribution to RDA (%)
		RDA	UL	
Ca	82.9	1000-1300	2500	6.4-8.3
Cr	0.02	0.024-0.035	ND*	57-83
Cu	1.40	0.9	8.00	156
Fe	6.00	8-18	45.0	33-75
Mg	55.9	310-320	350	17-18
Mn	3.70	1.6-2.3	9.00	161-231
Ni	0.43	ND	1.00	ND
Pb	0.01	ND	ND	ND
Se	0.12	55	400	0.2
Zn	3.18	8-11	34.0	29-40

* ND- Not determined.

People from rural provinces tend to depend on staple crops such as wheat and rice for their nutritional needs because they cannot afford to purchase or cultivate vegetables and fruits, as a result, malnutrition is common. In South Africa, rural families live near wild fruit-bearing trees; they can consume the wild fruits along with these staple crops to obtain a balanced diet.

Table 16 shows that consumption of 50 g of *C. latifolia* fruits contributes about 8% towards the RDA for Ca. Calcium is an essential element which helps strengthen bones and its deficiency can lead to poor blood clotting and osteoporosis (Dorozhkin and Epple, 2002). The fruits of *C. latifolia* can be utilised to supplement the body with the required amount of Ca needed for a healthy life, especially for people that consider their source of Ca from milk unfavourable due to lactose intolerance.

In 2005, the Fe status of children under the age of 1-9 years from rural and informal urban areas of South Africa was 16% (Moodley *et al*., 2012). Consumption of *C. latifolia* fruits can contribute to alleviating this problem as 50 g of the fruits contributes about 33-75% towards the RDA for this element (Table 16). Zinc being the only metal present in all classes of enzymes is distributed all over the human body and it helps to enhance learning (Takeda, 2001). Consumption of 50 g of *C. latifolia* fruits may contribute 29-40% towards RDA. Manganese was found to exceed the RDA for this element but not the Tolerable Upper Intake level (UL) therefore it is likely to pose no risk of adverse effects. Similarly, the element Cr, Ni, Pb and Se were all found to conform to the RDAs and could contribute to the RDAs for these elements.

The present study on the fruits of *C. latifolia* has demonstrated that the fruits are a good source of important dietary elements, which are needed for normal growth and development, especially to vulnerable communities who have closer access to this plant. The concentration of elements in *C. latifolia* fruits were found to be in increasing order of Pb < Cr < Se < Ni < Cu < Zn < Mn < Fe< Mg < Ca.

3.4 Conclusion

The study investigated the phytochemical constituents in the leaves and fruits of *C. latifolia* to determine their medicinal value. From the leaves, a new compound, 5-hexyltetrahydro-2*H*-pyran-2-one (**4**) was isolated together with known compounds, quercetin-3-*O*-rhamnoside (**1**), β-sitosterol (**2**), copaene (**3**) and nerolidol (**5**). However, some important monoterpenoids, diterpenoids, sesquiterpenoids and essential oils were identified with the aid of gas chromatography-mass spectrometry (GC-MS). Quercetin-3-*O*-rhamnoside (**1**) and β-sitosterol (**2**) was also isolated from the edible fruits of the plant. Anti-oxidant activity of the isolated compounds using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay indicated that all the compounds, except β-sitosterol (**2**), exhibited good anti-oxidant activity compared to ascorbic acid. This study shows that excessive removal of the plant's bark can be minimized by utilizing the leaves and fruits for ethnomedicinal practices and this can help in the management and conservation of this declining plant. The analytical study conducted on the fruits of *C. latifolia* indicates that they are a good source of important dietary elements and may contribute significantly to the recommended dietary intake for most individuals. These elements were found to be in increasing order of Pb < Cr < Se < Ni < Cu < Zn < Mn < Fe < Mg < Ca. This study confirms that consumption of the indigenous edible fruits of *C. latifolia* could be useful to the rural populace and other vulnerable communities and may be as an alternative source of important dietary elements.

CHAPTER FOUR

Evaluation of decontamination, bud break, in vitro and ex vitro rooting in Cryptocarya latifolia

Abstract

Cryptocarya latifolia (*Lauraceae)* is an evergreen woody plant growing along streams and rivers. It is a declining medicinal tree indigenous to South Africa used in ethnomedicinal practices. In the present study, an evaluation of decontamination, bud break, *in vitro* and *ex vitro* rooting was performed. Different types, combinations and concentrations of sterilants, fungicides and antibiotics were tested for the establishment of contaminant-free cultures. The most effective decontamination regime which resulted in 94% contaminant-free explants included the use of 70% ethanol, 1.75% sodium hypochlorite, Tween20®, Ampicillin®, Celest®, Heritage® and autoclaved distilled water at varying combinations, concentrations and time exposures. After establishing the most effective decontamination protocol, explants were grown in culture tubes containing MS nutrient medium supplemented with various concentrations and combinations of cytokinin (BAP) and auxin (NAA) to stimulate bud break. The treatment containing BAP:NAA at 1.0:0.01 mg L^{-1} resulted in the highest percentage of explants forming shoots (96%). This treatment also resulted in the longest shoot length (8.06 mm). Both *in vitro* and *ex vitro* rooting were unsuccessful after using various concentrations of the auxins IBA and IAA. Further research is necessary to investigate shoot multiplication and rooting in *C. latifolia*.

Keywords: broad-leaved quince, cytokinin, auxin, decontamination, bud break.

4.1 Introduction

Lauraceae is a family consisting of about 50 genera and 2500-3000 species (Nishida, 1998; Hu *et al.*, 2007; Bannister *et al.*, 2012; Cuca *et al.*, 2013; Miller and Tuck, 2013). The family consists mainly of aromatic evergreen trees and shrubs distributed worldwide (Miller and Tuck, 2013). The genus *Cryptocarya* is commonly found in some parts of southern Africa. In South Africa, *Cryptocarya latifolia* (broad-leaved quince) is found in the KwaZulu-Natal and Eastern Cape provinces (Hannweg, 1995) where the plant is believed to possess similar ethnomedicinal properties to *Ocotea bullata*. However, *O. bullata* is a highly endangered species; wild populations are rare; and this has caused South African traditional healers to utilize *C. latifolia* as a substitute for *O. bullata* (Zschocke and Van Staden, 2000) in the treatment of various ailments including headache, morning sickness, pulmonary disease, tuberculosis, bacterial and fungal infections, as well as for magical purposes (Drewes *et al.*, 1995; Wang *et al.*, 2009; Sabitha *et al.*, 2009; Yen, 2010; Arya, 2011; Kumar and Meshram, 2011). As a result of this substitutive use, *C. latifolia* is massively exploited and is listed among the declining indigenous medicinal plants in South Africa (Cunningham, 1993). Many plants are exploited by traditional healers within a large informal business system (Van Wyk, 1997). The plants are usually collected illegally through unsustainable harvesting techniques, resulting in the depletion of wild populations (Cunningham, 1993). Thus, it has become necessary to overcome the challenges of over-exploitation of *C. latifolia* by developing appropriate propagation methods to enhance its conservation. Plant tissue culture, which is the *in vitro* mass-propagation of plants on nutrient media under aseptic conditions (Bhojwani and Razdan, 1986; Chawla, 2002) has become the most widely used technique for rapid propagation of plants in need of conservation (Rout *et al.*, 2006). It is also used in scientific investigations to study the production of biochemical secondary metabolites, germ-plasm preservation and plant pathology (Hudson, 2002). However, one of the biggest challenges to

any tissue culture system is microbial contamination. Microorganisms such as fungi and bacteria can inhibit the growth of *in vitro* plants (Cassells *et al.,* 2000; Leifert and Cassells, 2001; Ryan, 2009). These contaminants may express themselves without delay after their introduction into the culture or they may appear after long periods of time after maturation in the *in vitro* system. Hence, the establishment of reliable and efficient decontamination (disinfection) protocols to eliminate microbial contaminants in culture systems is crucially important. *In vitro* cultures of woody tree species, such as *C. latifolia*, are especially difficult to establish (Han and Zhang, 2009). Therefore, this study aimed to develop an efficient protocol for decontaminating *C. latifolia* explants. Thereafter, the most efficient protocol was used on nodal explants to investigate bud break, and shoot tip explants to investigate *in vitro* rooting. *Ex vitro* rooting from stem cuttings was also studied.

4.2 Materials and methods

4.2.1 Plant material

Potted plants of *C. latifolia* (Figure 40-A) were purchased from Tropical Nursery (Durban, South Africa) and identified by herbarium technician, Mr Edward Khathi, of the Ward Herbarium, School of Life Sciences, UKZN. The potted plants were then transferred to a shadehouse facility and watered daily by an automatic sprinkler. In addition, the plants were treated with fungicides and nutrient mixtures weekly (see Tables attached in the appendices section).

4.2.2 Decontamination

Nodal segments from the mother plants in the shadehouse were cut and rinsed with tap water to remove dust particles. One-third of the leaf from the apex region was removed in order to reduce the effect of transpiration. Forty five nodal segments each were placed into different

decontamination treatments supplemented with various combinations of commercial sterilants, fungicides and antibiotics (Table 17). After the treatments, the ends of each nodal segment were trimmed with a sterile blade and placed onto Murashige and Skoog (MS) (1962) (4.4 g L^{-1}) nutrient media containing sucrose (30 g L^{-1}) and agar (10 g L^{-1}). Prior to the addition of agar, the pH of the media was adjusted to 5.8 with solutions of 1N hydrochloric acid and 1N sodium hydroxide. The media was autoclaved at 121 ^{o}C and 1.2 kg cm^{-2} for 20 min. One nodal segment per culture tube was used. The cultures were kept in a plant growth room at 25 ^{o}C, 16:8 hour light: dark photoperiod under diffuse white light at 55 µmol m^{-2} s^{-1}. Contamination data was recorded every three days for four weeks.

Table 17: Decontamination protocol details.

Protocol	Protocol details
Control	Nodal segments → autoclaved media [MS with vitamins (4.4 g L^{-1}) + sucrose (30 g L^{-1}) + agar (10 g L^{-1})]
A	Nodal segments → tap water rinse → autoclaved [MS with vitamins (4.4 g L^{-1}) + sucrose (30 g L^{-1}) + Benlate® (1 g L^{-1}) + Amphotericin B® (0.0025 g L^{-1})] → orbital shaker (30 hr) → 70% ethanol (30 s) → 1% sodium hypochlorite (20 min) → 3 rinse with autoclaved distilled water (30 s)
B	Nodal segments → tap water rinse → autoclaved [MS with vitamins (4.4 g L^{-1}) + sucrose (30 g L^{-1}) + Benlate® (1 g L^{-1}) + Ampicillin® (0.01 g L^{-1}) + Previcur® (35 ml L^{-1})] → orbital shaker (30 hr) → 70% ethanol (30 s) → 1% sodium hypochlorite (20 min) → 3 rinse with autoclaved distilled water (30 s)
C	Nodal segments → tap water rinse → autoclaved [MS with vitamins (4.4 g L^{-1}) + sucrose (30 g L^{-1}) + Benlate® (1 g L^{-1}) + Ampicillin® (0.01 g L^{-1}) + Previcur® (35 ml L^{-1})] → orbital shaker (30 hr) → 1.75% sodium hypochlorite (20 min) → $HgCl_2$ (0.02 g/100 ml) (3 min) → 3 rinse with autoclaved distilled water (30 s) → [Benlate® (1 g L^{-1}) + Ampicillin® (0.01 g L^{-1}) + 1% sodium hypochlorite] (20 min) → 1 rinse with autoclaved distilled water (30 s)
D	Nodal segments → tap water rinse → 70% ethanol (quick dip) → [1.75% sodium hypochlorite + Ampicillin® (0.01 g L^{-1}) + 1 drop Tween20®] (5 min) → 3 rinse with autoclaved distilled water (30 s) → autoclaved [MS with vitamins (4.4 g L^{-1}) + sucrose (30 g L^{-1}) + Previcur® (35 ml L^{-1}) + Early Impact® (10 ml L^{-1})] → orbital shaker (24 hr) → 3 rinse with autoclaved distilled water (30 s) → [1% sodium hypochlorite + Ampicillin® (0.01 g L^{-1}) + 1 drop Tween20®] (5 min) → 3 rinse with autoclaved water (30 s)
E	Nodal segments → tap water rinse → 70% ethanol (quick dip) → [1.75% sodium hypochlorite + Ampicillin® (0.01 g L^{-1}) + 1 drop Tween20®] (5 min) → 3 rinse with autoclaved distilled water (30 s) → autoclaved [MS with vitamins (4.4 g L^{-1}) + sucrose (30 g L^{-1}) + Celest® (4 ml L^{-1}) + Heritage® (10 g L^{-1})] → orbital shaker (24 hr) → 3 rinse with autoclaved distilled water (30 s) → [1% sodium hypochlorite + Ampicillin® (0.01 g L^{-1}) + 1 drop Tween20®] (5 min) → 3 rinse with autoclaved water (30 s)

4.2.3 Bud break

Nodal segments from the mother plants in the shadehouse were excised. They were rinsed using tap water to remove dust particles and one-third of the leaf sections were removed as before. Thereafter, the nodal segments were taken to the laminar flow and decontaminated according to protocol **E**. Subsequently, the cut ends of nodal segments were trimmed with a sterile blade and placed onto nutrient media comprising MS (4.4. g L^{-1}), sucrose (30 g L^{-1}) and agar (10 g L^{-1}), supplemented with various concentrations and combinations of plant growth regulators (PGRs) (Table 18). Prior to the addition of agar, the pH of all media was adjusted to 5.8 as before. PGR-free media was used as a control. The media was autoclaved at 121 ^{o}C and 1.2 kg cm^{-2} for 20 min. One nodal segment per culture tube was used. Sixteen explants were used for each treatment. The cultured explants were placed in the growth room as before and observed every three days for bud break.

Table 18: Different combinations and concentrations of PGR tested for bud break in *C. latifolia* nodal segments.

Bud break (BB) treatment	Plant growth regulator (PGR) mg L^{-1}
Control	PGR-free
BB1	0.5 BAP[a]
BB2	1.0 BAP
BB3	2.0 BAP
BB4	0.5:0.01 BAP:NAA[b]
BB5	1.0:0.01 BAP:NAA
BB6	2.0:0.01 BAP:NAA

[a]Benzylaminopurine

[b]Naphthaleneacetic acid

4.2.4 In vitro rooting

Shoot tips from mother plants in the shadehouse were excised. They were rinsed using tap water to remove dust particles and one-third of the leaf sections were removed as before. Thereafter, they were taken to the laminar flow and decontaminated according to protocol **E**. Subsequently, the cut ends of shoot tips were trimmed with a sterile blade and placed onto nutrient media comprising ½ strength MS (2.2 g L^{-1}), sucrose (30 g L^{-1}) and agar (10 g L^{-1}), supplemented with different concentrations of auxins (Table 19). The pH of the media was adjusted as before. Auxin-free media was used as a control. One shoot tip per culture tube was used. Fifteen explants were used for each treatment. The cultured explants were then placed in the growth room as before and observed every three days for rooting.

Table 19: Different concentrations of auxins tested for rooting in *C. latifolia* shoot tips.

Root (R) treatment	**Auxins (mg L^{-1})**	
	IBA[a]	IAA[b]
Control	auxin-free	auxin-free
R1	1.0	1.0
R2	2.0	2.0
R3	5.0	5.0

[a]Indole-3-butyric acid

[b]Indole-3-acetic acid

4.2.5 Ex vitro rooting

Stem cuttings of approximately 6 cm, each containing 2-3 nodes, were excised from shade-house plants. One-third of each of the leaf sections were removed as before. The basal ends of the cuttings were separately dipped in IBA and IAA at 500 and 1000 ppm for 5 and 10 min each, respectively. Thereafter, the cuttings were planted in two separate soil mixtures comprising potting soil:perlite and potting soil:vermiculite (50:50% w/w) in a non-misting shade-house. For the control, cuttings without IBA or IAA were used. Ten cuttings were used for each treatment including the control. The planted cuttings were watered twice a day by means of an automatic water sprinkler until the completion of the experimental period which was 30 days. Root and shoot formation was observed every 3 days.

4.2.6 Statistical analysis

Percentage contamination, shoot length and shoot number were analysed using one way ANOVA and Tukey's Post-Hoc (HSD) test using SPSS version 21.

4.3 Results and discussion

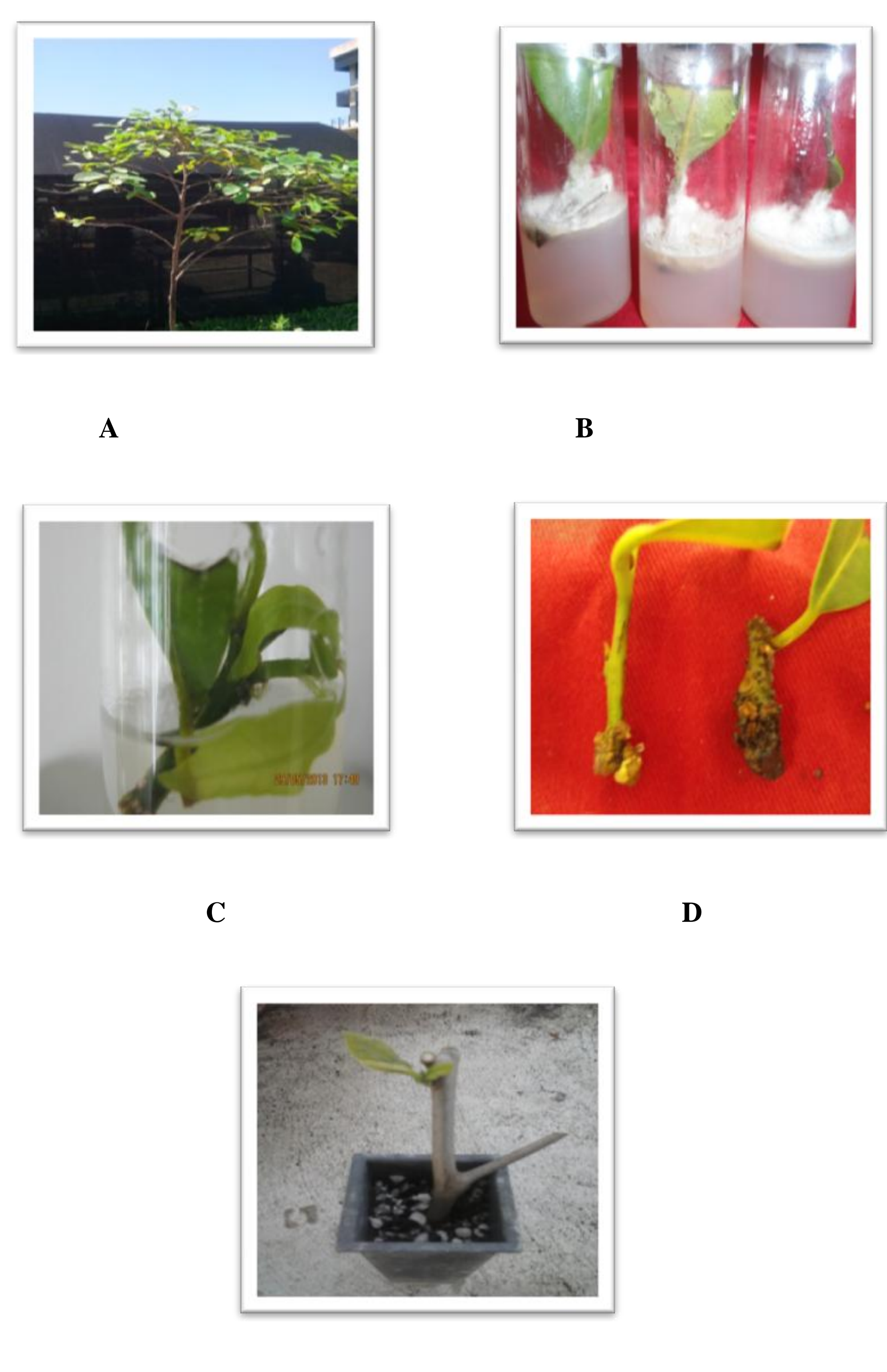


Figure 40: (A) *C. latifolia* potted plant in the shadehouse facility, (B) fungal contamination observed in nodal explants, (C) explant producing shoot in BB5, (D) effect of auxins on *in vitro* rooting and (E) effect of IBA on shoot induction in *ex vitro* experiment.

4.3.1 Decontamination

After 3 days of culture, 10% of explants were found to be infected with fungi in the control. By the end of the first week of culture, bacterial and fungal infections were detected in 50% of explants and by the end of two weeks, 100% of explants were contaminated (Figure 40-B). According to Cassells (2010) culture contamination begins with the introduction of endophytic plant pathogens and environmental microorganisms into the culture. These contaminants may kill the plants and result in economic losses (Cassells, 1990). Thus, commercial sterilants, fungicides and antibiotics were introduced in the subsequent decontamination experiments. Explants treated with 70% ethanol, Benlate® (1 g L^{-1}) and Amphotericin B® in protocol **A** had significantly lower ($P<0.05$) contamination (76%) compared to the control (Figure 41) after the culture period. This suggests that the sterilants, fungicides and antibiotics were able to inhibt the microorganisms and prevent infection (Hudson *et al*., 2002) to a certain extent. The role of fungicides and antibiotics has been explained by Chawla (2010). In order to further impede the microbial infection, protocol **B** was used, in which Previcur® was added and Amphotericin B® was replaced by Ampicillin®. In this protocol, fungal contamination was initially detected in the last days of the second week which agrees with the report of Leifert and Cassells (2001) that some contaminants may take a few days before affecting the culture. There was no bacterial contamination after replacing Amphotericin B® by Ampicillin®. There was a statistical difference between protocols **A** and **B** with regard to the level of contamination (76% and 27%, respectively). Protocol **C** was used in an effort to further reduce the fungal contamination. In this protocol, 70% ethanol was not used because some explants in protocol **B** became dehydrated. Consequently, an increase in fungal contamination (60%) was observed which was not significantly different compared to protocol **A**. Therefore, it was concluded that subsequent decontamination treatments of explants should contain 70% ethanol. Ethanol was found to be

a necessary component of decontamination by Renukdas *et al.* (2010). However, long exposure of the explants to ethanol has been reported to have a detrimental effect on the tissues and appropriate duration of the treatment has to be determined (Bhojwani and Razdan, 1986). This could explain the dehydration of the explants in protocol **B**. Thus, in the subsequent protocols (**D** and **E**), quick-dip pre-treatment of explants with 70% ethanol was adopted to avoid long exposure to prevent dehydration of plant tissues. In addition to the quick-dip pre-treatment, amendments to the previous protocols included the addition of Tween20® to the nutrient wash and increasing the number of rinses in autoclaved water. This resulted in a marked decrease in the level of fungal contamination (22% in **D** and 6% in **E**, respectively). Usually, contamination of explants can only be reduced to a minimal level (Ryan, 2009). Although there was no significant difference between protocols **D** and **E,** protocol **D** was not considered because use of the fungicide Early Impact® led to a substantial loss of explants due to necrosis.

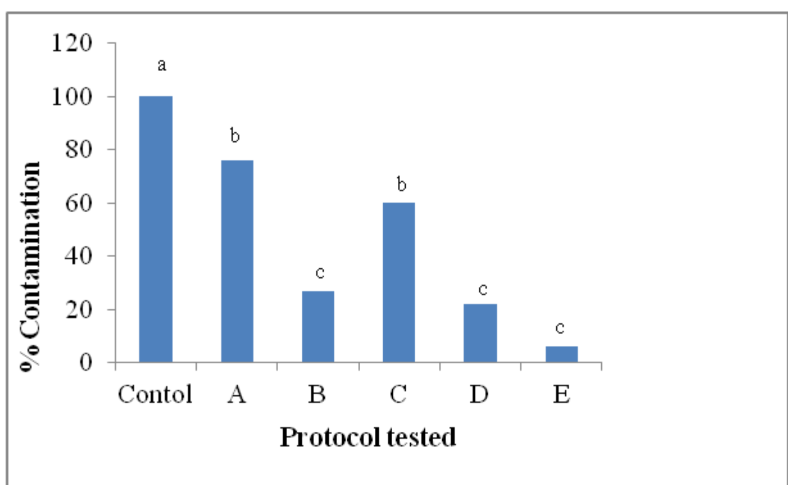


Figure 41: Different decontamination protocols tested on *C. latifolia* nodal explants. Different letters above the bars are significantly different at $P < 0.05$ using ANOVA and Tukey's HSD tests.

4.3.2 Bud break

The results showed that explants treated with PGRs (cytokinins and auxins) had a better shoot formation response compared to the control (Figure 42). This indicates the influence of PGRs in inducing shoots and has been reported in other studies (Hudson *et al*., 2002; Renukdas *et al*., 2010). However, the percentage of explants forming shoots at 0.5 (BB1), 1.0 (BB2) and 2.0 mg L^{-1} (BB3) BAP were lower (41%, 71% and 52%, respectively) than the percentage of explants treated with combination PGRs i.e. 0.5:0.01 (BB4), 1.0:0.01(BB5) and 2.0:0.01 mg L^{-1} BAP: NAA (BB6) (76%, 94% and 82%, respectively). This revealed the superior effect of combining PGRs to promote shoot development which was also reported in other studies (Shaik *et al*., 2010). But the results also showed that the number of shoots formed per explant was not dependent on the different concentrations of the combination PGRs because significant differences were not observed amongst these treatments. According to Shokri *et al*. (2012) the concentration of cytokinin (BAP) or auxin (NAA) at higher or lower amounts influences shoot development. Similarly Rout *et al*. (2006) recommended the use of the cytokinin BAP at moderate concentrations to induce shoots. It was recorded that BB5 resulted in the highest percentage of explants forming shoots (94%) compared to the other treatments (41-88%) and the control (29%). Table 20 shows that the highest average number of shoots forming per explant is only one and this was found for treatments BB2, BB4, BB5 and BB6. But the best shoot length was achieved in BB5 (Figure 40-C). Thus, BB5 could be considered as a suitable treatment for *in vitro* bud break in *C. latifolia*, however, multiplication of the shoots using various PGR combinations and concentrations needs further investigation.

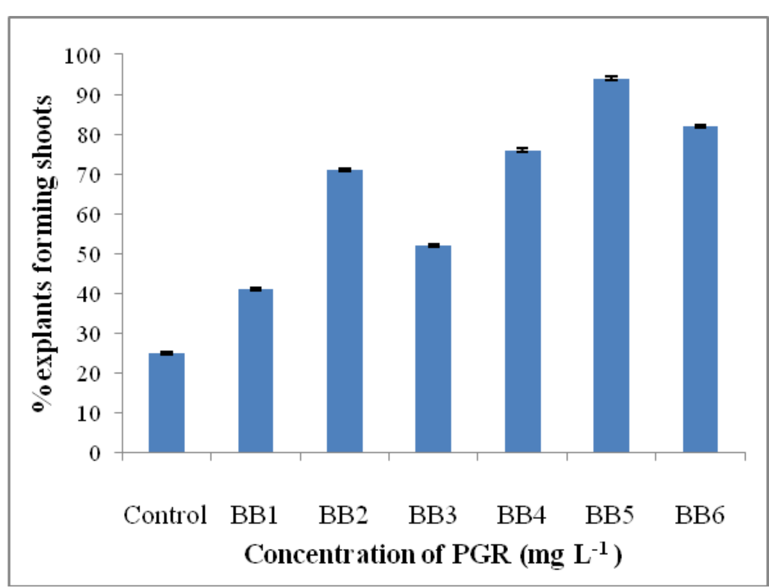


Figure 42: Effect of various combinations and concentrations of cytokinin and auxin on bud break in *C. latifolia* after four weeks of culture. Bars above each column represent mean ± SD.

Table 20: Effect of various combinations and concentrations of cytokinin and auxin on shoot number and shoot growth in *C. latifolia*.

Bud break (BB) treatment	Average number of shoots per explants	Average shoot length per explant (mm)
Control	$0.20^{a} \pm 0.4$	$0.25^{a} \pm 0.6$
BB1	$0.40^{a} \pm 0.5$	$1.63^{ab} \pm 2.1$
BB2	$1.00^{b} \pm 0.6$	$2.34^{ab} \pm 2.3$
BB3	$0.50^{a} \pm 0.5$	$2.06^{ab} \pm 2.6$
BB4	$1.00^{b} \pm 0.4$	$3.44^{b} \pm 3.0$
BB5	$1.00^{b} \pm 0.3$	$8.06^{c} \pm 3.7$
BB6	$1.00^{b} \pm 0.4$	$3.16^{b} \pm 2.3$

Different letters in a column are significantly different at $P<0.05$. Standard deviations are included after each mean value.

4.3.3 In vitro and ex vitro rooting

The *in vitro* rooting experiments using IBA and IAA (including the control) did not induce rooting at any of the concentrations tested. Auxins such as IBA have been found to stimulate production of roots better than IAA (Ozel *at el*., 2006). However, in this study, only abnormal swelling and tissue growth at the basal ends of the shoot tip explants was observed at all concentrations of auxins used (Figure 40-D). It is possible that the auxins did not undergo the necessary biochemical changes that lead to the formation of roots due to inappropriate positive carbon balance (Ozel *et al*., 2006). Poor rooting ability is a common phenomenon in woody species which are regarded as hard-to-root (Han and Zhang, 2009) because they possess high root inhibition substances (Hudson *et al*., 2002). Similarly, no rooting response was observed in the *ex vitro* experiments irrespective of the types of soil mixture used. Interestingly, shoot formation was observed in the planted stem cuttings (Figure 40-E) and the highest number of shoots per cutting was obtained in the treatment containing IBA at 1000 ppm for 5 min in potting soil:vermiculite (Table 21). Shoot formation could be explained by the acid growth hypothesis where cell walls loosen as a result of acidification (H^+) by auxins after which more water enters the cell by higher internal osmotic pressure which eventually expands the cell (Rayle *et al.,* 1992). Therefore, IBA may have had an indirect effect on shoot formation in the *ex vitro* experiment although no previous studies illustrating the effects of auxins on shoot induction have been reported. However, Riyadi (2013) showed the influence of auxins on shoot height and diameter. Further research is necessary to investigate the rooting mechanism in *C. latifolia*.

Table 21: Effect of IBA on shoot response in *ex vitro* stem cuttings of *C. latifolia* in two different soil mixtures. Standard deviations are included after each mean value.

Treatment with IBA (ppm/min)	**Average number of shoots per cutting in Soil A** [potting soil + vermiculite (50:50%)]	**Average number of shoots per cutting in Soil B** [potting soil + perlite (50:50%)]
Control	0.0 ± 0.0	0.2 ± 0.4
1000/10	0.8 ± 0.4	0.8 ± 0.4
1000/5	1.0 ± 0.0	0.5 ± 0.5
500/10	0.2 ± 0.4	0.4 ± 0.5
500/5	0.9 ± 0.3	0.5 ± 0.5

4.4 Conclusion

The present study investigated the development of protocols for decontaminating *C. latifolia* explants, bud break using nodal segments, *in vitro* rooting using shoot tip explants and *ex vitro* rooting using stem cuttings. The most effective decontamination regime which resulted in 94% contaminant-free explants included the use of 70% ethanol, 1.75% sodium hypochlorite, Tween20®, Ampicillin®, Celest®, Heritage® and autoclaved distilled water at varying combinations, concentrations and time exposures. The best cytokinin and auxin combination used to stimulate bud break and which resulted in the highest percentage of explants forming shoots (96%) with the longest shoot length (8.06 mm) was BAP:NAA at 1.0:0.01 mg L^{-1}. Both *in vitro* and *ex vitro* rooting experiments were unsuccessful after using various concentrations of the auxins IBA and IAA. Further research is necessary to investigate shoot multiplication and rooting mechanism in *C. latifolia*.

CHAPTER FIVE

5.1 Overall summary

This study focused on one plant species indigenous to South Africa namely; *Cryptocarya latifolia*. The plant is commonly used in ethnomedicinal practices in the treatment of various ailments and diseases including headache, morning sickness, pulmonary disease, tuberculosis, bacterial and fungal infections, as well as for magical purposes. It grows alongside streams and rivers and contains edible fruits which are enjoyed by the local people in Kwazulu-Natal and Eastern Cape provinces.

Because of the conservation status and claimed medicinal and nutritional benefit of the plant, this study aimed at investigating the leaves and fruits as a source of secondary metabolites and essential dietary elements. The isolation and characterization of the secondary metabolites was carried out primarily to investigate the kind of phytocompounds present and their biological activities in order to minimize the excessive removal of the plant's bark by utilizing the leaves and edible fruits of the plant for ethnomedicinal practices in an effort to help in the management and conservation of this declining plant species. However, the elemental investigation was done to validate the nutritional benefit of consuming the edible fruits; the elemental concentrations determined were compared to RDA. To further support conservation practices, the development of protocols for decontaminating *C. latifolia* explants, bud break using nodal segments, *in vitro* rooting using shoot tip explants and *ex vitro* rooting using stem cuttings was investigated.

5.2 Findings from C. latifolia

A new lactone, 5-hexyltetrahydro-2*H*-pyran-2-one, was isolated together with known flavonol (quercetin-3-*O*-rhamnoside), sterol (β-sitosterol) and sesquiterpenes (copaene and nerolidol). However, the leaves were found to be rich in monoterpenoids, diterpenoids and essential oils. The result highlighted the contributions of the isolated compounds in inhibition of oxidative stress that courses skin damage and aged related illness.

The same flavonol (Quercetin-3-*O*-rhamnoside) and sterol (β-sitosterol) was also isolated from the edible fruits of the plant. Elemental analysis indicated that the fruits are a good source of important dietary elements and if consumed, may contribute to the diet significantly.

5.3 General conclusions

The study investigated the phytochemical constituents in the leaves and fruits of *C. latifolia* to determine their medicinal value. From the leaves, a new compound, 5-hexyltetrahydro-2*H*-pyran-2-one (**4**) was isolated together with known compounds, quercetin-3-*O*-rhamnoside (**1**), β-sitosterol (**2**), copaene (**3**) and nerolidol (**5**). However, some important monoterpenoids, diterpenoids, sesquiterpenoids and essential oils were identified with the aid of gas chromatography-mass spectrometry (GC-MS). Quercetin-3-*O*-rhamnoside (**1**) and β-sitosterol (**2**) was also isolated from the edible fruits of the plant. Anti-oxidant activity of the isolated compounds using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay indicated that all the compounds, except β-sitosterol (**2**), exhibited good anti-oxidant activity compared to ascorbic acid. This study shows that excessive removal of the plant's bark can be minimized by utilizing the leaves and fruits for ethnomedicinal practices and this can help in the management and conservation of this declining plant. The analytical study conducted

on the fruits of *C. latifolia* indicates that they are a good source of important dietary elements and may contribute significantly to the recommended dietary intake for most individuals.

These elements were found to be in increasing order of Pb <Cr < Se < Ni < Cu < Zn < Mn < Fe< Mg< Ca. This study confirms that consumption of the indigenous edible fruits of *C. latifolia* could be useful to the rural populace and other vulnerable communities.

The results of the phytochemical and elemental study validated the need for alternative propagation methods of *C. latifolia*. In the biotechnological study, the most effective decontamination regime which resulted in 94% contaminant-free explants included the use of 70% ethanol, 1.75% sodium hypochlorite, Tween20®, Ampicillin®, Celest®, Heritage® and autoclaved distilled water at varying combinations, concentrations and time exposures. Thereafter, bud break was successfully achieved in nodal explants using various media formulations, however, the combination of BAP:NAA at 1.0:0.01 mg/L in full strength MS medium (4.4 g/L) supplemented with sucrose (30 g/L) and solidified with agar (10 g/L) produced the most explants (96%) that formed new shoots. Although both *in vitro* rooting experiments using shoot tips and *ex vitro* rooting experiments using stem cuttings were unsuccessful, this study has provided a bud break protocol that can be used to generate *C. latifolia* shoots for medicinal or conservation purposes.

5.4 Recommendations for further study

1. There is an urgent need for the determination of other biological activity such as anti-fungal, anti-cancer, anti-ulcer, anti-inflammatory and anti-bacterial activity on the new isolated compound from the leaves of *C. latifolia*.
2. *In vitro* and *ex vitro* rooting of *C. latifolia* needs further investigation to enable reliable plantlet regeneration techniques to be established.
3. Safety and efficacy of *in vitro* derived leaf material for medicinal purposes needs to be established.
4. Studies on the safety, efficacy, dosage control and potential health benefits of the herbal tonic of *C. latifolia*, sold in the streets markets needs thorough investigation.
5. Proper sensitization awareness programs more especially to rural populace and other vulnerable communities to realize the medicinal and nutritional benefits of the fruits thereby to incorporate the fruits in their diet.
6. There is a need for South African Government intervention to enforce conservation legislation that prohibits plant gatherers from excessive and uncontrolled collecting of *C. latifolia* bark and to alternately utilize the leaves or fruits in medicinal practices.
7. South African Government has to also introduce a sensitization awareness program to educate gatherers to understand the importance of plant conservation and to adopt sustainable harvesting techniques.

REFERENCES

Abdulla, M, Khan, AH, Reis, MF. 1996. Trace element nutrition in developing countries. Asia Pacific Journal of Clinical Nutrition. 3: 184-189.

Al-Achi, A. 2008. An Introduction to Botanical Medicines: History, Science, Uses, and Dangers. Praeger publishers, Westport, United States of America. 1: pp1-4.

Al-Daihan, S, Al-faham, M, Al-shawi, N, Al-mayman, R, Brnawi, A, Zargar, S, Bhat, RS. 2013. Antibacterial activity and phytochemical screening of some medicinal plants commonly used in Saudi Arabia against selected pathogenic microorganisms. Journal of King Saud University - Science. 25: 115-120.

Allard, PM, Dau, ET, Eydoux, C, Guillumeto, JC, Dumontet, CP, Canard, B, Gueritte, F, Litaudonm, M. 2011. Alkylated flavanones from the bark of *Cryptocarya chartacea* as dengue virus NS5 polymerase inhibitors. Journal of Natural Products 74: 2446-2453.

Amani, SA, Maitland, DJ, Soliman, GA. 2006. Hepatoprotective activity of *Schouwia thebica webb.* Bioorganic & Medicinal Chemistry Letters 16: 4624-4628.

Arya, V. 2011. A review on anti-tubercular plants. International Journal of Pharmacology Technology Research. 3: 872-880.

Baliga, MS, Baliga, BR, Kandathil, SM, Bhat, HP, Vayalil, PK. 2011. A review of the chemistry and pharmacology of the date fruits (phoenix dactylifera l). Food Research International. 44**:** 1812-1822.

Bannister, JM, Conran, JG, Lee, DE. 2012. *Lauraceae* from rainforest surrounding an early Miocene maar lake, Otago, Southern New Zealand. Review of Palaeobotany and Palynology. 178: 13-34.

BOLD System database 2013. *Cryptocarya* Genus. http://www.boldsystems.org/index.php/Taxbrowser_Taxonpage?taxid=179516

Bhojwani, SS, Razdan, MK. 1986. Plant tissue culture: theory and practice. Elsevier publishers, New York, United States of America. 5: pp1-91.

Bino RJ, Robert, DH, Fiehn, O, Kopka, J, Saito, K, Draper, Y. 2004. Pottential of metabolomics as a functional genomics tool. Trends in Plant Science. 9: 418-425.

Brand-Williams, W, Cuvelier, ME, Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. Food Science and Technology. 28: 25-30.

Cassells, AC. 2010. Contamination and its impact in tissue culture. Acta Horticulturae. 560: 353-359.

Cassells, AC, Doyle, BM, Curry, RF. 2000. Methods and markers for quality assurance in micropropagation. Acta Horticulturae. 530: 155

Cassells, A. 1990. Problems in tissue culture: culture contamination. Micropropagation. Springer publisher: United States of America. pp31-44.

Chaturvedula, VS, Prakash, I. 2012. Isolation of stigmasterol and β-sitosterol from the dichloromethane extract of *Rubus suavissimus*. International Current Pharmaceutical Journal. 1: 239-242.

Chawla, HS. 2002. Introduction to Plant Biotechnology. Science publishers, New Hampshire, United States of America. 2: pp5-42.

Chimicamo 1999. Lacromatografia liquido-solido su colonna: istruzioni per l'usohttps://www.google.co.za/search?q=column+chromatography&source=lnms&tbm=isch&sa=X&ei=IQMfU_ylEZPG7Aaf1oCoCQ&ved=0CAcQ_AUoAQ&biw=1366&bih=712#facrc=_&imgdii=_&imgrc=s5UdQa5k66HqEM%253A%3BXNSUGH1XF7qjIM%3Bhttp%253A%252F%252Fwww.chimicamo.org%252Fwpcontent%252Fuploads%252F2011%252F12%252Fcolumn chromatographyprocess.jpeg%3Bhttp%253A%252F%252Fwww.chimicamo.org%25

2Fchimica-analitica%252Fla-cromatografia-liquido-solido-su-colonna-istruzioni-perl%2525E2%252580%252599uso.html%3B373%3B252

Choi, HJ, Kim, JH, Lee, CH, Ahn, YJ, Song, JH, Baek, SH, Kwon, DH. 2009a. Antiviral activity of quercetin-7-rhamnoside against porcine epidemic diarrhea virus. Antiviral Research. 81: 77-81.

Choi, HJ, Song, JH, Park, KS, Kwon, DH. 2009b. Inhibitory effects of quercetin-3-rhamnoside on influenza virus replication. European Journal of Pharmaceutical Sciences. 37: 329-333.

Choi, YW, Kim, HJ, Park, SS, Chung, JH, Lee, HW, Oh, SO, Kim, BS, kim, JB, Chung, HY, Yu, BP, Kim, CD, Yoon, S. 2009c. Inhibition of endothelial cell adhesion by the new anti-inflammatory agent α-iso-cubebene. Vascular Pharmacology. 51: 215-224.

Chou, TH, Chen, JJ, Lee, SJ, Chiang, MY, Yang, CW, Chen, IS. 2010. Cytotoxic flavonoids from the leaves of *Cryptocarya chinensis*. Journal of Natural Products 73: 1470-1475.

Croteau, R, Kutchan, TM, Lewis, NG. 2000. Natural products (secondary metabolites). Biochemistry & Molecular Biology of Plants. 1250-1318.

Cuca, IE, Ramos, CA, Coy-Barrera, ED. 2013. Novel cadinane-related sesquiterpenes from nectandra amazonum. Phytochemistry Letters. 6: 435-438.

Cunningham, AB. 1993. African medicinal plants. Setting priorities at the interface between conseivation and primary healthcare. People and Plants Working Paper. 1-50.

Dahech, I, Farah, W, Trigui, M, Hssouna, AB, Belghith, H, Belghith, KS, Abdallah, FB. 2013. Antioxidant and antimicrobial activities of *Lycium shawii* fruits extract. International Journal of Biological Macromolecules. 60: 328-333.

De-Eknamkul, W, Potduang, B. 2003. Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units. Phytochemistry. 62: 389-398.

Dembitsky, VM, Poovarodom, S, Leontowicz, H, Leontowicz, M, Vearasilp, S, Trakhtenberg, S, Gorinstein, S. 2011. The multiple nutrition properties of some exotic fruits: biological activity and active metabolites. Food Research International. 44: 1671-1701.

Donath, J, Boland, W. 1995. Biosynthesis of acyclic homoterpenes: enzyme selectivity and absolute configuration of the nerolidol precursor. Phytochemistry. 39: 785-790.

Doniger, W, 1981. The Rig veda: an anthology: one hundred and eight hymns, selected, translated and annotated. Penguin book publishers. 402: 105:204.

Dorozhkin, SV, Epple, M. 2002. Biological and medical significance of calcium phosphates. Angew Chemie International Edition. 41: 3130-3146.

Drewes, SE, Sehlapelo, BM, Horn, MM, Scott-Shaw, R, Sandor, P. 1995a. 5,6-dihydro-α-pyrones and two bicyclic tetrahydro-α-pyrone derivatives from *Cryptocarya latifolia*. Phytochemistry. 38: 1427-1430.

Drewes, SE, Horn, MH, Mavi, S, Robert, S. 1995b. α-Pyrones and their derivatives from two *Cryptocarya species*. Phytochemistry. 40: 321-323.

Drewes, SE, Horn, MH, Mavi, S. 1996. *Cryptocarya liebertiana* and *Ocotea bullata*-their phytochemical relationship. Phytochemistry. 44: 437-440.

Duruibe, J, Ogwuegbu, M, Egwurugwu, J. 2007. Heavy metal pollution and human biotoxic effects. International Journal of Physical Sciences. 2: 112-118.

Edwards, RL, Lyon, T, Litwin, SE, Rabovsky, A, Symons, JD, Jalili, T. 2007. Quercetin reduces blood pressure in hypertensive subjects. The Journal of Nutrition. 137: 2405-2411.

Eid, SY, El-Readi, MZ, Wink, M. 2012. Digitonin synergistically enhances the cytotoxicity of plant secondary metabolites in cancer cells. Phytomedicine.19: 1307-1314.

Eloff, JN, McGaw, IJ. 2006. Plant extracts used to manage bacterial, fungal and parasitic infections in Southern Africa. Modern Phytomedicine: Turning Medicinal Plants into Drugs, 111.

Fay, MF. 1992. Conservation of rare and endangered plants using in vitro methods. In Vitro Cellular and Developmental Biology-Plant. 28: 1-4.

Fennell, CW, Lindsey, KL, McGaw, IJ, Sparg, SG, Stafford, GI, Elgorashi, EE, Grace, OM, Van staden, J. 2004. Assessing african medicinal plants for efficacy and safety: pharmacological screening and toxicology. Journal of Ethnopharmacology. 94: 205-217.

Gamborg, O, Murashige, T, Thorpe, T, Vasil, I. 1976. Plant tissue culture media. In vitro Cellular & Developmental Biology-Plant. 12: 473-478.

Gebrekidan, A, Weldegebriel, Y, Hadera, A, Van der Bruggen, B. 2013. Toxicological assessment of heavy metals accumulated in vegetables and fruits grown in Ginfel river near sheba tannery, tigray, Northern Ethiopia. Ecotoxicology and Environmental Safety. 95: 171-178.

George E, George, P. 1988. Plant culture media. Commentary and Analysis. Exegetics Edington. 2: 420.

George, EF, Hall, MA. De klerk, GJ. 2008. Plant tissue culture procedure-background. Plant Propagation by Tissue Culture. Springer. 3: 1-28.

George, EF. Sherrington, PD. 1984. Plant propagation by tissue culture. Springer publishers, Dordrecht, Netherlands. 1: pp1-227.

Ghani, A. 2003. Medicinal plants of Bangladesh: chemical constituents and uses. Asiatic Society of Bangladesh publishers, Dhaka, Bangladesh. 2: pp33.

Global Times News. 2013. Global times-discovery china, discover the world. www.globaltimes.cn/content/794757.shtml (accessed on 2nd November, 2013).

Hc-wydro, K. 2013a. Studies on β-sitosterol and ceramide-induced alterations in the properties of cholesterol/sphingomyelin/ganglioside monolayers. Biochimica et Biophysica Acta. 1828: 2460-2469.

Hąc-wydro, K. 2013b. The effect of β-sitosterol on the properties of cholesterol/phosphatidylcholine/ganglioside monolayers—the impact of monolayer fluidity. Colloids and Surfaces B: Biointerfaces. 110: 113-119.

Hammami, I, Triki, MA, Rebai, A. 2011. Chemical compositions, antibacterial and antioxidant activities of essential oil and various extracts of *Geranium sanguineum* flowers. Arch Applied Science Reaserch. 3: 135-144.

Han, H, Zhang, SS. 2009. A review on the molecular mechanism of plants rooting modulated by auxin. African Journal of Biotechnology. 8: 348-353.

Hannweg, KF. 1995. Development of micropropagation protocols for selected indigenous plant species. An M.Sc thesis submitted to Department of Biology, University of Natal. pp67-80.

Harrewijn, P, Van Oosten, AM, Piron, P. 2001. Natural terpenoids as messengers: a multidisciplinary study of their production, biological functions and practical applications. Kluwer academic publishers. Netherlands. pp1-54.

Hu, YQ, Ferguson, DK, Li, CS, Xiao, YP, Wang, YF. 2007. *Alseodaphne* (*Lauraceae*) from the pliocene of China and its paleoclimatic significance. Review of Palaeobotany and Palynology. 146: 277-285.

Hudson TH, Fred, TD, Robert IG. 2002. Plant propagation, principles and practices. Upper saddle river publisher, New Jersey, United States of America. 641-708.

Hyatt, DC, Youn, B, Zhao, Y, Santhamma, B, Coates, RM, Croteau, RB, Kang, C. 2007. Structure of limonene synthase, a simple model for terpenoid cyclase catalysis.

Proceedings of the National Academy of Sciences of the United States of America, 104: 5360-5365.

Ibelgaufts, H. 2012. Bioassays-cytokines and cells encyclopedia. http://www.copewithcytokines.de/cope.cgi?key=bioassays (accessed on 14th Octerber, 2013).

Iwu, M, Ezeugwu, C, Okunji, C, Sanson, DR, Tempesta, M. 1990. Antimicrobial activity and terpenoids of the essential oil of *Hyptis suaveolens*. Pharmaceutical Biology. 28: 73-76.

Juliawaty, LD, Kitajima, M, Takayama, H, Achmad, A, Aimi, N. 2000. A 6-substituted-5,6-dihydro-2-pyrone from *Cryptocarya strictifolia*. Phytochemistry. 54: 989-993.

Khan, M. 2010. Biological activity and phytochemical studies of selected medicinal plants. A PhD thesis submitted to the Department of Plant Sciences, Quaid-i-azam University, Islamabad. pp6-57.

Kirby EG, Lee. 1987. Nitrogen nutrition in cell and tissue culture in forestry. Nijhoff publishers, Netherland. pp1-67.

Klopell, FC, Lemos, M, Sousa, JB, Comunello, E, Maistro, EL, Bastos, JK, De Andrade, SF. 2007. Nerolidol, an antiulcer constituent from the essential oil of *Baccharis dracunculifolia dc* (*Asteraceae*). Z. Naturforsch. 62: 537-542.

Köksal, M, Zimmer, I, Schnitzler, JP, Christianson, DW. 2010. Structure of isoprene synthase illuminates the chemical mechanism of teragram atmospheric carbon emission. Journal of Molecular Biology. 402: 363-373.

Kumar, RN, Meshram, HM. 2011. Total synthesis of (−)-diospongin a and (+)-cryptofolione via asymmetric aldol reaction. Tetrahedron Letters, 52: 1003-1007.

Lange, M. 2003. Plant terpenoids: metabolism and function.

http://scholar.google.co.za/scholar?q=Plant+terpenoids%3A+metabolism+and+function&btnG=&hl=en&as_sdt=0%2C5. 1-6 (accessed on 4th September, 2013).

Lawrence, OM, Ivar, U, Peter, L. 2004. Further flavonol glycosides of *Embelia schimperi* leaves. Chemical Society of Ethiopia. 18: 51-57.

Lee, DK, Yoon, MH, Kang, YP, Yu, J, Park, JH, Lee, J, Kwon, SW. 2013. Comparison of primary and secondary metabolites for suitability to discriminate the origins of *Schisandra chinensis* by GC-MS and LC-MS. Food Chemistry. 141: 3931-3937.

Leifert, C, Cassells, A. 2001. Microbial hazards in plant tissue and cell cultures. In vitro Cellular & Developmental Biology-Plant. 37: 133-138.

Liu, HB, Yu, D, shin, SC, Park, HR, Park, JK, Bark, KM. 2009. Spectroscopic properties of quercetin derivatives, quercetin-3-O-rhamnoside and quercetin-3-O-rutinoside, in hydro-organic mixed solvents. Photochemistry and Photobiology. 85: 934-942.

Ma, X, Tian, W, Wu, L, Cao, X, Ito, Y. 2005. Isolation of quercetin-3-*O*- L-rhamnoside from *Acer truncatum bunge* by high-speed counter-current chromatography. Journal of Chromatography. 1070: 211-214.

Mabona, U, Van vuuren, SF. 2013. Southern African medicinal plants used to treat skin diseases. South African Journal of Botany 87: 175-193.

Mahlangeni, NT. 2012. Phytochemical and elemental analysis of *Cyrtanthus obliquus* and *Lippia javanica*. An M.Sc, submitted to the School of Chemistry, University of Kwazulu-Natal. pp5,67-89.

Malhotra, V. 1998. Biochemistry for students. Jaypee brothers medical publishers, New Delhi, India. pp102-253.

Mander, M. 1998. Marketing of indigenous medicinal plants in South Africa: A case study in Kwazulu-Natal.
http://scholar.google.co.za/scholar?hl=en&q=Mander%2C+M.+1998.+Marketing+of

+indigenous+medicinal+plants+in+south+africa%3A+a+case+study+in+Kwazulu-natal&btnG=&as_sdt=1%2C5&as_sdtp= (accessed on 5th September, 2013).

Mander, M. 2003. Indigenous medicinal plant trade sector analysis. Department of water affairs and forestry, Institute of Natural Resources. Report No: 248.1-17.

Mander, M, Ntuli, M, Diederichs, N, Mavundla, K. 2007. Economics of the traditional medicine trade in South Africa. 1-12.

Matsui, T, Matsushita, Y, Sugamoto, K, Yano, H. 2004. Isolation of terpenoids from *Sugi* (*Cryptomeria japonica*) wood and chemical conversion of ferruginol. Memoirs of the Faculty of Engineering, Miyazaki University. 33: 63-73.

McGarvey, DJ, Croteau, R. 1995. Terpenoid metabolism. The Plant Cell. 7, 1015-1026.

Mediani, A, Abas, F, Khatib, A, Maulidiani, H, Shaari, K, Choi, YH, Lajis, NH. 2012. ^{1}H-NMR-based metabolomics approach to understanding the drying effects on the phytochemicals in *Cosmos caudatus*. Food Research International. 49: 763-770.

Merfort, I. 2002. Review of the analytical techniques for sesquiterpenes and sesquiterpene lactones. Journal of Chromatography a. 967: 115-130.

Miller, RE, Tuck, KL. 2013. Reports on the distribution of aromatic cyanogenic glycosides in Australian tropical rainforest tree species of the *Lauraceae* and *Sapindaceae*. Phytochemistry. 92: 146-152.

Molyneux, P. 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin Journal of Science Technology. 26: 211-219.

Moodley, R, Koorbanally, N, Jonnalagadda, SB. 2012. Elemental composition and fatty acid profile of the edible fruits of *Amatungula* (*Carissa macrocarpa*) and impact of soil quality on chemical characteristics. Analytica Chimica Acta. 730: 33-41.

Moraes, PL. 2012. The *Lauraceae* collected in Brazil by ludwig riedel-i. Harvard Papers in Botany. 17: 185-216.

Moyo, M, Bairu, MW, Amoo, SO, Van staden, J. 2011. Plant biotechnology in South Africa: Micropropagation research endeavours, prospects and challenges. South African Journal of Botany. 77: 996-1011.

Murashige, T, Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco culture. Physiologia Plantarum. 15: 473-497.

Murray RK, DK, Granner, PA, Mayes, VW, Rodwell. 2000. Harper's biochemistry. McGraw-Hills publishers.United States of America. pp123.

NICD report 2013. South Africa influenza surveillance report. 2013, National institute for communicable disease. www.nicd.ac.za (accessed on 23rd September, 2013).

Nielsen, TR, Kuete, V, Jäger, AK, Meyer, JM, Lall, N. 2012. Antimicrobial activity of selected South African medicinal plants. BMC Complementary and Alternative Medicine. 12: 74.

Nishida, S. 1998. Revisional study of neotropical *Beilschmiedia* species (*Lauraceae*) with special reference to leaf anatomy. A Phd thesis submitted to the Depatment of Biology, Kyoto University. pp1-20.

Oladele AT, Oomobuwajo, OR. 2011. Conservation and cultivation of medicinal plants among traditional medicine practitioners (TMPs) in Aiyedade Local Government of Osun state of Nigeria a survey. Agriculture and Biology Journal of North America. 2: 476-487.

Ozel, CA, Khawar, K, Mirici, S, Arslan, O, Sebahattin, O. 2006. Induction of *ex vitro* adventitious roots on softwood cuttings of *Centaurea tchihatcheffii fisch* using indole-3-butyric acid and naphthalene acetic acid. Internation Journal of Agriculture and Biology. 1: 66-69.

Park, HR, Liu, HB, Shin, SC, Park, JK, Bark, KM. 2011. Spectroscopic properties of quercetin-3-*O*-rhamnoside and quercetin-3-*O*-rutinoside in aerosol reverse micelles. Bulletin of the Korean Chemical Society. 32: 981.

Parker, LA. 1915. A brief history of Materia Medica (continued). The American Journal of Nursing. 15: 729-734.

Parotta, J. 2002. Conservation & sustainable use of medicinal plant resources - an international perspective. Paper presented at the world ayurveda congress, kochi, kerala. 123.

Patricio, HP, Castaneto, YT, Vallesteros, AP, Castaneto, ET. 2006. Micropropagation of Shorea guiso using stem cuttimngs. Journal of Tropical Forest Science. 18: 198-201.

Rayle, DL, Cleland, RE. 1992. The Acid Growth Theory of auxin-induced cell elongation is alive and well. Plant physiology 99: 1271-1274.

Reed, BM, Tanprasert, P. 1995. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. Plant Tissue Culture and Biotechnology. 1: 137-142.

Renner, SS. 2005. Variation in diversity among *Laurales*, early cretaceous to present. Biologiske Skrifter/kongelike Danske Videnskabernes Selskab. 55: 441-458.

Renukdas, NN, Manoharan, M, Garner, JO. 2010. In vitro propagation of pecan (*Carya illioinensis* (*Wangenh*) *Kan*. Plant Biotecnology. 27: 211-215.

Rimm, EB, Stampfer, MJ, Ascherio, A, Giovannucci, E, Colditz, GA. & Willett, WC. 1993. Vitamin E consumption and the risk of coronary heart disease in men. New England Journal of Medicine. 328: 1450-1456.

Riyadi, I. 2013. *Ex vitro* rooting of oil palm (*Elaeis guineensis jacq*.) Plantlets derived from tissue culture. Indonesian Journal of Agricultural Science. 12: 57-62.

Rohwer, JG. 2000. Toward a phylogenetic classification of the *Lauraceae*: Evidence from matk sequences. Systematic Botany. 25. 60-71.

Rout, GR, Mohapatra, A, Jain, SM. 2006. Tissue culture of ornamental pot plant: a critical review on present scenario and future prospects. Biotechnology Advances. 24: 531-560.

Ruzicka, L. 1953. The isoprene rule and the biogenesis of terpenic compounds. Experientia. 9: 357-367.

Ryan, J. 2009. Understanding and managing cell culture contamination. Technical Bulletin. 1-24.

Sabitha, GN, Muddala NP, Jhillu SY. 2009. Stereoselective routes for the total synthesis of (+)-cryptocarya diacetate. Helvetica Chimica Acta. 92: 967.

Sarasan, V, Gripps, R, Ramsay, MM, Atherton, C, Monica, M, Grace, P, Rowntree, J. 2006. Conservation in vitro of threatened plants-progress in the past decade. In Vitro Cellular and Developmental Biology-Plant. 42: 206-214.

Schmeda, G, Astudillo, L, Bestida, J, Codina, C, Rojas, AD, Ferreira, ME, Inchausatti, A, Yaluff, G. 2001. Cryptofolione derivatives from *Cryptocarya alba* fruits. Journal of Pharmacy and Pharmacology 53: 563-567.

Sehlapelo, BM, Drewes, SE, Robert, SS. 1994. A 6-substituted 5,6-dihydro-α-pyrone from two species of *Cryptocarya*. Phytochemistry. 37: 847-849.

Shaik, S, Dewir, YH, Singh, N, Nicholas, A. 2010. Micropropagation and bioreactor studies of the medicinally important plant *Lessertia* (*Sutherlandia*) *frutescenes L.* South African Journal of Botany. 76: 180-186.

Shahidullah, AK. 2007. The role of medicinal plants in livelihood improvement and ecological sustainability in Bangladesh: An application of a participatory approach to

management and marketing. An M.Sc thesis submitted to the Faculty of Graduates Studies. University of Manitoba. pp1-68.

Sharma, OP, Bhat, TK. 2009. DPPH antioxidant assay revisited. Food Chemistry. 113. 1202-1205.

Shchepin, R, hornby, JM, Burger, E, Niessen, T, Dussault, P, Nickerson, KW. 2003. Quorum sensing in *Candida albicans*: probing farnesol's mode of action with 40 natural and synthetic farnesol analogs. Chemistry & Biology. 10: 743-750.

Shokri, S, Zarei, H, Alizadeh, M. 2012. Evaluation of rooting response of stem cuttings and *in vitro* micro-cuttings of bottlebrush tree (C*allistemon viminalis*) for commercial mass propagation. Journal of Agricultural Research. 1: 424-428.

Silicycle 2013. TLC plate visualization, http://www.silicycle.com/products/thin-layer-chromatography-tlc-plates/siliaplate-2.

Singh, G. 2007. Chemistry of terpenoids and carotenoids. Discovery publishing house, New Delhi, India. 1: pp1-3.

Skoog, DA, West, DM, Holler, FJ, Crouch, SR. 2004. Fundamentals of analytical chemistry. Brooks/Cole. 8th edition. USA.

Smith, RH. 2012. Plant tissue culture: Techiques and experiment. Elsvier publishers, Waltham, United States of America. 3: pp1-63.

Soetan, K, Olaiya, C, Oyewole, O. 2010. The importance of mineral elements for humans, domestic animals and plants: A review. African Journal of Food Science. 4: 200-222.

Sosińska, E, Przybylski, R, Hazendonk, P, Zhao, YY, Curtis, JM. 2013. Characterisation of non-polar dimers formed during thermo-oxidative degradation of β-sitosterol. Food Chemistry. 139: 464-474.

Sperotto, AR, Moura, DJ, Péres, VF, Damasceno, FC, Caramão, EB, Henriques, JA, Saffi, J. 2013. Cytotoxic mechanism of piper gaudichaudianum kunth essential oil and its major compound nerolidol. Food and Chemical Toxicology. 57: 57-68.

Steenkamp, V. 2003. Traditional herbal remedies used by South African women for gynaecological complaints. Journal of Ethnopharmacology. 86: 97-108.

Takeda, A. 2001. Zinc homeostasis and functions of zinc in the brain. Biometals. 14: 343-351.

Tang, S. 2005. Structural and synthetic studies of bioactive natural products. A Phd Thesis submitted to the Department of Chemistry. Virginia Polytechnic Institute and state University. pp56.

Tapiero, H, Townsend, DM, Tew, KD. 2003. Trace elements in human physiology and pathology. Biomedicine & Pharmacotherapy. 57: 386-398.

Taylor, J, Van Staden, J. 2001. Anti-inflammatory activity in extracts prepared from callus cultures of *Eucomis autumnalis*. Plant Growth Regulation. 34: 331-337.

Tesso, H. 2005. Isolation and structure elucidation of natural products from plants. A Phd thesis submitted to the Institute of Organic Chemistry. University of Hamburg. pp1-63.

Thakare, M. 2004. Pharmacological screening of some medicinal plants as antimicrobial and feed additives. An M.Sc thesis submitted to the Department of Chemistry. Virginia Polytechnic Institute and state University. pp1-45.

Thomas, DK. 2007. Isolation and characterizations of antioxidant compounds from *Combretum apiculatum* (sond.) *Subsp apiculatum* leaves extracts. An M.Sc thesis submitted to the Department of Paraclinical Sciences. University of Pretoria. pp 29-35.

Thomas, V. 2004. Sappi tree spotting KwaZulu-Natl and Eastern Cape. Jakana media press, Johannesburg, South Africa. 2: 23.

Thorpe, TA. 1980. Organogenesis *in vitro*: structural, physiological and biochemical aspects. International Review of Cytology Supplement. 11a: 71.

Thorpe, T A. 2007. History of plant tissue culture. Molecular Biotechnology. 37: 169-180.

UNICEF 2012. Improving child nutrition; the achievable imperative for global progress. www.unicef.org/publications/index.html (accessed on 6th Octorber, 2013).

Onibon, VO, Abulude, FO, Lawal, LO. 2012. Nutritional and anti-nutritional composition of some Nigerian fruits. Journal of Food Technology. 5: 120-122.

Van der werff, H, Richter, H. 1996. Toward an improved classification of *Lauraceae*. Annals of the Missouri Botanical Garden. 83: 409-418.

Van wyk, BE, Van Oudtshoorn, B, Gericke, N. 1997. Medicinal plants of South Africa. Briza Publisher, Pretoria. pp304.

Van wyk, BE. 2011. The potential of South African plants in the development of new medicinal products. South African Journal of Botany. 77: 812-829.

Veale, DJ, Furman, KI, Oliver, DW. 1992. South African traditional herbal medicines used during pregnancy and childbirth. Journal of Ethnopharmacology. 36: 185-191.

Verschaeve, L, Kestens, V, Taylor, JL, Elgorashi, EE, Maes, A, Van puyvelde, L, De Kimpe, N, Van staden, J. 2004. Investigation of the antimutagenic effects of selected South African medicinal plant extracts. Toxicology *in vitro*. 18: 29-35.

Vinholes, J, Rudnitskaya, A, Goncalves, P, Martel, F, Coimbra, MA, Rocha, SM. 2013. Hepatoprotection of sesquiterpenoids: a quantitative structure–activity relationship approach. Food Chemistry. 146:78-84.

Wang, X, Wang, W, Zheng, H, Su, Y, Jiang, T, He, Y, She, X. 2009. Efficient asymmetric total syntheses of cryptocarya triacetate, cryptocaryolone, and cryptocaryolone diacetate. Organic Letters. 11: 3136-3138.

Welch, RM, Graham, RD. 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. Journal of Experimental Botany. 55: 353-364.

Wiersum, KF, Dold, AP, Husselman, M, Cocks. 2006. Cultivation of medicinal plants as a tool for biodiversity conservation and poverty alleviation in the amatola region, South Africa. Medicinal and Aromatic Plants. 16:20-30.

Wink, M. 2013. Evolution of secondary metabolites in legumes (*Fabaceae*). South African Journal of Botany. 30: 30-33.

World Health Organisation. 2002. Traditional medicine Strategy 200-2005, Geneva. 1: p7

Xie, X, Kirby, J, Keasling, JD. 2012. Functional characterization of four sesquiterpene synthases from *Ricinus communis* (castor bean). Phytochemistry. 78: 20-28.

Xu, WL, Huang, YB, Qian, JH, Sha, O, Wang, YQ. 2005. Separation and purification of stigmasterol and β-sitosterol from phytosterol mixtures by solvent crystallization method. Separation and Purification Technology. 41:173-178.

Yang, JY. 1998. A flavonoid study of the *Lauraceae*. An M.Sc thesis submitted to the Department of Botany. University of British Columbia. pp1-118.

Yen, KH. 2010. Phytochemical study and biological activities on *Cryptocarya nigra*, *Litsea globularia* and *Uncaria pteropoda*. A Phd thesis submitted to the Faculty of Science and Technology. Universiti Kebangsaan Malaysia. pp1-24.

Žiauka, J, Kuusienė, S, Silininkas, M. 2013. Fast growing aspens in the development of a plant micropropagation system based on plant-produced ethylene action. Biomass and Bioenergy. 53: 20-28.

Zschocke, S, Van staden, J. 2000. *Cryptocarya* species-substitute plants for *Ocotea bullata*? A pharmacological investigation in terms of cyclooxygenase-1 and -2 inhibition. Journal of ethnopharmacology, 71: 473-478.

Appendix