Phytochemical evaluation of *Curtisia dentata* (Burm.f.) C.A.Sm. stem bark and seasonal and geographical region variability

by

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Declaration

I, Anna Susanna van Wyk, ID number 6205290027084, student number 45519560, hereby declare that the thesis, with the title: "**Phytochemical evaluation of *C. dentata* (Burm.f.)C.A. Sm. Stem bark and seasonal and geographic region variability**" which I hereby submit for the degree of Doctor of Philosophy at the University of South Africa, is my own work and has not previously been submitted by me for a degree at this or any other institution.

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ABSTRACT

The stem bark of the protected tree species, *Curtisia dentata* (Burm. f.)C.A.Sm., is one of the most popular plant species harvested and traded at traditional medicine markets in South Africa. The overexploitation of *C. dentata* trees lead to a "Near Threatened" conservation status and the population trend is portrayed as "declining". In the KwaZulu-Natal Province of South Africa, *C. dentata* is completely conservation dependent.

This study is not based on drug discovery or toxicological studies, but on the concern that the stem bark of *C. dentata* trees are harvested, prepared into remedies and consumed as traditional medicine without knowledge regarding the chemical compounds in the stem bark, particularly since the chemical composition of *C. dentata* stem bark was unknown to date. Phytochemical analyses were firstly conducted to determine the chemical composition of *C. dentata* stem bark using various solvents and various analytical methods, and secondly, to determine how seasons and regional separation of *C. dentata* trees affect the chemical profiles of *C. dentata* stem bark from an environmental and nature conservation perspective. Plants are known to contain numerous chemical compounds. Compounds isolated from a particular plant species are therefore not the only compounds present in that species, and although a plant has proven pharmacological properties, they can still cause harm. Previous studies on *C. dentata* aimed at validating the plant species as a medicinal plant by examining extracts of the leaves, twigs and stem bark's potentials against known pathogens and selected cancer cells *in vitro* and *in vivo*, and its anti-inflammatory, antioxidant and antiverotoxic properties. Four pentacyclic triterpenoids and one steroidal compound were also previously isolated from *C. dentata* leaves, however, the leaves are not used in traditional medicines, but were suggested as alternative for stem bark as the harvesting of leaves is less destructive. The efficacy of these compounds as therapeutic agents is, however, compromised by their low solubility in water and thus their potential to penetrate permeating biological membranes. Moreover, *in vitro* toxicity studies distort the picture of its actual potentials on human health as the whole human metabolome and all its processes, including uptake and phase I and phase II biotransformation are not included. *In vivo* toxicity studies on mammalian animal species may also not present a true picture of a chemical or extract's toxic effects on humans as animal metabolisms differ from those of humans. The chemical composition of leaves and stem bark may furthermore also be in contrast to some extent, and therefore chemical compounds were also isolated from *C. dentata* stem bark in this study.

Scientific studies on plant-based medicines generally involve the discovery or identification of compounds that may be beneficial, and which can be exploited in future. Chemical compounds in traditional medicines or other plant-based health products which may cause adverse effects are generally ignored. Moreover, scientific studies that consider that some compounds present in plant extracts may derive from contaminants are equally limited. Traditional plant-based medicines are neither standardized nor regulated in South Africa. Users of traditional plant-based traditional medicines therefore consume uncertain dosages of both beneficial and hazardous substances, as well as contaminants simultaneously. Certain chemical compounds are carcinogens or mutagens or have the ability to accumulate in human tissues. Adverse effects may therefore only manifest after several years of use and will subsequently not be connected to the use of a particular traditional plant-based medicine.

The goal of the thesis is therefore to provide a holistic portrayal of the full spectrum of chemical compounds in extracts of *C. dentata* stem bark and to discuss, where literature is available, the effect(s) each chemical compound may have on human health. Moreover, this thesis investigates variations in chemical composition and concentration in individual trees, seasonal variations and variations in composition and concentrations in the stem bark of *C. dentata* trees from geographically distinct regions. Most unexpected was that not all *C. dentata* stem bark samples contained chemical compounds with known beneficial potentials at each sampling date, and that chemical compounds may be region-specific and also tree-specific, which confirms that plants produce secondary metabolites according to the needs of each individual plant. Additional insight into the chemical composition and concentration of *C. dentata* trees is provided by the distribution profiles of amino acids in *C. dentata* stem bark. Extreme variations within populations and between geographical areas support the need for the cultivation of *C. dentata* trees to ensure sustainable production of homogenous material for chemical homogeneity.

Key Words: *Curtisia dentata*; chemical compound concentrations; chemical composition; Column Chromatography; GC-MS; LC-MS; NMR; regional variability; seasonal variability; secondary metabolites; stem bark; Thin Layer Chromatography.

ACRONYMS AND ABBREVIATIONS

DEFF	: Department of Environment, Forestry and Fisheries
DBH	: diameter at breast height
cGMP independent	: cyclic guanosine monophosphate independent
DCM	: dichloromethane
DNA	: deoxyribonucleic acid
ECHA	: European Chemical Agency
FAO	: Food and Agricultural Organization
GABA	: gamma amino butyric acid
GACP	: Good Agricultural and Collection Practices
GC-MS	: Gas Chromatography – Mass Spectrometry
GMP	: Good Manufacturing Practices
GKD	: glycerol kinase deficiency
HMDB	: Human Metabolome Database
LC-MS	: Liquid Chromatography – Mass Spectrometry
NEMBA	: National Environmental Management: Biodiversity Act
NEMPA	: National Environmental Management: Protected Areas Act
NFA	: National Forestry Act
mm	: millimeter
mM	: milliMol
KZN	: KwaZulu-Natal
NGO	: Non-Governmental Organization
NMR	: Nuclear Magnetic Resonance Spectrometry
OSC	: Orthogonal Signal Correction
OPLS	: Orthogonal Partial Least Squares
PCA	: Principle Component Analysis
ppm	: parts per million
SANBI	: South African National Biodiversity Institute
SANParks	: South African National Parks
TLC	: Thin Layer Chromatography
US EPA	: United States Environmental Protection Agency
US TSCA	: United States Toxic Substances Control Act
WHO	: World Health Organization

WWF : World Wide Fund for Nature

GLOSSARY

Abiotic:	Of or characterized by the absence of life or living organisms (https://www.dictionary.com/browse/abiotic, viewed: 6 May 2020).
Abscission:	Cells at the base of a leaf or fruit dies and tears, enabling the leaf or fruit to fall from the stem with minimum damage (Mauzeth, 1995: G – 1).
Adulteration:	The action of making something poorer in quality by the addition of other substances [adulteration, 1978(1):20].
Androgenic:	An organic compound which promotes development of masculine characteristics (androgen, 1978(1):53)
Aneugenic effects:	A substance's aneugenicity reflects its ability to induce aneuploidy (https://glosbe.com/en/en/aneugens, viewed: 6 May 2020).
Aneuploidy:	One or more extra or missing chromosomes resulting in an unbalanced chromosome complement; any number of chromosomes that is not an exact multiple of the haploid number (https://www.cancer.gov/publications/dictionaries/genetics-dictionary/def/aneuploidy, viewed: 6 May 2020).
Anoxia:	An abnormally low level of oxygen in the body tissues**. (**https://www.dictionary.com/browse/anoxia, viewed: 6 May 2020).
Apoptosis:	Cell death due to a series of molecular steps; Programmed cell death. Apoptosis may be blocked in cancer cells. (https://www.cancer.gov/publications/dictionaries/cancer-terms/def/apoptosis, viewed: 6 May 2020).

Ataxia:	The loss of control of co-ordinated movement due to damage to the sensory nerves [ataxia, 1978(1):88].
Atinociceptive:	The action or process of blocking painful or injurious stimulus by sensory neurons (https://www.definitions.net/definition/antinociceptive, viewed:19 July 2020).
Biodiversity:	The full range of species and biological communities, including their ecosystem interactions and genetic variation within species (Primack, 2012:315).
Biogenesis:	[1] Refers to the descent of living matter from living matter; [2] Hypothetical development of living matter from inanimate substances [biogenesis, 1978(1):147].
Biotic:	Relating to living organisms [biotic, 1978(1):147].
Cambium:	Layer of cells from which annual growth of woody tissue and bark takes place [cambium, 1978(1):226].
Carcinogen:	Cancer-producing substance or agent [carcinogen, 1978(1):243].
Clastogenic:	Capable of causing breakage of chromosomes (https://www.dictionary.com/browse/clastogenic, viewed: 6 May 2020).
Congeneric:	In chemistry it is a compound produced in the same process it produces another, often structurally similar compound (www.thefreedictionary.com/congeneric, viewed: 10 February 2020).
Cytotoxicity:	The quality of a substance to have a toxic effect on cells (https://www.dictionary.com/browse/cytotoxicity, viewed: 6 May 2020).

Denaturation:	The process of changing the nature of properties [denaturation, 1978(1):446].
Encephalopathy:	Any disease of the brain (https://www.dictionary.com/browse/encephalopathy, viewed: 6 May 2020).
Endemic:	A particular plant or animal species only be found in a specific country (Primack, 2012:317).
Enzymes:	A group of complex chemical substances produced by living organisms and promoting specific chemical reactions in the organism [enzymes, 1978(1):560].
Extrinsic:	Operating from without [extrinsic, 1978(1):589].
Genetic diversity:	Genetic variations found within a species (Primack, 2012:318).
Genotoxic:	A toxic agent that damages DNA molecules in genes, causing mutations, tumours etc. (https://www.dictionary.com/browse/genotoxic, viewed: 6 May 2020).
Girdling:	Ring around a tree made by the removal of bark [girdling, 1978(1):709].
Hepatotoxicity:	The capacity of a drug, chemical, or other exposure to produce injury to the liver (https://medicaldictionary.thefreedictionary.com/ hepatotoxicity, viewed: 6 May 2020).
Herbal monographs:	Documents that defines a plant-based drug and provides information that allows for proper identification (WHO, 2013:1-8).

Hormones:	Chemical signals that facilitate intercellular communication in plants. Also called growth regulators (Hopkins and Hüner, 2009:305).
Iatrogenic:	A medical disorder caused by a diagnosis manner or treatment by a physician (https://www.dictionary.com/browse/iatrogenic, viewed 7 May 2018).
Intrinsic:	Inherent; belonging naturally [intrinsic, 1978(1):873].
Lethargy:	Morbid drowsiness; prolonged and unnatural sleep [lethargy, 1978(2):969]
Metabolites:	Metabolites are the end products of cellular regulatory processes and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes (Fiehn, 2002:155).
Metabolome:	The set of small-molecule metabolites in a biological sample. It includes intermediates in primary metabolism, hormones and secondary metabolites (Robinson, 2009:4; Johansson, 2013:11).
Metaphysical:	Branch of philosophy dealing with first principles of things, including such concepts such as being, substance, space, time, identity etc. [metaphysical, 1978(2): 1067-1068].
Mutagen:	An agent which causes genetic mutation (https://www.dictionary.com/browse/mutagen, viewed:7 May 2020).
Mutation:	A change in heritable characteristics caused by a change in genes or chromosomes (https://www.dictionary.com/browse/mutation, viewed: 7 May 2020).

Narcolepsy:	Disease characterized by irresistible attacks of sleep, usually of brief duration [narcolepsy, 1978(2):1120].
Neurotoxin:	A poison which acts on the nervous system (https://www.dictionary.com/browse/neurotoxin, viewed: 7 May 2020).
Oligoanuria:	A condition in which less than 100ml of urine is produced per day (https://glosbe.com/en/en/oligoanuria, viewed: 7 May 2020).
Ontogeny:	History of the development of an individual organism [ontogeny, 1978(2):1177].
Orographic rain:	When moist air is lifted over a mountain range to produce rain (The New Encyclopaedia Britannica. Vol. 4, 1995:1010).
Pharmacodynamics:	The subdivision of pharmacology dealing with the course of action, effect, and breakdown of drugs within the body https://www.dictionary.com/browse/pharmacodynamics, viewed: 7 May 2020).
Pharmacokinetics:	The subdivision of pharmacology concerned with the movement of drugs within the body https://www.dictionary.com/browse/pharmacokinetics, viewed: 7 May 2020).
Pharmacopoeia:	An officially published book containing lists of drugs and directions for use [pharmacopoeia, 1978(2):1263]
Pharmacovigilance:	The relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem (https://www.who.int/medicines/areas/quality_safety/safety_efficacy/pharmvigi/en/, viewed: 7 May 2020).

Phenology:	The science dealing with the influence of climate on the recurrence annual phenomena of animal and plant life e.g. bird migrations or leaf budding https://www.dictionary.com/browse/phenology, viewed: 7 May 2020).
Phloem:	The downward conducting tissue of plants (Hopkins and Hüner, 2009: 498).
Proliferation:	Reproduce itself, grow, by multiplication of elementary parts [proliferation, 1978(2):1352]
Proton:	Fundamental atomic particle forming part of the nucleus, having a single positive electric charge equal and opposite to the electron [proton, 1978(2):1358].
Regeneration:	To reproduce or to renew itself (The New Encyclopaedia Britannica, Vol. 9, 1995:1000) (Viewed: 18 October 2015).
Senescence:	Growing old (www.dictionary.com/browse/senescence, viewed 18 October 2018).
Sustainability:	A method of using a resource that would allow for continued use without environmental degradation (Chapman and Reiss, 1999:310).
Toxicokinetic studies:	The study of the absorption, distribution, metabolism and elimination of hazardous substances by an organism (https://medical-dictionary.thefreedictionary.com/toxicokinetics, viewed: 7 May 2020).
Traditional health practitioners:	Medical practitioners with no formal medical training which are recognized as being proficient in dealing with basic healthcare needs (Fullas, 2007:102).

Transpiration: The vaporization of water from leaves (Hopkins and Hüner, 2009:502).

Xylem: The upward conducting tissue in plants (Hopkins and Hüner, 2009:503).

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CHAPTER 1. INTRODUCTION, BACKGROUND AND RESEARCH

1.1 INTRODUCTION

For centuries, plants were used as the primary source for healthcare (Van Wyk, Van Oudtshoorn and Gericke, 2013:8), however, the year 1805 marked the beginning of modern medicine, as it was the year in which Friedrich Sertürner isolated the chemical compound morphine from the opium plant, *Papaver somniferum*. Since then, numerous chemical compounds have been isolated from plants (Yuan, Ma, Ye and Piao, 2016:2). In 1899, yet another era in modern medicine commenced with the synthesis of the drug Aspirin (Verpoorte, 2000:253). Aspirin has a similar structure as that of salicin, a chemical compound present in the stem bark of *Salix* spp. (Islam and Dantas, 2017:264). The development of techniques to produce synthetic drugs gradually led to a reduction in reliance of natural medicines, however, it was soon realized that the development of synthetic drugs had severe limitations (Yuan *et al.,* 2016:2). The main limiting factor was time, since 12 to 15 years were required for the development of a new drug (Brewer, 2000:59). An additional limitation was that natural products are often too complex for chemists to explore their chemical structure relationships (Brewer, 2000:59), which therefore complicated the synthesis of the compounds.

The limitations experienced in synthetic drug development encouraged scientists in several fields to revert to traditional plant-based medicines. The arguments are firstly, that knowledge regarding plant medicines have been accumulated over many years through trial-and-error experiences, and secondly, that traditional plant-based medicines still remain the most important form of healthcare in many countries (Senanayake, 2015:88; Yuan *et al.*, 2016:1). Furthermore, the combination of secondary chemical defences, the evolutionary development of chemical receptors and the availability of biologically active chemical substances, make plants a rich source of functionally active leads for the development of a drug that may become a candidate for clinical trials (Brewer, 2000:63). There was, however, also a need for improved technology. During the 1800's, microscopic investigations were used for quality assessment of pharmaceutical products as it was the only analytical tools available at the time (Verpoorte, 2000:253). The development of modern technologies such as Thin Layer Chromatography (TLC) and Gas Chromatography (GC) opened new doors in drug discovery (Verpoorte, 2000:253). Additionally, the development of spectrometric

methods such as Mass Spectrometry (MS) and Nuclear Magnetic Resonance spectrometry (NMR) resulted in drastic increases in speed with which metabolites in a metabolome can be identified (Verpoorte, 2000:253; De Hoffmann and Stroobant, 2007:1-489).

From a nature conservationist's point of view, both drug discovery and the traditional use of medicinal plant material are matters of concern, even though it is acknowledged that both are important for human healthcare. The main concern is that large volumes of medicinal plant material are harvested from wild plants annually (Kuipers, 1997:48; Mander, Nthuli, Diederichs, and Mavundla, 2007:194). South African annual demand for traditional medicinal plant material in 2007 amounted to 20 045 tons, of which 5 tons were harvested from cultivated plants, and stem bark constitutes 27% of the total (Mander *et al.*, 2007). More recent bark collection data in South Africa is lacking, and support concerns regarding exploitation of natural plant resources for medicinal use, as an increase in use of medicinal plants are often reported (Grace, Prendergast, Van Staden, and Jäger, 2002:22). In 2017, the global value of medicinal plant material, which include functional foods and health products, were estimated to be one trillion US dollars, and annual growth is estimated to be between 8 and 10 percent (Ahn, 2017:112). The rapid global increase in the human population (Keinan and Clark, 2012:740), therefore results in increased demand, increased harvesting rates and a severe decrease in available plants to exploit. Cultivation has been proposed as a solution for the increasing demand of plants for medicinal use, although wild stocks are preferred over cultivated stocks (Schippmann, Leaman and Cunningham, 2002a:4).

With modern medicine development, drugs from plant sources are isolated, purified and standardized, and the dosages are carefully calculated (Kuipers, 1997:45). To the contrary, traditional medicines do not undergo the same rigorous processes (Nyazema, 1984:80), and is therefore a concern as these medicines are consumed by users without appropriate knowledge on the medicines' chemical constituents. According to the World Health Organization (WHO), raw and semi-processed plant material often contain contaminants which affect the quality of the plant material (WHO, 2007:13-15), and several recent scientific literature reported on the potential genotoxic, mutagenic and/or carcinogenic effects of plants used as food or traditional medicine (Steenkamp, Stewart, Van der Merwe, Zuckerman and Crowther, 2001:51-58; Elgorashi, Taylor, Maes, de Kimpe, Van Staden and Vershaeve, 2002:408-410; Fawole, Finnie and Van Staden, 2009:356-362; Prinsloo, Nogemane and Street, 2018:27-39; Makhuvele, Matshoga, Antonissen, Pieters, Verschaeve and Elgorashi, 2018:89-99).

The aim of this study is to use modern technologies and methods applied in drug discovery to identify potentially beneficial chemical compounds, but also to identify potentially harmful chemical constituents in the stem bark of *Curtisia dentata* (Burm.f.) C.A.Sm. trees. Furthermore, the concentrations of chemical compounds in plant material collected from wild resources vary due to various intrinsic and extrinsic factors (Street, Stirk and van Staden, 2008:706). This study therefore further determines seasonal variations in chemical compound composition and concentrations of *C. dentata* stem bark, and determine whether there are differences in compound composition and concentrations between *C. dentata* trees occurring in Groenkop in the Garden Route National Park (GRNP) and *C. dentata* trees occurring in Nkandla Forest in KwaZulu-Natal (KZN). The goal of the study is therefore to provide a holistic portrayal of the full spectrum of secondary compounds in *C. dentata* stem bark, including variations in individual trees, seasonal variations and variations in composition and concentrations according to geographical regions.

1.2 ASPECTS OF SUSTAINABLE BARK HARVESTING

An ecosystem approach to sustainability acknowledges that humans and ecosystems are interlinked. A society is regarded as sustainable when the utilization of natural resources occur at a rate that increase both human and ecosystem conditions. A sustainable harvest approach considers the following:

- the landscape in which the plants occur,
- both the plant communities and the ecosystems which the plants involved form a part of,
- the plant population sizes, and
- the genetic diversity of the plants (Schippmann et al., 2002b:77).

Cultural traditions prescribe that traditional health practitioners are the only persons to harvest plant material for medicines (Van Andel and Havinga, 2008:1541). Traditional health practitioners, whose actions are guided by high moral values, norms, taboos, and cultural beliefs, unconsciously conserved the plant species harvested from (Williams, Balkwill, and Witkowski, 2000:311; Kambizi and Afolayan, 2006:27; Van Wyk and Prinsloo, 2018:337), since traditional health practitioners generally remove patches of stem bark from only the eastern or western side of a stem (Cunningham, 1988:11, 20). The harvesting methods applied by traditional health practitioners are therefore sustainable. The use of medicinal

plants, however, shifted from subsistence use towards the commercialization of medicinal plant material, and therefore, the frequency with which medicinal plant material are harvested from wild habitats, as well as the amount of plant material harvested, increased substantially (Van Wyk *et al.*, 2013). Poverty and high unemployment rates forced unexperienced people from poor societies into commercial plant collection (Williams *et al.*, 2000:311). Commercial plant collectors thus often collect medicinal plant parts as their sole source of income. The harvesting methods applied by commercial harvesters have been designated the terms "total bark removal", "ring-barking" or "girdling" (Williams *et al.*, 2000:311; Delveaux, Sinsin, Darchambeau and Van Damme, 2009:705), as it includes the abstraction of all matter external to the vascular cambium (Delveaux *et al.*, 2009:705). Girdling is an environmentally destructive method of harvesting, and commercial harvesters do not care to harvest sustainably since there is no financial incentive for sustainable harvesting (Mander, 1998:41), and because sustainable harvesting limits their income. Girdling, most often results in the death of a tree as it affects the upward transportation of organic molecules and water from the root system to the tree canopy, and the downward transportation of photosynthates and growth hormones from the tree canopy to the root system (Moore, 2013:88). The abstraction of phloem (in which downward movement takes place) may further result in bacterial and/or fungal infections. Girdled areas further provide access to the inner sapwood area of a stem, and insects, especially wood borers, can enter trees, cause decay and the subsequent deaths of trees (Shigo, 1985:101). The conservation of tree species is complicated by the fact that traditional health practitioners, particularly those practicing in cities, discontinued the harvesting of plant material themselves, firstly, because it is convenient to purchase plant material from commercial harvesters, and secondly, because cities are most often very far from available resources (Mander, 1998:49).

Sustainable harvesting rates depend on the plant type or life form, such as trees or herbaceous plants, the size of the population harvested from and their growth rates. Smaller populations generally result in low yields, and sustainable rates of harvesting is particularly low if the plant species is an indigenous tree species with slow growth and reproduction rates, such as *C. dentata* trees. Additionally, a connection exists between the plant part collected, the method used and the effect of plant material collection on the plant (Cunningham, 1997:119). The removal of stem bark, the digging of roots and the uprooting of whole plants are generally regarded as destructive since such activities lead to plant death. The gathering of seeds, fruit or leaves are less damaging, however, severe cutting often affect reproduction (Van Andel

and Havinga, 2008:1540). The regeneration rate of stem bark after removal determines how much can be harvested sustainably (Delvaux *et al.*, 2009:704), however, for most tree species, statistics and literature on sustainable rates of harvesting are scant (Van Andel and Havinga, 2008:1540). The only manner in which sustainable rates of bark harvesting from medicinal trees can be determined is through the development of yield systems, which in turn, can only be achieved by conducting controlled harvesting experiments, and by monitoring tree responses to bark harvesting over an extended period of time (Vermeulen 2009:49).

1.3 RISKS OF USING RAW OR SEMI-PROCESSED PLANT MATERIAL AS MEDICINE

Due to both inherent and extrinsic factors, such as genetic variability, plant age, seasonal variation and geographical differences in harvesting sites, medicinal plant trade markets are flooded with medicinal plant material of capricious quality, even though the plant material is collected from the same species (Street *et al.*, 2008:706). The drying processes applied after harvesting and the time period of storage also contribute towards inconsistent quality (Liang, Xie and Chan, 2004:54; Kamboj, 2012:25; Fennel, Light, Sparg, Stafford and Van Staden, 2004b:118).

Local communities argue that medicinal plants are natural and therefore pose no health threat. However, natural does not guarantee safety, since plants produce secondary metabolites as chemical defences to harm, even kill species that pose a threat to their survival. Plant extracts may therefore be hazardous to human health, particularly when consumed (Street *et al.*, 2008:705). Raw and semi-processed plant material may also contain contaminants deriving from insects, bacteria, fungi and several other pathogens (WHO, 2007:13-15). The remedies prepared by traditional health practitioners are not standardized, the dosages are not specified, and do not relate to the age or weight of a patient (Nyazema, 1984:80). A fact that traditional health practitioners should consider is that any chemical substance is a potential toxin. The difference between a toxin and a medicine lies in the dose administered (McGaw, Elgorashi, and Eloff, 2014:223; Knöss, 2017:4). The Traditional Health Practitioners Act 22 of 2007 (South Africa, 2007a) regulates traditional medical practices, yet, medicinal formulations prepared and vended by traditional health practitioners are not registered in compliance to the Medicines and Related Substances Control Act (Act 101 of 1965) (South

Africa, 1965) and are thus sold without any authoritative control (Le Roux-Kemp, 2010:281, 282).

1.4 RESEARCH PROBLEM, QUESTIONS AND OBJECTIVES

The bark of numerous *C. dentata* trees are required to meet demand. Despite the environmental laws which prohibits the harvesting of bark from protected tree species, the harvesting of stem bark from *C. dentata* trees continues. Even more bark will be harvested to meet future demand due to the national and global population growth rate, the general unaffordability of western medicines by people in poor communities, the renewed interest in traditional medicines and the cultural preference and acceptability of traditional medicines. With constantly declining *C. dentata* populations and an interminably increasing human population, maintaining supply is a challenge for both medicinal plant collectors and traders of medicinal plant material, and has become a serious concern for nature conservationists. Cultivation of *C. dentata* trees should therefore become a priority issue. Cultivation will ensure the survival of the species and secure plant material for future generations.

Recent literature on the volumes of *C. dentata* stem bark harvested is extremely limited, therefore, three representatives from different branches of the Department of Environment, Forestry and Fisheries (DEFF) (previously The Department of Agriculture, Forestry and Fisheries – DAFF) were approached, as application for licenses to collect plant material from protected tree species need to be submitted to DEFF. None of the representatives could provide statistics regarding the volumes of stem bark harvested from trees, nor do they keep record of the number of traditional health practitioners or commercial harvesters that are in possession of licenses to collect bark from protected tree species, but stated that the numbers are very low. The representatives, however, stated that many traditional health practitioners are aware that they need to acquire licenses to collect plant material from protected tree species, but do not attempt to obtain it. The question of whether traders of medicinal plant material, such as wholesalers and street vendors, who do not necessarily collect plant material themselves, need licenses to be in possession of stem bark harvested from protected tree species for trading purposes, could not be answered.

Due to the limited information regarding the chemical composition of *C. dentata* stem bark, user knowledge on what chemical compounds are being consumed when using *C. dentata* stem bark as traditional medicine is dangerously low. Point 8.1e of the Medicines and Related

Substances Act 101 of 1965 (South Africa, 1965), states that: "*labels of medicines intended for administration to humans must contain the approved name of each active ingredient and that the quantity thereof must be contained in a dosage unit, or per suitable mass or volume or unit*,….", and point 8.1k of the Act states that: "*the labels must include the recommended dosage of the medicine*". Traditional plant material is, however, sold unpackaged and unlabeled in open street markets and muthi shops (Mander *et al.*, 2007:192-193; Pers. Obs., 18 September 2019). Moreover, due to the lack of standardization and control of traditional medicines, extracts from raw or semi-processed plant material result in inconsistent dosage prescriptions, which may affect the medicines' potency, efficacy and safety. Furthermore, the risk of combining plant material of several plant species may result in adverse health effects in humans, since a multitude of both potentially beneficial and potentially harmful chemical substances, including contaminants, are consumed simultaneously. The interactions of the different chemical compounds are unknown, and uptake, phase I and phase II biotransformation in the human body are also unknown, and therefore raise serious health concerns. Issues regarding safety, quality assessment and regulatory control of medicinal plant products should be priority issues for health authorities, national government and the general public.

Scientific literature on the full spectrum of chemical compounds present in the stem bark of *C. dentata* trees is extremely limited. Chemical compounds previously isolated from *C. dentata*, were isolated from the leaves, and only the classes of compounds were mentioned in studies pertaining to *C. dentata* stem bark, without specifying the compounds in each class. Even less is known about the concentrations of bioactive compounds in *C. dentata* stem bark, how much concentrations vary seasonally and how much it varies in trees from different South African regions. Furthermore, literature on *C. dentata* trees' potential to cause adverse effects are equally limited.

The research questions for this study are:

1. Are there chemical compounds in *C. dentata* stem bark with beneficial potentials?
2. Are there chemical compounds in the stem bark of *C. dentata* that are potentially hazardous to human health?
3. Do the chemical compound composition and concentrations in *C. dentata* stem bark vary seasonally?

4. Are there significant differences in composition and concentrations of chemical compounds in the stem bark of *C. dentata* trees occurring in KZN and *C. dentata* trees occurring in the southern Cape forest area?

The objectives of the study are:

1. To identify both potential beneficial and potential harmful compounds in *C. dentata* trees by means of GC-MS analyses of the crude extracts obtained from the stem bark,
2. To isolate and identify chemical compounds in the stem bark of *C. dentata* trees by means of column chromatography, TLC, NMR and GC-MS through the separation of compounds, the elucidation of their chemical structures and the determination of their exact masses,
3. To conduct ^{1}H NMR analysis to determine seasonal changes in *C. dentata* metabolic profiles,
4. To conduct q-NMR analyses to determine seasonal variation of chemical compound content in *C. dentata* stem bark,
5. To perform q-NMR analyses to determine regional differences in chemical compound concentrations in *C. dentata* stem bark of trees occurring in KZN and of trees occurring the southern Cape forest area.
6. To compare the chemical profiles of individual *C. dentata* trees occurring at the same location, as well as the regional chemical composition profiles of *C. dentata* trees from the southern Cape and *C. dentata* trees from KZN by means of GC-MS and LC-MS analyses,
7. To compare both the potential beneficial and potential harmful compounds' spectral data, chromatograms and chemical structures with compounds recorded in the Human Metabolome Database (HMDB), PubChem, and other databases,
8. To validate results through statistical analyses in order to interpret data.

1.5 HYPOTHESES

The hypotheses relevant to the research questions are as follows:

1.5.1 Hypothesis A

H_0 – *Curtisia dentata* tree stem bark do not contain chemical compounds beneficial to human health.

H_1 – *Curtisia dentata* tree stem bark do contain chemical compounds beneficial to human health.

1.5.2 Hypothesis B

H_0 – *Curtisia dentata* tree stem bark do not contain chemical compounds that are hazardous to human health.

H_1 – *Curtisia dentata* tree stem bark do contain chemical compounds that are hazardous to human health.

1.5.3 Hypothesis C

H_0 – There is no seasonal variation in chemical compound composition and concentrations in *C. dentata* stem bark.

H_1 – There is seasonal variation in chemical compound composition and concentrations in *C. dentata* stem bark.

1.5.4 Hypothesis D

H_0 – The chemical composition in the stem bark of *C. dentata* trees do not vary between individual trees or between geographical regions.

H_1 – The chemical composition in the stem bark of *C. dentata* trees vary between individual trees and between geographical regions.

1.6 LIMITATIONS, DELIMITATIONS AND ASSUMPTIONS

Limitations of the study include:

- Bark samples of *C. dentata* occurring in the southern Cape forest area had been collected for a previous study (van Wyk, 2017). The remaining sample material was stowed in a -80º C freezer to prevent the degradation of the metabolites, and were also used for this research study. The limitations imposed by SANParks, the managing authority of the GRNP, included:
    - The number of individual trees to be used

    - The diameters at breast height (DBH),
    - The sites in which research may be conducted, and
    - The specific trees on which research could be conducted (Appendix A). Trees were limited to those that were subjected to previous sustainable harvesting experiments.
- No limitations, except for the research site, were imposed on the researcher by Ezemvelo KZN Wildlife.
- Due the limitations imposed on the researcher by SANParks, and because this is a comparative study, the same limitations regarding the number of trees used and diameters at breast height (DBH) (where possible) were maintained.

Delimitations included:

- Research on *C. dentata* did not cover the whole population of trees occurring in the southern Cape forest area as a single, specific research site was allocated to the researcher by SANParks and specific boundaries were set.
- Similarly, research on *C. dentata* in KZN did not represent the whole population of trees occurring in KZN as a single, specific research site was allocated to the researcher by Ezemvelo KZN Wildlife.
- No tree in KZN was girdled.
- *Ocotea bullata* was initially included in the study, however, two weeks prior to the commencement of research, and after the permit from KZN and the license from DEFF was issued, the *O. bullata* trees had to be excluded from this study due to the lack of sufficient numbers of trees at the location allocated to the researcher by KZN Wildlife (Appendix B).
- This study was perceived from a nature conservationist's perspective. Therefore, the aim of this study does not include screening the plant material for biological activity or to discover any chemical compound that can be exploited for future drug research.
- Aqueous extracts of *C. dentata* stem bark showed no toxicity (Wintola and Afolayan, 2018:97-104). Toxicity studies using plant material extracted with other solvents were also not conducted in this study.
- There is a lack of reliable biological assays to determine the quality of the extract. Pharmacological studies are also currently very general, however, this study also did not aim to conduct a pharmacological investigation.

Assumptions:

It is assumed that:

- The trees allocated in each research site are representative of the populations in the specific areas of stem bark sample collection.
- The chemical compounds extracted from the purchased *C. dentata* stem bark is representative of the chemical content of *C. dentata* stem bark in general, irrespective of the trees' locations.
- Different solvents and different analytical methods employed yield different chemical profiles.

1.7 RESEARCH JUSTIFICATION

In South Africa, initial studies on medicinal plants centered around user needs in terms of plant species and products used (Maroyi, 2013:1-18; Cheikhyoussef, Shapi, Matengu and Ashekele, 2011:1-11; Fullas, 2007:102-112), and the impacts of harvesting on resource stocks (Botha, Witkowski and Shackleton, 2004b:1-18). As medicinal plants became increasingly commercialized, research emphasized the commercial value and the marketing of medicinal plants (Mander, 1998:1-151; Krog, Falcão and Smith Olsen,2006:1-39; Mander *et al.*, 2007:189-196), as well as on sustainable harvesting aspects of medicinal plant material and forest management (Van Andel and Havinga, 2008:1540-1545; Vermeulen 2009:1-197).

Recent studies on *C. dentata* were driven by the need to justify the use of the species as a medicine by testing the crude extracts of *C. dentata* plant material against known pathogens (McGaw, Jäger and Van Staden, 2000:247-263; Elgorashi, Taylor, Maes, de Kimpe, Van Staden and Vershaeve, 2003:195-207; Shai, 2007:1-188; Doughari, Ndakidemi, Human and Benade 2012:1041-1050; Oyedemi, Oyedemi, Arowosegbe and Afolayan, 2012:6189-6203; Fadipe, Mongalo and Opoku, 2015:971-983; Fadipe, Mongalo, Opoku, Dikhoba and Makhafola, 2017:1-6). McGaw *et al*. (2000:255), for example, concluded that disc diffusion assays and dilution assays of *C. dentata* stem bark exhibited limited activity against the Gram positive bacterium *Bacillus subtilus*, and no activity against the Gram positive bacterium *Staphylococcus aureus*, or the Gram negative bacteria *Escherichia coli* and *Klebsiella pneumonia*. *Curtisia dentata* extracts were also not highlighted for their anti-helminthic or anti-amoebic activity (McGaw *et al*. 2000:247-263). On the other hand, Doughari *et al*. 2012:

1041-1050) conducted general tests for the presence of classes of compounds in *C. dentata* root bark, stem bark and leaves, and conducted antioxidant, antibacterial and antiverotoxic tests on 112 stock cultures of test bacteria, and concluded that the mixture of classes of compounds possessed antimicrobial, antioxidant and antiverotoxic activity against various strains of *Escherichia coli* and *Acinetobacter* spp. None of these studies included lists of the chemical compounds present in *C. dentata*, and all studies generally exclude the fact that plants also contain compounds that may be hazardous to human health. Currently, chemical compounds with beneficial potentials in traditional medicines are not isolated from the potential harmful compounds and/or contaminants. When using raw or semi-processed plant material for the holistic treatment of ailments, both the potential beneficial and potential harmful compounds, including contaminants, are therefore consumed. Furthermore, the seasonal variability of chemical compound concentrations in plants, which affects dosage application, as well as the regional variability thereof in plants of the same species, have been neglected. Furthermore, the uncontrolled and unregistered sales of traditional plant-based medicines, the reported number of people dying annually after the use of traditional medicines and the limited information that could be accumulated on compound concentrations in medicinal plants in general, motivated research in this field.

1.8 IDENTIFICATION OF THE DEFICIT IN EXISTING KNOWLEDGE

String searches on the Google Scholar and Science Direct search engines revealed that scientific studies on human ailments using traditional medicine (60 100 hits), diarrhoeal medicines used in South African folk medicine (10 400 000 hits), poisoning due to the use of traditional remedies (22 800 000 hits) and screening of South African plants used in traditional medicines (68 600 hits) are abundant, however, many of which are duplicates. The opposite is true for the quantification of chemical compounds in South African medicinal plant material.

Additional Google Scholar and Science Direct string searches using search terms such as "*Curtisia dentata*" and "bioactive compounds" resulted in 1140 hits, however, the majority of literature related to medicinal plants in general and were not studies relating to only *C. dentata.* In one study, four compounds in *C. dentata* leaves were isolated and tested against *Candida albicans* Shai, 2007:1-188). Another study on the leaves isolated an additional compound: *β*-sitosterol (Fadipe, *et al.*, 2017:1-6). A few studies related to *C. dentata* activity

against parasitic and free-living nematodes, as well as the potential anti-microbial, anti-fungal, anti-verotoxic, anti-bacterial, anti-helminthic, anti-amoebic and antioxidant properties of *C. dentata* extracts have been reported (McGaw *et al.*, 2000:247-263; Elgorashi *et al.*, 2003:195-207; Shai, 2007:1-188; Doughari *et al.*, 2012:1041-1050; Oyedemi *et al.*, 2012:6189-6203; Fadipe *et al.*, 2015:971-983; Fadipe *et al.*, 2017:1-6. None of these studies, however, specified or quantified the specific chemical compounds responsible for these properties and activities. Two additional studies on the phytochemical analysis of *C. dentata* stem bark report on the classes of compounds present but do neither specify nor quantify the compounds in each class of compounds. Compounds that may be hazardous to human health were excluded in all studies.

1.9 SIGNIFICANCE OF THE STUDY

The significance of this study includes the following:

- Identification of the potentially beneficial as well as potentially hazardous chemical compounds in the stem bark of *C. dentata* trees.
- The identification of the potential beneficial bioactive compounds may lead to the formulation and production of safe and efficient synthetic medicines.
- The identification of potential harmful compounds may serve as motivation for government to insist on the fractionation and isolation, purification and standardization of beneficial compounds in plant-based traditional medicines.
- Provide knowledge regarding the seasonal variations of chemical compound concentrations occurring in *C. dentata* trees, as well as its variability in individual trees.
- Provide knowledge on the differences in concentrations of chemical compounds present in the bark of *C. dentata* trees occurring in KZN and of those occurring in the southern Cape forest area.
- Result in recommendations regarding the appropriate harvesting times of *C. dentata* bark in the different geographical regions of South Africa.
- Could be used to encourage government to finalize and promulgate legislation regarding South African traditional medicines, and to enforce the registration of traditional medicines in compliance to the Medicines and Related Substances Control Act 101 of 1965 (South Africa, 1965).

- Encourage government and NGO's, and with WHO and WWF as possible funding coordinators, in planning and implementing initiatives to provide upcoming farmers (possibly current commercial harvesters) with suitable state-owned land in all Provinces of South Africa (preferably adjacent to existing forests as trees often have specific habitat requirements) to cultivate large numbers of traditionally important and endangered medicinal plant species for sufficient supply of plant material for the medicinal plant trade market. Monocultures should, however, be prevented. Intercropping or taungya systems could possibly be implemented and could provide such farmers with a steady income while newly planted traditional medicinal plants reach maturity.
- Encourage the construction of traditional medicinal plant processing facilities where cultivated traditional medicinal plant material could be dried and processed hygienically. The appropriately processed cultivated traditional medicinal plant material should also be packaged with appropriate labelling for hygienic presentation in the trade market. Packaging could increase shelf life, prevent contamination by external sources such as motor vehicle emissions, dirt, bacteria and other impurities, and prevent the addition of adulterants which may be harmful to human health.
- Such initiatives would not only reduce harvesting pressure on wild populations, reduce extinction probability, increase tree numbers and maintain genetic variability, but would also provide job opportunities to many people from rural communities.

1.10 ETHICAL CONSIDERATIONS

1. Since the GRNP is managed by SANParks, permission to work on endangered tree species had to be obtained from SANParks. Formal approval to conduct research on these trees for a previous study was obtained from SANParks (Appendix C). A permit to collect plant material from the forest was issued (Appendix D).
2. An ethical clearance document (Ref 2015/CAES/070) was received from UNISA for the previous study (Appendix E). Point 5 in the ethical clearance document which stated that the researcher must provide periodic feedback was adhered to. Reports to UNISA's CAES Research Ethics Review Committee were submitted in January 2016, July 2016 and January 2017 (Appendix F).

3. An ethical clearance document (Ref 2018/CAES/114) was received from UNISA for the current study (Appendix G). Ethical clearance was granted for between 11/09/2018 and 31/08/2019 (Appendix H). A research progress reports was submitted to the ethics committee in compliance to point 7 of the ethical clearance document, after which ethical clearance was extended to 2020.
4. An application for permission to work on endangered species in KZN Nature Reserves, as well as an application for a permit has been submitted to Ezemvelo KZN Wildlife. A confirmation letter of project registration was received (Appendix H), as well as the permit to collect stem bark samples within a KZN Wildlife reserve (Appendix I).
5. Point 6 on Appendix I was adhered to as two voucher specimens were submitted to the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria (PRU 125493 and PRU 125494).
6. Since *C. dentata* is on the DEFF list of protected tree species, a license to collect bark samples from protected tree species in KZN was obtained (Appendix J).
7. Although this research study is purely for academic purposes, the study triggers Bioprospecting, Access and Benefit-Sharing (BABS) regulations. A notice for discovery phase bioprospecting has thus been completed and submitted to the DEA and acknowledgement has been received (Appendix K).
8. The study was conducted with strict adherence to the College of Agricultural and Environmental Sciences Research and Higher Degrees of the University of South Africa Ethics Committee's Research Ethics document.

1.11 THESIS STRUCTURE

The thesis consists of eight chapters. The introductory chapter precedes the chapter comprising the literature review. The third chapter identifies compounds in the hexane, dichloromethane (DCM) and ethanol crude extracts of *C. dentata* stem bark, and reports on the individual compounds' potentially beneficial or harmful effects. In the fourth chapter, the focus shifts to the chemical compounds isolated from the hexane crude extracts of *C. dentata* stem bark. The fifth chapter reports on the seasonal and regional variability of compounds in the stem bark of *C. dentata* trees occurring in KZN and the southern Cape, whereas the sixth chapter focuses on chemical compound composition variability in the stem bark of individual

C. dentata trees, and compares the chemical profiles of *C. dentata* trees occurring in KZN and in the GRNP. The seventh chapter discusses the seasonal and regional variability of amino acid composition and distribution, as well as variability of amino acid distribution in individual trees. Chapter 8 is the concluding chapter.

Chapter 1: The background provides an introduction, followed by an overview of the sustainability of bark harvesting. The background continues by providing an overview of the risks involved when using raw or semi-processed medicinal plant material. The first chapter also describes the research problem, questions and objectives and states the hypotheses, which is followed by the limitations, delimitations and assumptions. The research is justified, the gap in the existing knowledge is identified and the significance of the study is highlighted. Ethical considerations are pointed out, and finally, the first chapter provides a thesis structure and concluding remarks.

Chapter 2: This chapter provides a thorough literature review on the following: The species used for the study and the research sites from which *C. dentata* stem bark samples were collected. The chapter further reviews the traditional importance of *C. dentata* as a medicine, the volumes of *C. dentata* bark harvested annually, the ailments treated, the chemical compounds previously isolated from the leaves, including their pharmacological significance, and the classes of compounds previously identified in *C. dentata* stem bark. South African environmental legislation, legislation regarding traditional medicines in South Africa and the challenges regarding the legal framework and the regulation of South African traditional medicine, as well as legislation compliance in South Africa are included in the review. This is followed by the harmful effects associated with the use of plant-based medicines and the factors affecting variations in secondary metabolite composition and concentrations, which include examples of abiotic and biotic effects with respect to secondary metabolite production. Challenges and suggestions regarding medicinal plant cultivation are reviewed and an overview on the analytical methods used for this study is provided. A concluding statement completes this chapter.

Chapter 3: The introduction precedes a comprehensive description of the methodology used to extract and identify the chemical compounds in *C. dentata* stem bark purchased at a traditional medicine trade market. This chapter further provides the results obtained from the analytical methods applied (GC-MS), which includes lists of the full spectrum of compounds present in the hexane, DCM and ethanol crude extracts. An overview of the classes of compounds in plant essential oils and the classes of compounds' therapeutic potentials is

discussed, after which the potentials of the compounds identified in the purchased *C. dentata* stem bark is listed, classified into: 1) compounds with possible beneficial potentials, 2) compounds which may be a potential risk to human health and/or which are environmental hazards and, 3) possible contaminants in *C. dentata* stem bark, and discussed. A conclusion completes this chapter.

In **Chapter 4**, the focus shifts to the separation and isolation of compounds in the hexane crude extracts of *C. dentata* stem bark. The introduction is therefore followed by the methods applied to separate and isolate chemical compounds from the hexane crude extracts. The results obtained are provided, which include the TLC plates as visualized under shortwave and longwave UV exposure and the ^{1}H NMR and ^{13}C NMR spectra. The fourth chapter continues by characterizing the elucidated structures obtained from the ^{1}H NMR and ^{13}C NMR analysis.

Chapter 5: The introduction is followed by the methods applied to process, extract compounds and analyze (^{1} H NMR and ^{q}NMR) the *C. dentata* stem bark collected from both the KZN and southern Cape research sites in order to determine seasonal and geographical region variability of chemical compounds in *C. dentata* stem bark, including concentration variability, as well as the regional variations of chemical compound concentrations in individual trees. The fifth chapter continues by providing a complete report on the results obtained, and by discussing the effects of seasons and geographical region variability on the metabolite profiles of *C. dentata* trees, as well as its implications for use as a traditional medicine. A conclusion summarizes the findings presented in this chapter.

Chapter 6: This chapter compares the chemical profiles of *C. dentata* trees growing in KZN and *C. dentata* trees occurring in the GRNP. The introduction is therefore followed by a methodology section in which the methods applied to process, extract and analyze (GC-MS) the plant material collected from the southern Cape and KZN are described. The sixth chapter continues by exhibiting the results obtained, after which the similarities and differences in chemical compound composition between *C. dentata* trees occurring in KZN and *C. dentata* trees occurring in the Western Cape Province are highlighted and discussed. The discussion also includes the similarities and differences in chemical composition of individual trees growing at the same location, and explain the possible causes of these differences. The implications for the use of *C. dentata* stem bark from different trees and different regions are briefly highlighted, after which the chapter is concluded.

Chapter 7: The amino acid profiles of *C. dentata* stem bark of trees from the Western Cape and KwaZulu-Natal Provinces respectively, are compared in this chapter. The introduction is therefore followed by the methodologies applied to extract amino acids (LC-MS), after which the results obtained is displayed. The variations in composition and distribution of amino acids in individual trees, and the seasonal and regional variability of amino acids are discussed, as well as the use of amino acids as therapeutic drugs and the amino acids with potential toxic effects, after which the chapter is concluded.

Chapter 8 is the concluding chapter and therefore summarizes all the findings, discusses the achievement of objectives and relate the achievements to the hypotheses stated in the first chapter. The last Chapter further concludes the study, provides suggestions on burning issues such as the cultivation of medicinal trees, the harvesting of *C. dentata* stem bark and the improvement of the current permit system, and also provides recommendations for future research.

1.12 CONCLUDING REMARKS

Vast amounts of *C. dentata* stem bark is harvested annually to satisfy current demand in the medicinal plant trade markets. More bark will be needed to meet future demand due to the human population growth rate, the general unaffordability of western medicines by people in poor communities, the renewed interest in traditional medicines and health products, and the cultural preferences and acceptability of traditional medicines. *Curtisia dentata* is a protected tree species, however, the harvesting of the stem bark nevertheless continues due to poor law enforcement and people's general perception that trees are open-access resources. The overexploitation of stem bark from wild resources is a deeply concerning challenge for the conservation of nature. The effects of medicinal bark harvesting are devastating, with diminishing medicinal tree populations, narrowing genetic diversity and a loss of biodiversity being the major concerns. With incessantly declining *C. dentata* populations and a constantly increasing human population, maintaining supply is a challenge. The cultivation of medicinal trees is therefore essential.

Considering that little is known about the chemical compounds in *C. dentata* bark, the use of *C. dentata* stem bark may pose health risks as trees may contain both beneficial and harmful compounds. Traditional plant-based medicines are neither standardized nor regulated and are therefore vended unpackaged and unlabeled at open street markets and muthi shops without

any authoritative control. Variations in bioactive compound composition and concentrations in individual trees, seasonal variations and regional variations of harvesting sites may result in the prescription and administration of dosages that are inconsistent, thereby increasing the risk of over- or under dosage.

As one of the most valued traditional medicinal tree species, *C. dentata* stem bark therefore needs to be analyzed and the potential beneficial and potential hazardous chemical compounds identified. Chemical evaluation is one of the key factors for the standardization of medicinal plant material. Furthermore, the identified beneficial compounds in *C. dentata* stem bark may be synthesized to ensure efficacy and safety, and to prevent the extinction of the species. Alternatively, *C. dentata* stem bark should at least undergo the processes of isolation and purification of beneficial compounds, which would firstly exclude both contaminants and potentially harmful compounds, and secondly ensure consumer safety. This can, however, only be achieved through the cultivation of the species, as dwindling wild *C. dentata* populations would not support such activities in a sustainable manner.

CHAPTER 2. LITERATURE REVIEW

2.1 INTRODUCTION

Overexploitation of plant species for medicinal use is a great concern for the conservation of nature. The overexploitation of medicinal plant species is caused by increases in the human population, urbanization, land use issues which entail the competition between natural vegetation and other equally necessary land uses such as forestry, agriculture, urbanization, infrastructure development, and also by decreases in the area of distribution of natural vegetation (Cunningham 1997:117; Sibly and Hone, 2002:1; Primack, 2012:92). The increase in the commercialization of traditional medicine, an increase in demand for medicinal plant material in the local and global trade markets, the lack of appropriate legislation, as well as the failure to enforce laws put further pressure on medicinal plant resources. Poverty and high unemployment rates further cause increases in the number of commercial harvesters (Maundu, Kariuki and Eyog-Matig, 2006:51-52). Furthermore, the low rates paid for plant material force collectors to harvest large quantities of medicinal plant material to earn a reasonable salary (Monakisi, 2007:17). Increasing scarcity of plant products, including stem bark, from overexploited medicinal plant species lead to severe price increases, which in turn stimulates higher collection rates (Cunningham, 1993:16). Commercial harvesters in particular, often use unsustainable and damage-causing methods of harvesting and also put unjustifiable pressure on preferred species. Slow growth of indigenous tree species prevents the rapid replacement of depleted resources and invasive plants from countries such as Australia and the US are a threat to South African indigenous plant diversity (Maundu *et al.*, 2006:51-52). Overgrazing and trampling by animals bred for human consumption puts additional pressure on plants (Sibly and Hone, 2002:1; Primack, 2012:92). The effects of overexploitation include local and global plant extinctions, reduced genetic diversity and dwindling regeneration potential (Maundu *et al.*, 2006:51-52). In South Africa, non-sustainable harvesting rates were reported by several authors (Mander 1998; Botha, Witkowski and Shackleton, 2004a:1675; Kambizi and Afolayan 2006:26; Van Andel and Havinga 2008:1541). Mander (1998:7, 11, 53) further stated that increased pressure on habitats and ecosystems resulted in several local extinctions.

The harvesting of stem bark from *C. dentata* and other South African protected indigenous tree species continues despite comprehensive environmental laws. The second chapter of this

thesis therefore describes the species used for the study and include descriptions of the sites from which *C. dentata* stem bark samples were collected. The second chapter continues by reviewing the traditional importance of *C. dentata*, the volumes of *C. dentata* stem bark harvested annually, the ailments treated, the chemical compounds isolated from *C. dentata* leaves and the classes of compounds identified in stem bark. The second chapter further reviews issues pertaining to South African environmental legislation, legislation regarding traditional medicines in South Africa, the challenges regarding the legal framework and the regulation of traditional medicines in South Africa, as well as legislation compliance. This is followed by the adverse effects associated with the use of traditional medicines and the factors affecting variations in secondary metabolite composition and concentrations, with reference to the abiotic and biotic effects of secondary metabolite production. The cultivation of plants used medicinally with regard to the challenges encountered and the recommendations described in literature follows, after which an overview of the analytical methods used for the study is provided. A concluding statement finalizes this chapter.

2.2 THE SPECIES USED FOR THE STUDY

Curtisia dentata trees (Curtisiaceae – formerly Cornaceae), commonly known as Assegai trees, are attractive, evergreen medium to large trees reaching heights between 10 and 20 m. These trees generally occur in coastal and montane forest (Van Wyk and Gericke, 2007:292; Van Wyk *et al.*, 2013:110). The bark of younger trees is brown and smooth, but in older trees, the bark becomes dark brown to black, coarse and heavily fissured. The leaves are leathery and have shiny dark green upper surfaces. The lower surfaces of the leaves are characterized by prominent venation and dense wooly grey-brownish hairs. The leaf margins are sharply toothed (Figure 2.1) (Van Wyk and Gericke, 2007:292; Van Wyk *et al.,* 2013:110).

Figure 2.1: The sharply toothed and explicitly veined leaves of *C. dentata* trees (www.pza.sanbi.org/curtisia-dentata) (Accessed: 20 February 2019).

Flowers are terminally located, are cream-coloured and inconspicuous. Fruit (Figure 2.2), is a greenish-white berry of approximately 10 mm in size (Van Wyk and Gericke, 2007:292; van Wyk *et al.*, 2013:110). *Curtisia dentata* trees have no specific flowering time (Van Wyk and Van Wyk, 2009:15), and is therefore described as being between spring and autumn (Van Wyk and Van Wyk, 2009:292). .

Figure 2.2: The berry-type fruits of *C. dentata* trees (www.pza.sanbi.org/curtisia-dentata) (Accessed: 20 February 2019).

Curtisia dentata trees are threatened by bark collection for the medicinal plant markets. This threat follows after suffering historical declines resulting from timber harvesting, particularly in the forests of the GRNP. The South African National Biodiversity Institute (SANBI) marks *C. dentata* stem bark as one of the most frequently traded bark products in South African medicinal plant trade markets (SANBI, 2017). *Curtisia dentata* is not an endemic species as *C. dentata* trees also occur naturally in Zimbabwe and Mozambique. In South Africa, they

occur in six of the nine Provinces, i.e. the Limpopo, Mpumalanga, Free State, KZN, Eastern Cape and Western Cape Provinces. *Curtisia dentata* is on the SANBI Red List of plants where a 'Near Threatened' status is assigned. The *C. dentata* population trend is noted as declining (SANBI, 2017). The species is further on the DEFF list of protected tree species (notice 734 of 2011) (South Africa, 2011), which is a subsection of the National Forest Act (Act 84 of 1998) (South Africa, 1998).

2.3 TRADITIONAL IMPORTANCE OF *Curtisia dentata*, VOLUMES OF *C. dentata* STEM BARK HARVESTED, AILMENTS TREATED AND COMPOUNDS PREVIOUSLY ISOLATED

Despite the fact that *C. dentata* is listed in both the South African Red List of Plants and the DEFF list of protected species, the stem bark of *C. dentata* is ranked 6th on the top 10 list of medicinal plant material harvested, traded and used in both KZN and the Eastern Cape (Dold and Cocks, 2002:594) (Table 2.1). The Provincial differences in the ranks of plants traded is most probably due to ethnic differences in health care practices (Dold and Cocks, 2002:594).

Table 2.1: The 10 top-ranked medicinal plant species traded and used in three of South Africa's Provinces (Dold and Cocks, 2002: 594).

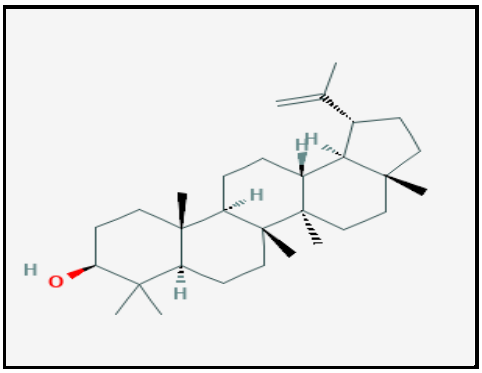
Rank	Eastern Cape	KwaZulu-Natal	Mpumalanga
1	*Hypoxis hemerocallidea*	*Merwilla plumbea*	*Alepidea amatymbica*
2	*Ilex mitis*	*Alepidea amatymbica*	*Warburgia salutaris*
3	*Rhoicissus digitata*	*Ocotea bullata*	*Acridocarpus natalitius*
4	*Rubia petiolaris*	*Warburgia salutaris*	*Siphonochilus aethiopicus*
5	*Helichrysum odoratissimum*	*Eucomis autumnalis*	*Vachellia xantophloea*
6	*Curtisia dentata*	*Curtisia dentata*	*Terminalia sericia*
7	*Protorhus longifolia*	*Haworthia limifolia*	*Bersama tysoniana*
8	*Bulbine latifolia*	*Bowiea volubilis*	*Maesa lanceolata*
9	*Haworthia attenuata*	*Siphonochilus aethiopicus*	*Cephelaria humulus*
10	*Myrsine melanophloeos* (formerly known as *Rapanea melanophloeos)*	*Secamone gerrardii*	*Turraea floribunda*

Literature regarding the volumes of stem bark harvested from medicinal tree species is extremely limited. The only available literature on the volumes of *C. dentata* stem bark harvested for the medicinal plant trade date back to 1993 and 2002, and statistics are available only for one Province in South Africa, namely KZN. Cunningham (1993:17) reported that

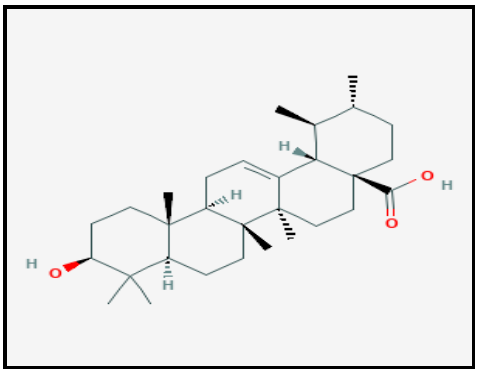
197 bags of *C. dentata* stem bark, with each bag containing 50 Kg of stem bark, were collected and sold in KZN alone in 1993. This amounts to 9.85 tons in one year. Nine years later, Grace *et al.,* (2002:23) announced an increase in the volume of *C. dentata* stem bark collected and traded in KZN, which was 23.9 tons. It is thus an increase of 140% over this nine-year period, which is a clear indication of the extent of increase in demand.

The remedies or tonics traditional health practitioners prepare from *C. dentata* stem bark are generally not prepared as a single-species medicine. The stem bark is used as part of a mixture of plant parts from several other medicinal plant species in complex formulas (van Wyk, 2013:110), and is used for the treatment of stomach ailments, diarrhoea and sexually transmitted diseases (Shai, 2007:26; Venter and Venter, 2012:22; Van Wyk *et al.*, 2013:110; Fadipe *et al.*, 2015: 976). The medicine prepared from *C. dentata* stem bark is further used as an aphrodisiac and to rid blood of potential impurities. It also has a use as a veterinary medicine as people in the Eastern Cape Province of South Africa use *C. dentata* stem bark to treat heartwater in cattle. Fadipe *et al.,* (2017:1-6) also reported that *C. dentata* is used to treat malaria and tuberculosis amongst the Sotho tribes of South Africa.

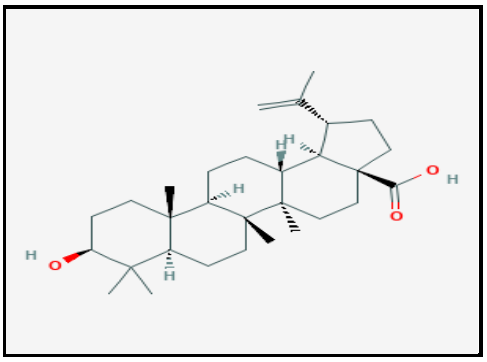
According to Van Wyk *et al.*, (2013:110), the chemical composition of *C. dentata* stem bark is largely unknown. Oyedemi, *et al.* (2012:6191), however, reported the presence of classes of compounds in *C. dentata* stem bark, which include saponins, steroids, alkaloids and tannins. Doughari *et al.*, (2012:1041-1050) also reported the presence of anthraquinones, glycosides, phenols, quinones, anthocyanins, amines and carboxylic acids in *C. dentata* stem bark. Shai (2007:1-188), reported the presence of saponins, anthraquinones and glycosides in the leaves of *C. dentata.* Shai (2007:1-188) also isolated four pentacyclic triterpenoids i.e. lupeol, betulinic acid, ursolic acid and 2α-hydroxyursolic acid from *C. dentata* leaves. Fadipe *et al.,* (2017:1-6) on the other hand, isolated *β*-sitosterol, also from *C. dentata* leaves. The chemical structures of these compounds are displayed in Figure 2.3. Fadipe *et al.*, (2017:4) further prepared ursolic and betulinic derivatives from the parent compounds, which evidently proved to be more potent against *Mycobacterium tuberculosis* than the parent compounds. All four the triterpenoids isolated from *C. dentata* leaves are, however, commonly found in plants, including fruits, vegetables, spices and herbs such as carrots, tomatoes, olives, figs, peas, cabbage, strawberries, grapes, guavas, cucumbers, rosemary, oregano, marjoram and sage, as they are structural components of plant cell membranes (Wal, Srivastava, Wal, Rai and Sharma, 2015:142; Pironi, De Araújo, Fernandes, Salgado and Chorilli, 2018:86).



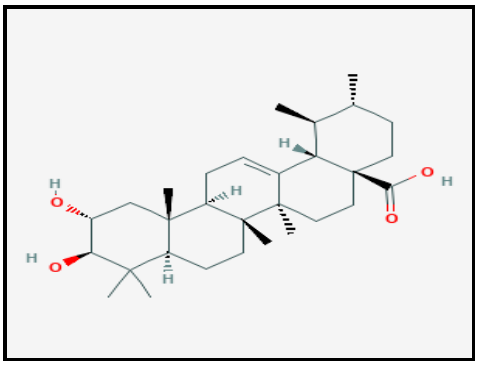
Lupeol ($C_{30}H_{50}O$)



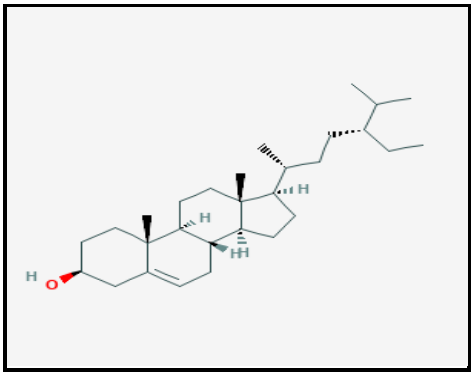
Ursolic acid ($C_{30}H_{48}O_3$)



Betulinic acid ($C_{30}H_{48}O_3$)



2α-hydroxyursolic acid ($C_{30}H_{48}O_4$)



β-sitosterol ($C_{29}H_{50}O$)

Figure 2.3: The chemical structures of the compounds isolated from *C. dentata* leaves (Source: PubChem).

Lupeol is a common compound also found in black tea, cucumber, carrots, figs, soya beans, tomatoes, mulberries, olives, dates, peas, guavas, grapes and mangoes (Wal et al., 2015:134, 135). Lupeol displayed therapeutic properties against cancer cells, inflammation, heart disease, diabetes and arthritis (Ruiz-Rodríguez, Vedani, Flores-Milires, Cháirez-Ramírez, Gallegos-Infante and González-Laredo, 2017:1562). In silico studies using *VirtualToxLab* showed that lupeol and its analogues, which includes betulinic acid, do not display high toxic potential and binding to nuclear receptors. The administration of lupeol and betulinic acid to mice did not show any mortality or toxic effects, no matter the manner of administration nor the dose administered (Ruiz-Rodríguez *et al.*, 2017:1568,1569). Lupeol and its analogues have shown to bind with progesterone and estrogen receptors (two of the proteins known to trigger adverse effects), and particularly its bond with estrogen receptors could generate adverse fertility effects (Ruiz-Rodríguez *et al.*, 2017:1569).

Betulinic acid is found in more than 2500 plant species (Gauthier, Leghault, Piochon-Gauthier and Pichette, 2011:552). Betulinic acid induces apoptosis in melanoma cancer cells *in vitro*. It has low *in vivo* toxicity and has even undergone phase II clinical trials for the treatment of human melanoma (Gauthier *et al.*, 2011:522). Betulinic acid further showed anti-HIV, anti-bacterial, anti-helminthic, anti-inflammatory, anti-protozoal, anti-obese and anti-malarial potentials and showed an inhibitory effect on *Candida albicans* (Moghaddam, Ahmad and Samzadeh-Kermani, 2012:119-123). Despite all these potential therapeutic properties, further pharmacological development of lupane-type triterpenoids is problematic due to its insolubility in water, which results in poor absorption and therefore limited pharmacological effect (Gauthier *et al.*, 2011:522).

Ursolic acid is a constituent of *C. dentata* leaves but also occur in apple peels and coffee, and in the leaves of herbs such as marjoram, rosemary, sage thyme and lavender (Woźniak, Skapska and Marszalek, 2015:20615). Ursolic acid showed anti-inflammatory, anti-cancer, cardioprotective, hepatoprotective, neuroprotective, antimicrobial, antidiabetic, antifungal and antiviral potentials (Pironi *et al.*, 2018:86; Seo, Lee, Heo, No, Rhee, Ko, Kwak and Han, 2018:235). Ursolic acid also showed anti-bacterial potential, but activity was limited to only Gram-positive bacteria (Wang, Chen, Wu, Jhan, Shyo and Chou, 2016:1). Ursolic acid is, however, classified as a Class IV drug in the biopharmaceutics classification system due to its low solubility in water and its problematic penetration of permeating biological membranes, which results in limited pharmacological effects (Pironi *et al*. 2018:86). Problematic absorption of both ursolic acid and betulinic acid therefore compromises the

efficacy of these chemical compounds as medicines, even though they have shown considerable potentials.

Literature on the pharmacological activities of 2α-hydroxyursolic acid, also known as corosolic acid, is limited. However, it was found that corosolic acid has anti-diabetic potentials (Miura, Ueda, Yamada, Fukushima, Ishida, Kaneko, Matsuyama and Seino, 2006:585-587). As a compound isolated from the leaves of *Perilla frutescens,* corosolic acid also showed considerable anti-inflammatory potential (Banno, Akihisa, Tokuda, Yasukawa, Higashihara, Ukiya, Watanabe, Kimura, Hasegawa and Nishino, 2004:88).

As a steroid-type compound in plants, *β*-sitosterol has a structure and functionality similar to that of cholesterol in mammals (Saeidnia, Manayi, Gohari and Abdollahi, 2014:590). Pharmacological potentials of *β*-sitosterol include anti-inflammatory, antioxidant, antidiabetic and apoptosis inducing activities, and it also showed chemoprotective and chemo preventative effects (Saeidnia *et al.*, 2014: 590-609; Pierre and Moses, 2015:89).

Oral bioavailability of a drug can be defined as the rate and extent at which a drug reaches the systemic circulation and is an essential parameter affecting the drug's efficacy, as well as its adverse effects (Hu and Li, 2011:1). The drug has to overcome several barriers before reaching the systemic circulation and therapeutic target. Many factors influence a drug molecule's ability to overcome barriers in order to reach and remain in the circulation system, which include physiochemical factors such as solubility and dissolution rates, and biological factors such as the physiology of the gastrointestinal tract and the metabolism of drugs in absorption and transport, diet and food effects and drug interactions (Hu and Li, 2011:2-4).

2.4 GEOGRAPHICAL REGIONS FROM WHICH *C. dentata* STEM BARK SAMPLES WERE COLLECTED

Large quantities of plant material are required for the extraction and isolation of chemical compounds. *Curtisia dentata* trees are protected, and the required licence and permits obtained (Appendices D and J) limited the quantity of bark that could be harvested from *C. dentata* trees to approximately 2 g per tree at each sampling date (1 x 25 mm x 8 mm cube per tree x 12 trees x 7 sampling dates). For isolation of compounds, 1.7 Kg of *C. dentata* stem bark was thus purchased at the Faraday traditional medicine trade market in Johannesburg. An application for discovery phase bioprospecting was submitted to the

Department of Environmental Affairs (DEA), and a letter of acknowledgement has been received, which is attached as Appendix K.

For the determination of seasonal and regional differences and similarities in composition and concentration of chemical compounds in *C. dentata* stem bark, bark sample material was collected from two research sites, one of which bark samples were collected during 2015/2016 and were used for a previous study (Van Wyk, 2017). The remainder of the sample material was stored in a -80° C freezer to prevent the degradation of metabolites. The research site used for the previous study is situated in the Western Cape Province of South Africa. The second research site is in KZN and is the only site from which bark sample material was collected for this study. The two research sites are indicated in Figure 2.4.

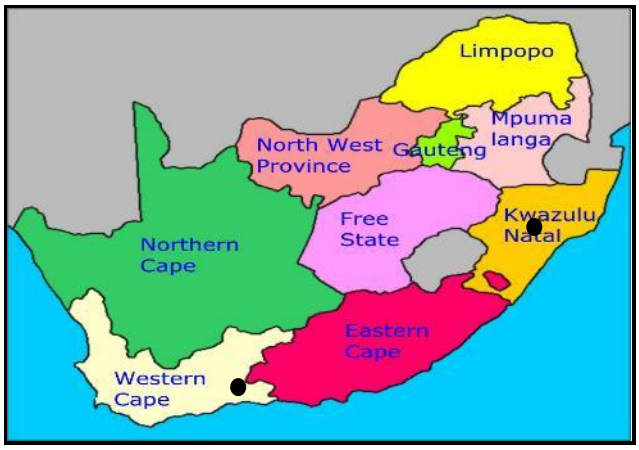


Figure 2.4: Map of South Africa indicating the location of the research sites in the Western Cape Province and in KZN. Available from: https://www.southafrica/provinces/provinces.htm (Retrieved: 10 December 2017).

2.4.1 Southern Cape forest area climate and vegetation

The southern Cape forest area has a moist, moderate climate as it borders the Indian Ocean. The area receives orographic rain and receives rain throughout the year. Peak rainfall, however, occur during autumn and early summer (Vermeulen, 2009:4). Mean annual rainfall is approximately 980 mm and mean daily maximum temperatures are between 18.7°C and 24.7°C (Vermeulen, 2009:102). South eastern and south western winds predominate in summer, whereas north westerly and south westerly winds prevail in winter (Geldenhuys, 1991:55; Vermeulen, 2009:4).

The vegetation of the southern Cape forest area is classified as mountain, coastal and scarp forest vegetation (Vermeulen, 2009:50). Geldenhuys (1991:59) describe the forest area by differentiating between forest species that occur in different landscape zones, e.g. mountains,

foothills, coastal platforms, river valleys, coastal scarp and dunes. Vermeulen (2009:4, 5), however, classifies the southern Cape forest area according to vegetation characteristics:

- Extremely dry scrub. The vegetation in this category is generally between 2m and 5m in height. This category has high species-richness and *Buddleia saligna, Cassine peragua, Chryssanthemoides monilifera* are among the plant species in this forest type.
- Very dry scrub forest. Dense shrubs between with a height of between 3 and 6m characterize this category, but trees reaching heights up to 9m are also present. The occasional large tree, e.g. *Podocarpus falcatus*, also occur in this classification.
- Dry high forest. This category of forest is relatively dense, however, the canopy is irregular, and vegetation reach 10 to 18m heights. Plant species of this forest type include *Cassine peragua, Maytenus acuminata* and *Canthium inerme.*
- Medium-moist high forest. Trees with a 16 m to 22m canopy in height characterized this forest type. Lower layers include dense shrub, representing mostly *Trichocladus crinitus*. Ground flora is abundant in this forest type. *Curtisia dentata* trees are among the upper canopy species.
- Moist high forest. Canopies of trees in this forest type reach heights of between 20 and 30m, however, tree trunk diameters are larger and tree density is lower. *Ocotea bullata* is a canopy species in this forest type while *Olea capensis* subsp. *Capensis* and *Halleria lucida* constitute the main sub-canopy species, and *Rumorha adiantiformis* and *Plectranthus fructicosis* are the main understory species.
- Wet high forests. Relatively few species occur in this forest type and the tree canopy heights range between 12 and 30m. *Cunonia capensis* and *Ocotea bullata* form part of the main canopy, whereas *Halleria lucida* and *Gonioma kamassi* are sub-canopy species and a variety of ferns occur, of which the tree fern *Cyathea capensis*, is characteristic.
- Very wet scrub. The general canopy height of forest species occurring in this category is 6m to 9m, and *Cunonia capensis* is named as the dominant species. Other species characteristic of this forest type includes *Ocotea bullata, Myrsine melanophloeos* and *Podocarpus latifolius*. Shrubs include *Sparrmannia africana* and *Diospyros glabra*, and the prominent ferns are *Blechnum capense* and *Todea barbara.*

2.4.2 Southern Cape research site location and geology

The GRNP is managed by South African National Parks (SANParks). The research site for *C. dentata* was at Groenkop (Figure 2.5), which is situated in the Wilderness section of the GRNP in the Western Cape Province of South Africa (S33°59,498' E022°32,850'). Groenkop lies at 260 m above sea level on the Outeniqua mountain range.

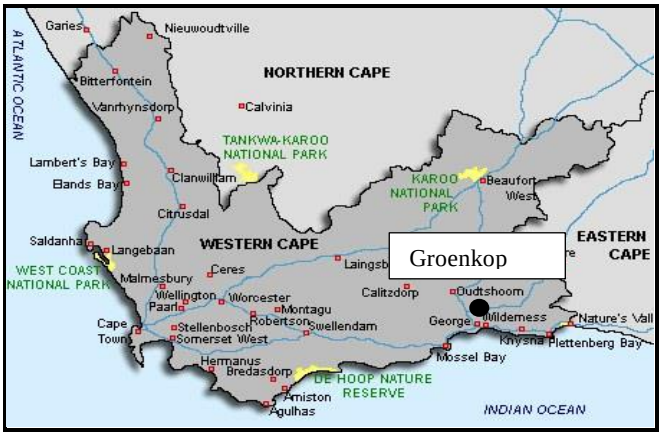


Figure 2.5: Map of the Western Cape Province indicting the location of the Groenkop research site in the George/Wilderness area of the Western Cape Province. Available from: www.aboutsouthafrica.com/maps_westerncape.htm (Retrieved: 18 March 2018).

The Cape Supergroup rock formations are characteristic of the GRNP. Pre-Cape rocks consisting of Maalgaten Granite and Cretaceous rocks, as well deposits of recent age occupy smaller areas to the west and east of George, which is further separated by a variety of metamorphic and sedimentary rocks of the Kaaimans Formation (Geldenhuys, 1991:54; SANParks, 2014:16). The Kaaimans Formation include rocks consisting of phyllite, grit, quartzite, hornfels and schist. On the Outeniqua Mountains, soils are acidic, leached, and basically infertile with poor buffering capacity. In wetter areas and at higher altitudes, topsoils are dark and acidic with high organic matter content (SANParks, 2014:19).

2.4.3 KwaZulu-Natal area climate and vegetation

Forests in KZN are limited to regions with high water availability. The determinant factor is rainfall, although riverine and kloof forests exist outside the normal rainfall pockets associated with forests. Groundwater, flood water and shelter phenomena (deep gorges with low solar irradiation, hence lower evaporation and evapotranspiration) play a role at these locations. Water availability to forest vegetation is thus a function of the amount of precipitation, evapotranspiration and groundwater availability. Soil structure and seasonality of precipitation also play a role (Mucina and Geldenhuys, 2006:10).

The Nkandla Forest Reserve is situated within the Usulu-Mhlaluze water catchment area and receives mainly summer rainfall. According to the Provincial Planning Commission (PPC) of KwaZulu-Natal, annual average rainfall in the KZN midlands vary between 827mm and 912mm (PPC, 2011:9-31), and there is a high occurrence of mist (Wirminghaus, 1990:159). Northeasterly winds prevail in summer, while in winter, northwesterly berg winds predominate. Mean annual summer temperatures in the KZN midlands vary between 18°C and 27.1°C and mean annual winter temperatures vary between 4.4°C and 10.1°C (Wirminghaus, 1990:159; PPC, 2011:33).

According to Wirminghaus, (1990:159) most of the KZN midlands mistbelt region fall under the Acocks's veld type 5 (Ngongoni veld). The forest types occurring in the midlands mistbelt is described as Mistbelt Mixed *Podocarpus* Forests and is classified under the Vegmap code Foz 3 (Mucina and Geldenhuys, 2006:18). Upper canopy trees occurring in the midlands mistbelt forests include *Afrocarpus falcatus, Celtis africana, Cunonia capensis, Curtisia dentata, Poducarpus henkelii, Protorhus longifolia, Ocotea bullata* and *Myrsine melanophloeos* (formerly – *Rapanea melanophloeos*). Among the sub canopy trees are species such as *Canthium ciliatum, Eugenia capensis, Ochna serrulata, Scutia myrtina* and *Trimelia grandifolia*. Tall shrubs include *Grewia occidentalis, Carissa bisponosa* subsp. *zambesiensis* and *Hyperacanthus amoenus*. Low shrubs include *Azima tetracantha* and herbs include *Streptocarpus daviesii* and *Streptocarpus haygarthii*. Geophytes include *Dietes iridioides* and *Dryopteris inaequalis*.

2.4.4 KwaZulu-Natal research site location and geology

The Nkandla Forest Reserve is managed by Ezemvelo KZN Wildlife. The forest, located at S28°43'0" E031°7'60", is approximately 20km from Nkandla village within the Uthungulu region in the north eastern part of KZN midlands mistbelt area (Figure 2.6)., and lies at an elevation of between 1100 m and 1300 m above sea level (Ngcobo, 2002:14, 15).

The Nkandla forest is situated at the upper edge of the Ngongoni veld type and is surrounded by wiregrass dominated grasslands. The 2480 ha area of forest is fragmented by numerous incised valleys that lie along the Natal monocline, and the topography is very rugged.

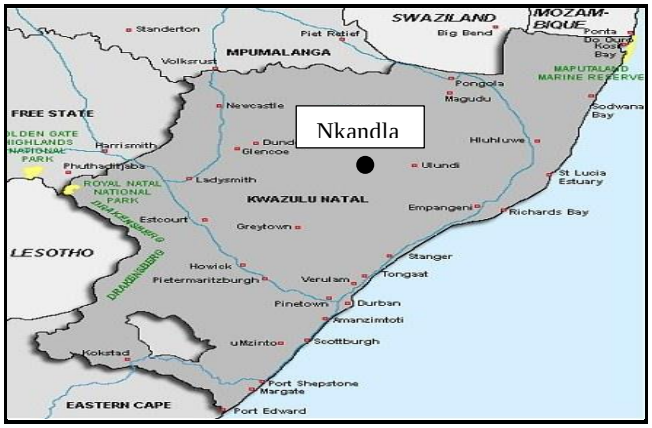


Figure 2.6: Map of the KwaZulu-Natal Province indicating the location of the Nkandla Forest Reserve. Available from: www.aboutsouthafrica.com/maps_kzn.htm (Retrieved: 18 March 2018).

The geology of the Nkandla forest area consists of complex granitic soils deriving from the deformation and metamorphism of the original geological formations. Hot molten magma from below penetrated into these rocks and produced granite exposures of the Nkandla area. Most of the rock formations have been deformed and altered by the effects of pressure and temperature and has led to a very complex geology consisting primarily of metamorphic igneous rocks. The underlying rock consists mainly of quartzite and granite. Within the Nkandla forest boundaries, quartzite and quartz schists of the Nkandla series underlie about 45% of the area. Approximately 30% of the area are represented by quartzite, with occasional thin conglomerates of the Nsuzi series while the granite and granite gneiss make up most of the remaining areas. Soils are often shallow and usually have weathered underlying parent material due to the high rainfall of the area. The grassland slopes have soils which are acidic and leached, derived from Insuzi, Nkandla and Archaic granite geological formations. In the less steep midslope, shallow to moderately deep soils have formed. Fairly deep soils occur at the bottom of some of the minor valleys (Ngcobo, 2002:16).

2.5 SOUTH AFRICAN ENVIRONMENTAL LEGISLATION

Pre-democratic South African environmental laws were reviewed after the abandonment of Apartheid and the change to a democratic system in 1994 and whereby the Constitution of South Africa (Act 108 of 1996) (South Africa, 1996) was promulgated, which advocates equal rights for all South Africans. South African environmental laws were thus transformed, promulgated and implemented at both national and provincial levels. Among the new

environmental laws are the National Environmental Management: Biodiversity Act 10 of 2004 (NEMBA) (South Africa, 2004), National Forests Act 84 of 1998 (NFA) (South Africa, 1998), and the National Environmental Management: Protected Areas Act 57 of 2003 (NEMPA) (South Africa, 2003), which facilitates access to natural resources for all South Africans, based on the principle of sustainability. The NEMBA (South Africa, 2004) Notice on Bioprospecting, Access and Benefit-sharing Notice 329 of 2007 (South Africa, 2007b) contains draft regulations on bioprospecting, access and the sharing of benefits to protect South African Indigenous Knowledge. Traditional health practitioners gained recognition through the promulgation and implementation of the Traditional Health Practitioners Act 22 of 2007 (South Africa, 2007a).

The NFA (Act 84 of 1998) (South Africa, 1998) protects South African forests and trees by means of a licensing system whereby it states that:

"*no person may without a license collect, remove, transport, export, purchase, sell, donate or in any other manner acquire or dispose of any protected tree without a license*".

The act, however, provides for exceptions in favour of the use of natural resources, and exempts local communities from its licensing provisions:

"*if the intention for the activity is for domestic, cultural, health or spiritual purposes*".

These exemptions do not include the use of the protected tree species listed in Notice 734 of 2011 (South Africa, 2011), which include *C. dentata* trees (South Africa, 2011; Strydom and King, 2013:110).

2.5.1 Legislation concerning traditional medicine in South Africa

In 1998, the WHO (of which South Africa is a member state) urged its member states to:

- "*critically evaluate their traditional medicinal systems,*
- *draft an inventory and to assess through pre-clinical and clinical studies, the traditional medicines used by traditional healers in their practices and by the public,*
- *introduce channels for the regulation and control of medicinal plant products,*
- *establish and maintain suitable standards,*
- *identify medicinal plants (or the products derived from them) with a satisfactory efficacy/side-effect ratio in order to include them into a national pharmacopoeia*" (WHO, 1998:6).

A draft policy for African traditional medicine in South Africa (Notice 906 of 2008) (South Africa, 2008) was thus formulated which recognized the need for:

- ”*Official support and acceptance of traditional medicine in the formal healthcare sector,*
- *Establishment of a system to regulate, register and license traditional health practitioners and the formal training of such practitioners,*
- *The establishment of a system to develop, regulate and register traditional medicine to ensure safety, quality and efficacy, including scientific research,*
- *The development of a national pharmacopeia or the updating of existing ones as part of the regulatory system, and*
- *Collaboration with other countries and the World Health Organization (WHO) in order to exchange information and promote policies and regulation according to international standards (South Africa, 2008:3)”.*

The draft policy for African traditional medicine (South Africa, 2008:9) further states that: “*institutionalization of African traditional medicine should take place, among others, by taking the following actions:*

- *Policy finalization and adoption*
- *The establishment of a National Institute of African Traditional Medicine for South Africa (ATMSA),*
- *Protecting traditional health practitioners and the users of traditional medicine against unqualified or incompetent individuals selling medicine and/or acting as traditional health practitioners,*
- *Protection of African traditional medicine knowledge and intellectual property rights,*
- *Conservation of medicinal plants and animals and counteracting unsustainable harvesting practices,*
- *Development of acceptable standards of safety and quality for African traditional medicines and raw materials,*
- *Establishment of an African traditional medicine pharmaceutical industry for the production and processing of African traditional medicine to ensure a sustainable supply of high quality, affordable products, and*
- *Education of street vendors regarding sanitation, conservation and harvesting of medicinal plants”*

2.5.2 Challenges regarding the legal framework and the regulation of traditional medicine in South Africa

The registration of African traditional medicines is hindered by challenges regarding the formulation of appropriate regulations, for example: the draft policy for African traditional medicine in South Africa (Notice 906 of 2008), (South Africa, 2008) states that: "*the application of the Patent Amendment Act (Act 20 of 2005) (South Africa, 2005) to traditional medicines is challenging due to the following reasons:*

1) *Knowledge of traditional medicine have been passed down within the context of a defined communal system without any identifiable creator or inventor,*
2) *Where a traditional healer may wish to patent his/her knowledge, he/she may have difficulty in proving his/her novelty,*
3) *If it is a plant-based medicine it is not patentable as natural material as plants are not patentable without processing,*
4) *No method of treatment of human or animal body or surgery is patentable, and*
5) *Lack of written records creates difficulties in terms of international protection as patent offices in other countries are not able to access such information for the purpose of establishing its novelty and inventiveness".*

The draft policy for African traditional medicine in South Africa (Notice 906 of 2008), (South Africa, 2008) further states that: "*the quality and safety of African traditional medicine has become a concern for health authorities and the public with potential pitfalls relating to the following:*

- *Intentional addition of an active drug responsible for therapeutic/adverse effect (adulteration),*
- *Unintentional substitution of a plant with a toxic species,*
- *Environmental contamination of the plant with a chemical or pathogen,*
- *Suboptimal or varying amounts of active compounds within plant matrix,*
- *Adverse effects,*
- *Drug-herb interactions (concurrent use of multiple products), and*
- *Confounding factors leading to misinterpretation of effect*" (South Africa, 2008:34, 35).

2.5.3 Legislation compliance

Despite comprehensive environmental laws, the illegal harvesting of protected South African indigenous plant species continues, and the plant parts collected are freely traded at traditional medicine trade markets (Botha *et al*., 2004a:1676; Pers. Obs. 18 September 2019). Legislation slowed down the rate at which exploitation increases, but it did not provide a feasible, long-term solution (Cunningham 1993:7). Legislation also did not prevent the practicing of unlicensed traditional health practitioners (Le Roux-Kemp, 2010:279). The Traditional Health Practitioners Act (Act 22 of 2007), states that: "*no person may practice as a traditional health practitioner within the Republic of South Africa unless he or she is registered in terms of the Act*". However, very few South African traditional health practitioners are registered with the Traditional Healer's Association (Le Roux-Kemp, 2010:279).

Environmental laws in South Africa are the responsibility of national and provincial governments, however, the fragmentation of responsibilities is an obstacle for the implementation and enforcement of the laws. Additional challenges include government officials' attitudes, i.e. their ability and willingness to act against those people who do not comply with the law (Strydom and King, 2013:124).

2.6 ADVERSE EFFECTS ASSOCIATED WITH THE USE OF PLANT-BASED TRADITIONAL MEDICINES

There is no foundation for the supposition that plants, including those with longstanding popular use, are either safe or beneficial, or that the chemical products derived from plants require fewer pre-clinical and clinical studies (Moreira, Texeira, Monteiro, De Oliveira and Paumgartten, 2014:249). A common misconception amongst users of traditional medicine, and also the users of so-called "health products" or "dietary supplements", is that plant-based medicines are safe to use because it is a natural product (Calixto, 2000:183). Plants produce secondary metabolites in response to stresses such as herbivory, and most of the secondary metabolites act as chemical defences to harm species that may pose a survival threat. It can thus not be taken for granted that extracts from medicinal plant material, or health products, are safe for consumption (Street *et al.*, 2008:705). Since the late 1970's, Germany subjected thousands of herbal medicines to pharmacovigilance. Most of these herbal medicines were removed from the market due to toxic effects and potential risks to human health (Calixto,

2000:183). There may be African traditional medicinal plants that are not hazardous to human health, however, plant-based medicines are frequently associated with severe iatrogenic complications, such as acute renal and liver dysfunction, diarrhoea, metabolic acidosis, jaundice, seizures, vomiting, fever, dehydration, encephalopathy, oligoanuria, shock, Kassmaul breathing and respiratory distress (Nyazema, 1984:81; Luyckx, Steenkamp, Rubel and Stewart, 2004:48).

Dosage requirements should be taken into consideration when a patient is treated for an ailment, illness or disease (Nyazema, 1984:80). However, traditional medicines are not standardized, plants may contain both beneficial and harmful substances, potential beneficial compounds that may occur in traditional plant medicines are not sequestered from the harmful substances, and even beneficial substances have an underlying promise of being a toxin. The dosage applied makes it either a poison or a remedy (McGaw et al., 2014:223). Furthermore, traditional health practitioners do not consider the age or weight of a patient, the seriousness of the disease, the nature of the plant, the speed of absorption and the particular circumstances relative to a patient when prescribing plant-based medicines. They are, for example, unaware of the fact that certain medicines may be highly unadvisable for pregnant women, people suffering from high blood pressure or people suffering from heart problems (Njume and Goduka, 2012:3925). Information on the potential toxicity of raw or semi-processed traditional plant-based medicine is limited as the secrecy enveloping the use of traditional medicine obstructs the methodical study of the spectrum of clinical presentations, the nature of the toxic substances involved and the number of incidences of hospital admissions resulting from the use of traditional medicine. If a patient denies the use of traditional medicine, it does not necessarily exclude the use thereof (Luyckx *et al.*, 2004:49). Traditional plant-based medicines are often self-administered, and some incidences of plant poisonings may be due to misidentification of plants, incorrect preparation, inappropriate administration and incorrect dosages (Maroyi, 2012:46). Acute poisoning in South Africa is not unusual, however, due to a lack of sufficient data, mortality rates resulting from the use of traditional medicine vary widely between 8000 and 20 000 each year (Fennell, Lindsey, McGaw, Sparg, Stafford, Elgorashi, Grace and Van Staden 2004a:213). Many cases of poisoning, however, remain unrecorded. Mortality rates may thus be significantly higher than what is known (Fennell *et al.*, 2004a:213). Acute gastro-intestinal and dermatological symptoms are short term toxic effects, and are more likely to be associated with traditional plant medicines than diseases and complications that develop over

longer periods of time such as cancer, liver and kidney damage, reproductive dysfunctions, birth defects and other difficult-to-detect morbidities. The plant-based cause of these complications can only be confirmed by well-designed epidemiology studies (Moreira *et al.*, 2014:250). Additionally, the absence of evidence linking plant-based traditional medicines to adverse effects, is not evidence that there are no chemical substances in plant material that have the potential to cause harm (Moreira *et al.*, 2014:250).

Contaminants are additional factors of concern for herbal remedies (Steveć, Pavlović, Stanković, and Šavikin, 2012:56). The WHO described the contamination of medicinal plant products as "*the undesired introduction of impurities of a chemical or microbiological nature, or of foreign matter, into or onto a starting material, intermediate product or finished medicinal plant product during production, sampling, packaging or repackaging, storage or transport*" (WHO, 2007:6). Contaminants may include chemical substances such as heavy metals, persistent organic pollutants (POPs), radioactive material, endotoxins, mycotoxins and solvents occurring as contaminants. Contaminants may also have biological origins, such as microbes and parasites, or may result from agricultural practices where residues of pesticides, herbicides and fertilizers, which are highly mobile, particularly by means of wind and water dispersion, contaminate large areas of land (WHO, 2007:13-15).

Contaminants in medicinal plant products may lead to a variety of hazardous conditions in humans and may vary in severity. Effects may include mild reactions such as allergic reactions, breathing difficulty, pain, fatigue, nausea, upset stomach, muscle weakness or mood disturbances, or effects can be moderate, such as vomiting, confusion, sensory disturbances, seizures, compression fractures, persistent hypoglycaemia, convulsions, Cushing's syndrome, burns and dermatitis. Severe effects resulting from contaminants include liver and/or renal failure, cerebral oedema, intracerebral haemorrhage, coma or death (Okem, 2014:14). Determining which traditional medicines are implicated in deaths is challenging as people are unwilling to provide information, partly due the secrecy surrounding traditional medicines and partly because medical staff frown upon the use thereof (Fennel, 2004a:213).

The chemical compounds with pharmacological potential are produced by plants as secondary metabolites (Azmir, Zaidul, Rahman, Sharif, Mohamed, Sahena, Jahurul, Ghafoor, Norulaini and Omar, 2013:427). Certain classes of chemical compounds such as alkaloids, for example, have been tested for its potential as anti-microbial agents, analgesics and/or narcotics or as stimulants. Furthermore, certain anthraquinones are used for the treatment of constipation. Tannins in particular have the potential to inhibit microbial proliferation by

denaturation of enzymes involved in microbial metabolism. Some tannins also show potential anti-viral, anti-bacterial, anti-parasitic and anti-cancer activities and essential oils often have decongestive, anti-viral, antiseptic and anti-microbial properties (Doughari, 2012:1043). Several studies, however, focused on the mutagenic, genotoxic and/or carcinogenic effects of South African medicinal plants (Steenkamp *et al.*, 2001:51-58; Elgorashi *et al.*, 2002:408-410; Fennel *et al.*, 2004a:205-217; Fawole *et al.*, 2009:356-362; Prinsloo *et al.*, 2018: 27-39). Fennel *et al*., (2004a:212), for example, studied the mutagenic effects of several commonly used South African medicinal plants, and Steenkamp *et* al., (2001:51-58) discussed the toxicity of pyrrolizidine alkaloids in *Senecio latifolius*, which could result in carcinogenic effects. Fawole *et al*., (2009:361) on the other hand, stated that plant extracts that showed good microbial activity had no indication of mutagenic potential. However, the safety of a drug can only be assumed after it had been tested by means of comprehensive pre-clinical and clinical studies (Moreira *et al.*, 2014:250).

2.7 FACTORS AFFECTING SECONDARY METABOLITE COMPOSITION AND CONCENTRATIONS IN PLANTS

Secondary metabolites in plants are compounds non-essential for cell survival, even though they are often synthesized from products of primary metabolism, however, they are essential for the plant's survival in the environment. Secondary metabolites aid in plant fitness by preventing pathogen infestations and herbivorous feeding and assist in plant reproduction by attracting pollinator through floral scent or colouration (Kliebenstein, 2004:675). Secondary metabolites are further responsible for the tastes, odours and colours in plants and are the sources of flavours, food additives and medicinal compounds (Akula and Ravishankar, 2011:1720-1731; Van Wyk and Prinsloo, 2020:56). Additional roles of secondary metabolites include temporary nutrient storage, protection from UV light, structural support and phytohormone regulation (Van Wyk and Prinsloo, 2020:56). They also protect plants during drought, facilitate nutrient uptake, aid in decomposition and are the mediators of plant relationships with nitrogen-fixing bacteria (Herms and Mattson, 1992:288).

The synthesis of secondary metabolites skeletons depend on the carbon assimilated during photosynthesis and therefore, the photoperiod and light intensity a plant is exposed to. The spectrum quality of the available light, water and nutrient availability may further influence chemical compound composition and concentrations (Zobayed, Afreen and Kozai,

2005:246). The structures of secondary metabolites often derive from a common backbone however, differential modifications of the structures often result in differential biological activities (Kliebenstein, 2004:675). Secondary metabolite concentrations also vary according to season, between harvesting sites, regions, plant age, post harvesting treatment, storage and between wild and cultivated plants (Fennel *et al.*, 2004b:113-121; Schippmann *et al.*, 2002a:5), while genetic variability is a proven factor affecting the composition and concentrations of phytochemicals in plants (Zobayed, *et al.*, 2005:245).

Secondary metabolite production often increase when plants experience stress because stress conditions inhibit growth more than it inhibits photosynthesis. The carbon fixed is then largely allocated to the synthesis of secondary metabolites (Akula and Ravishankar, 2011:1725). Prinsloo and Nogemane (2018:2), however, highlighted that stress conditions may also affect secondary metabolite concentrations negatively, resulting in lower concentrations of secondary metabolites. Two distinct stimuli regulate the biogenesis of secondary metabolites, i.e. abiotic and biotic effects (Pavarini, Pavarini, Niehues and Lopes, 2012:7).

2.7.1. Abiotic effects

Abiotic effects relate to all the physical factors that could impose stress conditions on a habitat (Pavarini *et al.*, 2012:5). Abiotic stress factors such as temperature, salinity, both excess water and water deficits, alkalinity, and UV radiation are potentially harmful to plants. Chemical stress factors include mineral salts, pollutants, gaseous toxins, heavy metals, aerosols and pesticides, and mechanical stress factors such as wind and soil movement may add to the effects (Akula and Ravishankar, 2011:1720, 1721). Plants may either increase or decrease secondary metabolite concentrations when exposed to stress conditions (Prinsloo and Nogemane, 2018:1). Important to note is that the effects caused by abiotic factors cannot be generalized as true for all plants. Seasonal differences, the differential expressions of genes, differences in species and cultivars, the different metabolic pathways and the interactions of two or more simultaneous environmental stress factors complicate conclusions (Prinsloo and Nogemane, 2018:1). However, examples of abiotic stress effects on plants, including their effects on the production of secondary metabolites, are provided below:

Salt stress – Salt stress results in cellular dehydration and causes ionic and osmotic stress, subsequently resulting in either increases or decreases of secondary metabolites in

plants. For example, anthocyanins increase distinctly in plants under salt stress conditions, however, in salt-sensitive plant species, anthocyanin concentrations decrease. In *Lycopersicon esculentum* proline concentrations increase, whereas polyphenol concentrations increase in several plant species, and tropane alkaloids increase in *Daturra innoxia*. Trigonelline further increases in *Glycine max* and flavonoids increase in *Hordeum vulgare* under salt stress conditions (Akula and Ravishankar, 2011:1721, 1722). Studies have also shown that osmotic shock stimulated the production of anthocyanins in *Vitis vinifera*, whereas saponins increase in *Panax notoginseng*, alkaloids increase in *Catharanthus roseus* and paclitaxel in *Taxus chinensis* (Wu, Wong, Ho and Zhou, 2005:133-138).

Drought stress – Plant responses to drought stress responses can be positively or negatively modified by the superimposition of other environmental stress factors such as temperature and/or salt stress (Chaves, Pereira, Maroco, Rodrigues, Ricardo, Osório, Carvalho, Faria and Pinheiro, 2002:907-916). Drought stress causes oxidative stress, which may subsequently result in increases/decreases of secondary metabolites in plants. For example, changes in chlorophyll "a" and "b" carotenoids were reported in plants under drought stress conditions whereas saponin concentrations decrease in *Chenopodium quinoa*. Drought stress increase anthocyanin concentrations in several plant species. Anthocyanin is produced by the plants to assist in drought-tolerance. Drought stress lowered monoterpene emissions from *Quercus ilex* (Akula and Ravishankar, 2011:1722), and further increase phenolic compound concentrations, where it has a specific role in the increase of lipophilic resins in *Diplacus* and *Larrea* species, which in turn, assumes a UV defensive role. There is also a link between xylem pressure and tannin synthesis, and may be either positive or negative, depending on the degree of drought stress a plant suffers (Ncube, Finnie and Van Staden, 2012:15).

Flooding – Flooding results in the accumulation of H_2O, the decrease of O_2, the anaerobic decomposition of organic matter, increased solubility of mineral substances, and the formation of toxic compounds (Kozlowski, 1997:2; Dat, Capelli, Folzer, Bourgeade and Badot, 2004:273,274). Unavailability of oxygen induces anaerobically induced polypeptides (ANPs) and includes the induction of 1-Aminoacylcyclopropane-1-carboxylic acid (ACC), which is a precursor of ethylene biosynthesis. ACC in conjunction with O_2 and ACC-oxidase forms ethylene (Irfan, Hayat, Afroz and

Ahmad, 2010:2-17). Increases in the phenols benzoic-2-hydroxybenzoic acid and 4-hydroxybenzoic acid were reported, and increases in monocarboxylic acids such as formic acid, propionic acid and acetic acid occur, which causes a shift towards and efflux of K^+ and an influx of H^+ in sensitive species (Irfan *et al.*, 2010:3-17).

Heavy metals – Ni is necessary for plant development, however, excessive Ni concentrations inhibit the biosynthesis of anthocyanins by inhibiting activity of l-phenylalanine ammonia-lyase (PAL). Accumulation of the metals Cr, Fe, Zn and Mn increase oily substances in *Brassica juncea* with up to 35%. Both Cu^{2+} and Cd^{2+} induce higher yields of shikonin and digitalin. Cu^{2+} further stimulates the production of betalains in *Beta vulgaris*. Lanthanum affect the production of taxol in *Taxus* spp., while both Cd^{2+} and Cu^{2+} increase putriscine concentrations in beans and oats and decrease putriscine and spermidine concentrations in *Helianthus annuus* (Akula and Ravishankar, 2011:1722,1723).

Cold stress – The metabolisms of temperate plants' are redirected toward the production of chemical compounds that protect the plants against cold-induced damage during winter. Such chemical compounds include sugar alcohols such as sorbitol and ribitol, soluble sugars such as raffinose and stachyose, and low-molecular weight nitrogenous compounds such as proline, glycine and betaine (Akula and Ravishankar, 2011:1723). Cold stress also increases production of phenolic compounds which are incorporated into cell walls as suberin or lignin. Lignification and suberin accumulation protect plants from freeze damage. Lower soil temperatures cause an increase in levels of steroids such as furostanol and saponins such as spirostanol. In *Artemisia* spp., cold temperatures increased the levels of artemisinin, and in both *Nicotiana tabacum* and *Malus* spp., increased levels of anthocyanins have been reported in plants exposed to extreme cold conditions (Rivero, Ruiz, García, López-Lefebre, Sánchez and Romero, 2001:315-321, Akula and Ravishankar, 2011:1723; Pavarini *et al.*, 2012:8).

UV-B radiation, heat and light stress – UV-B, light, heat, and also drought are often related/synergistic environmental stress factors (Chaves *et al.*, 2002:907-916). Heat stress reduces the rate of photosynthesis and also affects biomass production, however, storage ginsenoside production is improved during heat stress. Heat stress increased leaf senescence and root secondary metabolite production in *Panax quinquefolius*. Exposure to UV-B stimulate the production of catharantine, as well as vincristine and

vinblastine in *Catharanthus roseus*. These chemical compounds is used for the treatment of cancer and lymphoma, whereas in *Populus x canescense*, shifts on transcript levels of terpene biosynthesis-related genes take place. There is also a positive correlation between increasing light intensity and levels of phenolic and flavanols production. Willow leaves in shaded areas decrease the production of foliar tannins and phenolic glycosides. Low light intensities (301–600 lx) induce higher production of anthocyanins. UV-B increases flavonoid concentrations in barley, and polyamine concentrations in cucumber (Akula and Ravishankar, 2011:1723, 1724; Pavarini *et al.* 2012:8).

Nutrient stress – Nutrient stress affect phenolic compound concentrations in plants. Nitrogen and phosphate deficiencies lead to the accumulation of phenylpropanoids and results in lignification. In tomato, a three-fold increase in anthocyanidin levels occur under nutrient stress conditions. At the same time, quercetin-3-O-glucoside content doubled. Sulphur, potassium and magnesium deficiencies also increase phenolic compound concentrations, and calcium concentrations have been implicated in response to several abiotic stress factors, including drought, cold and salinity stresses. Higher levels of copper and manganese in *Eugenia uniflora* lead to decreases in the production of tannin and flavonoids (Akula and Ravishankar, 2011:1725).

Climate change – Plants are generally sensitive to climate change and do not readily adapt to elevated temperatures, or the higher or lower incidences of rainfall etc. associated with climate change. Furthermore, exposure to ozone resulted in increased conifer phenolic concentrations, however, low ozone exposure had no effect on monoterpene and resin acid concentrations. Plants growing in high carbon dioxide (CO_2) levels exhibited significant changes of their chemical composition. A prominent example of the effects of elevated CO_2 levels is the decrease of the N concentration in vegetative plant parts, as well as in seeds and grains, subsequently resulting in the decrease of protein levels. Studies have shown that elevated CO_2 increases phenolics and condensed tannins in leaves. In coniferous species, elevated CO_2 influenced a decrease/increase in concentrations of several monoterpenes. For example: the monoterpene, a-pinene increased in coniferous species whereas b-pinene decreased under elevated CO_2 levels. Higher levels of CO_2 further resulted in an increase in total phenolics (Akula and Ravishankar, 2011:1725, 1726).

2.7.2 Biotic effects

Biotic effects entail complex relations with plant physiology and biochemistry. Biotic effects relate to plant/insect relationships such as pollinator attraction, or the plant's defence against pathogens and/or herbivorous insects (Kliebenstein, 2004:675; Pichersky and Gang, 2000:439). Biotic effects also relate to plant physiological attributes such as phenology, which describes all ultradian (rhythmic activities occurring over a period between one and 24 hours e.g. expansion and shrinkage of tree stem diameter due to differences in xylem pressure resulting from transpiration), circadian (processes occurring on an approximate 24 hour cycle, e.g. photosynthetic activity) and infradian (rhythmic activities with a period of recurrence longer than a day, e.g. seasons) cycles in plants, and ontogeny, which is the determinant of physiological differences of an organism within its lifetime (Pavarini *et al.*, 2012:8).

Changes in both secondary metabolite composition and concentrations also occur during a plant's different developmental stages (Çirak, Radušiene, Ivanauskas and Janulis, 2007:200). Additionally, phytochemical constituents are generally not evenly distributed throughout a plant. Tanko, Carrier, Duan and Clausen (2005:2,5) for example, reported that alkamides and echinacosides were mainly detected in the root bark of *Echinacea angustifolia*, and that the hypericin content in the stems, leaves, flower buds and flowers of *Hypericum perforatum* were recorded as 0, 0, 21 and 79%, respectively. Ontogenic changes in hypericin, chlorogenic acid and quercetin of plant material were significant. The highest levels of hypericin, chlorogenic acid and quercetin in *H. origanifolium* were recorded during both the floral budding and full flowering stages, whereas in *H. brasiliense*, quercetin and rutin concentrations peaked at the full flowering stage. The concentrations of both compounds decreased during fruit formation (Çirak *et al.*, 2007:200), and corroborate the findings of Tanko *et al*., (2005:5), where they report that floral parts had the highest level of hypericin as well as quercitrin, quercetin and apigenin-7-O-glucoside. However, they further reported that leaves were superior to flowers and seeds with respect to chlorogenic acid and hyperoside accumulation in *H. origanifolium*. Leaves also had the highest levels of rutin and hyperoside.

Plants may release higher levels of bioactive compounds in response to leaf of stem bark damage caused by feeding insects, and concentrations may vary between plant species and the herbivorous insect species involved (Paré and Tumlinson, 1999:325). Compounds released may include monoterpenes, aromatic substances, sesquiterpenes, alcohols, esters and aldehydes and may repel or intoxicate insects, or it may lure the natural enemies of the damage

causing insects, while defence proteins may interfere with an insect's digestive system (Paré and Tumlinson, 1999:325).

Allelopathy refers to processes that involve the production of toxic secondary substances by one plant which interferes with the growth of another plant sharing the same habitat (Chou, 2006:1; Mallik, 2008:2), and can also be regarded as a biotic effect. Plant allelopathic chemicals may also affect soil microbes (Cipollini, Rigsby and Barto, 2012:714-727). Allelochemicals, which include flavonoids, phenolics, terpenoids, alkaloids and cyanogenic glycosides, are released into the environment through leaching, volatilisation, the decomposition of plant material and root exudates (Teixeira da Silva, Karimi, Mohsenzadeh, Dobránszki, 2015:109). These chemicals may interfere with the signal transduction chain, alter gene expression in the other plants, alter the structure of cell walls and membranes, affect seed germination and may increase the production of reactive oxygen species (Dhole, Lone, Dhole and Bodke, 2013:254-260; Teixeira da Silva *et al*., 2015:110; Bakhshayeshan-agdam, Salehi-Lisar and Motafakkerazad, 2015: 193-202; Prinsloo and Du Plooy, 2018:1-12). Allelopathy is more pronounced when the target plants are affected by abiotic or other biotic stresses (Gawronska and Golisz, 2006). For example: when a plant is exposed to pathogenic organisms, allelopathic activity may be higher due to the production of both higher concentrations and a wider spectrum of allelopathic chemicals in defence of the organisms causing harm (Gawronska and Golisz, 2006).

Allelochemicals may also have a positive effect on plants sharing the environment, provided that concentrations of allelopathic chemicals released are low (Teixeira da Silva *et al*., 2015:110). A positive effect is that allelochemicals are involved in plant adaptation processes through an evolutionary increase of the target plant's tolerance and/or resistance to environmental stress conditions (Gawronska and Golisz, 2006).

2.8 CULTIVATION OF MEDICINAL PLANT SPECIES: CHALLENGES AND RECOMMENDATIONS

The volumes of medicinal plants harvested from the various South African biomes, including forests, to meet the demands of expanding medicinal plant trade markets are increasing annually (Rao, Palada and Becker, 2004:109). On the other hand, urban development, the cultivation of crops and forestry practices, infrastructure development, as well as overgrazing and overexploitation result in a reduction of available wild medicinal plants (Cunningham,

1988:1; Cunningham, 1997:117; Rao *et al.*, 2004:109), which thus increases pressure on the remaining medicinal plants (Sibly and Hone, 2002). In light of the fact that many wild medicinal plant species are harvested to the brink of extinction while the human population is increasing, international organizations such as the WHO, the International Union for the Conservation of Nature (ICUN) and the World Wide Fund for Nature (WWF) suggested that medicinal plant cultivation programs be initiated (Schippmann *et al.*, 2002a:1).

In South Africa, large scale cultivation of medicinal plants has not been undertaken since first suggested in 1938 (Cunningham, 1988:62). Three main factors account for the non-existence of medicinal plant cultivation programs: 1) institutional support for the production and distribution of species for cultivation is lacking, 2) low prices paid to plant harvesters by traders, and 3) the most valued medicinal plant species only reach maturity after a few decades (Cunningham, 1997). The success of cultivation programs depend on the size and cost of such undertakings. If the number of plants of a specific species cultivated do not meet current market demand, cultivation will only be a front for the continued exploitation of wild populations (Cunningham, 1997:126).

Among the factors that should be considered when initiating cultivation programs are root system size, land area required and rotation period. The area of land required for cultivation increase according to plant size. For example: bulbous plants require a smaller area of land as the roots do not spread widely and can thus be planted densely. Rotation varies between six and ten years. Trees, on the other hand, require larger areas of land for healthy root growth. Their rotation area required is much greater and the time period for rotation is much longer, particularly if the tree species is slow growing. The total area of land required for the cultivation of trees also depends on demand, and thus the number of trees required (Cunningham, 1993:26).

Without exception, the species selected for cultivation in European countries, Asia and in other African countries, are species which are profitable, or which provide high levels of resource returns (multiple use species). These species are either fast growing, or species of which the plant parts used cause little or no damage to the plant, such as leave or resins (Cunningham, 1997:126). The number of species selected for cultivation is therefore much lower than the number of species used medicinally (Schippmann *et al.*, 2002b:4).

Cultural beliefs may hinder the cultivation of medicinal plants. For example: rural community members believe that metaphysical dangers will destroy the healing power of

medicine, such as when the plants are touched by so-called polluted people (Wiersum, Dold, Husselman and Cocks, 2006:49, 50). However, when asked whether the planting of medicinal plant species in a separate and secluded place in a home garden to reduce possible contact with polluted people would be acceptable, these community members agreed. The majority of people in communities, however, indicated that the use of cultivated material for healing or protection purposes is acceptable (Wiersum, *et al.* 2006:49, 50). Challenges regarding home garden cultivation, however, include scarce water resources for irrigation purposes, propagation difficulties and limited knowledge regarding plant requirements such as soil and light conditions (Wiersum *et al.*, 2006:54).

Communities further regard the quality of cultivated plant material as inferior to that of wild resources. Conservative traditional health practitioners in South Africa believe that plants planted in straight rows and the addition of fertilizers affect the medicinal properties of the plants (Fennel, 2004b:114). Scientific studies partly support this. Bioactive compounds in fast growing cultivated stock may be lower than the bioactive compounds in wild populations because wild populations may be older due to slow growth (Schippmann *et al.*, 2002b:5).

Juxtaposed to wild-growing plant resources, cultivation provide a number of advantages:

- Plant material collected from wild resources may become adulterated through the addition of harmful plant species to boost potency, whereas cultivated plants provides for reliable botanical identification.
- Cultivation provides a steady source of raw material.
- Growers, wholesalers and pharmaceutical companies can agree on volumes and prices.
- Controlled post-harvest handling of cultivated plants ensures appropriate quality control.
- Standards of cultivated plant material can be adjusted in accordance with regulations and consumer preferences.
- Cultivation allows for the implementation of product certification.
- Cultivation allows for the production of homogenous material, which guarantees chemical homogeneity
- Cultivation may safeguard species threatened with extinction (Rates 2001:606; Schippmann, *et al.*, 2002b:5)

Biological characteristics and/or ecological requirements such as slow growth rate, soil requirements, low germination rates, susceptibility to pests, water requirements, etc. may present challenges for the successful cultivation of medicinal plant species (Schippmann, *et al.*, 2002b:5; Rao *et al*., 2004:110). The motivation for cultivation is economic feasibility, but feasibility may also be a limitation. Commercial harvesters harvesting their material from wild populations and thus have no input costs, whereas input costs of cultivation is high. At trade markets, the wild stock will thus have an advantage over the cultivated plant material. An additional limitation is that only a few species could be marketed at high enough prices to make cultivation profitable, as prices of plant material at medicinal plant trade markets are generally low (Schippmann, *et al.*, 2002b:6).

Wiersum *et al.*, (2006:45) recommended that the interpretation of poverty alleviation and the research approach applied are to be considered when research options for stimulating cultivation of medicinal plants with a biodiversity conservation and poverty alleviation approach, are designed. Poverty alleviation ought to refer to a person's ability to make a living, to make life meaningful and to liberate. It is thus important that research projects involve participatory research. The selection of both the species for cultivation and the cultivation practices applied should complement existing local experiences on the cultivation of medicinal plants. Local knowledge could incite innovation (Wiersum *et al.*, 2006:45, 46). Suggestions provided by Rao *et al.*, (2004:111) include that traditional medicinal species that require or tolerate partial shade, moist soils high in organic matter, humidity and mild temperatures be cultivated in thinned forests and cleared forest patches. Rao *et al.*, (2004:111) further suggested a taungya system, where newly established plantations, such as pine plantations, be intercropped with herbaceous medicinal plants until the trees' canopies cover the ground area. It should, however, be considered that exotic plant species such as pine trees often contain allelochemicals which may impact on the germination, growth and development of cultivated indigenous medicinal plant species (Fernandez, Lelong, Vila, Mévy, Robles, Greff, Dupouyet, and Bousquet-Mélou, 2006:97). In Asian countries however, the participation of local people and the granting of rights to share benefits culminated from plantations, and particularly ownership to crops, helped governments to establish and protect large-scale tree plantations without conflict (Rao *et al.*, 2004:111, 113).

The high cultural value of *C. dentata* stem bark as a traditional medicine necessitates the cultivation of this tree species to continue the supply in demand for *C. dentata* stem bark and wood (Shaik, 2012:448). *Curtisia dentata* natural reproduction success is, however, low, as

only approximately 50% of the seeds dispersed reach favourable germination sites and develop into seedlings (Shaik, 2012:449). Germination probability is further affected by pre- and post-dispersal seed predation by frugivorous animals such as birds, monkeys, baboons and wild pigs, however, these animals also act as seed dispersers. Consumption of fruits facilitates the scarification of seed coats by metabolic acids in the digestive system before the seeds are excreted. Baboons process the seeds mechanically by chewing off the pulp, scarring the seed coat in the process, before the seed is spitted out (Shaik, 2002:451). The natural seed germination period for *C. dentata* is between 6 and 18 months, which further suggests that poor germination may be due to either exogenous dormancy imposed by the hard seed coat, or endogenous dormancy imposed by the embryo (Shaik, 2012:449). *Curtisia dentata* therefore requires pre-sowing treatment in the form of scarification. Scarification of the seed coat using sulphuric acid (H_2SO_4) for example, destroys the tough cuticle and suberin layers, and also the thickened palisade cells and osteosclereids, thereby improving the permeability of the seed coat to essential gases.

2.9 AN OVERWIEW OF THE ANALYTICAL METHODS USED FOR THIS STUDY

2.9.1 Chromatography

Chromatography is a technique used for the separation of a mixture. The mixture is dissolved in a fluid, called the mobile phase, which carries it through a structure holding another material, called the stationary phase. The separation of components depends on the differential partitioning between the mobile and the stationary phases (Patel, 2018:289). Chromatographic methods such as column chromatography (CC), thin layer chromatography (TLC), preparative TLC (PTLC), liquid chromatography (LC) and gas chromatography (GC) are used for the separation, purification and identification of compounds in extracts of, for instance plant material, for qualitative and quantitative analysis (Coskum, 2016). GC is a powerful tool for the analysis and identification of essential oils such as terpenoids and phenolic compounds (Agostini-Costa, Vieira, Bizzo, Silveira and Gimenes, 2012:131; Ingle, Deshmukh, Padole, Dudhare, Moharil and Khelurkar, 2017:34). GC is a column chromatography method in which the mobile phase is a gas and the stationary phase is either an immobilized liquid or a solid packed in a closed tube (Ismael and Nielsen, 2010:476). GC has the advantage of high sensitivity and resolution, and its coupling to a mass spectrometer allows for the identification of new and minor compounds of a solution without laborious

isolation procedures (Chierrito, De Castro Cunha, Koike, Gonçalves and De Oliveira, 2012:119,120). HPLC-MS is used for the separation and identification of organic and inorganic solutes in samples. Modern HPLC uses a non-polar solid phase and a polar liquid phase. High pressure up to 400 bars is required to elute the analyte through the column before they pass through a detector, such as a diode array detector (DAD). A DAD measures the absorption spectra of the analytes to aid in their identification. HPLC is useful for compounds that cannot be vaporized or that decompose under high temperature, and it provides a good complement to gas chromatography for detection of compounds (Ingle *et al.*, 2017:34). TLC is frequently used for the analysis of complex mixtures as it contributes greatly to the preliminary characterization of extracts and fractions. TLC plates' stationary phase contain fluorescence indicators. Under UV light, substances with a chromophore absorb radiation and become fluorescent, and therefore allow for prior identification of the presence of a substance (Chierrito et al., 2012: 123). Where TLC is employed for the separation and determination of the presence of compounds, without retrieval of the sample, PTLC is a purification process which allows for the isolation of pure compounds in a mixture. PTLC is regarded as a simple and inexpensive technique for the separation and purification of small quantities of substances. The compounds can be retrieved from the preparative TLC plates and analyzed further by means of GC-MS and/or NMR analysis (Chierrito et al 2012:124). CC is a method used to separate a mixture of substances into individual compounds by means of fractionation (Ismael and Nielsen, 2010:479). Fractionation of solutes occur as a result of differential migration through a closed tube of stationary phase. The stationary phase must be prepared by making use of packing material such as resin or gel, which is then packed into the column. Columns may be either dry or wet packed. For the wet packed model (used in this study), a slurry is prepared and added to the column until the desired bed height is achieved. Solvent is continuously added for the mobile phase to settle. The sample to be fractionated is mixed with a small volume of mobile phase and applied in a layer at the top of the column. In case of a gravity-fed system (used in this study), the eluent is simply siphoned from a reservoir to the column. The flow rate is governed by the hydrostatic pressure, measured as the distance between the level of liquid in the reservoir and the level of the column outlet. The process of passing the mobile phase through the stationary phase is called elution, and the portion that emerges from the outlet end of the column is called the eluate, or the fraction. As elution proceeds, components of the sample are selectively retarded

by the stationary phase based on the strength of interaction with the stationary phase. They are thus eluted at different times (Ismael and Nielsen, 2010:479).

2.9.2 Metabolomics

A metabolome encompasses the whole set of metabolites found in a biological sample, which include intermediates of primary metabolism, as well as secondary metabolites and growth regulators (Robinson, 2009:4; Johansson, 2013:11). Metabolites are the products of cellular regulatory processes. Their concentrations, however, depend on responses to genetic and/or environmental changes (Fiehn, 2002:155). Metabolomic research thus provides wide-ranging qualitative and quantitative information about changes in small-molecule metabolites, and simplifies perceptions on metabolic processes (Xie, 2007:30). Metabolomic research does therefore not only emphasized a select few compounds produced by a plant but focuses on the whole metabolome.

Metabolomic analysis can be divided into non-targeted and targeted analyses. Targeted analysis or metabolic profiling, centres around the quantification of a defined group of metabolites that are linked by a class of molecules or a metabolic pathway. To the contrary, non-targeted analysis targets as many metabolites as possible, thereby creating a universal metabolic fingerprint (Robinson, 2009:5). Non-targeted and target analyses are interdependent because of their shared objectives, which include improved biological understanding and diagnostic capabilities (Robinson, 2009:5).

NMR spectra are required for both targeted non-targeted analyses and interpretation, and also for specific metabolite identification and quantification. ^{1}H NMR signals are generated by the excitement of protons or other nuclei in a magnetic field with a high-frequency pulse, which in turn causes motion of magnetic moments of these protons or nuclei (Zheng, Zhang, Ragg, Raftery and Vitek, 2011:1637). A chemical shift is achieved through the transformation of the signal and is a measure in which the dependence of nuclear magnetic energy on the chemical and electronic environment in the molecule, is expressed (Zheng *et al.*, 2011:1637). A spectrum displays chemical shifts in parts per million or ppm, which is the difference between a resonance frequency and that of a reference substance in hertz , over a frequency of magnetic field in megahertz (Zheng *et al.*, 2011:1637).

A metabolite contains one or more protons. Each proton produces one or more peaks and the number of peaks generated by a metabolite, as well as their location and heights are reproducible and are determined by the chemical structure of the molecule (Zheng *et al.*,

2011:1637). Researchers can therefore identify metabolites by matching the observed spectra to reference spectra in a database. The area of the peaks from a metabolite is directly related to its abundance (concentration), thus, as the abundance changes, the heights of the peaks of metabolites change (Zheng *et al.*, 2011:1637).

2.9.3 Mass spectrometry

NMR can measure all small molecules from a metabolome, and the sample can be recovered for further analysis, however, NMR has significant sensitivity limitations. It is therefore only applied to high concentration compounds. In comparison to NMR, mass spectrometry (MS) is more sensitive, and can thus be used for compounds in lower concentrations (De Hoffmann and Stroobant, 2007:387, 388).

MS also has the ability to identify compounds through elucidation of their chemical structure by MS/MS (tandem mass spectrometry) and determination of their exact masses. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. The processes and methods are extensively discussed by De Hoffmann and Stroobant, (2007: 15-170). Mass spectrometry allows relative concentration determinations to be made between samples with a dynamic range of approximately 10 000. Absolute quantification is also possible, but reference compounds need to be used (De Hoffmann and Stroobant, 2007: 388).

Although mass spectrometry can be used as a standalone technique, it is more commonly coupled with other techniques, for example gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS) and high performance liquid chromatography–mass spectrometry (HPLC-MS) (Want, Cravatt and Siuzdak, 2005:1941-1951; De Hoffmann and Stroobant, 2007: 388).

Mass spectrometry's characteristics, i.e. unequalled sensitivity, detection limits, speed and diversity of its applications, have raised it to an outstanding position among analytical methods (de Hoffmann and Stroobant, 2007:1).

2.10 CONCLUDING STATEMENT

This chapter highlights the cultural importance of *C. dentata* tree bark as medicine and the subsequent continued harvesting of *C. dentata* stem bark. *Curtisia dentata* trees are, however, listed on both the DEFF List of Protected Tree Species and the South African Red List of Plant Species, which renders the harvesting of bark from *C. dentata* without a license an illegal activity. South Africa has comprehensive legislation regulating the harvesting of material from protected plant species, however, legislation failed to provide a sustainable long-term solution for the overexploitation of protected medicinal trees such as *C. dentata*. Large scale cultivation of *C. dentata* should therefore be considered for the conservation of the species and for the maintaining genetic diversity, species diversity and biodiversity. The cultivation of *C. dentata* trees is also necessary if the bark is to be used as medicine by future generations. However, establishing cultivated *C. dentata* trees through cultivation programs requires the drafting of appropriate management plans, including security options, to prevent the poaching of stem bark from the cultivated plants.

Several risks are involved when using raw or semi-processed plant material and many adverse effects were reported after the use of traditional medicines. The harvesting of medicinal plant material from different locations, different tree sizes (ages) and through all seasons result in inconsistent plant material being delivered to the medicinal trade markets. A draft policy on African tradition medicine was formulated in 2008, but has not been finalized or adopted to date, as several challenges are hindering its finalization. The development of acceptable standards of safety and quality for African traditional medicines, and the regulation thereof is, however, essential to ensure consumer safety.

It is furthermore essential to determine the variations of bioactive compound concentrations in the plant parts used as traditional medicines as variations in bioactive compounds may affect dosages, and any substance may be a potential toxin, depending on the dose consumed. It is, however, important to realize that safe and effective dosages can only be determined by pre-clinical and clinical studies. It is further important to determine how much the chemical compound concentrations vary seasonally and how much it varies when collected from different localities in South Africa. Differences in climatic conditions and other environmental factors such as soil type and structure, photoperiod, light intensity and light quality, and water and nutrient availability and chemotypes may affect both the composition

and concentrations of the chemical compounds. Changing environmental conditions due to the effects of climate change may also affect secondary metabolite production.

The identification and quantification of chemical compounds in *C. dentata* tree stem bark is necessitated by the importance of the bark as medicine, the reported factors affecting secondary metabolite production and the limited body of knowledge regarding both the beneficial and potential harmful compounds present in the bark. Identifying potential beneficial compounds may serve as motivation for the cultivation of *C. dentata* trees, whereas the identification of potential harmful compounds may serve as motivation for the isolation, purification and standardization of the beneficial compounds to ensure consumer safety.

CHAPTER 3. GC-MS ANALYSIS AND EVALUATION OF PHYTOCHEMICALS IN *Curtisia dentata* (Burm.f.) C.A.Sm. STEM BARK EXTRACTS

3.1 INTRODUCTION

Nature offers plants, however, none can be labelled as 'medicinal' unless validated for its activity and its therapeutic usefulness (Feliciano, Castro, Lopéz-Peréz and Del Olmo: 2014:127, 128). Plants have, however, been used therapeutically since time immemorial. Secondary metabolites produced in plants are involved in physiological responses during the plants' interactions with their biotic and abiotic environments. Of particular importance for plants is their role in plant defence (Gunatilaka, 2012:4). Even though secondary metabolites are produced from only a few primary metabolite precursors, secondary metabolites display substantial chemical diversity, often with specific compound classes restricted to particular plant phylogenetic clades. Additionally, secondary metabolites are produced through a variety of biosynthetic modes (Bednarek, 2014:5). Throughout the ages, man's search for well-being and pleasure stimulated man's approach to nature. The current upsurge in the interest in plants as sources of medicine is today one of the most frequently mentioned reasons for preserving biodiversity, as the discovery of novel compounds may result in new leads for drug development (Barreiro, Fraga and Lima, 2012:81, 83).

The stem bark of *C. dentata* trees is traditionally used for the treatment of stomach ailments, diarrhoea, sexually transmitted diseases, as an aphrodisiac, and to clean the blood. In the Eastern Cape Province of South Africa, the stem bark of *C. dentata* is also used to treat heartwater in cattle (Shai, 2007:26; Venter and Venter, 2012:22; Van Wyk *et al.*, 2013:110; Fadipe *et al*., 2015: 976). Fadipe *et al.*, (2017:2) also reported its use as treatment of malaria and tuberculosis among the Sotho communities. Even though some compounds were isolated from the leaves of *C. dentata* trees (which are not generally used medicinally) (Shai, 2007:1-188; Fadipe *et al.*, 2017:1-6), and its stem bark has shown therapeutic potentials (McGaw *et al.* 2000:247-263; Doughari *et al.* 2012: 1042-1051; Wintola and Afolayan, 2017: 237-246), the chemical composition of *C. dentata* stem bark remains largely unknown. The chemical compounds consumed when using the stem bark as traditional medicine, are therefore also unknown. It is further unknown whether there are chemical compounds that may cause adverse effects, or which may for instance be genotoxic, mutagenic or carcinogenic, as reported for many commonly used food and medicinal plants (Prinsloo, Nogemane and Street

2018:27-39). Chemical compounds which may be life threatening over time due to the accumulation of potential hazardous substances in the human body after consumption is an additional factor to consider. Traditional health practitioners prepare the stem bark of *C. dentata* into remedies as part of a collection of plant species. This mixture, called *khubalo*, consists of the stem bark of *Harpephyllum caffrum, Eckebergia capensis, Myrsine melanophloeos* (previously *Rapanea melanophloeos*), *Protorhus longifolia* and *Sclerocarya birrea*, and also *Cassine papillosa, Pterocelastrus* spp. and *C. dentata*. Due to the scarcity of the last three species, these mixtures are regarded as special mixes (Cunningham, 1988:26; Van Wyk *et al*., 2013:110). Concerns therefore include how the phytochemicals of the different plant species interact, and what these chemicals are metabolized to once inside the human body (Rietjens, Al Huseiny and Boersma, 2011:87-95; Boonpawa, Spenkelink, Rietjens and Punt, 2014:287-299). The fact that the same plant may contain both beneficial and hazardous substances has been ignored thus far. Additionally, as traditional medicines are neither standardised nor regulated, the possibility of naturally occurring contaminants, such as bacteria, fungi, insects and their excreta, may therefore also be additional concerns. Furthermore, the widespread use of herbicides and pesticides may contaminate the soils in which the trees grow due to its dispersion potential (Carvalho, 2017:48), which in turn may be taken up by the trees through their roots, or it can be absorbed from the air through the leaves. Other toxic substances such as heavy metals, which either occur naturally in soil or are dispersed into the environment through anthropogenic activities (Nagajyoti, Lee and Sreekanth, 2010:199-216), may also pose serious health risks when medicinal plant material containing these substances are consumed. In this Chapter, the aim is therefore to list the whole spectrum of metabolites found in *C. dentata* stem bark as identified by GC-MS analysis, using different extraction solvents, and to report, where available, the documented potentials of these chemical substances, as well as the possible risks involved when consuming extracts of raw or semi-processed *C. dentata* stem bark.

3.2. METHODS AND MATERIALS

3.2.1 Plant material used for extraction of chemical compounds

Due to the conservation status of *C. dentata*, collection of large volumes of stem bark harvested from wild trees is neither permitted nor advisable. *Curtisia dentata* stem bark was therefore purchased at the Faraday traditional medicine market in Johannesburg on the 18th of September

2019, using the Zulu name for the material (*Umlahleni*) (Figure 3.1). The bark was first chiselled into smaller chips using a hammer and chisel before grinding it to a powder in a blender.

Figure 3.1: The *C. dentata* stem bark (*Umlahleni*) purchased at the traditional medicine market.

The homogenised material was weighed (1.0002 Kg) and placed into two conical flasks (2 x 500 g). A flow chart of the extraction process (Figure 3.2), which involves sequential extraction [(2 x 1 L) x 2], using analytical grade hexane (97%), DCM (99.8%), ethanol (99.9%) (Sigma, St. Lois, USA), and water as solvents, in that order, is shown below. With each solvent, the mixtures were shaken in an orbital shaker (United Scientific, Johannesburg, South Africa) for 24 hours and the extract filtered through a Buchner funnel fitted with Munktell Ahlstrom filter paper (65 g/m^2). To remove the solvents from the filtrates, each of the hexane, DCM and ethanol filtrates were individually steam distilled and condensed using a Stuart distillation system. The hexane and ethanol filtrates were distilled and condensed using a Cometa Ivymen vacuum pump, whereas the DCM filtrate was distilled and condensed without the use of the vacuum pump. After distillation, the concentrated extracts were poured into pre-weighed beakers and air-dried. The dried crude extracts yielded an orange-yellow coloured, thick, oily substance as crude extract of hexane (4.3 g), a dark turmeric-coloured powder as a crude extract of DCM (4.7 g), and a dark wine-red coloured sticky substance as a crude extract of ethanol (104.1 g). The aqueous filtrate was dried in a, E2-2 plus Genevac

(United Scientific, Johannesburg, South Africa) set at a temperature of 26° C, which yielded 21.4 g of crude extract.

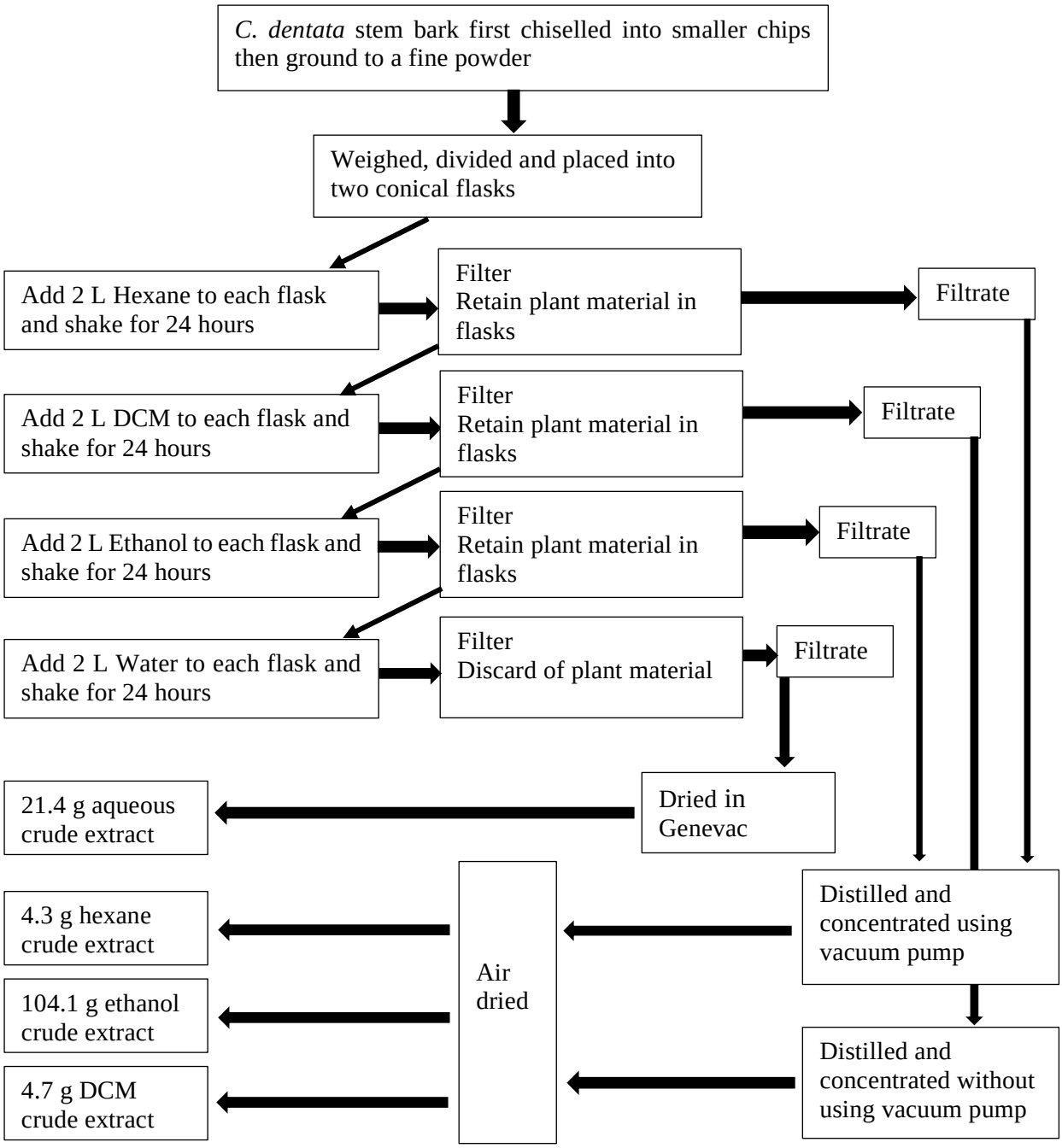


Figure 3.2: A flow diagram of the chemical compound extraction process.

3.2.2 Thin layer chromatography of the crude extracts

For thin layer chromatography (TLC), samples of the hexane, DCM, and ethanol crude extracts dissolved in DCM, methanol and acetone respectively, were spotted onto aluminium-backed TLC plates (Merck silica F_{254} plates) and allowed to dry before development in separate glass TLCs tanks with solvents of varying polarities, namely hexane/ethyl acetate (9:1), hexane/ethyl acetate (6:4), DCM/methanol (9:1) and DCM/methanol/acetone (6:3:1). The plates were removed from the solvents and air dried for both the shortwave (254 nm) and longwave (365 nm) UV visualization of the compounds in a Spectraline CM-10A fluorescent analyst. Visible spots were marked. The plates were further immersed in an acid stain consisting of sulfuric acid and methanol in a ratio of 1:9, after which the plates were air-dried, and then developed using the lower setting of a Master ProHeat PH-2100 dual temperature heat gun.

3.2.3 GC-MS analysis of crude extracts

From each of the dried hexane, DCM and ethanol extracts, 200 mg was weighed and put into separate Eppendorf tubes. In the Eppendorff tube containing the hexane crude extract, 2 ml hexane was added to dissolve the extract. Similarly, 2 ml DCM was added to the tube containing the DCM crude extract, and 2 ml ethanol was added to the tube containing the ethanol crude extract. The tubes were vortexed for 3 minutes each on a Labsmart MX-5 vortex mixer and sonicated for 20 minutes in a Branson 1800 ultrasound bath, after which 0.5 ml of the concentrated supernatants were filtered and poured into separate Restek GC/LC analytical bottles. As the supernatants were highly concentrated, 1 ml pure hexane, DCM and ethanol were added to the respective analytical bottles for dilution, and to increase the volume to 1.5 ml for GC-MS analysis.

GC-MS analysis was executed according to the methods applied by Wang *et al.* (2016), with some slight modifications. A Leco® 7890B GC chromatograph with a Gerstel multisampler and Chroma Time of Flight spectrometer software (TOF-MS) optimized for a Pegasus®4D mass spectrometer, was used for the analysis of the crude extracts and each sample was run twice. The extracted compounds were separated with two columns. The main column (Restek®) has a length of 32 m, an internal diameter of 250 µm and a film thickness of 0.025 µm, and the secondary column has a length of 0.790 m with an internal diameter of 250 µm. Analysis of the samples was carried out under the following conditions: splitless injection of 0.2 ml was employed; injection temperature was 33° C, held for 3 minutes, then increased at

a rate of 10° C per minute to the target temperature of 180° C; without a holding period, the rate of temperature increase was increased to a rate of 40°C per minute until a temperature of 220° C is reached. Helium (99.99%) was the carrier gas used, with a flow rate of 1.0 µl per minute (constant flow). The ionizing energy was 70 eV. The scan rate was 50 scans per second and the mass spectral scan range was 60 to 600 (Mhz). The relative constituent content was expressed as normalized percent peak area. Samples of pure hexane, DCM and ethanol were used as controls and were included and run as blanks when each of the respective crude extract samples were run. Each crude extract sample was analyzed twice.

3.2.4 Criteria for the consideration of chemical compounds for evaluation

Only the chemical compounds of which the chromatograms have an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer were considered for evaluation. The inclusion of chemical compounds is further limited to compounds on which information could be found from either chemical databases such as PubChem (Kim, Chen, Cheng, Gindulyte, He, He, Li, Shoemaker, Thiessen, Yu, Zaslavsky, Zhang and Bolton, 2019), the Human Metabolome Database (Wishart, Feunang, Marcu, Guo, Liang, Vázquez-Fresno, Sajed, Johnson, Li, Karu, Sayeeda, Lo, Assempour, Berjanskii, Singhal, Arndt, Liang, Badran, Grant, Serra-Cayuella, Liu, Mandal, Neveu, Pon, Knox, Wilson, Manach and Scalbert, 2018:D608-D617), the National Institute of Standards and Technology (NIST) (Shen, Siderius, Krekelberg, and Hatch, 2017) and/or peer reviewed literature. Hazardous effects were sourced from the European Chemicals Agency (ECHA), EU regulations, the Agency for Toxic Substances and Disease Registry (ATSDR), the Toxic Substances Control Act (TSCA), and ThermoFischer Scientific Safety Data Sheets.

3.3 RESULTS

The TLC plates for the hexane, DCM and ethanol crude extracts of *C. dentata* stem bark are shown in Figure 3.2, indicating the presence of compounds in solvents ranging from low polarity to high polarity.

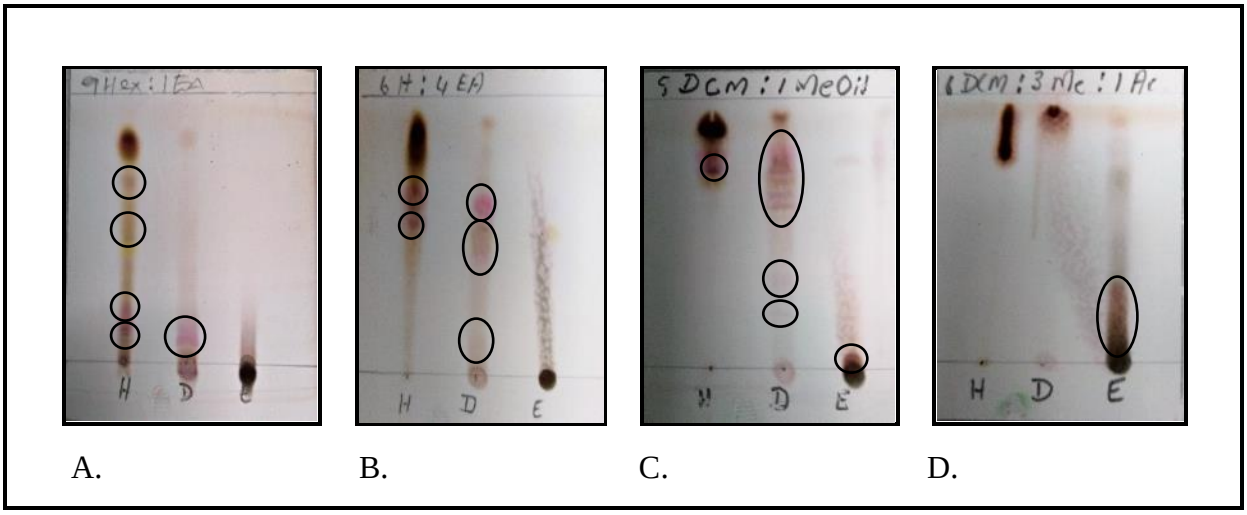


Figure 3.3: The TLC plates of the hexane (left), DCM (middle) and ethanol crude extracts (right) of *C. dentata* stem bark, processed using solvents of increasing polarity. The mobile phase used were A, hexane and ethyl acetate with a ratio of 9:1, B, hexane and ethyl acetate with a ratio of 6:4, C, DCM and methanol with a ratio of 9:1, and D, DCM, methanol and acetone with a ratio of 6:3:1.

The complete list of chemical compounds detected in the hexane crude extracts of *C. dentata* stem bark (195 chemical compounds) is shown in Table 3.1. The list of chemical compounds detected in the DCM crude extracts of *C. dentata* stem bark (11 chemical compounds) is shown in Table 3.2, whereas the list of chemical compounds detected in the ethanol crude extracts of *C. dentata* stem bark (13 chemical compounds) is shown in Table 3.3. In all three tables, the chemical formula for each compound is included. For correct identification of compounds, the GC-MS fragmentation pattern of the compounds identified were compared to the fragmentation patterns in databases such as the database of the National Institute of Standards and Technology (NIST).

Table 3.1: List of chemical compounds detected in the hexane crude extracts of *C. dentata* stem bark, including the chemical formulas.

	Compound	Formula
1	(-)-1,2,2à,3,3,4,6,7,8,8à-decahydro-2à,7,8-trimethylacenaphthylene	unknown
2	(1R,4aS,8aR)-1-Isopropyl-4,7-dimethyl-1,2,4a,5,6,8a-hexahydronaphthalene	$C_{15}H_{24}$
3	2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	$C_{11}H_{14}O_3$
4	4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	$C_{10}H_{12}O_3$
5	á-Famesene	$C_{15}H_{24}$
6	.tau.-Muurolol	$C_{15}H_{26}O$
7	1-(1,2,3-Trimethyl-cyclopent-2-enyl)-ethanone	$C_{10}H_{16}O$
8	1,2,5-Oxadiazole	$C_4H_4N_4O_2$
9	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	$C_{20}H_{30}O_4$
10	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	$C_{20}H_{32}$
11	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-	$C_{15}H_{24}$
12	1,4-benzenediol, 2,5-dimethoxy-	$C_8H_{10}O_4$
13	1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate]	$C_{18}H_{24}N_2O_4S_4$
14	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	$C_{15}H_{26}O$
15	1,7-Octadiene-3,6-diol, 2,6-dimethyl-	$C_{10}H_{18}O_2$
16	1,E-11,Z-13-Octadecatriene	$C_{18}H_{32}$
17	10,18-Bisnorabieta-8,11,13-triene	$C_{18}H_{26}$
18	10-Methyl-8-tetradecen-1-ol acetate	$C_{17}H_{32}O$
19	11-Methyldodecanol	$C_{13}H_{28}O$
20	1b,5,5,6a-Tetramethyl-octahydro-1-oxa-cyclopropa[a]inden-6-one	$C_{13}H_{20}O_2$
21	1-Butanamine, 3-methyl-	$C_5H_{13}N$
22	1-Butanone, 1-(2,4,6-trihydroxy-3-methylphenyl)-	$C_{11}H_{14}O_4$
23	1-Butoxy-2-propanol acetate	$C_9H_{18}O_3$
24	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$
25	1-Eicosanol	$C_{20}H_{42}O$
26	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-	$C_{18}H_{20}$
27	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene	$C_{15}H_{24}$
28	1-Methyl-10,18-bisnorabieta-8,11,13-triene	$C_{19}H_{28}$
29	1-Naphthalenepropanol, à-ethenyldecahydro-à,5,5,8a-tetramethyl-2-methylene-, [1S-[1à(R*),4aá,8aà]]-	$C_{20}H_{34}O$
30	1-Octadecanesulphonyl chloride	$C_{18}H_{37}ClO_2S$
31	1-Octanol, 2-butyl-	$C_{12}H_{26}O$
32	1-Octen-4-ol	$C_8H_{16}O$
33	1-Phenanthrenecarboxaldehyde, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1à,4aà,10aá)]-	$C_{20}H_{28}O$
34	1-Propanone, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-	$C_{10}H_{12}O_4$
35	1-Undecene, 7-methyl-	$C_{12}H_{24}$
36	2(1H)-Pyridinethione, 3-hydroxy-	$C_5H_5NO_S$
37	2(5H)-Furanone, 5,5-dimethyl-	$C_6H_8O_2$
38	2,2-Dimethyl-5-hexen-3-ol	$C_8H_{16}O$
39	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$

40	2,4-Pentadien-1-ol, 3-pentyl-, (2Z)-	$C_{10}H_{18}O$
41	2,5-Furandione, 3,4-dimethyl-	$C_6H_6O_3$
42	2,6-Dimethoxybenzoquinone	$C_8H_8O_4$
43	2,8-Dioxatricyclo[5.3.0.0(3,9)]decane	unknown
44	2-Acetoxy-1,1,10-trimethyl-6,9-epidioxydecalin	$C_{15}H_{24}O_4$
45	2-Butanamine, (S)-	$C_4H_{11}N$
46	2-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	$C_{10}H_{16}O$
47	2-Ethyl-oxetane	$C_5H_{10}O$
48	2-Heptanol, 3-methyl-	$C_8H_{18}O$
49	2-Hexanone, 4-methyl-	$C_7H_{14}O$
50	2-Hexenoic acid	$C_6H_{10}O_2$
51	2-Hexyl-1-octanol	$C_{14}H_{30}O$
52	2H-Pyran-2-one, 5,6-dihydro-6-propyl-	$C_8H_{12}O_2$
53	2-Isopropenyl-5-methylhex-4-enal	$C_{10}H_{18}O$
54	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$
55	2-Nonanol	$C_9H_{20}O$
56	2-Pentanol	$C_5H_{12}O$
57	2-Propynoic acid, methyl ester	$C_4H_4O_2$
58	3-Butyn-1-ol	C_4H_6O
59	3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	$C_{14}H_{16}O_2S_2$
60	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$
61	4-(6-Methyl-4-methylene-3,4,5,6-tetrahydro-2H-pyran-2-yl)-1-butanol	$C_{11}H_{20}O_2$
62	4-Chloro-3-n-hexyltetrahydropyran	$C_{10}H_{21}ClO$
63	4H-1,3,2-Dioxaborin, 6-ethenyl-2-ethyl-4-methyl-4-(2-methylpropyl)-	$C_{12}H_{21}BO_2$
64	4-Methoxycarbonyl-4-butanolide	$C_6H_8O_4$
65	5,9-Dimethyl-2-(1-methylethyl)cyclodecane-1,4-dione	$C_{15}H_{26}O_2$
66	5-Iodo-nonane	$C_9H_{19}I$
67	6-[3,4-Dichlorophenyl]-N-ethyl-N-methyl-1,2,4,5-tetrazin-3-amine	$C_{13}H_{20}Cl_2N_2$
68	7-Methyl-Z,Z-8,10-hexadecadien-1-ol acetate	$C_{19}H_{34}O_2$
69	9,19-Cyclolanostan-3-ol, acetate, (3á)-	$C_{32}H_{54}O_2$
70	9-Hexadecen-1-ol, (Z)-	$C_{16}H_{32}O$
71	9-Oxa-bicyclo[3.3.1]non-6-en-2-ol	$C_8H_{12}O_2$
72	à-Calacorene	$C_{15}H_{20}$
73	Acetamide, 2,2,2-trichloro-N-(3-methylphenyl)-	$C_9H_8Cl_3NO$
74	á-copaene	$C_{15}H_{24}$
75	Allopregnan-3à-ol-20-one	$C_{21}H_{34}O_2$
76	à-Muurolene	$C_{15}H_{24}$
77	Aniline	$C_6H_5NH_2$
78	Aromandendrene	$C_{15}H_{24}$
79	à-ylangene	$C_{15}H_{24}$
80	Baccharane	$C_{30}H_{54}$
81	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	$C_9H_{10}O_4$
82	Benzene, (isothiocyanatomethyl)-	C_8H_7NS
83	Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$
84	Benzene, 1,3-dimethoxy-	$C_8H_{10}O_2$

85	Benzene, 1,3-dimethyl-	$C_6H_4(CH_3)_2$ or C_8H_{10}
86	Benzene, 1-methyl-3-(1-methylethyl)-	$C_6H_4(CH_3)_2$ or C_8H_{10}
87	Benzene, isocyanato-	C_7H_6NO
88	Benzenemethanol, à,à-dimethyl-	$C_9H_{12}O$
89	Benzothiazole	C_7H_5NS
90	Benzyl alcohol	C6H5CH2OH or C_7H_8O
91	Bicyclo[2.2.2]oct-5-en-2-one	$C_8H_{10}O$
92	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	$C_{15}H_{24}$
93	Bromopride, N4-trifluoroacetyl-	unknown
94	Butane, 2-methyl-	C_5H_{12}
95	Butanoic acid, 4-hydroxy-	$C_4H_8O_3$
96	Butyrolactone	$C_4H_6O_2$
97	Carbonic acid, phenyl propyl ester	$C_6H_{12}O_3$
98	Chloromethanesulfonyl chloride	$CH_2Cl_2O_2S$
99	cis-á-Farnesene	$C_{15}H_{24}$
100	cis-Calamenene	$C_{15}H_{22}$
101	ç-Muurolene	$C_{15}H_{24}$
102	Coniferyl aldehyde	$C_{10}H_{10}O_3$
103	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	$C_{15}H_{24}$
104	Cyclopentane, methyl-	C_6H_{12}
105	Cyclopropanecarboxylic acid, oct-3-en-2-yl ester	$C_{10}H_{14}O_2$
106	Cyclopropanemethanol, 2-methyl-2-(4-methyl-3-pentenyl)-	unknown
107	Cyclotetradecane	$C_{14}H_{28}$
108	Decyl trifluoroacetate	$C_{12}H_{21}F_3O_2$
109	Dibutyl phthalate	$C_{16}H_{22}O_4$
110	Di-epi-1,10-cubenol	$C_{15}H_{26}O$
111	Diisooctyl phthalate	$C_{24}H_{38}O_4$
112	Divinyl sulfide	C_4H_6S
113	dl-à-Tocopherol	$C_{29}H_{50}O_2$
114	Docosane, 11-butyl-	$C_{26}H54$
115	Dodecane, 1-iodo-	$C_{12}H_{25}I$
116	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$
117	Dodecane, 2-methyl-	$C_{13}H_{28}$
118	Dodecyl acrylate	$C_{15}H_{28}O_2$
119	Eicosane	$C_{20}H_{42}$
120	Eicosane, 2-methyl-	$C_{21}H_{44}$
121	Erucic acid	$C_{22}H_{42}O$
122	Ethanone, 1-(1,3-dimethyl-3-cyclohexen-1-yl)-	$C_{10}H_{16}O$
123	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	$C_{10}H_{12}O_4$
124	Ethyl 2-acetamido-3,3,3-trifluoro-2-(4-[(5-methyl-3-isoxazolyl)sulfamoyl]anilino)propionate	$C_{17}H_{19}F_3N_4O_6S$
125	Ethyl à-d-glucopyranoside	$C_8H_{16}O_6$
126	Formamide, N,N-dibutyl-	$C_9H_{19}NO$
127	Geranyl acetate, 2,3-epoxy-	$C_{12}H_{20}O_3$
128	Glycoldial, bis-O-pentafluorobenzyloxime	$C_{16}H_6F_{10}N_2O_2$
129	Heneicosane	$C_{21}H_{44}$

130	Heptacosane	$C_{27}H_{56}$
131	Heptadecane	$C_{17}H_{36}$
132	Heptadecane, 2,6,10,14-tetramethyl-	$C_{21}H_{44}$
133	Heptadecane, 2,6-dimethyl-	$C_{19}H_{40}$
134	Heptadecane, 2-methyl-	$C_{18}H_{38}$
135	Heptanoic acid	$C_7H_{14}O$
136	Hexacosane	$C_{26}H_{54}$
137	Hexadecane	$C_{16}H_{34}$
138	Hexadecen-1-ol, trans-9-	$C_{16}H_{32}O$
139	Hexadecenoic acid, Z-11-	$C_{16}H_{30}O_2$
140	Hexane, 2,2,5-trimethyl-	C_9H_{20}
141	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$
142	Hexanedioic acid, mono(2-ethylhexyl)ester	$C_{16}H_{24}O_4$
143	Hexanoic acid	$C_6H_{12}O_2$
144	Hexanoic acid, 3-tridecyl ester	$C_{19}H_{38}O_2$
145	Homovanillyl alcohol	$C_9H_{12}O_3$
146	Humulene	$C_{15}H_{24}$
147	Isoledene	$C_{15}H_{24}$
148	Kaur-16-en-18-oic acid, methyl ester, (4á)-	$C_{21}H_{32}O_2$
149	Methoxyolivetol	$C_{12}H_{18}O_2$
150	Methyl 2-butynoate	$C_5H_6O_2$
151	Methyl kolavenate	$C_{21}H_{34}O_2$
152	Methylene chloride	CH_2Cl_2
153	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aá,8aà)-	$C_{15}H_{24}$
154	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	$C_{15}H_{24}$
155	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	$C_{15}H_{18}$
156	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
157	Nonanal	$C_9H_{18}O$
158	Nonanoic acid	$C_9H_{18}O_2$
159	Octacosane	$C_{28}H_{58}$
160	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$
161	Octane, 2,2,6-trimethyl-	$C_{11}H_{24}$
162	Octane, 2,3,3-trimethyl-	$C_{11}H_{24}$
163	Oleic Acid	$C_{18}H_{34}O_2$
164	Oxacycloheptadec-8-en-2-one, (8Z)-	$C_{16}H_{28}O_2$
165	Oxalic acid, isobutyl nonyl ester	$C_{15}H_{28}O_4$
166	o-Xylene	$C_6H_4(CH_3)_2$ or C_8H_{10}
167	Pentadecane	$C_{15}H_{32}$
168	Pentadecanoic acid	$C_{15}H_{30}O_2$
169	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$
170	Pentadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$
171	Pentane	C_5H_{12}
172	Pentane, 2,2,3,4-tetramethyl-	C_9H_{20}
173	Phenol	C_6H_5OH
174	Phenol, 2,5-bis(1,1-dimethylethyl)-	$C_{14}H_{25}O_2$

175	Phenol, 2-methoxy-4-(1-propenyl)-	$C_{10}H_{12}O_2$
176	Phthalic acid, 4-chloro-3-methylphenyl undecyl ester	$C_{26}H_{23}ClO_4$
177	Phytol	$C_{20}H_{40}O$
178	Propane, 1-bromo-2-methyl-	C_4H_9Br
179	Propylure	$C_{18}H_{32}O_2$
180	Scopoletin	$C_{10}H_8O_4$
181	Silane, cyclohexyldimethoxymethyl-	$C_9H_{20}O_2Si$
182	Silanediamine, 1,1-dimethyl-N,N'-diphenyl-	$C_{14}H_{28}N_2Si$
183	Succinic anhydride	$C_4H_4O_3$
184	Tetracosane	$C_{24}H_{50}$
185	Tetradecane	$C_{14}H_{30}O$
186	Tetradecanoic acid	$C_{28}H_{56}O_4$
187	Tetrahydropyran Z-10-dodecenoate	$C_{17}H_{30}O_3$
188	Thiophene, 2,5-dihydro-	C_4H_6S
189	trans-3-Methyl-4-octanolide	$C_9H_{16}O_2$
190	trans-Calamenene	$C_{15}H_{22}$
191	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$
192	Undecanoic acid	$C_{11}H_{22}O_2$
193	Ursolic aldehyde	$C_{30}H_{48}O_2$
194	Vanillin	$C_8H_8O_3$
195	Vitamin E	$C_{29}H_{50}O_2$

Table 3.2: List of chemical compounds detected in the DCM crude extracts of *C. dentata* stem bark, including their chemical formulas.

	Compound	Formula
1	2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	$C_{11}H_{14}O_3$
2	1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis-	$C_{15}H_{26}O$
3	2-Ethyl-oxetane	$C_5H_{10}O$
4	3-Butyn-1-ol	C_4H_6O
5	4H-1,3,2-Dioxaborin, 6-ethenyl-2-ethyl-4-methyl-4-(2-methylpropyl)-	$C_{12}H_{21}BO_2$
6	Cyclobutane, ethyl-	C_6H_{12}
7	Methylene chloride	CH_2Cl_2
8	n-Hexane	C_6H_{14}
9	Oxeladin	$C_{20}H_{32}NO_3$
10	Phenol, 2-methoxy-4-(1-propenyl)-	$C_{10}H_{12}O_2$
11	Pregnan-3,11-diol-20-one	$C_{30}H_{60}O_3Si_3$

Table 3.3: List of chemical compounds detected in the ethanol crude extracts of *C. dentata* stem bark, including their chemical formulas.

	Compound	Formula
1	2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	$C_{11}H_{14}O_3$
2	3-Butyn-1-ol	C_4H_6O
3	4H-1,2,4-Triazol-4-amine	$C_2H_4N_4$
4	4H-1,3,2-Dioxaborin, 6-ethenyl-2-ethyl-4-methyl-4-(2-methylpropyl)-	$C_{12}H_{21}BO_2$
5	á-Sitosterol	$C_{29}H_{50}O$
6	Benzaldehyde, 3-bromo-5-methoxy-4-[(4-methylphenyl)methoxy]-	$C_{10}H_{11}BrO_4$
7	Ethanol, 2-nitro-	$C_2H_6NO_3$
8	Glycerin	$C_3H_8O_3$
9	Lactic acid	$HC_3H_5O_3$
10	Methylene chloride	CH_2Cl_2
11	Oxalic acid	$C_2H_2O_4$
12	Oxirane, (fluoromethyl)-	C_3H_5FO
13	Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester	unknown

Based on the criteria stipulated, a total number of 94 chemical compounds in the *n*-hexane crude extracts are included in Tables 3.4, 3.5 and 3.6. Table 3.4 (26 chemical compounds), lists the chemical compounds in the *n*-hexane crude extracts with potential beneficial properties, whereas Table 3.5 (47 chemical compounds), lists the chemical compounds with potential risks to human health and/or which are documented as environmental hazards, and Table 3.6 (21 chemical compounds), lists possible contaminants present in the *n*-hexane extracts of the *C. dentata* stem bark analyzed. In each of the tables, a brief overview of each

compound's documented characteristics is provided, which also explains the categorization of the compounds. Chemical compounds of biological origin, such as from insects, bacteria and fungi are considered as contaminants as it may not be part of the matrix of chemical compounds in the stem bark of *C. dentata* trees, but may be due to the presence of bacteria and/or fungi etc. in or on the stem bark. This is particularly considered since the stem bark was purchased from a traditional medicine market where both plant material and animal parts are being displayed unwrapped, either on the ground or on top of cardboard covered crates or tables or on shelves in an open air environment. The probability of biological contamination is therefore high. However, freshly collected plant material may also host a variety of microorganisms and insects.

The main chemical compounds in the n-hexane crude extracts of *C. dentata* stem bark are: 1,4-S,S-2,5-bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate] (22.8%), 2-ethyl-oxetane (16.8%), butane, 2-methyl (8.0%), bromopride, N_4-trifluoroacetyl- (7.9%), octacosane (4.4%) and benzene, 1-methyl-3-(1-methylethyl)- (M-xylene) (3.3%). No information is available for bromopride, N4-trifluoroacetyl-, however, bromopride, as a single constituent is a dopamine antagonist used as an anti-emetic (www.drugbank.ca/drugs/DB09018).

The DCM crude extracts of *C. dentata* stem bark contain 11 chemical compounds (Table 3.2), of which five compounds have an 80% or higher similarity to the chemical compounds in the main database of the equipment. These compounds are 2-ethyl-oxetane (44.7%), oxeladin (4.3%), 2,6-dimethoxy-4-(prop-1-en-1-yl)phenol (0.1%), phenol, 2-methoxy-4-(1-propenyl)- (0.01%) and cyclobutane, ethyl- (0.005%). The characteristics of these compounds are discussed in Table 3.7.

The ethanol crude extracts of *C. dentata* stem bark contain 13 chemical compounds (Table 3.3), of which the only compounds with an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer are pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester (4%), oxalic acid (0.2%), 2,6-dimethoxy-4-(prop-1-en-1-yl)phenol (0.09%) and glycerin (0.02%). The characteristics of these chemical compounds are discussed in Table 3.8.

Table 3.4: Chemical compounds in the *n*-hexane extracts of *C. dentata* stem bark with possible beneficial potentials.

Compound	Description	Source
1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate]	This compound is the major compound in the n-hexane extracts of C. *dentata* stem bark, but it is also one of the major compounds in the leaves of *Cinnamomum iners*. It was suggested that 1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate] supports the cytotoxicity of naphthalene derivatives in the HCT 116 cell line	Ghalib, Hashim, Sulaiman, Mehdi, Anis, Rahman, Ahamed, Malik and Majid, 2011:2157
2-ethyl-oxetane	2-ethyl-oxetane also occurs in *Clinacanthus nutans* (Acanthaceae) and *Cyperus esculentus* (Cyperaceae) leaves. The methanolic extract matrix of *C. nutans* showed antinociceptive properties via the opiod/nitric oxide mediated, but cGMP-independent pathways.	Rahim, Zakaria, Sani, Omar, Yakob, Cheema, Ching, Ahmad and Kadir, 2016:4 Imo, Uhegbu, Arowora, Ezeonu, Opara, Nwaogwugwu and Anigbo, 2019:411
(1R,4aS,8aR)-1-Isopropyl-4,7-dimethyl-1,2,4a,5,6,8a-hexahydronaphthalene Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aá,8aà)-	Also known as zizanene or alpha amorphene, it is a member of the cadinene family of sesquiterpenes. It has a role as a plant metabolite and has been identified in various plant species as a component of essential oils. Amongst the plants reported to contain either alpha amorphene or zizanene are *Santolina Chamaecyparissus, Vetivera zizinoides and Centaurea* spp. However, in none of these species, the compound zizanene or alpha amorphene were isolated from these essential oils.	https://pubchem.ncbi.nlm.nih.gov/compound/101708 Khubeiz and Mansour, 2016:372-378 Raja, Rajamani, Suresh, Joel and Uma, 2018:1709-1713 Erdoğan, Sümer, Özçinar, Çakilcioğlu, Demirci, Başer, and Kivçak, 2017: 69-73
α-Copaene α-Muurolene ç-Muurolene Aromandendrene α-ylangene Humulene (α-Caryophyllene)	These components in *C. dentata* stem bark are components of essential oils of various plant species, including *Cinnamomum* spp., *Pimenta* sp., and *Piper* spp. Aromandendrene, humulene and nerolidol (nerolidol is an environmental hazard) are also found in *Cannabis* spp. All these components are sesquiterpenes and have the same chemical formula – $C_{15}H_{24}$. Although these essential oil matrices showed anti-microbial, antioxidant, anti-inflammatory, antiseptic, anti-fungal and immunomodulatory properties, there may be a possibility of synergistic action of two or more components in these essential oils	Hazekamp, Tejkalova and Papadimitriou, 2016:1-16 Takeara, Gonçalves, Dos Santos Ayres and Guimarães, 2017:81-93 Paula, Ferri, Bara, Tresvenzol, Sá and Paula, 2011:643-650 Kumar, Kumari and Mishra, 2019:1735-1761

α-Calacorene	α-Calacorene is a sesquiterpene also present in the essential oils of *Ageratum conyzoides, Ficus carica, Pelargonium citrosum* and more. No literature on the pharmacological potentials of only α-calacorene is available.	Koame, Toure, Kablan, Bedi, Tea, Robins, Chalchat and Tonzibo, 2018:163 Badgujar, Patel, Bandivdekar and Mahajan, 2014:1490 Matsuda, Surgeoner, Heal, Tucker and Maciarello, 1996:73
2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	This chemical compound is a methoxyphenol and is used as flavouring agent in food. Biologically it has a role as a nutrient. The compound has no hazard statement. 2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol is also found in *Olea europaea* waste (after oil extraction), and suggestions were made as to its use in the food, pharmaceutical and cosmetic industries.	https://pubchem.ncbi.nlm.nih.gov/compound/4-Propenyl-2_6-dimethoxyphenol https://webbook.nist.gov/cgi/cbook.cgi?ID=C20675950 Del Pozo, Bartoli, Puy and Fàbregas, 2018:160-167
Heneicosane Heptacosane Hexacosane	These chemical components are aliphatic alkanes with roles as plant metabolites. Heneicosane, heptacosane and hexacosane are also present in *Glandora rosmarinifolia* essential oil. The essential oil matrix showed *in vitro* anti-tumour activity against various cancer cell lines, and pro-oxidant activity. Essential oils from *Campanula olympica*, which also contain heneicosane, showed activity against several microbial organisms. The results obtained may be due to the synergistic activity of two or more chemical components in the essential oil matrix.	Poma, Labbozzetta, Notarbartolo, Bruno, Maggio, Rosselli, Sajeva and Zito, 2018:1-11 Tosun, Kahriman, Çoskunçelebi, Genç, Karaoglu and Yayli, 2011:2389-2391
trans-Calamenene cis-Calamenene	This sesquiterpenoid is also a constituent in the essential oils of *Piper peltata* leaves and the fruits and leaves of *Ficus carica*. No further information on the component is available.	Soltana, Flamini and Hammami, 2017:162 Pino, Marbot, Bello and Urquiola, 2004:126
1,E-11,Z-13-Octadecatriene	This fatty acid is present in various plant species, including the seed oil extracts of *Persia americana* (avocado) and the leaf and seed extracts of *Senna tora.* The compound matrices showed activity against both Gram positive and Gram negative bacteria. No literature is available on the pharmacological activities of 1,E-11,Z-13-Octadecatriene as a single component of oils.	Omeje, Ozioko and Opmeje, 2018:1-3 Alao, Ololade and Nkeonye, 2018:1-4

2-Isopropenyl-5-methylhex-4-enal	This compound is also present in *Piper nigrum* (black pepper) and *Mentha piperita* (mint). Both these essential oils have therapeutic properties, but its activities may be attributed to the synergistic action of two or more compounds. No information is available for only 2-Isopropenyl-5-methylhex-4-enal and the compound has no ECHA warnings.	Mohammed, Omran and Hussein, 2016:977-996 Afridi, Ali, Abbas, Rehman, Khan and Shahid, 2016:90-97
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1methylethenyl)-	Also known as (-)-beta-elemene, the compound is currently undergoing clinical trials (CT) for lung cancer treatment (CT phase 2) and as maintain treatment for complete remission patients of newly diagnosed malignant gliomas following standard treatment (CT phase 3).	https://pubchem.ncbi.nlm.nih.gov/compound/10583 https://pubchem.ncbi.nlm.nih.gov/compound/10583#section=Clinical-Trials
Cyclotetradecane	Cyclotetradecane is a cycloalkane present in various herbs and spices, including *Capsicum annuum* (paprika) and *Vanilla planiflora.* Cyclotetradecane is also a constituent of dog saliva. The dog saliva matrix inhibited the growth of *Bacillus pumilus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis, Salmonella typhi and Serratia marcescens*, but not of *Bacillus subtilis.*	Kocsis, Amtmann, Mednyánszki and Korány, 2002:195-203 Gurnani, Kapoor, Mehta, Gupta and Mehta, 2014:769-776 Dhasarathan, Arunkumar, Blessy, Sowmaya and Sowmaya, 2013:1-6
Di-epi-1,10-cubenol	Other names for Di-epi-1,10-cubenol include (1R,4R,4Ar,8aR)-4,7-dimethyl-1-propan-2-yl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-4a-ol. It is a sesquiterpene also present in *Cedrus atlantica*, a medicinal plant occurring in Marocco. The essential oil mixture of *Cedrus atlantica* showed insecticidal activity. Additional literature on di-epi-1,10-cubenol is extremely limited.	https://pubchem.ncbi.nlm.nih.gov/compound/91748749 Ainane, Charaf, Elabboubi, Elkouali, Talbi, Benhima, Cherroud and Ainane, 2019:474-485
dl-à-Tocopherol	dl-à-Tocopherol is a form of vitamin E also present in *Cassia siamea* stem bark. It has potent anti-oxidant properties, however, the matrix of 17 compounds found in *Cassia siamea*, including dl-à-tocopherol, showed anti-fertility properties in male Wistar rats.	https://pubchem.ncbi.nlm.nih.gov/compound/DL-alpha-Tocopherol Dewal, Lakhne and Gupta, 2018:3050-3053

Homovanillyl alcohol	Homovanillyl alcohol (HA), also known as 3-O-methyl-hydroxytyrosol, is the product of the catechol-O-methyltransferase (COMT) enzyme. Hydroxytyrosol is a strong anti-oxidant, anti-proliferative, pro-apoptic, anti-platelet and anti-inflammatory polyphenol. HA is, however, chemically far more stable in biological fluids than hydroxytyrosol, which suggests that HA can exert benefits for longer. HA occurs in olive oil and red wine. The moderate use of these products have a protective effects on cardiovascular disease.	De la Torre, Corella, Castañer, Martínez-González, Salas-Salvador, Vila, Estruch, Sorli, Arós, Fiol, Ros, Serra-Majem, Pintó, Gómez-Gracia, Lapetra, Ruiz-Canela, Basora, Asensio, Covas and Fitó, 2017:1297-1304
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	Also known as beta-cadinene, the compound is also a constituent of all spice. This component is used as flavouring agent or flavour modifier. Additional information on the chemical is extremely limited.	https://pubchem.ncbi.nlm.nih.gov/compound/52138
n-Hexadecanoic acid	Also known as palmitic acid, n-Hexadecanoic acid is a saturated long-chain fatty acid, a plant metabolite, a *Daphnia magna* metabolite and a fungal metabolite. N-Hexadecanoic acid has shown anti-inflammatory properties.	https://pubchem.ncbi.nlm.nih.gov/compound/Palmitic-acid Vasudevan, Kalarickal, Pradeep, Ponnuraj, Chittalakkotto and Madathilkovilakathu, 2012:434-439
Ursolic aldehyde	Ursolic aldehyde is a derivative of ursolic acid. Ursolic acid (UA) is a constituent of various fruits and herbs and is therefore a component of the human diet. Pharmacological effects of UA include anti-inflammatory, hepatoprotective, anti-tumour, cardio-protective, neuroprotective, anti-microbial, anti-hyperlipidemic, anti-diabetic, anti-fungal, anti-viral and trypanocidal effects. *Verbena bonariensis*, a plant containing ursolic acid, lupeol and *β*-sitosterol showed anti-fertility activity in female hamsters. However, the efficacy of ursolic acid is compromised by its low solubility in water and resultant difficulty in penetrating permeating biological membranes.	Pironi *et al.*, 2018:86 Pathak, Mallurwar, Kondalkar and Soni, 2005:4

Table 3.5: Chemical compounds in the *n*-hexane extracts of *C. dentata* stem bark which pose potential risks to human health and/or are environmental hazards, including each compound's percentage area (concentration).

Compound	Description	Source
1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl- (0.006%)	Also known as nerolidol, it is a sesquiterpenoid, a component of essential oils but an environmental hazard. It has a biological role as nutrient, energy storage and energy source. Nerolidol showed anti-malarial activity and activity against airborne microbes and the dimorphic fungus *Candida albicans*. Nerolidol is also a known pheromone used for pest control. Nerolidol causes skin, eye, and respiratory irritation and is very toxic to aquatic life.	https://pubchem.ncbi.nlm.nih.gov/compound/Nerolidol https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/16160 Saito, Rodriguez, Vega, Sussman, Kimura and Katzin, 2016:641-646 Krist, Banovac, Tabanca, Wedge, Gochev, Wanner, Schmidt and Jirovetz, 2015:143-148 Thacker and Train, 2010:158
2,6-Dimethoxybenzoquinone (0.035%)	2,6-Dimethoxybezoquinone is a quinone (phenolic compound) and a haustorium-inducing factor released by host plants such as sorghum, which results in the growth and spread of parasitic plants with subsequent large-scale damage to crop plants. The compound was also isolated from the leaves of *Caesalpinea crista* and showed anthelminthic activity. The compound carries several ECHA warnings, which include: acute toxicity if swallowed, serious eye irritation and eye damage, causes skin irritation, acute toxic if inhaled and respiratory tract irritation. Older literature found that at physiological concentrations, 2,6-dimethoxybenzoquinone has anti-bacterial potential, however, in higher concentrations it is mutagenic, cytotoxic, genotoxic and hepatotoxic.	https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/131760 https://hmdb.ca/metabolites/HMDB0029673 Wang, Steele, Murdoch, Lai and Yoder, 2019:1878 Suryawanshi and Patel, 2017:1-7 Brambilla, Robbiano, Cajelli, Martelli, Turmolini and Mazzei, 1988:1011-1015
2-Heptanol, 3-methyl- (0.033%)	3-methyl-2-butanol is an alcohol component of the bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* isolated from fermented milk. The bacteria are used for the production of yoghurt. The compound is corrosive and an irritant. It therefore causes skin irritation, serious eye damage, and may cause respiratory tract irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/35784 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/186492 Dan, Wang, Wu, Jin, Ren and Sun, 2017:1-14

O-Xylene (0.009%) Benzene, 1,3-dimethyl- (also known as M-Xylene) (0.009%) Benzene, 1-methyl-3-(1-methylethyl)- (also known as M-Xylene) (3.333%)	These chemical substances belongs to the family of tuolenes and carry several ECHA warnings: fatal when swallowed or passes through airways, acute dermal and inhalation toxicity, causes serious eye damage, specific target organ toxicity, particularly the respiratory tract. It is therefore a health hazard. Both O-xylene and M-xylene are, however, constituents of the essential oils of *Chrysophyllum albidum* leaves, seeds and stem bark. The plant is used as traditional medicine in Nigeria for the treatment of yellow fever, malaria, diarrhoea, vaginal and dermatological infections. Both O-xylene and M-xylene are present in *Artocarpus camansi* leaves, stem bark and nuts, with the stem bark containing exceptionally high concentrations. M-Xylene is one of the major constituents in *C. dentata* stem bark.	https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/40127 https://pubchem.ncbi.nlm.nih.gov/compound/meta-xylene Ishola, Aboaba, Chaudhary and Ekundayo, 2017:234-245 Ante, Aboaba, Siddiqui and Chaudhary, 2016:203-210
Pentane (0.366%)	Pentane is a straight chain alkane and a volatile oil component. It is a component also present in *Humulus lupus* (hops), celery sticks and soy beans, and is present in alcoholic beverages. Pentane is a potentially toxic chemical and is both a health hazard and an environmental hazard as it may be fatal if swallowed and enters airways, it affects the peripheral nervous system and is toxic to aquatic life with long-term effects. It can cause anorexia, euphoria, dizziness, headache, depression, confusion, anoxia, narcosis and loss of consciousness and coma at high concentrations. Ingestion may also cause pulmonary toxicity.	https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 https://pubchem.ncbi.nlm.nih.gov/compound/Pentane https://hmdb.ca/metabolites/HMDB0029603
Pentadecane (0.008%)	Pentadecane is an acyclic hydrocarbon, a plant metabolite and a volatile oil component but is a health hazard since swallowing it may be fatal. Pentadecane as a single chemical component showed inhibitory growth effects on the protozoa *Leishmania infantum.*	https://pubchem.ncbi.nlm.nih.gov/compound/Pentadecane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/77760 Bruno, Castelli, Migliazzo, Piazza, Galante, Lo Verde, Calderone, Nucatolo and Vitale, 2015:701-705

Phenol (0.002%)	Phenol is a 1-hydroxy-4-unsubstituted benzenoid and a toxic compound. The occurrence of phenols in the environment stems from the use of pesticides. Phenol may, however, also be formed by natural processes e.g. the decomposition of organic matter. Phenol accumulates in the brain, muscles, kidneys and liver and health hazards include necrosis, kidney, liver, muscle and eye damage, mutagenicity and carcinogenicity.	https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 https://pubchem.ncbi.nlm.nih.gov/compound/Phenol Michalowicz and Duda, 2006:347-362 Mishra and Kumar, 2017:17-22
Tetradecane (0.006%)	Tetradecane is a straight chain alkane with roles as plant metabolite and volatile oil component. Tetradecane is also found in walnuts, lemon balm, buckwheat, cucumbers, all spice and green bell peppers. Tetradecane is a health hazard and in humans, it is associated with diseases such as Crohn's disease, ulcerative colitis, non-alcoholic fatty liver disease, asthma and celiac disease. Old literature indicated that a noncarcinogenic dose of tetradecane promoted papilloma growth in the skins of Swiss mice. Tetradecane is also a major constituent of the roots of *Limonium gmelinii*, which is introduced into the medicine and State Pharmacopoeia of Kazakhstan.	https://hmdb.ca/metabolites/HMDB0059907 https://pubchem.ncbi.nlm.nih.gov/compound/Tetradecane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/36082 Ikhsanov, Zhussopova, Kasymova, Ross and Zhusopova, 2019:160 Sicé, 1966
Hexanoic acid (0.0009%)	Also known as caproic acid, it is a medium chain fatty acid and a plant metabolite. Hexanoic acid was also found in the aerial parts of *Echinops cephalotes*, which is used as medicinal plant in Iran. Hexanoic acid has antifungal properties against *Botrytis cinerea*, *Cucumerinum lagenarium*, *Fusarium oxysporum*, *Myrothecium verrucaria* and *Trichoderma viride.* Hexanoic acid is, however, corrosive and have the health hazards of being acute toxic when skin is exposed to the compound and can cause severe skin burns and eye damage. No ECHA warning exists for its exposure via ingestion, however, the high number of reports on hexanoic acid acute toxicity suggests that a high probability exists that the chemical may be fatal if swallowed.	https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/30551 https://pubchem.ncbi.nlm.nih.gov/compound/Hexanoic-acid Mohammadi, Piri and Dinarvand, 2019:169 Pohl, Kock and Thibane, 2011:62

Aniline (0.0108%)	Aniline is a primary aromatic amine, first isolated from the destructive distillation of indigo. Aniline is a highly acrid poison and can be toxic if ingested, or by skin contact. Routes of exposure include food. Aniline damages hemoglobin and the damaged hemoglobin cannot carry oxygen, a condition called methemoglobinemia. Its severity depends on the dose and length of exposure. Dizziness, headaches, irregular heartbeat, convulsions, coma, and death may occur. Long-term exposure may cause symptoms similar to those experienced in acute high-level exposure. The available studies in humans are inadequate to determine whether exposure to aniline can increase the risk of developing cancer in people. Rats that ate food contaminated with aniline developed cancer of the spleen.	https://hmdb.ca/metabolites/HMDB0003012 https://pubchem.ncbi.nlm.nih.gov/compound/Aniline https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 Agency for Toxic Substances and Disease Registry (ATSDR), 1995
(1R,4aS,8aR)-1-Isopropyl-4,7-dimethyl-1,2,4a,5,6,8a-hexahydronaphthalene (0.003%)	Also known as Cadina-1(10), 4-diene, delta amorphene or delta cadinene and it also has the chemical formula $C_{15}H_{24.}$ This chemical is a sesquiterpene and has the hazard code of X1, which means that is an irritant. The risks include that it may cause skin, eye and respiratory irritation.	www.chemicalbook.com/RiskAndSafety.htm#risk https://pubchem.ncbi.nlm.nih.gov/compound/Cadina-1_10_4-diene
Butane, 2-methyl (8.076%)	2-Methylbutane, also known as Isopentane is a volatile component emitted by some species of pine and oak trees, ferns and mosses. It is one of the major components in the *n*-hexane extracts of *C. dentata* stem bark. 2-Methylbutane is a health hazard and an environmental hazard. The compound is a skin, eye, and respiratory tract irritant. Coughing, dizziness, drowsiness, headache, shortness of breath, sore throat, irregular heartbeat, and loss of consciousness have been reported in humans following inhalation of high levels of isopentane in the air. Stomach pain, nausea and vomiting may occur following ingestion.	https//:pubchem.ncbi.nih.gov/compound/Isopentane#section=Overview

Octacosane (3.196%)	Octacosane is a straight-chain alkane found in at least 13 other plant species. Octacosane is one of the major components in the *n*-hexane extracts of *C. dentata* stem bark and has a role as a plant metabolite. Octacosane can cause skin, eye and respiratory irritations. No report indicates safety risks for ingestion of Octacosane. Octacosane isolated from *Moschosma polystachyum* showed mosquitocidal activity.	https://pubchem.ncbi.nlm.nih.gov/compound/Octacosane#section=Safety-and-Hazards https://pubchem.ncbi.nlm.nih.gov/compound/Octacosane Rajkumar and Jabenesan, 2004: 87-89
a-famesene (0.038%) cis-farnesene (0.032%)	A sesquiterpenoid that is also a component of the oil of *Perilla frutescens.* It poses a health risk as it may be fatal if swallowed. Farnesene is also an aphid pheromone and trials with the aphid alarm pheromone, farnesene, have shown that combinations of reduced-rate insecticide and this pheromone are effective at controlling cereal aphids. This pesticide is, however, not available commercially yet.	https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/18205 https://pubchem.ncbi.nlm.nih.gov/compound/alpha-Farnesene#section=Safety-and-Hazards Thacker and Train, 2010:164
Benzothiazole (0.006%)	The parent of the class of benzothiazoles and an alkaloid. It has a role as a plant metabolite, a xenobiotic and an environmental contaminant. Benzothiazole was first isolated from cranberries but has since been found as components in the tail gland of the red deer, as a sulphur component of wine, as well as the French Oak wood wines are aged in, and is present in the aroma fraction of tea leaves. Benzothiazole as a single component is, however, acute toxic when swallowed, when in contact with skin and when inhaled.	https://pubchem.ncbi.nlm.nih.gov/compound/Benzothiazole https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/37086 Le Bozec and Moody, 2009:639
Phenol, 2,5-bis(1,1-dimethylethyl)- (0.124%)	Also known as 2,5-di-tert-butylphenol it has a role as a plant metabolite and a mammalian metabolite. This compound may cause serious skin, eye and respiratory tract irritation and may cause long-lasting harmful effects to aquatic life.	https://pubchem.ncbi.nlm.nih.gov/compound/2_5-Di-tert-butylphenol https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/64161

Phenol, 2-methoxy-4-(1-propenyl)- (0.038%)	Also known as isoeugenol, it is a phenylpropanoid that is an isomer of eugenol. Occurs in ylang-ylang oil, cinnamon oil, clove oil and other essential oils. Isoeugenol is used in the manufacturing of perfumeries, flavourings and essential oils. Medicinally it is used as local antiseptic and analgesic, as well as the synthetic production of vanillin. However, isoeugenol carries several warnings: acute toxic if swallowed, acute dermal toxicity, cause skin corrosion/irritation, may cause allergic skin reactions and cause serious eye irritation. Isoeugenol showed carcinogenic activity in mice and rats.	https://pubchem.ncbi.nlm.nih.gov/compound/Isoeugenol https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/82994 Gunnels, Harper, Rathman, Serbus and Simmons (2010:62)
Phytol (0.005%)	Phytol is a diterpene fatty alcohol with a role as a plant metabolite and an algal metabolite. Phytol is used as schistosomicide drug and as precursor for the synthetic manufacturing of vitamins E and K1. Phytol is also reported to have antioxidant, anti-inflammatory, antimicrobial neuroprotective and anti-diabetic properties, however, it also showed cytotoxic and genotoxic activities. Phytol carries several warnings: it causes skin corrosion/irritation and is very toxic to aquatic life with long lasting effects to aquatic organisms.	https://pubchem.ncbi.nlm.nih.gov/compound/Phytol https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/7367 Islam, Streck, Barros de Alencar, Silva, Da Conceição Machado, Da Conceição Machado, Gomes, Paz, Ferreira da Mata, De Castro e Sousa, Da Costa, Rolim, Da Silva and De Carvalho Mel Cavalcante, 2017:93-101
Vanillin (0.003%)	Vanillin is methoxyphenol and a benzaldehyde and is the major component of vanilla bean extract. It has a role as a plant metabolite. Synthetic vanillin, instead of natural vanilla extract, is used as a flavouring agent in foods, beverages, and pharmaceuticals. Vanillin has shown neuroprotective activity in male Wistar rats. The study showed that vanillin alleviated motor and non-motor impairments, oxidative stress and neurochemical deficits in the rats. Vanillin also showed promising potentials for the treatment of Alzheimer's disease, Huntington's disease and depression. In humans, however, vanillin may cause severe eye irritation, skin allergy reactions and migraine.	https://pubchem.ncbi.nlm.nih.gov/compound/Vanillin https://hmdb.ca/metabolites/HMDB0012308 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/125259 ThermoFischer Scientific Safety Datasheet CAS No. 121-33-5 Anand, Kuranha, Wahal, Mahajan, Mehta, Satija, Sharma, Vyas, Kurhana, 2019:1000-1004

1-Naphthalenepropanol, à-ethenyldecahydro-à,5,5,8a-tetramethyl-2-methylene-, [1S-[1à(R*),4aá,8aà]]-(0.083%)	Also known as 13-epimanool, it is a labdane diterpenoid and an anti-fungal agent and a metabolite. Warnings include skin irritation/corrosion, serious eye irritation and respiratory irritation. The compound is very toxic to aquatic life.	https://pubchem.ncbi.nlm.nih.gov/compound/238792 https://pubchem.ncbi.nlm.nih.gov/compound/13-Epi-manool https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/169899
1-Octanol, 2-butyl-(0.004%)	This fatty alcohol is a metabolite observed in cancer metabolism but also has a role as a human metabolite. 1-Octanol, 2-butyl- is also found in alcoholic beverages and is a constituent of *Humulus lupus*. Distinct warnings as to its long-lasting toxicity to aquatic organisms were issued.	https://pubchem.ncbi.nlm.nih.gov/compound/19800 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/87735 https://hmdb.ca/metabolites/HMDB0041288
1-Propanone, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-(0.003%)	Also known as propriovanillone, this constituent is acute toxic if swallowed. It is also acute toxic to aquatic life with long-lasting effects. 1-Propanone, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)- was also found in *Leontodon hispidus* subsp. *hispidus*. Old literature suggested that this compound plays the same role in the plant respiratory system as is performed by citric (or isocitric) acid in the animal system.	https://pubchem.ncbi.nlm.nih.gov/compound/4-hydroxy-3-methoxypropiophenone https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/236592 Michalska, Pieron and Stojalowska, 2018:393-394 West and Hibbert, 1943:1170
2,5-Furandione, 3,4-dimethyl-(0.004%)	Also known as 2,3-dimethylmaleic anhydride. This compound has been isolated from *Colocasia esculenta* corms and leaves in two different studies and tested for its potentials as eco-friendly insecticide. This compound is however acute toxic if swallowed, may cause skin irritation/corrosion and serious eye irritation. It may also cause respiratory irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/2_3-Dimethylmaleic-anhydride ttps://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/111380 Rajeshekar, Tonsing, Shantibala and Manjunath, 2016:1-7 Devi, Mayanglambam, Chanu, Singh, Sougrakpan, Nameirakpam, Singh, Potshangbam, Sahoo and Rajeshekar, 2018:1-6

4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (0.018%)	Also known as coniferyl alcohol, this compound is a phenylpropanoid with roles as monolignol, mouse metabolite and volatile oil component. Coniferyl alcohol is an intermediate in the biosynthesis of eugenol, coumarin and stilbene and is also a component of *Zingiber officinale* (ginger) extracts. This compound has been reported to exhibit antioxidant, anti-microbial and anti-inflammatory properties, however, the compound also has the warnings that it may cause skin corrosion/irritation, serious eye irritation and respiratory irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/Coniferyl-alcohol https://hmdb.ca/metabolites/HMDB0012915 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/269265 Shareef, Muhammed, Hussein and Hameed, 2016:817-837
Benzaldehyde, 4-hydroxy-3,5-dimethoxy- (0.011%)	Also known as syringaldehyde, it is hydroxybenzaldehyde and a plant metabolite. The metabolite was isolated from *Pisonia aculeata* and *Panax japonicus* var. major. Syringaldehyde exhibits hypoglycemic activity, and also has potent antioxidant and anti-inflammatory effects on peripheral blood mononuclear cells from patients of myocardial infarction, however it is acute toxic if swallowed and may cause skin, eye and respiratory tract irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/8655 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/110296 Shahzad, Mateem, Kauser, Naeem, Hasan, Abidi, Nayeem, Faizy and Moin, 2020:691-704
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]- (0.005%)	Also known as beta-caryophyllene. It is a bi-cyclic sesquiterpenoid commonly associated with the presence of α-Humulene (previously α-caryophyllene) and is found in the essential oils of many plant species, including *Cannabis sativa*, *Syzigium aromaticum, Origanum vulgare*, *Cinnamomum* spp., *Piper* spp. *Aegle marmelos* and *Humulus lupus.* Beta caryophyllene has anti-inflammatory properties and can induce apoptosis in lymphoma and neuroblastoma cells. Beta caryophyllene does, however, pose a health risk. It may be fatal if swallowed and cause allergic reactions.	https://pubchem.ncbi.nlm.nih.gov/compound/6887 https://hmdb.ca/metabolites/HMDB0036792 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/107662 Gertsch, Leonti, Raduner, Racz, Chen, Xie, Altmann, Karzak and Zimmer, 2008:1 Sain, Naoghare, Devi, Daiwile, Krishnamurthi, Arrigo and Chakrabarti, 2014:45-55

Butanoic acid, 4-hydroxy- (0.00001%)	4-hydroxybutanoic acid or 4-hydroxybutyric acid (HBA) is a naturally occurring short-chain fatty acid and is the immediate precursor of gamma amino butyric acid (GABA) with neuromodulatory and anaesthetic properties. HBA is illegal in many countries, but its use is legal in patients with narcolepsy. Its street name is juice or ecstasy and is used illegally as a date rape drug. In low doses it is a euphoriant and at high doses it inhibits the central nervous system, thereby inducing sleep and inhibiting respiratory drive. In high levels HBA can act as acidogen, neurotoxin and metaboxin with several adverse effects ranging from headaches and vomiting to ataxia, seizures, heart and kidney abnormalities, liver damage, coma and possibly death.	https://pubchem.ncbi.nlm.nih.gov/compound/4-Hydroxybutanoic-acid http://www.hmdb.ca/metabolites/HMDB0000710 https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/223990
Butyrolactone (0.0004%)	An isomer of butyrolactone, beta-butyrolactone is an irritant, a health hazard and is flammable. It causes skin and eye irritation and is suspected of being a carcinogen. Gamma butyrolactone has a role as a metabolite and a neurotoxin. It is acute toxic if swallowed, causes skin and eye irritation and can cause eye damage. It is further toxic if inhaled and have narcotic effects.	https://pubchem.ncbi.nlm.nih.gov/compound/beta-Butyrolactone ttps://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/3816
Coniferyl aldehyde (0.011%)	Coniferyl aldehyde or 4-hydroxy-3-methoxycinnamaldehyde is a phenolic compound with roles as plant metabolite and anti-fungal agent. Coniferyl aldehyde has radioprotective properties but may cause skin irritation or corrosion, eye and respiratory tract irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/4-Hydroxy-3-methoxycinnamaldehyde https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/90940 Mun, Kim, Choi, Kim and Lee, 2018:1038
2-Hexenoic acid (0.10%)	2-hexenoic acid is a metabolite involved in fatty acid biosynthesis. It is also a compound present in the aerial parts of ferns used in the perfumery industry. 2-hexenoic acid is corrosive and can cause serious skin and eye damage.	https://pubchem.ncbi.nlm.nih.gov/compound/2-Hexenoic-acid https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/41416 Froissard, Fons, Bessiére, Buatois and Rapior, 2011:1723-1726

Cyclopentane, methyl- (0.033%)	Methylcyclopentane is a cycloalkane isolated from *Helianthus annuus* and is also a constituent of the methanolic extracts of wild ginger. The chemical is, however, highly flammable and may be fatal if swallowed. It causes narcotic effects and also skin corrosion or irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/methylcyclopentane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/75118 Nayak, Jena, Mittal and Joshi, 2014:1
Eicosane (0.021%)	Eicosane is an acyclic alkane present in plant species such as *Limonium gmelinii*, the methanol extracts of *Pimpinella anisum*, or it may derive from petroleum products. Lesions in the liver that did not cause liver dysfunction, have been associated with the ingestion of mineral oils (a natural mixture of long chain alkanes) used for dietary or medicinal reasons. Eicosane may be fatal if swallowed. The potential for long chain alkanes, such as Eicosane, to cause cancer in humans has not been assessed by the International Agency for Research on Cancer or the U.S. National Toxicology Program 14th Report on Carcinogens.	https://pubchem.ncbi.nlm.nih.gov/compound/Eicosane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/97581 Ikhsanov et al. 2019:161 Sun, Shahrajabian and Cheng, 2019:5
Erucic acid (0.009%)	Erucic acid is a monounsaturated omega-9 fatty acid mainly found in plants of the Brassicaceae family such as brussel sprouts, cabbage, broccoli, rapeseed, canola and mustard seed. Many Brassica species' erucic acid content range between 40 and 50%. However, erucic acid may cause skin, eye and respiratory irritation. Processing of oils such as rapeseed oil involves the reduction of erucic acid to levels less than 2%. The < 2% concentration of erucic acid is an evaluation index for edible rapeseed products.	https://pubchem.ncbi.nlm.nih.gov/compound/Erucic-acid https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/73863 Lu, Aziz, Sturtevant, Chapman and Guo, 2020:1
Hexadecane (0.012%)	Hexadecane is a straight chain hydrocarbon with roles as plant metabolite and volatile oil component. The E681 strain of the rhizobacteria *Paenibacillus polymyxa* produces hexadecane that triggers the induction of systemic resistence against phytopathogens. The compound, however, poses a health risk due to its potential to be fatal if swallowed.	https://pubchem.ncbi.nlm.nih.gov/compound/Hexadecane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/23463 Park, Lee, Kloepper and Ryo, 2013:1-4

Nonanal (0.02%)	Nonanal or nonanaldehyde is a medium chain saturated fatty acid occurring in various essential oils. It has roles as plant metabolite and human metabolite. It is also a metabolite observed in cancer metabolism. Warnings regarding nonanal include that it can cause skin and eye damage and that it is harmful to aquatic life with long-lasting effects. Data is lacking for any further health hazard classification.	https://pubchem.ncbi.nlm.nih.gov/compound/Nonanal https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/107406
Nonanoic acid (0.013%)	Nonanoic acid or pelargonic acid straight chain fatty acid occurring as esters in the oils of *Pelargonium* sp. The compound has roles as plant metabolite, *Daphnia magna* metabolite, fungal metabolite and antifeedant. Nonanoic acid is used in the preparation of plasticiser and lacquers. The ammonium salt form of nonanoic acid is used as herbicide. Nonanoic acid showed anti-fungal activity against *Crinipellis pernicosa*, *Microsporum gypseum, Moniliophthora roreri, Myrothecium verrucaria* and *Trichoderma viride.* The chemical, however, causes skin irritation and serious eye damage. It is further harmful to aquatic life with long-lasting effects.	https://pubchem.ncbi.nlm.nih.gov/compound/Nonanoic-acid https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 Pohl *et al.* 2011:62
Tetradecanoic acid (0.023%)	Also known as myristic acid, this compound is a long chain saturated fatty acid also found in palm oil, coconut oil and butter fat. It is used as flavouring agent but also showed anti-fungal activity against *Alternaria solani, Aspergillus niger, Candida albicans, Emericella nidulans, Fusarium oxysporum, Penicillium glabrum* and *Penicillium italicum.* Tetradecanoic acid causes skin and eye irritation and can cause eye damage and allergic reactions. It is also hazardous to aquatic life with long lasting toxic effects. Myristic acid is also suspected to cause cancer.	https://pubchem.ncbi.nlm.nih.gov/compound/Myristic-acid https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/21713 Pohl *et al.* 2011:62
Undecanoic acid (0.022%)	Undecanoic acid is a straight chain saturated fatty acid which showed anti-fungal activity against *Myrothecium verrucaria, Saccharomyces cerevisiae, Trichoderma virid* and *Trichophyton rubrum*. Undecanoic acid warnings include severe skin burns and eye damage, and it may cause respiratory tract irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/Undecanoic-acid https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/36950 Pohl *et al.*, 2011:63

Octadecanoic acid, ethyl ester (0.007%)	Also known as ethyl stearate, it is a long chain fatty acid ethyl ester and a plant metabolite. Octadecanoic acid, ethyl ester or ethyl stearate is also present in coriander and in the needles of C*edrus deodara*. Chloroform and acetone extracts of the needles and cones showed anti-tubercular potentials. Ethyl stearate is further used as flavouring ingredient, but has some warnings attached to it as it may cause skin, eye and respiratory tract irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/Ethyl-stearate http://www.hmdb.ca/metabolites/HMDB0034156 ttps://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/65425 Gupta, Walia and Malan, 2011:2015
Oleic Acid (0.697%)	Oleic acid is an *Escherichia coli* metabolite, a plant metabolite, *Daphnia galeata* metabolite and a mouse metabolite. The chemical is also a constituent in the fixed oil of *Annona muricata* fruit. Oleic acid has moderate anti-fungal activity and moderate termite repellent activity and its toxicity is described as moderately toxic. Oleic acid also has antioxidant properties but did not exhibit significant antiproliferative effects on the various cancerous cell lines. Oleic acid is an eye, skin and respiratory tract irritant.	https://pubchem.ncbi.nlm.nih.gov/compound/Oleic-acid https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/78531 Alabi, Lajide and Owolabi, 2018:9-18 Elagbar, Naik, Shakya and Bardaweel, 2016:1-7
Oxacycloheptadec-8-en-2-one, (8Z)- (0.265%)	Oxacycloheptadec-8-en-2-one, (8Z)- is also known as ambrettolide. It was once used as fruit flavouring, but its use has been discontinued due to its toxic nature by oral administration and skin exposure. It also causes eye and respiratory tract irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/Ambrettolide https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/39509 https://chem.nlm.nih.gov/chemidplus/sid/0000123693
Oxalic acid, isobutyl nonyl ester (0.0006%)	Literature on oxalic acid, isobutyl nonyl ester is limited, however, in one study, oxalic acid, isobutyl nonyl ester is present in high concentrations in *Rhus semialata* seeds, and the chemical matrix showed activity against the bacterial strains *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoneae* and the fungal strains *Aspergillus niger* and *Penicillium* spp. Oxalic acid is, however, acute toxic if swallowed or when in contact with skin.	https://pubchem.ncbi.nlm.nih.gov/compound/Oxalic-acid https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 Sreedharan, Gothe, Aier, Shivasharanappa, Kumar and Patil, 2019:10-17

Hexane, 2,2,5-trimethyl- (0.004%)	Hexane, 2,2,5-trimethyl- is a volatile organic compound (VOC) and an environmental pollutant. VOCs have many sources, such as vehicle exhaust fumes, diesel service stations, dry cleaners, building materials, dust, pesticides, cleaning products, cigarette smoke, adhesives, paint strippers, moth balls, water-chlorination by-products, pollen, bacteria, fungi, trees and human breath. Conflicting reports exist on its toxicity. A *Baccilus methylotropicus* strain which produces natural volatile organic compounds (VOCs), including 2,2,5-trimethylhexane, was patented for its potent anti-bacterial and anti-fungal properties. The authors stated that these produced VOCs are harmless to both humans and animals and can be used for the control of plant diseases in an environmentally friendly manner. To the contrary, PubChem and ECHA classifies 2,2,5-trimethylhexane as a health hazard and an environmental hazard. The compound may be fatal if swallowed and enters airways, it causes skin irritation and has narcotic effects. The compound further has long lasting toxic effects on aquatic life.	https://pubchem.ncbi.nlm.nih.gov/compound/2_2_5-Trimethylhexane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/135690 Nowak, Poudyal and McNulty, 2017:49-63 Batterman, Su, Mukherjee and Jia, 2014:3-63 Phillips, Herrera, Krishnan, Zain, Greenberg and Cataneo, 1999:75-88 Kim, Yu, Jeon, Im and Kim, 2019:1
Dodecyl acrylate (0.003%)	Dodecyl acrylate is an aroma constituent of white *Rosa rugosa* flowers and of the aerial parts of the herbaceous plant *Gynura cusimbua*, of which the sap is used medicinally in Manipur, India. The extract of rose flowers showed strong anti-microbial activity which may have been due to cooperative synergism between the volatiles and the phenolic compounds in the rose flowers. Dodecyl acrylate has warnings that include skin corrosion and irritation warnings. It can also cause serious eye and respiratory irritation. Dodecyl acrylate is also an environmental hazard with long-term effects on aquatic life.	Joo, Kim and Lee, 2010:57-62 Rana and Blazquez, 2007:22 https://pubchem.ncbi.nlm.nih.gov/compound/Dodecyl-acrylate https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/43726

Table 3.6: Possible contaminants in the *n*-hexane extracts of *C. dentata* stem bark.

Compound	Description	Source
2,4-di-tert-butylphenol	2,4-di-tert-butylphenol has a role as a bacterial metabolite, an antioxidant and a marine metabolite. It is an alkylbenzene and a member of phenols. Additionally, 2,4-di-tert-butylohenol is used industrially as UV stabilizer and an antioxidant for hydrocarbon-based products ranging from petrochemicals to plastics. The compound has been isolated from the bacterium *Lactococcus* sp., as well as the marine bacterium *Vibrio alginolyticus* associated with the red seaweed *Gracilaria gracilis*. 2,4-di-tert-butylhenol has been found to be effective in controlling the quorum sensing regulated phenotypes of the Gram-negative uropathogen, *Serratia marcescens*. Furthermore, 2,4-di-tert-butylphenol was also tested against the fungi *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* as potential biocontrol of fungi on wheat grains.	https://pubchem.ncbi.nlm.nih.gov/compound/2_4-Di-tert-butylphenol Varsha, Devendra, Shilpa, Priya, Pandey and Nampoothiri, 2015:44-50
Hexane, 2,2,5-trimethyl- (as contaminant)	Hexane, 2,2,5-trimethyl- is a volatile organic compound (VOC) and an environmental pollutant. VOCs have many sources, such as vehicle exhaust fumes, diesel service stations, dry cleaners, building materials, dust, pesticides, cleaning products, cigarette smoke, adhesives, paint strippers, moth balls, water-chlorination by-products, pollen, bacteria, fungi, trees and human breath. It can therefore be a contaminant. Conflicting reports exist on its toxicity. A *Baccilus methylotropicus* strain which produces natural volatile organic compounds (VOCs), including 2,2,5-trimethylhexane, was patented for its potent anti-bacterial and anti-fungal properties. The authors stated that these produced VOCs are harmless to both humans and animals and can be used for the control of plant diseases in an environmentally friendly manner. To the contrary, PubChem and ECHA classifies 2,2,5-trimethylhexane as a health hazard and an environmental hazard. The compound may be fatal if swallowed and enters airways, it causes skin irritation and has narcotic effects. The compound further has long lasting toxic effects on aquatic life.	Nowak, Poudyal and McNulty, 2017:49-63 Batterman, Su, Mukherjee and Jia, 2014:3-63 Phillips, Herrera, Krishnan, Zain, Greenberg and Cataneo, 1999:75-88 Kim, Yu, Jeon, Im and Kim, 2019:1 https://pubchem.ncbi.nlm.nih.gov/compound/2_2_5-Trimethylhexane https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/135690

Dibutyl phthalate Phthalic acid, 4-chloro-3-methylphenyl undecyl ester Diisooctyl phthalate	Dibutyl phthalate is used as plasticiser in cosmetics, pharmaceutical coatings, medical devices, food containers, paints, floor and wall coverings and as ectoparasiticide but is a ubiquitous environmental contaminant that poses a serious health risks to humans. Phthalates do not occur naturally. It is a human xenobiotic metabolite and an alpha-glucosidase inhibitor. Plants and herbal preparations may be contaminated by the absorption of phthalates from water or soil or by the migration of phthalates from inexpensive recycled plastics. Phthalates possess various toxic effects in the kidneys, liver, testes and thyroid. Dibutyl phthalate may harm an unborn child, cause reproductive toxicity and is very toxic to aquatic life.	https://pubchem.ncbi.nlm.nih.gov/compound/Dibutyl-phthalate https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 Omidpanah, Saeidnia, Hadjiakhondi and Manayi, 2018:51-67
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	Also known as Cembrene or Thumbergene, the compound is a diterpenoid isolated from *Pinus oleoresins*. Other forms of Cembrene, Cembrene A (neocembrene, neocembrene A) are found in *Commiphora* and *Boswellia* spp., but has also been found to be the trail pheromone of the Australian subterranean termite, *Nasutitermes exitiosus*, the queen recognition pheromone of the Pharaoh's ant, *Monomorium pharaonis*, and it has been isolated from soft corals (*Nephthea* spp.) as well as the paracloacal glands of the Chinese alligator (*Alligator sinensis*). Furthermore, (3*Z*)-cembrene A has been isolated from the heads of soldier termites, *Cubitermes umbratu*. No additional information is available on 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	https://pubchem.ncbi.nlm.nih.gov/compound/5377896 Villanueva and Setzer, 2010:115
1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-	This compound is a constituent of the adhesives used in the food packaging industry and has the potential to migrate, either into food or the environment (litter). The compound is also a constituent in the blue-green alga *Nostoc* sp., which in turn may serve as biomarker for petroleum-related environmental contamination.	Canellas, Vera and Nerín, 2015:79-87 Dembitsky, Shkrob and Dor, 1999:221-229
Benzene, 1,3-bis(1,1-dimethylethyl)-	Also known as 1,3-di-tert-butylphenol. Both 1,3 di-tert-butylphenol and 2,4-di-tert-butylphenol are also decomposition products of polymer additives like Irgafos 168, which are typical for polyolefins in plastic food packaging material.	Welle, Mauer and Franz, 2002:841

9,19-Cyclolanostan-3-ol, acetate, (3á)-	9,19-Cyclolanostan-3-ol, acetate, (3á)- is one of three major compounds in the bacterium *Bacillus cereus*. The metabolites in *Bacillus cereus* were tested for its potential as agent against rice fungal pathogens. No additional information on this compound is available. The compound's presence in *C. dentata* stem bark may derive from bacterial contamination.	Suryadi, Samudra, Priyatno, Susilowati and Sutoro, 2015:35-42
2H-Pyran-2-one, 5,6-dihydro-6-propyl-	5,6-dihydro-6-propyl-2H-pyran-2-one was isolated from *Prosopis* spp. (Mesquite) flour, a volatile component that contributes to the odour of mesquite flour, however, 5,6-dihydro-6-propyl-2H-pyran-2-one is also the major volatile component produced by *Lasiodiplodia theobromae*, a strain of filamentous fungi that cause food products to spoil. The compound's presence in *C. dentata* stem bark may thus also be due to the presence of fungi containing this chemical compound since the stem bark was purchased from an unhealthily managed environment.	Matsumoto and Nago,1994:1262-1266 Takeoka, Wong, Dao and Felker, 2009:1025-1027
4-Chloro-3-n-hexyltetrahydropyran	This compound is also a constituent of *Punica granatum* peels and has shown anti-inflammatory activity, however, 4-Chloro-3-n-hexyltetrahydropyran is also a hydrocarbon type fuel produced by the thermal decomposition of plastics in the presence of calcium carbonate, which means that it has the potential to be an environmental contaminant. The recycling of plastics also cause air pollution which may lead to increased incidences of respiratory diseases in humans.	Huang, Zhou, Feng, and Tao, 2013:327-334 Mohammed, Al-Jassani and Hameed, 2016: 480-494 Sarker, Rashid and Rahman, 2012:114-123
9-Hexadecen-1-ol, (Z)-	This compound is used in the cosmetics industry and as anti-hair fall agent, however, it is a component of the sex pheromones of *Heliothis virescens* and *Heliothis subflexa* moths. The compound was present in the ethanolic extracts of *Ipomoea staphylina* but may have been present due to contamination.	Groot, Nojima, Heath, Ammagarahalli, van Wijk, Claβen, Santangelo, Lopez and Schal, 2018:621-630 Padmashree, Ashwathanarayana, Raja and Roopa, 2018:473-492

Benzene, 1,3-dimethoxy-	Benzene, 1,3-dimethoxy- is a methoxybenzene found in fungi and is responsible for the odour of mushrooms. Benzene, 1,3-dimethoxy- is, however, also and alarm substance found in *Neanura muscorum* (springtail), which is a hexapod frequently inhabiting rotting wood.	https://pubchem.ncbi.nlm.nih.gov/compound/1_3-Dimethoxybenzene Messer, Dettner, Schultz and Francke, 1999:174-182
Benzene, isocyanato-	Isocyanato benzene is formed by the photodegradation of fenuron pesticides and is extremely toxic. It is flammable, corrosive, acute toxic, an irritant, a health hazard and an environmental hazard. It is also a hapten and an allergen. It is very toxic by ingestion or skin absorption and causes severe skin burns and eye damage. It is fatal when inhaled. Benzene, isocyanato- cause allergy or asthma symptoms and is extremely toxic to aquatic life with long-lasting effects.	https://pubchem.ncbi.nlm.nih.gov/compound/Phenyl-isocyanate ttps://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/104564 Diaw, Mbaye, Thiaré, Oturan, Gaye-Seye, Coly, Le Jeune, Giamarchi, Oturan and Aaron, 2019:467
Benzenemethanol, à,à-dimethyl-	Also known as 2-phenyl-2-propanol or α-cumyl alcohol, it is a tertiary alcohol. The compound was also found in *Camellia sinensis* (tea) and is believed to contribute to tea's flavour. The compound is, however, a *Mycoplasma genitalium* bacterium metabolite and a human xenobiotic, acute toxic if swallowed, and may cause skin and eye irritation.	https://pubchem.ncbi.nlm.nih.gov/compound/2-Phenyl-2-propanol https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/49439 Kfouri, Scott, Orians, Ahmed, Cash, Griffin, Matyas, Stepp, Han, Xue, Long and Robbat, 2019:8
Docosane, 11-butyl-	11-Butyldocosane is a monoalkyl alkane also present in the essential oil of *Cycas revoluta*. However, 11-butyldocosane is also a constituent in the cuticle of the green stinkbug *Nezara viridula* and is present in the fungus *Aspergillus nomius.* It is therefore possible that the chemical's presence in plants is due to contamination by insects or fungi.	Olabisi and Olubunmi, 2019:106 Rafaat, Meshrif, Husseiny, El-Hariry and Seif, 2015:83 Azeez, Muid and Hasnul, 2016:757
Hexadecen-1-ol, trans-9-	Hexadecen-1-ol, trans-9- is also a constituent of *Pseudarthria viscida* leaves and is produced by *Serratia* spp. rhizobacteria. These bacteria are bacterial antagonists, i.e. bacteria that negatively affect the growth of other organisms, such as the plant pathogen *Rhizoctonia solani.* Additional information on the chemical is extremely limited.	Mallesh, Amareshwari, Roja and Nirmala, 2019:2104 Kai, Effmert, Berg and Piechulla, 2007:358

N,N-Dibutyl formamide	N,N-Dibutyl formamide is a contaminant deriving from anthropogenic activities involving technical industrial applications/origins, and which is often found in wastewater, even after treatment of the wastewater. In another study an analysis of honey revealed the presence of N,N-Dibutyl formamide deriving from the pollens of the plants the bees visited, which in turn derived from an oil extraction plant nearby. In this study, it was suggested that honey be used as marker for environmental pollution. N,N-Dibutyl formamide is acute toxic if swallowed, causes skin burns, serious eye damage and allergic reactions and is harmful to aquatic life with long-lasting effects.	https://pubchem.ncbi.nlm.nih.gov/compound/N_N-Dibutylformamide https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/128391 Wluka, Coenen and Schwarzbauer, 2017:838 Bentivenga, D'Auria, Fedeli, Mauriello and Racioppi, 2004:1079-1086
Heptadecane, 2,6,10,14-tetramethyl-	2,6,10,14-tetramethylheptadecane is also a constituent of *Petiveria alliacea* (whole plant) and *Pseudarthria viscida* leaves, however, the compound is also a known component of archaebacterial lipids found in sediments and of bacteria associated with mucilaginous cyanobacterial blooms in freshwater ecosystems. The component's presence in plants may thus be due to the presence of bacteria. Additional information on Heptadecane, 2,6,10,14-tetramethyl- is extremely limited.	Sathiabalan, Packia, Muthukumarasamy and Mohan, 2014:390 Vella and Holzer,1992:209 Mallesh, Amareshwari, Roja and Nirmala, 2019:2104 Wang, Shen, Shi, Chen, Ni and Xie, 2015:10
1-Undecene, 7-methyl-	1-Undecene, 7-methyl- is also found in the essential oil of the weed plant *Laggera pterodonta* which is used as insecticide in Cameroon. Furthermore, 1-Undecene, 7-methyl- is a constituent of the flowers of *Acacia auriculiformis.* The floral scent showed anti-oxidant and anti-fungal potentials. However, 1-Undecene, 7-methyl- is also a constituent of the sex pheromone of the *Spodoptera exigua hubner* moth species, which are regarded as pests. This pheromone is suggested as agent for the control of these pests. No further information on the single component is available, however, its use as pesticide may indicate toxicity and may therefore pose potential risks to human health but has not been evaluated. The compound's occurrence in moth pheromones may also be indicative of its origin, i.e. insects and not plants. The compound's presence in floral scent may be due to pollinator visits. Much more research is necessary on this compound.	Omoregie, Oluyemisi, Koma and Ibumeh, 2012:198-202 Samling and Umaru, 2018:646-650 Mujiono, Witjaksono and Putra, :146-150

Heptadecane, 2,6-dimethyl-	Heptadecane, 2,6-dimethyl- is also a constituent of the essential oil of *Adiantum flabellulatum*. However, 2,6-dimethylheptadecane has a role as a fungal metabolite. No additional information is available on 2,6-dimethylheptadecane.	https://pubchem.ncbi.nlm.nih.gov/compound/2_6-Dimethylheptadecane Kang, Ji and Wang, 2009:576

Table 3.7: Characteristics of the chemical compounds in the DCM crude extracts of *C. dentata* stem bark.

Compound	Description	Source
Phenol, 2-methoxy-4-(1-propenyl)-	This compound is also present in the *n*-hexane extracts of *C. dentata* stem bark. Also known as Isoeugenol, it is a phenylpropanoid that is an isomer of eugenol. Occurs in ylang-ylang oil, cinnamon oil, clove oil and other essential oils. Isoeugenol is used in the manufacturing of perfumeries, flavourings and essential oils. Medicinally it is used as local antiseptic and analgesic, as well as the synthetic production of vanillin. However, isoeugenol carries several warnings, which include: Acute toxic if swallowed, acute dermal toxicity, cause skin corrosion/irritation, may cause allergic skin reactions and cause serios eye irritation. Isoeugenol showed carcinogenic activity in mice and rats.	https://pubchem.ncbi.nlm.nih.gov/compound/Isoeugenol https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/82994 Gunnels *et al.*, (2010:62)
2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	This chemical compound is also in the *n*-hexane extracts of *C. dentata* stem bark. It is a methoxyphenol and is used as flavouring agent in food. Biologically it has a role as a nutrient. The compound has no hazard statement. 2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol is also found *Olea europaea* waste (after oil extraction), and suggestions were made as to its use in the food, pharmaceutical and cosmetic industries.	https://pubchem.ncbi.nlm.nih.gov/compound/4-Propenyl-2_6-dimethoxyphenol https://webbook.nist.gov/cgi/cbook.cgi?ID=C20675950 Del Pozo, Bartoli, Puy and Fàbregas, 2018:160-167
2-Ethyl-oxetane	2-ethyl-oxetane is also occurs in *Clinacanthus nutans* (Acanthaceae) and *Cyperus esculentus* (Cyperaceae) leaves. The methanolic extracts of *C. nutans* showed antinociceptive properties via the opiod/nitric oxide mediated, but cGMP-independent pathways.	Rahim, Zakaria, Sani, Omar, Yakob, Cheema, Ching, Ahmad and Kadir, 2016:4 Imo, Uhegbu, Arowora, Ezeonu, Opara, Nwaogwugwu and Anigbo, 2019:411
Oxeladin	Oxeladin is used as non-opioid anti-tussive (cough-suppressing) medicine in various countries, however, oxeladin is acute toxic if swallowed, through skin contact or if inhaled. This chemical was withdrawn from the US, UK and Canadian markets due to its carcinogenicity.	https://pubchem.ncbi.nlm.nih.gov/compound/Oxeladin Lin, Wang, Huang, Ke, Chao, Chen and Hsiao, 2017:3 Enache and Sarafoleanu, 2016:72
Cyclobutane, ethyl-	Ethylcycobutane is a basic structural element in a wide range of naturally occurring compounds in bacteria, fungi, plants and marine invertebrates. Some cyclobutane-containing compounds such as amino acids, peptides and nucleosides showed protective properties against UV radiation.	Sergeiko, Poroikov, Hanus and Dembitsky, 2008:1

Table 3.8: Characteristics of the chemical compounds in the ethanol crude extracts of *C. dentata* stem bark.

Compound	Description	Source
2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	This chemical compound is also detected in both the *n*-hexane and DCM extracts of *C. dentata* stem bark. It is a methoxyphenol and is used as flavouring agent in food. Biologically it has a role as a nutrient. The compound has no hazard statement. 2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol is also found *Olea europaea* waste (after oil extraction), and suggestions were made as to its use in the food, pharmaceutical and cosmetic industries.	https://pubchem.ncbi.nlm.nih.gov/compound/4-Propenyl-2_6-dimethoxyphenol https://webbook.nist.gov/cgi/cbook.cgi?ID=C20675950 Del Pozo, Bartoli, Puy and Fàbregas, 2018:160-167
Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester	Drugs containing pyrrole ring system are known to have many biological properties, which include antipsychotic, *β*-adrenergic antagonist, anxiolytic, anticancer (leukemia, lymphoma and myelofibrosis etc.), antibacterial, antifungal, antiprotozoal and antimalarial properties. Some Pyrroles, such as 1Hpyrrole-2-carboxilic acid, 1[1-(3,4,5-trimethoxyphenyl)ethyl] are, however, listed in the US List of Toxic Substances Control Act (TSCA). No literature provides the chemical formula or describes the potentials of Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester.	Bhardwaj, Gumber, Abbot, Dhiman and Sharma, 2015:2 TSCA, 1977:187
Glycerin	Glycerin is a trihydroxyalcohol with localised osmotic diuretic and laxative effects. Also known as Glycerol, it has roles as an osmolyte, a solvent, a detergent, a human metabolite, an algal metabolite, a *Saccharomyces cerevisiae* metabolite, an *Escherichia coli* metabolite and a mouse metabolite. It is widely used in the food industry as a sweetener and humectant and in pharmaceutical formulations. It is mostly non-toxic, however, chronically high levels of glycerol in the blood are associated with glycerol kinase deficiency (GKD). GKD causes the condition known as hyperglycerolemia. The infantile form of GKD is the most severe and is associated with vomiting, lethargy, severe developmental delay, and adrenal insufficiency. The mechanisms of glycerol toxicity in infants are unknown, however, it appears to shift metabolism towards chronic acidosis. In infants with acidosis, the initial symptoms include poor feeding, vomiting, loss of appetite, weak muscle tone (hypotonia), and lack of energy (lethargy). These can progress to heart, liver, and kidney abnormalities, seizures, coma, and possibly death. These are also the characteristic symptoms of untreated GKD.	https://pubchem.ncbi.nlm.nih.gov/compound/Glycerol https://hmdb.ca/metabolites/HMDB0000131

Table 3.8 cont.

Oxalic acid	Oxalic acid is a human metabolite, a plant metabolite and an algal metabolite. In plants, oxalic acid plays a role in plant defence against insect pests and grazing animals. Oxalic acid is also known for its phytoremediation properties by assisting in the accumulation of heavy metals by hyperaccumulating plants such as *Brassica juncea* (black mustard), *B. napus* (rapeseed), *B. rapa* (field mustard) and other species from the cabbage family such as *Thlaspi caerulscens* (alpine penny cress), and also *Hordeum vulgare* (barley), *Avena sativa* (oats), *Salix* spp. (willows), *Sedum alfredii*, commonly known as stonecrops and *Arabidopsis* spp. (rock cress). Oxalic acid is, however, acute toxic if swallowed, and has acute dermal toxicity.	https://pubchem.ncbi.nlm.nih.gov/compound/Oxalic-acid https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32008R1272 Chen, Lin, Luo, He, Zhen, Yu, Tian, and Wong, 2003:807-811 Lone, He, Stoffella and Yang, 2008; 210-220 Pulford and Watson, 2003: 529-540 Prasad and Shivay, 2017:1665-1667

3.4 DISCUSSION

Not all *C. dentata* stem bark used in traditional medicinal preparations are freshly harvested material. Traditional health practitioners often collect more bark than what is immediately needed and dry it for later use in preparations, or purchase bark from commercial harvesters. Commercial harvesters also deliver to traditional medicine markets in cities. It is not ideal to purchase stem bark from traditional medicine markets as it may encourage the continued harvesting of bark. However, not purchasing bark from the traditional medicine market will also not prevent the harvesting of bark. Purchasing stem bark at the traditional medicine market does, however, provide scientists with an opportunity to study the bark without causing more damage to the environment. In traditional medical systems, chemical compounds are generally extracted using either water or alcohol, or a combination of both (Kamboj, 2012:23; Liang et al., 2004:54). However, Kotze and Eloff (2000:64) determined that compounds extracted using water as solvent showed very low activity against known drug-resistant microorganisms. As a result of sequential extraction in this study, most chemical compounds were present in the hexane fraction, which was the first solvent used. However, the majority of compounds extracted in the hexane fraction are also soluble in other solvents. Most of the compounds with adverse effects will dissolve equally well in water or alcohol and will therefore be present in most traditional preparations.

Various chemical compounds in the apolar crude extracts of *C. dentata* stem bark are compounds also present in the essential oils of other plant species, including fruits, vegetables, herbs and spices. Essential oils are complex, light- and heat sensitive mixtures of volatile and non-volatile compounds produced by living organisms (Franz and Novak, 2010:39). Volatile organic compounds in plants are lipophilic, low molecular weight compounds which facilitate interactions with the environment, such as pollinator attraction, seed dispersion and as chemical defence against parasites, bacteria and other pathogens, and herbivores (Tholl, 2006:297; Dudareva, Klempien, Muhlemann and Kaplan, 2012:16). Based on the biosynthetic origin, volatile organic compounds, which are also present in the stem bark extracts of *C. dentata* trees, are divided into different classes such as terpenoids (monoterpenoids and sesquiterpenoids), phenylpropanoids, benzenoids, fatty acid derivatives and amino acid derivatives (Widhalm, Jaini, Morgan and Dudureva, 2015: 545).

- Terpenoids represents the largest and most diverse group of secondary metabolites and include many volatile constituents (Dudareva *et al.* 2013:17). In older literature,

the term ‘terpenes’ was used for all compounds with isoprene units (C_5H_3). Nowadays, terpenes refer to only monoterpenoid hydrocarbons (Sell, 2010:129). The name ‘isoprene units” for five-carbon units remained, even though it became known that the five-carbon building blocks *in vivo* are the inconvertible isomers isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Humphrey and Beale, 2006:47).

- Phenylpropanoids include the following groups of compounds: anthocyanidins, coumarins, flavonoids, hydroxycinnamoyl derivatives, isoflavonoids, lignans, phenolenones, proanthocyanidins, stilbenes and tannins (Hassanpour, Maheri-Sis, Eshratkhah and Mehmander, 2011:48).
- Benzenoids are organic compounds possessing a six-carbon aromatic ring. These compounds act as environmental contaminants, even at very low concentrations. Benzene, the simplest of the benzenoid compounds are classified as a group ‘A’ human carcinogen by the United States Environmental Protection Agency (US EPA) (Mishra and Kumar, 2017:17).
- Fatty acids are precursors for other significant components of plant metabolism (Ohlrogge, Browse, Jaworski and Somerville, 2000:956) and serve roles as energy substrates and integral membrane components (Anthwal, Sati, Thapliyal, Verma, Kumar and Thapliyal, 2018:271). Fatty acids also play a role in plant defence (Lim, Singhal, Kachroo and Kachroo, 2017:508), and are generally categorized as saturated fats, unsaturated fats, polyunsaturated fats, monounsaturated fats, essential fatty acids (fatty acids not biosynthesized by the body and therefore has to be taken in through food), trans fatty acids and more (Anthwal *et al.*, 2018:271).
- Fatty alcohol is a generic term for a range of aliphatic hydrocarbons containing a hydroxyl group at the terminal position.
- Only 2% of the dry weight of plants is composed of nitrogen, however, there is a large number of nitrogen-containing substances known in plants. The 20 protein amino acids are involved in the biosynthesis of all other plant nitrogenous substances. Nitrogen-containing compounds include certain amino acids, amines, alkaloids, cyanogenic glucosides, porphyrins, purines, pyrimidines and the hormone classes cytokinins and auxins. Many non-protein amino acids, particularly the cyanogenic glucosides and glucosinolates, are toxic to life (Harbourne, Baxter and Moss, 1998:72).

The stem bark of *C. dentata* trees contain more chemical compounds with hazardous potentials (Table 3.5) and potential contaminants (Table 3.6) than chemical compounds with beneficial potentials (Table 3.4). Even though the majority of hazardous compounds in this study are only present in the hexane extracts, some of these compounds might be forced into the preparation when extracted with a mixture of other plants, as is generally done with *C. dentata* stem bark when preparing *khubalo*. There is also a possibility of more compounds with adverse effects in the plants that are not identified with GC-MS. These compounds might be present in more polar fractions, but these require analytical methods appropriate for compounds that are not volatile. These compounds may therefore have been extracted in this study but could not be detected with GC-MS as this method is the preferred method for detecting volatiles.

Phenolic compounds exhibited anti-allergenic, anti-inflammatory, anti-diabetic, anti-artherogenic, anti-microbial, anti-thrombotic, antioxidant, anti-pathogenic, anti-viral, cardioprotective and vascodilatory potentials and also have the potential to prevent diseases such as cancer, heart problems and Alzheimers disease (Balasundram, Sundram and Samman, 2006:191; Huyut, Beydemir and Gülçin, 2017:1). Phenol, otherwise known as hydroxybenzene, is present in *C. dentata* stem bark (0.002% area, mass 94 g/mol, retention time 403.48) and is the parent compound of all phenolic compounds. Phenol is a major environmental pollutant and is included in the United States Environmental Protection Agency's (US EPA) list of pollutants (Mishra and Kumar, 2017:17). Phenol is synthesized industrially, but also occurs naturally, where it is biosynthesized by plants, microorganisms or the decomposition of organic matter (Michalowicz and Duda, 2006:348). Phenol may be fatal by ingestion, inhalation and skin absorption, and is considered to be a human carcinogen (Mishra and Kumar, 2017:17; Michalowicz and Duda, 2006:356) and a mutagen (Michalowicz and Duda, 2006:355). The most systemic effect is central nervous system depression (Lyutyy, Bekhta, Ortynska and Sedliačik, 2017:108), but it may also cause damage to the kidneys, liver, muscles and eyes (Michalowicz and Duda, 2006:354). Phenols, after cell penetration, undergo active transformation, and sometimes these transformation processes lead to increases in toxicity of individual compounds by the formation of electrophilic metabolites that may bind and damage DNA and enzymes. The noxious effects of phenols and their derivatives (e.g. catechol) are related to concerns regarding acute toxicity, histopathological changes, mutagenicity and carcinogenicity (Michalowicz and Duda, 2006:347).

Of the chemical compounds with beneficial potentials, very few assays have been conducted on single components (Sendker and Sheridan, 2017:30). Assays of essential oils or extracts from plants generally encompass the compound matrices, and therefore the possibility that two or three, or even a range of compounds act synergistically cannot be ruled out. Furthermore, many assays using complex mixtures are conducted *in vitro* on cell cultures in test tubes or petri dishes, which may distort the picture of its actual potentials on humans as the whole human metabolome and all its processes, including uptake and phase I and phase II biotransformation are not included in such studies (Prinsloo, Papadi, Hiben, De Haan, Louisse, Beekmann, Vervoort and Rietjens, 2017:1187-1120; Boonpawa et al. 2014:287-299; Rietjens, Tyrakowska, Van den Berg, Soffers and Punt, 2015:1-38). *In vivo* toxicity studies on mice, rats and rabbits etc. may also be deceiving as certain chemical compounds may be toxic to humans but not to animals, and vice versa (e.g. in Garlick, 2003: 1633S-1639S) due to differences in metabolisms.

Fatty acids have known antifungal effects and medium chain fatty acid in particular, have shown antibacterial activities (Ma *et al.*, 2019:1, 2). Short chain fatty acids affect local and systemic immune functions, regulate systemic bone mass and protect from pathological bone loss (Lucas, Omata, Hoffman, Böttcher, Iljazowic, Sarter, Albrecht, Schulz, Krishnakumar, Krönke, Herrmann, Mougiakakos, Strowig, Schett and Zias, 2108:1-10). Exogenous fatty acids inhibit fatty acid biosynthetic pathways in *Plasmodium falciparum*, the parasite that causes malaria and causes the deaths of many people, and exposure to 2-hexadecanoic acid blocks fatty acid biosynthesis as well as its degeneration in mycobacteria, which is an indication that fatty acids could be employed for the treatment of tuberculosis. Furthermore, undecylenic acid or undec-10-enoic acid is sold as an over-the-counter medicine to treat oral thrush and denture stomatitis (Ma *et al.*, 2019:2). The fatty acid, n-Hexadecanoic acid, was isolated from *C. dentata* stem bark in Chapter 4.

Many beneficial compounds are, however, also fatty, oily or waxy substances with low solubility in water, such as the four pentacyclic triterpenoids previously isolated from *C. dentata* leaves, with subsequent problematic penetration of permeating biological membranes, which therefore limits efficacy (Pironi *et al*., 2018:86). Furthermore, chemical compounds in plant extracts may be bacterial or fungal metabolites, however, studies on essential oils or plant extracts do not consider that plants may host a myriad of microbacteria, fungi and other pathogenic organisms when collecting sample material, and that these metabolites may reflect in the analysis of the plant material. Chemical compounds that may

have both natural and anthropogenic origins (e.g. 2,2,5-trimethylhexane) may simply be regarded as a compound with natural origins because it was detected in plant material, without researching its potential origins as a contaminant. Also, because a compound is a constituent of essential oils, it is simply perceived as beneficial, without researching each compound's potential harmful effects.

Curtisia dentata stem bark has been used as traditional medicine for a long time. Historically, traditional "risk assessments" involved trial-and-error experiences. Plants with rapid adverse effects were seldom used afterwards but were sometimes applied as poisons in hunting (Knöss, 2017:7), and the species with no immediate adverse effects were accepted as medicinal. However, in South Africa, *Callilepis laureola* (Ox-eye daisy or Impila), a highly poisonous plant, continues to be used medicinally, despite the high number of deaths reported after using the tubers as a medicine (Cunningham, 1988:14,15; Kotsiou and Christine, 2017:81) In *C. dentata* stem bark, there are six known or suspected carcinogens (nerolidol, oxeladin, butyrolactone, isoeugenol, tetradecanoic acid and phenol), two compounds are mutagens (2,6-dimethoxybenzoquinone and phenol), whereas phytol, which is also present is *C. dentata* stem bark, showed genotoxic potentials. Additionally, *C. dentata* stem bark contain chemical compounds with narcotic effects (e.g. butyrolactone, methylcyclopentane, 4-hydroxybutanoic acid and 2,2,5-trimethylhexane). Phenol further accumulates in human tissue such as the brain, liver and kidneys. The health hazards associated with chemical compounds depend on the dose taken and several other factors relevant to the patient treated, but since traditional medicines in South Africa are neither standardized nor regulated, the dosages consumed are uncertain. However, with the potential of phenol to accumulate in human tissues, the dose consumed at each administration becomes irrelevant. The adverse effects of these potential hazardous compounds may only express itself after a number of years of use (Moreira *et al.*, 2014:250), and will therefore not be associated with the use of the traditional medicinal plant material.

Traditional medicines harvested from wild stocks in traditional medicinal systems lack the good agricultural and collection practices (GACP), good manufacturing practices (GMP) pharmacopoeias and herbal monographs that define and ensure high quality herbal formulations. The safety and efficacy of any plant-based medicinal product is governed by their quality (WHO, 2003, 2007). The misidentification of plant material, adulterants, contaminants, the generation of artifacts caused by enzymic activity and the fumigation of

plant material can lead to serious and potentially fatal consequences (Sendker and Sheridan, 2017:30).

The toxicity of traditional herbal medicines has not been evaluated completely (Sponchiado, Adam, Silva, Soley, Mello-Sampayo, Cabrini, Correr and Otuki, 2016:290). The specific challenge regarding the safety assessment of herbal medicines is the limited availability of data. Toxicology studies involve more than only testing a compound or mixture's effect on known bacterial and fungal pathogens. It also involves single-dose toxicity, repeat-dose toxicity, genotoxicity, *in vitro* and *in vivo* (including supportive toxicokinetics evaluations), carcinogenicity, long-term studies, short- or medium-term studies, other studies, reproductive and developmental toxicity, fertility and early embryonic development, embryofetal development, prenatal and postnatal development, studies in which the offspring of juvenile animals are investigated and/or further evaluated, and local tolerance. Toxicology studies further involve antigenicity, immuno-toxicity, mechanistic studies, dependence, metabolites and impurities (Knöss, 2017:7). Pharmacology and toxicology studies are based on the foundation that there is a direct relationship between the concentration of a biologically active constituent in a medicine, the site of action and the extent of the effect. After a given dose, the concentration of that bioactive constituent at the site of action is extensively modulated by several factors, which include variations in the formula, the body build of the patient, metabolism and excretion. The active constituents in traditional medicines are most often not characterized or even known, which complicates pharmacokinetic and toxicokinetic studies (Pelkonen and Ahokas, 2017:70), particularly where it concerns ill-defined products and products with questionable efficacy.

As a hypothetical claim, it is often said that the whole plant-based medicinal product is biologically active and that the whole is more than the sum of its parts, or in the case of toxicity, less than the sum of its parts. This statement is, however, used as an excuse not to perform kinetic studies on isolated components as the results can often not be extrapolated to the situation in which the whole herbal medicinal product is being used (Pelkonen and Ahokas, 2017:70). Clinically or experimentally observed beneficial or adverse effects and their tentative linkages with identified components of the mixture is considered a prerequisite and starting point for kinetic studies. However, since many herbal medicinal products and traditional medicines are being used without proof of efficacy, it is necessary to conduct pharmacokinetic and toxicokinetic studies to address selected safety concerns of these products. With modern medicinal products, toxicology is included at different stages e.g.

drug discovery, drug evaluation, environmental toxicology and pharmacovigilance and clinical toxicology. However, toxicology evaluations for raw plant materials in traditional medical systems is complicated by the complexity of the mixtures (Knöss, 2017:7). To overcome the question of which components to measure in complex mixtures, pharmacodynamic and toxicodynamic studies and efficacy trials of traditional, herbal and complementary medicines should precede extensive kinetic studies (Pelkonen and Ahokas, 2017:70). These studies, however, require adequate knowledge of the chemical composition of the mixture. Products with proven efficacy and/or observed adverse effects should be automatically considered for pharmacokinetic studies. However, safety concerns often arise from the presence of a known toxic substance in the product. Within a complex herbal preparation, there is a large number of components that may potentially affect the toxicity of other components (Pelkonen and Ahokas, 2017:72). Interactions between different components can be inhibitory, additive, or synergistic and should be taken into consideration during the observation of beneficial and adverse effects.

Genotoxicity or carcinogenicity are two outcomes that are difficult to detect in conventional toxicity tests or by pharmacovigilance, and they can have severe adverse outcomes that must be addressed even if the evidence is based on a single component study. Several protocols have been developed based on studies demonstrating the correlation between carcinogenicity and mutagenicity, and the correlation of both parameters with genotoxicity (Sponchiado *et al.*, 2016:290). Obtaining reliable safety data on medicinal plant extracts requires using more than one methodology, a wide range of genotoxic tests using a range of solvents and various target organisms, including both bacterial and mammalian organisms, and each test should ideally be repeated at least two or three times (Sponchiado *et al.*, 2016:293, 294). Also, the tests should be capable of detecting outcomes that include mutation induction, clastogenic and aneugenic effects, and structural chromosome abnormalities (Sponchiado *et al.*, 2016:294).

3.5 CONCLUSION

This study showed that the stem bark of *C. dentata* trees contain 26 chemical compounds with beneficial potentials, however, a derivative of only one of the compounds previously isolated from *C. dentata* leaves were detected in the stem bark by GC-MS analysis, i.e. ursolic aldehyde. The pentacyclic triterpenoids lupeol, betulinic acid and 2α-hydroxyursolic acid

previously isolated from *C. dentata* leaves were not detected in the stem bark samples analyzed and *C. dentata* leaves contained *β*-sitosterol whereas the GC-MS analysis of the stem bark analyzed in this study detected α-sitosterol. The majority of the chemical compounds with beneficial potentials identified with GC-MS in the stem bark of the scarce and near threatened *C. dentata* trees, including the chemical compounds previously isolated from the leaves are also present in readily available fruits, vegetables, herbs and spices, which renders the harvesting of *C. dentata* stem bark and the resultant population declines as unjustifiable, particularly since no lead that could result in clinical trials and the development of a new drug has been isolated thus far, and the compounds isolated to date has limited efficacy due to their non-solubility in water.

Plants are known to contain numerous compounds, and although they have proven pharmacological properties, as is the case with *C. dentata,* they can still cause harm. The chemical compounds which may lead to adverse effects in humans are not removed during the preparation of traditional medicinal remedies. This study therefore further showed that users of traditional remedies prepared from the stem bark of *C. dentata* trees also consume several chemical compounds with potentials to cause adverse effects in humans as the extracts contain carcinogens, mutagens, chemical compounds with narcotic and genotoxic potentials, chemical compounds that are acute toxic and chemical compounds that may derive from contaminants, including biological contaminants and contaminants deriving from anthropogenic activities such as those that are present in pesticides, plastics and vehicle exhaust fumes. The presence of known toxic substances in the *C. dentata* stem bark extracts therefore raises concerns, although the concentration, biotransformation and matrix effects in extracts are poorly studied and may present a system where adverse compounds are biosynthesized, eliminated or transformed to inactive forms or compounds with little adverse outcomes. The findings of this study emphasize the need for authoritative regulation of traditional plant-based medicines. Additionally, primary and secondary pharmacodynamic and toxicodynamic studies followed by pharmacokinetic and toxicokinetic studies should be conducted on the extracts of *C. dentata* stem bark and these tests should be capable of detecting outcomes that include mutation induction, clastogenic and aneugenic effects, and structural chromosome abnormalities.

CHAPTER 4. ISOLATION OF CHEMICAL COMPOUNDS FROM THE HEXANE CRUDE EXTRACTS OF *Curtisia dentata* (Burm.f.) C.A.Sm. STEM BARK

4.1 INTRODUCTION

Traditional medicine has become a competitive business venture with medicinal plant trade markets booming in cities and several thousand commercial harvesters collecting medicinal plant material for these trade markets (Mander *et al.*, 2007:192). *Curtisia dentata* stem bark is harvested from different regions, throughout the year, and from trees of different ages to maintain supply to these markets. Medicinal plant trade markets are therefore flooded with medicinal plant material of unequal quality, even though collected from the same species (Street *et al.*, 2008:705-710).

Reports concerning specific chemical compounds from *C. dentata* trees are limited to two studies conducted on the leaves. In the first study, four pentacyclic triterpenoids were isolated from *C. dentata* leaves, i.e. lupeol, ursolic acid, betulinic acid and 2α-hydroxyursolic acid (Shai, 2007:1-208). In the second study, the phytosterol *β*-sitosterol was isolated from the leaves (Fadipe *et al.*, 2017:106). However, in traditional medicinal systems, only the stem bark of *C. dentata* trees is used as medicine (Van Wyk *et al.*, 2013:110), and the chemical composition of *C. dentata* stem bark is largely unknown (Van Wyk *et al.*, 2013:110) since only the classes of compounds that occur in *C. dentata* stem bark were reported (Oyedemi *et al.*, 2012: 6191; Doughari *et al.*, 2012:1041-1050). Phytochemical constituents are not evenly distributed throughout a plant (Tanko *et al.* 2005:3). For example: in defence of wood borers attacking the stem of a tree, the tree may produce insect-specific metabolites, however, where a variety of insects damage leaves, the tree may produce broad-spectrum metabolites to ward off the attacks (Kortbeek, Van der Gragt and Bleeker, 2019:67). The metabolites produced by the tree may thus differ between the leaves and the stem bark of the same tree. Metabolites in trees are produced to aid in their survival in an environment they cannot escape from when either biotic or abiotic threats jeopardise their safety (Kliebenstein, 2004:675; Prinsloo and Nogemane, 2018:1). Versatility and adaptability are thus essential for the tree's survival but may affect the chemical composition of traditional medicines. To ensure consumer safety, it is necessary to standardize traditionally used formulations, document its uses and back it up with scientific evidence. The isolation and identification of chemical constituents in the stem bark is the first step in achieving this objective.

In this study, the hexane crude extract of *C. dentata* stem bark is thus subjected to thin layer chromatography, column chromatography, GC-MS analysis and both ^{1}H and ^{13}C NMR analysis to isolate, identify and elucidate the chemical structures of chemical compounds.

4.2 METHODS AND MATERIALS

4.2.1 Plant material used

The *C. dentata* stem bark (*Umlahleni* in Zulu) purchased at the Faraday traditional medicine market in Johannesburg on the 18th of September 2019 was ground to a fine powder and the chemical compounds in the stem bark exhaustively extracted using the method described in section 3.2.1.

4.2.2 Isolation and purification of compounds in *C. dentata* stem bark

Liquid column chromatography was selected as method for the isolation and purification of compounds in the *n*-hexane crude extracts. In preparation of the stationary phase, cotton wool was placed in the base of a 650 x 50 mm column and 200 ml of pure hexane was added to the column. The tap was set to a low flow rate for the release of hexane. For the stationary phase, a packing of silica gel slurry was prepared by adding 150 ml hexane to 140 mg silica with a pore size of 60A and mesh 70-230 (Sigma Aldrich, St. Louis, Missouri, USA), after which it was stirred with a glass rod. The slurry was added to the column to a height of approximately 300 mm and was allowed to settle while continuously adding more 200 ml additions of hexane. The column is never allowed to become dry. The 3.4 g hexane crude extract was re-dissolved in a 50:50 mixture of hexane and DCM (6 ml), after which dry silica powder was added and the mixture stirred to transform the oily crude extract into a powder. The column tap was closed, and the dried extract added to the column to form a thin, even layer of approximately 12 mm on top of the silica gel. A layer of cotton wool was carefully placed on top of the crude extract layer to prevent the suspension of crude extract particles when adding more solvent mixtures. Figure 4.1 shows the completed stationary phase.

Figure 4.1: The stationary phase complete and ready for the mobile phase to be added.

For the mobile phase, the outlet was opened, and the first solvent mixture added to the column was adjusted to a ratio of 9:1 hexane and ethyl acetate (180:20 ml) to slightly increase polarity. The first fraction was excluded as it consisted of solvents. As soon as the yellow coloured elutes reached the bottom of the column, fractionation commenced. Solvent mixtures at 200 ml per addition were continuously added to the column, gradually increasing polarity of the solvents. The quantities and ratios of additions were as follows: 2 x 8:2 hexane and ethyl acetate, 1 x 7.5:2.5 hexane and ethyl acetate and 1 x 7:3 hexane and ethyl acetate, after which the volume of solvents added were reduced to 100 ml per addition. The ratios for the 100 ml additions were: 1 x 6:4 hexane and ethyl acetate, 1 x 5:5 hexane and ethyl acetate, 1 x 3:7 hexane and ethyl acetate, 1 x 100 ml pure ethyl acetate, 1 x 9:1 ethyl acetate and methanol, and 1 x 100 % methanol. The hexane and ethyl acetate mixtures removed most of the non-polar compounds in the column. Ratios of hexane and ethyl acetate were used until most of the light and dark yellow coloured bands of compounds eluted from the column. As the polarity of solvents was increased gradually, a green band eluted from the column, after which another yellow and an orange-yellow band followed. After all the colour bands were eluted from the column, the concentration of ethyl acetate was reduced, and methanol was added to increase polarity. Pure methanol was used to clear compounds that were still trapped in the column. In Figure 4.2, the mobile phase is approximately halfway through and the

fractions already collected are numbered and placed in order of collection. The colour bands are clearly visible in the column. The separation yielded 25 fractions of varying volumes.

Figure 4.2: The mobile phase with approximately half the fractions already collected.

4.2.3 Thin Layer Chromatography of the hexane fractions

The fractions were dried overnight. A white coloured precipitate formed in fraction 11, which was separated from the liquid. The liquid was thus annotated fraction 11a and the precipitate 11b. The fractions, including the precipitate, were reconstituted in one or a mixture of any two of hexane, DCM, ethyl acetate and methanol before spotting onto TLC plates (Merck silica F_{254} plates). The plates were allowed to dry before development in separate glass TLC tanks with solvents of varying polarities. For fractions 2 to 6, hexane and ethyl acetate with a ratio of 9.2:0.8 were used in the TLC tank. For fractions 7 to11, a ratio of 9:1 hexane and ethyl acetate were used and for fractions 10 to 17, a ratio of 7.5:2.5 hexane and ethyl acetate were used. The precipitate separated from fraction11 was spotted onto a separate TLC plate and developed in the tank with the 7.5:2.5 ratio of hexane and ethyl acetate. Fractions 17 to 21 was developed in a ratio of 5:5 hexane and ethyl acetate and fractions 21-25 were developed in a ratio of 9.5:0.5 DCM and methanol. The plates were removed from the tanks with solvents and air dried for both the shortwave (254 nm) and longwave (365 nm) UV visualization of the compounds in a Spectraline CM-10A fluorescent analyst. Visible spots were marked. The plates were further immersed in an acid stain consisting of sulfuric acid and methanol in a ratio of 1:9, after which

the plates were air-dried and developed using the lower setting of a Master ProHeat PH-2100 dual temperature heat gun. Fractions with similar TLC profiles were pooled together, which yielded 11 major fractions. Fractions were pooled as follows: **1)** fraction 2, **2)** fractions 3 and 4, **3)** fractions 5 to 7, **4)** fractions 8 and 9, **5)** fraction 10, **6)** fraction 11, **7)** fractions 12 and 13, **8)** fractions 14 to 16, **9)** fractions 17 and 18, **10)** fractions 19 to 21, and **11)** fractions 22 to 25. Upon standing, additional solids precipitated from six of the pooled fractions, and these were separated from the liquid by pouring the liquids through small Buchner funnels fitted with Munktell Ahlstrom filter paper (65 g/m^2). The solids were allowed to dry completely before carefully scraping the dry, white and cream coloured powders off the filter paper and placing each precipitate into separate glass vials.

4.2.4 Identification of compounds

The identification and determination of the structures of compounds is a lengthy and difficult process as several methods are often used to determine the structures. The purified fractions, including the purified and semi-purified compounds were further subjected to ^{1}H-NMR, ^{13}C-NMR and GC-MS analyses to ascertain the chemical structures. The settings applied in 3.2.3 was also applied for the GC-MS analysis of the fractions.

One dimensional proton and carbon nuclear magnetic resonance (NMR) spectra were obtained to get an indication of the types of compounds. In preparation, deuterated chloroform (1.5 ml) was added to each of the fractions, including the compounds that precipitated from the fractions, stirred and heated where necessary to dissolve the dried fractions. At least 750 µl of the supernatant from each sample was poured into separate 5 mm NMR tubes (Norell, Sigma-Aldrich) using pipettes. NMR lids were placed onto each NMR tube to reduce evaporation. A Varian 600 nuclear magnetic resonance spectrometer was used for analysis. The experimental data obtained were compared with published data on the compounds isolated.

4.3 RESULTS

The masses and percentages of the extracts obtained with each solvent as described in section 3.2.1 is displayed in Figure 4.3. Only the hexane crude extracts were subjected to column chromatography as the GC-MS analysis conducted in Chapter 3 revealed that the hexane crude extracts yielded the most chemical compounds, even though the DCM, ethanol and water yielded larger quantities of crude extracts.

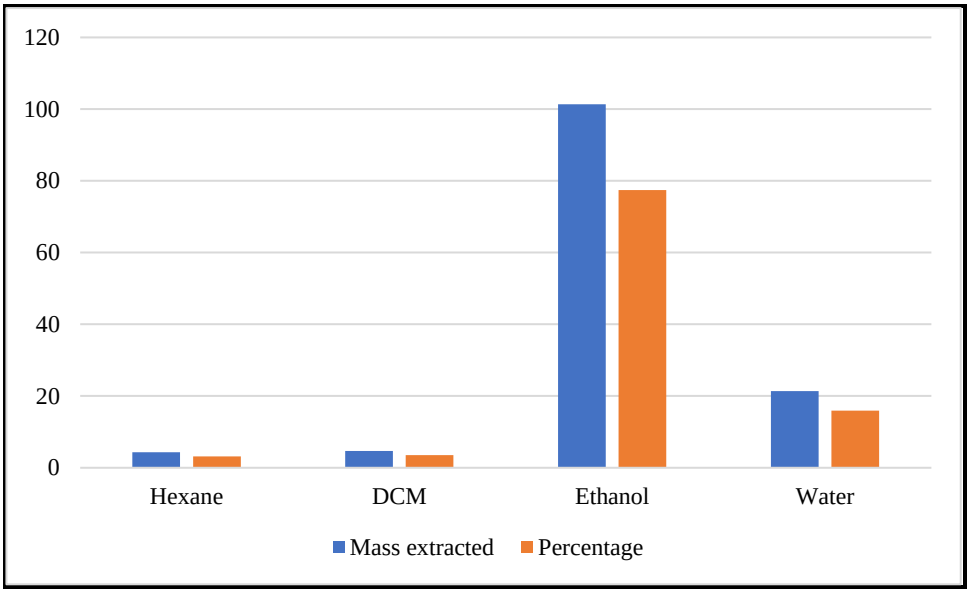


Figure 4.3: The yields of crude extracts obtained from 1.002 Kg *C. dentata* stem bark using solvents of increasing polarities and the percentages of each extract as part of the total yield.

Figure 4.4 shows the TLC plates of the fractions in numerical order from fraction 2 to fraction 25.

The presence of compounds in each fraction was evaluated by using TLC plates (Figure 4.5). The basic TLC parameter is retardation factor (R_f,) which can be mathematically described by the following equation: $R_f = a/b$, where a is the distance from the origin to the spot center and b is the distance from the origin to mobile phase front (Ciura, Dziomba, Nowakowska and Markuszewski, 2017:3). With shortwave and longwave visualization and the development of the TLC plates in the acid stain, the compounds could be marked. Fractions 2 – 9 did not show a significant presence of compounds, which was confirmed by the GC-MS analysis of the fractions. However, the retention factors (R_f) for fractions 10 and 11a were the same (Figure 4.5B). This observation suggests that this is either the same compound or two compounds that fall within the same solubility range. ^{13}C NMR analysis, however, showed that fraction 11a was a different compound than that of fraction 10. The compound in fraction 11a was annotated compound **1**. The precipitate in fraction 11b only shows a faint presence on the TLC plate as the concentration was too low. No structure could successfully be elucidated from this precipitate. Fractions 12 and 13 were pooled together since the R_f's were the same (Figure 4.5D), however, two structures were elucidated from this pooled fractions and they were annotated compounds **2** and **3**. Compound **4** is present in pooled fraction 14-16. These fractions were pooled together on the basis of their and their R_f and their distinct colour change under longwave UV exposure (Figure 4.5D). Compound **5** is a

more polar compound present in the pooled fractions 19-21 (Figure 4.5E). The structures of two compounds, annotated compounds **6** and **7** respectively**,** were elucidated from the spectral data of fraction 10. The GC-MS analysis of fractions 10 – 25 collectively yielded 879 chemical compounds (Appendix L).

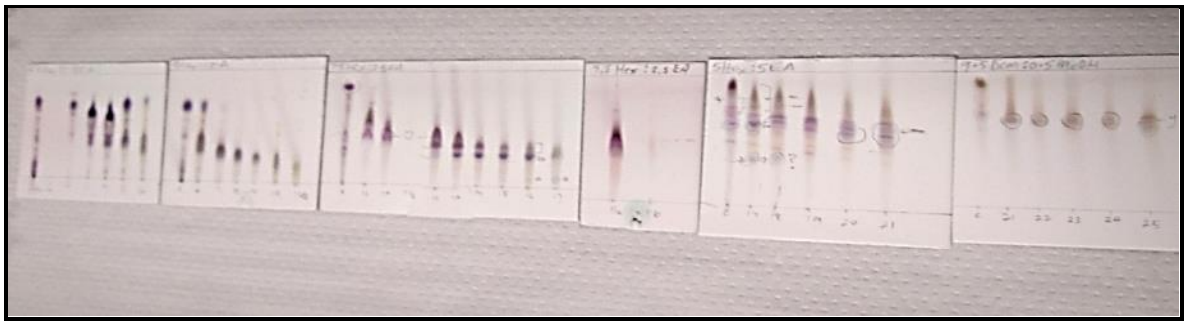
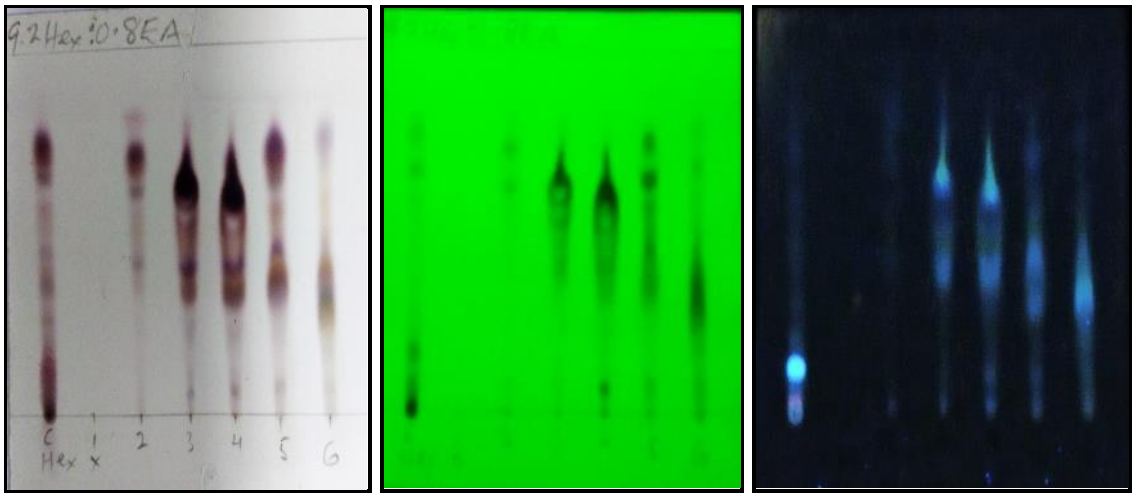
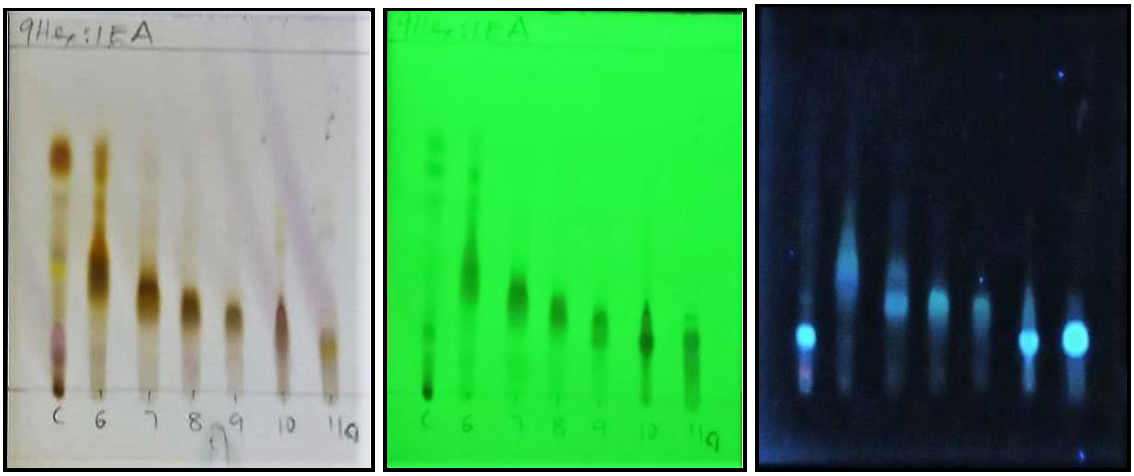


Figure 4.4: The TLC plates of the fractions in numerical order from fraction 2 (far left) to fraction 25 (far right). The furthest left sample on each plate is the control sample.

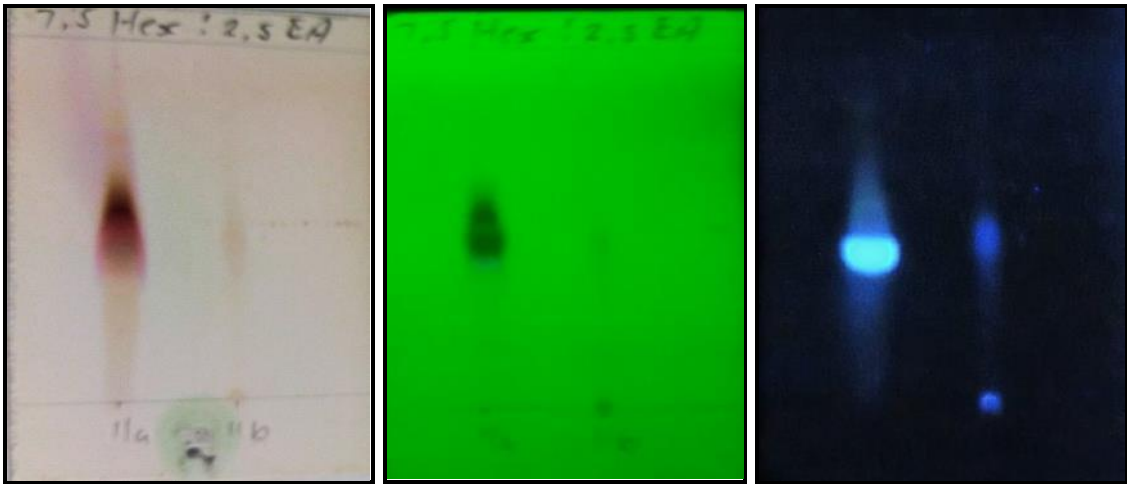
A.



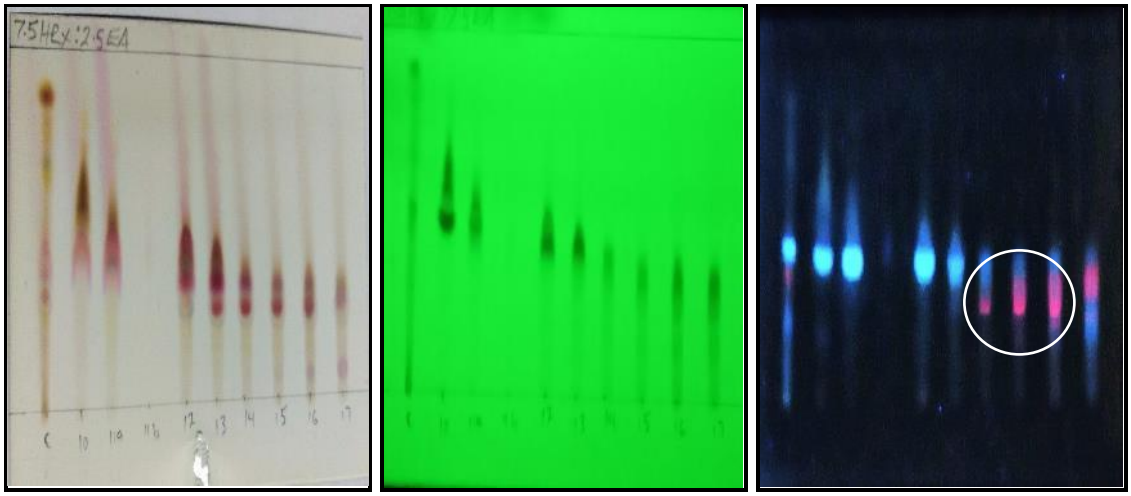
B.



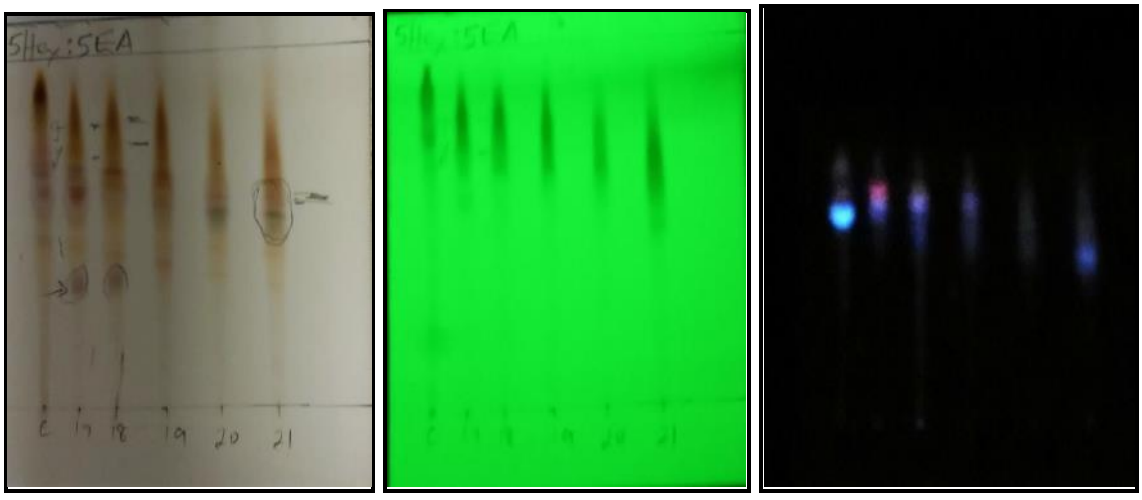
C.



D.



E.



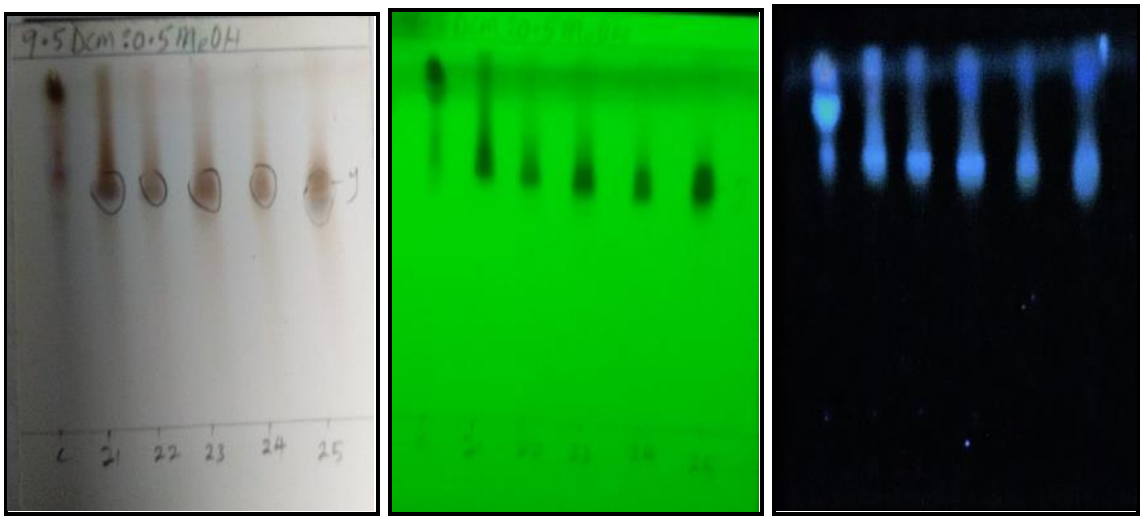
F. 

Figure 4.5: The TLC plates of the hexane fractions of *C. dentata* stem bark as visualized when stained (left), under shortwave (center) and longwave (right) UV exposure. The sample marked "C" on the left of each (stained) plate (except in Figure 4.5C) is a control sample of the hexane crude extracts. Figure 4.5A shows the presence of compounds in fractions 2 to 6. Figure 4.5B shows the presence of compounds in fractions 6 to 11a, and Figure 4.5C visualizes the compound in fraction 11b which consists the precipitate from fraction 11a. On the left of Figure 4.5C is fraction 11a and on the right is fraction 11b. Figure 4.5D visualizes the presence of compounds in fractions 10 to 17. Note the distinct colour change of fractions 14 to 16 under longwave UV exposure (encircled). Figure 4.5E shows the presence of compounds in fractions 17 to 21 and Figure 4.5F shows the presence of compounds in fractions 21 to 25.

Figure 4.6 shows the ^{1}H NMR spectrum of compound **1** and Figure 4.7 shows the ^{13}C NMR spectrum of compound **1.** The compound has an oleanane or ursane type skeleton and was identified as 3β-hydroxy-urs-12-en-28-oic acid (ursolic acid).

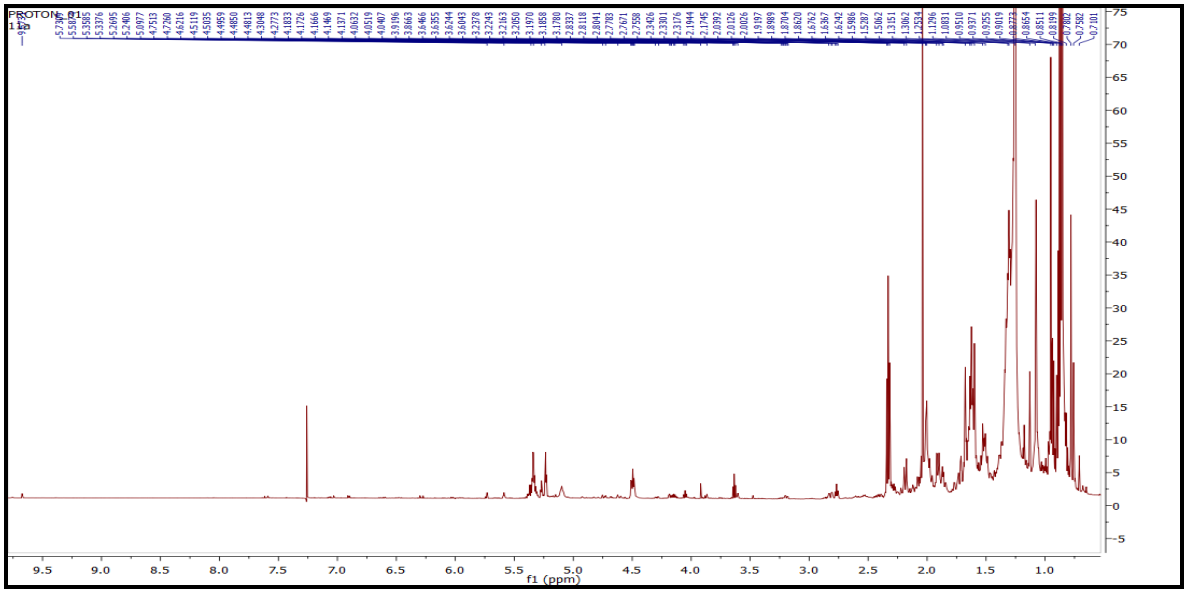


Figure 4.6: ^{1}H NMR spectrum of compound **1** in deuterated chloroform**.**

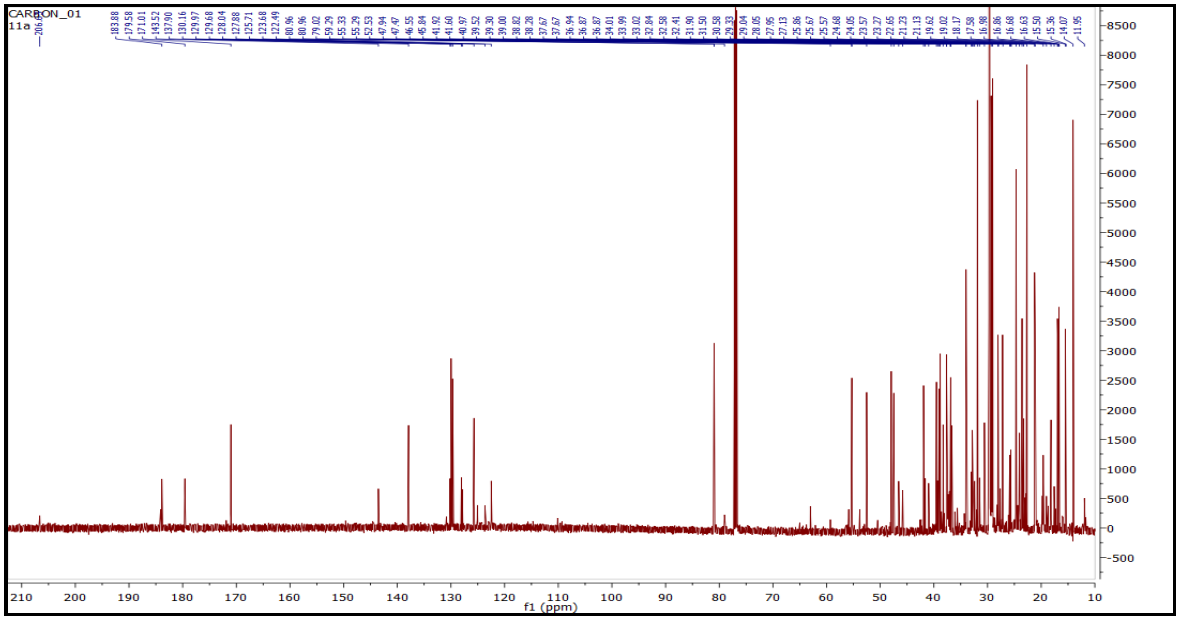


Figure 4.7: ^{13}C NMR spectrum of compound **1** in deuterated chloroform.

Fraction 12-13 contained two compounds, of which the first has a lupane type skeleton and the second a steroid-type skeleton. Figure 4.8 shows the ^{1}H NMR spectrum of compounds **2** and **3** and Figure 4.9 shows the ^{13}C NMR spectrum of compounds **2** and **3.** The compounds were identified as betulinaldehyde and stigmasterol respectively.

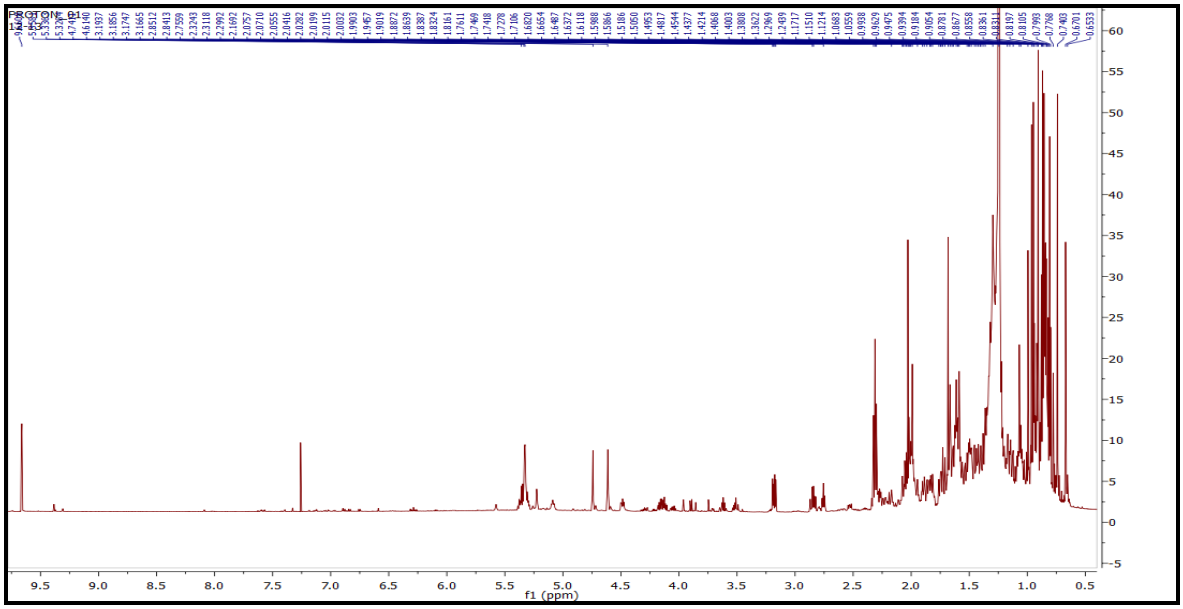


Figure 4.8: ^{1}H NMR spectrum of compounds 2 and 3 in deuterated chloroform.

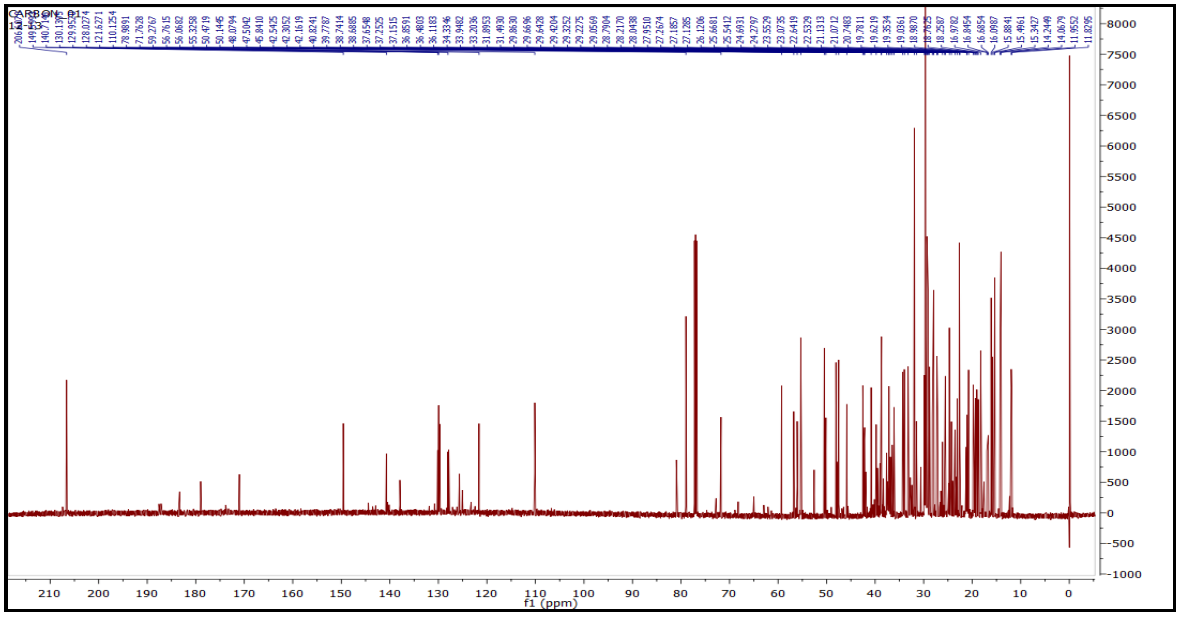


Figure 4.9: ^{13}C NMR spectrum of compounds **2** and **3** in deuterated chloroform.

Figure 4.10 shows the ^{1}H NMR spectrum of compound **4** and Figure 4.11 shows the ^{13}C NMR spectrum of compound **4.** The compound had a steroid skeleton and was identified as *β*-sitosterol.

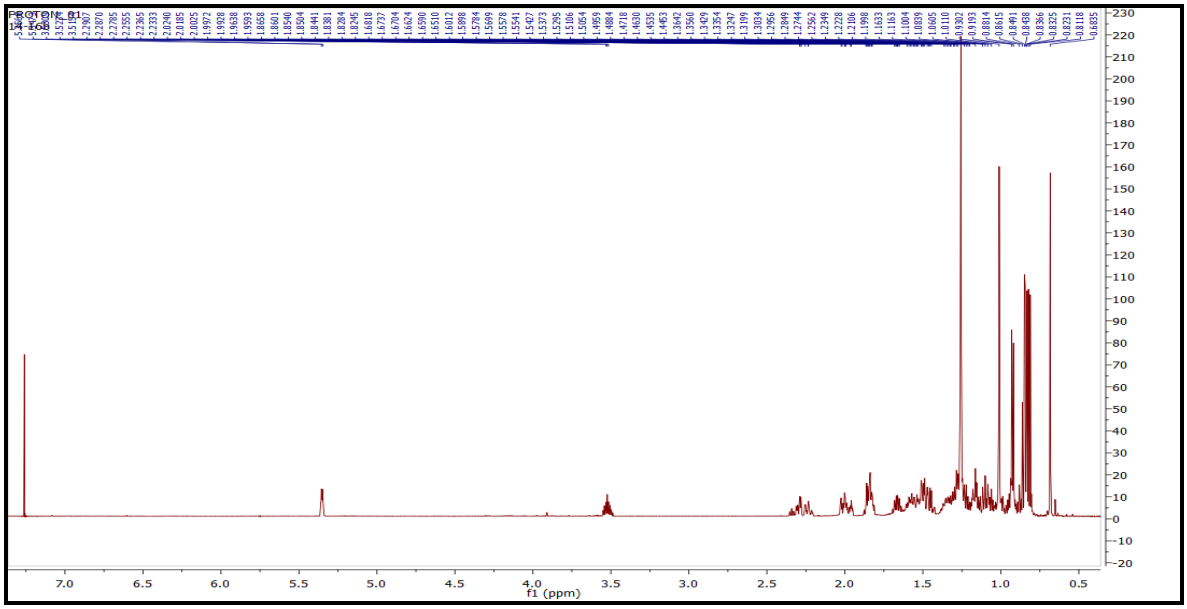


Figure 4.10: ^{1}H NMR spectrum of compound **4** in deuterated chloroform.

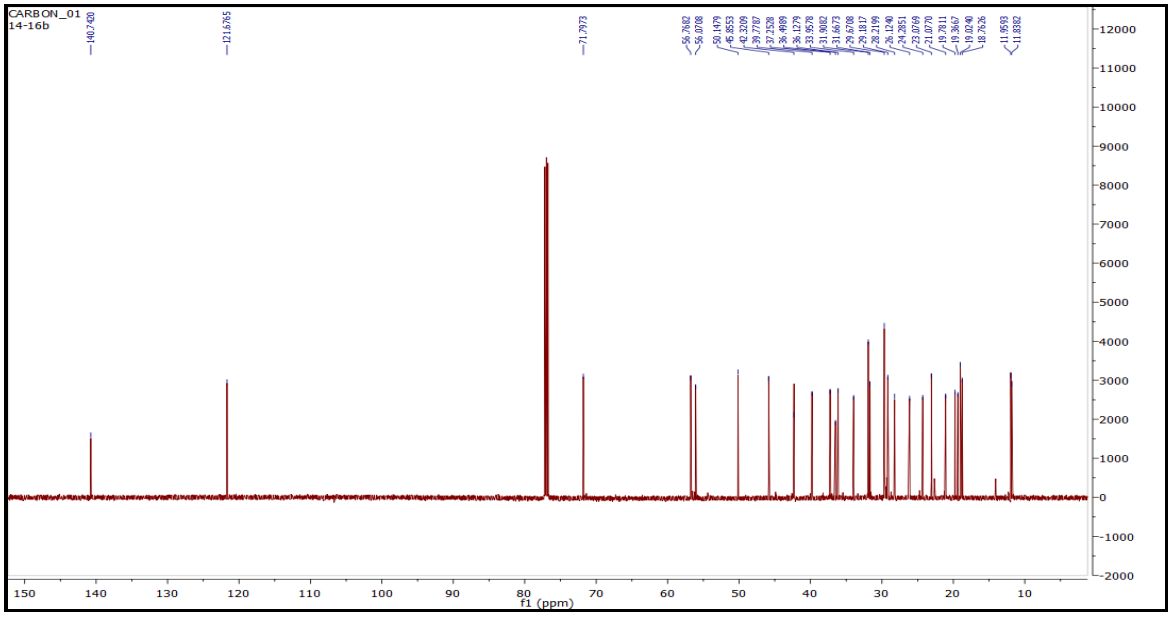


Figure 4.11: ^{13}C NMR spectrum of compound **4** in deuterated chloroform.

Figure 4.12 shows the ^{1}H NMR spectrum of compound **5** and Figure 4.13 shows the ^{13}C NMR spectrum of compound **5**. The compound has a lupane skeleton and was identified as 3β-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid).

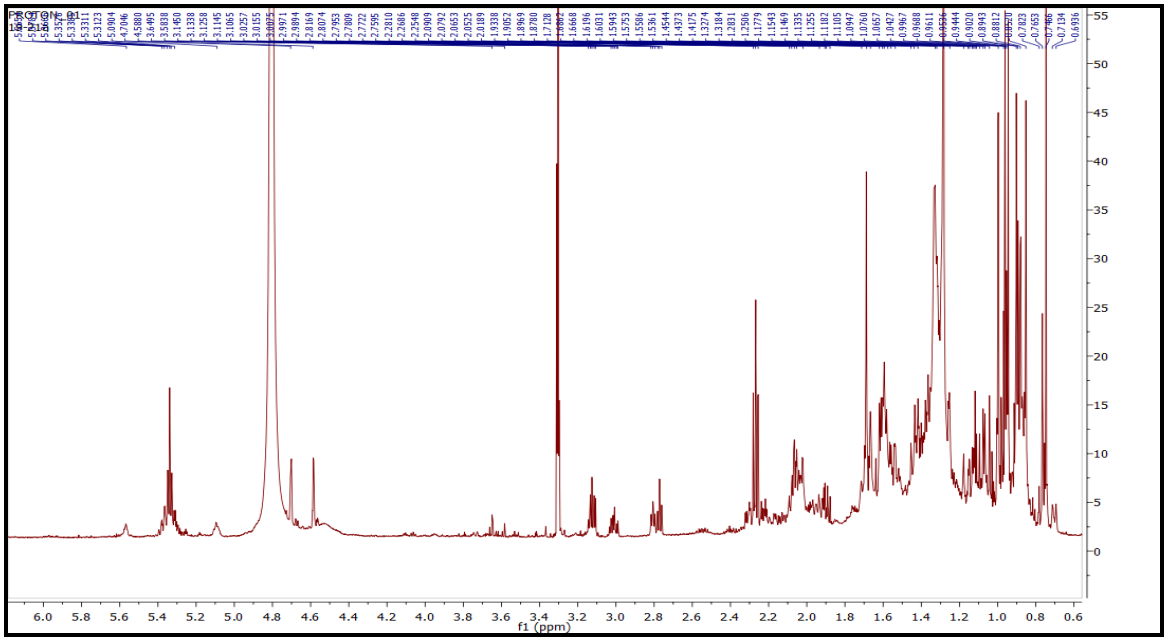


Figure 4.12: ^{1}H NMR spectrum of compound **5** in deuterated chloroform but with the addition of small amounts of deuterium oxide and deuterium methanol to improve solubility**.**

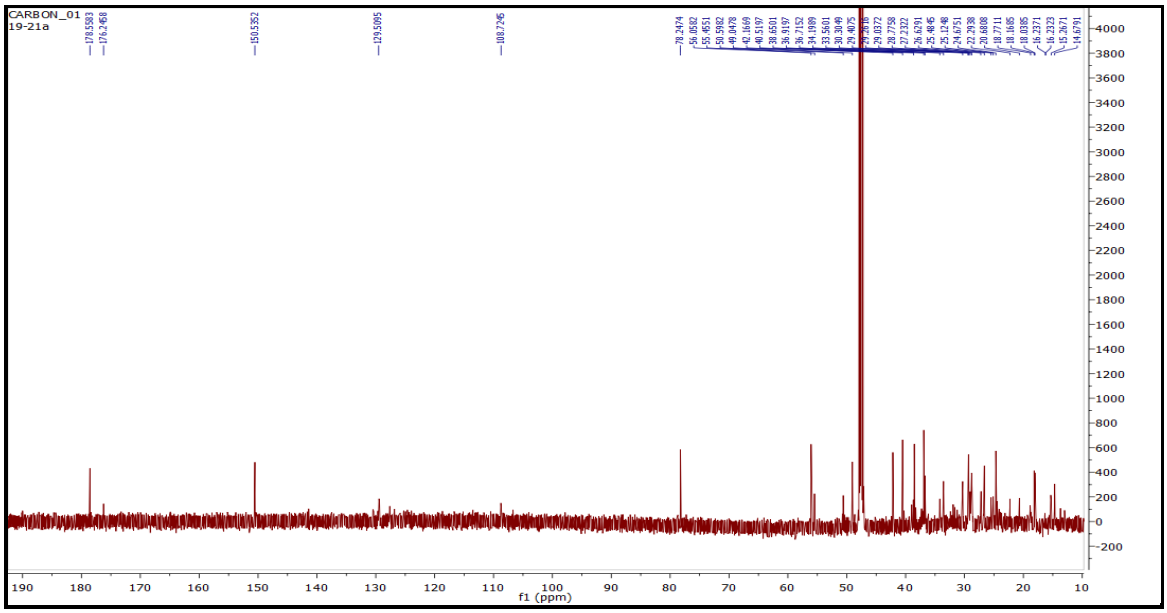


Figure 4.13: ^{13}C NMR of compound **5** in deuterated chloroform**.**

Figure 4.14 shows the ^{1}H NMR spectrum of compounds **6** and **7** and Figure 4.15 shows the ^{13}C NMR spectrum of compounds **6** and **7.**

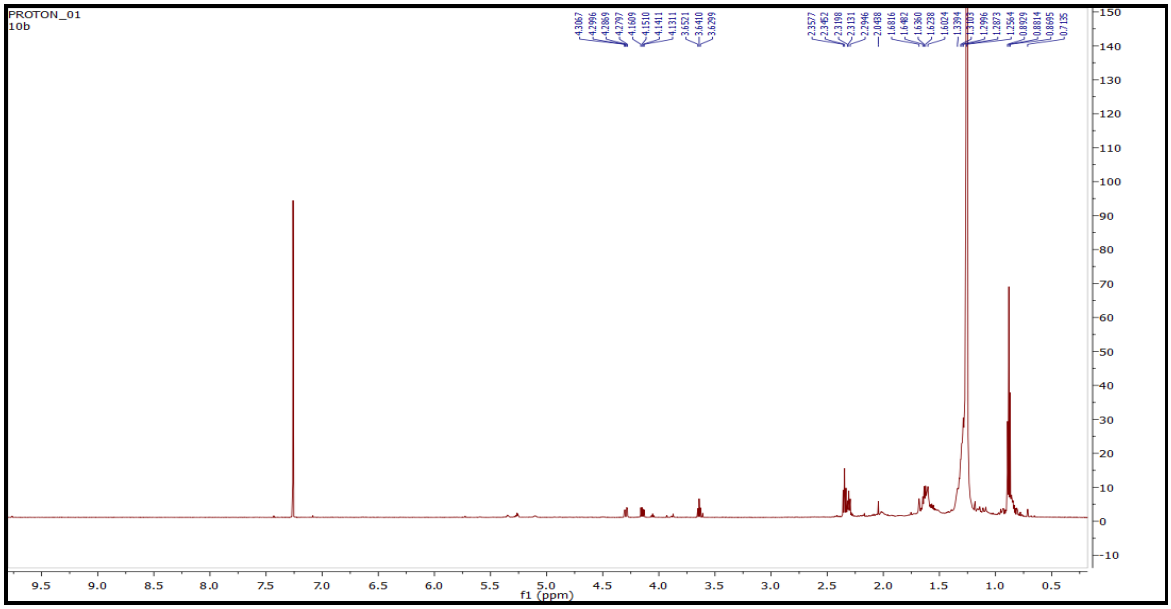


Figure 4.14: ^{1}H NMR spectrum of compounds **6** and **7** in deuterated chloroform.

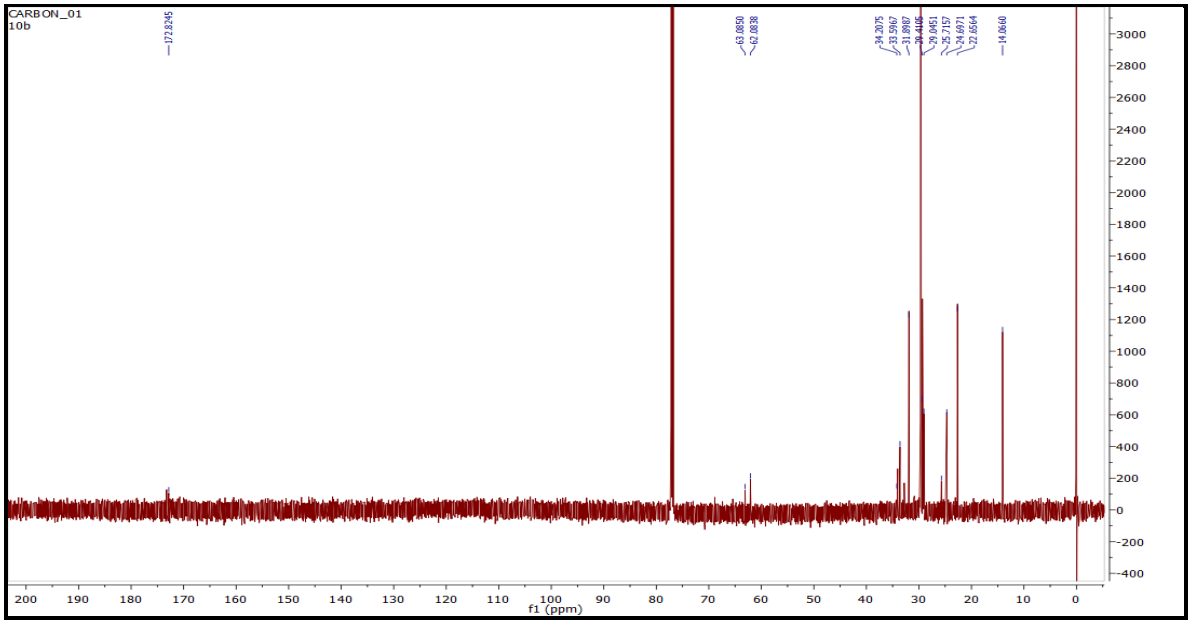


Figure 4.15: ^{13}C NMR spectrum of compounds **6** and **7** in deuterated chloroform.

Table 4.1 compares experimental data for compound **1** with reported data on ursolic acid published by Silva, Vieira, Mendes, Albuquerque, Dos Santos, Silva and Morais (2008:2486) and Shai (2007:91).

In Table 4.2, the experimental data for compound **2** perfectly corresponds to the values for betulinaldehyde as published by Monako and Previtera (1984:675) and by Lee, Jung, Park, Yoo, Chung and Baek (2010:143).

Table 4.3 compares experimental data for compound **3** with reported data on stigmasterol published by Habib, Nikkon, Rahman, Haque and Karim (2007:4175) and Xie, Wang, Ye and Li (2000:165).

Table 4.4 compares experimental data for compound **4** with reported data on *β*- sitosterol as published by Nweze, Ibrahim and Ndukwe (2019:1047) and Okoro, Tor-Anyiin, Igoli, Noundou and Krause (2017:3).

Table 4.5 compares experimental data for compound **5** with reported data on betulinic acid as reported by Shai, (2007:90) and Egbubine, Adeyemi and Habila (2020:5).

Table 4.1: The ^{13}C NMR chemical shifts of compound **1** is compared with literature on ursolic acid (Silva *et al.* 2008:2486; Shai, 2007:91).

Carbon	**Ursolic acid** (Silva *et al.* 2008:2486)	**Ursolic acid** (Shai, 2007:91)	**Compound 1 experimental**
1	38.2	39.1	39.3
2	26.8	28.2	28.0
3	76.8	77.5	79.0
4	38.3	39.8	39.5
5	54.7	55.5	55.3
6	18.0	18.7	18.1
7	30.7	33.4	33.9
8	39.1	41.1	40.9
9	47.0	47.5	47.4
10	36.5	38.9	38.2
11	23.8	23.5	23.5
12	124.5	125.2	125.7
13	138.2	138.9	137.9
14	41.6	42.3	41.9
15	32.7	27.7	28.0
16	22.8	24.5	24.6
17	46.8	47.5	47.4
18	52.4	53.0	52.5
19	38.4	37.0	39.0
20	38.5	37.2	38.2
21	27.5	30.9	30.5
22	36.3	37.2	37.6
23	28.2	28.9	28.0
24	16.9	16.8	16.8
25	16.0	15.9	15.5
26	11.2	17.6	17.5
27	23.2	23.9	24.0
28	178.2	179.0	179.5
29	16.9	17.7	17.0
30	21.1	21.8	21.2

Table 4.2: The ^{13}C NMR chemical shifts of compound **2** is compared with literature on betulinaldehyde (Monako and Previtera 1984:675; Lee *et al.*, 2010:143)

Carbon	**Betulinaldehyde** (Monako and Previtera 1984:675)	**Betulinaldehyde** (Lee *et al.* 2010:143)	**Compound 2 Experimental**
1	38.7	39.3	38.7
2	27.3	27.3	27.2
3	78.9	78.0	78.9
4	38.8	39.6	38.7
5	55.5	55.8	55.3
6	18.2	18.8	18.2
7	34.3	34.7	34.3
8	40.8	41.1	40.8
9	50.4	50.7	50.4
10	37.1	37.5	37.1
11	20.7	21.0	20.7
12	25.5	25.9	25.5
13	38.7	39.0	38.7
14	42.5	42.8	42.5
15	29.2	28.7	29.2
16	28.8	29.1	28.8
17	59.3	59.4	59.2
18	48.0	48.0	48.0
19	47.5	48.3	47.5
20	149.7	150.1	149.5
21	29.8	29.4	29.8
22	33.2	33.3	33.2
23	27.9	28.4	27.9
24	15.4	16.2	15.4
25	15.9	16.5	15.8
26	16.1	16.6	16.1
27	14.2	14.4	14.2
28	205.6	206.4	206.6
29	110.1	110.5	110.1
30	19.0	19.1	19.0

Table 4.3: The ^{13}C NMR chemical shifts of compound **3** is compared with literature on stigmasterol (Habib *et al.*, 2007:4175; Xie *et al.*, 2000:165).

Carbon	**Stigmasterol** (Habib *et al.*, 2007:4175)	**Stigmasterol** (Xie *et al.*, 2000:165)	**Compound 3 Experimental**
1	32.9	37.1	33.2
2	34.5	31.5	34.3
3	79.0	71.7	78.9
4	42.0	42.2	42.1
5	154.6	140.6	149.5
6	124.4	121.6	121.6
7	31.2	31.5	31.4
8	28.7	31.8	28.9
9	42.0	50.0	42.3
10	39.6	36.4	39.7
11	19.4	21.1	19.3
12	31.9	39.6	31.9
13	40.8	42.1	40.8
14	47.7	56.7	47.5
15	21.4	24.3	21.1
16	21.3	28.8	21.0
17	48.2	55.8	48.1
18	18.3	12.1	18.7
19	18.2	18.9	18.2
20	33.4	40.4	33.2
21	17.4	21.0	18.2
22	107.1	129.2	110.1
23	139.5	138.2	140.1
24	47.7	51.1	47.5
25	30.6	31.8	29.8
26	20.2	21.0	20.7
27	20.2	19.3	21.7
28	25.4	25.3	25.5
29	12.2	11.9	11.9

Table 4.4: The ^{13}C NMR chemical shifts of compound **4** is compared with literature on *β*-sitosterol (Nweze *et al.*, 2019:1047; Okoro *et al.*, 2017:3).

Carbon	***β*-sitosterol** (Nweze *et al.*, 2019:1047)	***β*-sitosterol** (Okoro *et al.*, 2017:3)	**Compound 4 experimental**
1	37.2	37.2	37.2
2	31.6	31.6	31.7
3	71.8	71.8	71.8
4	42.3	42.2	42.3
5	140.7	141.0	140.7
6	121.7	121.6	121.7
7	31.9	31.9	31.9
8	31.9	30.9	29.7
9	50.0	50.1	50.1
10	36.4	36.5	37.0
11	21.0	24.3	23.1
12	39.7	39.7	39.8
13	42.2	40.5	45.8
14	56.7	56.7	56.8
15	24.2	23.0	24.3
16	28.2	28.9	28.2
17	55.9	56.0	56.1
18	11.8	12.0	11.8
19	19.3	19.1	19.4
20	36.1	39.6	36.1
21	18.7	21.0	19.8
22	33.9	138.3	33.9
23	25.9	129.3	26.1
24	45.7	51.2	45.8
25	29.0	36.1	29.2
26	19.8	18.6	19.0
27	18.9	18.7	18.7
28	23.0	25.4	21.1
29	11.9	11.9	11.9

Table 4.5: ^{13}C NMR chemical shifts of compound **5** is compared with literature on betulinic acid (Shai, 2007:90; Egbubine *et al.*, 2020:5)

Carbon	**Betulinic acid** (Shai, 2007:90)	**Betulinic acid** (Egbubine *et al.*, 2020:5)	**Compound 5 Experimental**
1	37.6	39.4	38.6
2	27.2	27.3	27.2
3	76.8	77.4	78.2
4	38.5	38.7	38.6
5	55.4	55.1	55.4
6	18.0	18.2	18.7
7	33.9	34.1	33.5
8	40.3	40.0	40.5
9	49.9	50.1	50.5
10	38.3	36.9	36.7
11	20.5	20.5	20.6
12	25.1	25.30	25.4
13	36.7	37.8	36.9
14	42.0	42.2	42.1
15	31.7	30.3	30.3
16	33.9	31.9	44.5
17	54.9	55.6	55.4
18	46.6	46.8	49.0
19	48.5	48.7	49.0
20	150.3	150.5	150.5
21	30.1	36.6	36.7
22	36.4	29.4	29.4
23	28.1	28.3	28.7
24	15.9	15.9	16.2
25	15.8	15.9	15.2
26	15.7	16.1	16.2
27	14.4	14.6	14.6
28	177.2	177.5	176.2
29	109.7	109.8	108.7
30	18.9	19.4	18.7

Compound **6** is a long chain fatty alcohol with 24 carbon atoms and a hydroxy group that replaces a hydrogen atom at one of the terminal carbons. The structure of the compound was compared to literature (Makhafola, Elgorashi, McGaw, Awouafak, Verschaeve and Eloff, 2017:4) and was identified as n-tetracosanol.

Compound **7** is a long chain fatty acid with a 16-carbon backbone. The structure of the compound was compared to literature (Ravi and Krishnan 2017:20-27) and was identified as n-hexadecanoic acid.

4.4 DISCUSSION

Liquid column chromatography was employed to isolate compounds in *C. dentata* stem bark. The presence of compounds was evaluated using TLC plates. After shortwave and longwave UV visualization, staining and heating, compounds present could be marked and fractions with similar retention factors could be pooled. ^{1}H NMR and ^{13}C NMR were employed to elucidate the structures of the isolated compounds. For compounds **1, 2** and **5,** 30 carbon atoms were assigned to each of the respective compounds. For compounds **3** and **4**, 29 carbon atoms were assigned to each of the respective compounds. Compound **6** has 24 carbon atoms and compound **7** has 16 carbon atoms.

4.4.1 Compound 1

The ^{1}H NMR spectrum for compound **1** showed signals of an olefinic proton at δ 5.1 ppm and seven methyls which are characteristic of an oleanane or ursane type structure. This structure is supported by ^{13}C NMR as it contains seven methyls (δ_C 28.0 [C23], 16.8 [C24], 15.5 [C25], 17.5 [C26], 24.0 [C27], 17.0 [C29], 21.2 [C30]), olefinic carbons (δ_C 125.7 [C12], 137.9 [C13], a carboxylic acid at δ_C 179.5 (C28) and a beta hydroxy group at δ 79.02 (C3) which is characteristic of 3*β*-hydroxyurs-12-en-28-oic acid (Table 4.1). The structure of ursolic acid is displayed in Figure 4.16. Ursolic acid was isolated from several plant species, including the peels of apples (Yamaguchi, Noshita, Kidachi, Umetsu, Hayashi, Komiyama, Funayama and Ryoyama, 2008:654-660). Ursolic acid is also present in coffee and the leaves of herbs such as marjoram, rosemary, sage thyme and lavender (Woźniak, Skapska and Marszalek, 2015:20615). Ursolic acid is known for its anti-inflammatory, anti-cancer, cardioprotective, hepatoprotective, neuroprotective, antimicrobial, antidiabetic, antifungal and antiviral potentials (Pironi *et al.*, 2018:86; Seo *et al.*, 2018:235). A derivative of ursolic acid, ursolic aldehyde, was detected by GC-MS analysis in Chapter 3. Ursolic acid was isolated from the leaves of *C. dentata* (Shai, 2007:1-208) but was not isolated from *C. dentata* stem bark before.

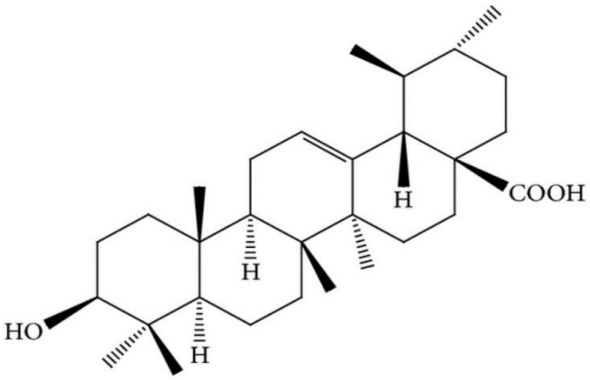


Figure 4.16: The chemical structure of ursolic acid.

4.4.2 Compound 2

The ^{1}H NMR spectrum of compound **2** showed the presence of five tertiary methyl groups at δ 0.74, 0.85, 0.86, 0.79 and 0.91 ppm and one isoprenyl moiety at δ 1.76, 4.60 and 4,70 ppm, indicating a lupane type skeleton. A characteristic aldehyde hydrogen was present at 9.66 ppm. The carbonyl group moved downfield to affect an adjacent aldehyde group at δ 2.32 ppm. The ^{13}C NMR of compound **2** supports this structure with methyl signals at δ 15.4 (C24) 15.8 (C25), 16.1 (C26), 14.2 (C27), and 19.0 (C30), an olefinic carbon at δ 149.5 (C20) and a carbonyl group at 206.6 (C28) (Table 4.2). The compound was identified as betulinaldehyde. The chemical structure of betulinaldehyde is shown in Figure 4.17. Betulinaldehyde was previously isolated from *Quercus suber* (Monako and Previtera,1984: 673-676) and *Cornus cosa* fruits (Lee *et al*., 2010:142-145) and showed significant inhibitory activity against human cancer cell lines (Lee *et al.*, 2010:156). Betulinaldehyde has not been isolated from *C. dentata* before.

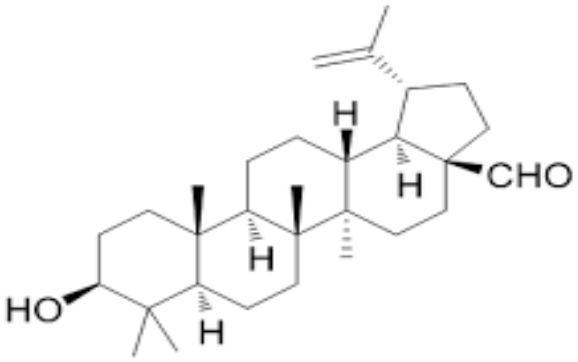


Figure 4.17: The chemical structure of betulinaldehyde.

4.4.3 Compound 3

The ^{1}H NMR spectrum of compound **3** showed the existence of signals for olefinic protons at δ 5.32, 4.68, 4.61 and 2.32. Methyl protons at δ 0.67, 0.81 and 1.05 corresponds to C18 and C19 respectively. ^{13}C NMR downfield resonances at δ 149.5 (C5) and 121.6 (C6) and signals at δ 110.1 (C22) and 140.7 (C23) indicate unsaturated double bonds. The values at δ 18.2 and 18.7 correspond to the angular carbon bonds at C19 and C18 respectively. The resonance at δ 79.0 is due to the C3 *β*- hydroxyl group which is characteristic of stigmasterol (Table 4.3). The chemical structure of stigmasterol is shown in Figure 4.18. Researchers claim that stigmasterol may be useful in prevention of certain cancers, including ovarian, prostate, breast, and colon cancers. It is further claimed that stigmasterol possesses antioxidant, hypoglycemic and thyroid inhibiting properties (Panda *et al.* 2009:123-126), and also laxative properties (Pierre and Moses, 2015:88-96). Stigmasterol was previously isolated from various plant species, including *Ageratum conyzoides* (Asteraceae) (Kamboj and Saluja, 2011: 1-3) and *Odontonema strictum* (Acanthaceae) (Pierre and Moses, 2015:88-96). Stigmasterol was, however, not isolated from *C. dentata* before.

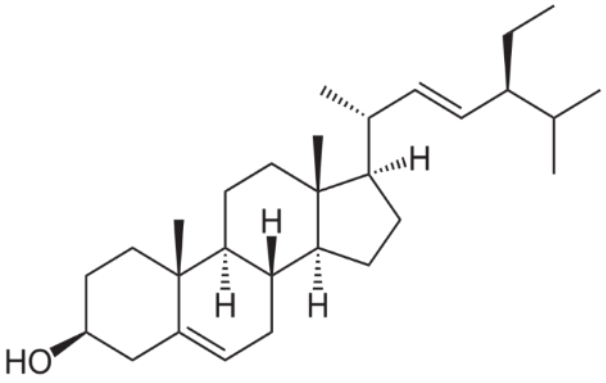


Figure 4.18: The chemical structure of stigmasterol.

4.4.4 Compound 4

The structures of compounds **3** and **4** are very similar. ^{1}H NMR of compound **4** showed six high intensity peaks indicating the presence of methyl groups at δ 0.68, 0.82, 0.84, 0.86, 0.88 and 0.93. An olefinic proton signal showed at δ 5.36. These assignments agree with the structure of *β*-sitosterol. This structure is supported by ^{13}C NMR with signals at δ 140.7 and 121.7 for the C5 = C6 double bond respectively, a signal at δ 71.8 for the C3 hydroxyl group and signals at δ 19.4 and 11.8 for angular methyl carbon atoms at C19 and C18 respectively (Table 4.4). The chemical structure of *β*-sitosterol is exhibited in Figure 4.19. The GC-MS analysis of the hexane crude extract in Chapter 3 detected α-sitosterol in *C. dentata* stem

bark, whereas *β*-sitosterol was isolated from the extract with column chromatography. With GC-MS analysis, it is possible that not all chemical compounds are detected due to compounds overlapping. However, fractionation methods employed to attempt the isolation of pure constituents from plant extracts sometimes also modifies the chemistry of a compound (Bodede, Shaik, Govinden and Moodley, 2017:6). Pharmacological potentials of *β*-sitosterol include anti-inflammatory, antioxidant, antidiabetic and apoptosis inducing activities, and it also showed chemoprotective and chemo preventative effects (Saeidnia *et al.*, 2014: 590-609; Pierre and Moses, 2015:89). Concerning is that *in vivo* studies on fish indicated that *β*-sitosterol is an endocrine disrupting compound where it acts as an estrogen receptor agonist. It is also androgenic after metabolization (Hilscherova, Machala, Kannan, Blankenship and Giesy, 2000:162). *B*-Sitosterol was also previously isolated from *C. dentata* leaves (Fadipe *et al*., 2017:1-6), but was not isolated from *C. dentata* stem bark before.

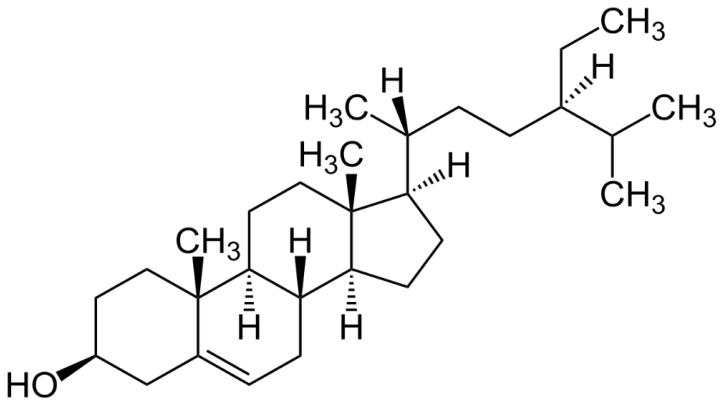


Figure 4.19: The chemical structure of *β*-sitosterol.

4.4.5 Compound 5

The ^{1}H NMR spectrum for compound **5** showed signals for tertiary methyl groups at δ 0.74, 0.85, 0.87, 0.69 and 0.90, one isoprenyl moiety at δ 1.72, 4.60 and 4.73, indicating a lupane type skeleton. The ^{13}C NMR spectrum of compound **5** supports the structure with methyls at δ 16.2 (C24), 15.2 (C25), 15.2 (C26), 14.6 (C27) and 18.7 (30) and a carboxylic acid at δ 178.5 (C28). The compound was identified as 3β-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid) by direct comparison of the spectral data with reported values (Table 4.5). The chemical structure for betulinic acid is shown in Figure 4.20. Betulinic acid showed anti-HIV, anti-bacterial, anti-helminthic, anti-inflammatory, anti-protozoal, anti-obese and anti-malarial potentials and showed an inhibitory effect on *Candida albicans* (Moghaddam *et al.*, 2012). Betulinic acid is present in numerous plant species. Betulinic acid was isolated from *C. dentata* leaves, but was not previously isolated from *C. dentata* stem bark.

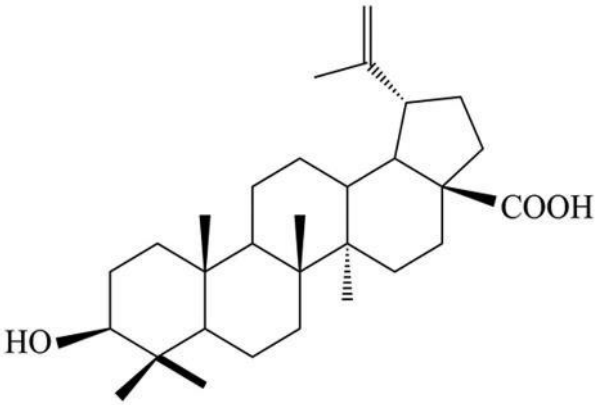


Figure 4.20: The chemical structure of betulinic acid.

4.4.6 Compound 6

The proton NMR of compound **6** showed the presence of a two-proton triplet at δ 3.62, 3.64 and 3.65, and a three-proton triplet at δ 0.86, 0.88 and 0.89 adduced to hydroxy methylene H_2-1 and terminal C24 primary methyl protons respectively. The methylene protons were found to resonate as multiplets at δ 1.68 and 1.31 and as broad singlet at δ 1.34. The ^{13}C NMR spectrum of compound **6** displayed signals for hydroxy methylene at δ 63.08 (C1), methylene carbons between δ 34.3 and δ 22.65 and methyl carbon at δ 14.06 (C24). The 24 carbon atoms and the hydroxy group that replaces a hydrogen atom at one of the terminal carbons is characteristic of n-tetracosanol (Fig. 4.21). The NMR values for n-tetracosanol was compared with literature (Gowtham, Asharani and Paridhavi, 2019) and the structure compared with Makhafola, Elgorashi, McGaw, Awouafak, Verschaeve and Eloff (2017:4). N-Tetracosanol isolated from the roots of *Premna latifolia* showed promising antidiabetic activity, reno and hepatoprotective effects and antioxidant activity (Gowtham Asharani and Paridhavi, 2018, 2019). To the contrary, n-tetracosanol isolated from *Origanum onites* showed no effects against any of the 14 phytobacteria used in assays (Kotan, Cakir, Ozer, Kordali, Cakmakci, Dadasoglu, Dikbas and Kazaz, 2014: 210-220). A similar compound, tetracosane, was detected by GC-MS analysis in Chapter 3. N-Tetracosanol was not previously isolated from *C. dentata.*

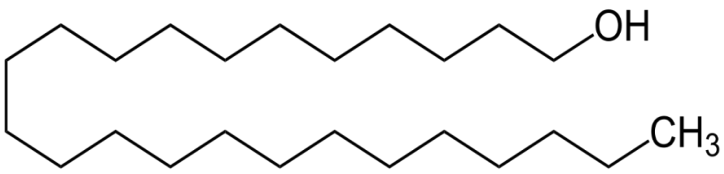


Figure 4.21: The chemical structure of n-tetracosanol.

4.4.7 Compound 7

The ^{1}H NMR analysis revealed the positioning and number of hydrogen atoms within the molecule, which is 32 hydrogen atoms. The ^{13}C NMR spectrum showed that compound **7** has 16 carbon atoms. The absence of aromatic hydrogen atoms in the spectrum suggests that the compound is aliphatic in nature. The structure of the compound (Fig. 4.22) was compared to literature on n-hexadecanoic acid (Ravi and Krishnan, 2017:20-27). Ravi and Krishnan (2017:20-27) isolated n-hexadecanoic acid from *Kigelia pinnata* and determined that n-hexadecanoic acid showed significant cytotoxicity against human colorectal carcinoma cells (HCT-116) with an IC_{50} value of 0.8 µg/mL^{-1}. This compound was also detected by GC-MS analysis in Chapter 3. The application of exogenous fatty acids inhibit fatty acid biosynthetic pathways in the malarial parasite, *Plasmodium falciparum*, and exposure to 2-hexadecanoic acid blocks fatty acid biosynthesis as well as its degeneration in mycobacteria. (Ma *et al.*, 2019:2). N-Hexadecanoic acid, also known as palmitic acid, was not previously isolated from *C. dentata.*

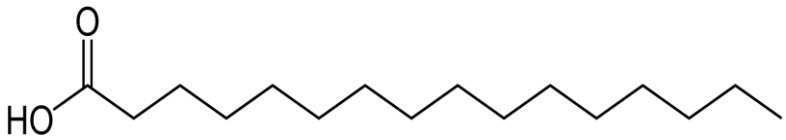


Figure 4.22: Chemical structure of n-hexadecanoic acid.

The traditional use of *C. dentata* stem bark to treat stomach ailments, diarrhea and the use as an aphrodisiac cannot be linked to any of the isolated and identified compounds as these activities have not been reported for any of the compounds. The treatment of sexually transmitted diseases might be linked to the pharmacological studies indicating anti-microbial activity for ursolic acid and betulinic acid. Both these compounds however are reported with low solubility and limited absorption, and it is therefore unlikely that these compounds contribute to the traditional uses of the stem bark. Ursolic acid and *β*-sitosterol have reported anti-diabetic activity. Except for *β*-sitosterol with reported anti-diabetic activity, none of isolated compounds can therefore be used as biological markers and cannot be used in determining the biological activity of the bark.

It is however interesting that five compounds namely ursolic acid, betulinaldehyde, stigmasterol, betulinic acid and n-hexadecanoic acid, have reported pharmacological activity against various cancer cell lines (Lee *et al*. 2010:142-145; Pironi *et al*. 2018:86-93; Panda *et al*. 2009:123-126; Gauthier *et al*. 2011:521-544; Ravi and Krishnan, 2017:20-27), although

there is no report of the use of *C. dentata* stem bark to treat cancer. The chemical compounds isolated from *C. dentata* stem bark (as well as those previously isolated from the leaves) have limited associations to the ailments the stem bark of *C. dentata* trees are traditionally used for. More research is therefore required before the traditional use of the species can be validated or supported.

4.5 CONCLUSION

Exhaustive extraction of compounds from *C. dentata* stem bark, and TLC, column chromatography, ^{1}H NMR and ^{13}C NMR analysis of the hexane crude extracts were employed to isolate and identify compounds in *C. dentata* stem bark. The compounds of which the structures were elucidated include ursolic acid, betulinaldehyde, stigmasterol, *β*-sitosterol, betulinic acid, n-tetracosanol and n-hexadecanoic acid. Ursolic acid, betulinic acid and *β*-sitosterol were previously isolated from *C. dentata* leaves but were not previously isolated from *C. dentata* stem bark. The compounds betulinaldehyde, stigmasterol, n-tetracosanol and n-hexadecanoic acid were not previously isolated from *C. dentata.* The traditional use of *C. dentata* stem bark to treat stomach ailments, diarrhea and the use as an aphrodisiac cannot be linked to any of the isolated and identified compounds as these activities have not been reported for any of the compounds. The limited association of the isolated compounds to the medicinal use of the stem bark provide substantial opportunities for the isolation and identification of compounds which can support and validate the species' traditional use.

CHAPTER 5. SEASONAL AND REGIONAL VARIABILITY OF CHEMICAL COMPOUNDS IN *Curtisia dentata* (Burm.f.) C.A.Sm. STEM BARK

5.1 INTRODUCTION

Medicinal plants and plant parts are harvested throughout the year to satisfy the increasing demand in the medicinal plant trade markets. In the second chapter, it was established that secondary metabolite production varies between individual trees of the same species due to genetic variability and age differences (Zobayed, *et al.*, 2005:245; Çirak *et al.*, 2007:200) and due to both biotic and abiotic environmental stress factors (Çirak *et al.*, 2007:200; Tanko *et al.*, 2005:2; Gershenzon and Dudareva, 2007:408; Street *et al.*, 2008; Akula and Ravishankar, 2011:1720-1731; Pavarini *et al.*, 2012:8; Prinsloo and Nogemane, 2018:1). Furthermore, secondary metabolite production may also vary due to differences in species and cultivars, the differential expressions of genes, the different biosynthetic pathways, the different enzymes involved and the interactions of two or more simultaneous environmental stress factors (Prinsloo and Nogemane, 2018:1).

This chapter, however, focuses on the seasonal variability of metabolite profiles and the regional differences in seasonal responses of metabolites in the stem bark of *C. dentata* trees. To determine seasonal effects on secondary metabolite production in different geographical regions, comparisons are firstly made between trees occurring in the same geographical region. The metabolite profiles of *C. dentata* trees occurring in two geographically separated regions are then compared. Stem bark samples from *C. dentata* trees in the two geographical regions were not collected in the same year, but three years apart, and time differences in sample collection is also considered. Plant material used as traditional medicines are collected during all seasons, in different years and from different locations, but are sold at the same market and used for the same purpose, even though no information on the date of collection is available. The comparison of the metabolite profiles of *C. dentata* stem bark samples from different geographical regions collected over a 13-month period therefore provide more insight into the variability of the material as it is harvested during all seasons, from different regions and during different years. Seasonal and regional variability relate to the differences in concentrations (dosages) of secondary metabolites extracted and consumed when using plant-based material as traditional medicine.

5.2 METHODS AND MATERIALS

5.2.1 Research sites

Stem bark samples from *C. dentata* trees were collected from two research sites in South Africa. The first research site from which bark samples were collected (2015-2016) was at Groenkop (Figure 5.1), which is situated between the towns of George and Wilderness in the Wilderness section of the GRNP, and can be located at S33°59,498' E022°32,850'. Groenkop lies at 260m above sea level. The area receives rainfall throughout the year with peaks in autumn and early summer (Vermeulen, 2009:4). Soils derive from rocks of the Cape Supergroup and constitute Maalgaten granite and rocks from the Kaaimans formation (SANParks, 2014:16). The second research site from which stem bark sample s were collected (2018-2019) was at the Nkandla Forest Reserve (Figure 5.2), which is situated in the KZN midlands. The research site is 1100m above sea level and can be located at S28°43'0" E031°7'60". The Nkandla forest area receives rainfall in summer and soils mainly derive from granite and quartzite type rocks (Ngcobo, 2002:16).

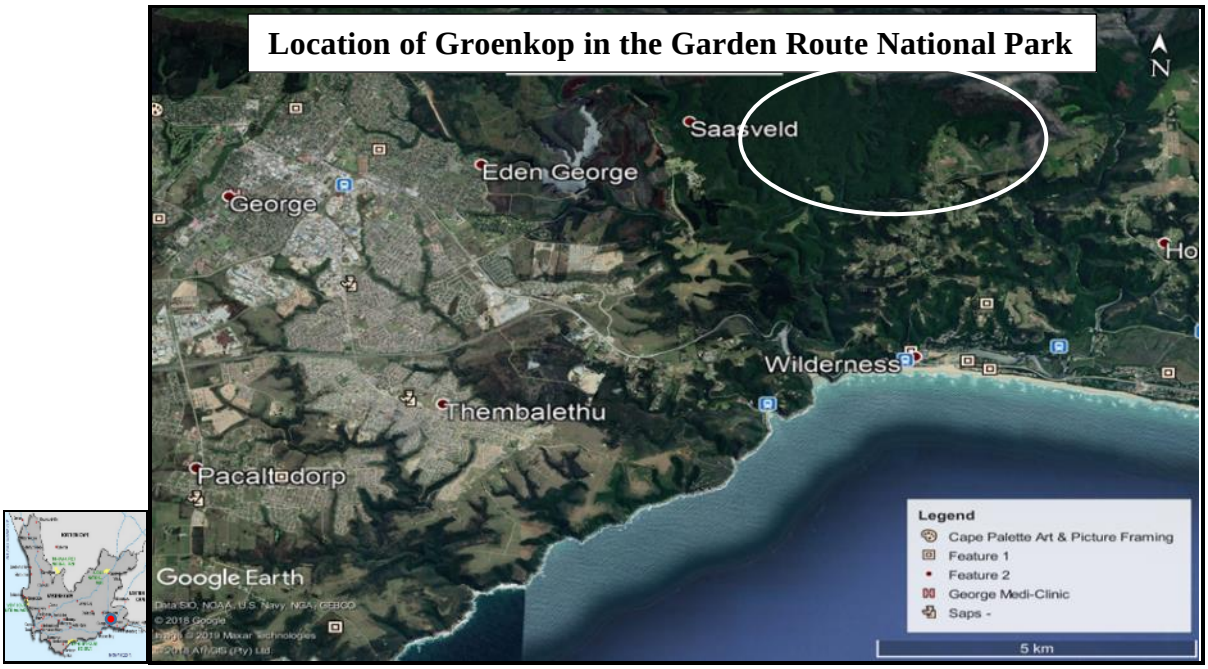


Figure 5.1: Map of the George/Wilderness area with the Groenkop section of the GRNP encircled (Google Earth). To the left is a map of the Western Cape Province of South Africa, indicating the location of the Groenkop section of the GRNP.

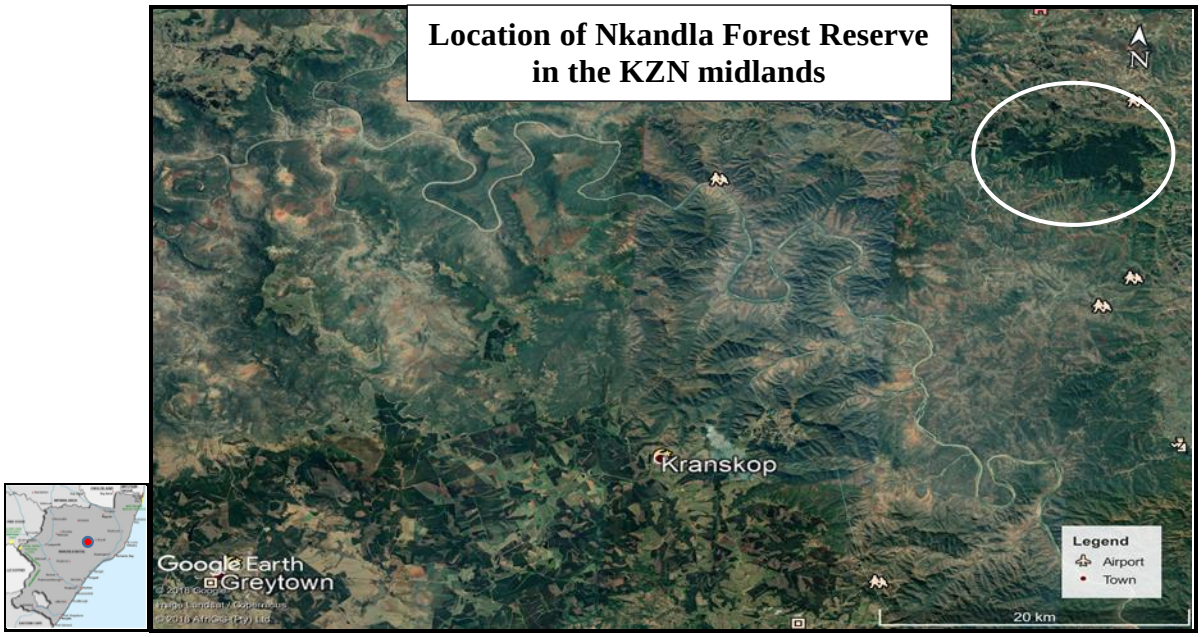


Figure 5.2: Map of the KZN midlands with the location of Nkandla forest encircled (Google Earth). To the left is a map of the KZN Province of South Africa, indicating the location of the Nkandla Forest Reserve.

5.2.2 Tree selection, frequency and sample sizes

At both research sites, *C. dentata* trees were selected according to the limitations imposed on the researcher by SANParks during the 2015/2016 sampling period. The limitations with respect to tree size and number of trees were upheld as far as possible during the 2018/2019 sampling period to maintain uniformity. At the Groenkop research site, SANParks' Scientific Services identified 12 *C. dentata* trees from which barks samples could be collected, and similarly at Nkandla Forest Reserve, KZN Wildlife identified 12 *C. dentata* trees for sampling purposes. At the Groenkop site, 11 of the 12 trees allocated were trees on which previous sustainable harvesting experiments were conducted in 2001 (Vermeulen, 2009:1-197). At the Nkandla Forest Reserve, only one tree was unscathed, thus without any bark harvesting damage, however, the trees in general and the canopies of the harvested trees were healthy and green.

Stem bark samples from the allocated *C. dentata* trees at Groenkop were collected bi-monthly between September 2015 and September 2016 for a previous study, and thus entailed seven sampling periods. The remainder of the sample material was kept in a -80°C freezer at the University of South Africa science campus laboratory complex. Stem bark samples from the allocated *C. dentata* trees at Nkandla forest were collected bi-monthly between September

2018 and September 2019 to match the months in which samples were collected during the 2015/2016 sampling period. From the stem of each tree, 1 x 8mm x 25mm sample was collected. The total number of samples collected over both sampling periods are thus 168 samples (12 trees x 7 sampling dates = 84 samples per site x 2 research sites = 168 samples).

5.2.3 Method for bark sample collection

Stem bark samples at each sampling date and at both the research sites were collected by cutting one 8mm x 25mm piece from the stem using a hammer and chisel. The pieces were carefully lifted out using a flat-tipped screwdriver. Each sample was placed in a separate cryovial, marked and immersed into liquid nitrogen. The holes in the stems after bark removal were plugged with heated candle wax (Price's candles, South Africa) (Figure 5.3A), pressed, smoothed and painted over with grafting grade water-based bitumen grafting sealer and protector (abe® Tree Seal, Chryso Group, Boksburg North, South Africa) to prevent desiccation and bacterial, insect and fungal infestation (Figure 5.3B). For the flight to the Gauteng Province, all bark samples collected at each sampling period were transferred to a polystyrene container filled with 2,5Kg dry ice before boarding. The samples were taken to the laboratories at the University of South Africa science campus soonest after landing, where preparations for processing commenced immediately.

A.  B. 

Figure 5.3: Restoration of the *C. dentata* tree after bark sample collection. The holes left by bark sampling were plugged with candle wax (Fig. 5.3A) and painted with water-based bitumen sealer and protector (Fig. 5.3B).

5.2.4 Pre-processing and NMR analysis of *C. dentata* stem bark

After each sample collection, the frozen bark samples were freeze-dried (Labonco® Freezone 2.5) for 24 hours at -55.5º C with a vacuum of 0.010 millibar (0.001 kPa). Each sample was separately ground to a fine powder using a mortar and pestle, after which two 50 mg samples of each of the samples were weighed and placed into separate Eppendorff tubes. At each sampling period, 24 x 50 mg samples (two sets of 12 each) were prepared for untargeted ^{1}H NMR analysis. The first set of 50 mg samples was extracted by adding 750 µl of deuterium oxide and 750 µl of deuterium methanol (Merck, South Africa) to each Eppendorf tube. To 100 ml of deuterium oxide, 1.232 g potassium dihydrogen phosphate (KH_2PO_4) and 10 mg trimethylsilylproprionic acid sodium salt (TSP, internal standard) was added with the pH adjusted to 6 using sodium deuteroxide solution (NaOD). The samples were then macerated on a Labsmart M-X vortex mixer for 1 min each, sonicated for 20 min in a Branson 1800 ultrasound bath and centrifuged for 15 minutes in an Eppendorff Mini Spin to separate the particulate matter from the supernatant. From each sample, 600 µl of the supernatant was transferred into 5 mm NMR tubes (Norell, Sigma-Aldrich) using a pipette. NMR lids were placed onto each NMR tube to reduce evaporation. The samples were analyzed with a Varian 600 NMR spectrometer. For each sample, 64 scans were recorded with consistent settings throughout. The chemical shift ranges of δ 3.23 – 3.36 ppm representing methanol, as well as the chemical shift ranges of δ 4.6 – 5 ppm representing water, were excluded from further analysis.

The second set of 50 mg samples were extracted by adding 1,5 ml deuterium chloroform to the sample material in each of the Eppendorff tubes. The samples were vortexed and sonicated but not centrifuged, as centrifuging did not separate the particles from the supernatant. After treatment in the ultrasound bath, 600 µl supernatant from each sample was thus filtered by placing a small ball of cotton wool in each tube, thereby extracting the supernatant through the cotton wool using a pipette. The remainder of the procedure was executed in the same manner as for the first set of samples. However, with the samples extracted with deuterium chloroform, the chemical shift ranges of δ 7.25 – 7.29 ppm representing chloroform were excluded.

The ^{1}H NMR profiles obtained for each sample were used for metabolomic analysis. Profile data was pre-processed using MestReNova software (MestReLab, Spain) to ensure alignment of the sample spectra. The data was normalized, baseline corrected, phased and referenced according to the internal standard TSP and binned into 0.04 ppm bins. Multivariate data

analysis with a 95% confidence level was carried out using Soft Independent Modeling of Class Analogy (SIMCA, Umea, Sweden). Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) plots were constructed. The plots were used to visualize the changes in the metabolite profile of the trees over time, and to relate it to seasonal changes.

5.2.5 Visualization of the metabolic profiles of individual trees

MestReNova software (MestReLab, Spain) was used to layer the ^{1}H NMR profiles of the sample material collected from the single undamaged *C. dentata* tree in the southern Cape and the single undamaged *C. dentata* tree in the Nkandla Forest Reserve in KZN in order to visualize differences and bi-monthly changes in metabolite profiles. Metabolite profiles for all seven the corresponding sampling dates and for both the hydrophilic and lipophilic metabolites for the undamaged trees in the southern Cape and KZN were compiled. For both the hydrophilic and lipophilic metabolites, the TSP reference at δ 0.00 ppm were aligned and normalized to obtain equal heights, after which the layered profiles were baseline corrected.

5.3 RESULTS

With PCA, the objective is to arrive at a linear transformation that preserves as much of the variance in the original data as possible, and to identify class differences from a multivariate dataset. PCA is used to obtain an overview, find clusters, and to identify outliers (Trygg, Holmes and Lunstedt, 2007:472). Figure 5.4 therefore provides an overview of the bi-monthly changes in the hydrophilic metabolite profile for the *C. dentata* trees sampled from the southern Cape forest area over a period of 13 months, including both September 2015 and September 2016. The clusters for each sampling period are clearly separated and there are a few outliers outside the ellipse indicating the 95% confidence level, however, these outliers do not deviate too much from the mean, and were thus included (R2X = 0,796, Q2 = 0.156). The samples of which the means deviated too much and thus lay far outside the ellipse were excluded.

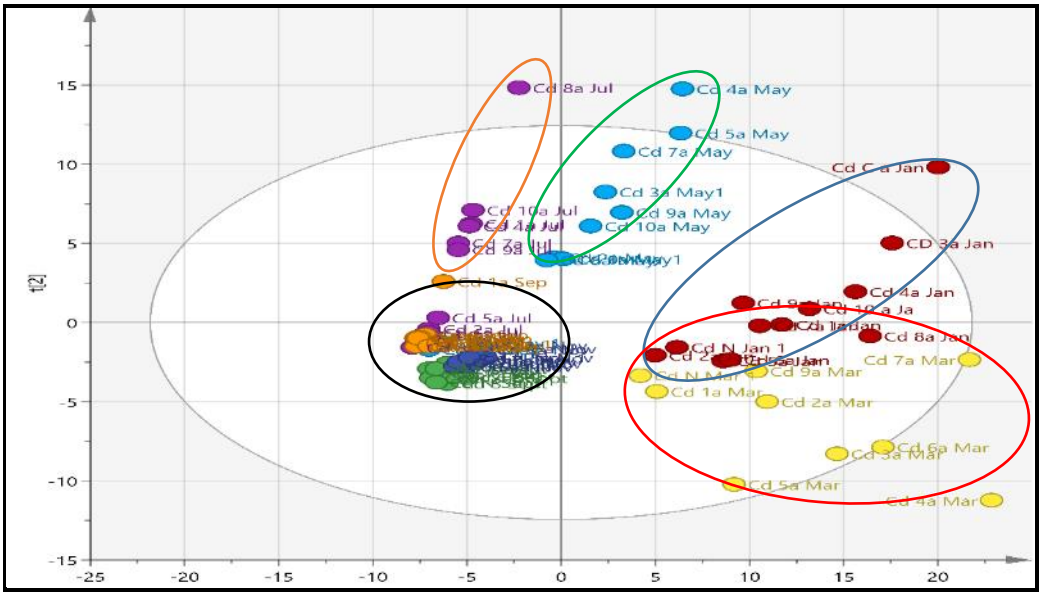


Figure 5.4: PCA-X model of the *C. dentata* stem bark samples collected from the southern Cape forest area provides an overview of the hydrophilic metabolite profile over a period of 13 months during 2015 and 2016. The cluster encircled in black represents the stem bark samples collected during September 2015, November 2015 and September 2016. The cluster encircled in blue represents the samples collected during the January 2016 sampling period. The cluster encircled in red represents the samples collected during March 2016, whereas the cluster encircled in green represents the samples collected during the May 2016 sampling period. The cluster encircled in brown represents the stem bark samples collected in July 2016.

Orthogonal Partial Least Squares (OPLS) addresses interpretability problems by incorporating the Orthogonal Signal Correction (OSC) filter (Worley and Powers, 2013:95) into a PLS model, effectively separating Y-prediction variation from y-uncorrelated variation in X (Trygg, *et al.*, 2007: 473). The OPLS-DA model for the hydrophilic metabolites in the *C. dentata* stem bark sample set collected in the southern Cape shows clear seasonal differentiation (Figure 5.5). The main cluster constitutes the September 2015, November 2018 and September 2016 samples, which grouped together to form one cluster, whereas the samples collected during the January 2016, March 2016, May 2016 sampling periods separated into individual clusters. The July 2016 samples overlapped the main cluster to some extent (R2X = 0.561, R2Y = 0.658, Q2= 0.549).

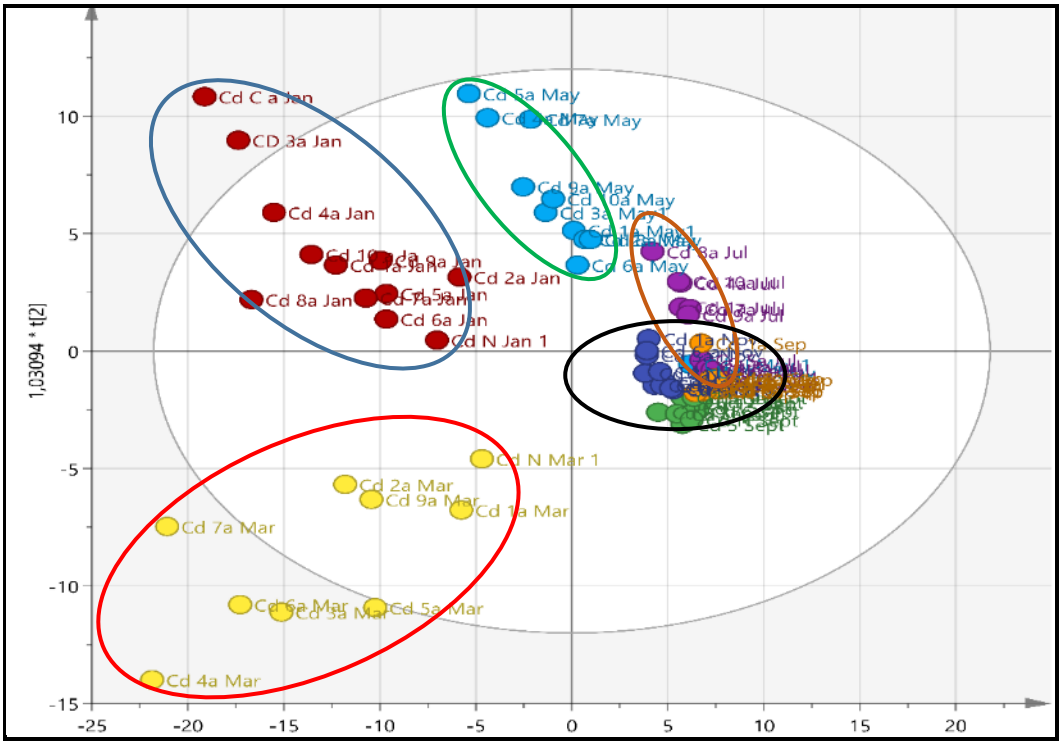


Figure 5.5: OPLS-DA score plot of the stem bark sample set collected from *C. dentata* trees in the southern Cape forest area during 2015 and 2016. Deuterium oxide and deuterium methanol was used for the extraction of hydrophilic metabolites. The cluster encircled in black represents the stem bark samples collected during September 2015, November 2015 and September 2016. The cluster encircled in blue represents the samples collected during the January 2016 sampling period. The cluster encircled in red represents the samples collected during March 2016, whereas the cluster encircled in green represents the samples collected during the May 2016 sampling period. The cluster encircled in brown represents the stem bark samples collected in July 2016.

Contrary to the PCA-X model of the *C. dentata* stem bark sample set collected in the southern Cape (Fig. 5.4), the PCA-X model for the hydrophilic metabolite profile of the *C. dentata* stem bark sample set collected in KZN does not show clear bi-monthly clustering (Figure 5.6). In this model, only two clear clusters can be identified, with one November 2018 outlier sample and a few January 2019 and March 2019 samples occurring outside the ellipse indicating the 95% confidence level. Since these outliers do not deviate too much from the mean, they were included (R2X = 0.829, Q2 = 0.611). The samples of which the means deviated too much and thus lay far outside the ellipse were excluded.

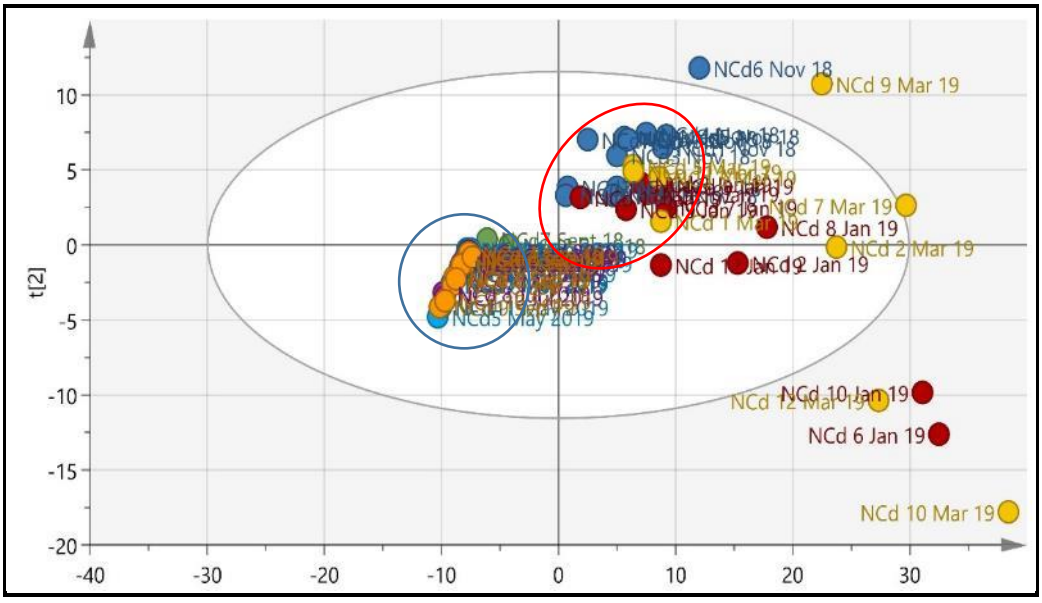


Figure 5.6: PCA-X model of the *C. dentata* stem bark sample set collected from KZN provides an overview of the hydrophilic metabolite profile over a period of 13 months during 2018 and 2019. The cluster encircled in blue represents the samples collected during the September 2018, May 2019, July 2019 and September 2019 sampling dates. The cluster encircled in red represent the samples collected during the November 2018 sampling date. The samples collected in January (red dots) and March (yellow dots) are scattered and overlap the November samples to some extent.

The OPLS-DA model for the hydrophilic metabolites in the *C. dentata* stem bark sample set collected in KZN (Figure 5.7) separated into three clusters with the samples collected during January and March 2019 grouping together into one cluster and the samples collected during the September 2018, May 2019, July 2019 and September 2019 sampling periods grouping together in another cluster. The November 2018 samples separated to form an individual cluster (R2X = 0.685, R2Y = 0.296, Q2=0.248).

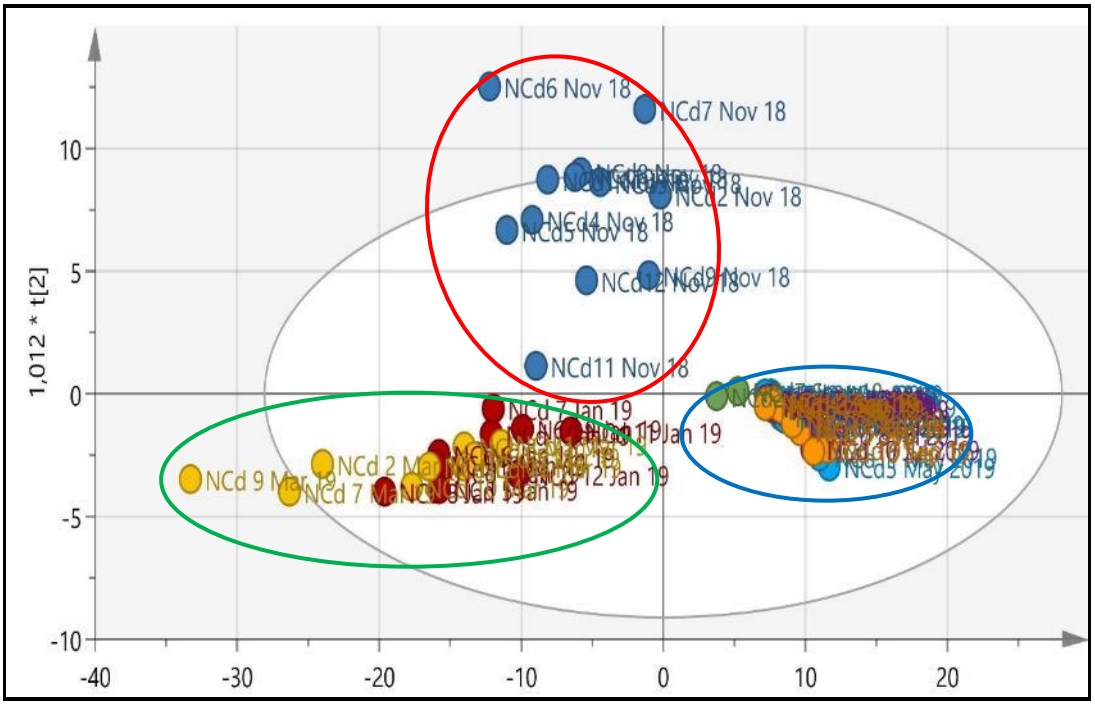


Figure 5.7: OPLS-DA score plot of the stem bark samples collected from *C. dentata* trees in KZN. Deuterium oxide and deuterium methanol was used for the extraction of hydrophilic metabolites. The cluster encircled in blue represents the samples collected during the September 2018, May 2019, July 2019 and September 2019 sampling dates. The cluster encircled in red represent the samples collected during the November 2018 sampling date whereas the cluster encircled in green represents the *C. dentata* stem bark samples collected during January and March 2019.

In Figure 5.8, the PCA-X model of the *C. dentata* stem bark samples collected from the southern Cape forest area shows bi-monthly separation of the lipophilic metabolites, however, separation of the metabolites for the months of November 2015, March 2016, May 2016, July 2016 and September 2016 are not so clear. The September 2015 and the January 2016 samples, however, shows clear clustering. A few of the September 2015 samples are outliers, but excluding these samples would exclude almost all of the September 2015 samples, and since their means did not deviated too much from the means of the samples inside the ellipse indicating the 95% confidence level, they were thus included. One September 2015 sample tended to cluster with the main cluster while two of the March 2016 samples grouped with the September 2015 cluster. One March 2016 sample is an outlier, however, this outlier was included since the mean did not deviate too far from the means of the samples in the ellipse representing the 95% confidence level (R2X = 0.602, Q2 = 0.105). The samples that were too far outside the ellipse were excluded.

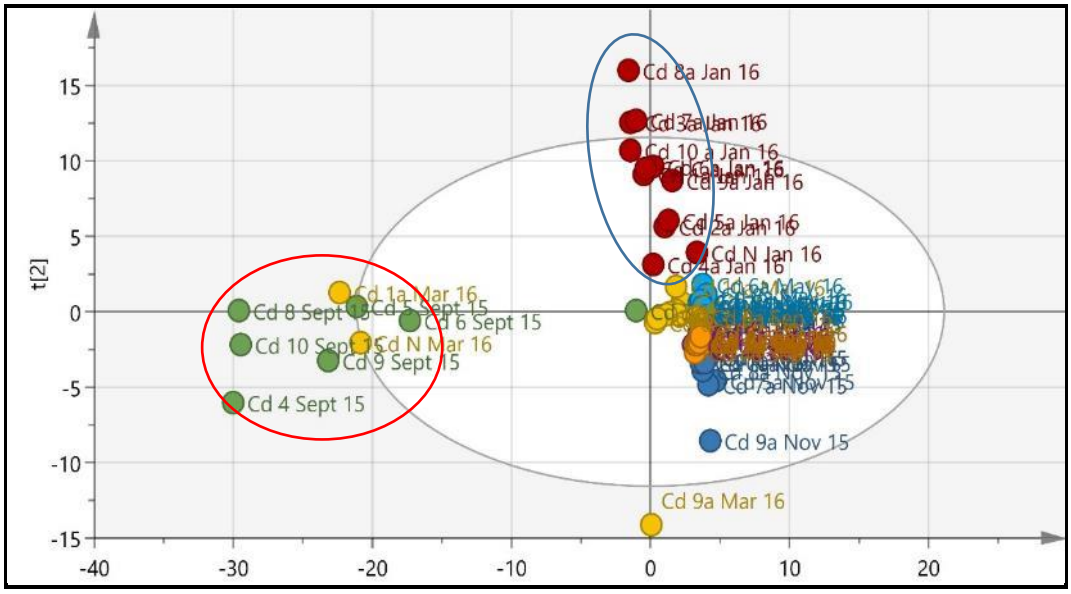


Figure 5.8: PCA-X model of the *C. dentata* stem bark samples collected from the southern Cape forest area provides an overview of the lipophilic metabolite profile over a period of 13 months during 2015 and 2016. The cluster encircled in red represents the stem bark samples collected during September 2015, however, there are a few March 2016 samples within the cluster. The cluster encircled in blue represents the stem bark samples collected during January 2016. The remaining samples representing the months of November 2015, March 2016, May 2016, July 2016 and September 2016. These samples seem to separate but separation is not clear.

The OPLS-DA model compiled for the lipophilic metabolites of the *C. dentata* stem bark sample set collected from the southern Cape forest shows clearer seasonal separation of clusters (Figure 5.9). The *C. dentata* stem bark samples collected during the September 2015 and January 2016 sampling periods formed clear individual clusters. The individual clusters representing the samples collected during the November 2015 and May 2016 sampling periods, separate, but are positioned very close to the collective cluster representing the March 2016, July 2016 and September 2016 samples (R2X = 0.547, R2Y = 0.679, Q2 = 0.490).

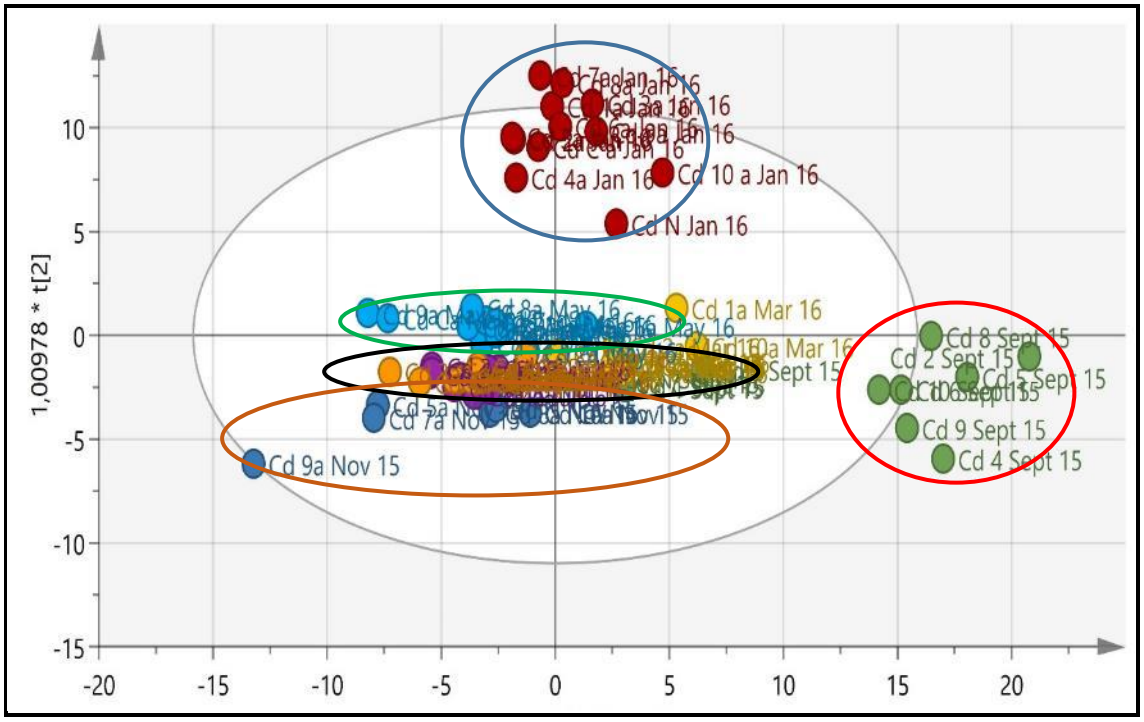


Figure 5.9: OPLS-DA score plot of the stem bark sample set collected from *C. dentata* trees in the southern Cape forest area. Deuterium chloroform was used for the extraction of lipophilic metabolites. The cluster encircled in red represents the stem bark samples collected during September 2015. The cluster encircled in brown represents the stem bark samples collected during the November 2015 sampling period, whereas the cluster encircled in blue represents the stem bark samples collected during January 2016. The cluster encircled in green represents the *C. dentata* stem bark samples collected during the May 2016 sampling period. The cluster encircled in black represents the stem bark samples collected during March, July and September 2016.

The PCA-X model of the *C. dentata* stem bark sample set collected from KZN shows bi-monthly clustering of the lipophilic metabolites for the months of September 2018, November 2018 and July 2019 (Figure 5.10). Six of the November 2018 and two of the September 2018 samples are outliers, however, excluding particularly the November 2018 outliers would exclude almost all samples collected during November 2018, and since both the September and November 2018 outlier samples' means did not deviate too much from the mean of the samples inside the ellipse representing the 95% confidence level, these samples were included. The remainder of the sampling periods' bi-monthly collected samples, however, appear to form a collective cluster. The samples represented in the collective cluster include the samples collected during the January 2019, March 2019, May 2019 and September 2019 sampling periods (R2X = 0.826, Q2 = 0.0776).

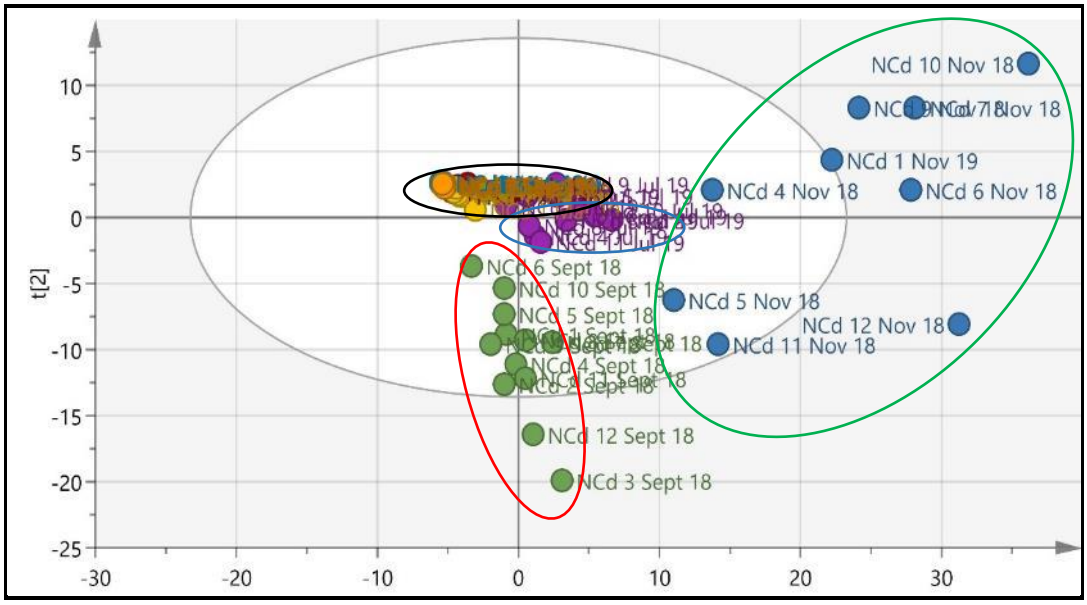


Figure 5.10: PCA-X model of the *C. dentata* stem bark samples collected from KZN provides an overview of the lipophilic metabolite profile over a period of 13 months during 2018 and 2019. The cluster encircled in red represents the stem bark samples collected during September 2018, whereas the cluster encircled in green represents the stem bark samples during November 2018. The cluster encircled in blue represents the samples collected during the July 2019 sampling period. The stem bark samples collected during the January, March, May and September 2019 sampling periods are encircled in black.

The OPLS-DA model of the *C. dentata* stem bark sample set collected from KZN shows clearer seasonal separation of clusters (Figure 5.11). As for the PCA-X model, the *C. dentata* stem bark samples collected during the January 2019, March 2019, May 2019 and September 2019 sampling periods clearly grouped into a collective cluster. The September 2018, November and July 2019 samples are grouped into individual clusters; however, the July 2019 cluster is positioned very closely to the collective cluster (R2X = 0.544, R2Y = 0.412, Q2 = 0.288).

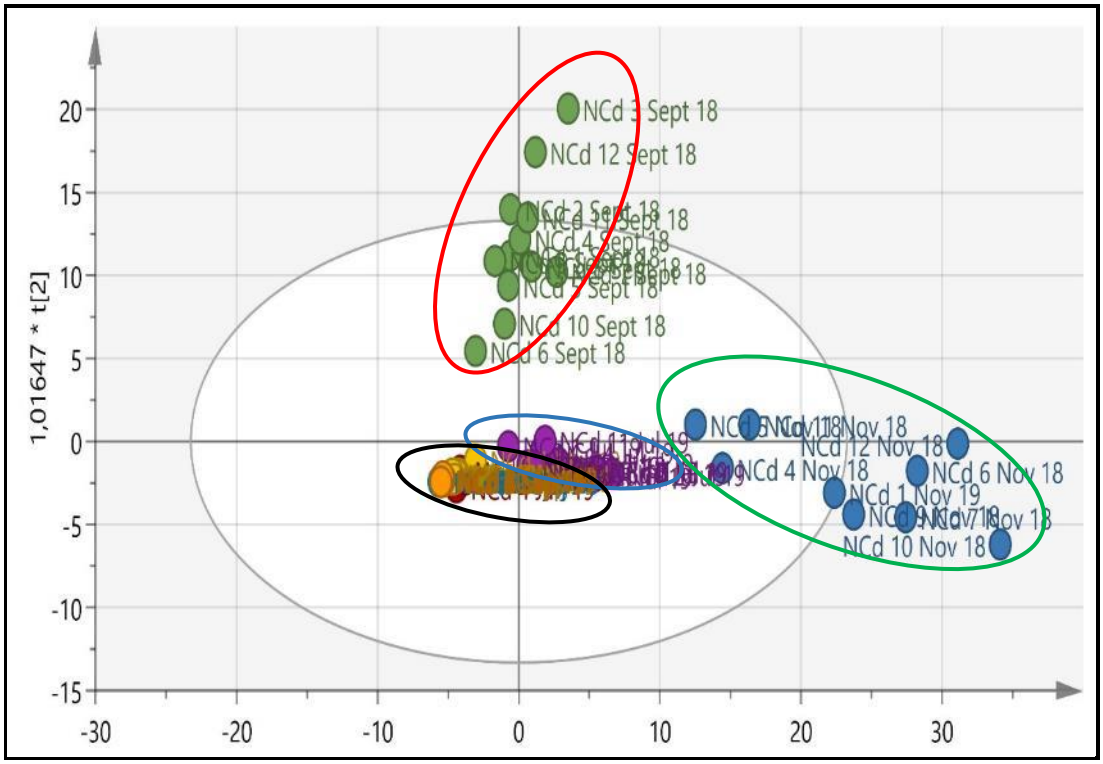


Figure 5.11: OPLS-DA score plot of the stem bark sample set collected from *C. dentata* trees in KZN. Deuterium chloroform was used for the extraction of lipophilic metabolites. The cluster encircled in red represents the stem bark samples collected during September 2018, whereas the cluster encircled in green represents the stem bark samples during November 2018. The cluster encircled in blue represents the samples collected during the July 2019 sampling period. The stem bark samples collected during the January, March, May and September 2019 sampling periods formed a collective cluster, and is encircled in black.

The OPLS-DA model of the regional hydrophilic responses show clear differentiation between the samples collected in the southern Cape (upper half) and the samples collected in KZN (lower half) (Figure 5.12) (R2X = 0.941; R2Y = 0.249; Q2 = 0.147). The separation of samples collected from the southern Cape in January 2016 (red dots), March 2016 (yellow dots) and May 2016 (blue dots) encircled in green (above), and the separation of samples collected in KZN in November 2018 (brown dots), January 2019 (green dots) and March 2019 (yellow dots) encircled in blue (blow) is emphasized, with the cluster encircled in red encompassing the hydrophilic compounds for the samples collected during September 2015, November, 2015, July 2016 and September 2016 (southern Cape) and September 2018 and May, July and September 2019 (KZN).

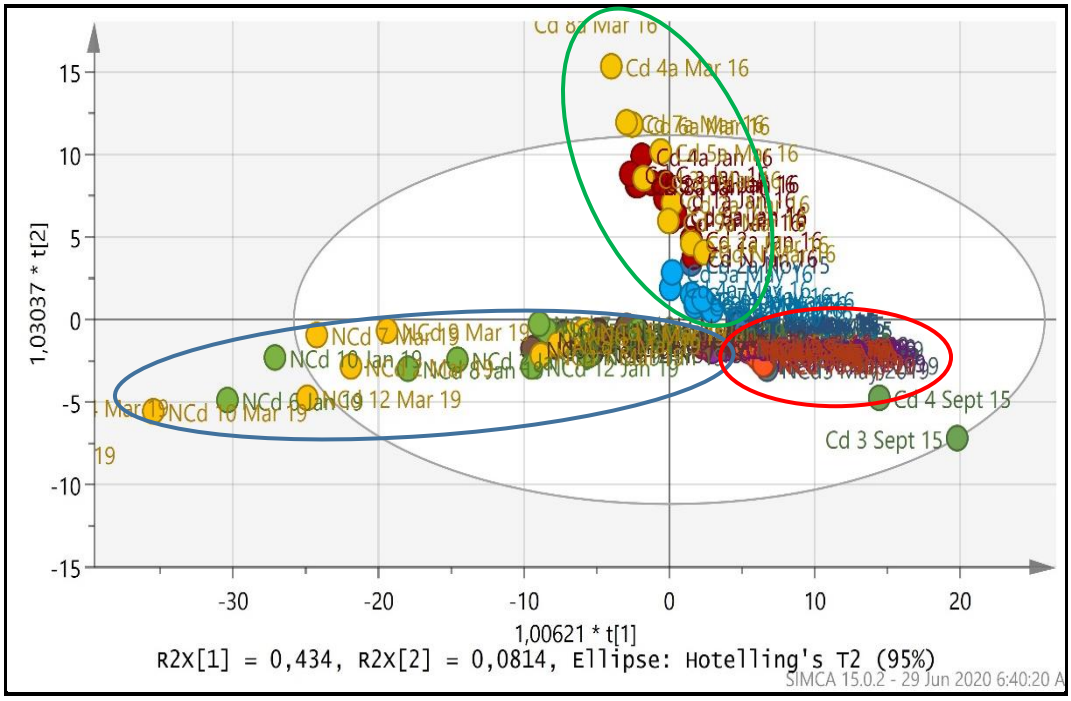


Figure 5.12: OPLS-DA model of the regional differentiation of hydrophilic compounds. The samples in the upper half (encircled in green) represent samples collected in the southern Cape during January, March and May 2016 whereas the samples in the lower half (encircled in blue) represent the samples collected in KZN during November 2018, January 2019 and March 2019. The samples encircled in red represent the samples collected during September 2015, November 2015, July 2016 and September 2016 (southern Cape) and September 2018 and May, July and September 2019 (KZN).

The OPLS-DA model of the regional lipophilic responses also show differentiation between the samples collected in the southern Cape (upper half) and the samples collected in KZN (lower half) (Figure 5.13) (R2X = 0.903; R2Y = 0.241; Q2 = 0.158). In this model, however, the samples collected in the southern Cape during November 2015 (blue dots) and in KZN during November 2018 (brown dots) grouped into an individual cluster (encircled in green), indicating similarities in responses, even though collected three years apart. A few November outliers (four in November 2015 and two in November 2018) indicate individual responses that deviate from the population mean for the month of November.

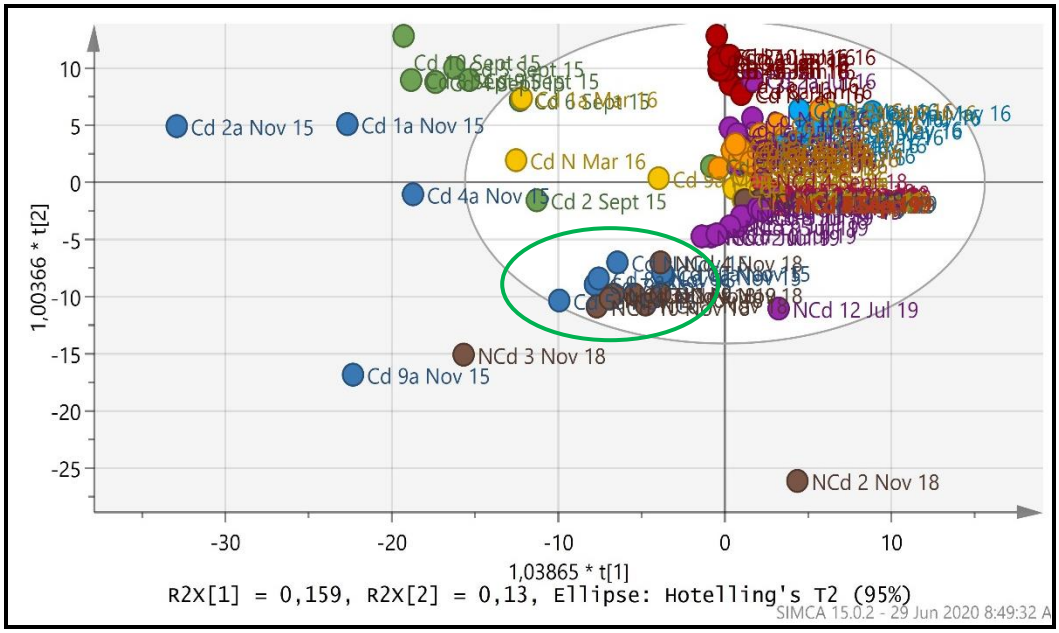
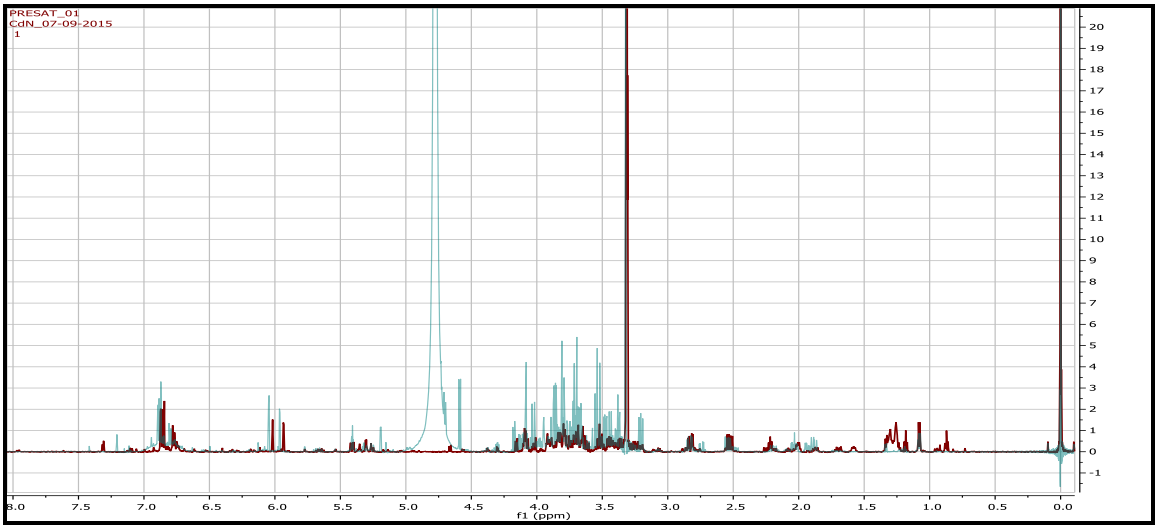


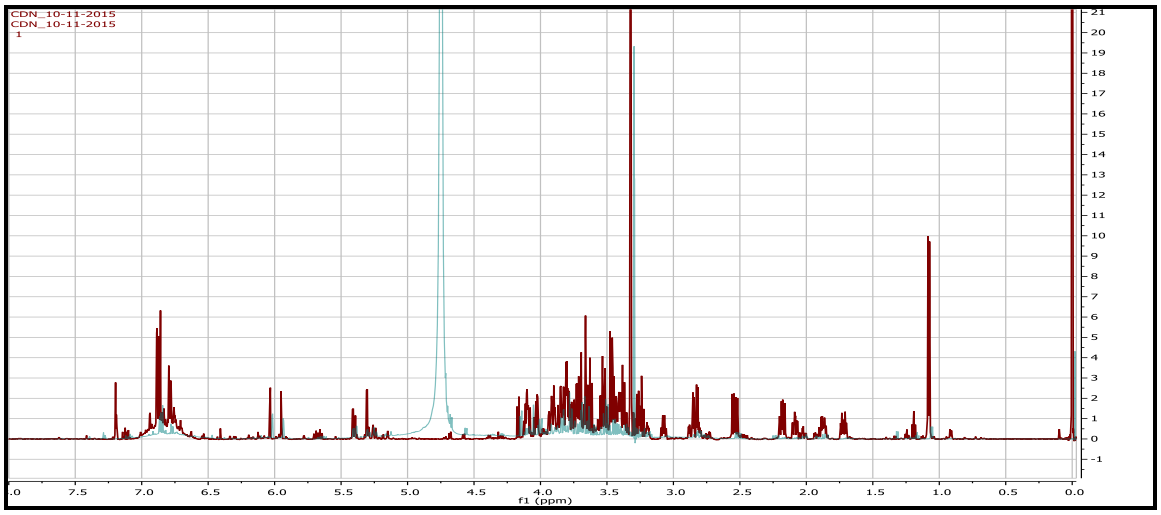
Figure 5.13: OPLS-DA model of the regional differentiation of lipophilic compounds. The samples collected from the southern Cape are in the upper half of the plot and the samples collected from KZN are in the lower half of the plot. The majority of the samples collected in the southern Cape during November 2015 and in KZN during November 2018 grouped into an individual cluster (encircled in green).

The only trees allocated for research at each site which had no prior damage, either experimental or through harvesting, were used as examples of how metabolite profiles in individual *C. dentata* trees may vary seasonally and regionally. Figures 5.14A-G therefore compare the bi-monthly profiles of the hydrophilic metabolites in the stem bark of the selected *C. dentata* tree in the southern Cape and the selected *C. dentata* tree in KZN, whereas Figures 5.15A-G compare the bi-monthly profiles of the lipophilic metabolites in the stem bark of the same *C. dentata* tree in the southern Cape and the same *C. dentata* tree in KZN.

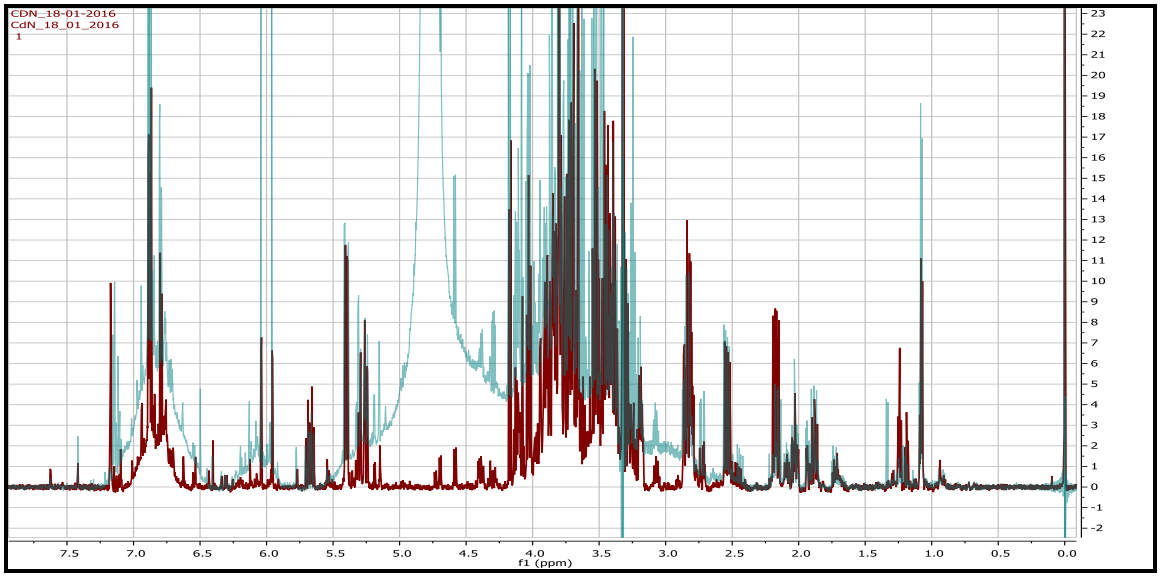
A



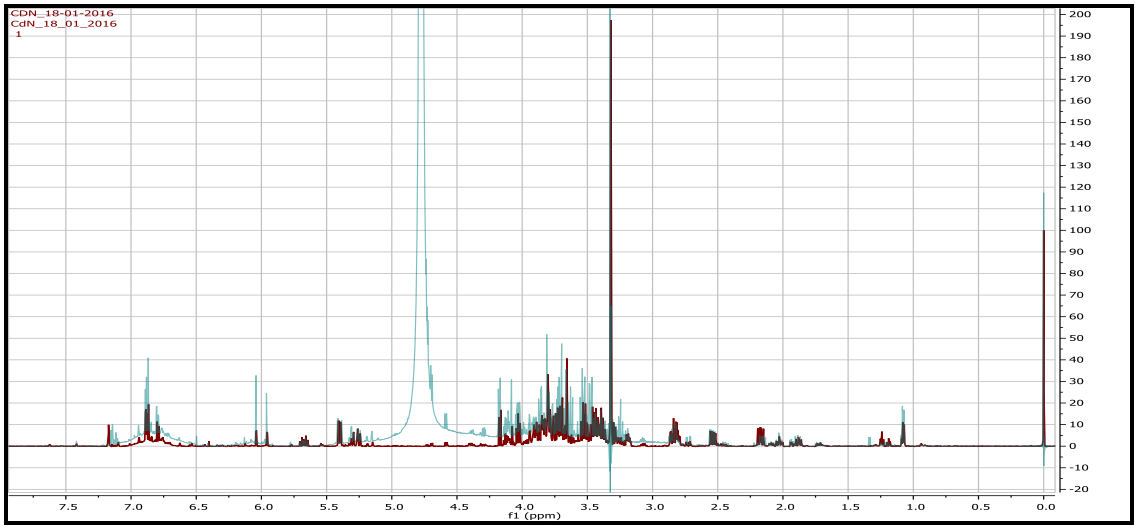
B



C1



C2



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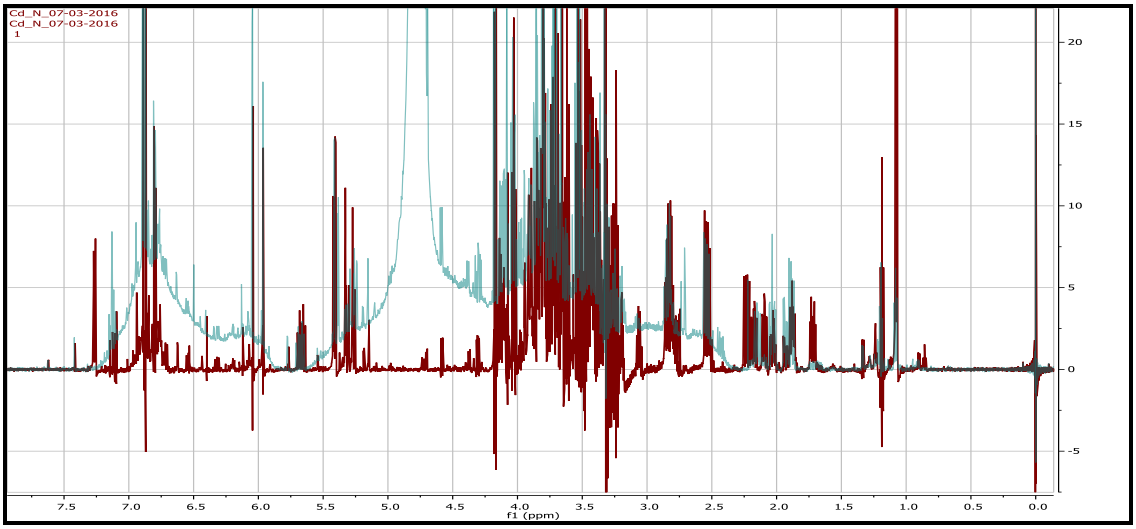

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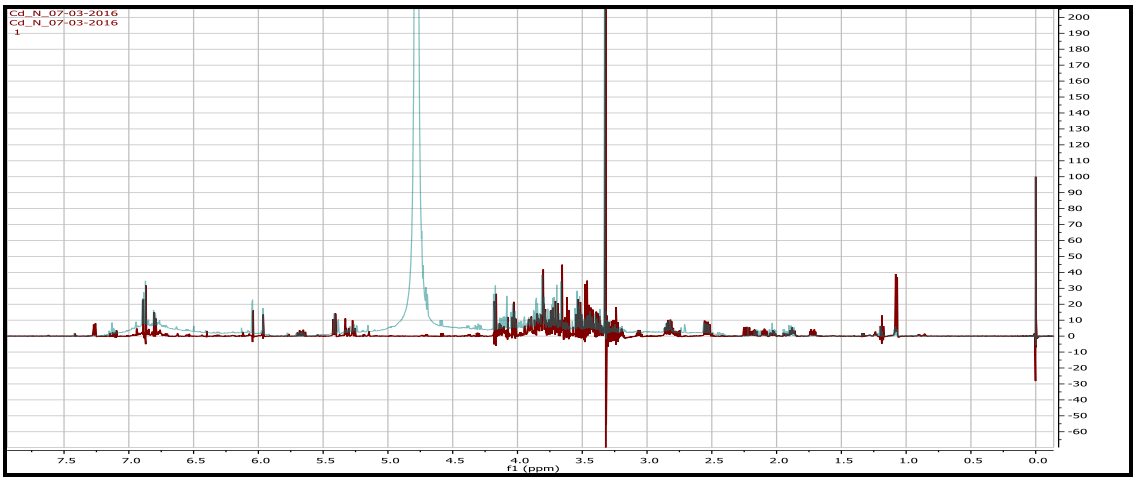

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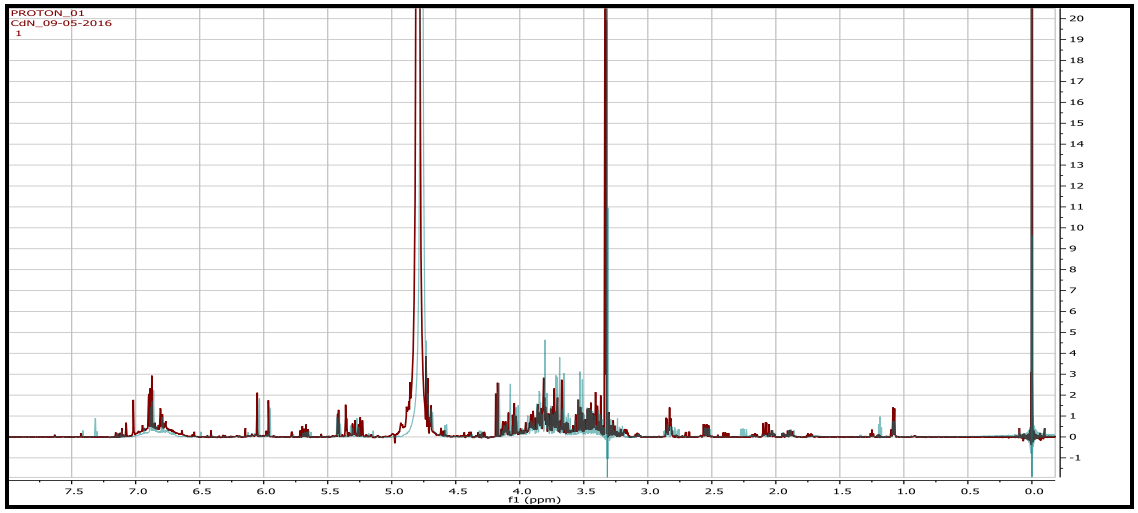
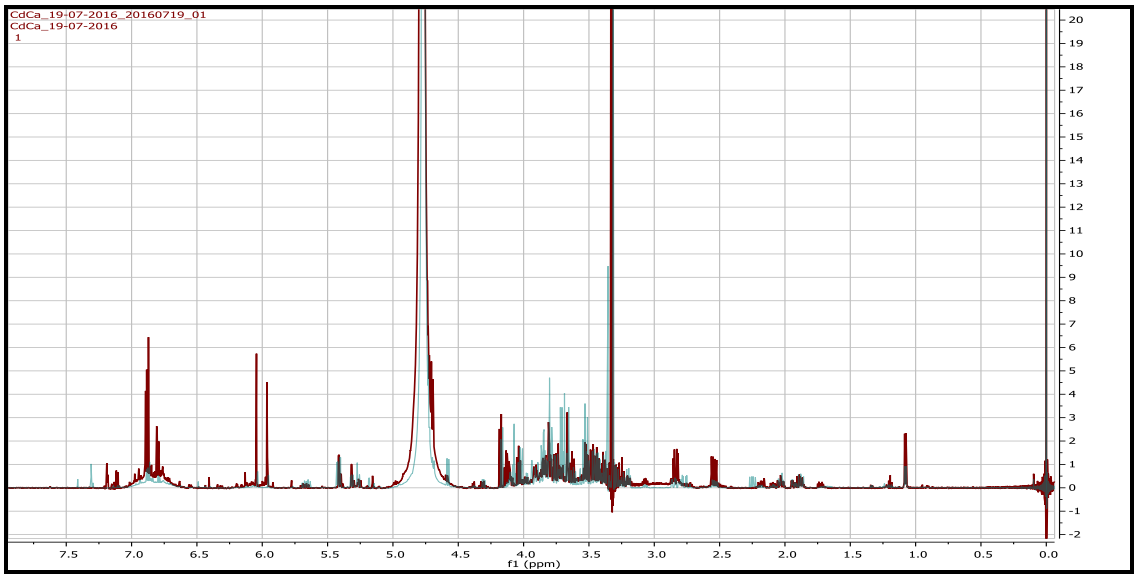


F



G

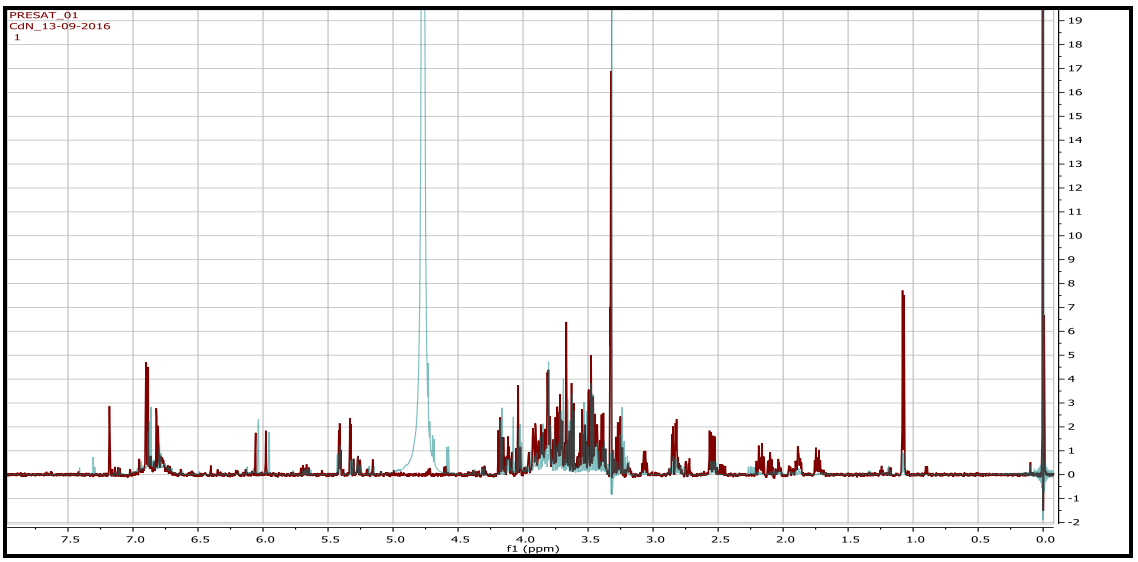
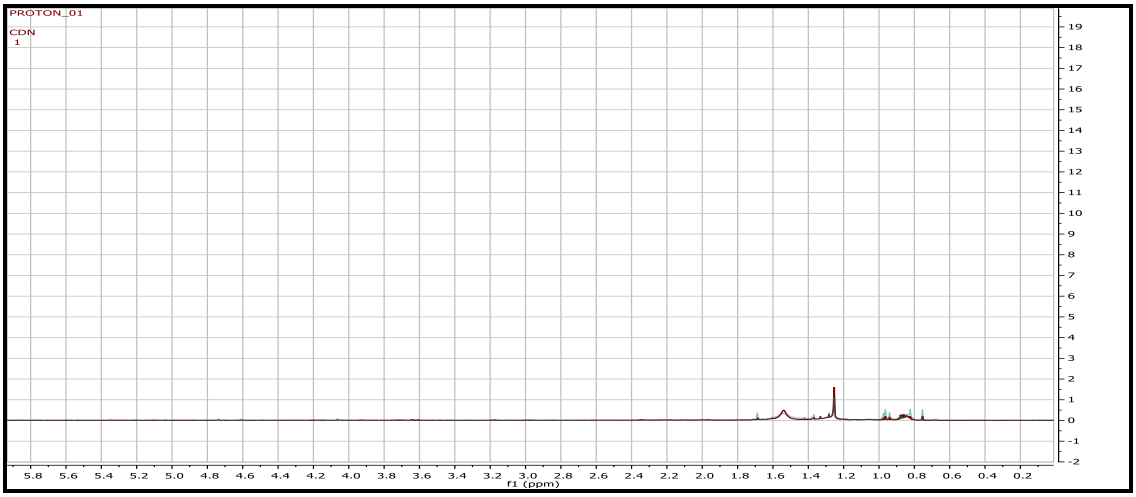


Figure 5.14: Seasonal and regional differences of the hydrophilic metabolites in the stem bark of the only undamaged *C. dentata* tree in the southern Cape and the only undamaged *C. dentata* tree in KZN. In all Figures, the red spectra represent the hydrophilic metabolites in the stem bark of the selected *C. dentata* tree in the southern Cape forest area, whereas the blue spectra represent the hydrophilic metabolites in the stem bark of the selected *C. dentata* tree in the Nkandla Forest Reserve in KZN. The differences in peak heights represent differences in concentrations. In Figure 5.14A, the profiles of the hydrophilic metabolites of the samples collected in September 2015 (southern Cape) and in September 2018 (KZN), which represents early spring, are shown. Figure 5.14B shows the profiles of the hydrophilic metabolites of the samples collected in November 2015 (southern Cape) and in November 2018 (KZN), which represents early summer, and Figures 5.14C1 and 2 show the profiles of the hydrophilic metabolites of the samples collected in January 2016 (southern Cape) and in January 2019 (KZN), which represents mid-summer. Figures 5.14D1 and 2 show the profiles of the hydrophilic metabolites of the samples collected in March 2016 (southern Cape) and in March 2019 (KZN), which represents early autumn, and Figure 5.14E shows the profiles of the hydrophilic metabolites of the samples collected in May 2016 (southern Cape) and in May 2019 (KZN), which represents late autumn. Figure 5.14F shows the profiles of the hydrophilic metabolites for July 2016 (southern Cape) and July 2019 (KZN), which represents mid-winter. Figure 5.14G shows the profiles of the hydrophilic metabolites for the samples collected in September 2016 (southern Cape) and September 2019 (KZN), which represents the early spring season of the following year. In Figures 5.14C1 and 5.14D1 the scales of the spectra are the same as for Figures 5.14A, B, E, F and G. As the concentrations of hydrophilic compounds in Figures 5.14C1 and 5.14D1 are very high during these two months, the scales of the spectra were adjusted (reduced) in Figures 5.14C2 and 5.14D2, to be able to visualize concentration differences between the trees. Note that PRESAT settings were applied to suppress the water peak for the samples collected from the southern Cape forest area.

A1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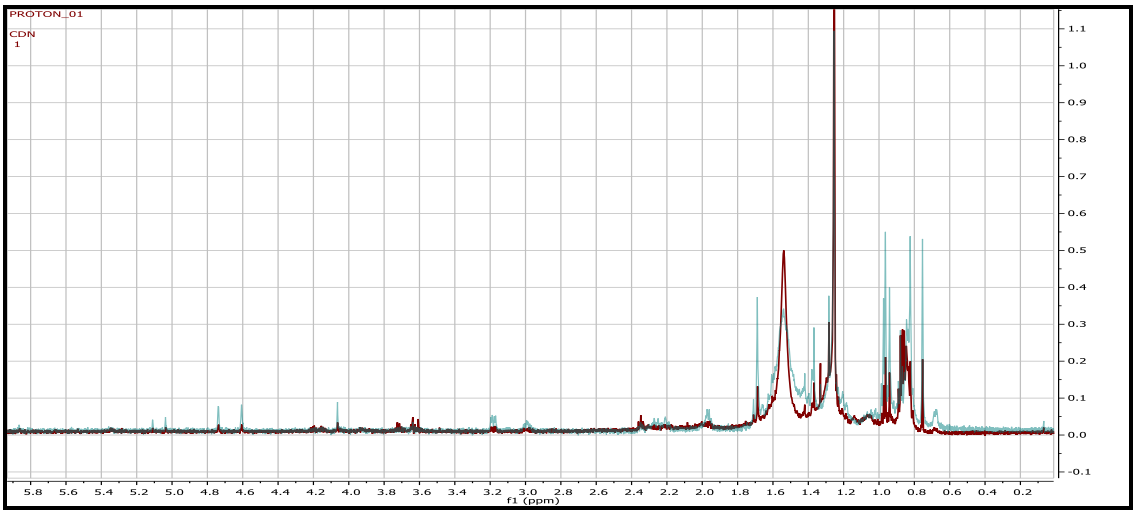
CDN
1
f1 (ppm)

A2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CDN
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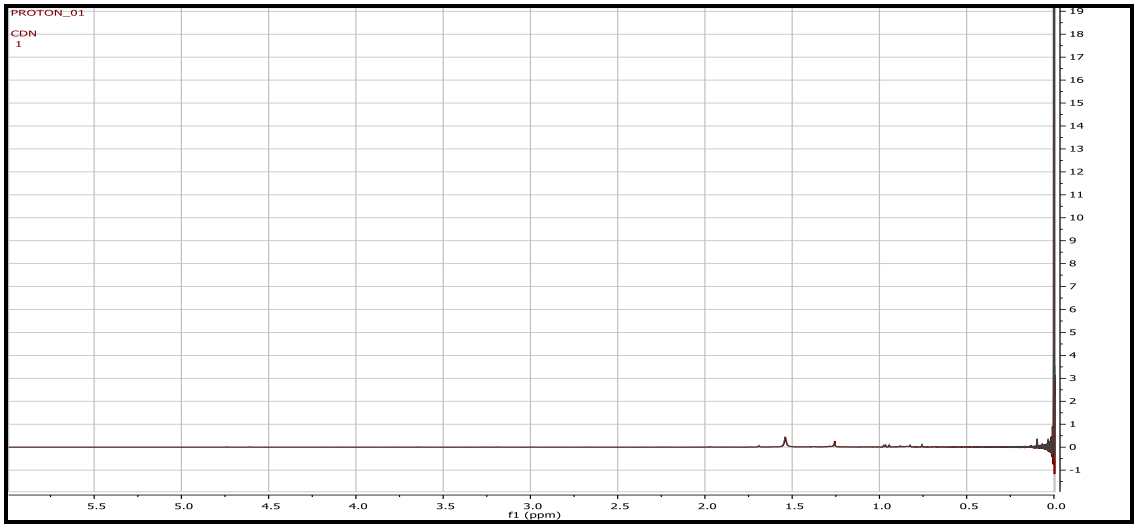
B1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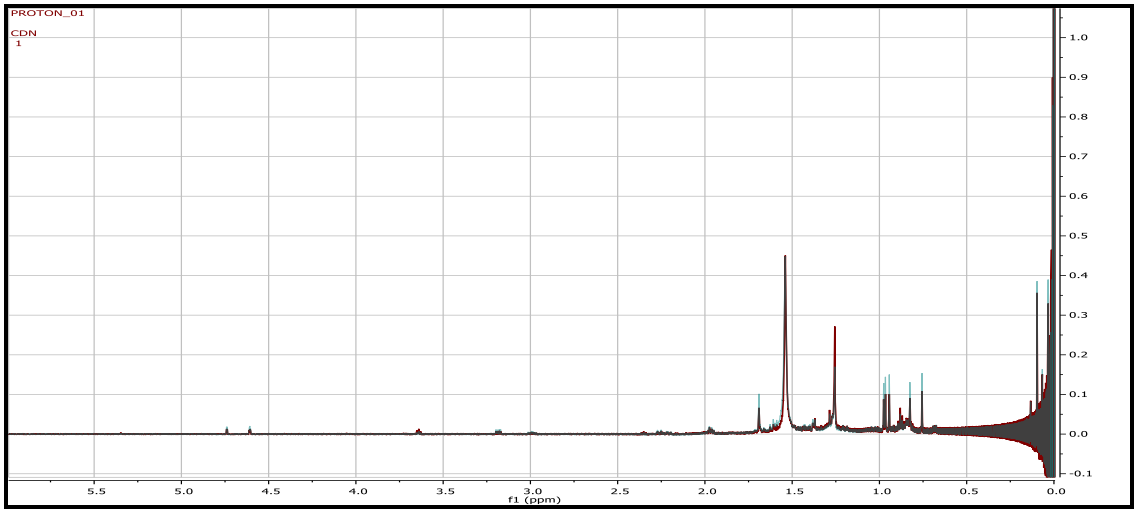
CDN
1
f1 (ppm)

B2



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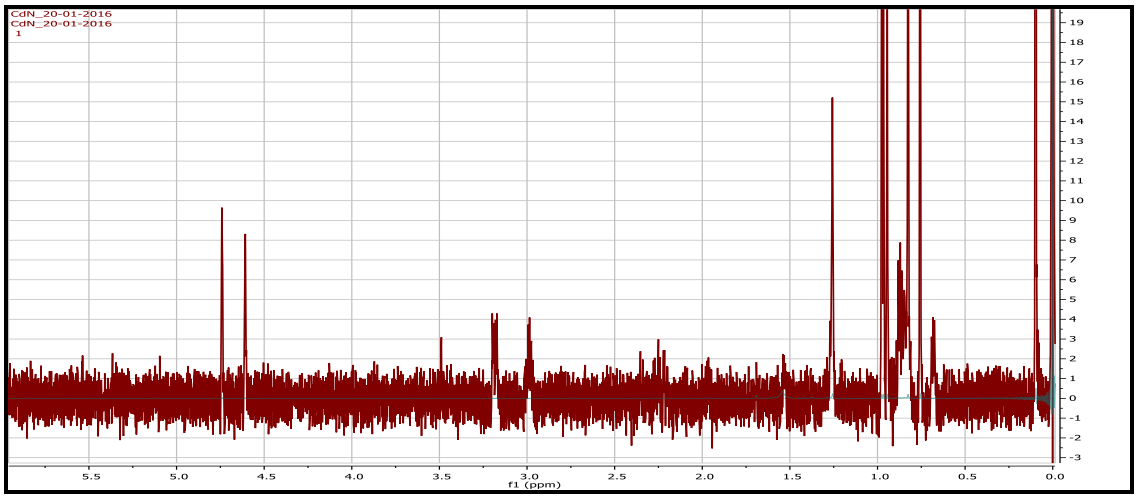
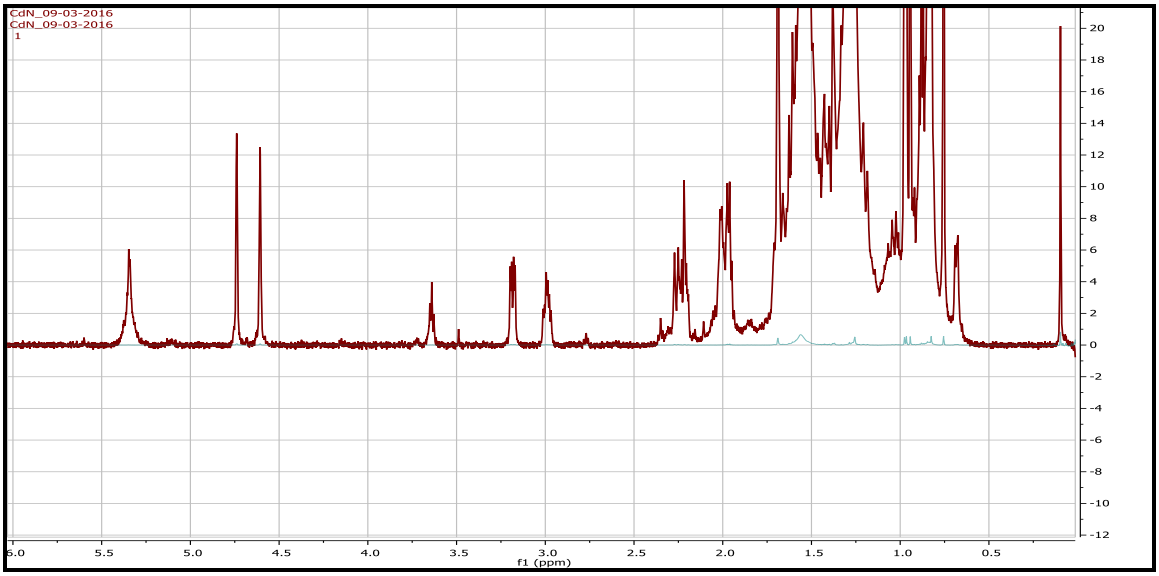
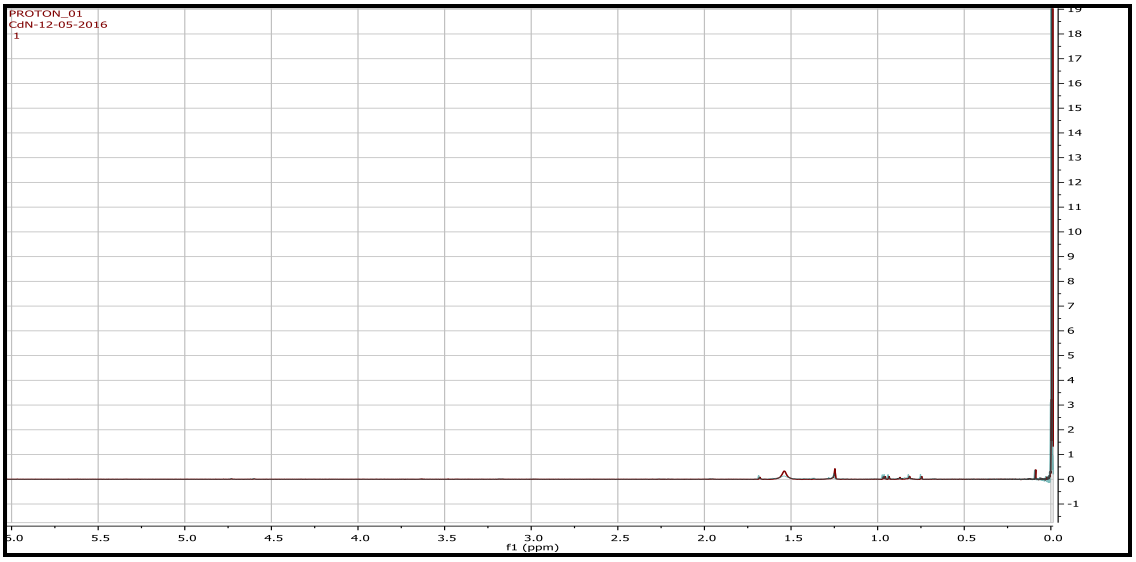


D



E1



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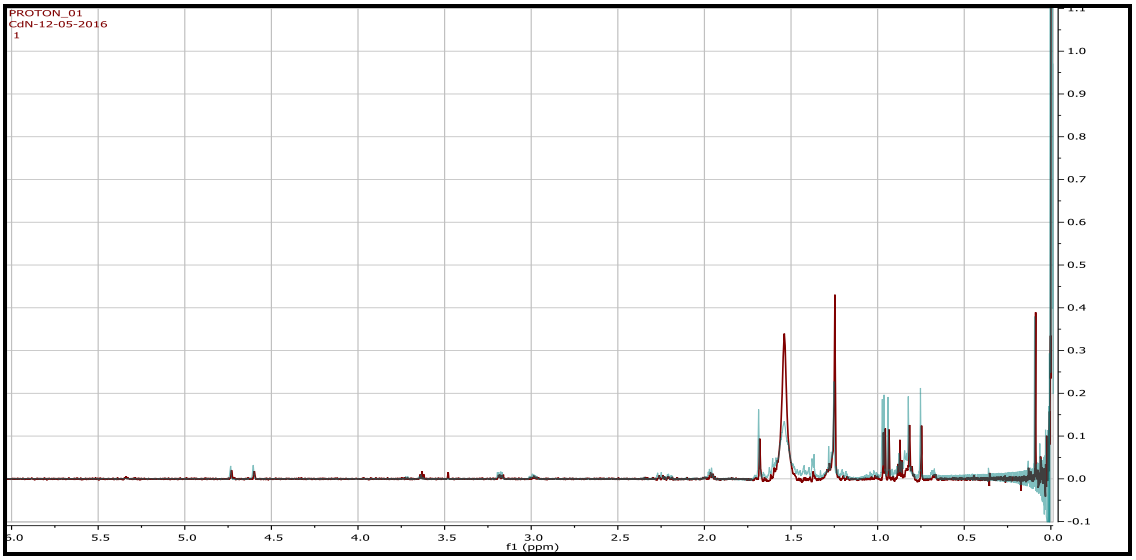
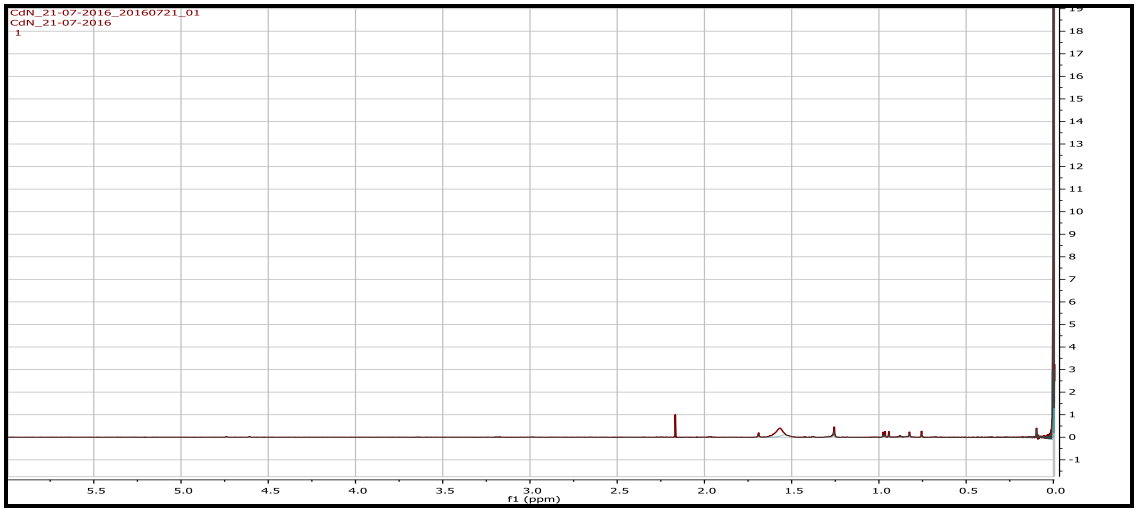
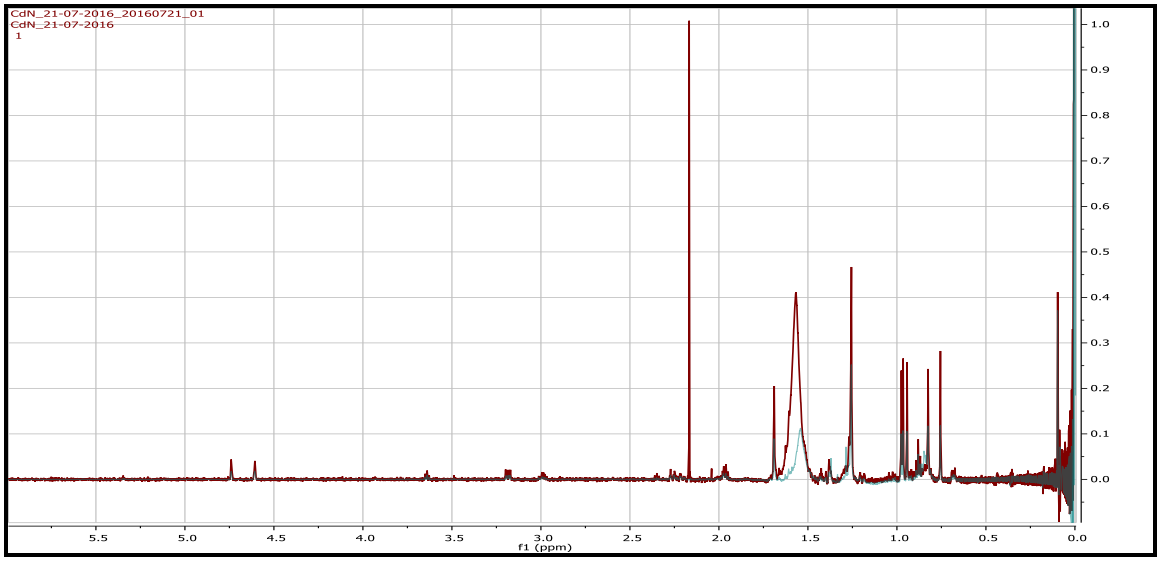


F1



F2



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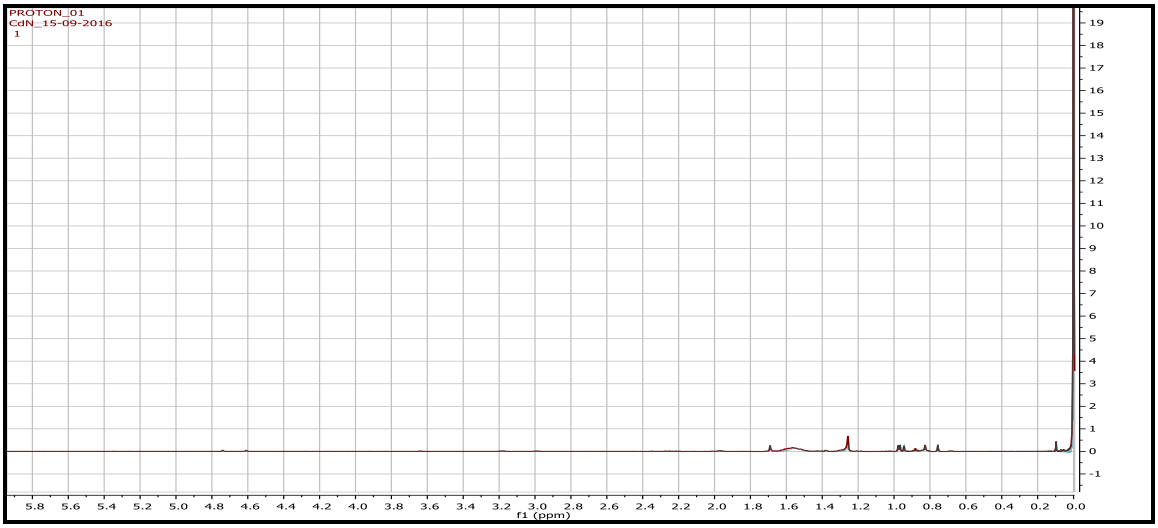


G2

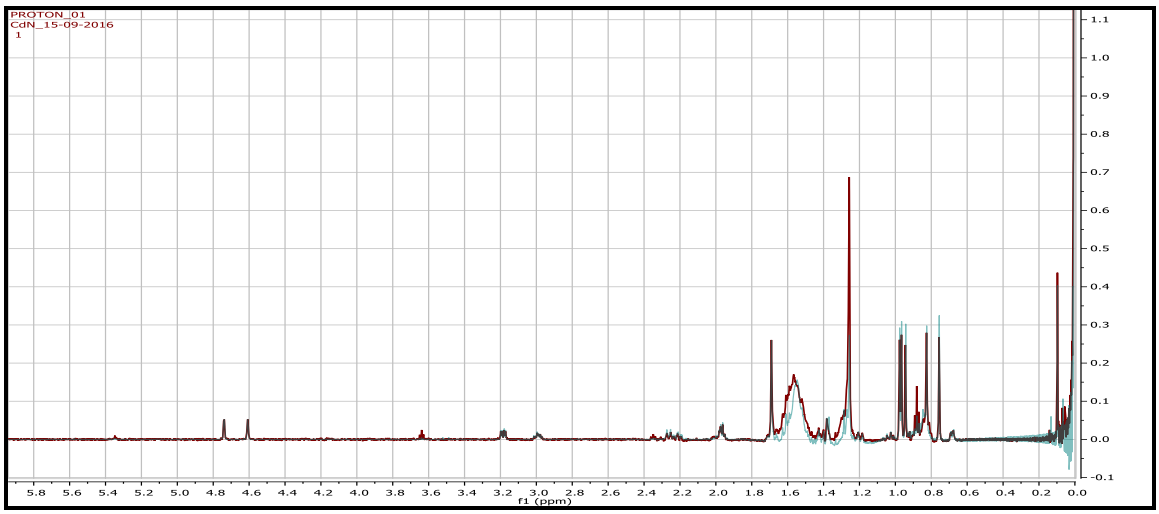


Figure 5.15: Seasonal and regional differences of the lipophilic metabolites in the stem bark of the only undamaged *C. dentata* tree in the southern Cape and the only undamaged *C. dentata* tree in KZN. In all Figures, the red spectra represent the lipophilic metabolites in the stem bark of the selected *C. dentata* tree in the southern Cape forest area, whereas the blue spectra represent the lipophilic metabolites in the stem bark of the selected *C. dentata* tree in the Nkandla Forest Reserve in KZN. The differences in peak heights represents differences in concentrations. Figures 5.15A1 and 2 show the profiles of the lipophilic metabolites of the samples collected in September 2015 (southern Cape) and in September 2018 (KZN), which represents early spring. Figures 5.15B1 and 2 show the profiles of the lipophilic metabolites of the samples collected during the early summer months of November 2015 (southern Cape) and November 2018 (KZN). Figure 5.15C shows the profiles of the lipophilic metabolites of the samples collected during the mid-summer months of January 2016 (southern Cape) and January 2019 (KZN). Figure 5.15D shows the profiles of the lipophilic metabolites of the samples collected during the early autumn months of March 2016 (southern Cape) and March 2019 (KZN). Figures 5.15E1 and 2 show the profiles of the lipophilic metabolites of the *C. dentata* stem bark samples collected in May 2016 (southern Cape) and May 2019 (KZN), which represents late autumn. Figures 5.15F1 and 2 shows the profiles of the lipophilic metabolites of the *C. dentata* stem bark samples collected in July 2016 (southern Cape) and July 2019 (KZN), which represents late mid-winter. Figures 5.15G1 and 2 show the profiles of the lipophilic metabolites of the *C. dentata* stem bark samples collected in September 2016 (southern Cape) and September 2019 (KZN), which represents the following year's early spring season. The spectra in Figures 5.15A1, B1, C, D, E1, F1 and G1 all have the same scale. Because the concentrations of lipophilic chemical compounds are generally very low, the spectra in Figures 5.15A2, B2, E2, F2 and G2 were adjusted (increased) to be able to visualize concentration differences between the trees.

5.4 DISCUSSION

Both the hydrophilic and lipophilic OPLS-DA metabolic profiles of the *C. dentata* stem bark samples in Figures 5.5, 5.7, 5.9 and 5.11 show seasonal variations, however, there are also significant regional differences (Figures 5.14 and 5.15). The concentrations of chemical compounds in the bark of *C. dentata* trees may also vary between individual trees. Variations in individual trees may result from soil properties of an area that are not necessarily uniform

throughout the area. Differences in spatial soil properties are caused by soil formation processes, hydrological regimes and temperature regimes, and are modified by topography, with subsequent variations in pH, organic matter, clay, silt and sand content, bulk density, water retention capacity and nutrient content (Keshavarzi, Tuffour, Bagherzadeh and Vasu, 2018:93-102). The biosynthesis of plant metabolites, and specifically secondary metabolites, are undeniably also influenced by regulatory genes, enzymes, hormones and transcription factors, which change according to the needs of the plant in its environment (Varma and Shukla, 2015:108). In plants, immunity against pathogens threatening a plant's survival is often age-dependent, with older plants exhibiting increased resistance and younger plants displaying less resistance against pathogens such as herbivorous insects, bacteria and fungi (Mao, Liu, Chen, Chen, Fang, Hong, Wang, Wang and Chen, 2016:2). In A*rabidopsis thaliana*, for example, the gene miR156 acts as regulator of age-dependent development by targeting squamosa promoter binding protein-like (SPL)19 transcription factors. The miR156 level is high during the juvenile stage, decreasing gradually towards advanced plant growth and development stages, resulting in progressive increases in SPLs levels. The SPLs regulate a wide range of processes, which include flowering, shoot regeneration, vernalization, shoot regeneration and secondary metabolite production (Mao *et al.*, 2016:2).

5. 4.1 Hydrophilic compounds

The stem bark sample set collected from *C. dentata* trees in the southern Cape (Figure 5.5) show that there are similarities in responses of the hydrophilic chemical compounds in the samples collected during September 2015, November 2015 and September 2016, since these three sampling periods' samples grouped into one cluster. The *C. dentata* stem bark samples collected during January 2016, March 2016 and May 2016, which represent the mid-summer, early and late autumn seasons respectively, clearly separated into individual clusters, which indicates significant changes in hydrophilic chemical compound composition and/or concentrations during the warm summer and milder autumn months. These differences may either be positive or negative. The July 2016 samples, which represents the samples collected during mid-winter, overlap the main cluster, which suggests that the hydrophilic chemical compound concentrations are actively returning to the September concentration levels. The September 2016 samples grouping with the September 2015 samples show that the hydrophilic chemical compound concentrations do not differ significantly on a year-to-year basis in *C. dentata* trees growing in the southern Cape.

The responses of the hydrophilic chemical compounds in the stem bark of *C. dentata* trees from the southern Cape were generally found to correspond to the seasonal climatic conditions of the area. Rainfall figures for the George area between September 2015 to September 2016 as provided by the South African Weather Service are provided in Table 5.1. By comparing the bi-monthly rainfall amounts with the hydrophilic metabolite responses in Figure 5.5, it becomes clear that during the months of September 2015, November 2015 and September 2016, rainfall was relatively high, and these three months' stem bark samples grouped into one cluster. In January 2016, rainfall decreased. Rainfall decreased again in March 2016, and again in May 2016. The stem bark samples collected during these three sampling dates grouped into individual clusters. In July 2016, the amount of rainfall increased, therefore grouping the samples collected during July 2016 in a cluster close to the main cluster, however, a few July 2016 samples also began grouping with the samples in the main cluster, as rainfall increased towards September 2016. Rainfall affects water and nutrient availability for the production of primary metabolites during photosynthesis, however, photoperiod, light intensity, the spectrum quality of light and temperature also contribute (Zobayed *et al*. 2005). The aim of the study was mainly untargeted analysis of the seasonal and regional variations of chemical concentrations, however, to demonstrate the seasonal and regional variations, examples of sucrose and isoeugenol concentrations are discussed. Therefore, to compare quantitatively, the sucrose concentrations in the stem bark of the only undamaged *C. dentata* tree in the southern Cape were measured at the doublet anomeric proton peaks at δ 5.4 ppm, and the two doublet proton peaks for isoeugenol at δ 6.9 and 6.6 were used to determine the concentration in each sample (Chenomx Inc., Northern Alberta, Canada, and TSP at 0.1% as a standard) (Table 5.1). Even though sucrose is not a medicinal compound or related to defence, it can be easily measured and provides a good indication of the variation of the compounds in the tree.

From Table 5.1 it can be deduced that warmer temperatures, longer days and drier conditions toward summer (January) and early autumn (March) favoured the production of sucrose in *C. dentata* trees in the southern Cape, and thus corroborate the observations of the authors reviewed by Zobayed *et al.* (2005). However, these observations are contrary to the observations of Terziev, Boutelje and Larsson (1997:216-224), who found that in Sweden, the content of low-molecular sugars in *Pinus sylvestris* are highest during autumn and winter. Sugar production in trees may therefore be both species-specific and region specific, depending on the needs of the plant.

Month	Rainfall (mm)	Sucrose concentrations (mM)	Isoeugenol concentrations (mM)
September 2015	**122**	**0.0481**	**0.2244**
October 2015	41.9	Decreases to:	Decreases to:
November 2015	**99.4**	**0.0323**	**0.1179**
December 2015	26	Increases to:	Increases to:
January 2016	**76.3**	**0.1325**	**0.1940**
February 2016	23.6	Decreases to:	Decreases to:
March 2016	**43.7**	**0.1305**	**0.1046**
April 2016	30.3	Decreases to:	Decreases to:
May 2016	**14.3**	**0.0518**	**0.0568**
June 2016	36,2	Decreases to:	Increases to:
July 2016	**34**	**0.0296**	**0.1748**
August 2016	16.2	Increases to:	Decreases to:
September 2016	**90.1**	**0.0633**	**0.1322**

Table 5.1: Rainfall for the George region from September 2015 to September 2016, as well as the sucrose and isoeugenol concentrations recorded from the undamaged *C. dentata* tree in the southern Cape forest. The highlighted rainfall figures correspond to the months of sample collection.

In Figure 5.14A, it is apparent that the hydrophilic compound concentrations, particularly in the sugar region between δ 3.0 and 4.5ppm, in the stem bark of the undamaged *C. dentata* tree in the southern Cape in September 2015 (red spectrum) were lower than the hydrophilic compound concentrations in the stem bark of the undamaged *C. dentata* tree in KZN in September 2018 (blue spectrum). In Figure 5.14B (November 2015) the concentrations of the hydrophilic compounds in the sugar region of the same *C. dentata* tree in the southern Cape increased. However, in the January sampling periods (2016 for the southern Cape and 2019 for KZN), the hydrophilic compounds in the sugar region for the undamaged trees from both regions increased dramatically (Figure 5.14C1). This dramatic increase is very clear as the scales of the spectra in these Figures (Figures 5.14A, B and C1) are the same. From Figure 5.14C1 it is, however, not clear which tree has the highest concentration. The scales of the spectra for January were thus reduced in Figure 5.14C2 to be able to visualize concentration differences. From Figure 5.14C2 it is thus clear that the concentrations of the hydrophilic compounds in the sugar region between δ 3.0 and 4.5 ppm, as well as peaks in the aliphatic and aromatic regions are higher for the undamaged tree in KZN than for the undamaged tree in the southern Cape. Towards March 2016, the hydrophilic compound concentrations in the sugar region remained high for both regions, however, the concentrations of hydrophilic compounds for the tree in the southern Cape are now slightly higher than the concentrations of the hydrophilic compounds for the tree in KZN. The concentrations of the hydrophilic

compounds in the sugar region for both trees decreased towards May and July, however, the concentrations in the tree in KZN is slightly higher at both these sampling periods. From Figures 5.14A and 5.14G, it is clear that concentrations of chemical compounds vary on a year-to-year basis (September 2015 and September 2016 for the southern Cape, and September 2018 and September 2019 for KZN), however, it should also be considered that sample collection from trees in the two areas occurred three years apart.

The profile of the hydrophilic chemical compounds of the *C. dentata* stem bark samples of the *C. dentata* trees in KZN (Figure 5.7), show that the samples collected during November 2018 (late spring) grouped into an individual cluster, whereas the samples collected during January 2019 (mid-summer) and March 2019 (early autumn) grouped together into another cluster and the stem bark samples collected in the September 2018 (early spring), May 2019 (late autumn), July 2019 (mid-winter) and September 2019 (early spring) sampling periods grouped together in the main cluster. It is therefore clear that the *C. dentata* stem bark samples collected from KZN during the warmer, late-spring, summer and early autumn seasons separated from the samples collected during the colder late autumn, winter and early spring seasons.

The responses of the hydrophilic chemical compounds in the stem bark of *C. dentata* trees in the Nkandla forest were also found to correspond to the seasonal climatic conditions of the area. The rainfall figures for the Nkandla forest between September 2018 and September 2019 as supplied by the South African Weather Service are provided in Table 5.2. The amount of rainfall in the Nkandla Forest in the September 2018/September 2019 sampling period was generally lower than amount of rainfall in the George area in the southern Cape during the September 2015/September 2016 sampling period. In the two months with high rainfall in KZN, i.e. February and April 2019, bark samples were not collected as bark samples were collected bi-monthly from September 2018 onward. In Figure 5.7, the November 2018 samples grouped into an individual cluster. Rainfall for the Nkandla Forest during November 2018 was higher than in September 2018. Both the samples collected during January and March 2019 grouped into another cluster, while the amounts of rain for the corresponding months were also relatively higher than in September 2018. For the months in-between, i.e. October and December 2018, rainfall was almost similar to rainfall for November 2018 and January 2019, therefore explaining these two months samples grouping together. However, during September 2018, May 2019, July 2019 and September 2019, rainfall was either very low, or absent, which clearly shows that the Nkandla Forest falls

within the summer rainfall region. These four months' samples therefore also grouped into one cluster.

To determine the effects of rainfall on the production of hydrophilic compounds in the *C. dentata* trees in the Nkandla forest, the sucrose concentrations in the only undamaged tree allocated in the Nkandla Forest, was again quantified (Table 5.2). From Table 5.2 it is observed that the Nkandla forest was generally drier than the southern Cape forest which may be due to differences in rainfall seasons, even though heavy rainfall incidences occurred during February and April 2019 in KZN. However, it may also be that 2015/6 was a generally wet year in the southern Cape and 2018/9 a generally dry year in KZN. Sucrose concentrations in the *C. dentata* trees in KZN were also at its higher levels during the months of January and March, and therefore correspond to the responses observed in the *C. dentata* trees occurring in the southern Cape. However, the sucrose concentrations in the stem bark of *C. dentata* trees in KZN are generally much higher than the sucrose concentrations in the stem bark of the *C. dentata* trees in the southern Cape from January through to July. Even though the hydrophilic profiles of the trees in the southern Cape (Figure 5.5) and KZN (Figure 5.7) correspond to the rainfall of the respective areas, the sucrose concentrations seemed to me more dependent on temperature than on rainfall since both areas' sucrose concentrations (Tables 5.1 and 5.2) were highest during summer and early autumn when warmer temperatures prevail, photoperiod is longer and light intensities and the spectrum quality of light are optimal.

Month	Rainfall (mm)	Sucrose concentrations (mM)	Isoeugenol concentrations (mM)
September 2018	**4.7**	**0.0492**	**0.2820**
October 2018	19.7	Increases to:	Decreases to:
November 2018	**23.7**	**0.0778**	**0.1476**
December 2018	28.2	Increases to:	Increases to:
January 2019	**21.8**	**0.3947**	**0.5840**
February 2019	145	Decreases to:	Decreases to:
March 2019	**41.1**	**0.2213**	**0.2562**
April 2019	224	Increases to:	Decreases to:
May 2019	**0**	**0.2729**	**0.1342**
June 2019	0	Decreases to:	Decreases to:
July 2019	**0**	**0.1147**	**0.1052**
August 2019	12	Decreases to:	Decreases to:
September 2019	**5.9**	**0.0531**	**0.0565**

Table 5.2: Rainfall for the Nkandla Forest between September 2018 and September 2019, as well as the sucrose and isoeugenol concentrations of the undamaged *C. dentata* tree in KZN. The highlighted rainfall figures correspond to the months of sample collection.

The hydrophilic compound concentrations, and particularly the compounds in the sugar region between δ 3.0 ppm and 4.5 ppm of the *C. dentata* trees in KZN (blue spectra) in Figures 5.14C-F, which represent the months of January, March, May and July 2019, also show higher concentrations than the hydrophilic compound concentrations of the *C. dentata* trees in the southern Cape (red spectra). Regional variations in hydrophilic compounds are further reflected in Figure 5.12, with these months' samples showing clear indications of separation between the samples collected from the southern Cape (upper half) and the samples collected in KZN (lower half). There is also separation of the samples for January and March for both regions from the main cluster of samples. The main cluster of samples grouping together indicate similar chemical profiles. These are the samples collected during September 2015, November 2015, July 2016 and September 2016 (southern Cape) and September 2018 and May, July and September 2019 (KZN), which are all colder months.

Not all hydrophilic chemical compounds in Figure 5.14 show the same pattern of increase and decrease at each sampling date. The concentrations of the phenylpropanoid, isoeugenol (2-methoxy-4-(1-propenyl)-phenol) was measured at the two doublet proton peaks for isoeugenol at δ 6.9 and 6.6. Isoeugenol is a compound with well-known pharmacological effects (Medina-Holguin, Holguin, Michelleto, Goehle, Simon and O'Connell, 2008:919-927) and toxicity (Kobets, Duan, Brunnemann, Etter, Smith and Williams, 2016: 301-311), and increases/decreases as shown in Tables 5.1 and 5.2 respectively, may therefore affect the medicinal properties and toxicity of the bark collected at different times in the year and from different localities. The concentrations for isoeugenol for the undamaged trees in both the southern Cape and KZN (Tables 5.1 and 5.2 respectively), show that isoeugenol concentrations in the stem bark of the trees in KZN are generally much higher than the isoeugenol concentrations in the stem bark of the trees in the southern Cape, and for neither of the regions the concentrations of isoeugenol can be linked to the seasonal factors of either rainfall or temperature, as increases/decreases do not necessarily correspond to colder or warmer months or to more or less rain. In the southern Cape forest, the patterns of increase and decrease in isoeugenol concentrations corresponded greatly to the patterns exhibited by sucrose (Table 5.1), however, in the July 2016 sampling period, an increase in the isoeugenol concentration was recorded whereas the sucrose concentration in the stem bark of the same *C. dentata* tree decreased. Two months later in September 2016, the opposite was observed with the sucrose concentration increasing and the isoeugenol concentration decreasing (Table 5.1). In KZN, uncorrelated changes in direction of sucrose and isoeugenol concentrations

occurred in November 2018 and May 2019 (Table 5.2). Even though the highest isoeugenol concentrations for both regions were recorded mid-summer (January) (Tables 5.1 and 5.2 respectively), the remaining uncorrelated changes according to season, and the differences in directional changes in isoeugenol concentrations to directional changes in sucrose concentrations (increases or decreases), highlight that metabolites are produced in plants according to the needs of the plant and that several factors, both external and internal, are involved.

Apart from differences in rainfall seasons and the differences in rainfall amounts for the two regions, differences in elevation and the differences in climatic and environmental conditions associated with differences in altitude such as temperature and humidity, wind velocity, and radiation intensity are important factors to consider. The *C. dentata* trees of the Groenkop area occur at 260 m above sea level and the *C. dentata* trees of the Nkandla Forest Reserve occur at 1100 m above sea level. Furthermore, the *C. dentata* trees in Groenkop are situated on a south-facing slope whereas the *C. dentata* trees of the Nkandla forest are situated close to the peak of a north-facing slope. Moreover, even though the two regions fall within the same timeline, the western Cape is very far west of KZN, which affects the times of sunrise and sunset and therefore the photoperiod of each region. Therefore, rainfall pattern and figure differences remain important, however, differences in temperature and humidity, and differences in environmental factors such as water, and nutrient availability, UV exposure, photoperiod and quality of light associated with differences in altitude and longitude may be factors of equal significance for the production of hydrophilic compounds in the two geographical regions.

5.4.2 Lipophilic compounds

The lipophilic chemical compound responses of the stem bark samples collected from *C. dentata* trees in the southern Cape (Figure 5.9) show that the September 2015 samples (early spring) and the January 2016 samples (mid-summer) grouped into individual clusters, whereas the main cluster constitutes the lipophilic chemical compound responses of the March 2016 (early autumn), July 2016 (mid-winter) and September 2016 (early spring) sample sets. The November 2015 samples (late spring) and the stem bark samples collected during the May 2016 sampling period (late autumn) separated from the main cluster but the lipophilic chemical compound responses of the November 2015 and May 2015 samples do not differ much from the lipophilic responses of the main cluster.

The lipophilic chemical compounds in the *C. dentata* stem bark samples collected from KZN separated into three clearly separated clusters, with the September 2018 and November 2018 samples grouping into separate individual clusters and almost all the samples collected during 2019 (January, March, May and September 2019) grouping into one cluster. The July 2019 samples seem to separate from the main cluster, but separation is trivial and a few of the July samples overlap the main cluster (Figure 5.11).

The connection between the lipophilic content and rainfall for both the southern Cape and KZN are less conspicuous than for the hydrophilic content. Furthermore, the concentrations of lipophilic compounds are much lower than the concentrations of hydrophilic compounds, considering that the scales of the spectra in Figures 5.15A-G are the same as for Figures 5.14A-G. However, where the scales of some spectra in Figures 5.14 had to be reduced to visualize difference in concentrations of the hydrophilic content, the scales of most spectra in Figures 5.15 had to be increased to visualize differences in concentrations of the lipophilic content.

In Figure 5.15A, it is clear that the concentrations of lipophilic compounds in the stem bark of the *C. dentata* trees in the southern Cape (red spectra) were highest during the January 2016, March 2016 and July 2016 sampling periods. This is particularly clear for the compounds in the aliphatic region of δ 0.5 to 2.0 ppm. In KZN (blue spectra), the lipophilic content in the aliphatic region seems to be higher during the September 2018, November 2018 and May 2019 sampling periods. Since rainfall cannot be linked directly to the lipophilic content, factors such as age and genetic factors such as chemotypes, the differential expressions of genes and differences in metabolic pathways are major contributing factors for variations in the lipophilic content. However, environmental factors cannot be ruled out since abiotic factors such as UV exposure, temperature, flooding or drought, and biotic environmental factors such as insects, fungi and pathogenic bacteria, or a combination of both abiotic and biotic factors are often environmental factors that causes stress in plants, and therefore influence secondary metabolite production.

Regional differentiation in lipophilic compounds is also observed in Figure 5.13, with the samples collected from the southern Cape differentiating towards the upper half and samples collected from KZN differentiating towards the lower half of the plot. The majority of samples are also in the right half of the scatter plot in Figure 5.13. A peculiar observation is that the November samples from both areas grouped into an individual cluster in the lower left quartile. The peculiarity of the observation arises from the time difference in sample

collection, which is three years. A contribution plot (which indicates the deviations from the normal correlation structure) generated from the OPLS-DA score plot of the regional variation in lipophilic compounds (Figure 5.16) illustrates the variables responsible for the observed clustering in Figure 5.13. Figure 5.17 illustrates the regions of a proton NMR spectrum from which variables contributing towards variability in different samples or regions can be interpreted

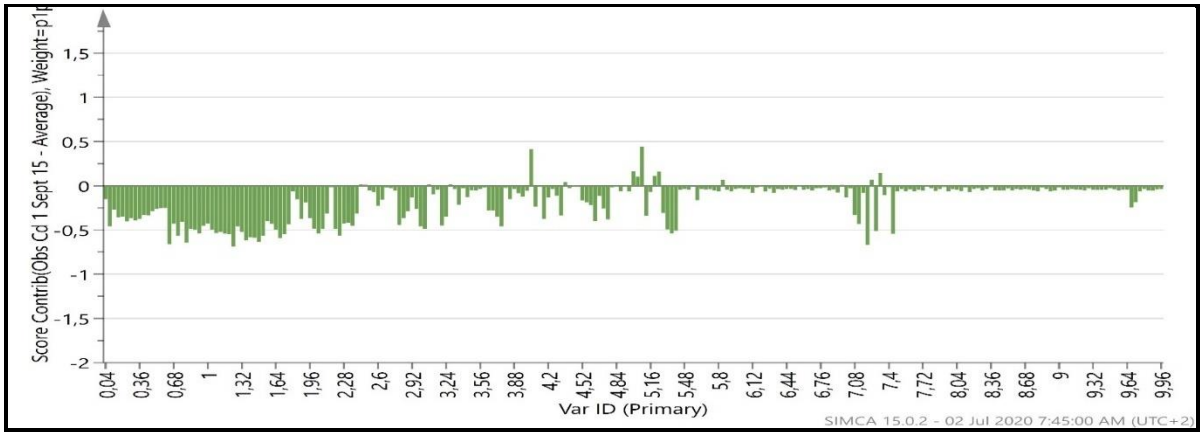


Figure 5.16. Contribution plot of the variables responsible for the regional differences in lipophilic compounds. The horizontal axis represents x-variables and the vertical axis represents the degree of contribution of each variable. The sign of the bar (down represents minus and up represents plus) indicates in which direction the variable deviates.

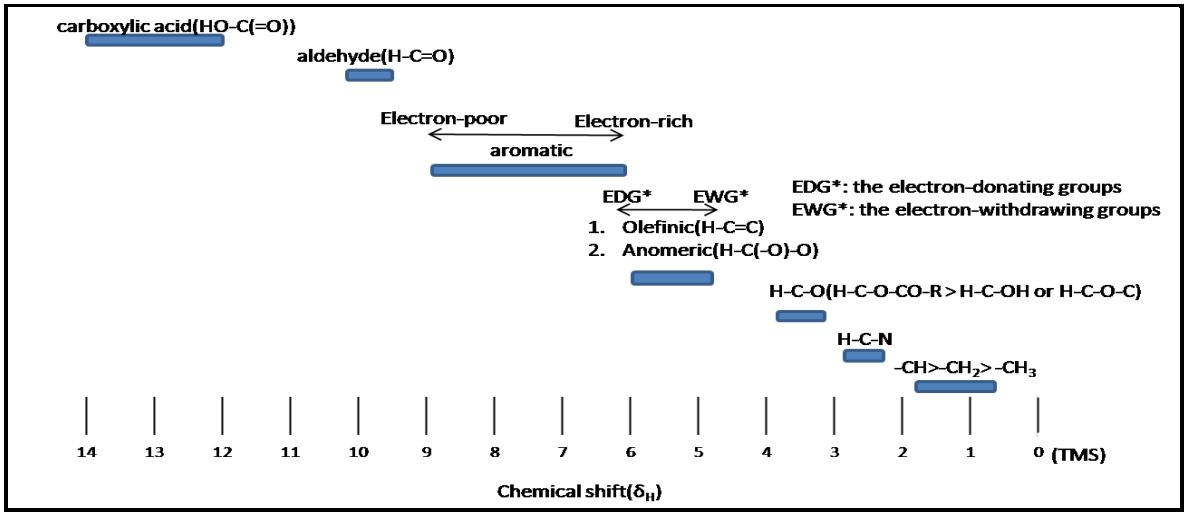


Figure 5.17: The regions of a proton NMR spectrum from which variables contributing towards variability in different samples or regions can be interpreted (Source: https://chem.libretexts.org/) (Accessed 3 July 2020).

Although no compounds have been isolated from the stem bark of *C. dentata* before, one of the compounds isolated from the leaves was used as example for variations in concentrations. Screening of the peaks in the lipophilic samples and comparing them to the peaks of known compounds detected in *C. dentata*, the peaks of betulinic acid can be clearly observed. By focusing on the peaks of the isoprenyl moiety at δ 1.71, 4.58 and 4.74 of betulinic acid, seasonal and regional fluctuations for betulinic acid becomes clear in Figures 5.15A-G. Differences in peak heights indicate differences in concentration. In Figure 5.15A2 (September), the peaks at these positions are higher for the sample collected from KZN (blue spectra), and in Figure 5.15B2 (November), these peaks (blue spectra) are also slightly higher than the southern Cape peaks (red spectra), however, it is clear that the concentration of all compounds (including betulinic acid) in the stem bark samples collected from both locations are extremely low. In January and March (Figures 5.15C and D), the concentrations of betulinic acid in the samples collected from the southern Cape (red spectra) increased significantly, and far exceeded the concentrations of betulinic acid in the samples collected from KZN (blue spectra). The increase is more than 100-fold, and for a compound such as betulinic acid with well-known medicinal properties, this increase will significantly affect the compound concentration in medicinal preparations using bark harvested form this region in these months. In May (Figure 5.15E2), however, the betulinic acid concentration in the stem bark sample collected from KZN (blue spectrum) again exceeded that of the sample collected from the southern Cape (red spectrum), as the concentration of betulinic acid again drops to levels observed in September and November for the southern Cape. In the July samples (Figure 5.15F2), the betulinic acid concentrations in the sample collected from the southern Cape was much higher than that of the sample collected from KZN. In September of the following year (Figure 5.15G2), the betulinic acid concentrations in the samples collected from the two regions increased again with concentrations that are almost equal, with the sample collected from the southern Cape showing only a slightly higher concentration. The two consecutive September month's samples for each of the research sites (September 2015 and September 2016 for the southern Cape and September 2018 and September 2019 for KZN) not showing similar concentrations shows that the concentrations of a particular compound, in this case betulinic acid, can vary not only on a month-to-month basis, but also yearly.

The undamaged tree in the southern Cape has a circumference of 89.2 cm (DBH – 28.38 cm) and the undamaged tree in KZN has a circumference of 111.5 cm (DBH – 35.47 cm). Age

differences between the two trees are thus apparent, however, the regional differences in elevation and the associated differences in environmental conditions, including the stress factors the trees are exposed to, are also factors responsible for differences in lipophilic metabolite concentrations between regions. Melito, Petretto, Podani, Foddai, Maldini, Chessa and Pintore (2016: 242-250) for example, determined that *Helichrysum italicum* produced higher percentages of essential oils such as geraniol, α-cis/trans bergamotene, linalool and ß-eudesmol at elevations of 600-1250 m above sea level than at elevations of 60 m above sea level. Similarly, Safari, Yadegari and Hamedi (2018:2719-2726) found that *Ferulago anugulate* growing at altitudes of 3000 m above sea level produced higher percentages of essential oils than at lower altitudes. Differences in elevation, and the resultant differences in stress factors trees experience in the different regions, may thus be the most probable factors for the production of lipophilic compounds. Environmental factors associated with elevation may also be beneficial for the production of certain lipophilic compounds in plants while inhibiting the production of others. Another factor that needs to be considered is that at higher altitudes, conditions for herbivores become less favourable, with a resultant decrease in the compounds that act as defence against these herbivores. The amount and diversity of secondary metabolites also drop from the equator to the poles (Gouvea, Gobbo-Neto and Lopes, 2012:432). Any secondary metabolite produced in plants is produced to aid in the plants' survival in the harsh environment the plants are exposed to. Determining the factors responsible for the production of lipophilic chemical compounds is therefore not an easily accomplished task, as many, often combined internal and external triggers can initiate the production of a plethora of lipophilic compounds using equally complex biosynthetic pathways, regulatory genes, hormones and transcription factors.

5.5 CONCLUSION

Seasonal changes in both the hydrophilic and lipophilic metabolite profiles of *C. dentata* trees are evident in this Chapter. Seasonal differences in the metabolite profiles of the *C. dentata* trees are clearly observed considering the clustering of the samples. The clustering can be explained by the climatic patterns and environmental conditions, which may include seasonal differences in the amount of rainfall, water and nutrient availability, and abiotic stress factors such as temperature, UV exposure, photoperiod, drought and flooding for the specific region. This is evident for the hydrophilic clustering patterns for both regions, clearly

representing the rainfall patterns in the specific region. However, this Chapter also showed that not all hydrophilic compound concentrations correlate with rainfall figures. Where sucrose concentrations corresponded with rainfall, isoeugenol concentrations did not. This Chapter further emphasizes differences in the metabolite profiles of *C. dentata* trees occurring in the southern Cape region and *C. dentata* trees occurring in the KZN region, which may be due to additional environmental differences such as differences in soil properties, regional differences in rainfall patterns (winter/all year and summer rainfall), elevation and topography. These have been clearly illustrated for both the lipophilic and hydrophilic compounds. Interesting to note is the clustering pattern for November in the samples for both regions for the lipophilic compounds. This study has further shown that there are differences in the production patterns of hydrophilic and lipophilic chemical compounds in the same tree. The hydrophilic and lipophilic chemical compounds do therefore not necessarily start increasing or decreasing during the same season, and the rates of increase or decrease between hydrophilic and lipophilic chemical compounds in the same tree also differ. Furthermore, these patterns of hydrophilic and lipophilic compound production in individual trees also vary regionally. Using the well-known medicinal compound betulinic acid as an example, an extreme increase was observed for January and March in the southern Cape, whereas the compound levels stayed generally constant for the KZN samples throughout the season. Similarly, differences in concentration of isoeugenol in response to month of collection and collection area might affect both the medicinal and toxicological profile of the collected material. It is therefore clear that the seasons in which plant material is collected, the regions from which plant material is collected and the specific tree from which plant material is collected will affect the dosages of chemical compounds consumed when using raw or semi-processed *C. dentata* stem bark as a medicine.

CHAPTER 6. COMPARISON OF THE CHEMICAL PROFILES OF *Curtisia dentata* (Burm.f.) C.A.Sm. STEM BARK OF TREES FROM THE SOUTHERN CAPE AND KWAZULU-NATAL

6.1. INTRODUCTION

Curtisia dentata (Burm.f.) C.A.Sm. stem bark is of particular importance as a traditional medicine in both the Eastern Cape and KZN Provinces of South Africa, where it is ranked sixth on the list of the top ten species harvested and traded at traditional medicine markets (Dold and Cocks, 2002: 594). Of particular concern is that the stem bark of *C. dentata* trees are being harvested, prepared into remedies or tonics and consumed without people knowing the chemical content of the medicine. The origins of *C. dentata* stem bark traded at medicinal trade markets such as the Faraday traditional medicine market, are mostly unknown and the date of harvest is equally unknown. The bark may also have been harvested at different locations in South Africa, or may have been imported from neighboring countries within their range of distribution since *C. dentata* has become scarce in some Provinces of South Africa, for example KwaZulu-Natal (Mander et al. 2007; SANBI, 2017).

In Chapter 3, the chemical compounds in the stem bark purchased at the Faraday medicine market were extracted exhaustively using hexane, DCM, ethanol and water, in that order. The *C. dentata* stem bark used in Chapters 5 and in this Chapter were, however, freshly harvested at each sampling period and the chemical compounds in the plant material collected were extracted using only DCM. In Chapter 5 the aim was to determine seasonal and regional variations in chemical compound concentrations, which is why NMR analyses were conducted. However, in this Chapter, the aim is to determine whether there are seasonal and regional differences in chemical compound composition, and also whether there are differences in the composition of chemical compounds in the stem bark of individual trees growing at the same location. The volatile chemical profiles of plants are best determined with GC-MS analysis. Plant material harvested for the traditional medicine market is collected during all seasons, in different years and from different locations, but is sold at the same market and used for the same purpose. The stem bark collected at the two selected geographical regions was collected three years apart, and therefore, time differences in collection dates are included.

6.2 METHODS AND MATERIALS

6.2.1 Stem bark samples and analytical method used

The *C. dentata* stem bark samples collected from Groenkop in September 2015, and from the Nkandla Forest Reserve in September 2018, were analyzed using GC-MS to determine similarities and differences in chemical composition in individual trees occurring at the same location. As the tree species is protected, authorities from the two regions only allocated 12 trees at each locality for sampling purposes. Stem bark samples collected and analyzed in September 2015, November 2015 and January 2016 from *C. dentata* trees in the southern Cape, and in September 2018, November 2018 and January 2019 from *C. dentata* trees in KZN, were used as example to demonstrate seasonal and regional changes in chemical composition.

6.2.2 Extraction of chemical compounds

From the pre-processed stem bark samples collected from both Groenkop and Nkandla Forest in Chapter 5, an additional 200 mg of homogenized sample material from each sample was weighed and transferred to separate Eppendorff tubes. For the extraction of chemical compounds for GC-MS analysis, 2 ml DCM was added to each tube containing the homogenized sample material. The samples were vortexed (Labsmart M-X vortex mixer), sonicated for 20 minutes (Branson 1800 ultrasound bath), and centrifuged for 15 minutes (Eppendorff Mini Spin), after which 1.5 ml of the supernatant in each tube was filtered through Whatman 13mm filters with a pore size of 0.2 µm and poured into individual Restek GC/LC analytical bottles.

6.2.3 GC-MS analysis of *C. dentata* stem bark samples

Each sample prepared was run twice in a Leco® 7890B GC chromatograph with a Gerstel multisampler and Chroma Time of Flight spectrometer software (TOF-MS) optimized for a Pegasus®4D mass spectrometer. The main column (Restek®) has a length of 32 m, an internal diameter of 250 µm and a film thickness of 0.025 µm, and the secondary column has a length of 0.790m with an internal diameter of 250 µm. Analysis of the samples was carried out under the following conditions: splitless injection of 0.2 ml was employed; injection temperature was 33°C, held for 3 minutes, then increased at a rate of 10°C per minute to the target temperature of 180°C; without a holding period, the rate of temperature increase was increased to a rate of 40°C per minute until a temperature of 220°C is reached. Helium (99.99%) was the carrier gas used, with a flow rate of 1.0 µl per minute (constant flow). The ionizing energy was 70eV. The scan rate was

50 scans/ second and the mass spectral scan range was 60 to 600 (MHz). The relative constituent content was expressed as normalized percent peak area.

6.2.4 Statistical analysis

Statistical analyses were conducted using SPSS. The significance of the bi-monthly differences in the total number of compounds in the stem bark of all 12 the *C. dentata* trees occurring at the same location was analyzed statistically using the Wilcoxon rank sum tests for dependent groups, whereas t-tests for two independent means were used to test the significance of the regional differences in the total number of compounds in the stem bark of all 12 the *C. dentata* trees at each research site.

6.3 RESULTS

Stem bark samples collected from *C. dentata* trees in the southern Cape and KZN over three consecutive sampling dates were used to compile Figures 6.1 and 6.2, which demonstrate changes in the number of chemical compounds detected by GC-MS, occurring in individual *C. dentata* trees over time. The bi-monthly differences in numbers of chemical compounds occurring in each individual tree reflects changes in chemical composition. Note that there are also regional differences in the number of chemical compounds in the stem bark of *C. dentata* trees (Figure 6.3).

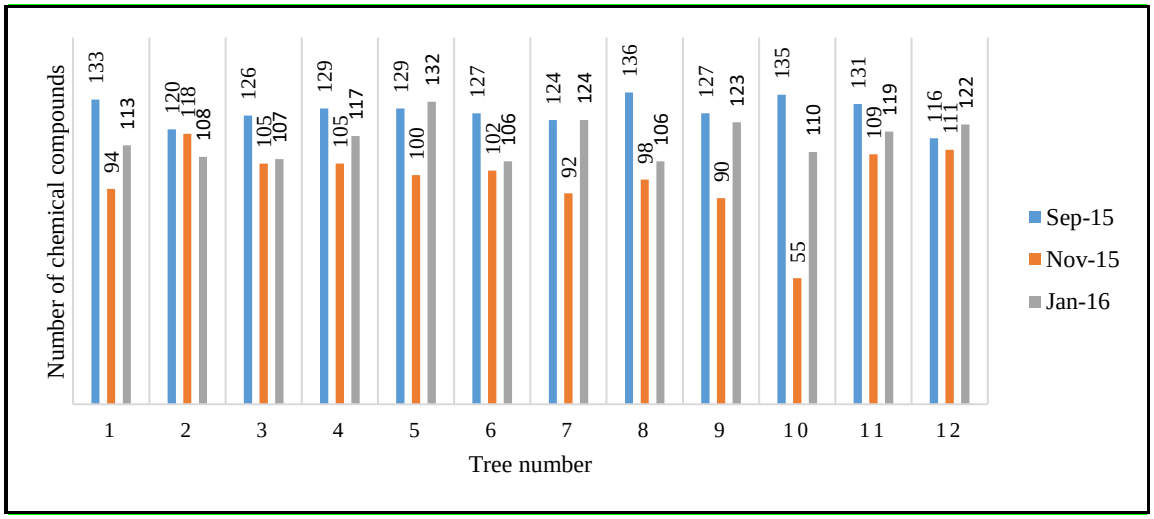


Figure 6.1: Changes in the number of chemical compounds in the stem bark of individual *C. dentata* trees in the southern Cape over three consecutive sampling dates.

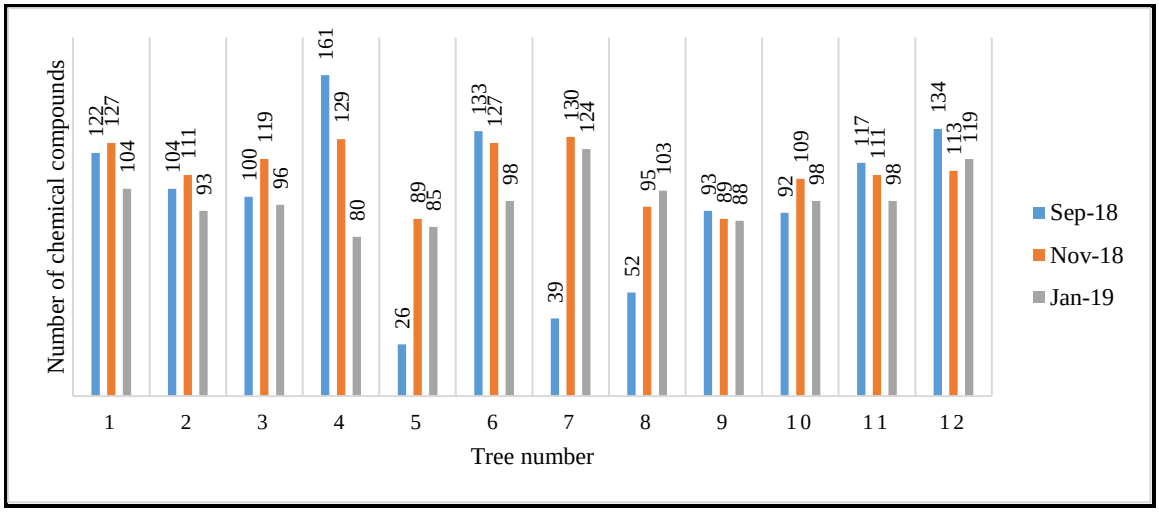


Figure 6.2: Changes in the number of chemical compounds in the stem bark of individual *C. dentata* trees in KZN over three consecutive sampling dates.

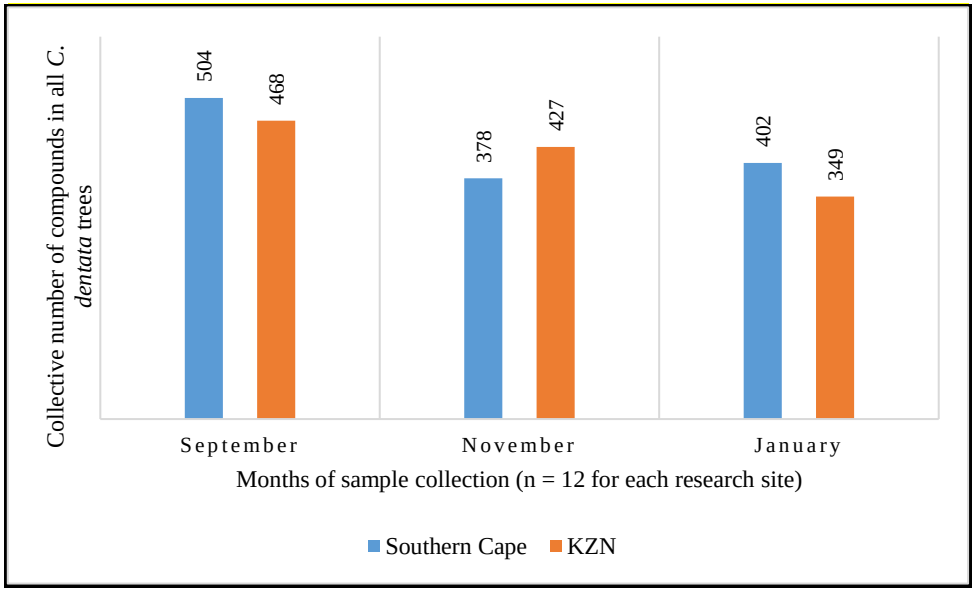


Figure 6.3: Comparison of the total number of chemical compounds in the stem bark of all 12 the *C. dentata* trees at both research sites over three consecutive sampling dates.

Statistically significant differences were obtained between the southern Cape and KZN samples ($p < 0.05$) for September ($p = 0.019$), November ($p = 0.035$) and January ($p = 0.001$) sampling periods.

Statistically significant differences ($p < 0.05$) were obtained in the total numbers of chemical compounds for the southern Cape samples; between September 2015 and November 2015 ($p = 0.002$) and from November 2015 to January 2016 ($p = 0.006$).

A statistically significant difference ($p < 0.05$) was not obtained for the total number of chemical compounds in the KZN samples between September 2018 and November 2018 ($p = 0.271$). A statistically significant difference was however obtained from November 2018 to January 2019 ($p = 0.016$).

Table 6.1, which is attached as Appendix M, compares the combined chemical composition of the stem bark samples of all the *C. dentata* trees at each research site in September (September 2015 for the southern Cape and September 2018 for KZN). Similarly, Table 6.2, which is attached as Appendix N, compares the combined chemical composition of the stem bark samples of all the *C. dentata* trees at each research site in November (November 2015 for the southern Cape and November 2018 for KZN), and Table 6.3 (attached as Appendix O), compares the combined chemical composition of the stem bark samples of all the *C. dentata* trees at each research site in January (January 2016 for the southern Cape and January 2019 for KZN). Note that the occurrence of Argon in the lists are due to impurities deriving from the carrier gas used for GC-MS analysis. To highlight the similarities and differences in chemical composition during each month, the compounds highlighted represent compounds that occur in trees at both sites, however, not necessarily in every tree. The remaining compounds are region-specific and may also include tree-specific compounds.

Two trees from each of the southern Cape and KZN research sites were used as examples to demonstrate similarities and differences in chemical composition in the stem bark of individual trees growing at the same location. The chemical compounds in the DCM stem bark extracts of the two individual *C. dentata* trees occurring in the southern Cape, of which the chromatograms have an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer, are therefore listed in Tables 6.4 and 6.5 respectively. For correct identification, the fragmentation patterns of the compounds identified by GC-MS analysis were compared to the fragmentation patterns in databases such as PubChem, the database of the National Institute of Standards and Technology (NIST) and the Human Metabolome database (HMDB). Note that there are similarities in chemical composition in the stem bark of the two *C. dentata* trees selected from each research site (highlighted), but also distinct differences. By comparing the percentage areas relative to the total area of corresponding chemical compounds, the concentration differences between the two individual trees occurring at the same location also becomes clear. For example, in Table 6.4, nonadecane in the stem bark of the first tree has a percentage area of 0.26 %, whereas in Table 6.5, nonadecane in the stem bark of the second tree has a percentage area of 2.16%.

Table 6.4: Chemical composition of the stem bark of one individual *C. dentata* tree from the southern Cape, including the chemical compounds' retention times, percentage areas and similarities to the chemical compounds in the mass spectrometer's main database. Compounds found in both the trees selected in the southern Cape are highlighted.

	R.T. (s)	Name	Area %	Similarity
1	102,22	(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane	0,27107	800
2	1044,94	11-Hexadecen-1-ol, (Z)-	0,11714	939
3	544,18	**11-Methyldodecanol**	0,022372	874
4	889,36	**1-Iodo-2-methylundecane**	1,0829348	946
5	348,8	1-Octene, 3,7-dimethyl-	0,02204	820
6	660,3	1-Pentanol, 2,4-dimethyl-, (ñ)-	0,035398	911
7	525,08	2,3,4,5,6-Pentamethyl acetophenone	0,00094602	817
8	156,38	**2,4-Dimethyl-1-heptene**	0,011139	918
9	702,22	**2,4-Di-tert-butylphenol**	0,4728957	926
10	665,1	**2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-**	0,0051509	846
11	284,1	2,6-Dimethyldecane	0,055625	861
12	958,3	2-Bromotetradecane	0,031865	879
13	1296,68	3,4-Hexanedione, 2,2,5-trimethyl-	0,080345	852
14	285,96	**3-Carene**	0,0028396	876
15	76,74	3-Methyl-2-butenoic acid, tridec-2-ynyl ester	5,6913	925
16	496,82	4-(Trifluoromethyl)benzyl alcohol, 1-methylpropyl ether	2,0089	956
17	1172,32	4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone	0,00016124	919
18	427,2	5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran	0,046079	872
19	222,68	à-Pinene	0,011325	903
20	496,42	**Benzene, 1,3-bis(1,1-dimethylethyl)-**	2,0089	910
21	601,14	**Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-**	0,0017765	810
22	194,48	**Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-**	0,0072852	821
23	211	**Cyclopentene, 1,2,3,4,5-pentamethyl-**	0,0095231	888
24	539,18	**Decane**	0,474632	912
25	667,24	**Decane, 2,3,5,8-tetramethyl-**	0,127899	951
26	764,44	Decane, 2,6,8-trimethyl-	0,264118	885
27	328,6	**Decane, 4-methyl-**	0,562146	966
28	523,12	Decane, 5-methyl-	0,15626	837
29	693,86	Docosane	0,063488	910
30	757,56	**Eicosane**	1,940597	939
31	1121	**Ether, 6-methylheptyl vinyl**	0,201243	895
32	1164,44	**Heneicosane**	0,2312679	932
33	1267,68	**Hentriacontane**	0,010632	884
34	1002,86	Heptacosane, 1-chloro-	0,0058911	888
35	679,22	**Heptadecane**	3,40337	940
36	527,52	**Heptadecane, 2,6,10,14-tetramethyl-**	0,797941	939
37	711,84	Heptadecane, 2,6-dimethyl-	0,136768	910

38	825,9	**Heptadecane, 2-methyl-**	0,655357	949
39	144,22	**Heptane, 2,4-dimethyl-**	2,143793	908
40	1315,56	**Hexacosane**	0,127431	931
41	686,56	**Hexadecane**	0,8211836	960
42	1058,8	**Hexadecen-1-ol, trans-9-**	0,017812	945
43	256,4	**Hexane, 2,4-dimethyl-**	0,11543	867
44	288,38	Hexyl isobutyl carbonate	0,18708	875
45	906,54	Isopropyl myristate	0,014543	803
46	149,84	Methylene chloride – solvent	1,884	978
47	896,82	n-Hexyl salicylate	0,0090411	862
48	1197,96	Nonacosane	0,317967	928
49	928,34	**Nonadecane**	0,264253	933
50	293,12	**Nonane, 2,5-dimethyl-**	0,072228	898
51	246,36	**Nonane, 2-methyl-**	0,059389	924
52	244,36	**Nonane, 4-methyl-**	0,011851	844
53	1070,78	**Octacosane**	0,8074256	943
54	807,76	**Octadecane**	2,08699	940
55	801,74	Octane, 1,1’-oxybis-	0,093765	891
56	358,56	**Octane, 2,3,6,7-tetramethyl-**	0,043865	907
57	217,78	**Octane, 2,7-dimethyl-**	0,0066424	953
58	376,56	**Octane, 3-methyl-**	0,03077	896
59	167,42	**Octane, 4-methyl-**	0,186104	938
60	1081,44	Oxalic acid, allyl hexyl ester	0,092751	826
61	428,38	**Oxalic acid, isobutyl octyl ester**	0,046079	914
62	658,12	**Pentadecane**	2,516976	946
63	535,48	Precocene I	0,0036112	825
64	375,82	**Sulfurous acid, hexyl 2-pentyl ester**	0,018574	870
65	1377,58	**Tetracosane**	0,2131065	940
66	486,3	**Tetradecane**	1,634447	951
67	583,06	**Tridecane, 2-methyl-**	0,037811	943
68	548,1	**Tridecane, 3-methyl-**	0,021301	892
69	614,1	**Tridecane, 4,8-dimethyl-**	0,014166	914
70	578,98	**Tridecane, 4-methyl-**	0,024225	917
71	573	Tridecane, 6-methyl-	0,00583	822
72	967,68	Tridecanoic acid, methyl ester	0,27301	839
73	712,4	Tridecanol, 2-ethyl-2-methyl-	0,09678	829
74	447,02	Undecane	0,1209049	905
75	458,32	**Undecane, 2,4-dimethyl-**	0,025819	944
76	461,24	Undecane, 2,6-dimethyl-	0,15134	908
77	418,52	**Undecane, 2-methyl-**	0,028505	962
78	479,46	Undecane, 4,6-dimethyl-	0,032828	915
79	467,88	**Undecane, 4,8-dimethyl-**	0,140884	924
80	414,74	**Undecane, 4-methyl-**	0,0084968	955

Table 6.5: Chemical composition of the stem bark of a second individual *C. dentata* tree from the southern Cape, including the chemical compounds' retention times, percentage areas and similarities to the chemical compounds in the mass spectrometer's main database. Compounds found in both the trees selected in the southern Cape are highlighted.

	R.T. (s)	Name	Area %	Similarity
1	222,62	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	0,006245	915
2	524,9	1-(4-tert-Butylphenyl)propan-2-one	0,002854	802
3	1063,94	1,3-Dioxane, 2-heptyl-	0,015892	852
4	544,06	**11-Methyldodecanol**	0,027469	870
5	345,14	1-Decene, 3,3,4-trimethyl-	0,018664	822
6	868,18	1-Dodecanol, 2-hexyl-	0,05238	842
7	889,46	1-**Iodo-2-methylundecane**	0,249119	954
8	348,76	1-Octanesulfonyl chloride	0,017686	833
9	874,68	1-Octanol, 2-butyl-	0,027757	896
10	156,24	**2,4-Dimethyl-1-heptene**	0,048862	848
11	702,18	**2,4-Di-tert-butylphenol**	0,47379	926
12	665,04	**2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-**	0,003219	862
13	658,14	2,6,10-Trimethyltridecane	0,34254	908
14	285,92	**3-Carene**	0,002618	860
15	1045,1	9-Hexadecen-1-ol, (Z)-	0,11363	925
16	127,3	Argon	9,535866	885
17	496,28	**Benzene, 1,3-bis(1,1-dimethylethyl)-**	2,0324	943
18	601,12	**Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-**	0,000828	825
19	194,38	**Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-**	0,006776	808
20	257,66	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0,11838	904
21	210,9	**Cyclopentene, 1,2,3,3,4-pentamethyl-**	0,009183	882
22	330,72	**Decane**	0,462734	891
23	667,18	**Decane, 2,3,5,8-tetramethyl-**	0,158381	946
24	295,56	**Decane, 4-methyl-**	0,310097	935
25	101,3	Desmethyldeprenyl	0,090303	844
26	1162,18	Docosane, 11-butyl-	0,018259	899
27	447,06	Dodecane, 4,6-dimethyl-	0,008397	909
28	461,2	Dodecane, 6-methyl-	0,16093	925
29	1108,72	Dotriacontane	0,16821	888
30	689,98	**Eicosane**	2,040928	937
31	1181,12	Eicosane, 2-methyl-	0,089357	913
32	964,64	**Ether, 6-methylheptyl vinyl**	1,112429	854
33	1080,84	**Heneicosane**	0,369573	927
34	1082,24	Heneicosanoic acid, methyl ester	0,092482	912
35	1212,4	**Hentriacontane**	0,033505	894
36	1317,76	Heptacosane	0,05296	936
37	781,1	**Heptadecane**	3,437354	932
38	516,16	**Heptadecane, 2,6,10,14-tetramethyl-**	2,482251	927

39	880,78	Heptadecane, 2,6,10,15-tetramethyl-	0,014444	914
40	679,1	Heptadecane, 2,6-dimethyl-	0,474484	922
41	712,28	**Heptadecane, 2-methyl-**	0,524492	920
42	141,04	**Heptane, 2,4-dimethyl-**	4,924068	910
43	620,88	Heptane, 3-ethyl-2-methyl-	0,13656	866
4445	427,16	Heptane, 4,4-dimethyl-	0,053356	842
46	288,2	Heptane, 4-ethyl-	0,19716	861
47	1314,74	**Hexacosane**	0,066636	928
48	650,98	**Hexadecane**	1,564417	939
49	1059,26	**Hexadecen-1-ol, trans-9-**	0,09446	900
50	256,32	**Hexane, 2,4-dimethyl-**	0,1196	874
51	149,94	Methylene chloride – solvent	3,1518	975
52	892,82	**Nonadecane**	2,168875	948
53	1266,82	Nonadecane, 2-methyl-	0,005773	894
54	293,02	**Nonane, 2,5-dimethyl-**	0,08658	900
55	246,26	**Nonane, 2-methyl-**	0,068	928
56	244,2	**Nonane, 4-methyl-**	0,018933	826
57	1103,52	**Octacosane**	0,066441	933
58	807,74	**Octadecane**	0,165574	931
59	358,54	**Octane, 2,3,6,7-tetramethyl-**	0,028807	906
60	217,68	**Octane, 2,7-dimethyl-**	0,003736	958
61	284,02	Octane, 3,5-dimethyl-	0,056998	878
62	376,56	**Octane, 3-methyl-**	0,010402	844
63	167,5	**Octane, 4-methyl-**	0,214491	932
64	428,46	**Oxalic acid, isobutyl octyl ester**	0,053356	914
65	1201,5	Oxalic acid, isobutyl pentyl ester	0,081056	873
66	627,86	**Pentadecane**	1,378593	944
67	116,38	Sulfurous acid, 2-ethylhexyl pentyl ester	1,2787	818
68	375,78	**Sulfurous acid, hexyl 2-pentyl ester**	0,019627	841
69	618,3	Sulfurous acid, hexyl octyl ester	0,016016	896
70	319,58	Sulfurous acid, nonyl pentyl ester	0,023577	871
71	1298,1	**Tetracosane**	0,140101	930
72	486,26	**Tetradecane**	0,331573	951
73	660,16	Tetradecane, 4-ethyl-	0,034878	880
74	1284,3	Triacontane	0,055785	818
75	326,08	Tridecane	1,1444	905
76	583,06	**Tridecane, 2-methyl-**	0,05732	939
77	548,1	**Tridecane, 3-methyl-**	0,020547	898
78	614,14	**Tridecane, 4,8-dimethyl-**	0,016249	916
79	771,68	**Tridecane, 4-methyl-**	0,011994	951
80	458,3	Undecane, 2,4-dimethyl-	0,098994	941
81	418,46	**Undecane, 2-methyl-**	0,034313	967
82	467,92	**Undecane, 4,8-dimethyl-**	0,08165	927
83	414,66	**Undecane, 4-methyl-**	0,007322	928
84	968,02	Undecanoic acid, methyl ester	0,34872	831

Chemical compounds in the DCM stem bark extracts of two individual *C. dentata* trees from the Nkandla Forest Reserve in KZN, of which the chromatograms have an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer are listed in Tables 6.6 and 6.7 respectively. Note that there are also similarities in chemical composition in the stem bark of the two trees growing in KZN (highlighted), however, as with the trees in the southern Cape, there are also distinct differences.

Table 6.6: Chemical composition of the stem bark of one individual *C. dentata* tree from the Nkandla Forest Reserve in KZN, including the chemical compounds' retention times, percentage areas and similarities to the chemical compounds in the mass spectrometer's main database. Compounds found in both the trees selected in KZN are highlighted.

	R.T. (s)	Name	Area %	Similarity
1	1380,6	1,2-Benzenedicarboxylic acid, decyl octyl ester	0,003907	964
2	622,52	1-Hexanol, 2,2-dimethyl-	0,034907	860
3	711,74	1-Pentanol, 2,4-dimethyl-, (ñ)-	0,064159	838
4	538,26	2,2-Dimethyl-3-heptene trans	9,14E-08	861
5	703,9	**2,4-Di-tert-butylphenol**	0,22905	939
6	1048,32	9-Hexadecen-1-ol, (Z)-	0,060073	942
7	620,16	Acetamide, N-tetrahydrofurfuryl-2,2,2-trifluoro-	0,033008	849
8	1171,36	Benz[a]anthracene	0,015635	959
9	497,14	**Benzene, 1,3-bis(1,1-dimethylethyl)-**	1,5309	956
10	1393,68	Benzo[b]fluoranthene	0,02835	964
11	1431,48	Benzo[k]fluoranthene	0,032873	948
12	1167,36	Benzyl butyl phthalate	0,002301	939
13	70,84	Binapacryl	4,1084	907
14	1179,66	Chrysene	0,00618	949
15	60,28	Cyclobutanol	3,3014	834
16	211,28	**Cyclopentene, 1,2,3,4,5-pentamethyl-**	0,013487	850
17	540,3	**Decane**	0,028307	897
18	766,18	Decane, 2,5-dimethyl-	0,021621	928
19	296,08	Decane, 2,6,7-trimethyl-	0,34722	920
20	288,82	**Decane, 5-methyl-**	0,2186	894
21	1294,56	Diisooctyl phthalate	0,021209	887
22	480,54	**Dodecane, 4,6-dimethyl-**	0,023548	913
23	838,84	**Eicosane**	0,064578	925
24	691,66	**Heptadecane**	0,506231	935
25	517,12	**Heptadecane, 2,6,10,14-tetramethyl-**	1,20169	939
26	496,66	**Heptadecane, 2,6-dimethyl-**	1,5309	877
27	584,3	**Heptadecane, 2-methyl-**	0,0061	957
28	524,04	**Hexadecane**	0,10675	942
29	1061,92	**Hexadecen-1-ol, trans-9-**	0,18421	951
30	370,52	**Hexane, 2,3,4-trimethyl-**	0,11581	897

31	256,82	**Hexane, 2,4-dimethyl-**	0,10699	878
32	674,3	Hexane, 3,3-dimethyl-	0,003276	910
33	1215,54	Hexanedioic acid, bis(2-ethylhexyl) ester	0,014934	900
34	87,9	Methane-d, trichloro-	2,4026	843
35	150,26	Methylene chloride – solvent	7,6673	951
36	1088,54	**Nonadecane**	0,749067	935
37	293,66	**Nonane, 2,5-dimethyl-**	0,083113	889
38	246,48	**Nonane, 2-methyl-**	0,077246	915
39	545,22	**n-Tridecan-1-ol**	0,015857	872
40	818,02	**Octadecane**	0,442843	947
41	320,18	**Octane, 2,3,6,7-tetramethyl-**	0,060961	895
42	713,3	Octane, 3,5-dimethyl-	0,064159	918
43	170,38	**Octane, 4-methyl-**	0,043837	935
44	462,28	Oxalic acid, isobutyl nonyl ester	0,082414	891
45	429,38	Oxalic acid, isobutyl octyl ester	0,002352	926
46	629,28	**Pentadecane**	1,177612	958
47	1306,22	Phthalic acid, 2H-octahydroquinazoline-1-methyl pentyl ester	0,052641	993
48	1190,8	Phthalic acid, hexyl nonyl ester	0,062045	951
49	91,34	Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester	6,1015	932
50	499,7	Sulfurous acid, hexyl pentyl ester	0,056642	817
51	642	**Tetradecane**	0,127479	957
52	569,64	**Tridecane**	0,003268	929
53	549,4	Tridecane, 3-methyl-	0,009579	860
54	468,88	**Tridecane, 4-methyl-**	0,053018	930
55	459,26	**Undecane, 2,4-dimethyl-**	0,01492	930
56	419,36	**Undecane, 2-methyl-**	0,014985	948
57	326,7	**Undecane, 3,7-dimethyl-**	1,1617	901
58	284,48	Undecane, 4,6-dimethyl-	0,087621	871

Table 6.7: Chemical composition of the stem bark of a second individual *C. dentata* tree from KZN, including the chemical compounds' retention times, percentage areas and similarities to the chemical compounds in the mass spectrometer's main database. Compounds found in both the trees selected in KZN are highlighted.

	R.T. (s)	Name	Area %	Similarity
1	161,4	1-Butanol, 2,2-dimethyl-	0,003252	804
2	889,84	1-Iodo-2-methylundecane	0,008421	927
3	525,78	2,3,4,5,6-Pentamethyl acetophenone	0,001602	807
4	157,3	2,4-Dimethylhept-1-ene	0,008033	919
5	703,26	**2,4-Di-tert-butylphenol**	0,24029	940
6	666,22	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	0,000548	850
7	349,64	2-Undecanethiol, 2-methyl-	0,020321	844
8	847,62	3,5-Dimethyldodecane	0,018844	871

9	872,38	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	0,000392	870
10	95,2	3-Methyl-2-butenoic acid, tridec-2-ynyl ester	2,2693	891
11	329,32	3-Nitropyrrole	0,035572	817
12	428,02	5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran	0,020782	826
13	1064,18	9,12-Octadecadienoic acid, methyl ester, (E,E)-	0,024091	828
14	1067,88	9,12-Octadecadienoyl chloride, (Z,Z)-	0,069117	829
15	497,06	**Benzene, 1,3-bis(1,1-dimethylethyl)-**	0,90487	947
16	223,66	Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	0,001341	808
17	212,14	**Cyclopentene, 1,2,3,4,5-pentamethyl-**	0,006475	852
18	941,46	Cyclotetradecane	0,040559	933
19	277,2	**Decane**	0,156127	923
20	345,96	Decane, 1-fluoro-	0,012007	851
21	667,84	Decane, 2,3,5,8-tetramethyl-	0,131612	947
22	677,74	Decane, 2,4,6-trimethyl-	0,032353	909
23	296,48	Decane, 4-methyl-	0,11709	926
24	523,8	**Decane, 5-methyl-**	0,073525	839
25	991,2	Dibutyl phthalate	0,11969	912
26	1294,98	Dicyclohexyl phthalate	0,000321	854
27	1299,9	Docosane, 11-butyl-	0,01264	916
28	1120,72	Dodecane, 1-iodo-	0,002037	939
29	447,86	**Dodecane, 4,6-dimethyl-**	0,014689	920
30	1082,38	Dodecanoic acid, methyl ester	0,034357	879
31	683,92	**Eicosane**	0,524886	943
32	1081,46	Eicosane, 2-methyl-	0,034357	929
33	1044,42	Heneicosane	0,359714	934
34	1280,76	Heptacosane	0,033522	924
35	781,8	**Heptadecane**	0,505608	942
36	516,82	**Heptadecane, 2,6,10,14-tetramethyl-**	0,793819	939
37	658,64	**Heptadecane, 2,6-dimethyl-**	0,178099	929
38	583,62	**Heptadecane, 2-methyl-**	0,079331	963
39	142,4	Heptane, 2,4-dimethyl-	5,20093	914
40	656,18	Heptane, 3,4-dimethyl-	0,013988	872
41	764,78	Heptane, 3,5-dimethyl-	0,032778	909
42	651,62	**Hexadecane**	0,078228	933
43	1059,24	**Hexadecen-1-ol, trans-9-**	0,035556	945
44	539,84	**Hexane, 2,3,4-trimethyl-**	0,088323	899
45	257,32	**Hexane, 2,4-dimethyl-**	0,14716	886
46	150,44	Methylene chloride – solvent	0,4259	948
47	832,26	**Nonadecane**	1,062951	936
48	1067,28	Nonadecane, 2-methyl-	0,069117	943
49	293,92	**Nonane, 2,5-dimethyl-**	0,042691	895
50	848,32	Nonane, 2,6-dimethyl-	0,018844	902
51	247,26	**Nonane, 2-methyl-**	0,031432	924
52	544,7	**n-Tridecan-1-ol**	0,008333	864
53	808,34	**Octadecane**	0,336921	943
54	320,48	**Octane, 2,3,6,7-tetramethyl-**	0,009673	885

55	218,7	Octane, 2,7-dimethyl-	0,002777	926
56	619,4	Octane, 3-ethyl-2,7-dimethyl-	0,008203	928
57	168,9	**Octane, 4-methyl-**	0,067767	935
58	1353,72	Oxalic acid, dodecyl 3,5-difluorophenyl ester	0,003333	663
59	428,84	Oxalic acid, isobutyl octyl ester	0,167521	919
60	763,46	Oxalic acid, isohexyl pentyl ester	0,032778	851
61	687,1	**Pentadecane**	0,768496	963
62	117,04	Pentane, 2-methyl-	3,3951	837
63	376,54	Pentane, 3,3-dimethyl-	0,020422	872
64	141,74	Pentanol, 5-amino-	1,0948	867
65	377,34	Sulfurous acid, dibutyl ester	0,021563	902
66	658,44	Sulfurous acid, hexyl heptyl ester	0,180711	863
67	703,78	Sulfurous acid, hexyl pentadecyl ester	0,24029	864
68	487,02	**Tetradecane**	0,336757	945
69	450,28	**Tridecane**	0,011673	974
70	614,7	Tridecane, 4,8-dimethyl-	0,003765	917
71	468,6	**Tridecane, 4-methyl-**	0,178081	959
72	573,52	Tridecane, 6-methyl-	0,002972	921
73	459,06	**Undecane, 2,4-dimethyl-**	0,01336	921
74	419,16	**Undecane, 2-methyl-**	0,016941	941
75	710,38	**Undecane, 3,7-dimethyl-**	0,752801	910
76	499,5	Undecane, 4,8-dimethyl-	0,021755	844
77	415,36	Undecane, 4-methyl-	0,005424	926
78	359,42	Undecane, 5,7-dimethyl-	0,011047	898
79	968,14	Undecanoic acid, methyl ester	0,1099	851

6.4 DISCUSSION

The biosynthesis of secondary metabolites in plants are influenced by internal and external factors which change according to the needs of the plants in their environment (Varma and Shukla, 2015:108). Changes in the number of compounds present in individual *C. dentata* trees at both research sites over three sample periods are evident in Figures 6.1 (Appendix M) and 6.2 (Appendix N) respectively. Evident from Figure 6.3 (Appendix O) is that the total number of compounds in the stem bark of all the study *C. dentata* trees, in both the southern Cape and KZN and over the same three consecutive sampling dates, differ both bi-monthly and regionally. Statistically, the differences in composition of chemical compounds in the stem bark of the *C. dentata* trees in the southern Cape and the *C. dentata* trees in KZN for September are significant at $p < 0.05$ as $p = 0.019$, which is also true for the November ($p = 0.035$) and January sampling periods ($p = 0.001$). The total number of compounds in the stem bark of all *C. dentata* trees in the southern Cape for September 2015 is 504 [Figure 6.3; Table 6.1 (Appendix M)], whereas in November 2015, the total

number of chemical compounds in the stem bark of all *C. dentata* trees in the southern Cape decreased to 378 [Figure 6.3; Table 6.2 (Appendix N)]. The differences in the total numbers of chemical compounds, and thus the changes in chemical composition of all study trees between September 2015 and November 2015 are therefore significant at $p < 0.05$ as $p = 0.002$. The total number of chemical compounds in the stem bark increased from 378 compounds in November 2015 to 402 compounds in January 2015 [Figure 6.3; Table 6.3 (Appendix O)]. Between November 2015 and January 2016, the changes in chemical composition are equally significant at $p < 0.05$ as $p = 0.006$. During the September 2018 sampling period, all *C. dentata* stem bark samples collected from KZN collectively constituted 468 chemical compounds [Figure 6.3; Table 6.1 (Appendix M)] whereas the number of compounds occurring in the stem bark decreased slightly to 427 chemical compounds in November 2018 [Figure 6.3; Table 6.2 (Appendix N)]. The differences in the numbers of chemical compounds, and thus the changes in chemical composition of all study trees in KZN between September 2018 and November 2018 are therefore not significant at $p < 0.05$ as $p = 0.271$. However, in January 2019, the total number of chemical compounds in the stem bark of all the *C. dentata* trees in KZN decreased sharply from 427 compounds in November 2018 [Figure 6.3; Table 6.2 (Appendix N)] to 349 compounds in January 2019 (Figure 6.3; Table 6.3 (Appendix O)]. Between November 2018 and January 2019, the differences in chemical composition are therefore significant at $p < 0.05$ as $p = 0.016$.

Of the 504 compounds in the stem bark of all *C. dentata* trees from the southern Cape and the 468 chemical compounds in the stem bark of all *C. dentata* trees in KZN during the month of September, 239 chemical compounds occur in trees at both research sites. Of the 378 compounds in the stem bark of all *C. dentata* trees from the southern Cape and the 427 chemical compounds in the stem bark of all *C. dentata* trees in KZN during the month of November, 198 chemical compounds occur in trees at both research sites. Of the 402 compounds in the stem bark of all *C. dentata* trees from the southern Cape and the 349 chemical compounds in the stem bark of all *C. dentata* trees in KZN during the month of January, 193 chemical compounds occur in trees at both research sites. The chemical compounds occurring in the stem bark of the *C. dentata* trees at both research sites at each sampling period, however, do not necessarily occur in all the *C. dentata* trees at a specific location. The chemical compounds not highlighted in Tables 6.1, 6.2 and 6.3 (Appendices L, M and N respectively) are therefore region-specific compounds for those particular months. The region-specific compounds may also not necessarily occur in all trees. Some compounds may thus be specific to certain trees. Important to note is that all Tables include contaminants originating from agricultural activities, vehicle emissions, plastics (litter), bacteria, and more, but again, they do not

necessarily occur in all trees. These contaminants were not removed from the Tables as the extracts of *C. dentata* stem bark used in traditional medicine are not standardized and beneficial chemical compounds are not isolated from the mixture, which therefore increases the possibility that these contaminants may enter the human body through consumption, and could lead to adverse effects.

Seasonal changes in environmental factors in a specific region, such as seasonal changes in soil nutrient and water availability, temperature, humidity, photoperiod, the quantity and quality of light and rainfall, and both the biotic and abiotic stress factors the trees are exposed to during a specific season, therefore not only affect the concentrations of chemical compounds in the stem bark of *C. dentata* trees, but also the chemical composition of the stem bark. Each individual tree's specific genetic factors may also play a role, while age differences between individual trees (Zobayed, *et al.*, 2005:245), and spatial differences in soil properties such as pH, organic matter, clay, silt and sand content and bulk density (Keshavarzi *et al.*, 2018:93-102), may contribute. Regional differences in the numbers of compounds, and therefore the chemical composition in the stem bark of *C. dentata* trees, may be due to regional differences in climatic patterns, soil properties, including soil type, organic matter content, bulk density and pH, differences in elevation, including the differences in environmental factors associated with altitude such as temperature and humidity, topography and the regional differences in biotic and abiotic stress factors the trees are exposed to at the time. For example: secondary metabolites produced as defence against insects and pathogens may either be broad-spectrum or insect-specific metabolites (Kortbeek *et al.*, 2019: 67). Appropriate defence responses therefore require initial recognition of the insect or pathogen feeding on, or attacking the plant. Plant hormone regulatory networks such as abscisic acid, auxin, brassinosteroids and gibberellins integrate different herbivore recognition cues, which in turn triggers the production of appropriate defence-related metabolites (Erb, Meldau and Howe, 2012:1-10). Plant defence metabolites produced may thus differ between individual plants, between the different months of the year (seasonally) and between regions, depending on the type and/or species of herbivorous feeder/ pathogen damaging the plant.

Two *C. dentata* trees approximately 100 m apart from one another in the southern Cape, were selected to demonstrate similarities and differences in chemical composition in individual trees occurring at the same location. The first *C. dentata* tree has a circumference of 89.2 cm (DBH = 28.38 cm) and the second *C. dentata* tree has a circumference of 70.4 cm (DBH = 22.4 cm). Age differences between the selected trees are therefore apparent. The first *C. dentata* tree from the southern Cape's stem bark constituted a total number of 133 chemical compounds, of which the chromatograms of 80 compounds had an 80% or higher similarity to the chemical compounds in

the main database of the mass spectrometer (Table 6.4). The second *C. dentata* tree from the southern Cape's stem bark constituted a total number of 120 chemical compounds, of which the chromatograms of 84 compounds had an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer (Table 6.5). Of these chemical compounds in the two trees, only 49 chemical compounds occurred in both trees (highlighted). Therefore, 31 chemical compounds were detected only in the stem bark of the first tree, and 35 chemical compounds were detected only in the stem bark of the second tree in the southern Cape (compounds not highlighted in Tables 6.4 and 6.5).

The two trees selected to demonstrate similarities and differences in chemical composition in individual trees occurring in KZN are approximately 50 m to 60 m apart. The first *C. dentata* tree has a circumference of 111.5 cm (DBH = 35.48 cm) and the second *C. dentata* tree has a circumference of 70 cm (DBH = 22.27 cm), which also indicate significant differences in tree ages. The first *C. dentata* tree from KZN's stem bark constituted a total number of 122 chemical compounds, of which the chromatograms of 58 compounds had an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer (Table 6.5). The second *C. dentata* tree from KZN's bark constituted a total number of 104 chemical compounds, of which the chromatograms of 79 compounds had an 80% or higher similarity to the chemical compounds in the main database of the mass spectrometer (Table 6.7). Of these chemical compounds in the two trees, only 30 chemical compounds were detected in both trees (highlighted). Therefore, 28 chemical compounds were detected only in the stem bark of the first tree, and 49 chemical compounds were detected only in the stem bark of the second tree in KZN (compounds not highlighted in Tables 6.6 and 6.7).

The differences in chemical composition of individual trees therefore demonstrate that the chemical compounds extracted from the stem bark of *C. dentata* trees may not be the same for each preparation when traditional health practitioners prepare medicines to treat patients. Of the chemical compounds occurring in *C. dentata* trees at both locations, only five compounds occur in all trees at both sites and at each sampling date, i.e. 1,3-bis(1,1-dimethylethyl)benzene, eicosane, 2,6,10,14-tetramethylheptadecane, nonadecane and pentadecane. According to the HMDB and PubChem, 1,3-bis(1,1dimethyethyl)benzene is an organic compound with the chemical formula $C_{14}H_{22}$ and a member of the phenylpropanes. Additional information on the compound is extremely limited. Eicosane ($C_{20}H_{42}$) is an acyclic alkane identified as a chemical constituent of *Agave attenuata* leaves (Rizwan, Zubair, Rasool, Riaz, Zia-Ul-Haq and De Feo, 2012:6442) and a volatile component. Eicosane is also produced by endophytic fungi isolated from the bark of *Ficus religiosa*

(Sundaramoorthy, Kalyanaraman, Vaithianathan, Kumar, Meenambiga, Meenashree and Arulmathi, 2018:5297). Sundaramoorthy *et al.* (2018:5297, 5302) also stated that eicosane is a bioactive secondary compound with possible activity against pathogenic microbes, however, according to the ECHA, eicosane poses a health risk as it may be fatal when swallowed. The compound 2,6,10,14-tetramethylheptadecane ($C_{21}H_{44}$) is a bioactive component also found in *Petiveria alliacea* (Sathiabalan *et al.*, 2014:587-592) and both eicosane and 2,6,10,14-tetramethyl heptadecane are found in *Polygala furcata* (Yang, Mao, Long, Sun and Guo, 2010:781, 782). PubChem noted 2,6,10,14-tetramethylheptadecane as a metabolite observed in cancer metabolism and is noted as having a role as a human metabolite. This component may, however, also be a bacterial metabolite (Vella and Holzer, 1992:209; Wang *et al*., 2015:10). Nonadecane ($C_{19}H_{40}$) is a straight chain alkane and an aliphatic hydrocarbon. According to PubChem, it is a component of essential oil from *Artemisia armeniaca*. It is a plant metabolite and a volatile component. Amiri, Yadegari and Hamedi (2018:251-262) found high levels of Nonadecane in the essential oils of *Cirsium arvense*. As discussed in Chapter 3, essential oils are known for their promising therapeutic properties. Pentadecane has the chemical formula $C_{15}H_{32}$ and is a hydrocarbon. According to PubChem, it has a role as an animal metabolite and a plant metabolite. It is also a volatile component. Rao, Rajesekharan, Roy and Kumar (2009:1) lists pentadecane as a component of essential oil from *Kaempferia galanga*. However, the ECHA classified pentadecane as a health hazard as it may be fatal if swallowed or if it enters airways.

Known components of essential oils, such as α-Pinene, were detected in *C. dentata* trees. The compound, however, also occur in rosemary, coriander, cumin, cinnamon, black pepper and citrus spp. (Salehi, Upadhaya, Orhan, Jugran, Jayaweera, Dias, Sharopov, Taheri, Martins, Baghalpour, Cho and Sharifi-Rad, (2019:2). This monoterpene has a wide range of pharmacological potentials, which include anti-tumour, anti-microbial, anti-malarial, antioxidant, anti-inflammatory, anti-*Leishmania* and analgesic effects (Salehi *et al.*, 2019:1-34). However, in September 2015, α-Pinene was detected in only seven of the 12 trees in the southern Cape forest area. In November 2015, α-Pinene was detected in only one tree, and in January 2016, no tree in the southern Cape had α-Pinene as a constituent in the stem bark extracts. Of the 12 *C. dentata* trees in the Nkandla forest, two trees had α-Pinene as a constituent in September 2018, three trees contained α-Pinene in November 2018, and two trees had α-Pinene as a constituent in January 2019. Other components with therapeutic potentials are also present in the stem bark of *C. dentata*, which include the sesquiterpenoids α-Copaene, α-Cubebene, α-Muurolene and cis-Calamenene and the

monoterpenoid ç-terpenine. As with α- Pinene, these components do also not occur in all *C. dentata* trees, nor do they occur in the stem bark throughout the year.

In nature, several factors hinder a homogenous and continuous production of secondary metabolites. These factors include physiological variations such as organ development, pollinator activity cycles, type of plant material, type of secretory structure and mechanical or chemical injuries, the environmental factors already mentioned, geographic variation and also pollution (Figueiredo, Barroso, Pedro1 and Scheffer, 2008:214), however, chemotypes is a factor often overlooked. A chemotype is a chemically distinct entity in a plant, with differences in the secondary metabolite composition. Chemotypes are often defined by a single dominant chemical an individual plant produces (Keefover-Ring, Thompson and Linhart, 2008:117). The popular herb, thyme (*Thymus vulgaris*) for example, has nine subspecies and varieties and as many as 13 chemotypes based on the predominance of certain monoterpenoids in the essential oils (Satyal, Murray, McFeeters and Setzer, 2016:1). These dominant chemicals can be used to delineate chemotypes according to geographically distinct locations (Satyal *et al*., 2016:1-12), for example: Spanish thyme represent the 1,8 cineole chemotype, French thyme represent the linalool chemotype and the geraniol chemotype is characteristic of thyme from Serbia (Wesolowska and Jadczak, 2019). Chemotypes for *C. dentata* have not been determined before. *Curtisia dentata* variants or chemotypes can, however, be determined by examining the volatile constituents of leaves or stem bark. Chemotype determination for *C. dentata* was not the goal of this study, but could be researched in future for better management of genetic resources of popular and overexploited medicinal plant species such as *C. dentata*, and to determine, amongst others, the most appropriate variety for propagation and cultivation, particularly since natural germination of *C. dentata* seeds is extremely poor (Shaik, 2012:449). Bioassays, or biological assays are employed to determine the effect of a substance on certain types of living matter. In a biological test system, tissue, cells etc. (or live animals) are exposed to a particular stimulus, such as a drug, extract or compound, of which the concentration (dose) is usually varied (Rosso, 2010:7). Bioassays are generally comparative, where the capacity of a substance to cause a specific effect is estimated relative to a standard. Since a response is dependent on living matter, this introduces large variability between measurements obtained by identical operations. Additionally, there is variability due to experimental factors such as variation in sample preparations, technicians, equipment, batches, etc. (Rosso, 2010:11). Important to note is that there is no bioassay linked to the activity or use of a plant species, for example *C. dentata* trees, and therefore selection for cultivation based on activity is also not clearly defined.

Of concern is that the medicinal preparations from *C. dentata* stem bark may contain pesticides such as binapacryl and chlorfenapyr, which are present in Tables 6.1, 6.2 and 6.3 (Appendices N, O and P respectively), and are environmental pollutants deriving from agricultural activities. These pesticides may have been taken up by the roots of the trees and are highly toxic to humans (Zaharia, Tudorachi, Pintilie, Drochioi, Gradiaru and Murariu, 2016:120-130; Periasamy, Deng and Liu, 2017:833-835). They may thus cause adverse effects when consuming medicines prepared from raw or semi-processed stem bark containing these substances. Equally concerning is the presence of the polycyclic aromatic hydrocarbons (PAHs) Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benz[a]anthracene and Chrysene in one of the two individual *C. dentata* trees selected from KZN to demonstrate similarities and differences in chemical composition. The origins of the PAH's may be pyrogenic, petrogenic or biological. Pyrogenic sources of PAHs are by means of incomplete combustion of organic substances such as coal, wood, and petroleum. Petrogenic sources of PAHs are petroleum products such as kerosene, diesel fuel, gasoline, lubricating oil, and asphalt. Biologically, PAHs can be formed by the degradation of vegetative matter. Forest fires, volcanoes, algal synthesis, and petroleum seeps are natural sources of PAHs while anthropogenic sources of PAHs include automotive emissions, petroleum product spills, incineration, cigarette smoke, and sewage sludge (Obanya, Omoarukhe, Amaeze and Okoroafor, 2019: 1). The Agency for Toxic Substances and Disease Registry (ATSDR) stated that some PAHs are both carcinogenic and mutagenic, and may also cause birth defects (ATSDR, 1995:4). Factors such as dose, duration and the route of exposure (breathing, eating, drinking, or skin contact), other chemicals people are exposed to, and an individual person's characteristics such as age, sex, nutritional status, family traits, lifestyle, and state of health will determine whether harmful health effects will occur and what the type and severity of those health effects will be (ATSDR, 1995:1).

There is no manner in which either a traditional health practitioner or a commercial harvester can know before harvesting that a specific tree contains pesticides and/or PAHs, or any other contaminant. Diseases and complications that develop over longer periods of time such as cancer, liver and kidney damage, reproductive dysfunctions and birth defects may thus not be linked to the use of traditional medicines (Moreira *et al.*, 2014:250), whereas in fact, chemical compounds such as these pesticides and PAHs may be the major causes of these diseases (ATSDR, 1995:4). However, pesticides and PAHs may not be the only sources of carcinogenic, mutagenic or toxic substances in trees. Trees may also biosynthesize compounds with human carcinogenic, mutagenic or toxic potentials. A tree produces chemical compounds, not for the benefit of mankind, but to aid in metabolic processes and decomposition, to lure pollinators, and particularly for the protection of

the tree against biotic and/or abiotic stresses (Herms and Mattson, 1992:288). The isolation or synthesis of beneficial chemical compounds and the standardization of traditional plant-based medicines are therefore matters that require serious consideration. However, using current wild *C. dentata* populations for these activities will not be sustainable as overharvesting of *C. dentata* stem bark already rendered the species vulnerable to extinction. The cultivation of *C. dentata* trees should therefore become a priority undertaking in South Africa.

6.5 CONCLUSION

In this Chapter, it was determined that the chemical composition of *C. dentata* tree stem bark vary between individual trees, seasonally and between regions due to intrinsic and extrinsic factors. Various known beneficial chemical compounds do occur in *C. dentata* stem bark, however, not necessarily in every tree. Beneficial chemical compounds also do not necessarily occur in the stem bark of *C. dentata* trees throughout the year. *Curtisia dentata* stem bark further contain chemical compounds that may affect human health over time due to bioaccumulation. Some wild growing *C. dentata* trees' stem bark may also contain contaminants such as pesticides deriving from agricultural activities, and PAHs which may derive from pyrogenic, petrogenic and/or biological sources. It is generally expected that all plants of a particular species used medicinally should contain the same beneficial chemical compounds. This study has, however, shown that only five chemical compounds occur in all 12 *C. dentata* trees at both the research sites at all the sampling dates, i.e. Benzene, 1,3-bis(1,1-dimethylethyl)-, Eicosane, Heptadecane, 2,6,10,14-tetramethyl-, Nonadecane and Pentadecane. Two of these compounds, i.e. eicosane and pentadecane, are acute toxic and may be fatal if swallowed and the compound 2,6,10,14-tetramethylheptadecane may derive from bacteria. Furthermore, the chemical compounds isolated from the leaves and the majority of compounds isolated from the stem bark in Chapter 4 (with the exception of n-tetracosanol, which is a derivative of tetracosane) were not detected in the DCM extracts of the stem bark samples collected from *C. dentata* trees from the southern Cape and KZN. These chemical components are therefore only be detected when using chromatographic separation and isolation techniques.

The chemical content of the medicinal formulations traditional health practitioners prepare from raw or semi-processed *C. dentata* stem bark may therefore also vary with each preparation and may contain chemical compounds with the potential to cause adverse effects in humans. It is impossible to know the chemical content of a tree before harvesting the bark, which emphasizes the need for the isolation or synthesis of beneficial compounds and the standardization of traditional plant-based

medicines. However, the standardization of traditional plant-based medicines from *C. dentata* trees require the cultivation of *C. dentata* trees, as the overharvesting of *C. dentata* stem bark resulted in a “Near Threatened” conservation status. The remaining wild stocks will therefore not support such activities sustainably.

CHAPTER 7. AMINO ACID DISTRIBUTION PROFILES OF INDIVIDUAL AND GEOGRAPHICALLY SEPARATED *Curtisia dentata* (Burm.f.) C.A.Sm. TREES

7.1 INTRODUCTION

Amino acids are essential units responsible for the formation of protein molecules and are organic carbon acids made up of the amine (NH_2) and carboxyl groups (COOH) with an additional alkyl group, known as a side chain, which is specific to each amino acid (Baqir, Zeboon and Al-behadili, 2019:1402). Amino acids are produced through the assimilation of ammonia, transamination, chemical transformations of acid amides or other N compounds and the hydrolysis of proteins by enzymes (Pallarday, 2008:240). Twenty amino acids are directly involved in protein structure, however, there are also numerous non-protein amino acids, or free amino acids, that do not play a role in protein structure (Vranova, Rejsek, Skene and Formanek, 2011:31). Amino acids have various metabolic roles in plants and are also necessary for the transport and storage of nutrients (Kumar, Sharma, Kaur, Thukral, Bhardwaj and Ahmad, 2017:2; Baqir *et al.*, 2019:1402-1410). A few amino acids are precursors to other amino acids, and others are essential for secondary plant metabolism and the biosynthesis of secondary metabolites such as phenolic compounds, glucosinolates and alkaloids which have roles in plant-environment interactions relating to plant stresses (Rai, 2002:481-487), including plant defence (Kumar *et al.*, 2017:2).

Kumar *et al.* (2017:1-49) determined that amino acids are differentially distributed in different species of plants. However, no consideration was given to variations in distribution between individual plants of the same species, or to seasonal and regional variations of amino acid distribution in plants of the same species. Since it was determined in Chapter 6, that secondary metabolites in the stem bark of *C. dentata* trees vary seasonally, regionally, as well between individual trees, the amino acid profiles in *C. dentata* trees from KZN and the southern Cape were also analysed and compared.

7.2 METHODS AND MATERIALS

7.2.1 Plant material used and extraction of compounds

The plant material collected and used for NMR and GC-MS analysis in Chapters 5 and 6 were also used for the analysis of the amino acids in *C. dentata* stem bark. The samples collected during the months of September and November (2015 for the southern Cape and 2018 for KZN) were used for

LC-MS analysis. The aim was to determine seasonal and regional differences in amino acid content and distribution, and also differences in individual trees. Of the dried and homogenized plant material of each sampling period, 200 mg material from each sample was weighed (Shimadzu ATX 124 scale) and placed into separate Eppendorff tubes (2 x 12 samples). Into each tube, 3 ml analytical grade methanol was added (Sigma-Aldrich), after which all samples were vortexed, sonicated in an ultrasound bath for 20 minutes, and centrifuged for 15 minutes. Of the supernatant in each tube, 1.5 ml was extracted using syringes and filtered through Whatman 13mm filters with a pore size of 0.2 µm and poured into individual Restek GC/LC analytical bottles.

7.2.2 LC-MS analysis of samples

HPLC was carried out using a Shimadzu 8040 system with a column oven. For chromatographic separation a Discovery HS F5 Supelguard column (2.0 x4 mm, 3 µm particle and an analytical column Discovery HS F5 (2.1 x150 mm, particle size 3µm) were used (Sigma Aldrich). Five microliters of each sample was injected into the system for LC-MS analysis. Degassed solutions of formic acid:ultrapure water (1:103 , v/v; eluent A) and formic acid:acetonitrile (1:103 , v/v; eluent B) were pumped at 0.19 mL min^{-1} into the HPLC system. The gradient applied started at 5% B and increased linearly to 95 % B in 20 min. Then, for 15 min the column was washed and equilibrated before the next injection. The column temperature was kept at 35° C and the samples at 20° C. The room temperature was maintained at 20°C. For MS detection, ESI conditions were set as follows: capillary voltage: 2.5 kV, sample cone voltage: 17 V, MCP detector voltage: 1600 V, source temperature: 120º C, desolvation temperature: 250º C, cone gas flow: 1.5 L/h, desolvation gas flow: 2.5 L/min, m/z range: 100–1000, scan time: 0.1 s, interscan delay: 0.02 s, mode: centroid, lockmass: leucine enkephalin (556.3 l g/mL), lockmass flow rate: 0.4 mL/min, mass accuracy window: 0.5 Da. Mass accuracy of all the m/z values in all the acquired spectra were automatically corrected during acquisition based on calibration curves, lockmass and dynamic range extended. The MS detector was set to collect both negative (ESI-) and positive (ESI+) ions.

7.3 RESULTS

Both protein and non-protein, or free amino acids, were considered in this study as up to 250 known free amino acids occur in plants (Vranova *et al.*, 2011:31). Therefore, of the 72 amino acids occurring in the stem bark samples of *C. dentata* trees, only 24 amino acids occurred in the stem bark samples of all 12 the *C. dentata* trees in KZN as well as all 12 the *C. dentata* trees in the southern Cape during September, and 19 amino acids occurred in the stem bark samples of all 12

the *C. dentata* trees in KZN as well as in all 12 the *C. dentata* trees in the southern Cape during November. The remaining amino acids did not occur in all *C. dentata* trees but were limited to certain trees at each research site at each of the harvesting dates. The mean percentage areas of amino acids occurring in the stem bark of all *C. dentata* trees from both regions during the September and November sampling dates are shown in Figures 7.1A and B.

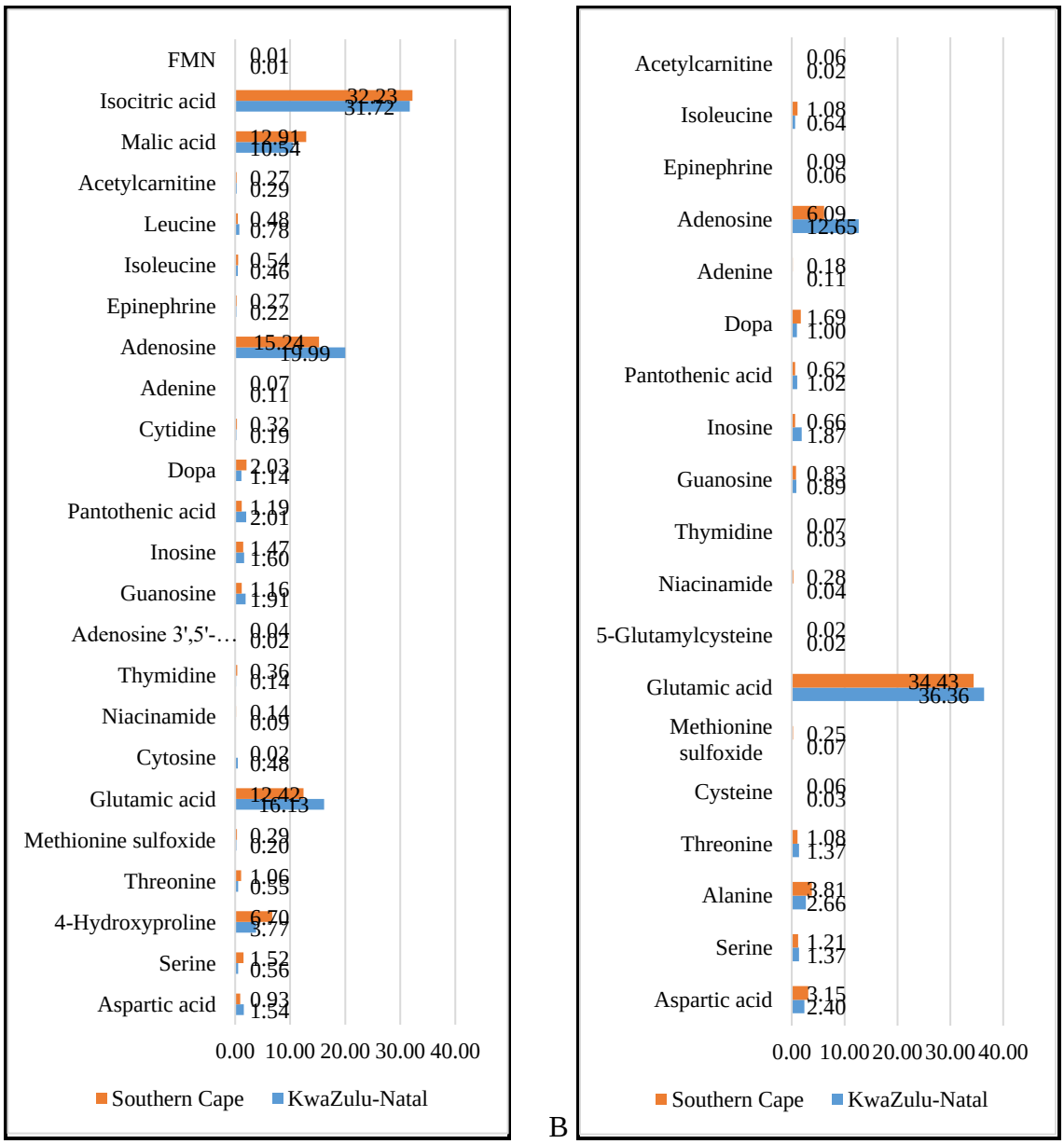
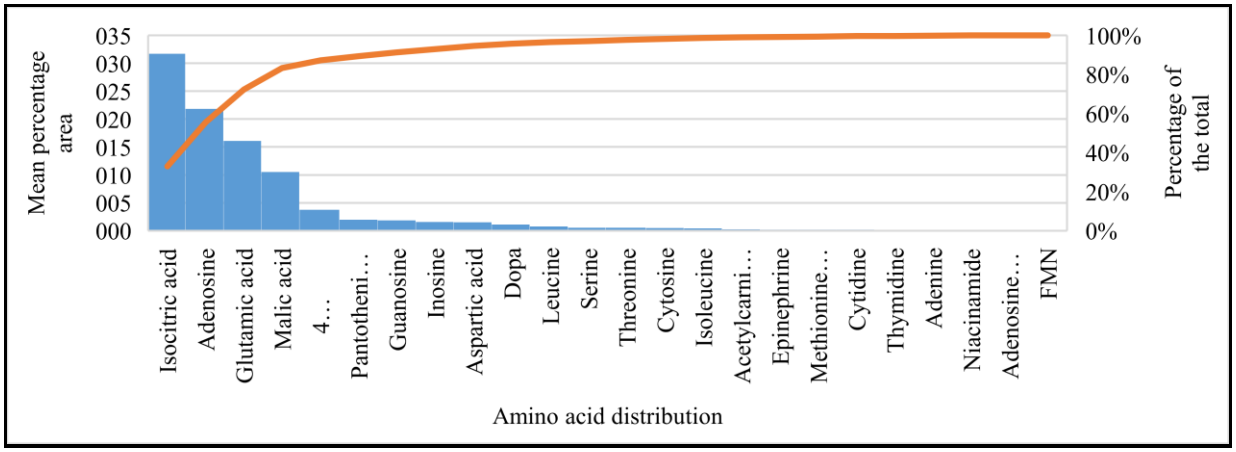


Figure 7.1: The profiles of the amino acids occurring in the stem bark samples of all *C. dentata* trees during (A) September and (B) November. Note that 24 amino acids occur in the stem bark of all *C. dentata* trees in September, but that the number of amino acids occurring in the stem bark of all *C. dentata* trees decreased to 19 in November.

In the pareto charts in Figures 7.2A and B and Figures 7.3A and B, a cumulative line which represents a percentage of the total, occurs on a secondary axis. Figure 7.2A therefore shows the 24

amino acids present in the stem bark samples of all *C. dentata* trees (all 12 trees from the southern Cape and all 12 trees from KZN), and its mean percentage areas and distribution in descending order in the stem bark samples of the *C. dentata* trees in KZN during September 2018, whereas Figure 7.2B shows the 19 amino acids in the stem bark of all *C. dentata* trees and its mean percentage areas and distribution in descending order in the stem bark samples of the *C. dentata* trees in KZN during November 2018. Figure 7.3A shows the 24 amino acids in the stem bark of all the *C. dentata* trees and its mean percentage areas and distribution in descending order in the stem bark samples of the *C. dentata* trees in the southern Cape during September 2015, whereas Figure 7.3B shows the 19 amino acids in the stem bark of all the *C. dentata* trees and its mean percentage areas and distribution in descending order in the stem bark samples of the *C. dentata* trees in the southern Cape during November 2015.

A



B

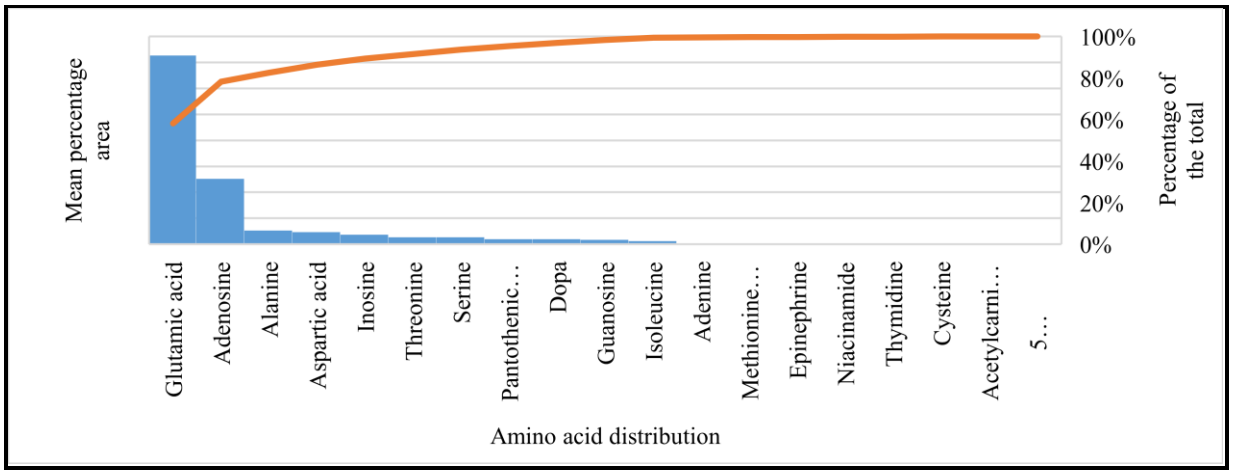
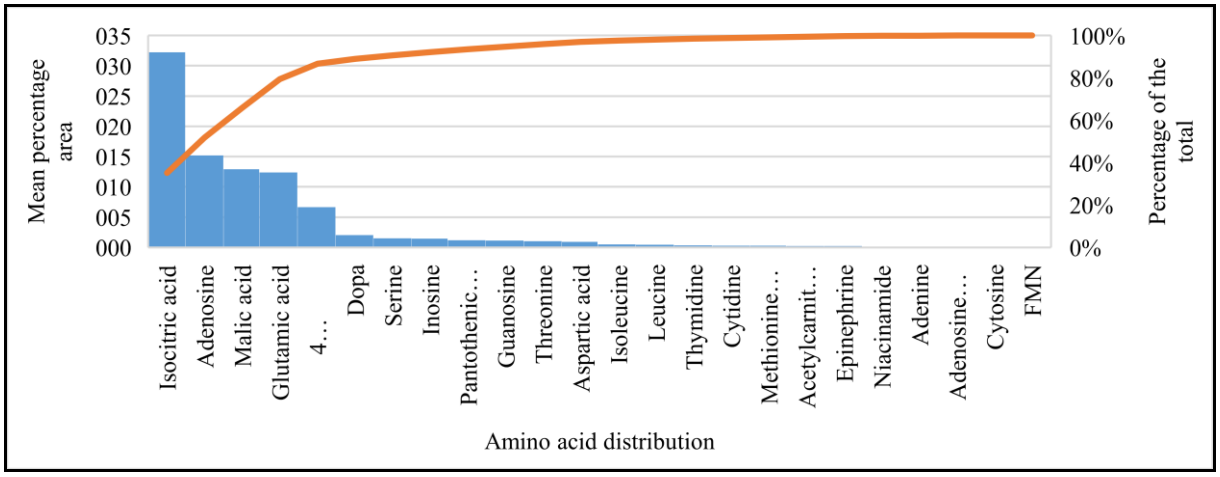


Figure 7.2: The amino acids occurring in the stem bark samples of all *C. dentata* trees during (A) September and (B) November, their mean percentage areas and their distribution in descending order in the stem bark samples of the *C. dentata* trees in KZN.

A



B

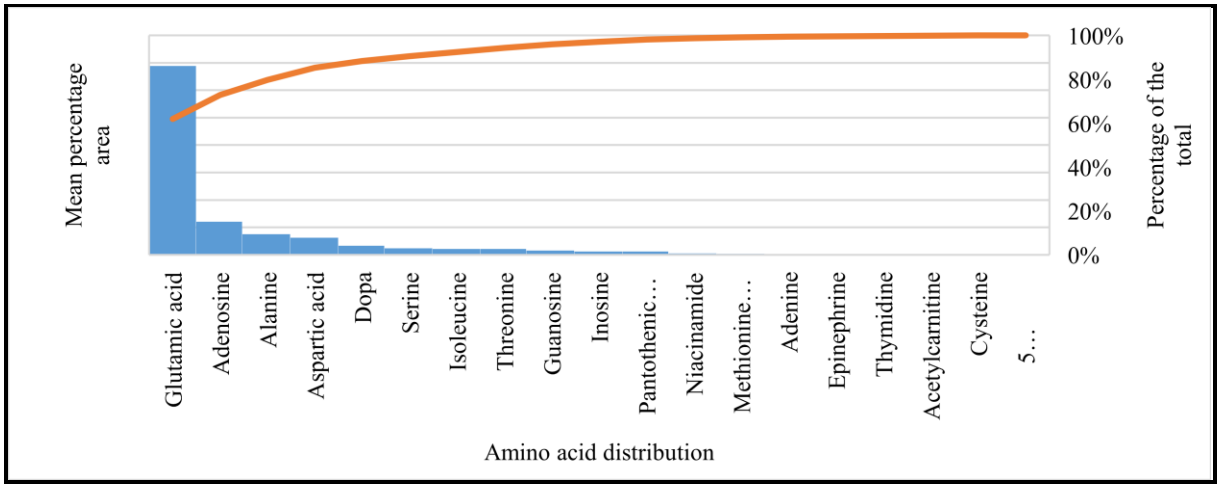
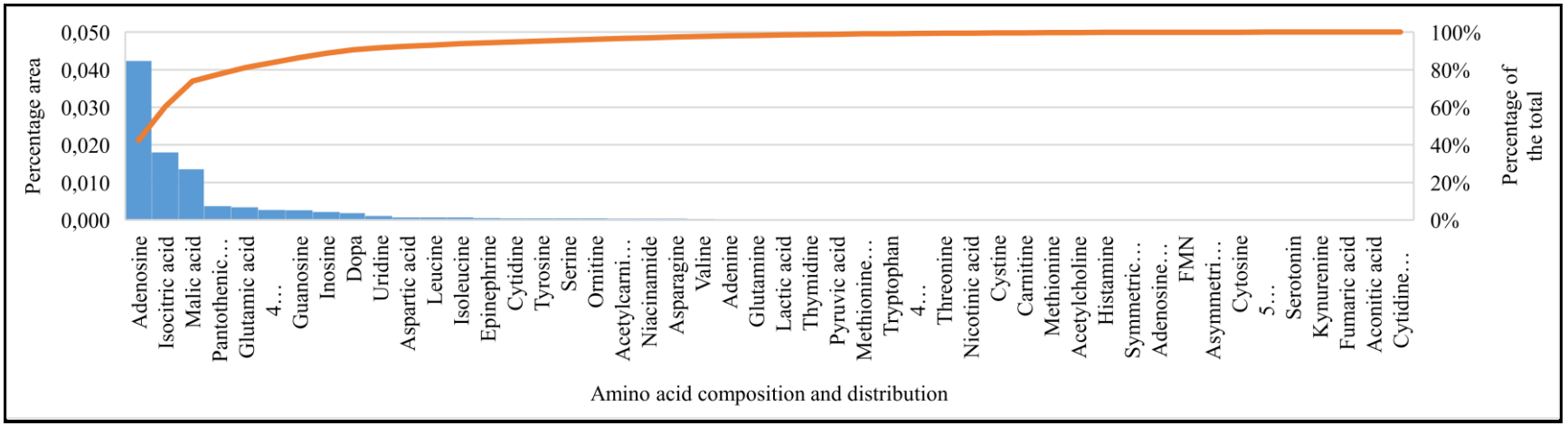


Figure 7.3: The amino acids occurring in the stem bark samples of all *C. dentata* trees during (A) September and (B) November, their mean percentage areas and their distribution in descending order in the stem bark samples of the trees in the Southern Cape.

Figures 7.4A and B show the distribution profiles of amino acids of the stem bark of two individual *C. dentata* trees in KZN during September 2018, whereas Figures 7.5A and B exhibit the distribution profiles of amino acids of the stem bark of the same two individual *C. dentata* trees in KZN during November 2018. Figures 7.6A and B show the distribution profiles of amino acids of the stem bark of two individual *C. dentata* trees in the southern Cape during September 2015 and Figures 7.7A and B show the distribution profiles of amino acids of the stem bark of the same two individual *C. dentata* trees in the southern Cape during November 2015.

A



B

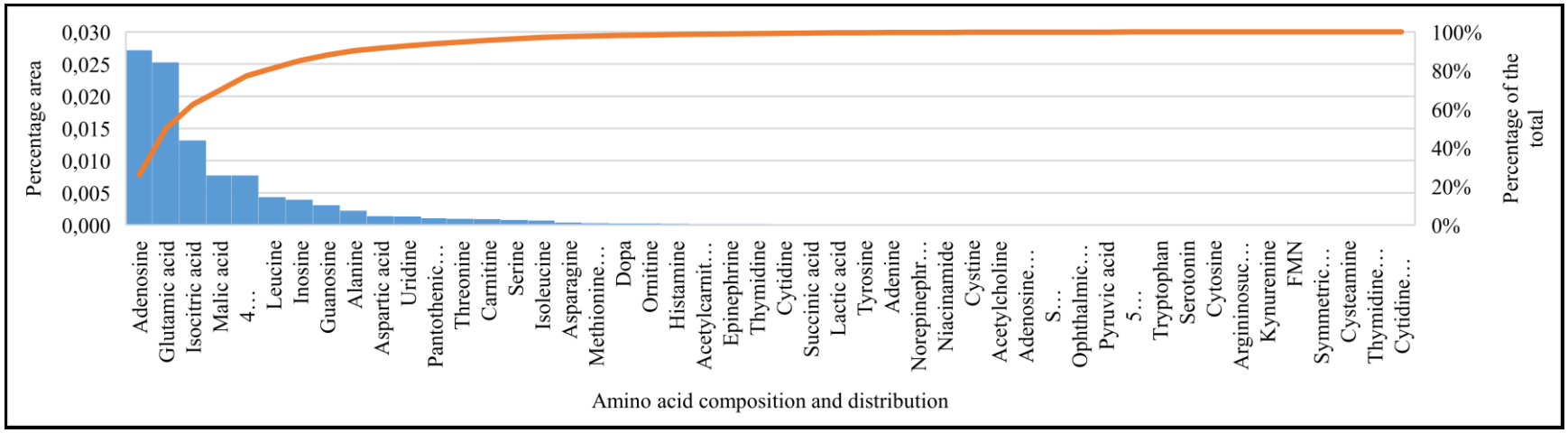
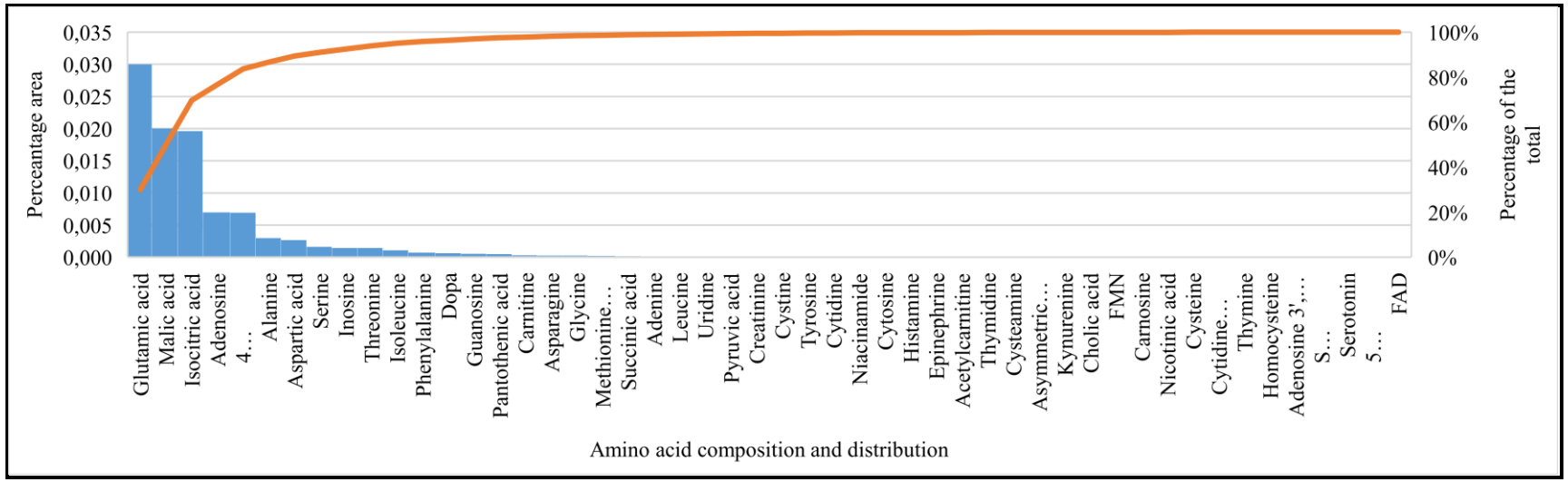


Figure 7.4: The distribution profiles of amino acids of the stem bark of two individual *C. dentata* trees in KZN during September 2018.

A



B

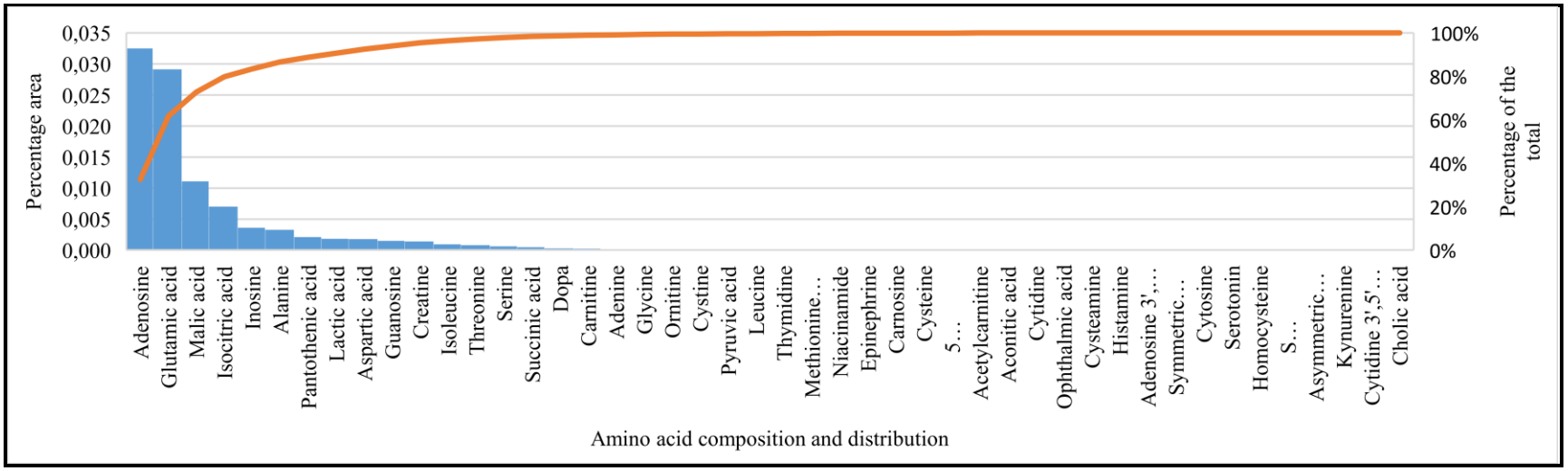
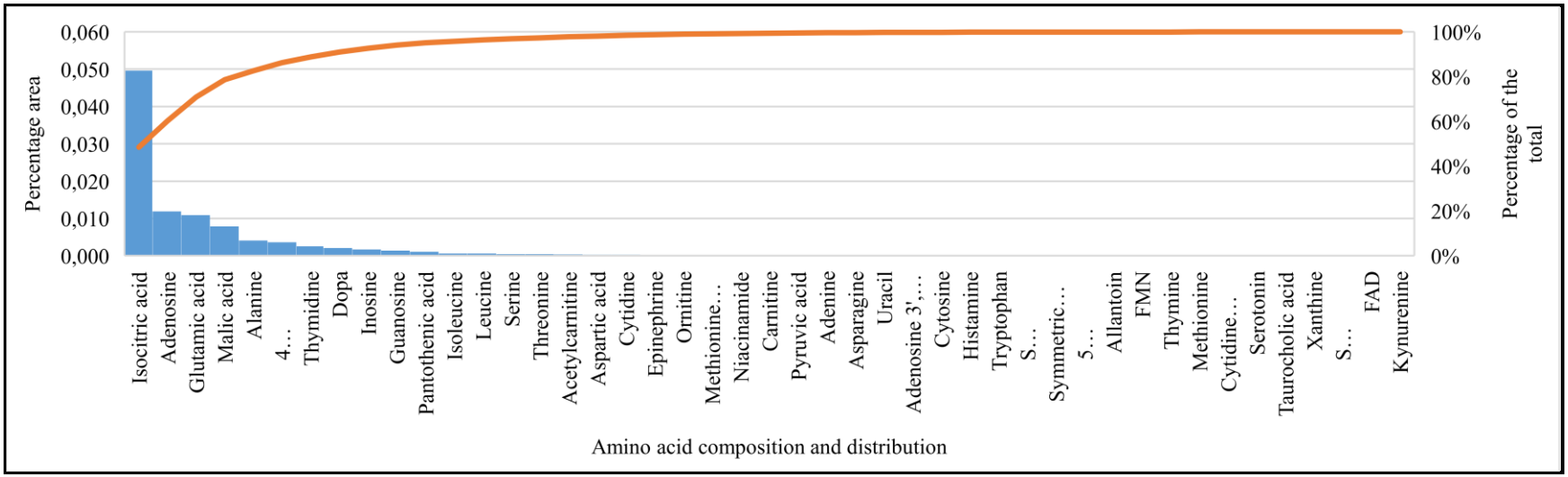


Figure 7.5: The distribution profiles of amino acids of the stem bark of the same two individual *C. dentata* trees in KZN during November 2018.

A



B

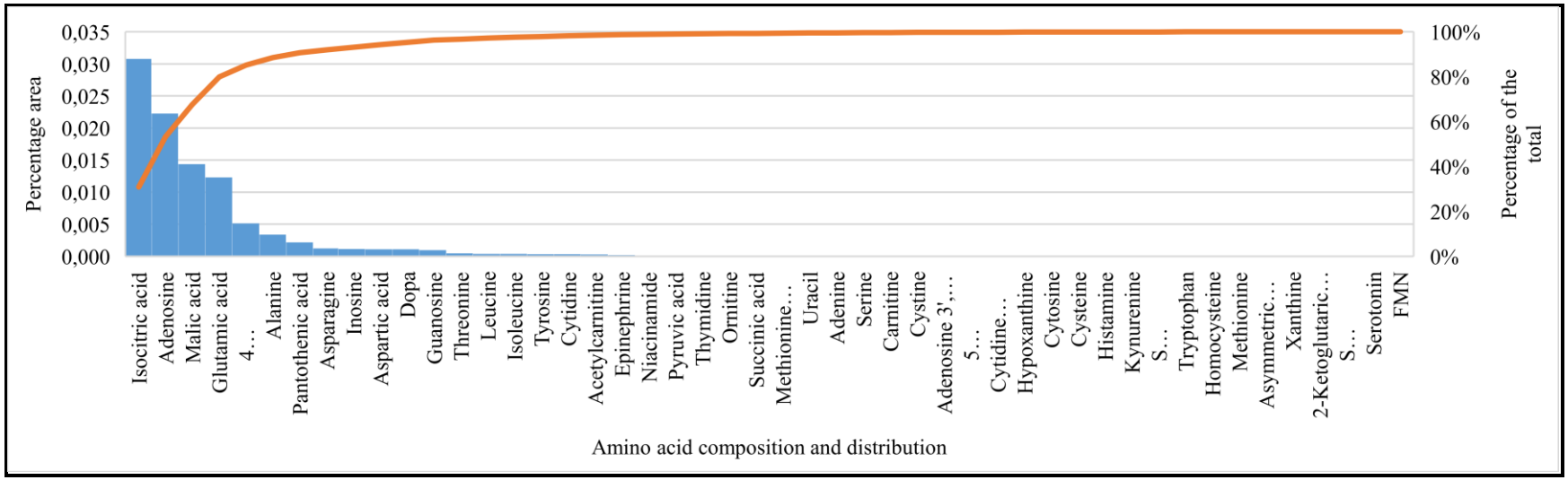
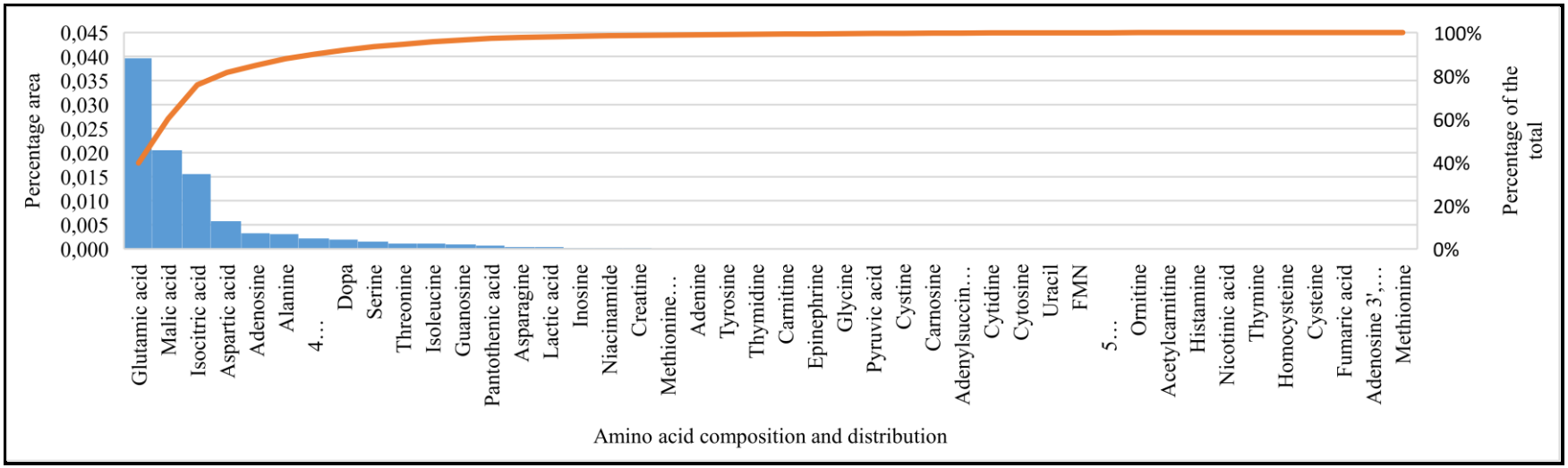


Figure 7.6: The distribution profiles of amino acids of the stem bark of two individual *C. dentata* trees in the southern Cape during September 2015.

A



B

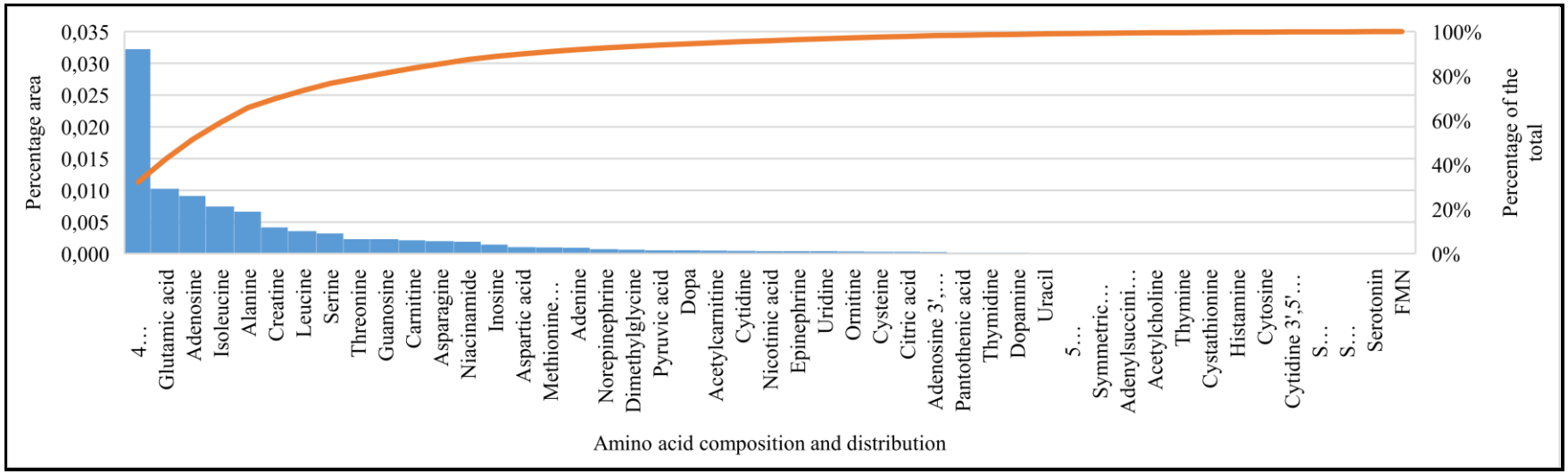


Figure 7.7: The distribution profiles of amino acids of the stem bark of the same two individual *C. dentata* trees in the southern Cape during November 2015.

7.4 DISCUSSION

The amino acid profiles of *C. dentata* stem bark in Figure 7.1A and B indicate that amino acids are also biosynthesized in plants according to the needs of the plants in its environment. As amino acids are also essential for secondary plant metabolism and the biosynthesis of secondary metabolites (Rai, 2002:481-487), plant stresses such as water stress and N stress, and a plant species' tolerance levels to a particular environmental stress factor may thus influence amino acid biosynthesis (Pallarday, 2008:245). Seasonal, and to a lesser extent regional differences in amino acid biosynthesis are evident from Figures 7.1A and B, which may be due to the same seasonal and regional differences in environmental factors responsible for variations in secondary metabolite production as discussed in Chapters 5 and 6. It is noteworthy that the major amino acids are remarkably similar in the different regions when the months of harvest are compared, even though sampled three years apart. In September isocitric acid, with adenosine, glutamic acid and malic acid are the main amino acids for both regions and similarly, glutamic acid and adenosine are the major amino acids for both regions in November. The concentration differences between the regions is however, still observed when for instance adenosine is compared between samples for the two regions.

Of the 72 amino acids detected in *C. dentata* stem bark, only 24 amino acids occur in all 12 the *C. dentata* trees from KZN (Figure 7.2A), as well as in all 12 the *C. dentata* trees from the southern Cape (Figure 7.3A) during September. In November, the number of amino acids that occurred in the stem bark of all *C. dentata* trees from both research sites decreased to 19 amino acids (Figure 7.2B and 7.3B), which undoubtedly reflects differences in plant needs according to both season and region. Also noticeable from these figures is that seasonal and regional variations include variations in distribution and therefore concentrations. Once again, the similarity on the major amino acid profiles were observed with isocitric acid, glutamic acid, malic acid and 4-hydroxyproline being the major amino acids for the two regions in September. When comparing the profiles for November of the two regions, glutamic acid, adenosine, aspartic acid and alanine were the major amino acids, indicating the change in amino acid profile from September to November, although similar changes were observed in both regions.

The remaining 47 amino acids (out of 72 amino acids) detected in the stem bark of *C. dentata* trees in September were not detected in all trees. For example, Cystine was detected in the stem bark of nine of the 12 *C. dentata* trees in KZN in September, whereas the stem bark of

only six of the 12 *C. dentata* trees in the southern Cape contained Cystine in September. In November, Cystine was present in the stem bark of eight *C. dentata* trees in KZN and in the stem bark of 10 *C. dentata* trees in the southern Cape. Cystine ($C_6H_{12}N_2O_4S_2$) is formed by the oxidation of two Cysteine ($C_3H_7NO_2S$) molecules linked via a disulphide bond between the -SH groups (HMDB). Interesting to note is that Cystine occurs in the stem bark of nine *C. dentata* trees in KZN in September, however, only five trees in KZN contain Cysteine, and in only four of these trees, both Cystine and Cysteine occur in the same tree. Similarly, Cystine occurs in the stem bark of six *C. dentata* trees in the southern Cape in September, however, five trees in the southern Cape contain Cysteine, and in only three of these trees, both Cystine and Cysteine occur in the same tree. In November, Cystine was present in the stem bark of eight *C. dentata* trees in KZN and in the stem bark of 10 *C. dentata* trees in the southern Cape, however, all trees from both sites contained Cysteine. Furthermore, sampling from the two areas were executed three years apart (2015 in the southern Cape and 2018 in KZN), therefore, in addition to seasonal and regional differences in environmental factors, environmental factors may also differ on a year-to year basis during the same month (season). No clear correlation between *C. dentata* tree age and amino acid content could be found, which at first suggests that seasonal and regional variations in environmental factors are more important factors for amino acid production than plant age. Nitrogen-containing amino acid biosynthesis for example, may depend on the distribution and seasonal fluctuations of N in the trees. Nitrogen concentrations in plant tissues tend to increase during autumn and winter, decrease when growth begins in spring, and increase again as growth slows towards dormancy (Pallarday 2008:235). The amount of N present in trees vary with tissue (leaves, bark, sapwood, heartwood etc), plant age, stage of plant development (spring flush, flowering, seeding etc.) and season (Pallarday, 2008:234).

The amino acid distribution profiles of two individual trees in KZN (Figures 7.4A and B and Figures 7.5A and B) and of two individual trees in the southern Cape (Figures 7.6A and B and Figures 7.7A and B) depict the differences in distribution, composition and concentrations during the months of September and November respectively, but also show seasonal and regional differences. Apart from the differences in seasonal and regional environmental factors the *C. dentata* trees in each of the two research sites are exposed to, spatial differences in chemical and physical soil properties such as pH, organic matter, nutrient content, clay, silt and sand content and bulk density (Keshavarzi *et al.*, 2018:93-102) may contribute toward the differences in distribution, composition and concentration in

individual trees, particularly since the *C. dentata* trees in each location are not situated far from one another. However, genetic diversity also needs to be considered.

The most prominent amino acids in the stem bark samples of *C. dentata* trees are Isocitric acid, Adenosine, Glutamic acid, Malic acid, Alanine, Aspartic acid, Dopa, 4-Hydroxyproline, and Serine, even though some of these amino acids, e.g. Alanine, Isocitric acid and Malic acid were not detected in the stem bark of all *C. dentata* trees at both research sites and at each sampling date. The prominence of these nine amino acids is reflected by their high mean percentage areas in the stem bark of *C. dentata* trees, and their mean percentage area covering approximately 80 % of the total area. The remaining 63 amino acids therefore cover the remaining 20 % of the total area, which means that they only occur in trace amounts in *C. dentata* stem bark, and often also not in all trees. For this reason, the *C. dentata* stem bark also show very similar amino acid profiles, both seasonally and regionally (Figures 7.2 and 7.3), and between individual trees (Figures 7.4 to 7.7), even though stem bark samples from *C. dentata* trees in the two regions were harvested three years apart.

Plants have to deal with climatic factors and develop strategies to adapt their metabolism in order to acclimate and survive under unfavourable growth conditions. Mechanisms of plant stress tolerance involve some changes in gene expression, protein modification and metabolic pathways. More particularly, plant amino acid composition is modified by environmental conditions and characterized by an elevated accumulation of specific amino acids involved in plant stress tolerance (Ali, Athar, Haider, Shahid, Aslam, Shehzad, Naseem, Ashraf, Ali and Hussain, 2019:175-203). Amino acids are synthesized by various metabolic networks and accumulate differentially in plant species exposed to various stresses. In response to these environmental stresses, amino acid metabolism plays an important regulatory role, not only because amino acids are constituents of proteins, but also because free amino acids are potential regulatory and signalling molecules and precursors for energy-associated metabolites as well numerous secondary metabolites that have several functions in plant growth and adaptive responses to various stresses (Okumoto, Funck, Trovato and Forlani, 2016:1).

Amino acids may have very specific roles in plants, but the possibility of two or more amino acids acting synergistically to perform one function may also be true. For example, Glycine plays a role in pollination, Alanine affects growth velocity, Arginine enhances root formation and cell division and Serine plays a role in hormone balance in plants, however, all four these amino acids also play a role in chlorophyll formation, which is vital for photosynthesis (Baqir

et al., 2019:1403, 1404). One amino acid may thus also have two or more functions in plants; or they may be complimentary, for example: both Cysteine and Aspartic acid increase plant resistance to diseases while Threonine, Serine and Tyrosine increase plant tolerance to disease. Another example of possible synergistic actions are the amino acids Arginine, Hydroxyproline and Proline, which all play a role in heat, cold, drought and/or salinity stress in plants (Baqir *et al.*, 2019:1403-1405). Glutamic acid, one of the major amino acids in *C. dentata* stem bark, play a role in plant growth (Baqir *et al.*, 2019:1404). Isocitric acid is a structural isomer of Citric acid, which is at the center of cell metabolism as it plays a prominent role in the Citric acid cycle, better known as the Krebs cycle in cell respiration (Wang, Cui, Zhao and He, 2017:25-29). The backbones of several other amino acids enter the Krebs cycle and form Glucogenic intermediates and Ketogenic intermediates which involve several processes and enzymes, and which ultimately produces energy and maintains nitrogen and carbon levels in plants (Bender, 2012:95).

In humans, amino acids are categorized as essential and non-essential amino-acids. The amino acids that need to be obtained from food intake are the essential amino acids whereas the amino acids the human body produces are the non-essential amino acids (Kumar *et al.*, 2017:1, 2). Essential amino acids such as Leucine, Isoleucine, Methionine, Phenylalanine, Arginine, Histidine, Tryptophan, Valine, Threonine and Lysine are produced only by plants, whereas non-essential amino acids such as Alanine, Asparagine, Cysteine, Glutamine, Aspartic acid, Glycine, Proline, Serine and Tyrosine are produced by both plants and animals, including humans (Kumar *et al.*, 2017:1). Of the most prominent amino acids in the stem bark of *C. dentata* trees, none are among the essential amino acids that needs to be taken by humans as part of their diet. Additional subsets of amino acids based on their structural features are also recognized, e.g. Leucine, Isoleucine and Valine are referred to as branched-chain amino acids whereas Phenylalanine, Tyrosine and Tryptophan are regarded as aromatic amino acids. Methionine, Cysteine and Homocysteine are classified as sulphur-containing amino acids, and Arginine, Histidine, Ornithine and Threonine amongst others, are nitrogen-containing amino acids, while yet another subset, the excitatory amino acids, include Glutamate and Aspartate (D'Mello, 2003:1, 2).

Several amino acids are employed as drugs, or as drug conjugates. Many drugs, including low-molecular weight anti-cancer drugs, are delivered to all parts of the body and get excreted rapidly, which leads to repeated high doses with undesired effects and organ damage (Venkatraj, Nanjan and Chandrasekar, 2017:63). Additionally, many drugs exhibit poor bio-

availability due to insolubility. The conjugation of a hydrophobic anti-cancer drug for example, to hydrophilic polymeric carriers can improve these drugs' water solubility, allowing for easy formulation and better administration of these drugs (Venkatraj *et al.*, 2017:63). An example of such carrier is (polymeric) Malic acid, which has recently been tested as drug carrier for site-specific targeting of anti-cancer drugs (Venkatraj *et al.*, 2017:63). An example of an amino acid used as drug is the purine nucleoside Adenosine, which is a neurotransmitter and a vasodilator. It is commonly used as antiarrhythmic agent (El-Menyar and Gehani, 2010:433). Among the cardioprotective properties of Adenosine are its ability to improve cholesterol homeostasis, impact platelet aggregation and inhibit the inflammatory response (Reiss, Grossfeld, Kasselman, Renna, Vernice, Drewes, Konig, Carson sans DeLeon, 2019:449).). Both Malic acid and Adenosine are major amino acids in the stem bark of *C. dentata*.

Amino acids are expansively metabolized, and for this reason, it is widely assumed that any surplus are disposed of without any adverse effects. However, there is now undisputable evidence that amino acids may cause profound deleterious effects due to imbalances, antagonisms and toxicities (D'Mello, 2003: 125). For example, an overdose of Adenosine intake as a drug lead to side effects such as chest pain, faintness, shortness of breath, and tingling of the senses. More serious side effects may include a worsening dysrhythmia, low blood pressure and cardiac arrest (El-Menyar and Gehani, 2010:433-436). According to the databases PubChem and the HMDB, L-Dopa, or Levodopa, is the naturally occurring form of Dihydroxyphenylalanine and the immediate precursor of Dopamine. L-Dopa is the most effective and commonly used drug in the treatment of Parkinson disease, and is present in all the trees (Figures 2 and 3). Some discrepancies, however, exist as to its safety as a drug. The HMDB states that L-Dopa, unlike Dopamine, can be taken orally, and that it crosses the blood-brain barrier, however, the European Chemical Agency (ECHA) warns that L-Dopa is acute toxic when swallowed. Alanine and Arginine, which are both non-essential amino acids, taken exogenously have serious interferences with glucose oxidative metabolism (Dioguardi, 2011:78). Alanine is one of the major amino acids detected, in contrast to Arginine detected in much lower concentrations in all samples tested. However, Arginine, and also Glutamine, Asparagine, Glycine, Proline and Serine may become "conditionally essential" amino acids when the body does not produce adequate amounts during demanding pathological conditions. Metabolism may therefore not be able to maintain their concentrations at sufficient levels to match metabolic requirements (Dioguardi, 2011:75, 78;

Bender, 2012:86). Methionine, also present in low concentrations in all samples, is regarded as the most toxic amino acid due to its transformation into a toxic intermediate, homocysteine, when cysteine synthesis is required by metabolic needs (Dioguardi, 2011:75). In animals, low-protein diets that contain a range of Cysteine levels between 0.5 and 10%, resulted in reduced weight gain and food intake, and also resulted in high mortality. In humans, 5 to 10 g doses of Cysteine induced nausea, dizziness and dissociation. Doses of up to 20 g Cysteine induced, dizziness, nausea, fatigue and insomnia (Garlick, 2003:1634S). Other amino acids that cause reduced growth and food intake depression in animals include Glycine, Histidine, Lysine, Serine and Tryptophan. Lysine was only detected in one of the 12 trees in KZN in September 2018 and in one tree (a different tree) in KZN in November 2018. Lysine was not detected in any of the 12 trees in the southern Cape in either September or November 2015. Tryptophan, which was detected in all *C. dentata* trees in KZN in September 2018 and in only four *C. dentata* trees in the southern Cape in September 2015, also causes depressed motor activity in animals (Garlick, 2003:1637S). In November 2015 for the southern Cape and November 2018 for KZN, tryptophan was not detected in any of the studied trees at each of the respective research sites. In humans, Glycine is often given as an irrigant during transurethral prostate resection. Adverse effects reported include nausea, transient blindness and visual impairment. Central nervous system symptoms occurred when > 0.5 g per kg of glycine was absorbed (Garlick, 2003:1637S). Histidine is used to treat obesity, rheumatoid arthritis and chronic uremia, however, doses exceeding 4.5 g per day increased urinary zinc and caused headaches, weakness, drowsiness, nausea, anorexia, painful eyes, changed visual acuity, mental confusion, poor memory and depression (Garlick, 2003:1637S). Lysine showed no toxicity in humans and is widely used as treatment for herpes virus. Excessive dosages may, however, cause upset stomach (Garlick, 2003: 1636S). Glutamic acid, the most abundant amino acid detected in November in both regions, has various applications. It is administered to humans as Glutamate and causes nausea and vomiting in proportion to the serum Glutamate level. Concentrations of > 1 Mm /L resulted in vomiting in 50 % of the subjects tested (Garlick, 2003:1635S). In view of the neurotoxic effects of Glutamate, there is concern about the use of the monosodium salt (MSG) as a flavour-enhancing agent. A large proportion of people seem to be sensitive to MSG, where the administration of less than 3 gram of MSG resulted in symptoms such as a burning sensation or numbness at the back of the neck, forearms, back and chest, chest pain, headache,

nausea, facial pressure or tightness, tingling of the upper body, palpitations, drowsiness and bronchospasms (Garlick, 2003:1635S).

For many amino acids, the data relevant to humans are very limited. Unanticipated adverse consequences when consuming large amounts, for example by means of supplementation or the consumption of raw or semi-processed plant material believed to have medicinal properties, cannot be ruled out. Additionally, no data exists that would confidently enable the establishment of a maximum intake of a specific amino acid (Garlick, 2003: 1637S), which therefore warrants systematic studies of amino acid intake and effects on humans, particularly studies which has safety as a primary objective.

7.5 CONCLUSION

The amino acid profiles in the stem bark of *C. dentata* trees vary according to season, year of harvest and region. Seasonal and regional amino acid variability also include variations in composition, distribution and concentrations. Furthermore, the amino acid profiles in the stem bark of *C. dentata* trees also vary between individual trees. Additionally, not all the amino acids in the stem bark *C. dentata* trees occur in all trees, which clearly suggests that each individual tree biosynthesizes amino acids according to its metabolic requirements and its individual interactions with the environment. The same seasonal and regional factors generally involved in variations of secondary metabolites may also affect amino acid biosynthesis, particularly since amino acids are involved in plant stress tolerance. Site-specific factors may also contribute, for example the availability of N in soil, which affects the concentrations available in plant tissues for the biosynthesis of nitrogen-containing amino acids.

Several amino acids are employed as therapeutic agents. However, amino acids may also cause adverse health effects in humans, particularly when excessive amounts are consumed by means of supplementation, which may cause imbalances, antagonisms and toxicities. The consumption of traditional medicines such as the stem bark of *C. dentata* trees, which contains high levels of amino acids such as adenosine, dopa, histidine, methionine, glycine, cysteine and tryptophan may therefore result in unexpected adverse effects.

CHAPTER 8. SUMMARY, CONCLUSION AND RECOMMENDATIONS

8.1 INTRODUCTION

Plants were used as medicine for generations, however, unsustainable harvesting practises is resulting in declining populations and available material for traditional use. Material is harvested from all possible sources, and traditional health practitioners and consumers do not have knowledge on what chemical compounds are being consumed when formulations are prepared from plants as plant material is not from the same area, age or population. The chemical composition of *C. dentata* stem bark, which is the plant part used as medicine, was unknown until date. From a nature conservationist's perspective, no plant can be termed "medicinal" unless validated for its activity and its therapeutic usefulness, and until the medicinal usefulness of *C. dentata* stem bark is proven, the harvesting of the bark is unjustified. Even though the stem bark is in high demand, no studies have been performed on the chemical profiles, or the effect of harvesting practises, area, time and population. Previous studies on *C. dentata* trees involved the isolation of five compounds from the leaves. These compounds are also commonly found in fruits vegetables, herbs and spices. "Why then the destruction of these trees? Furthermore, the medicinal value of a plant cannot be judged on only five compounds of which at least three are insoluble in water and thus have limited absorption and effect. Plants, including tree species such as *C. dentata*, contain numerous compounds, many of which may be beneficial, but could also be hazardous to human health.

Additional studies on *C. dentata* trees reported on the classes of compounds in the stem bark without listing the chemical compounds in the bark. In another study, the crude extracts of *C. dentata* stem bark, roots and leaves were used to determine its antioxidant, anti-microbial and antiverotoxic potentials, with varying outcomes. No scientific study, however, confirmed that extracts of *C. dentata* stem bark has therapeutic effects on a patient with malaria, or with tuberculosis or sexually transmitted diseases or on any of the other ailments *C. dentata* stem bark is traditionally used for. The stem bark's effect on cattle with heartwater also needs scientific confirmation. Furthermore, anti-microbial screening tests previously conducted using *C. dentata* plant parts were very basic and mostly related bacteria with potential to cause gastrointestinal ailments. No previous study conducted a phytochemical analysis of *C. dentata* stem bark, neither were the beneficial and the potentially hazardous components or contaminants present in the extracts identified. Traditional plant-based medicines are neither

standardized nor quality regulated, and the trade in traditional medicines is not controlled. Consumers thus consume uncertain dosages of potentially beneficial compounds, potentially hazardous chemical compounds and contaminants simultaneously.

The aim of this study was to present a comprehensive overview of the chemical compounds in *C. dentata* stem bark to guide management and conservation strategies of this highly utilised tree species. This study focused on the tree species from an environmental and nature conservation viewpoint, and therefore focused on both the beneficial and hazardous compounds, as well as contaminants, and how the location and season affect the chemical profiles. However, the findings of this study also concern the users of medicines prepared from *C. dentata* stem bark as users generally have no knowledge on the chemical compounds consumed. Lists of the chemical compounds identified in extracts were compiled in Chapters 3, 4 and 6 and their variability according to season, region and between individual trees highlighted in Chapter 5, 6 and 7. The potentials of the chemical compounds in the hexane, DCM and ethanol crude extracts were described in Chapter 3. Furthermore, in Chapter 4, seven chemical compounds were isolated from the hexane crude extracts from *C. dentata* stem bark. Therefore, the achievements of objectives described in Chapter 1 are summarized in the concluding Chapter, followed by the conclusions, a list of suggestions, as well as recommendations for future research.

8.2 ACHIEVEMENT OF OBJECTIVES

8.2.1 Hypothesis A

H_0 – *Curtisia dentata* tree stem bark do not contain chemical compounds beneficial to human health.

H_1 – *Curtisia dentata* tree stem bark do contain chemical compounds beneficial to human health.

The *C. dentata* stem bark used in Chapter 3 was purchased from the Faraday traditional medicine market whereas the *C. dentata* stem bark used in Chapters 5, 6 and 7 was collected personally from two research sites of which one site was in the southern Cape forest area and the other in the KZN midlands. Whether the bark bundles purchased at the traditional medicine market all originated from the same tree or from different trees is unknown, and the date of collection is equally unknown. Comparison between the collected bark and the purchased bark show similarities in chemical content to some extent, but also shows chemical

variation, which is due to either differences in the solvents used and/or the purchased stem bark originating from a location not included in this study. However, chemical variability between individual trees is also considered. Extraction of compounds in Chapter 3 was done exhaustively using hexane, DCM and ethanol, in that order, whereas only DCM was used as solvent in Chapters 5, and 6 and methanol was used in Chapter 7. In Chapter 3, a total number of 125 compounds in the hexane crude extracts were researched for its potentials. From this list of 125 compounds, information on the compounds' potentials could be found for 94 compounds. Of these 94 compounds, 26 compounds were identified as compounds with possible beneficial potentials. However, in the literature cited, the whole matrix of compounds in the essential oils or extracts of a plant species are generally used to screen the plant's potential activity against pathogens, its radical scavenging activity, antioxidant potentials or its activities against cancer cell lines. In only a few studies, the beneficial chemical compounds are isolated and used in bioassays. The probability of two or more chemical compounds acting either synergistically or agonistically, to achieve the results in studies is thus high. In Chapter 6, it was determined that selected chemical compounds with beneficial potentials did not occur in all *C. dentata* trees studied. The chemical compounds with beneficial potentials also did not necessarily occur in the same tree at each sampling date. The DCM crude extracts in Chapter 3 yielded a low number of chemical compounds as the majority of compounds were extracted with hexane. The DCM crude extracts had five chemical compounds with an 80% or higher similarity to the compounds in the main database of the spectrometer. Of the five compounds, three had beneficial potentials. Similarly, the ethanol crude extracts also yielded a low number of compounds, of which four compounds had an 80% or higher similarity to the compounds in the main database of the spectrometer. Only one of these compounds may be potentially beneficial. Six of the seven compounds isolated from *C. dentata* stem bark has proven beneficial potentials however, all seven compounds are insoluble in water, which affects the efficacy of these compounds as therapeutic agents. Trees produce secondary metabolites according to the trees' needs in the environment, not for the benefit of mankind. Therefore, the null hypothesis can be rejected as the stem bark of *C. dentata* trees do contain chemical compounds with beneficial potentials, however, not all *C. dentata* trees contain chemical compounds with beneficial potentials at a specific harvesting date. Chemical compounds of which the efficacy as therapeutic agent is compromised cannot be counted as beneficial before conjugates to increase their solubility in water are found. The fact that it is impossible to know before

harvesting which trees contain these beneficial compounds and which trees do not, as well as the fact that the compounds isolated from *C. dentata* (both leaves and stem bark) are all insoluble in water, necessitates the rejection of the alternative hypothesis.

8.2.2 Hypothesis B

H_0 – *Curtisia dentata* tree stem bark do not contain chemical compounds that pose a risk to human health.
H_1 – *Curtisia dentata* tree stem bark do contain chemical compounds that pose a risk to human health.

Plants produce secondary metabolites according to the needs of the plants in their environment, which include compounds that assist in the plants' survival in a harsh environment, for example where the plants may be exposed to abiotic stress factors such as water deficits, high UV exposure and more, and biotic stress factors such as herbivorous insects and pathogens. Many of these secondary metabolites are produced to deter, stun, poison, kill or react with proteins in the insect or pathogen's metabolisms. Humans can therefore not assume that plant material is safe to use because it is natural. Of the 94 compounds on which information on its potentials could be found in Chapter 3, 47 chemical compounds have the potential to cause adverse effects in humans and 21 chemical compounds may originate from contaminants such as bacteria, fungi, plastics, pesticides and petrochemical products. Additionally, of the 47 chemical compounds which may be hazardous to human health, six compounds are either known or suspected carcinogens, i.e. nerolidol, oxeladin, phenol, butyrolactone, isoeugenol and tetradecanoic acid. Nerolidol and phenol are also mutagenic, while phytol showed genotoxic potentials. Of the 47 chemical compounds listed in Table 3.5, 14 compounds are reported to be acute toxic and/or fatal if swallowed. One of the major compounds in the hexane extracts of *C. dentata* stem bark, i.e. Benzene, 1-methyl-3-(1-methylethyl)-, also known as M-Xylene (3.3%), is a chemical that may be fatal if swallowed. Additional potential adverse effects may result from chemical compounds having narcotic effects, such as 4-hydroxy-butanoic acid, chemical compounds that accumulate in the human body, such as Phenol, or chemical compounds that can induce coma and death in higher dosages, such as Aniline. Compounds which may cause severe liver and/or kidney damage, anorexia, euphoria and other serious conditions are also present. Several of these compounds may cause serious eye, skin and respiratory tract irritation, while others are environmental hazards with particular reference to their acute and long-lasting toxic effects on aquatic life. Of the five chemical compounds in the DCM crude extracts, one is acute toxic if swallowed and another is a carcinogen. Of the four compounds in the ethanol

crude extracts, oxalic acid is acute toxic if swallowed and an overdose or chronic use of glycerin can cause adverse effects, particularly in infants. No information could be found on Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-,2-ethyl ester, however, several other pyrrole derivatives are listed in the US List of Toxic Substances Control Act (TSCA). The potentials of each of the compounds in Chapter 6 were not evaluated but several of the compounds detected in the stem bark extracts in Chapter 3 are also listed in Tables 6.1, 6.2 and 6.3. The concentrations that will cause adverse effects are not known and therefore, it is not known whether these compounds will have an adverse effect when consumed as part of a preparation. Also, the matrix effect is well known, and the effects of these compounds might be reduced/eliminated by the matrix. The biotransformation of these compounds are also not known and might not be absorbed/present in active form. The opposite may, however, also be true, where seemingly harmless compounds become highly toxic during biotransformation. However, it is important to identify the compounds and to know their presence in a particular plant species for consideration in future studies. The null hypothesis which states that *Curtisia dentata* tree stem bark do not contain chemical compounds that pose a risk to human health can thus be rejected.

8.2.3 Hypothesis C

H_0 – There is no seasonal variation in chemical compound composition and concentrations in *C. dentata* stem bark.
H_1 – There is seasonal variation in chemical compound composition and concentrations in *C. dentata* stem bark.

In Chapter 5 the metabolic profiles of *C. dentata* stem bark clearly showed seasonal differentiation. This study investigated both the hydrophilic and lipophilic chemicals of the tree. Seasonal effects of hydrophilic compound concentrations largely correspond with rainfall figures. Rainfall therefore seems to be a major driver of seasonal variation, however, rainfall is probably not the only determining factor. Several other environmental factors such as photoperiod, temperature, UV exposure, water and nutrient availability and quality of light also contribute. Lipophilic compound concentrations cannot be directly connected to rainfall patterns. Lipophilic concentrations are therefore determined by other factors such as seasonal abiotic and biotic stress factors. Particularly clear are the seasonal variations in concentrations of the lipophilic compound betulinic acid, which was isolated from *C. dentata* stem bark in Chapter 4. The fluctuations in concentrations of this compound, which is known for its medicinal properties, provide a clear example of how season and locality might influence the

medicinal compounds in plants. This example is, however, an example of a compound with beneficial properties, but it could easily be applicable to compounds with adverse effects. Furthermore, the hydrophilic and lipophilic compounds do not show similar responses (increases or decreases) during a specific season, for example, sucrose concentrations (a hydrophilic compound) may increase towards autumn (March) and decrease towards mid-winter (July) whereas betulinic acid concentrations (a lipophilic compound) may show opposite responses. Furthermore, not all hydrophilic compounds (or all lipophilic compounds) may show similar responses during a specific season. This is clearly demonstrated in Chapter 5 with the compound, isoeugenol, showing different responses than those exhibited by sucrose. Chapter 6 emphasizes the seasonal effects by exhibiting seasonal variation in chemical composition in Tables 6.1, 6.2 and 6.3 respectively. Similarly, Chapter 7 shows seasonal variability in the distribution of amino acids in the stem bark of *C. dentata* trees. Therefore, the null hypothesis which states that there is no seasonal variation in chemical compound composition and concentrations in *C. dentata* stem bark can be rejected.

8.2.4 Hypothesis D

H_0 – The chemical composition in the stem bark of *C. dentata* trees do not vary between individual trees or between geographical regions.
H_1 – The chemical composition in the stem bark of *C. dentata* trees vary between individual trees and between geographical regions.

The general assumption that all plants of the same species contain the same chemical compounds is highly disputable. Chapter 6 particularly highlights the variability in chemical composition in *C. dentata* stem bark. Several chemical compounds occurred in the stem bark of *C. dentata* trees at both the Groenkop research site and the Nkandla Forest research at each sampling date site but did not necessarily occur in all 12 the trees at each of the research sites. Similarly, the chemical compounds that did not occur in all the trees at each of the research sites did also not occur in all the trees, but in only specific trees. For this reason, the total number of compounds in *C. dentata* trees at a specific location at a specific sampling date (Tables 6.1, 6.2 and 6.3) are much higher than the number of compounds that are present in the stem bark in individual trees (Tables 6.4 and 6.5 for the southern Cape and Tables 6.6 and 6.7 for KZN). Tables 6.4, 6.5, 6.6 and 6.7 further showed variations in chemical compound composition in *C. dentata* trees occurring in the same location. Only five chemical compounds in the DCM extracts of the collected *C. dentata* stem bark occur in all *C. dentata* trees at each of the two research sites and at each harvesting date, i.e. Benzene, 1,3-bis(1,1-

dimethylethyl)-, also known as 1,3-di-tert-butylphenol, which is a contaminant, eicosane, which maybe fatal if swallowed, 2,6,10,14-tetramethylheptadecane, which may be a bacterial metabolite and thus derive from a biological contaminant, nonadecane, which, as part of a matrix of chemical compounds, may contribute towards beneficial potentials, and pentadecane, which may also be fatal if swallowed. The amino acid distribution profiles of the stem bark of *C. dentata* trees (Chapter 7) also vary between individual trees and between regionally separated trees. Differences in the amino acid profiles include both the composition and concentrations of amino acids. These differences are reflected by the percentage area of each individual amino acid in a specific sample or set of samples, as well as the order or distribution. The regional and individual amino acid distribution profiles of the stem bark of *C. dentata* trees in the southern Cape and KZN, however, also show remarkable similarities regarding the most prominent amino acids, with the nine most abundant amino acids: Isocitric acid, Adenosine, Glutamic acid, Malic acid, Alanine, Aspartic acid, Dopa, 4-Hydroxyproline, and Serine occupying approximately 80 % of the total area and the remaining 63 amino acids occupying the remaining 20 % of the total area. The null hypothesis which states that the chemical composition in the stem bark of *C. dentata* trees do not vary between individual trees or between geographical regions can thus be rejected.

8.2.5 Additional outcomes of the research study

In Chapter 4, seven chemical compounds were isolated from the stem bark of *C. dentata* trees, their structures elucidated and characterized, which could be used for targeted analysis since no compounds were previously isolated from the stem bark. Three of these compounds, i.e. ursolic acid, betulinic acid and *β*-sitosterol, were previously isolated from *C. dentata* leaves, however, it was the first time that these compounds were isolated from *C. dentata* stem bark. The structures of four additional chemical compounds were elucidated from *C. dentata* stem bark, i.e. betulinaldehyde, stigmasterol, n-tetracosanol and n-hexadecanoic acid. These four compounds were not previously isolated from *C. dentata* trees. All seven these compounds are, however, well-known and common compounds found in plants, and therefore do not provide sufficient explanation for the high demand of *C. dentata* stem bark for the preparation of medicinal formulations. These compounds do not justify the use of *C. dentata* stem bark for the ailments it is traditionally used for.

8.3 CONCLUSIONS

In this study it is concluded that:

- The different solvents used yielded different chemical compounds, and different equipment targeted different compound groups. It was therefore of importance to include various solvents and equipment to provide a thorough overview of the chemical profile of *C. dentata* trees. The lipophilic compounds were studied in Chapters 3, 4, 5 and 6 and the hydrophilic compounds in Chapters 3, 5 and 7, thereby providing a good overview of the chemical profile and the changes due to seasonal and regional factors.
- In this study, the focus was not on drug discovery, but rather on the phytochemical variation in compounds consumed when people use preparations made from *C. dentata* stem bark as traditional medicine. Because plants contain numerous chemical compounds, many of the chemical compounds serially extracted were researched for their potential effects on human health. In the literature cited, very few of the beneficial chemical compounds listed in Chapter 3 were used in bioassays as single compounds. In the majority of cases, these beneficial compounds were part of a matrix of chemical compounds. Since the majority of the beneficial compounds are well known compounds, the information provided may be regarded as reliable. However, not all compounds in a matrix are necessarily beneficial. Some chemical compounds may have adverse effects on human health, and in Chapter 3, this fact is highlighted.
- There are potentially hazardous chemical compounds in the hexane crude extracts of *C. dentata* stem bark that may be beneficial as natural crop protectants against insects or pathogens, but which are acute toxic if swallowed as constituent of a non-standardized, non-regulated traditional medicine. However, many chemicals with potential negative effects are unknowingly consumed daily (e.g. in herbs, spices and alcoholic beverages), however, low dosages and the irregular consumption of such chemicals when using the stem bark as medicine, also the consumption thereof as part of a matrix and their modification during biotransformation may detoxify these compounds. Effects of such compounds on human health, however, also depend on consumer-related factors such as weight, age, underlying conditions and the seriousness of the disease, and also on the nature of the plant and the speed of absorption etc. For hazardous compounds that can be extracted from plants to use as

crop protectants, consideration must be given to their effect on the environment as several hazardous chemical components have long-lasting toxic effects on aquatic life.

- It is of essence that the six carcinogens and the two mutagens in the hexane crude extract and the carcinogen in the DCM crude extract of the purchased *C. dentata* stem bark are highlighted. The chemical compounds detected in the DCM extracts of the *C. dentata* stem bark samples collected from the southern Cape and KZN in Chapter 6 were not researched for their characteristics and potentials. However, with carcinogens detected in the sequentially extracted crude extracts, it is advisable to also conduct literature researches on each of those compounds' characteristics and potentials. An additional known carcinogen was also detected with GC-MS of the fractions, i.e. methyl glyoxal. There may even be more, but the whole list of 879 chemical compounds in Appendix L were also not researched for its characteristics and potentials. Another chemical compound of concern detected in *C. dentata* stem bark is aniline, which is a highly acrid poison. The available studies in humans are inadequate to determine whether exposure to aniline can increase the risk of developing cancer in people. Rats that ate food contaminated with aniline, however, developed cancer of the spleen. Phenol, which is the parent compound of all phenolic compounds, is both a carcinogen and mutagen. In the case of phenol, dosages consumed, and all other patient-related factors become irrelevant due to the compound's ability to accumulate in human tissues. Even though the toxicity of the stem bark has been reported as not toxic, it was performed on cell lines and for a short time duration. Long-term use of *C. dentata* stem bark may thus be detrimental and users of preparations from *C. dentata* stem bark must be cautioned in this regard.
- In Chapter 4 , seven chemical compounds were isolated from stem bark, not for drug discovery purposes but to compare compounds isolated from *C. dentata* stem bark with the compounds previously isolated from the leaves. Three compounds, namely ursolic acid, betulinic acid and the steroid, *β*-sitosterol were isolated from both *C. dentata* leaves and stem bark. However, lupeol and 2α-hydroxyursolic acid, which were previously isolated from *C. dentata* leaves were not detected in any of the stem bark samples analyzed. These two compounds are also not among the 879 compounds detected by GC-MS analysis of the fractions (Appendix L). It is possible that they may be present but were not detected with the equipment used, or isolated as only

seven compounds were isolated. Betulinaldehyde, stigmasterol, n-tetracosanol and n-hexadecanoic acid isolated from the stem bark in this study were not isolated from the leaves. The same principle, however, also applies for these four compounds. More information is needed to make an informed decision on the substitution of plant parts for medicinal use, however, the use of *C. dentata* leaves, instead of stem bark, is definitely preferred.

- ^{1}H NMR and GC-MS analysis of *C. dentata* stem bark in Chapters 5 and 6 respectively, showed that the concentrations and composition of chemical compounds in *C. dentata* stem bark vary between individual trees, seasonally and regionally. Even though the objective of Chapter 5 was untargeted NMR analysis, the seasonal variations in concentration in individual trees and regionally separated trees were highlighted with the targeted analysis of sucrose and isoeugenol. The peaks for the isolated lipophilic compound, betulinic acid, reiterated these findings. However, these findings also showed that not all hydrophilic compounds necessarily exhibit the same response at any given sampling date, nor did all lipophilic compounds exhibit similar responses at any given sampling date. Moreover, hydrophilic compounds and lipophilic compounds also showed differences in the seasons at which concentrations increased/decreased. The implications of such variations are that the concentrations and composition of chemical compounds extracted when preparing traditional medicines from *C. dentata* stem bark therefore vary with each preparation, which therefore also mean that the dosages and the composition of chemical compounds consumed vary with each new batch of medicines prepared from *C. dentata* stem bark.
- The chemical compounds isolated from the stem bark in this study cannot be linked to the ailments the stem bark is used for. Furthermore, particularly in Chapter 6 it was determined that not all *C. dentata* trees at a specific location contain potentially beneficial chemical compounds at a specific harvesting date, which raises the question of whether *C. dentata* stem bark can be termed “medicinal”? However, this also provides ample opportunity to continue research on this species to find that “missing link” between the compounds and the ailments treated. This may be challenging since two or more compounds may be involved, however, the chemical profiles of *C. dentata* stem bark may greatly assist in achieving this objective in future.
- The pesticides and PAHs detected in the stem bark of some of the *C. dentata* trees from both research sites in Chapter 6 reflects the state of health of the environment.

Only these two hazardous classes of components were highlighted, but there are probably a few more compounds in the collected stem bark that can directly be linked to anthropogenic activities. Of concern is that these chemicals are also extracted when traditional medicines are prepared from *C. dentata* stem bark, and consumed. These compounds may have deleterious effects on human health. Furthermore, analysis of the purchased *C. dentata* stem bark also revealed the presence of several metabolites of potential bacterial origin. Even freshly harvested stem bark may unknowingly contain bacteria and other pathogens that are too small to observe with the naked eye. The potentials of the compounds in the collected stem bark were not researched, as was done with the purchased stem bark in Chapter 3, however, it is possible that a few of the compounds listed in Chapter 6 are also of bacterial, fungal or other pathogenic origin, and bacteria in particular are often responsible for stomach ailments.

- The amino acid profiles of *C. dentata* stem bark is disclosed for the first time in this study. Most interesting was that not all 72 amino acids detected in *C. dentata* stem bark occurred in all studied *C. dentata* trees at every sample collection date, that the order, composition and concentrations of amino acids also change according to season and that the amino acid profiles of individual and regionally separated *C. dentata* trees differ to some extent. There were, however, also surprising similarities, whereby the nine most prominent amino acids in *C. dentata* stem bark occupy the majority part of the total amino acid profile for both research sites, even though bark samples were collected three year apart. The study on amino acids reiterated the fact that plants produce metabolites according to their needs in the environment, however, it also shows that some amino acids are necessary for normal plant growth, development and protection (development of tolerance to abiotic environmental factors). Of the most prominent amino acids in the stem bark of *C. dentata* trees, none are among the essential amino acids that needs to be taken by humans as part of their diet. Humans therefore also produce these prominent amino acids in their bodies, and supplementation through the consumption of traditional medicines such as the stem bark of *C. dentata* trees, could result in excessively high levels of these amino acids in the human body. Previous studies determined that there are undisputable evidence that amino acids may cause profound deleterious effects due to imbalances, agonisms and toxicities.

- Particularly noticeable from the literature researched in this study is that the majority of chemical compounds which are reported to have beneficial therapeutic and/or pharmacological properties are present in fruits, vegetables, herbs and spices. Such fruits, vegetables, herbs and spices are more readily available than so-called medicinal plant species containing these chemical compounds and could therefore provide the necessary therapeutic effects at a much lower cost to the environment.

8.4 SUGGESTIONS

- Ideally, until uncontested proof exists for *C. dentata* stem bark's medicinal properties, including the specific chemical compounds involved, the harvesting of *C. dentata* stem bark should be urgently reconsidered. The species is a protected species in terms of the environmental laws of South Africa, however, environmental laws did not prevent the continued harvesting of *C. dentata* stem bark due to its long-term cultural use thereof as a traditional medicine. Environmental laws should therefore be actively enforced and people transgressing these laws should be prosecuted.
- Since tradition is an important factor in South Africa, the large-scale cultivation of popular threatened and endangered plant species used as traditional medicines, such as *C. dentata* trees, should be seriously considered. A suggestion in this regard would be to adopt a forest expansion approach, whereby a stretch of land is made available adjacent to current forested areas, for example, a stretch of land of approximately 500 m or more in width (the wider the better), encircling a current forested area, or at least a large part thereof. A variety of tree species which are similar to the tree species occurring in the natural forest, or which historically occurred in that forest, can be planted in a manner which mimics the composition of trees in the natural forest, thereby creating a heterogenous man-made forest which is similar but complimentary to the natural forest.
- Considering that many traditional medicines have been used for many generations, consumers of traditional medicines need to be made aware of the potential risks involved, particularly when the complete chemical composition of a specific plant part of a particular plant species is known, and even more so if those plant parts are prepared into remedies for consumption. It is therefore vital that environmental education and awareness programs include awareness on plant-based traditional

medicines in an understanding and sensitive manner. It is further essential that environmental education and awareness programs include awareness on the consequences of overharvesting, particularly where plant parts such as stem bark and roots are collected.

- The current permit system to collect plant material from protected species is both inadequate and poorly managed. DEFF officials do not know how many traditional healers or commercial harvesters have permits to collect plant material from protected species and admit that only a few have permits. It is debatable whether DEFF officials know how many traditional health practitioners and commercial harvesters there are in South Africa. It is also questionable whether DEFF officials follow up on those traditional healers and commercial harvesters who do not comply with the environmental laws of South Africa. DEFF officials also do not know whether traders of plant material collected from protected plant species need permits to be in possession of plant material collected from protected species. The current permit system therefore needs to be redesigned and databases upgraded to include the names and physical addresses of all traditional health practitioners and commercial harvesters. Alternatively, a geographical information system (GIS) could be employed to pinpoint the residential locations of traditional healers and commercial harvesters, particularly those residing in rural areas. The permit system and databases should also include traders of traditional plant medicines as they are often in possession of plant parts collected from protected plant species. Unannounced follow-up operations could be employed to ensure collectors and traders of plant material comply with the stipulations of their permits, particularly with regard to the species and quantities of plant material specified in their permits. Ideally, permits should also specify times (specific months) in which plant material from specific plant species may be collected, and stipulate "bag limits" per person per harvesting season (i.e. the maximum volume of bark that each person may harvest).
- Since the production of potentially beneficial chemical compounds in *C. dentata* stem bark depends on several environmental factors and the biotic and abiotic stresses the trees are exposed to at a particular time and at a particular site, it is challenging to suggest an ideal time or season for the harvesting of *C. dentata* stem bark at a specific site. The stem bark samples collected from the southern Cape forest area and Nkandla forest were collected three years apart. Plant stresses, and thus also the chemical

composition of the stem bark of *C. dentata* trees may differ on a year-to-year basis, a month-to-month basis, and may even differ on a week-to-week or day-to-day basis. Plant stresses also differ between regions and between individual trees. However, from the results in this study, it appears that autumn is the best time for harvesting in the southern Cape, and spring for harvesting in the Nkandla forest. However, since not all trees always contain the beneficial chemical compounds generally sought after, no guarantee can be provided for the presence of the beneficial chemical compounds in the stem bark of the *C. dentata* trees when harvested.

8.5 RECOMMENDATIONS FOR FUTURE RESEARCH

- Because of its scarcity, *C. dentata* stem bark is used as part of a mixture of stem bark from eight tree species for the formulation of therapeutic preparations, a mixture called *khubalo*. Firstly, the stem bark of the other seven tree species used for the *khubalo* mixture should also be studied individually, and the chemical compounds identified evaluated, as was done with *C. dentata* stem bark in Chapter 3. Secondly, the chemical composition of the extracts of *khubalo* should be determined and each compound identified evaluated for its properties and potentials. It is then of the utmost importance to compare the chemical compositions of the individual tree species, including *C. dentata*, with the chemical composition of *khubalo* to evaluate and compare the potential effects of the mixture when consumed as traditional medicine. Long term toxicity is especially advised since individual compounds with possible long-lasting toxic effects have been detected in *C. dentata* stem bark. Aqueous extracts extract of *C. dentata* stem bark showed no toxicity in one study, however, in another study, it was also determined that aqueous extracts generally have limited biological activity. Toxicity studies should therefore be conducted on extracts obtained from solvents other than water, and particularly on extracts obtained with solvents that are less polar, such as hexane and DCM.
- It is essential to continue and expand studies pertaining to seasonal effects on secondary metabolite production to include *C. dentata* trees from all areas within their distribution range, and to determine their respective chemical compositions to compare, evaluate, determine and finally suggest the best options for harvesting, as

this study has shown that the optimal seasons for harvesting between the trees in the southern Cape and the trees in KZN vary.

- Before suggestions can be made on the substitution of stem bark with leaves for use in traditional medicinal preparations, it is of the utmost importance to conduct a full phytochemical analysis of *C. dentata* leaves. The chemical compounds in the profiles of the leaves must then also be evaluated and compared to the chemical profiles of the stem bark to be able to make an informed decision in this regard.
- It is important to find a link between the ailments treated and the chemical compounds detected/isolated in/from *C. dentata* stem bark. It is therefore imperative to conduct directed bioassays on the species to determine pharmacological effects on the diseases (stomach ailments, malaria, tuberculosis and sexually transmitted diseases) the stem bark of *C. dentata* trees are used for. If no connection can be made, the harvesting of *C. dentata* stem bark can rightfully be declared unjustified.
- Since *C. dentata* trees have become relatively scarce and is conservation-dependent in KZN as a result of overexploitation, the cultivation of the species is of the utmost importance. However, cultivation requires the determination of the most appropriate propagation methods, particularly since a previous study determined that natural germination of *C. dentata* seeds is very poor. The cultivation of large numbers of *C. dentata* trees is necessary to maintain tree species diversity, genetic diversity and biodiversity in an environment dominated by humans.
- *Curtisia dentata* stem bark contain several potentially harmful chemical compounds, which include carcinogens, mutagens, a chemical compound with genotoxic effects, chemical compounds with narcotic effects and a chemical compound which can induce coma. Primary and secondary pharmacodynamic and toxicodynamic studies followed by pharmacokinetic and toxicokinetic studies on *C. dentata* stem bark extracts are therefore vital, and these tests should be capable of detecting outcomes that include mutation induction, clastogenic and aneugenic effects, and structural chromosome abnormalities.
- Currently, no data exists that would confidently enable the establishment of a maximum intake of amino acids. It is therefore important to conduct systematic studies of amino acid intake and their effects on humans.

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APPENDIX A: All communication between the researcher and SANParks between 30/04/2015 and 17/05/2015 to obtain trees to conduct research on in the southern Cape, and the limitations imposed.

1) E-mail received from Jessica Hayes 30/04/2015

Hi Anne,

Thanks for your enquiry. We have received feedback from the relevant scientists, and it includes the following comments for your consideration and comment:

1. It was acknowledged that the method could be a useful technique in specific situations where one wants to save individual trees damaged by destructive bark harvesting, however the wide application of the technique in forest management or sustainable resource use is possibly not practical due to various management constraints. Considering the above, the excessive damage that would be caused to more than 80 trees cannot be justified. In summary, the project was not supported in its current form.

2. If the number of trees are drastically reduced (it was suggested by one scientist – reducing sample size to a maximum of 5 trees per species, if still statistically viable), and treatments are confined to smaller trees at the Groenkop research site that have already been exposed to experimental bark harvesting, the project could be reconsidered.

3. It is however also appreciated that this could impact on the soundness of the project in terms of research methodology and sample size.

The following comments and questions regarding methodology of the proposal were also raised:

- **Site**: The researcher refers to Wilderness; it is not clear if the researcher has a specific site in mind – probably not. However, a suitable site could be selected through consultation between the managers and Scientific Services, probably located at Groenkop or Bergplaas. How uniform must the site be? The boundaries of the site will have to be clearly indicated to the researcher.
- **Species**: Ocotea bullata Stinkwood, Curtisia dentata Assegai and Myrsine melanophloeos Boekenhout (formerly Rapanea melanophloeos) are relatively common on suitable sites and sufficient trees should be relatively easy to locate. Ilex

mitis Cape holly is often less common and favours wetter sites, e.g. along streams, and it may be more difficult to locate sufficient trees.

- **Sizes of trees** required are not specified by the researcher. Smaller trees would be preferable, but she may need to include some larger trees.
- **Scions** cut from the previous year's growth will be used for the grafting. These presumably come from the tree's canopy, and may be difficult to collect. What are the researcher's plans to deal with this?
- What size are the **bark samples** that will be collected every 3 months (about 6 sampling occasions)?

Please do not hesitate to contact me should you have any queries regarding the comments.

Kind regards,

Jessica

Jessica Hayes

Regional Ecologist – Garden Route

2.) Reply E-mail to Jessica Hayes 01/05/2015

Jessica

The samples will be collected using a hammer and chisel and the depth will depend on bark thickness. I need approximately 2g of sample material per sample. I am not sure of the weight of one such sample as yet, though. I do have a little scale with which I can measure the weight. I have reduced the sampling dates from 6 to 4 times due to the costs involved in the analysis thereof. It is extremely expensive.

I do not mind working with smaller trees as long as they are not so small that when they are girdled there would be insufficient carbohydrates left below the girdle wound for carbohydrate metabolism by the roots. I certainly do not want to kill trees!! Girdling and grafting during spring (in the active growing season) would favour rapid callus development. I am a little unsure about working on trees that have been used for bark harvesting experiments before because the experiments may have influenced the carbohydrate regime and auxin allocation to the trunks, but I am willing to compromise if needed. This would most probably be one of the things that I would need some feedback on from my supervisors. This and of the reduction of sample sizes, of course.

When the scientists suggested 5 trees, did they mean 5 trees in total or 1 normal, 1 control and 5 grafted one week after girdling and another 5 two weeks after girdling? This is a total of 12 trees per species. A total of 5 trees will not be enough for statistical validity and reliability. I am also willing to reduce the number of species to 3 species (remove Ilex mitis) if necessary, but including it would provide information on more species (if there are enough trees available – I know they grow along streams).

I will be perfectly happy with any site the scientists recommend, and I will comply with the boundaries that will be set for me. No problem there.

I have sent you another e-mail regarding the collection of scion material.

I hope we can sort this out as soon as possible. Let me know if there are more questions and I will get back to you as soon as I have received feedback from my supervisors.

Anne

3.) E-mail from Jessica Hayes to Scientific services Knysna 05/05/2015

Kind regards

Dear Colleagues,

I have received comment back from Anne van Wyk regards her project, and I also believe she chatted to Wessel this morning regarding sample size. Please see my comments hereunder and her response in the email below that. Please could you provide me with further comment following Anne's feedback. Once we have finalized the details, I will ask her for the amended project proposal.

1. It was acknowledged that the method could be a useful technique in specific situations where one wants to save individual trees damaged by destructive bark harvesting, however the wide application of the technique in forest management or sustainable resource use is possibly not practical due to various management constraints. Considering the above, the excessive damage that would be caused to more than 80 trees cannot be justified. In summary, the project was not supported in its current form.

2. If the number of trees are drastically reduced (it was suggested by one scientist – reducing sample size to a maximum of 5 trees per species, if still statistically viable), and treatments are confined to smaller trees at the Groenkop research site that have already been exposed to experimental bark harvesting, the project could be reconsidered.

3. It is however also appreciated that this could impact on the soundness of the project in terms of research methodology and sample size.

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· Site: The researcher refers to Wilderness; it is not clear if the researcher has a specific site in mind – probably not. However, a suitable site could be selected through consultation between the managers and Scientific Services, probably located at Groenkop or Bergplaas. How uniform must the site be? The boundaries of the site will have to be clearly indicated to the researcher.

· Species: Ocotea bullata Stinkwood, Curtisia dentata Assegai and Myrsine melanophloeos Boekenhout (formerly Rapanea melanophloeos) are relatively common on suitable sites and sufficient trees should be relatively easy to locate. Ilex mitis Cape holly is often less common and favours wetter sites, e.g. along streams, and it may be more difficult to locate sufficient trees.

· Sizes of trees required are not specified by the researcher. Smaller trees would be preferable, but she may need to include some larger trees.

· Scions cut from the previous year's growth will be used for the grafting. These presumably come from the tree's canopy, and may be difficult to collect. What are the researcher's plans to deal with this?

· What size are the bark samples that will be collected every 3 months (about 6 sampling occasions)?

With thanks and regards,

Jessica Hayes

4.) E-mail from Dr. Wessel Vermeulen, chief scientist at Scientific services, Knysna, to Jessica Hayes and forwarded to the researcher 06/05/2015

Hi Jessica

With reference to the revised project proposal, I support the project **provided that**:

- The number of trees per species be limited to 7 (5 for treatment, 1 control and 1 normal tree);

- The study be confined to the trees that have already been exposed to experimental bark harvesting;

- Only trees ≤35 cm DBH be selected for the study.

Further comments on study site and species:

- Witelsbos (Tsitsikamma Section of the GRNP) would be the best site to study *Ocotea bullata* and *Curtisia dentata*;

- *Myrsine melanophloeos* at Groenkop (Wilderness Section of the GRNP) could be added as a third species if the sample size is reduced to seven.

5.) E-mail sent to Jessica Hayes 14/05/2015

Jessica

1. With the revised proposal I will only be working with *Ocotea bullata* and *Curtisia dentata* – ***Myrsine Melanophloeos is not included.***

2. I will work with trees that have been used in bark harvesting experiments before. I have indicated that.

3. I asked Wessel telephonically to reconsider if I used 1 normal tree, 1 girdled control tree, 5 trees grafted 1 week after girdling and 5 trees grafted 2 weeks after girdling. That is 1+1+5+5 = 12 trees. He said that that would be much better so I asked advice from my statistician and he said to rather graft the 10 trees either 1 week or 2 weeks after girdling (remember they will be wrapped immediately after girdling – a proven way of preventing trunk desiccation, insect and pathogen attack and to promote callus development) and cut out the time differences of grafting. That would still yield relatively valid and reliable results. Doing it with 5 trees only would not- too few repetitions, according to the statistician. They wanted me to have 30 repetitions for valid and reliable results and to prevent me from being torn apart during the presentation of my dissertation.

4. Growth of trees in the hypothesis means **canopy responses** (does cover health remain the same, or decrease over time) and I was advised by the statistician to rather keep it at percentages and not add the classes of 0 to 5 to it. He knows why he advised me to do that.

5. Trees with <35cm DBH is fine.

6. The idea is to see whether callus development will take place. It is not about edge- or sheet phellogen growth as may be the case when only the outer bark is being removed. Callus cells differentiates from non-distinct cells into phloem, xylem and cork cells which over time closes a wound – scientifically proven in scientific literature regarding fruit trees. How would we know if we do not do it on medicinal trees?

As a final point – a reminder that I have done this before and I have really compromised where I could – from 88 trees to 24 of which 22 will be girdled and 20 grafted again. Please let me do this. I am pleading!!

Apart from compromising statistical results if fewer trees are used, the lab costs will increase because I will no longer get the discount offered. Less than 40 samples per sampling date will result in the full prices being charged and that is extremely expensive.

I have attached the proposal again.

Kind regards

Anne

6.) Another E-mail to Jessica Hayes 14/05/2015

Jessica

I am really down-in-the-dumps this morning. Wessel himself worked on 180 *Curtisia dentata* and 89 *Ocotea bullata* – trees with no previous damage – in his study. All I am asking is 11 trees of each of the 2 species that have previously been used in his experiments (that is apart from the 1 normal tree of each species), of which 10 each will be grafted again, in the site he suggests, with trunk DBH as he suggests – medium sized trees is fine (his description). I will do **everything** I can to prevent the death of these trees, even re-graft if necessary. I will insert enough scions, see to it that each and every scion is placed correctly, and I will monitor them every second month to make sure they are okay. I did not even include repetitions of control tree responses because they may also differ – as my previous study has shown.

Please help me!!!

Anne

7.) Jessica Hayes forwarded the researcher's previous two e-mails to the scientists and wrote the following:

Hi Graham, Zodwa and Wessel,

Thanks for your comment to date on this project. Please see below the applicant's response to the recent detailed comment from Wessel. The main dilemma at this stage is the number of trees that she needs to work on to make her project worthwhile.

Please can you give her latest comments, in terms of numbers and the other details, some thought so that we can conclude the comments from our side.

With thanks and kind regards,

Jessica

8.) Jessica Hayes received a reply from Graham Durrheim in which he wrote the following on 14/05/2015:

From: Graham Durrheim
Sent: 14 May 2015 03:25 PM
To: Jessica Hayes; Ntombizodwa Ngubeni; Wessel Vermeulen
Subject: RE: Research proposal: van Wyk, A.S. – Determination of the success of bridge-grafting as technique to restore growth in four girdled medicinal tree species in Wilderness, Western Cape

I could accept 12 trees of each species. I expect that several could survive.

Logistically it would be best if all trees are at the same site.

The collection of scions will be difficult if taken from standing trees, due to the crown height. She may need to harvest these from coppice shoots and saplings.

Regards

Graham Durrheim

Scientist (Forest Ecology)

South African National Parks (SANParks)

9.) Dr. Vermeulen replied with this e-mail 14/05/2015:

Hi Jessica

I've discussed this with Graham and Zodwa.

We would accept 12 trees per species, assuming that several of them would survive.

The study site for *Ocotea bullata* would have to be Witelsbos. We would prefer *Curtisia dentata* to be done at Groenkop as no trees were felled there for Zodwa's studies, but could accept Witelsbos if this is problematic.

Regards

Wessel

10.) E-mail sent to researcher from Jessica Hayes 17/05/2015

Good morning Anne,

Please see the closing comments below from the forest scientists in regards to your research project. It seems the situation regarding numbers has been settled, and 12 trees per species accepted.

Please could you now put your project into the attached template format. Please could you address all comments and detail discussed via email over the last couple of days and weeks (which will mostly be addressed under the 'work procedure') in the application form. All research applications need to be submitted in this format for audit purposes.

Once I have this document, I will obtain the final approval from our GM at scientific services and pass onto to park management for their approval. This should hopefully wrap up during the course of next week.

I am also attaching the research agreement and indemnity forms. Please could you also complete these and return them to me.

Kind regards,

Jessica

APPENDIX B: Communication between the researcher and John Roff.

E-mail dated 18 July 2018

Hi Anne

I'm John Roff, Kevin Penderis gave me your information. I'm the person who would be helping you find Ocotea trees in the Karkloof Nature reserve. I have a few points and thoughts:

The Karkloof Nature Reserve, while portions are managed by KZN Wildlife, is composed of several pieces of land, owned by several landowners. I know most of them.

I have found Ocotea bullata populations in two places in the Karkloof, one on Rockwood, and the other fairly nearby in a piece of forest on SAPPI land. Both these places are hard to get to.

At Rockwood, I recall finding about 6 trees, not on a path, about 20 years ago. It would take some time to relocate them, and there is no guarantee that they will still be alive. Ring-barking for medicinal use means that some trees die from over-harvesting. All the trees that I have seen at Rockwood have had bark removed from them. I have only seen quite old trees at Rockwood, most with coppicing due to over-harvesting. This does not mean you should not look there, but I am trying to paint a realistic picture of the effort required and what may or may not be found.

The other place where I have seen Ocotea in the Karkloof is on SAPPI land, about 6 years ago, and I found several trees which were much less disturbed, and I recall finding at least one that appeared to have not been harvested for bark at all. I do not recall how many I found, but there may have been as many as 8 or 10. I did not explore this forest extensively, and there may be more trees in it. This area is also far from a path and requires significant effort

to reach. I have the phone number of the manager of that area, should you wish to approach him for permission. He has met me.

A further issue is that of the conservation value of these trees. In the Karkloof, much more so than in the Southern Cape, the trees are highly threatened by over-harvesting for medicinal use. There appears to be no recruitment of seedlings, and I have seen one tree in the last 30 years of exploring these forests that looked under 50 or 60 years old. This is a species in real danger of local extinction.

Your proposal requires regular visiting of sites and marking and GPS marking of trees for return visits. I am very concerned that these activities may endanger the trees by drawing attention to them, and I do not want to do that. It would be essential, in my view, that the location and location data of the trees be kept secret at all times, and at no stage be made available on a detailed map or shared publicly, even after the publication of the thesis. In fact, it would be a requirement that if I take you to any Ocotea trees, and you sample them, you do not mark them in a way that any passer-by would notice anything different about them.

Having said all this, I am certainly available as a guide to help you try to locate trees on either of these properties. I think allocating only one day to find trees and sample them is too optimistic – we may not find enough trees, and I think it is extremely unlikely that we would find trees matching the requirements outlined in your proposal, the relevant section of which I have quoted below:

Diameters at breast height (DBH) of the trees selected will be similar to the DBH's of the trees used in the southern Cape forest area.

Further points:

You mention the regional ecologist of KZN Wildlife – will they accompany us on the search for the trees? They may know the locations of more trees than I do.

I have seen one tree in the wild in the Karkloof that had no sign of having been harvested for bark, but I think your study stated that no trees previously used for medicinal harvesting would be sampled.

To summarise, I offer the suggestion that we would need to allocate a day to locate suitable trees in the SAPPI location. A separate day would be needed for Rockwood. I think there is more chance of finding enough trees at SAPPI. I think there is virtually no chance of finding 12 trees of the suitable diameter, in either location.

Sorry to sound pessimistic – I am happy to keep chatting about this project. I would love to take you into these forests and look for the trees. I wonder if you shouldn't consider modifying your proposal according to the reality of the situation on the ground.

All the best

John

E-mail Dated 12 August 2018

Hi John,

Sorry, I did not receive your email earlier. Kevin forwarded it to me now.

Regarding your email: I have realized that both Ocotea bullata and Curtisia dentata are in a worse state of endangerment in KZN than in the Southern Cape. Most of the Curtisia trees we found in Nkandla were also harvested from. I have no choice but to use them. I state in

my thesis that only 1 unharvested tree could be found.

One question: the Sappi land, is it also in the Karkloof Nature Reserve? I know they do operate there, but my permit allows me to collect Ocotea bark samples in the Karkloof Reserve only.

I will never publish their individual locations in a thesis ever! I also did not reveal the specific locations of the trees in the Southern Cape in my Master's dissertation. The regional ecologist assigned to my project is the regional ecologist of the area in which Nkandla forest is situated. She cannot help me find trees in Karkloof. It is outside of her area. The KZN wildlife ranger responsible for overseeing Karkloof, and who left me hanging in June, Richard Zuma, apparently does not know his trees – that's what I was told, so he will also not know where they are.

Another thing- my planes, accommodation and everything else are booked. I cannot stay in the Karkloof area for more than one day. My plane will leave without me if I do. So, I am extremely time limited.

Let me first forward your email, and my answer to you, to my supervisor to hear what he suggests. He knows that I do have a problem regarding the Ocotea trees. I will let you as soon as I receive an answer from him.

Kind regards

Anne

Thanks Anne. The Sappi land is in the Karkloof area, but not in the nature reserve.

Regards

John

Then I will not be able to use that area. My permit limits me to the reserve.

I will let you know what my supervisor suggests as soon as I received an answer from him.

Regards

Anne

Email dated 13 August 2018

Hi John

My supervisor just answered my email. He suggested that in light of the scarcity and ecological status of Ocotea in KZN, and the difficulty of reaching these trees in a limited time, we should leave O bullata out of the study and then do an in-depth study on only C. dentata.

I want to thank both you and Kevin for willingness to assist. I am sorry things did not work out as I had planned, but that is research! Thank you again.

Kind regards

Anne

APPENDIX C: The SANParks approval letter.

To acquire and manage a system of national parks which represents the indigenous wildlife, vegetation, landscapes and significant cultural assets of South Africa for the pride and benefit of the nation.

South African NATIONAL PARKS

2015/06/12

Dear Madam,

APPLICATION TO DO RESEARCH IN THE GARDEN ROUTE NATIONAL PARK

PROJECT TITLE: DETERMINATION OF THE SUCCESS OF BRIDGE-GRAFTING AS TECHNIQUE TO RESTORE GROWTH IN TWO GIRDLED MEDICINAL TREE SPECIES IN THE SOUTHERN CAPE FOREST AREA

It is my pleasure to confirm that your application to do long-term research in the Garden Route National Park: Wilderness and Tsitsikamma Areas has been successful. The attached front page of your application (approved by the Area Managers and General Managers) is your permit valid from 12 June 2015 – 31 December 2017. You must keep it handy at all times when in the park and it must be produced on request.

This approval grants you (A.S. van Wyk) free entrance to the Park. You are required to abide by the Parks' rules and regulations, which are available from the Area Manager.

This approval is subject to the standard conditions below. The Park Management staff must be contacted prior to entry into the park (see list of staff members below). Use may only be made of accepted roads and pathways, unless otherwise agreed with the Park Management staff. This permit gives access only to the Wilderness and Tsitsikamma Sections of the Garden Route National Park.

Area	Park Management Staff	Telephone Number:
Wilderness	Sandra Taljaard	044 877 0046
Tsitsikamma	Lesley-Ann Meyer	042 281 1653

Kind Regards,

Jessica Hayes
Regional Ecologist
Garden Route: Scientific Services
PO Box 176
Sedgefield
6573
Tel: 044 343 1302
Fax: 044 343 2331
e-mail: jessica.hayes@sanparks.org

Standard Conditions:
No fires, littering and overnight camping shall be permitted;
No parking at entrances to fire fighting access roads;
No damage may be caused to indigenous flora and fauna or geological formations;
Any other instruction, issued in writing by any GRNP staff, to safe guard the environment or to protect persons against injury, will have to be adhered to.

APPENDIX D: The permit issued by SANParks**.**

APPLICATION TO REGISTER A RESEARCH PROJECT

South African
NATIONAL PARKS

This application form must be completed by all researchers wishing to visit a national park to conduct research and must be submitted to the relevant contact person for the park in which research will be conducted.

FOR OFFICIAL USE ONLY

The signatures below indicate the project has been approved.

General Manager Scientific Services *(where applicable)* ..... Date: 29/05/2015

Manager Social Science *(where applicable)* N/A Date: .....

Area Manager(s) *(where applicable)*

1. Wilderness ..... Date: 11/06/2015
2. Knysna N/A Date: .....
3. Tsitsikamma ..... Date: 29/05/15

General Manager (GRNP) ..... Date: 12/06/15

Animal Use & Care Committee *(where applicable)* ..... Date: .....

Project Rating: .....

Senior Researcher: A.S. van Wyk

Project Title: **Determination of the success of bridge-grafting as technique to restore growth in two girdled medicinal tree species in Wilderness and Tsitsikamma, Western Cape.**

Area in which the study will be conducted: **Garden Route National Park**

Wilderness [X] Knysna [ ] Tsitsikamma [X]

APPROVED [X] NOT APPROVED [ ]

APPENDIX E: The ethical clearance document Ref 2015/CAES/070.

UNISA university of south africa

CAES RESEARCH ETHICS REVIEW COMMITTEE

Date: 18/08/2015

Ref #: **2015/CAES/070**
Name of applicant: **Ms AS Van Wyk**
Student #: **45519560**

Dear Ms Van Wyk,

Decision: Ethics Approval

Proposal: Determination of the success of bridge-grafting as technique to restore growth in two girdled medicinal tree species in the Southern Cape forest area

Supervisor: Mrs E Van Staden

Qualification: Postgraduate degree

Thank you for the application for research ethics clearance by the CAES Research Ethics Review Committee for the above mentioned research. Final approval is granted for the duration of the project.

Please note points 4 and 5 below for further action.

The application was reviewed in compliance with the Unisa Policy on Research Ethics by the CAES Research Ethics Review Committee on 18 August 2015.

The proposed research may now commence with the proviso that:

1) *The researcher/s will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.*
2) *Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study, as well as changes in the methodology, should be communicated in writing to the CAES Research Ethics Review Committee. An amended application could be requested if there are substantial changes from the existing proposal, especially if those changes affect any of the study-related risks for the research participants.*
3) *The researcher will ensure that the research project adheres to any applicable*

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www.unisa.ac.za

national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study.

4) *The Committee acknowledges that the researcher is still awaiting approval from SANBI for the project. The permission letter must be submitted to the Committee as soon as it is obtained.*
5) *As this is a high-risk project, the researcher must provide periodic feedback on the progress made and on any negative repercussions of the research. Feedback must be submitted to the Committee every six months, with the first report to be submitted in January 2016.*

Note:
The reference number [top right corner of this communiqué] should be clearly indicated on all forms of communication [e.g. Webmail, E-mail messages, letters] with the intended research participants, as well as with the CAES RERC.

Kind regards,

Signature
CAES RERC Chair: Prof EL Kempen

Signature
CAES Executive Dean: Prof MJ Linington

NOTE CONDITION 4 HJ

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APPENDIX F: The reports submitted to the ethics committee during 2016 and 2017.

Report 1

12 January 2015

Work on the trees commenced on Monday the 31st of August 2015 where I first girdled the eleven *Ocotea bullata* at the Witelsbos site and covered them in plastic sheeting, securing the edges with duct tape (except for the control tree-thus the 10 trees of each species that would be grafted was covered but not the control tree). Bark sample were cut from the removed bark, placed into cryo-vials and immersed into liquid nitrogen immediately. From the normal tree (not girdled) I cut a 30 x 8 mm cube from the trunk of the tree, plugged the hole with wax and melted it so that it would not fall out, and so that it could properly seal off the hole, preventing desiccation, insect and other pathogen attacks. The sealed hole was then painted with Tree Seal. The following day I girdled the eleven *Curtisia dentata* and treated them exactly the same as for *Ocotea bullata.*

The Wednesday I repacked the samples onto dry ice for the flight to Johannesburg, and from O. R. Tambo airport I drove directly to UNISA Florida campus where preparation work on the samples started. After freeze-drying, grinding and measuring off two sets of 50mg of sample material of each sample (2 x 24 = 48 samples-which would all yield results regarded as "normal", as the trees were not damaged before girdling, except for the previous harvesting experiments conducted on them), the samples were taken to the CSIR where the Nuclear Magnetic Resonance spectrometer is located.

The two sets of samples were divided, and into the first set, 750 microliters of de-ionized water (deuterium oxide) and deuterium methanol each was added to each sample for the metabolite extraction process. These two chemicals extract all metabolites present in the bark except for lipids and fatty acids. Into the second set of samples 1.5 millilitres of deuterium chloroform was added into each sample, which extracts the lipids and fatty acids present in the sample material.

Each sample was then individually analyzed with the NMR spectrometer, and yielded graphs which will in future be used to analyse the samples statistically on a computer programme specifically designed specifically for NMR analysis.

After I flew back to George airport, I directly went back to the sites to do the grafting work. Each tree's plastic sheeting was removed and then first painted with a grafting grade sealer and protector (Tree seal). Scions were cut from saplings and young trees and the grafting procedure was done exactly as described in the proposal. All unions were also painted with Tree Seal but the scions (now inserted into the trees) were not painted.

On the 8th of October my supervisor, Mrs Elize van Staden, and Professor Stoffberg accompanied me to the Witelsbos site so that they can familiarize themselves with what I have done and to inspect my work. It was unfortunately not possible to take them to Groenkop as well because that site has three gates which need to be unlocked, SANParks staff were not available that day and time was running out, but I took them to the site entrance to show them where it was situated, and I assured them that treatment on *Curtisia dentata* was exactly the same as for *Ocotea bullata*. Mrs Van Staden and Prof. Stoffberg agreed that the scion material inserted into the trees that they have seen were still alive and that everything is going well.

In November I returned to the sites again to collect sample material. This time one sample was again collected from the normal trees but from the rest of the trees (control and 10 x grafted), two sets of samples were collected, one above and one below each girdle wound (2 x 23 = 46 samples). From these, and future samples, the variation in metabolite levels will be determined. No tree showed any signs of leaves yellowing or browning, scions were green, well secured and showed no signs of water stress.

I collected my next sets of bark samples the 11th of January 2016 at Witelsbos and the 12th of January 2016 at Groenkop. All grafted trees of both species are callusing whereas there were still no calluses in November whatsoever on either of the two species. The *Curtisia dentata* control tree is also callusing. Seven of the *Ocotea bullata* and three of the *Curtisia dentata* are also coppicing.

My next sample collection will be in March 2016 and my next report will be submitted in July 2016.

Anne van Wyk

Student number: 45519560

21 Myrtle Rigg Avenue

Bonnievale

6730

Cell number: 083 271 0669

Supervisors: Elize van Staden

Prof. W.A.J. Nel

Report 2.

5 July 2016

In January all grafted trees of both species have initiated callus development. A few of the calluses already connected the top and bottom girdle margins. The *Curtisia dentata* girdled control tree was, however, also callusing. In the *Ocotea bullata* girdled control tree callus initiation only occurred on the upper and lower girdle margins. The tree canopies were healthy, and scions still seemed to be alive. A few trees of both species had basal shoots.

During both the March and May monitoring and bark sample collection dates I had a horticulturist accompanying me. It was noted that a few of the very thin scions cut from the previous year's growth had died. A few thicker scions cut from the previous year's growth and all the scions cut from two year old growth were green, alive and growing. By May the scion unions had developed calluses and a few trees of both species were between 50 and 75% covered in calluses (figures 1 and 2). In only one *C. dentata* and one *O. bullata* calluses did not yet connect the top and bottom of the girdle wounds. All grafted trees of both species had callus nodular growth (figure 3). Basal shoots on both species were strong and healthy, therefore providing the root systems with additional carbohydrates. On the basal shoots of some of the *C. dentata* it was noted that some of the young leaves were eaten. This may have been either a herbivorous animal, or insects.

Figure 1. *Ocotea bullata* callus cover. Source: Own

Figure 2. *Curtisia dentata* callus cover. Source: Own.

Figure 3. Callus nodular growth on *O. bullata.* Source: Own.

Some leaf fall was noted but very strong winds were reported for two to three days prior to monitoring. This will be monitored carefully with future monitoring and bark sampling dates. However, the canopies were still within the category “healthy” as described by Vermeulen (2009:57). Table 1 describes the classes to estimate canopy responses.

Table 1. Description of classes to estimate tree canopy responses to girdling and bridge-grafting. Source: Vermeulen (2009:57)

	Class description
0	0% - tree is dead
1	1– 20% healthy crown (few leaves present)
2	21– 40% healthy crown
3	41– 60% healthy crown
4	61– 80% healthy crown
5	81– 100% healthy crown (dense foliage cover, no apparent dieback)

The next monitoring and bark sample collections will be 11 July 2016 for *O bullata* and 12 July 2016 for *C. dentata.*

A.S. van Wyk

Student number: 45519560

21 Myrtle Rigg Avenue

Bonnievale

6730

Cell number: 083 271 0669

Supervisors: Elize van Staden

Prof. W.A.J. Nel

Report 3

1 January 2017

After May 2016, no significant development occurred on the trees as it is the dormant season. There were, however, some callus improvement on one *O. bullata* and one *C. dentata*. All canopies were still healthy, including that of the girdled control trees, and no further abscission occurred. Scions were healthy and basal shoots were strong although *C. dentata* is slower in development.

After reviewing my results around July 2016, Dr. Prinsloo mentioned that my study has "matured", and my supervisors and I decided to only continue the monitoring and bark collection until September 2016 to complete a full year's data collection on the bark samples.

My dissertation is sent to all my supervisors for review and I have submitted a 'Notice of Intention to Submit Dissertation' form on the 8th of December 2016.

Anne van Wyk

Student number: 45519560

21 Myrtle Rigg Avenue

Bonnievale

6730

Cell number: 083 271 0669

Supervisors: Elize van Staden

Prof. W.A.J. Nel

APPENDIX G. The ethical clearance document Ref 2018/CAES/114

UNISA university of south africa

CAES HEALTH RESEARCH ETHICS COMMITTEE

Date: 30/08/2018

NHREC Registration # : REC-170616-051
REC Reference # : 2018/CAES/114
Name : Ms AS Van Wyk
Student #: 45519560

Dear Ms Van Wyk

Decision: Ethics Approval from 01/09/2018 to 31/08/2019

Researcher(s): Ms AS Van Wyk
anne.vanwyk@hotmail.com

Supervisor (s): Prof G Prinsloo
prinsg@unisa.ac.za; 011-471-2167

Working title of research:

Quantification of bioactive compounds in the bark of *Ocotea bullata* and *Curtisia dentata* trees from different South African locations

Qualification: PhD Environmental Science

Thank you for the application for research ethics clearance by the Unisa CAES Health Research Ethics Committee for the above mentioned research. Ethics approval is granted for a one-year period. After one year the researcher is required to submit a progress report, upon which the ethics clearance may be renewed for another year.

Due date for progress report: 31 August 2019

*The **medium risk application** was **reviewed** by the CAES Health Research Ethics Committee on 29 August 2018 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.

University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane
PO Box 392 UNISA 0003 South Africa
Telephone +27 12 429 3111 Facsimile: +27 12 429 4150
www.unisa.ac.za

2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:
*The reference number **2018/CAES/114** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

Prof EL Kempen
Chair of CAES Health REC
E-mail: kempeel@unisa.ac.za
Tel: (011) 471-2241

Prof MJ Linington
Executive Dean : CAES
E-mail: lininmj@unisa.ac.za
Tel: (011) 471-3806

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Preller Street, Muckleneuk Ridge, City of Tshwane
PO Box 392 UNISA 0003 South Africa
Telephone: +27 12 429 3111 Facsimile: +27 12 429 4150
www.unisa.ac.za

APPENDIX H: The confirmation of project registration letter from Ezemvelo KZN Wildlife.

SCIENTIFIC SERVICES ECOLOGICAL ADVICE

Ezemvelo KwaZulu-Natal Wildlife

Scientific Services

1 Peter Brown Drive, Montrose

Pietermaritzburg, 3202

20 June 2018

Mrs. Anne van Wyk

UNISA

Dept. of Agriculture and Environmental Sciences

Florida

Johannesburg

Dear Anne,

RE: CONFIRMATION OF PROJECT REGISTRATION

Thank you for your interest in conducting research in Ezemvelo KwaZulu-Natal Wildlife's (Ezemvelo) protected areas. We are pleased to inform you that your project, "Quantification of bioactive compounds in the bark of *Ocotea bullata* and *Curtisia dentata* trees from different locations in South Africa" at Nkandla Forest Reserve and Karkloof Nature Reserve has been approved and registered, with Mrs. Sharon Louw as the project coordinator. Your project registration number is **E/5143/02** and should be quoted on all correspondence and reports relating to the project.

The following Conservation Manager will need to be contacted and informed of planned field trips prior to your arrival.

Protected Area	**Conservation Manger**	**Cell**	**Email**
Nkandla Forest Reserve	Mr Elliackim Zungu	079 0290 004	Elliackim.Zungu@kznwildlife.com
Karkloof Nature Reserve	Mr Richard Zuma	072 1446 600	Richard.Zuma@kznwildlife.com

The Conservation Managers have been informed of your project. Please make a point of always contacting the Conservation Managers telephonically at least 48 hrs before making a visit to their protected area and present yourself to them upon arrival. The Conservation Manager is responsible for security and discipline in their protected area, and researchers are expected to acquaint themselves with the local regulations before starting their work.

Please ensure that you are in possession of the relevant sampling and collecting permits. Please carry your permit(s) with you in the original and be ready to present them if asked.

Please also take the time to carefully read the conditions and scope of the permit as if any of these are not fulfilled, the permit is not valid.

Data requests from EKZNW need to be made on the official Data Request Form and submitted to database@kznwildlife.com. The collecting records and sample data you will be asked to submit as a condition of your project's registration is incorporated into our biodiversity database. Even if you request that this data not be distributed outside of Ezemvelo, by submitting your information you will be contributing directly to nature conservation efforts as this information forms the basis of the KwaZulu-Natal Systematic Biodiversity Conservation Plan.

As a condition of being allowed to conduct work in Ezemvelo's protected areas you will be asked for annual progress reports and a copy of your final report. The format for the annual progress report, due in April every year, will be emailed to you in due course.

I wish you success with your project. Please contact me if you require any further assistance.

Yours Sincerely,

Mrs Sharon Louw

District Ecologist: iLembe & King Cetshwayo

APPENDIX I: The permit issued by Ezemvelo KZN Wildlife.

ORIGINAL

ORDINARY PERMIT

Fee: R 50.00 Permit No: OP 2826/2018

Receipt No: 3036/2018 Contact: Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 11 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 6205290027084

Mrs Anna Susanna Van Wyk	**Residential Address**
Dept of Agriculture and	**Dept of Agriculture and**
Environmental Sciences	**Environmental Sciences**
Florida JHB	**Florida JHB**
Environmental Sciences	**Environmental Sciences**
21 Myrtle Rigg Avenue	**21 Myrtle Rigg Avenue**
Bonnievale	**Bonnievale**
6730	**6730**

Province: Western Cape

In the capacity of Researcher

To Collect the following species of Plants

THIS PERMIT REPLACES CANCELLED PERMIT OP2653/2018

ASSEGAI *(CURTISIA DENTATA)*

Collect bark samples (30mmx8mm), over 7 collection periods (12x7=84 samples per site)

12 trees between 18 and 35 DBH throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Nkandla Forest.

BLACK STINKWOOD *(OCOTEA BULLATA)*

Collect bark samples (30mmx8mm), over 7 collection periods (12x7=84 samples per site)

12 trees between 18 and 35 DBH throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Karkloof Nature Reserve.

Please read the Terms and Conditions under which this Permit is issued

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

for CHIEF EXECUTIVE Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
Tel +27 33 845 1320 / 1324. Fax: +27 33 845 1747. Fax to Email: 086 529 3320
Email: permits@kznwildlife.com. Website: www.kznwildlife.com

1. It is valid only:

   (i) from : 01 September 2018 to : 30 September 2019

   (ii) in the original

   (iii) if all **4** pages are signed by the permit holder named above

   (iv) to the permit holder named above and the following Nominees :

2. By signing the permit or licence the holder accepts, and agrees to comply with the conditions under which it is issued.

3. Permit shall be carried by holder, or the specified nominees, at all times during use.

4. Outside of E KZN Wildlife areas, use of this permit is subject to landowner's or controlling authority's written permission.

5. Prior to collecting in areas under the control of the E KZN Wildlife the holders shall contact the Officer-in-Charge of the area at least 48 (Forty-eight) hours before commencing, and shall comply with any conditions which the Officer may impose at his discretion. The officer may refuse collection or capture at his or her discretion.

6. At least one representative specimen (preferably at least one male and one female) of each species collected from each locality must be lodged with a recognised South African museum/herbarium. Holotype specimens, and half the number of paratype specimens, of any new species MUST BE DEPOSITED with a recognised South African museum/herbarium, and may only leave South Africa on a loan basis. These specimens are to be deposited in the SA museums within a year of publishing the description of the new species. The holder shall provide the Chief Executive Officer, KZNNCS with the name of the museum at which the specimens have been lodged, and the accession number of each specimen. This condition relates to unavoidable by-catch of non-target organisms as well.

7. A copy or copies of any publication arising from the authority herein contained will be made available to E KZN Wildlife.

8. Should renewal of this permit be desired, a minimum of one month's notice is required.

9. (i) Reserving accommodation within E KZNWildlife areas is entirely the responsibility of the permit holder. Booking is obtainable at the Central Booking Office, Telephone 033 8451000.(ii) Any assistance required from Board staff will be subject to other demands on the Officer's time and must be arranged in advance with him/her.

10. Failure to comply with the terms and conditions of this permit could result in the cancellation thereof and jeopardise the re-issue of a permit in the future.

11. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE

(museum (ddmmyy) (Seconds (Seconds

Accession) Accuracy) Accuracy).

Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

12. PERMIT MUST BE CARRIED BY HOLDER, OR SPECIFIED NOMINNES, AT ALL TIMES DURING USE.

13. PRIOR TO COLLECTING IN EZEMVELO'S PROTECTED AREAS THE HOLDERS OF THIS PERMIT MUST CONTACT THE NKANDLA FOREST RESERVE OFFICER -IN-CHARGE (MR

ELLIACKIM ZUNGU - CELL 0790290004) AND KARKLOOF NATURE RESERVE OFFICER –

IN-CHARGE (MR RICHARD ZUMA CELL : 0721446600) OF THE PROTECTED AREAS

ATLEAST 48 (FOURTY EIGHT) HOURS BEFORE ARRIVING AT THE PROTECTED AREA,

PRESENT THEMSELVES TO THE OFFICER -IN-CHARGE UPON FIRST ARRIVAL AND MUST COMPLY WITH ANY CONDITIONS WITH THE OFFICER MAY IMPOSE AT HIS DISCRETION.

14. AUTHORIZATION IS ACCEPTED ON THE UNDERSTANDING THAT THE RESEARCHER WILL NOT TRIGGER BIO PROSPECTING REGULATIONS.

15. EZEMVELO KZN WILDLIFE FIELD RANGERS AT INKANDLA CAN ASSIST WITH LIMITED SUPPORT, HOWEVER THERE IS NO FIELD RANGER SUPPORT AT KARKLOOF NATURE RESERVE.

16. THE RESEARCHER WILL HAVE TO MAKE THE NECESSARY ARRANGEMENTS FOR PRIVATE SUPPORT IN FIELD , INCLUDING THE VERIFICATION OF TARGET SPECIES FOR RESEARCH PURPOSES.

17. ALL COLLECTING RECORDS MUST BE SUBMITTED TO THE EZEMVELO DISTRICT ECOLOGIST : KING CETSHWAYO AND ILEMBE, SHARON LOUW ON THE EZEMVELO BIODIVERSITY DATABASE DATA IMPORT TEMPLATE.

18. IT IS CRITICAL THAT EACH HOLE LEFT BY SAMPLING WILL BE PLUGGED WITH PARAFFIN WAX(CANDLE WAX) AND PAINTED WITH WITH WATER-BASED BITUMEN

SEALER AND PROTECTOR TO PREVENT THE INVASION OF INSECTS, FUNGI AND

POSSIBLE OTHER DISEASE-CAUSING PESTS AS SPECIFIED IN THE RESEARCH METHODOLOGY.

19. A COPY OR COPIES OF ANY PUBLICATION ARISING FROM THE AUTHORITY HEREIN CONTAINED MUST BE MADE AVAILABLE TO EZEMVELO WITHIN FOUR WEEKS OF PUBLICATION.

20. SHOULD A RENEWAL OF THIS PERMIT BE DESIRED, A MINIMUM OF ONE MONTH'S NOTICE IS REQUIRED.

APPENDIX J: The licence to collect bark samples from endangered tree species from DEFF.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

F 033 342 8783
033 392 7722
AyandaMny@nda.agric.za

DAFF
Forestry Regulations & Support
Private Bag X 9029
Pietermaritzburg
3200

Mr. A.A. Mnyungula
Assistant Director

LICENCE: KZN0027/07/18-19

TO AUTHORISE ACTIONS AND ACTIVITIES AFFECTING a) INDIGENOUS TREES IN NATURAL FORESTS **AND/OR b) PROTECTED TREES, OR THE ACQUISITION OR DISPOSAL IN ANY MANNER OF SUCH TREES OR THEIR PRODUCTS [SECTIONS 7(1) AND 15(1) OF THE NATIONAL FORESTS ACT, 1998, AS AMENDED]**

1.1.1 This licence grants authority under the National Forests Act, 1998 (Act No. 84 of 1998), as amended, to carry on one or more of the activities, upon such conditions, and for such a period, as specified in more detail below; does not exempt the licensee from adhering to the provisions of any other law; is valid for the **26 months** as from the date issued, must re-apply at Pietermaritzburg Forestry Office a month before the expiry of the specified period, if still required.

A	**PARTICULARS OF LICENSEE**
Applicant	Anna Susanna van Wyk
Applicant I.D. No.	6205290027084
Postal address	21 Myrtle Ri g Avenue Bonnievale 6730
Residential address	Same as above
Tel. / Cell. / Fax numbers	Tel N/A Cell. 0832710669 Email Anne.vanw k hotmail.com
Name of areas affected	**Nkandla Forest reserve near Nkandla village and Karkloof Nature Reserve near Howick, KwaZulu-Natal**

B	LICENSED ACTIVITIES

1. **In respect of indigenous trees in natural forests**

~~Cut/Prune~~

~~-Collect: bark / branches / logs~~

~~-Sell / donate cut wood or logs/ produce / products~~

~~-Remove / transport: cut wood or logs/ produce / products~~

~~-Purchase / receive / possess: trees / produce / products~~

~~-Export: trees / produce / products (to: _____[country])~~

2. **In respect of protected trees**

~~-Damage / disturb~~

~~-Cut/Prune~~

Collect: bark ~~/ branches / logs~~

~~-Sell / donate cut wood or logs / produce / products~~

~~-Remove / transport: cut wood or logs/ produce / products~~

~~-Purchase / receive / possess: bark/ wood / produce / products~~

~~-Export: trees / produce / products (to: _____[country])~~

If a declared champion tree: **None**

3. Cutting of live trees / collection of produce from live trees:

According to sustainable management plan? Yes ~~/ No / N/A~~

4. Numbers and sizes of trees per species: **24 protected trees namely: 12 *Ocotea Bullata* (Black Stinkwood) and 12 *Curtisia Dentata* (Assegai).**

5. ~~Canopy trees[1] / under-storey trees[2] / saplings[3] / senile trees /~~ **mature tree/** ~~vigorous tree / dead trees / windfalls~~

6. Estimated quantity / volume of products per species: **02 protected tree species: 2g sample per tree (12 samples per species) bimonthly between September 2018 and September 2019 (7 x sampling dates). Thus the total will be 12x7 samples=84 samples x 2 species = 168 samples over a period of one year.**

7. Origin: **Nklandla Forest reserve near Nklandla village and Karkloof Nature Reserve near Howick, KwaZulu-Natal.**

8. Destination: **Gauteng, University of South Africa, Florida campus, CAES laboratory.**

9. Specifications, prescriptions and remarks: **Samples will be collected bimonthly between September 2018 and September 2019.**

C	LICENCE CONDITIONS

1. General license rules

This license –

(a) is not transferable (you cannot pass, or cede it to another person), and

(b) is only valid for the period it was issued for.

2. Showing this license

(a) You must produce this license together with your valid identity document on demand to any forest officer or police officer.

(b) The person(s) moving or transporting timber or other products on your behalf must at all times be in possession of a certified / stamped copy of this licence.

(c) Distributors/wholesalers of firewood / braai wood must provide all of their retail clients with a certified / stamped copy of this licence, who have to produce it on demand to any forest or police officer.

3. Any further conditions:

3.1. This licence is for the collection of 168 bark samples of *Ocotea bullata* and *Curtisia dentata* (84 samples per species) over a period of one year for research purposes i.e. to analyse the variability of bioactive compounds in the bark of these medicinal tree species at Nkandla Forest reserve near Nkandla village and Karkloof Nature reserve near Howick, KwaZulu-Natal.

3.2. Only barks of 8x25mm in size are permitted to be collected.

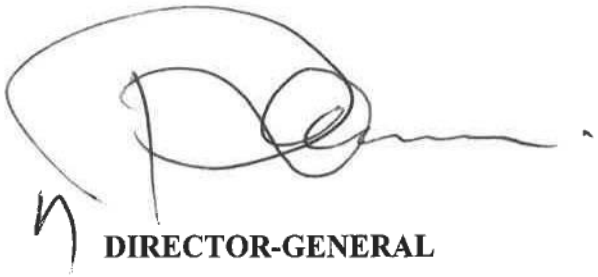
3.3. The sampling activity should not disturb the vascular cambium of the trees as this will negatively affect the tree survival.

3.4. The holes left by sampling must be plugged with wax and sealed with a water-based bitumen grafting sealer and protector to prevent rotting of the trees.

3.5. It is the responsibility of the applicant to liaise with the land owner if the affected tree(s) is or are on private land.

3.6. DAFF officers and other relevant stakeholders should at all times be permitted to monitor the compliance of the licence conditions.

3.7. Failure to comply with the rules and conditions stated herein will be subjected to the withdrawal of this license or legal action be taken against the applicant.



DIRECTOR-GENERAL

DATE STAMP

APPENDIX K: Acknowledgement of Discovery Phase Bioprospecting Notice.

environmental affairs

Department:
Environmental Affairs
REPUBLIC OF SOUTH AFRICA

Private Bag X 447· PRETORIA · 0001· 437 Steve Biko Road· Acardia · PRETORIA
Tel (+ 27 86) 111 2468

Ref: BABS/000418P
Enquiries: Ms Nomusa Mbuyazi
Tel: 012 399 9614 **Fax:** 086 530 9607 **E-mail:** NMbuyazi@environment.gov.za

Dr Gerhard Prinsloo
Department of Agriculture and Animal Health
C/o Christiaan de Wet and Pioneer Street
Florida
JOHANNESBURG
1709

E-mail: prinsg@unisa.ac.za

Dear Dr Prinsloo,

RE: NOTIFICATION FOR BIOPROSPECTING DISCOVERY PHASE BY UNIVERSITY OF SOUTH AFRICA REFERENCE BABS/000418N

The Department of Environmental Affairs (DEA) would like acknowledge receipt of the e-mailed Notification for bioprospecting discovery phase from University of South Africa on the 31 May 2018, submitted in compliance with the National Environmental Management: Biodiversity Act, 2004 (Act No. 10 of 2004) and the Bioprospecting Access and Benefits Sharing (BABS) Amendment Regulation of 2015. The title of the project is "*Quantification of bioactive compounds in the bark of Ocotea bullata and Curtisia dentanta from different locations in south Africa*".

The following documents were received:

1) Annexure 1: Notification for bioprospecting discovery phase;
2) ID copy of the project leader, Ms Anne van Wyk;
3) ID copy of Dr Gerhard Prinsloo; and
4) Annexure A: Methodology.

Your notification has been captured in the Department database and has been assigned a reference number: BABS/000418N. Kindly quote this reference number in any future correspondence with respect to this notification. You are reminded to comply with the NEMBA section 81(1) (a) once the project reaches commercialization phase.

1

The proposed timeframe of the project is September 2018 to September 2019. Given that, you are requested to submit an annual status/progress report of the project to the Department of Environmental Affairs.

Once again the Department wishes to express its sincere appreciation of the efforts made by the University of South Africa to ensure compliance with the requirements of NEMBA and the BABS regulation of 2015.

Should you have any further enquiry in this regard, please contact the responsible official in the department as detailed in this enquiry section or Mr Ntambudzeni Nepfumembe, e-mail: NNepfumembe@environment.gov.za, tel: 012 399 9612 or Mrs Lactitia Tshitwamulomoni, e-mail: LMabadahane@environment.gov.za, tel: 012 399 9611.

Yours sincerely

Ms Nosipho Ngcaba
Director-General
Department of Environmental Affairs
Letter signed by: Ms Natalie Feltman
Designation: Director: Bioprospecting and Biodiversity Economy
Date: 08/06/18

APPENDIX L: The list of 879 chemical compounds detected with GC-MS analysis in fractions 10 to 25.

Compound	Compound
((1R,4aR,4bS,10aR)-1,4a-Dimethyl-7-(propan-2-ylidene)-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthren-1-yl)methanol	Benzene, 1,2,4-trimethyl-
((1S,4aR,4bR,10aR)-7-Isopropyl-1,4a-dimethyl-1,2,3,4,4a,4b,5,6,10,10a-decahydrophenanthren-1-yl)methanol	Benzene, 1,2-diethyl-
((4aS,8S,8aR)-8-Isopropyl-5-methyl-3,4,4a,7,8,8a-hexahydronaphthalen-2-yl)methanol	Benzene, 1,2-diethyl-
((8R,8aS)-8-Isopropyl-5-methyl-3,4,6,7,8,8a-hexahydronaphthalen-2-yl)methanol	Benzene, 1,2-dimethoxy-4-[[(4-methylphenyl)sulfonyl]methyl]-
(-)-Spathulenol	Benzene, 1,3-diethyl-
(+)-3-Carene, 10-(acetylmethyl)-	Benzene, 1,3-dimethyl-
(1aR,4aS,8aS)-4a,8,8-Trimethyl-1,1a,4,4a,5,6,7,8-octahydrocyclopropa[d]naphthalene-2-carbaldehyde	Benzene, 1,3-dimethyl-5-(1-methylethyl)-
(1R,2R,4S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decan-4-ol	Benzene, 1,4-diethyl-
(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	Benzene, 1-ethenyl-3-ethyl-
(1R,4aR,4bS,10aR)-1,4a-Dimethyl-7-(propan-2-ylidene)-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene-1-carbaldehyde	Benzene, 1-ethyl-2,3-dimethyl-
(1R,4S)-4-Isopropyl-1,6-dimethyl-1,2,3,4-tetrahydronaphthalen-1-ol	Benzene, 1-ethyl-2-methyl-
(1R,5S,6R)-2,7,7-Trimethylbicyclo[3.1.1]hept-2-en-6-yl acetate	Benzene, 1-ethyl-3,5-dimethyl-
(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	Benzene, 1-ethyl-3-methyl-
(1R,9R,E)-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene	Benzene, 1-ethyl-4-(1-methylethyl)-
(1S,3aR,4R,8R,8aS)-1-Isopropyl-3a-methyl-7-methylenedecahydro-4,8-epoxyazulene	Benzene, 1-ethyl-4-methyl-
(1S,7S,8aR)-1,8a-Dimethyl-7-(prop-1-en-2-yl)-1,2,3,7,8,8a-hexahydronaphthalene	Benzene, 1-methyl-2-(2-propenyl)-
(2R,8R,8aS)-8,8a-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,7,8,8a-hexahydronaphthalene	Benzene, 1-methyl-2-propyl-
(3E,7E,11E)-1-Isopropyl-4,8,12-trimethylcyclotetradeca-3,7,11-trienol	Benzene, 1-methyl-3-(1-methylethyl)-
(3-Methylphenyl) methanol, 2-methylpropyl ether	Benzene, 1-methyl-3-propyl-
(3R,3aR,3bR,4S,7R,7aR)-4-Isopropyl-3,7-dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2]319ydrazi-3-ol	Benzene, 1-methyl-4-(1-methylpropyl)-

(3R,4aS,8aS)-8a-Methyl-5-methylene-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	Benzene, 1-methyl-4-(2-propenyl)-
(3R,6R)-3-Hydroperoxy-3-methyl-6-(prop-1-en-2-yl)cyclohex-1-ene	Benzene, 1-methyl-4-propyl-
(3R,6S)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	Benzene, 2,4-dimethyl-1-(1-methylethyl)-
(5R,10R)-10-Methyl-6-methylene-2-(propan-2-ylidene)spiro[4.5]dec-7-ene	Benzene, 2-ethyl-1,3-dimethyl-
(9Z,12Z,15Z)-3,7-Dimethyloct-6-en-1-yl octadeca-9,12,15-trienoate	Benzene, 2-ethyl-1,4-dimethyl-
2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	Benzene, 4-ethyl-1,2-dimethyl-
4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	Benzene, n-butyl-
Tetradec-2-enal	Benzene, pentamethyl-
2,7,8-Trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)chroman-6-ol	Benzenecarbothioic acid, 2,4,6-triethyl-, S-(2-phenylethyl) ester
(R,1E,5E,9E)-1,5,9-Trimethyl-12-(prop-1-en-2-yl)cyclotetradeca-1,5,9-triene	Benzeneethanamine
(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	Benzeneethanamine, á-methyl-
.tau.-Cadinol	Benzeneethanol, 2-methoxy-à-methyl-
.tau.-Muurolol	Benzenehexanenitrile, á,á-dimethyl-î-oxo-
[3,3-Dimethyl-2-(3-methylbuta-1,3-dienyl)cyclohex-1-enyl]methanol	Benzenemethanesulfonyl chloride
{Methanediylbis[(3,4,6-trichlorobenzene-2,1-diyl)oxy]}bis(trimethylsilane)	Benzenemethanol, 2-methyl-
1-(1-Butoxypropan-2-yloxy)propan-2-yl 2-methylbutanoate	Benzenemethanol, 4-methyl-
1-(1-Butoxypropan-2-yloxy)propan-2-yl pentanoate	Benzenemethanol, à-(1-phenylaminoethyl)-
1-(2-Methoxyethoxy)-2-methyl-2-propanol, methyl ether	Benzenemethanol, à,à,4-trimethyl-
1-(3,4-Dimethoxyphenyl)propan-1-one	Benzenemethanol, à-methyl-
1-(3-Methyl-cyclopent-2-enyl)-cyclohexene	Benzenemethanol, à-methyl-, ®-
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	Benzenesulfonamide, N-benzyl-2-ethoxy-5-(tetrazol-1-yl)-
1,1,1-Trichloro-4,4-dimethyl-4-vinyldisilethylene	Benzo[b]thiophene, 2,5,7-trimethyl-
1,1,2-Triacetoxyethane	Benzocycloheptatriene
1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulene-4,7-diol	Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester
1,1,7,7a-Tetramethyl-1a,2,6,7,7a,7b-hexahydro-1H-cyclopropa[a]naphthalene	Benzoic acid, 3-formyl-2,4-dihydroxy-6-methyl-, methyl ester
1,10-Dichlorodecane	Benzoic acid, 4-ethoxy-, ethyl ester
1,1-Dimethoxy-2-phenylpropane	benzoic acid, 4-hexyl-, 4-(1-oxooctyl)phenyl ester
1,2,3,4-Tetrahydro-3-isopropyl-5-methyl-1-oxonaphthalene	Benzoic acid, silver(1+) salt
1,2,3-Propanetriol, 1-acetate	Benzonitrile, 3-(4-benzyloxybenzylidenamino)-
1,2,4,5-Tetroxane, 3,3,6,6-tetramethyl-	Benzothiazole
1,2,5-Oxadiazole	Betulinaldehyde

1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester

1,2-Benzenediol, o-(3-cyclopentylpropionyl)-o'-(2-methylbenzoyl)-

1,2-Benzenediol, o-(4-methoxybenzoyl)-o'-(4-methylbenzoyl)-

1,2-Benzenediol, O,O'-di(2-methylbenzoyl)-

1,2-Benzisothiazole, 3-butoxy-

1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-

1,2-Dimethoxy-4-(1,2,3-trimethoxypropyl)benzene

1,2-Diphenylethylamine

1,2-Ethanediol, dipropanoate

1,2-Propanedione, 1-phenyl-

1,3(2H)-Benzodioxole, 2-[(diethylamino)methyl]-5-[(2,4-diamino-5-pyrimidinyl)methyl]-

1,3,5-Cycloheptatriene

1,3,5-Cycloheptatriene, 3,7,7-trimethyl-

1,3,5-Cycloheptatriene, 7-ethyl-

1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-

1,3-Benzenediol, 4-propyl-

1,3-Benzenediol, o-(2-bromopropionyl)-o'-(3-methylbenzoyl)-

1,3-Benzenediol, O,O'-di(2-methylbenzoyl)-

1,3-Benzenediol, oo'-di(3-methylbenzoyl)-

1,3-Bis-(2-cyclopropyl,2-methylcyclopropyl)-but-2-en-1-one

1,3-Cyclohexadiene-1-methanol, à,2,6,6-tetramethyl-, (.+-.)-

1,3-Cyclopentadiene, 1,2,3,4-tetramethyl-5-(2-methoxyethyl)-

1,3-Dioxane

1,3-Dioxolane, 2-(1-phenylethyl)-

1,3-Dioxolane, 2-(5-bromopentyl)-

1,3-Dioxolane, 2-methyl-2-phenyl-

1,3-Dioxolane, 2-pentadecyl-

1,3-Dithiolo[4,5-b]furan, tetrahydro-3a-methyl-

1,3-Propanediol, 2-dodecyl

1,4-Benzenedimethanol

1,4-benzenediol, 2,5-dimethoxy-

1,4-Butanediamine

Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl-

Bicyclo[3.1.1]heptane, 6,6-dimethyl-3-methylene-

Bicyclo[4.2.0]octa-1,3,5-trien-7-ol

Bicyclo[5.1.0]octane

Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol, 4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R*,12R*)]-

Bicylo[4.1.0]heptane, 7-bicyclo[4.1.0]hept-7-ylidene-

Biphenylene, 1,2,3,6,7,8,8a,8b-octahydro-4,5-dimethyl-

Borane, trimethyl-

Boron trifluoride

Boron, diethyl(4-methyl-3,5-heptanediiminato-N,N')-, (t-4)-

Bufa-20,22-dienolide, 14,15-epoxy-3,16-dihydroxy-, (3á,5á,15á,16á)-

Butanamide, 3,3-dimethyl-

Butane, 1-methoxy-3-methyl-

Butanenitrile

Butanenitrile, 4-chloro-

Butanoic acid, tridec-2-ynyl ester

Butyl ethylphosphonofluoridate

Butylphosphonic acid, 2-cyclohexylethyl heptyl ester

Butyrovanillone

Cadala-1(10),3,8-triene

Carbon disulfide

Carbonic acid, decyl ethyl ester

Carbonic acid, ethyl 2-propenyl ester

Carda-16,20(22)-dienolide, 3-[(6-deoxy-3,4-O-methylenehexopyranos-2-ulos-1-yl)oxy]-7,8-epoxy-11,14-dihydroxy-12-oxo-, (3á,5á,7á,11à)-

Carvone oxide, cis-

Caryophyllene oxide

Catechol

Cedren-13-ol, 8-

Cetene

CH3C(O)CH2CH2OH

Chloromelfoquine

Cholest-4-en-3-one

1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate]

1,5-Heptadien-4-ol, 3,3,6-trimethyl-

1,5-Undecadiene, 6,7,7,8,8,9,9,10,10,11,11,11-dodecafluoro-3-methyl-

1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-

1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-

1,6-Dimethyl-5-oxo-1,2,3,5-tetrahydroimidazo[1,2-a]pyrimidine

1,7-Hexadecadiene

1,7-Nonadiene, 4,8-dimethyl-

1,8,11,14-Heptadecatetraene, (Z,Z,Z)-

1,8-Nonadiene, 2,8-dimethyl-

1-[3-(2-Bromophenyl)-2-thioureido]-1-deoxy-á-d-glucopyranose 2,3,4,6-tetraacetate

10-(2-Hydroxyethyl)-3,7,8-trimethyl-10H-benzo[g]322ydrazine-2,4(3H)-dione

10,11-Dimethyl-tricyclo[4.3.1.1(2,5)]undecane-10,11-diol

10,18-Bisnorabieta-8,11,13-triene

10-Undecenal

10-Undecenoic acid, methyl ester

10-Undecyn-1-ol, TBDMS derivative

11-Dodecenol

11-Hexadecynal

12-Methyl-E,E-2,13-octadecadien-1-ol

13-Octadecenal, (Z)-

13-Tetradecen-1-ol acetate

1-Acetoxynonadecane

1-Butanamine

1-Butanamine, 3-methyl-

1-Butanamine, 4-methoxy-

1-Butanol, 4-[(tetrahydro-2H-pyran-2-yl)oxy]-

1-Butanone, 1-(2,4,6-trihydroxy-3-methylphenyl)-

1-Buten-3-yne, 1-chloro-

1-Butoxy-2-propanol acetate

1-Cyclohexyl-1-pentyne

Cholesta-8,24-dien-3-ol, 4-methyl-, (3á,4à)-

Cholestan-3-amine, N,N,4,4-tetramethyl-, (3á,5à)-

cis-5-Dodecenoic acid, methyl ester

cis-à-Copaene-8-ol

cis-á-Farnesene

cis-Calamenene

cis-Linaloloxide

cis-Muurola-4(15),5-diene

cis-Z-à-Bisabolene epoxide

Cobalt, [(1,2,5,6-ü)-1,5-cyclooctadiene][(1,2,3,4,5-ü)-1,2,3,4-tetraphenyl-2,4-cyclopentadien-1-yl]-

ç-Sitosterol

ç-Tocopherol

Cyclobutene, 2-propenylidene-

Cyclododecyne

Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-

Cyclohexasiloxane, dodecamethyl-

Cyclohexene, 2-ethenyl-1,3,3-trimethyl-

Cyclohexyl methylphosphonofluoridate

Cyclooctasiloxane, hexadecamethyl-

Cyclopentane, heneicosyl-

Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester

cyclopentanone, 2-decyl-

Cyclopropane, 1-methylene-2-phenyl-2-trimethylsilyl-

Cyclopropanecarboxamide, N-benzoyloxy-

Cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-, 2-methyl-4-oxo-3-(2-pentenyl)-2-cyclopenten-1-yl ester, [1R-[1à[S*(Z)],3á]]-

Cyclopropanecarboxylic acid, 3-(3-buten-2-yl)-3-methyl-, methyl ester

Cyclopropanemethanol, 2-methyl-2-(4-methyl-3-pentenyl)-

Cyclopropanemethanol, à,2-dimethyl-2-(4-methyl-3-pentenyl)-, [1à(R*),2à]-

Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester

Cyclotetradecane

d,l-trans-4-Methyl-5-methoxy-1-(1-methoxy-1-isopropyl)cyclohex-3-ene

1-Cyclohexyldimethylsilyloxybutane

1-Docosene

1-Dodecanamine

1-Dodecanamine, N,N-dimethyl-

1-Dodecanol

1-Eicosanol

1H-1,3-Benzimidazole, 5-methoxy-1-methyl-2-(4-morpholinylmethyl)-

1H-3a,7-Methanoazulene-6-methanol, 2,3,4,7,8,8a-hexahydro-3,8,8-trimethyl-, [3R-(3à,3aá,7á,8aà)]-

1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis-

1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1aà,4á,4aá,7à,7aá,7bà)]-

1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aà,4aà,7á,7aá,7bà)]-

1H-Cycloprop[e]azulene, decahydro-1,1,4,7-tetramethyl-, [1aR-(1aà,4á,4aá,7á,7aá,7bà)]-

1-Heneicosanol

1-Heptafluorobutyryloxy-10-undecene

1-Heptatriacotanol

1-Hexadecanol

1-Hexadecyn-3-ol, 3,7,11,15-tetramethyl-

1-Hexanol, 6-amino-

1H-Indene, 1-ethylidene-

1H-Indene, 1-ethylideneoctahydro-7a-methyl-, cis-

1H-Indene, 1-methyl-3-propyl-

1H-Indene, 1-methylene-

1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-

1H-Indene, 2,3-dihydro-1,6-dimethyl-

1H-Indene, 2,3-dihydro-2-methyl-

1H-Indene, 2,3-dihydro-4-methyl-

1H-Indene, octahydro-, cis-

1H-Pyrazole-4-carboxylic acid, 3-amino-

1H-Pyrazolo[3,4-d]pyrimidin-4-amine

1-Methoxy-3-methyl-3-butene

1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2one

1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-, [1R-(1à,4á,4aá,8aá)]-

1-Naphthalenol, 5,6,7,8-tetrahydro-2,5-dimethyl-8-(1-methylethyl)-

1-Nonanol

1-Octadecanesulphonyl chloride

1-Octadecyne

Dammarane-3,12,25-triol, 20,24-epoxy-, 3-(hydrogen propanedioate), (3à,12á,24R)-

Decanal

Decanal dimethyl acetal

Decane

Decane, 3-bromo-

Decanoic acid, methyl ester

Dehydroelsholtzia ketone

Dehydroergosterol 3,5-dinitrobenzoate

Di®-but-2-enyl phthalate

Diallyl isophthalate

Diazoprogesterone

Dibutyl phthalate

Diethyl Phthalate

Difluorochloromethane

Difluoromethane

Dihydroxanthin

Dimethyl sulfone

Dimethyl-(isopropyl)-silyloxybenzene

Dimethyl,fluoromethyl,phenylsilane

Diphenyl sulfone

Disulfide, bis[1-(methylthio)ethyl]

dl-7-Azatryptophan

dl-à-Tocopherol

Doconexent

Dodecane, 1,1-dimethoxy-

Dodecane, 1-iodo-

Dodecanoic acid

Dodecanoic acid, methyl ester

Duroquinone

E-8-Hexadecen-1-ol acetate

Eicosane

Eicosane, 2-methyl-

endo-1,5,6,7-Tetramethylbicyclo[3.2.0]hept-6-en-3-ol

Epicubenol

Epi-Inositol

Ergosta-5,7,9(11),22-tetraen-3-ol, (3á,22E)-

1-Pentanone, 1-(4-methylphenyl)-

1-Phenanthrenecarboxaldehyde, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1à,4aà,10aá)]-

1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,4a,7-trimethyl-, methyl ester, [1R-(1à,4aá,4bà,7à,10aà)]-

1-Propanone, 1-phenyl-

1-Propene, 3,3-dichloro-

1-Tridecene

2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol

2-(1-{[4-(Trifluoromethyl)phenyl]methyl}-1,2,3-triazol-4-yl)ethanamine

2(2-Bromoethyl)-1,3-dioxane

2(3H)-Furanone, 5-butyldihydro-4-methyl-, cis-

2(3H)-Furanone, 5-heptyldihydro-

2(3H)-Furanone, 5-hexyldihydro-

2(3H)-Furanone, dihydro-5-pentyl-

2(3H)-Furanone, dihydro-5-propyl-

2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-

2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-

2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid

2,10-Dodecadien-1-ol, 3,7,11-trimethyl-, (ñ)-

2,10-Dodecadien-1-ol, 3,7,11-trimethyl-, (Z)-

2,2’-(Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) dibenzoate

2,2,4,4-Tetramethyl-1,3-dithia-2,4-disilacyclobutane

2,2,4-Trimethyl-1,3-pentanediol diisobutyrate

2,2’-Bi-2H-pyran, octahydro-

2,2-Dimethylindene, 2,3-dihydro-

2,3,3-Trimethyl-2-(3-methyl-buta-1,3-dienyl)-cyclohexanone

2,3,4-Trifluorobenzoic acid, 2-fluorophenyl ester

2,4,6-Trimethyl-2-(4-methyl-pent-3-enyl)-2H-pyran

2,4,7,9-Tetramethyl-5-decyn-4,7-diol

2,4-Decadienal

2,4-Decadienal, (E,E)-

2,4-Diamino-6-[[p-chlorobenzyl]nitrosoamino]-5,6,7,8-tetrahydroquinazolne

Estran-3-one, 17-(acetyloxy)-2-methyl-, (2à,5à,17á)-

Ethanamine, 2-methoxy-

Ethane, 1,1,1-trimethoxy-

Ethaneperoxoic acid, 1-cyano-1,4-diphenylpentyl ester

Ethanol, 2-(dodecyloxy)-

Ethanol, 2-(eicosyloxy)-

Ethanol, 2-(trimethylsilyl)-

Ethanol, 2-nitro-

Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-

Ethanone, 2-(acetyloxy)-1-phenyl-

Ethyl 2-(benzylamino)-2-(2-chloroacetamido)-3,3,3-trifluoropropionate

Ethyl Acetate

Ethylbenzene

ë-Tocopherol

Eudesma-4(15),7-dien-1á -ol

Farnesene epoxide, E-

Flopropione

Formamide, N-methyl-

Fumaric acid, 3,5-dichlorophenyl ethyl ester

Fumaric acid, ethyl 2,3,5-trichlorophenyl ester

Fumaric acid, ethyl 3-methylbut-2-yl ester

Furan, 2-propyl-

Geraniol

Geranyl acetate, 2,3-epoxy-

Germacrene D

Globulol

Glutaraldehyde bis-(O-pentafluorophenylmethyl-oxime)

Glutaric acid, di(1-phenylpropyl) ester

Glycerol 1-palmitate

Glycine

Glycine, methyl ester

2,4-Dichlorophenethylamine
2,4-Diethyl-5-methyloxazole
2,4-Difluorobenzoic acid, 2-formyl-4,6-dichlorophenyl ester
2,4-Difluorobenzoic acid, 3-methylbutyl-2 ester
2,4-Dihydroxybenzoic acid, 3TMS derivative
2,4-Heptadienal, (E,E)-
2,5-Dihydrothiophene sulfone
2,5-Dimethylhex-5-en-3-yn-2-ol
2,5-Furandione, 3,4-dimethyl-
2,5-Hexanediol
2,5-Octadecadiynoic acid, methyl ester
2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-
2,6,9,11-Dodecatetraenal, 2,6,10-trimethyl-
2,6,9,11-Dodecatetraenal, 2,6,10-trimethyl-, (E,E,E)-
2,6-Difluorobenzoic acid, 3,5-difluorophenyl ester
2,7-Octadien-4-ol, 2-methyl-6-methylene-
2,7-Octadien-4-ol, 2-methyl-6-methylene-, (S)-
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde
2-[5-Diethylamino-2-pentylamino]-4,6-bis[perfluoropropyl]-1,3,5-triazine sulfamate
22-Desoxycarpesterol
2-Amino-4-isopropyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile
2-Benzyloxy-3-methyl-1,4-butanediol
2-Butanone, 1-(2-furanyl)-3-methyl-
2-Butene-1,4-diol, diacetate
2-Butyloxycarbonyloxy-1,1,10-trimethyl-6,9-epidioxydecalin
2-Chloroethylamine
2-Cyanobenzaldehyde
2-Cyclobutyl-2-propanol
2-Decenal
2-Decenal, (Z)-
2-Ethyl-5-hydroxy-3,6-dimethyl-pyran-4-one
2-Ethyl-hexoic acid
2-Ethyl-oxetane
2-Fluorobenzoic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester
2-Heptenal
2-Heptenal, (Z)-
2-Heptenoic acid, 7-(methylenecyclopropyl)-, methyl ester
2-Hexanol
Glycolaldehyde dimethyl acetal
Glycoldial, bis-O-pentafluorobenzyloxime
Glycyl-dl-alanine
Heneicosanoic acid, methyl ester
Heptacosane
Heptadecane
Heptadecane, 2,6,10,15-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
Heptane, 3,5-dimethyl-
Heptanoic acid
Heptanoic acid, methyl ester
Heptasiloxane, hexadecamethyl-
Heptyl methyl ethylphosphonate
Hexacosanoic acid, methyl ester
Hexadecane
Hexadecane, 1,1-dimethoxy-
Hexadecanoic acid, 15-methyl-, methyl ester
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Hexadecanoic acid, methyl ester
Hexadecen-1-ol, trans-9-
Hexadecenoic acid, Z-11-
Hexane, 1-(3-butenyloxy)-
Hexano-dibutyrin
Hexanoic acid
Hexanoic acid, 2-ethyl-
Hexanoic acid, 3-(2,2,3,3-tetramethylcyclopropylidenmethylidene)-4-methyl-
Hexasiloxane, tetradecamethyl-
Homovanillyl alcohol
Hydratropic acid, 3-methylbut-2-en-1-yl ester
Hydroxyurea
Indan, 1-methyl-
Inositol
Iridomyrmecin
Isoaromadendrene epoxide
Isobutyl (3-(methylthio)propyl) carbonate
Isobutylamine
Isolongifolene, 4,5-dehydro-

2-Hexanone, 5-methyl-

2H-Oxireno[3,4]cyclopenta[1,2-c]furan-2-one, 1a,1b,4,4a,5,5a-hexahydro-4-(dimethoxymethyl)-, (1bR,1a-cis,4-trans,4a-cis,5a-cis)-

2H-Pyran-2-one, 5,6-dihydro-6-pentyl-

2H-Pyran-2-one, 5,6-dihydro-6-propyl-

2'-Hydroxy-4'-methoxyacetophenone, butyl ether

2-Isopropenyl-5-methylhex-4-enal

2-Methyl(pentamethylene)silyloxytetradecane

2-Methyl-2-propyl methylphosphonofluoridate

2-Methyl-5-oxohexanethioic acid, S-t-butyl ester

2-Methyl-7-oxo-4,7-dihydro-triazolo(3,2-c)triazine

2-Methylheptanoic acid

2-Methyl-Z,Z-3,13-octadecadienol

2-Naphthalenemethanol, 1,2,3,4,4a,8a-hexahydro-à,à,4a,8-tetramethyl-, [2R-(2à,4aà,8aà)]-

2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-à,à,4a,8-tetramethyl-, [2R-(2à,4aá,8á)]-

2-n-Heptylcyclopentanone

2-Nonadecanone

2-Nonen-1-ol,

2-Nonenoic acid

2-Octanol, 2,6-dimethyl-

2-Octenoic acid, 4-isopropylidene-7-methyl-6-methylene-, methyl ester

2-Pentadecanone, 6,10,14-trimethyl-

2-Pentanol, 2,4-dimethyl-

2-Pentanol, 4-methyl-

2-Pentanone, 5-(2-methylenecyclohexyl)-, stereoisomer

2-Pentene, 1-(pentyloxy)-,

2-Pentene, 5-butoxy-,

2-Phenyl-propane-1,1-diol diacetate

2-Piperidinone, N-[4-bromo-n-butyl]-

2-Propen-1-ol

2-Tridecanone

2-Undecanone, 6,10-dimethyl-

3,3-Dimethyl-hepta-4,5-dien-2-one

3,4-Bis(Methoxycarbonyl)furan

3,5-Hexadien-2-ol, 2-methyl-

3,6-Dimethyl-6-hepten-4-yn-3-ol

3,7,11,14,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-

Isophthalic acid, butyl undec-2-en-1-yl ester

Isophytol

Isopropyl 5,11-dihydroxy-3,7,11-trimethyl-2-dodecenoate

Isoquinoline, 1-butyl-

Isospathulenol

Kauran-18-al, 17-(acetyloxy)-, (4á)-

Kauran-19-oic acid, methyl ester

Khusimyl methyl ether

Kolavenol

L-Alanyl-L-norleucine, N-dimethylaminomethylene-, methyl ester

Lanceol, cis

Ledene oxide-(II)

Ledol

Lilial

Linalool, methyl ether

L-Leucine, methyl ester

Longiverbenone

Malonic acid, bis(2-trimethylsilylethyl ester

Mandelic acid, 3,4-dimethoxy-, methyl ester

m-Cresol, TMS derivative

Megastigma-4,6®,8®-triene

Methane, isocyanato-

Methane, trimethoxy-

Methane-d, trichloro-

Methaniminium, N,N-dimethyl-, [[(4,7,7-trimethyl-3-oxobicyclo[2.2.1]heptan-1-yl)carbonyl]amino]-, hydroxide, inner salt

Methanol, (1,4-dihydrophenyl)-

Methanol, TBDMS derivative

Methoxyacetic acid, butyl ester

Methoxyolivetol

Methyl (Z)-5,11,14,17-eicosatetraenoate

Methyl 11,12-tetradecadienoate

Methyl 5,11,14,17-eicosatetraenoate

Methyl 6,9,12,15,18-heneicosapentaenoate

Methyl 6,9,12,15-hexadecatetraenoate

Methyl 8,11,14-heptadecatrienoate

Methyl 9-cis,11-trans-octadecadienoate

3-[1-(4-Cyano-1,2,3,4-tetrahydronaphthyl)]propanenitrile	Methyl glyoxal
3-Buten-2-ol, 2-methyl-	Methyl octadec-6,9-dien-12-ynoate
3-Buten-2-ol, 2-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]hept-2-yl)-	Methyl stearate
3-Buten-2-ol, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-, (3E)-	Methyl steviol
3-Buten-2-one, 4-(3-hydroxy-6,6-dimethyl-2-methylenecyclohexyl)-	Methyl tetradecanoate
3-Butoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	Methylene chloride
3-Butyn-1-ol	Methyl-methoxy-hydroxymethyl-amine
3-Cyclohexene-1-carboxylic acid, 2-(dimethylamino)-1-phenyl-, ethyl ester, trans-	Molybdenum, di-æ-chlorobis[(1,2,3,4,5,6-ü)-methylbenzene]bis(ü3-2-propenyl)di-
3-Cyclohexene-1-methanol, 5-hydroxy-à,à,4-trimethyl-	Monobenzone
3-Cyclopentylpropionic acid, 4-pentadecyl ester	Morpholine, 4-octadecyl-
3-Decen-1-ol,	Muramic acid
3-Ethoxy-2-bromo-1-propanol	Muurola-4,10(14)-dien-1á-ol
3-Ethyl-2-methyl-2-heptanol	Myristoleic acid
3-Ethyl-5-hexen-3-ol	N-([3-(3-Chlorophenyl)-1,2-oxazol-5-yl]methyl)-4-fluorobenzamide
3-Heptanone, 2,4-dimethyl-	N-(1H-Tetrazol-5-yl)benzamide
3-Hepten-1-ol	N-(2-Methylacryloyl)imidazola
3-Hexanol	N-[2-(Adamantan-1-yloxy)-ethyl]-2,4,6-trimethyl-benzenesulfonamide
3-Hexanone, 2,2-dimethyl-	N1-(2-Dimethylamino-ethyl)-3,N1,N2,N2-tetramethyl-butane-1,2-diamine
3-Hexen-1-ol, 2-ethyl-	Naphthalene
3-Hydroxy-á-damascone	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-
3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-
3-Methoxy-2,4,5-trifluorobenzoic acid, 2,3-dichlorophenyl ester	Naphthalene, 1-methyl-
3-Methoxy-2,4,5-trifluorobenzoic acid, 2,4-dichloronaphthyl-1 ester	N-Benzyl-N-methyl-5,7-dinitro-2,1,3-benzoxadiazol-4-amine
3-Methoxy-2-methyl-1-pentene	n-Decanoic acid
3-Methoxy-3-methyl-1-pentene	Neophytadiene
3-Methyl-1-hexen-3-ol	Neric acid
3-Methylbenzyl alcohol	Nerolidol
3-Nonen-1-ol, (Z)-	N-Formyl-d-threo-O-methylthreonine
3-Oxatricyclo[20.8.0.0(7,16)]triaconta-1(22),7(16),9,13,23,29-hexaene	n-Hexadecanoic acid
3-Oxo-androsta-1,4-dien-17á-spiro-2'-3'-oxo-oxetane	n-Hexyl methylphosphonofluoridate
3-Phenylbut-1-ene	N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide

3-Tetradecyn-1-ol

3-Ureidopropionic acid, N-dimethylaminomethylene-, butyl ester

4-(1-Hydroperoxy-2,2-dimethyl-6-methylene-cyclohexyl)-pent-3-en-2-one

4-(2-Aminoethyl)pyridine

4,4-Dimethyladamantan-2-ol

4,5-Bis-dimethoxymethyl-octanedioic acid, dimethyl ester

4,5-di-epi-aristolochene

4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (Z)-

4,8,12,16-Tetramethylheptadecan-4-olide

4,8,12-Tetradecatrien-1-ol, 5,9,13-trimethyl-

4,8,12-Trimethyltridecan-4-olide

4[h]-Pyridone, 1-benzyl-3,5-dichloro-2,6-dimethyl-

4a,5-Dimethyl-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalen-1-ol

4-Acetoxy-3-methoxyacetophenone

4-Amino-3-methoxypyrazolo[3,4-d]pyrimidine

4-benzoxazolol, 2-methyl-, acetate (ester)

4-Dimethylsilyloxypentadecane

4H-1,3,2-Dioxaborin, 6-ethenyl-2-ethyl-4-methyl-4-(2-methylpropyl)-

4H-Cyclopropa[5',6']benz[1',2':7,8]azuleno[5,6-b]oxiren-4-one, 8-(acetyloxy)-1,1a,1b,1c,2a,3,3a,6a,6b,7,8,8a-dodecahydro-3a,6b,8a-trihydroxy-2a-(hydroxymethyl)-1,1,5,7-tetramethyl-, (1aà,1bá,1cá,2aá,3aá,6aà,6bà,7à,8á,8aà)-

4-Hexen-1-ol, 6-(2,6,6-trimethyl-1-cyclohexenyl)-4-methyl-,

4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene

4-Methoxy-1-pentene

4-Methoxy-3-methylbenzyl chloride

4-Methyl-3-(2-methylpropyl)-6-isopropyl-2,5-dioxomorpholine

4-Nonenal

4-Nonene, 5-nitro-

4-Pentyn-2-ol

4'-Phenylpropiophenone

4-Trifluoroacetoxyhexadecane

5-(2-Methoxypropan-2-yl)-2-methyl-2-vinyltetrahydrofuran

5-(2-Nitrophenylthio)-1,2,3,4,5-pentamethylcyclopentadiene

Nonadecane, 2-methyl-

Nonanal

Nonanal dimethyl acetal

Nonane, 1-iodo-

Nonane, 2,2,4,4,6,8,8-heptamethyl-

Nonanoic acid

Nonanoic acid, methyl ester

Norfenfluramine

n-Propyl acetate

n-Propyl chloride

n-Tetracosanol-1

o-Anisic acid, 4-benzyloxyphenyl ester

Octadecane

Octadecane, 1,1-dimethoxy-

Octadecanoic acid, 9,10-epoxy-18-(trimethylsiloxy)-, methyl ester, cis-

Octanal, 7-hydroxy-3,7-dimethyl-

Octane

Octane, 1-chloro-

Octanoic acid

Octanoic acid, methyl ester

o-Cymene

o-Ethylhydroxylamine

Oleic Acid

o-Toluic acid, tridec-2-ynyl ester

Oxalic acid

Oxalic acid, 2-isopropylphenyl pentyl ester

Oxalic acid, cyclobutyl pentadecyl ester

o-Xylene

p-Cumenol

p-Cymene

p-Dioxane-2,3-diol

5,7-Decadien-3-yne, 2,9-dihydroxy-5-(1-hydroxy-1-methylethyl)-2,9-dimethyl-, (Z,E)-

5,9-Dimethyl-9-decen-3-ol

5,9-Methano-5H-benzocycloheptene, 8-bromo-8,9-dihydro-

5,9-Undecadien-2-one, 6,10-dimethyl-

5à-Androstan-3-one, 17á-hydroxy-4à-methyl-, cyclic ethylene acetal

5-Cholestene-3-ol, 24-methyl-

5-Hepten-2-one, 6-methyl-

5H-Pyrrolo(3,2-d)pyrimidine-2,4-diamine

5-Phenoxymethyl-furan-2-carboxylic acid

6-(2-propynyloxy)-1-hexanol

6,10,14,18,22-Tetracosapentaen-2-ol, 3-bromo-2,6,10,15,19,23-hexamethyl-, (all-E)-

6,10-Dodecadien-1-ol, 3,7,11-trimethyl-

6,10-Dodecadien-1-yn-3-ol, 3,7,11-trimethyl-

6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene

6-Heptadecyne, 1-chloro-

6-Heptene-2,4-diol

6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one

6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2,3-diol

6-Isopropyl-1,4-dimethylnaphthalene

6-Nitroundec-5-ene

6-Octen-1-ol, 3,7-dimethyl-, formate

6-Octenoic acid, 3,7-dimethyl-, 3,7-dimethyl-6-octenyl ester

7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione

7-Acetyl-2-hydroxy-2-methyl-5-isopropylbicyclo[4.3.0]nonane

7-Benzoyloxybicyclo[2.2.1]hepta-2,5-diene

7-Chloro-1,3,4,10-tetrahydro-10-hydroxy-1-[[2-[1-pyrrolidinyl]ethyl]imino]-3-[3-(trifluoromethyl)phenyl]-9(2H)-acridinone

7-Hexadecenoic acid, methyl ester, (Z)-

7-Hydroxycadalene

7-Hydroxyfarnesen

7-Methyl-8-oxo-1,2,3,4-tetrahydro-8H-pyrimido[1,2-a]pyrimidine

7-Methyl-Z-tetradecen-1-ol acetate

7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-

Pentadecane

Pentadecanoic acid

Pentadecanoic acid, 14-methyl-, methyl ester

Pentafluorobenzoic acid, tridec-2-ynyl ester

Pentan-2-one, 4-(2-naphthylsulfonyl)-4-methyl-

Pentane, 1-methoxy-

Pentane, 2,2,3,4-tetramethyl-

Pentanoic acid, 4-methyl-, 1-buten-1-yl ester

Perillyl benzoate

Phenethyl alcohol, p-methyl-à-phenyl-

Phenol

Phenol, 2-(1-methylethyl)-

Phenol, 2,5-bis(1,1-dimethylethyl)-

Phenol, 2,6-dimethoxy-4-(2-propenyl)-

Phenol, 2,6-dimethyl-4-[[5-(4-pyridinyl)-1H-1,2,3,4-tetrazol-1-yl]methyl]-

Phenol, 2-methoxy-4-(1-propenyl)-

Phenol, 3,5-bis(1,1-dimethylethyl)-

Phenylethanolamine

Phenylethyl Alcohol

Pholedrine

Phosphoric acid, dibutyl 1,1-dimethyl-2,2,3,3-tetrafluoropropyl ester

Phthalic acid, 4-chloro-3-methylphenyl ethyl ester

Phthalic acid, butyl 2-ethylcyclohexyl ester

Phthalic acid, butyl isohexyl ester

Phthalic acid, heptyl 4-nitrophenyl ester

Phthalic acid, hex-2-yn-4-yl hexyl ester

Phytol

Pipazethate

Plastoquinone 3

Platambin

Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl-

Pregn-4-en-18-oic acid, 11-(acetyloxy)-6,7-epoxy-9,20-dihydroxy-3-one-, ,gamma.-lactone, (6à,7à,11à,20R)-

7-Tetradecenal, (Z)-
8,11,14-Eicosatrienoic acid, (Z,Z,Z)-
8-Decen-1-ol, 5,9-dimethyl-
8-Methoxy-5,5,8-trimethyl-3-nonen-2-one
8-Methylene-3-oxatricyclo[5.2.0.0(2,4)]nonane
9(11)-Dehydroergosteryl benzoate
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-
9,12,15-Octadecatrienoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ester, (Z,Z,Z)-
9,12,15-Octadecatrienoic acid, 2,3-bis(acetyloxy)propyl ester, (Z,Z,Z)-
9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
9,12-Octadecadien-1-ol, (Z,Z)-
9,12-Octadecadienoic acid (Z,Z)-
9,12-Octadecadienoic acid, methyl ester
9,12-Octadecadienoic acid, methyl ester, (E,E)-
9,12-Octadecadienoyl chloride, (Z,Z)-
9,17-Octadecadienal, (Z)-
9,19-Cyclolanostan-3-ol, acetate, (3á)-
9-Dodecenoic acid, methyl ester
9-Eicosyne
9-Fluorenone, thiosemicarbazone
9-Hexadecen-1-ol, (Z)-
9-Methoxycalamenene
9-Octadecen-1-ol
9-Octadecenoic acid (Z)-, 2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ester
9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester
9-Octadecenoic acid (Z)-, methyl ester
9-Oxononanoic acid
9-Tetradecen-1-ol
9-Tetradecen-1-ol, acetate
9-Undecenal, 2,10-dimethyl-
á-Alanine
à-Cadinol
à-Calacorene
Acetamide, N,N-dibutyl-
Acetate, 4-hydroxy-3-methyl-2-butenyl-
Acetic acid, (acetyloxy)-
Pregn-4-en-3-one, 20-hydroxy-, (20R)-
Pregna-5,9(11)-dien-20-ol-3-one ethylene ketal
Pregnan-3,11-diol-20-one
Propan-2-ol, 1-(2-adamantyloxy)-3-(4-morpholyl)-
Propanamide
Propane, 1-ethoxy-2-methyl-
Propane-1,2-dione, 3-(3,3-dimethyl-3,4-dihydro-2H-isoquinolin-1-ylidene)-1-(morpholin-4-yl)-
Propanedioic acid, dihydroxy-
Propanetrione, diphenyl-
Propanoic acid, 2-(benzoylamino)-3,3,3-trifluoro-2-[[2-(trifluoromethyl)phenyl]amino]-, ethyl ester
Propanoic acid, 2-hydroxy-2-methyl-, ethyl ester
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester
Propanoic acid, 2-oxo-
Propanoic acid, 2-oxo-, methyl ester
Propylamine
Propylure
Pteridine, 4,6,7-trihydroxy-
p-Toluic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester
p-Toluic acid, 2-bromo-4-fluorophenyl ester
p-Xylene
Pyridazin-3(2H)-one, 2-cyclohexyl-5-hydroxy-4-methoxy-
Pyrimidine-2,4(1H,3H)-dione, 5-bromo-1-(1-methoxyethyl)-
Pyrolo[3,2-d]pyrimidin-2,4(1H,3H)-dione
pyrrolidine, 1-(4-fluoro-2-methylphenyl)-
Quassin
Ritalinic acid, TMS derivative
S-(Neopentyloxythiocarbonyl)thiohydroxylamine
Santalol, E-cis,epi-á-
sec-Butyl nitrite
Selin-6-en-4à-ol
Silane, (2-methoxyethyl)trimethyl-
Silane, [1-(5-hexenyl)-2-methylenecyclopropyl]trimethyl-
Silane, cyclohexyldimethoxymethyl-
Silane, dimethoxydimethyl-
Silanol, allyldimethyl-
Silanol, trimethyl-
Silicon tetrafluoride

Acetic acid, [(aminocarbonyl)amino]oxo-
Acetic acid, 4-methylphenyl ester
Acetic acid, hydroxy-
Acetic acid, hydroxy-, ethyl ester
Acetophenone
á-Citronellol, chlorodifluoroacetate
á-copaene
á-D-Glucopyranose, 1,6-anhydro-
à-D-Glucopyranoside, methyl 2,3,4-tri-O-methyl-
á-Eudesmol, TMS derivative
à-Furil
Alloaromadendrene
Alloaromadendrene oxide-(1)
Andrographolide
Androsta-1,4-dien-3-one, 17-hydroxy-17-methyl-, (17à)-
Androstan-4-one, (5á)-
Androstan-7-one, 3-(acetyloxy)-, (3á,5à)-
Aniline
Anthracene, 1,4-bis(phenylmethoxy)-
Apocynin
Aromadendrene oxide-(1)
Aromadendrene oxide-(2)
á-Sitosterol
Aspartylglycine ethyl ester
à-Tocopherolquinone
à-Tocopheryl acetate
Azulene
Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1à,3aá,4à,7á)]-
Azulene, 1,2,3,3a-tetrahydro-
Azulene, 1,4-dimethyl-7-(1-methylethyl)-
Behenic alcohol
Benzadehyde O-benzyloxime
Benzaldehyde, 2-methyl-
Benzaldehyde, 3,4-dimethoxy-, methylmonoacetal
Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-
s-Indacene, 1,2,3,5,6,7-hexahydro-1,1,7,7-tetramethyl-
s-Indacene-1,7-dione, 2,3,5,6-tetrahydro-3,3,5,5-tetramethyl-
Spiro(1,3-dioxolane)-2,3'-(5'-androsten-16'-ol), TMS derivative
Spiro[4.5]dec-8-en-7-ol, 4,8-dimethyl-1-(1-methylethyl)-
Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-
Stigmast-4-en-3-one
Stigmasterol
Sulfone, 2-hydroxypropyl t-butyl
Sulfurous acid, butyl pentyl ester
Sulfurous acid, isobutyl pentyl ester
Sulphuric acid dibutyl ester
Terephthalic acid, allyl 4-fluoro-2-methoxyphenyl ester
Terpineol
tert-Butyldimethylsilanol
Tetradecane
Tetradecanoic acid
Tetraethyl ammonium fluoride
Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]-
Thiazol-5(4H)-one, 2-(4-methylphenyl)-4-(3-nitrobenzylideno)-
Thiophene, 2,5-bis(1,1-dimethylethyl)-
Thiosemicarbazone-5,16-pregnadienolone, 3-acetate
Thunbergol
Toluene
trans-13-Octadecenoic acid
trans-3-Methyl-4-octanolide
trans-Ascaridol glycol
trans-Calamenene
trans-Farnesol
trans-Z-à-Bisabolene epoxide
Trichlor-(1,2,3,4,5-pentamethylcyclopenta-2,4-dienyl)-german
Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-
Tricyclo[3.3.1.1(3,7)]decane-1,3-dicarboxylic acid, 4-oxo-
Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, à,à,6,8-tetramethyl-, stereoisomer
Trideca-1,7,11-triene-1,1-dicarbonitrile, 4,8,12-trimethyl-
Tridecane

Benzaldehyde, 3-chloro-5-methoxy-4-[(4-methylphenyl)methoxy]-

Benzaldehyde, 3-methyl-

Benzaldehyde, 4-benzyloxy-3-methoxy-2-nitro-

Benzaldehyde, 4-hydroxy-3,5-dimethoxy-

Benzaldehyde, 4-methyl-

Benzamide, N-[5-(4-pyridinyl)-1H-1,2,4-triazol-3-yl]-

Benzamide, o-(á-hydroxyphenethyl)-

Benzene, (1,1-dimethylpropyl)-

Benzene, (1-methyl-1-propenyl)-

Benzene, (1-methylethyl)-

Benzene, (2-methylpropyl)-

Benzene, (bromomethyl)-

Benzene, (iodomethyl)-

Benzene, (phenoxymethyl)-

Benzene, 1-(bromomethyl)-2-methyl-

Benzene, 1,2,3,4-tetramethyl-

Benzene, 1,2,3-trimethyl-

Benzene, 1,2,4,5-tetramethyl-

Tridecane, 2,2,4,10,12,12-hexamethyl-7-(3,5,5-trimethylhexyl)-

Tridecane, 2-methyl-

Tridecane, 5-propyl-

Tridecanoic acid, methyl ester

Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-

Undecane

Undecane, 2,10-dimethyl-

Undecane, 3-methyl-

Undecanoic acid

Undecanoic acid, methyl ester

Urea, ethyl-

Urs-12-en-28-al

Ursolic aldehyde

Uvaol

Valerenol

Vanillin

Vitamin E

Ylangenol

Z,E-7,11-Hexadecadien-1-yl acetate

APPENDIX M

Table 6.1: Comparison of chemical compounds in the stem bark of all 12 the *C. dentata* trees from the southern Cape and all 12 the *C. dentata* trees from KZN, collected September 2015 and September 2018 respectively. The highlighted compounds occur in trees from both sites but not necessarily in every tree at each of the respective sites.

Southern Cape	KZN
(-)-Neoclovene-(II), dihydro-	((4aS,8S,8aR)-8-Isopropyl+A2:A25-5-methyl-3,4,4a,7,8,8a-hexahydronaphthalen-2-yl)methanol
(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	**(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene**
(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane	**(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane**
®-N-(4-Hydroxy-3-methoxybenzyl)-9-methyldec-7-enamide	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol
1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone	(1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
1-(4-tert-Butylphenyl)propan-2-one	(2S,3S)-(-)-3-Propyloxiranemethanol
1,1,1,3,5,5,5-Heptamethyltrisiloxane	(Aminomethyl)cyclopropane
1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	.alfa.-Copaene
1,1-Di(prop-2-enyl)-1-silacyclobutane	1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone
1,2,3,4,5-Cyclopentanepentol	**1-(4-tert-Butylphenyl)propan-2-one**
1,2,3,4-Tetrahydro-1-naphthylamine, N-heptafluorobutyryl-	**1,1,1,3,5,5,5-Heptamethyltrisiloxane**
1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline	1,13-Tetradecadiene
1,25-Dihydroxyvitamin D3, TMS derivative	1,15-Pentadecanediol
1,2-Benzenedicarboxylic acid, bis(4-methylpentyl) ester	**1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline**
1,2-Benzisothiazol-3-amine, TBDMS derivative	1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl-5-(1-methylethyl)-, [1S-(1à,2à,3aá,4à,5à,7aá,8S*)]-
1,2-Octadecanediol	1,2,5-Oxadiazole-3-carboxamide, 4-amino-N-[2-[[(2,3-dihydro-1,3-dimethyl-2-oxo-1H-1,3-benzimidazol-5-yl)methyl]amino]ethyl]-
1,3,5,7-Tetrazaadamantane borane	**1,25-Dihydroxyvitamin D3, TMS derivative**
1,3,5-Trisilacyclohexane	**1,2-Benzisothiazol-3-amine, TBDMS derivative**
1,3-Bis-t-butylperoxy-phthalan	1,2-Bis(tert-butylimino)ethane
1,3-Butadiyne	1,2-Bis(trimethylsilyl)benzene
1,3-Dioxane, 2-heptyl-	1,3,2-Dioxaborinane, 2,4-diethyl-5-methyl-6-propyl-
1,3-Dioxane, 2-pentadecyl-	**1,3-Bis-t-butylperoxy-phthalan**
1,3-Dioxolane, 2-(1-methylethyl)-	**1,3-Butadiyne**
1,3-Dioxolane, 2-(5-bromopentyl)-	1,3-Dioxolane, 2-heptyl-4-methyl-
1,3-Dioxolane, 4-hexyl-2,2-bis(trifluoromethyl)-	1,3-Dioxolane, 4-methyl-2-pentadecyl-
1,3-Dioxolane, 4-methyl-2-pentadecyl-	1,4-Bis(trimethylsilyl)benzene
1,4-Cyclohexadiene, 1,3,6-tris(trimethylsilyl)-	1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate]
1,4-di-iso-propylnaphthalene	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-
1,7-di-iso-propylnaphthalene	**1,7-di-iso-propylnaphthalene**
1,7-Dimethyl-4-(1-methylethyl)cyclodecane	**11-Hexadecen-1-ol, (Z)-**
10-Bromodecanoic acid, ethyl ester	**11-Methyldodecanol**
10-Undecynoic acid, TMS derivative	16-Hexadecanoyl hydrazide
11-Hexadecen-1-ol, (Z)-	1à,2á,3à,4á-Tetramethylcyclopentane
11-Methyldodecanol	1-Benzamido-pyrrolidine
11-Tricosene	1-Butanamine
1-Acetoxynonadecane	**1-Butanamine, 3-methyl-**
1-Butanamine, 3-methyl-	**1-Butanol, 2,2-dimethyl-**
1-Butanol, 2,2-dimethyl-	1-Buten-3-yne, 1-chloro-, ®-
1-Butanol, 2-ethyl-	**1-Buten-3-yne, 1-chloro-, (Z)-**
1-Buten-3-yne	1-Butene, 4-chloro-3-methyl-
1-Buten-3-yne, 1-chloro-, (Z)-	
1-Decanol, 2-hexyl-	
1-Decene, 3,3,4-trimethyl-	

1-Decene, 3,4-dimethyl-
1-Decene, 4-methyl-
1-Docosene
1-Dodecanol, 2-hexyl-
1-Ethanone, 1-[3-methoxy-4-[(4-methylphenyl)methoxy]phenyl]-
1-Ethenyl-3-(1-hexenyl)-4-trimethylsilylcyclopentane
1H-Cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan-2-yl)-, (3aR,1-trans,6a-cis)-
1-Heptanamine
1-Heptanol, 2-propyl-
1-Hepten-4-ol
1-Hexadecanol
1H-Indene, 1-hexadecyl-2,3-dihydro-
1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl-
1H-Indole, 1-methyl-
1-Iodo-2-methylundecane
1-Methyl-1-(5-nonyn-3-yloxy)-1-silacyclohexane
1-Naphthalenol, 5,6,7,8-tetrahydro-2,5-dimethyl-8-(1-methylethyl)-
1-Naphthalenol, 5,6,7,8-tetrahydro-2,5-dimethyl-8-(1-methylethyl)-
1-Octanesulfonyl chloride
1-Octanol, 2-butyl-
1-Octanol, 3,7-dimethyl-, (S)-
1-Octene, 3,7-dimethyl-
1-Pentanol, 2,4-dimethyl-, (ñ)-
1-Pentanol, 2-ethyl-4-methyl-
1-Penten-3-ol
1-Pentene, 3,4-dimethyl-
1-Undecene, 4-methyl-
2-((Pent-4-enyloxy)carbonyl)benzoic acid
2(2-Bromoethyl)-1,3-dioxane
2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid
2-(Heptyloxycarbonyl)benzoic acid
2,2,4,4-Tetramethyloctane
2,2,4-Trimethyl-3-hexene
2,2'-Bifuran, octahydro-
2,2-Dichloroethyl methyl ether
2,2-Diethyl-3-methyl-N-ethylpyrrolidine
2,2-Dimethyl-2-sila-1,3-dioxacyclohexane
2,2-Dimethylpropionic acid, 4-methylpentyl ester
2,3,4,5,6-Pentamethyl acetophenone
2,3-Epoxyhexanol
2,4,4-Trimethyl-1-hexene
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
2,4-Dimethyl-1-heptene
2,4-Dimethyldodecane
2,4-Dimethylhept-1-ene
2,4-Disilapentane, 2,4-dimethyl-
2,4-Di-tert-butylphenol
2,4-Pentadien-1-ol, 3-propyl-, (2Z)-
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
2,5-di-tert-Butyl-1,4-benzoquinone
2,5-Hexanediol, 2,5-dimethyl-
2,5-Monoformal-l-rhamnitol triacetate
1-Cyclohexene, 1,3,3-trimethyl-2-(1-methylbut-1-en-3-on-1-yl)-
1-Decanol, 2-ethyl-
1-Decanol, 2-hexyl-
1-Decene, 3,3,4-trimethyl-
1-Docosene
1-Ethenyl-3-(1-hexenyl)-4-trimethylsilylcyclopentane
1H-Cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan-2-yl)-, (3aR,1-trans,6a-cis)-
1-Heptanol, 2-propyl-
1-Hexadecanol
1-Hexanol, 2,2-dimethyl-
1-Hexene, 6-phenyl-4-(1-phenylethoxy)-
1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)-
1H-Indene, 1-hexadecyl-2,3-dihydro-
1H-Indole, 1-methyl-
1H-Indole, 2-methyl-
1-Hydroxy-2-butanone
1-Iodo-2-methylundecane
1-Octanesulfonyl chloride
1-Octanol, 2-butyl-
1-Pentanol, 2,4-dimethyl-, (ñ)-
1-Pentyn-3-ol, 3-methyl-
1-Trifluorosilyltridecane
1-Undecene, 4-methyl-
1-Undecene, 7-methyl-
2(2-Bromoethyl)-1,3-dioxane
2-(2-Hydroxyethyl)piperidine
2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid
2,2,4-Trimethyl-3-hexene
2,2-Dichloroethyl methyl ether
2,2-Dimethyl-3-heptene trans
2,3,4,5,6-Pentamethyl acetophenone
2,3-Dimethyldecane
2,3-Epoxyhexanol
2,4,4-Trimethyl-1-hexene
2,4,6,8-Tetramethyl-1-undecene
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
2,4,6-Trimethyl-1-nonene
2,4-Dimethyl-1-heptene
2,4-Dimethylhept-1-ene
2,4-Disilapentane, 2,4-dimethyl-
2,4-Di-tert-butylphenol
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
2,5-Hexanediol, 2,5-dimethyl-
2,5-Monoformal-l-rhamnitol triacetate
2,6,10-Trimethyltridecane
2,6-Dimethyldecane
2,9-Decanedione
2-Amino-4-isopropyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile
2-Bromo dodecane
2-Butene
2-Butenoic acid, 4-hydroxy-, methyl ester
2-Cyclopenten-1-one, 3-(acetyloxy)-
2-Decene, 5-methyl-, (Z)-

2,6,10-Trimethyltridecane
2,6-Dihydroxyacetophenone, 2TMS derivative
2,6-Diisopropylnaphthalene
2,6-Dimethyldecane
2,9-Decanedione
2-Amino-4-isopropyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile
2-Azido-2,4,4,6,6,8,8-heptamethylnonane
2-Bromo dodecane
2-Bromotetradecane
2-Cyano-2-O-fluorosulfatofluoropropane
2-Cyclopenten-1-one, 3-(acetyloxy)-
2-Diethylamino-N-naphthalen-1-yl-acetamide
2-Ethyl-3-ketovalerate, 2TMS derivative
2-Heptafluorobutyroxytetradecane
2-Hexanol
2-Hexanone, 4-methyl-
2-Methyltriacontane
2-Penten-1-ol, (Z)-, TBDMS derivative
2-Piperidinone, N-[4-bromo-n-butyl]-
2-Propanol, 1-cyclohexyloxy-(1-piperidinyl)-
2-Propanone, 1,1-dibutoxy-
2-Propyl-1-Pentanol, trifluoroacetate
2-Propyn-1-amine, N,N-dimethyl-
2-Pyrrolidinone
2-Pyrrolidinone, 5-(ethoxymethyl)-
2-Tridecen-1-ol, ®-
2-Undecanethiol, 2-methyl-
3,4-Diethyl hexane
3,4-Hexanedione, 2,2,5-trimethyl-
3,5-Dimethyldodecane
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
3,7,11,15,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-
3-Carene
3-Ethyl-3-methylheptadecane
3-Ethyl-3-methylheptane
3-Heptene, 2,2,3,5,6-pentamethyl-
3-Heptene, 2,6-dimethyl-
3-Methyl-1-tripropylsilyloxybut-2-ene
3-Methyl-2-butenoic acid, tridec-2-ynyl ester
3-Nitropyrrole
4-(Trifluoromethyl)benzyl alcohol, 1-methylpropyl ether
4,4-Dipropylheptane
4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone
4-Decenal, ®-
4-Imidazolidinone, 2,2-dimethyl-3-[(1-methylethylidene)amino]-
4-Methoxy-4-methyl-2-pentanol
4-Methyl-3-heptanol, trifluoroacetate
4-Nonene, 3-methyl-, (Z)-
4-Octanol, 7-methyl-, acetate
4-Octene, 2,3,6-trimethyl-
4-Propionyloxypentadecane
4-Propionyloxytridecane
5,8,11,14,17-Eicosapentaenoic acid
5-Hexen-2-one
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
2-Hexanone, 5-methyl-5-nitro-
2-Nitro-1,3-bis-octyloxy-benzene
2-Octanol, ®-
2-Octene, 2,6-dimethyl-
2-Penten-1-ol, (Z)-, TBDMS derivative
2-Piperidinone, N-[4-bromo-n-butyl]-
2-Propanol, 1-amino-
2-Propanone, 1,1-dibutoxy-
2-Propenamide
2-Propyl-1-pentanol
2-Propyl-1-Pentanol, trifluoroacetate
2-Propyn-1-ol, propionate
2-Tridecen-1-ol, ®-
2-Trifluoromethylbenzoic acid, tridec-2-ynyl ester
2-Undecanethiol, 2-methyl-
3,5-Dimethyldodecane
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
3,7,11,15,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-
3,7-Dimethyl-2,3,3a,4,5,6-hexahydro-1-benzofuran #
3-Buten-2-ol
3-Buten-2-ol, 2-methyl-
3-Butyn-1-ol
3-Carene
3-Ethyl-3-methylheptane
3-Heptene, 4-methyl-
3-Methyl-1-tripropylsilyloxybut-2-ene
3-Methyl-2-butenoic acid, tridec-2-ynyl ester
3-Nitropyrrole
3-Undecene, 6-methyl-, ®-
4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone
4-Carene, (1S,3S,6R)-(-)-
4-Decene, 7-methyl-, ®-
4-Heptafluorobutyryloxyhexadecane
4-Hexyn-3-ol
4-Methoxy-2-nitro-N-[á-diethylaminoethyl]aniline
4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene, 2TMS derivative
4-Octanol, 7-methyl-, acetate
4-Octene, 2,3,6-trimethyl-
5-Ethyl-5-methylnonadecane
5-Ethyldecane
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
5-Undecene, 7-methyl-, (Z)-
6-(1'-Hydroxyethyl)-2,2-dimethylchromene
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
8,9,9,10,10,11-Hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo[5.4.1.0(2,6).0(8,11)]dodecane
8-Heptadecanol
8-Hydroxy-2-octanone
9,12-Octadecadienoic acid, methyl ester, (E,E)-
9,12-Octadecadienoyl chloride, (Z,Z)-
9,15-Octadecadienoic acid, methyl ester
Acetamide, N-ethyl-
Acetic acid, 2,2,3,4,4,4-hexafluorobutyl ester
Acetic acid, chloro-
Acetic acid, hydroxy-

5-Undecene, 7-methyl-, (Z)-
7-Chloro-3-[3,4-dichlorophenyl]-1-[[2-[diethylamino]ethyl]imino]-acridin-9(2H)-one
7-Hexadecenoic acid, methyl ester, (Z)-
8,9,9,10,10,11-Hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo[5.4.1.0(2,6).0(8,11)]dodecane
8-Hexadecanol
9,12-Hexadecadienoic acid, methyl ester
9-Hexadecen-1-ol, (Z)-
9-Methyl-Z-10-pentadecen-1-ol
9-Octadecenoic acid (Z)-, methyl ester
Acetic acid, dichloro-, ethyl ester
Acetic acid, rubidium salt
Acetic acid, trifluoro-, 2,2-dimethylpropyl ester
à-Cubebene
à-D-Glucopyranoside, methyl 2,3,4-tri-O-methyl-
á-d-Mannofuranose, 2,3:5,6-di-O-ethylboranediyl-1-O-(10-undecen-1-yl)-
à-D-Xylofuranoside, methyl 5-O-methyl-
Allo-Inositol
Aminocaproic acid
à-Muurolene
Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime], (3à,5à)-
à-Pinene
Argon
Arsenous acid, tris(trimethylsilyl) ester
Aspidofractinine-1-carboxaldehyde, 3-oxo-, (2à,5à)-
à-Tocopheryl acetate
Benzene, 1,1'-(2-butene-1,4-diyl)bis-
Benzene, 1,3-bis(1,1-dimethylethyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-
Benzene, 1,4-bis(1,1-dimethylethyl)-
Benzene, 1-ethyl-4-methyl-
Benzene, 1-tert-butyl-4-cyclopropylmethyl-
Benzene, tert-butyl-
Benzo[b]dihydropyran, 6-hydroxy-4,4,5,7,8-pentamethyl-
Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
Binapacryl
Borane, diethyl[1-ethyl-2-(methoxymethyl)-1-butenyl]-, (Z)-
Boron trifluoride
Butane, 1-chloro-2-methyl-
Butane, 1-chloro-3,3-dimethyl-
Butanoic acid, 2-methylbutyl ester
Butyric acid, neopentyl ester
Carbonic acid, dimethyl ester
Carbonic acid, octadecyl vinyl ester
Chlorfenapyr
Chloromethanesulfonyl chloride
cis-9-Tetradecen-1-ol
Cyclohexane, 1,1'-(1,2-dimethyl-1,2-ethanediyl)bis-
Cyclohexane, 1-ethyl-2-propyl-
Cyclohexane, propyl-
Cyclooctane, 1,4-dimethyl-, cis-
á-copaene
à-Cubebene
à-Muurolene
à-Pinene
Argon
Arsenous acid, tris(trimethylsilyl) ester
Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-
Benzene, (1-methylethyl)-
Benzene, (1-nitropropyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-
Benzene, 1,4-bis(1,1-dimethylethyl)-
Benzene, tert-butyl-
Benzo[b]dihydropyran, 6-hydroxy-4,4,5,7,8-pentamethyl-
Benzoic acid, 3-(3-hydroxy-3-methyl-1-butynyl)-
Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
Bicyclosesquiphellandrene
Binapacryl
Bis(2-ethylbutyl) diselenide
Borane, diethyl(decyloxy)-
Borane, diethyl[1-ethyl-2-(methoxymethyl)-1-butenyl]-, (Z)-
Boron trifluoride
Butane, 1-chloro-2-methyl-
Butane, 2,2'-[methylenebis(oxy)]bis[2-methyl-
Butanoic acid, 2-methylbutyl ester
Butylamine, N-acetyl-1-cyano-2-ethyl-
Carbamic acid, (cyanomethyl)-, 1,1-dimethylethyl ester
Carbonic acid, nonyl vinyl ester
Carbonic acid, octadecyl vinyl ester
Chlorfenapyr
Chlorine
Chloromethane
Chloromethanesulfonyl chloride
cis-Calamenene
cis-Inositol
ç-Terpinene
Cyclobutene, 3,4-dichloro-
Cyclohexane, 1-ethyl-2-propyl-
Cyclohexane, decyl-
Cyclohexanespiro-5'-(2',4',4'-trimethyl-2'-oxazoline)
Cyclohexylphenylacetonitrile
Cyclooctane, 1,4-dimethyl-, cis-
Cyclopentane, 1-butyl-2-propyl-
Cyclopentane, hexyl-
Cyclopentanecarboxamide, N-(2-fluorophenyl)-
Cyclopentanone
Cyclopentene, 1,2,3,3,4-pentamethyl-
Cyclopropanemethanol, à,à-dimethyl-2-methylene-

Cyclopentane, 1,2-dimethyl-, cis-
Cyclopentane, 1-butyl-2-ethyl-
Cyclopentane, 1-butyl-2-propyl-
Cyclopentane, decyl-
Cyclopentane, hexyl-
Cyclopentaneundecanoic acid, methyl ester
Cyclopentanol, 2-(aminomethyl)-, trans-
Cyclopentene, 1,2,3,3,4-pentamethyl-
Cyclopropanecarboxylic acid, 1-(phenylmethyl)-, 2,6-bis(1,1-dimethylethyl)-4-methylphenyl ester
Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester
Cyclotetradecane
Cyclotrisiloxane, hexamethyl-
d,l-trans-4-Methyl-5-methoxy-1-(1-methoxy-1-isopropyl)cyclohex-3-ene
Decane
Decane, 1-fluoro-
Decane, 2,3,5,8-tetramethyl-
Decane, 2,3,5-trimethyl-
Decane, 2,4,6-trimethyl-
Decane, 2,5-dimethyl-
Decane, 2,6,8-trimethyl-
Decane, 4-methyl-
Decane, 5-methyl-
Desmethyldeprenyl
Desulphosinigrin
Dibutyl phthalate
Dicyclohexyl phthalate
Difluoromethane
Difluorophosphoric acid
Dimethyl 2-hydroxy-2-methylbutane-1,4-dioate
Dimethyl tetradecanedioate
Diphenyl isophthalate
D-Limonene
d-Mannitol, 1-decylsulfonyl-
D-Mannopentadecane-1,2,3,4,5-pentaol
Docosane
Docosane, 11-butyl-
Dodecane, 1-iodo-
Dodecane, 2,6,10-trimethyl-
Dodecane, 2,6,11-trimethyl-
Dodecane, 4,6-dimethyl-
Dodecane, 4-methyl-
Dodecane, 6-methyl-
Dodecanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ester
Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester
Dodecanoic acid, ethyl ester
Dodecanoic acid, methyl ester
Dotriacontane
Eicosane
Eicosane, 2,4-dimethyl-
Eicosane, 2-methyl-
Eicosyl octyl ether
Erythro-3-bromo-2-pentanol
Ethane, fluoro-
Ethanethioic acid, S-(2-methylbutyl) ester
Ethanol, 1-(methylenecyclopropyl)-1-(methylene-1-trimethylsilylcyclopropyl)-
Cyclopropanepentanoic acid, 2-undecyl-, methyl ester, trans-
Cyclotetradecane
Cyclotrisiloxane, hexamethyl-
d,l-trans-4-Methyl-5-methoxy-1-(1-methoxy-1-isopropyl)cyclohex-3-ene
Dasycarpidan-1-methanol, acetate (ester)
Decane
Decane, 1-fluoro-
Decane, 2,3,5,8-tetramethyl-
Decane, 2,4,6-trimethyl-
Decane, 2,5-dimethyl-
Decane, 2,6,7-trimethyl-
Decane, 2,6,8-trimethyl-
Decane, 2,9-dimethyl-
Decane, 3,3,4-trimethyl-
Decane, 4-methyl-
Decane, 5-methyl-
Dibutyl phthalate
Dicyclohexyl phthalate
Dimethyl tetradecanedioate
Disulfide, diheptyl
di-t-Butylacetylene
Di-tert-butyl peroxide
dl-3,4-Dehydroproline methyl ester
dl-3,4-Dehydroproline methyl ester
D-Limonene
d-Mannitol, 1-decylsulfonyl-
Docosane
Docosane, 11-butyl-
Dodecane
Dodecane, 1-iodo-
Dodecane, 2,6,11-trimethyl-
Dodecane, 4,6-dimethyl-
Dodecane, 4-methyl-
Dodecane, 6-methyl-
Dodecanoic acid, ethyl ester
Dodecanoic acid, methyl ester
Dotriacontane
E-10-Pentadecenol
Eicosane
Eicosane, 2,4-dimethyl-
Eicosane, 2-methyl-
Erythro-3-bromo-2-pentanol
Ethane, fluoro-
Ethanedioic acid, bis(3-methylbutyl) ester
Ethanol, 1-(methylenecyclopropyl)-1-(methylene-1-trimethylsilylcyclopropyl)-
Ethanol, 2,2-dichloro-
Ethanone, 1-(3-methylphenyl)-
Ether, 2-ethylhexyl tert-butyl
Ether, 6-methylheptyl vinyl
Ether, hexyl pentyl
Ethoxyacetylene
Ethyl 3-hydroxydocosanoate
Ethyl 3-methylbut-3-enyl carbonate
Formamide, N-(cyanomethyl)-
Formic acid, 2,2-dimethylpent-3-yl ester
Furan, 2-butyltetrahydro-
Germacrene D

Ethanol, 2-(1,1-dimethylethoxy)-
Ethanol, 2,2-dichloro-
Ethanol, 2-[2-(2-methoxyethoxy)ethoxy]-
Ethanone, 1-(2,4,5-triethylphenyl)-
Ethanone, 1-(3,4-dihydro-1H-isoquinolin-2-yl)-2-(1-methyl-1H-tetrazol-5-ylsulfanyl)-
Ether, 2-ethylhexyl tert-butyl
Ether, 6-methylheptyl vinyl
Ether, heptyl hexyl
Ether, hexyl pentyl
Ethyl 2-acetamido-3,3,3-trifluoro-2-(4-[(5-methyl-3-isoxazolyl)sulfamoyl]anilino)propionate
Ethyl 2-chloro-3,3,3-trifluoro-2-propionamidopropionate
Ethyl 3-hydroxydocosanoate
Ethyl 3-hydroxyicosanoate
Ethyl 3-hydroxytetracosanoate
Ethyl 3-methylbut-3-enyl carbonate
Fenpipramide
Floxuridine
Formic acid, 2,2-dimethylpent-3-yl ester
Glycine
Glycyl-L-tryptophylglycine
Heneicosane
Heneicosane, 11-(1-ethylpropyl)-
Heneicosanoic acid, methyl ester
Hentriacontane
Heptacosane
Heptacosane, 1-chloro-
Heptadecane
Heptadecane, 2,6,10,14-tetramethyl-
Heptadecane, 2,6,10,15-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
heptadecanoic acid, 2-methoxyethyl ester
Heptane, 2,2,3,5-tetramethyl-
Heptane, 2,4,6-trimethyl-
Heptane, 2,4-dimethyl-
Heptane, 2,6-dimethyl-
Heptane, 3,3,5-trimethyl-
Heptane, 3,3-dimethyl-
Heptane, 3,4-dimethyl-
Heptane, 3,5-dimethyl-
Heptane, 3-ethyl-2-methyl-
Heptane, 4-(1-methylethyl)-
Heptane, 4,4-dimethyl-
Heptane, 4-ethyl-
Heptane, 4-propyl-
Hexacosane
Hexadecane
Hexadecane, 2,6,10,14-tetramethyl-
Hexadecane, 2,6,11,15-tetramethyl-
Hexadecanoic acid, 15-methyl-, methyl ester
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Hexadecen-1-ol, trans-9-
Hexane, 1-nitro-
Hexane, 2,2,3,3-tetramethyl-
Hexane, 2,3,4-trimethyl-
Hexane, 2,3,5-trimethyl-
Glucitol, 6-O-nonyl-
Glutamic acid, N-pivaloyl-, dimethyl ester
Glycerol, 1-tert-butyl ether
Glycine
Glycyl-L-tryptophylglycine
Heneicosane
Hentriacontane
Heptacosane
Heptacosane, 1-chloro-
Heptadecane
Heptadecane, 2,6,10,14-tetramethyl-
Heptadecane, 2,6,10,15-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
heptadecanoic acid, 2-methoxyethyl ester
Heptane, 2,2,3,3,5,6,6-heptamethyl-
Heptane, 2,4,6-trimethyl-
Heptane, 2,4-dimethyl-
Heptane, 3-(chloromethyl)-
Heptane, 3,4-dimethyl-
Heptane, 3,5-dimethyl-
Heptane, 3-ethyl-
Heptane, 3-ethyl-2-methyl-
Heptane, 4,4-dimethyl-
Heptane, 4-ethyl-
Heptane, 4-propyl-
Heptyl isobutyl carbonate
Hexacosane
Hexacosanoic acid, methyl ester
Hexadecanal
Hexadecane
Hexadecane, 1-chloro-
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Hexadecanoic acid, methyl ester
Hexadecen-1-ol, trans-9-
Hexan-3-yl trifluoroacetate
Hexane, 2,2,3,3-tetramethyl-
Hexane, 2,3,3-trimethyl-
Hexane, 2,3,5-trimethyl-
Hexane, 2,4-dimethyl-
Hexane, 3,3,4,4-tetramethyl-
Hexane, 3,3,4-trimethyl-
Hexane, 3,3-dimethyl-
Hexane, 3-ethyl-
Hexane, 3-ethyl-4-methyl-
Hexane, 4-ethyl-2-methyl-
Hexasiloxane, tetradecamethyl-
Hexatriacontane
Hexyl isobutyl carbonate
Hydroxylamine, O-decyl-
Indole, 3-methyl-
Isophthalic acid, isohexyl 1-isopropyl-2-methylpropyl ester
Isopropyl myristate
Krypton
Mercaptamine
Methane-d, trichloro-
Methanol, TMS derivative
Methoxyacetic acid, 3-tridecyl ester

Hexane, 2,4-dimethyl-
Hexane, 2-nitro-
Hexane, 3,3,4-trimethyl-
Hexane, 3,3-dimethyl-
Hexane, 3-ethyl-
Hexane, 3-ethyl-2-methyl-
Hexane, 3-ethyl-4-methyl-
Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-
Hexasiloxane, tetradecamethyl-
Hexatriacontane
Hexyl isobutyl carbonate
Hexyl octyl ether
Hydroxylamine, O-(2-methylpropyl)-
Indole, 3-methyl-
Isobutylamine
Isophthalic acid, isohexyl 1-isopropyl-2-methylpropyl ester
Isopropyl 5,11-dihydroxy-3,7,11-trimethyl-2-dodecenoate
Isopropyl myristate
Kaur-16-en-18-oic acid, methyl ester, (4á)-
Kaur-16-ene
Malonic acid, 10-chlorodecyl isobutyl ester
Mercaptamine
Mercaptoacetic acid, 2TMS derivative
Methane-d, trichloro-
Methanethiol
Methyl 11,12-tetradecadienoate
Methyl 1-acetylpyrrolidine-2-carboxylate
Methyl 3-butynoate
Methylene chloride- solvent
Methyl 8-methyl-nonanoate
N-(2-Octyl)heptafluorobutyramide
Naphthalene, 1,2,3,4-tetrahydro-1-methyl-8-(1-methylethyl)-
n-Butyric acid 2-ethylhexyl ester
n-Decanoic acid
n-Hexyl salicylate
N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide
Nonacosane
Nonadecane, 2-methyl-
Nonane
Nonane, 2,2,4,4,6,8,8-heptamethyl-
Nonane, 2,5-dimethyl-
Nonane, 2-methyl-
Nonane, 4,5-dimethyl-
Nonane, 4-ethyl-5-methyl-
Nonane, 4-methyl-
Nonane, 5-methyl-
Nonane, 5-methyl-5-propyl-
Norfenfluramine
n-Tetradecyltrichlorosilane
n-Tridecan-1-ol
Octacosane
Octadecane
Octadecane, 1-iodo-
Octadecane, 2-methyl-
Octadecane, 4-methyl-
Methyl 1-acetylpyrrolidine-2-carboxylate
Methyl 3-butynoate
Methylene chloride -solvent
Methyl isobutyrate
Morpholine, 4-[[(dimethylamino)thioxomethyl]thio]-
N,N-Diethyl-11H-indolo[3,2-c]quinoline-11-ethanamine
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-
Nitrous acid
N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide
Nonacosane
Nonadecane
Nonadecane, 2-methyl-
Nonane
Nonane, 2,3-dimethyl-
Nonane, 2,5-dimethyl-
Nonane, 2,6-dimethyl-
Nonane, 2-methyl-
Nonane, 4-methyl-
Nonane, 5-methyl-
Norfenfluramine
n-Tridecan-1-ol
o-Anisic acid, tridec-2-ynyl ester
Octacosane
Octadecane
Octadecane, 1-iodo-
Octadecane, 6-methyl-
Octane, 2,3,6,7-tetramethyl-
Octane, 2,3-dimethyl-
Octane, 2,4,6-trimethyl-
Octane, 2,7-dimethyl-
Octane, 2-bromo-
Octane, 3,5-dimethyl-
Octane, 3-ethyl-
Octane, 3-ethyl-2,7-dimethyl-
Octane, 3-methyl-
Octane, 4-methyl-
Oleic Acid
Oxalic acid, dineopentyl ester
Oxalic acid, dodecyl 3,5-difluorophenyl ester
Oxalic acid, heptyl isohexyl ester
Oxalic acid, heptyl propyl ester
Oxalic acid, hexyl isohexyl ester
Oxalic acid, hexyl octadecyl ester
Oxalic acid, hexyl propyl ester
Oxalic acid, isobutyl hexyl ester
Oxalic acid, isobutyl nonyl ester
Oxalic acid, isobutyl octyl ester
Oxalic acid, isohexyl neopentyl ester
Oxalic acid, isohexyl pentyl ester
Oxirane, [(hexadecyloxy)methyl]-
Oxirane, hexadecyl-
Pentaborane(11)
Pentacosane
Pentadecane
Pentadecane, 2,6,10-trimethyl-

Octadecane, 5,14-dibutyl-
Octadecane, 6-methyl-
Octadecanoic acid, 3-hydroxypropyl ester
Octahydropyrano[3,2-b]340ydrazin-6-one
Octan-2-one, 3,6-dimethyl-
Octane, 1,1'-oxybis-
Octane, 2,3,6,7-tetramethyl-
Octane, 2,3-dimethyl-
Octane, 2,7-dimethyl-
Octane, 2-methyl-
Octane, 3,3-dimethyl-
Octane, 3,5-dimethyl-
Octane, 3-ethyl-
Octane, 3-methyl-
Octane, 4,5-diethyl-
Octane, 4-methyl-
Oleic Acid
Oxalic acid, 2TMS derivative
Oxalic acid, allyl dodecyl ester
Oxalic acid, allyl hexyl ester
Oxalic acid, allyl octyl ester
Oxalic acid, bis(2-ethylhexyl) ester
Oxalic acid, decyl neopentyl ester
Oxalic acid, dineopentyl ester
Oxalic acid, dodecyl hexyl ester
Oxalic acid, heptyl propyl ester
Oxalic acid, hexyl isohexyl ester
Oxalic acid, isobutyl hexyl ester
Oxalic acid, isobutyl octyl ester
Oxalic acid, isobutyl pentyl ester
Oxirane, [(hexadecyloxy)methyl]-
Oxirane, tetradecyl-
Pent-1-yne, 5-benzyloxy-
Pentaborane(11)
Pentaborane(9)
Pentacosane
Pentadecane
Pentadecane, 2-methyl-
Pentadecanoic acid, 14-methyl-, methyl ester
Pentane, 1,3-epoxy-4-methyl-
Pentane, 1-bromo-5-methoxy-
Pentane, 2,3,4-trimethyl-
Pentane, 2-methoxy-2,4,4-trimethyl-
Pentane, 3-ethyl-
Pentane, 3-ethyl-2-methyl-
Pentanoic acid, 2-acetyl-4-methyl-, methyl ester
Pentanol, 5-amino-
Phenol, 2,4,6-tris(1-methylethyl)-
Phenol, 2,5-bis(1,1-dimethylethyl)-
Phenol, 4,6-di(1,1-dimethylethyl)-2-methyl-
Phthalic acid, 2-chloropropyl isohexyl ester
Phthalic acid, 2-chloropropyl propyl ester
Phthalic acid, 3,3-dimethylbut-2-yl isohexyl ester
Phthalic acid, 8-chlorooctyl heptyl ester
Phthalic acid, di(hept-2-yl) ester
Phthalic acid, di(hept-4-yl) ester
Pivalic acid vinyl ester
Precocene I
Pregn-5-ene-3,20-diol, (3à)-, 2TMS derivative
Pregn-5-ene-3,20-diol, (3á,20S)-, 2TMS derivative
Pentadecane, 2-methyl-
Pentane, 1-bromo-5-methoxy-
Pentane, 2,3,4-trimethyl-
Pentane, 2-methyl-
Pentane, 3,3-dimethyl-
Pentanol, 5-amino-
Pentyl tetradecyl ether
Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,1,4a,7-tetramethyl-, [4aS-(4aà,4bá,7á,10aá)]-
Phenol, 2,5-bis(1,1-dimethylethyl)-
Phenol, 4-ethyl-2-methoxy-
Phosphonous dichloride, [[chloro(1,1-dimethylethyl)phosphino]methyl]-
Phthalic acid, 2-chloropropyl propyl ester
Phthalic acid, butyl 2-chloropropyl ester
Phthalic acid, di(hept-2-yl) ester
Phthalic acid, hex-2-yn-4-yl nonyl ester
Phthalic acid, hexyl tridec-2-yn-1-yl ester
Pivalic acid vinyl ester
Pregn-5-ene-3,20-diol, (3à)-, 2TMS derivative
Pregn-5-ene-3,20-diol, (3á,20S)-, 2TMS derivative
Pregna-3,5-dien-20á-ol, O-trimethylsilyl
Pregnan-20-one, 3-(acetyloxy)-5,6-epoxy-, cyclic 20-(1,2-ethanediyl acetal), (3á,5à,6à)-
Propanamide, 3-(1-pyrrolidinyl)-N-(2-thiazolyl)-
Propane, 1-nitro-
Propane, 2-chloro-2-methyl-
Propane, 2-nitro-
Propanoic acid, 2-methyl-, 1,2,3-propanetriyl ester
Propiolonitrile
Pyrazolo[3,4-b]pyran-5-carbonitrile, 1,4-dihydro-6-amino-4-(3,4-dichlorophenyl)-3-methyl-
Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester
Salvial-4(14)-en-1-one
Silane, trichlorooctadecyl-
Stearic acid hydrazide
Succinic acid, 3-methylbut-2-yl tetrahydrofurfuryl ester
Succinic acid, isohexyl tetrahydrofurfuryl ester
Sulfone, 2-hydroxybutyl t-butyl
Sulfone, butyl isopropyl
Sulfurous acid, 2-ethylhexyl pentyl ester
Sulfurous acid, 2-ethylhexyl undecyl ester
Sulfurous acid, butyl 2-ethylhexyl ester
Sulfurous acid, butyl octadecyl ester
Sulfurous acid, butyl octyl ester
Sulfurous acid, butyl pentyl ester
Sulfurous acid, dibutyl ester
Sulfurous acid, dipentyl ester
Sulfurous acid, hexyl heptyl ester
Sulfurous acid, hexyl nonyl ester
Sulfurous acid, hexyl octyl ester
Sulfurous acid, hexyl pentadecyl ester
Sulfurous acid, hexyl pentyl ester
Sulfurous acid, hexyl tridecyl ester
Sulfurous acid, isobutyl pentyl ester
Sulfurous acid, isohexyl 2-pentyl ester
Sulfurous acid, nonyl 2-pentyl ester

Propanamide, 3-(1-pyrrolidinyl)-N-(2-thiazolyl)-
Propane, 2,2'-[methylenebis(oxy)]bis[2-methyl-
Propane, 2-methyl-1-nitro-
Propane, 2-nitro-
Propanoic acid, 2,2-dimethyl-
Propanoic acid, 2,2-dimethyl-, 2,4-dinitrophenyl ester
Propanoic acid, 2,2-dimethyl-, methyl ester
Propanoic acid, 2,2-dimethyl-, propyl ester
Propanoic acid, 2-methyl-, 3-methylbutyl ester
Propanoic acid, 2-propenyl ester
Propene
Pyrazolo[3,4-b]pyran-5-carbonitrile, 1,4-dihydro-6-amino-4-(3,4-dichlorophenyl)-3-methyl-
Pyrido[2,3-d]pyrimidine-2,4(1H,3H)-dione, 6,7-dichloro-5-[(1-ethylpyrrolidin-2-yl)methylamino]-1,3-dimethyl-
Pyrimidine-2,4(1H,3H)-dione, 5-bromo-1-(1-methoxyethyl)-
Scyllo-Inositol
Silane, (diphenylmethyl)trimethyl-
Silane, trichlorooctadecyl-
Silicic acid, diethyl bis(trimethylsilyl) ester
Silicon tetrafluoride
Stearic acid hydrazide
Succinic acid, isohexyl tetrahydrofurfuryl ester
Sulfone, 2-hydroxypropyl t-butyl
Sulfurous acid, 2-ethylhexyl pentyl ester
Sulfurous acid, butyl dodecyl ester
Sulfurous acid, butyl hexadecyl ester
Sulfurous acid, butyl nonyl ester
Sulfurous acid, butyl pentyl ester
Sulfurous acid, dodecyl 2-propyl ester
Sulfurous acid, hexyl 2-pentyl ester
Sulfurous acid, hexyl heptyl ester
Sulfurous acid, hexyl nonyl ester
Sulfurous acid, hexyl octyl ester
Sulfurous acid, hexyl pentadecyl ester
Sulfurous acid, hexyl pentyl ester
Sulfurous acid, hexyl tridecyl ester
Sulfurous acid, isohexyl 2-pentyl ester
Sulfurous acid, nonyl pentyl ester
Sulfurous acid, pentyl tridecyl ester
t-Butyl n-hexyl disulfide
tert-Hexadecanethiol
Tetracosane
Tetradecane
Tetradecane, 1-fluoro-
Tetradecane, 1-iodo-
Tetradecane, 4-ethyl-
Thymol, TBDMS derivative
trans-2,3,4-Trichloro-2-butene nitrile
Triacontane
Triacontanoic acid, methyl ester
Tridecane
Tridecane, 2-methyl-
Tridecane, 3-methyl-
Tridecane, 4,8-dimethyl-
Tridecane, 4-methyl-
Tridecane, 6-methyl-
Sulfurous acid, nonyl pentyl ester
Sulfurous acid, pentyl tridecyl ester
Sulfurous acid, pentyl undecyl ester
tert-Butyl 2-chloro-3-(4-chloro-2-vinyl-1,3-oxazol-5-yl)-1H-indol-1-carboxylate
tert-Butyl Hydroperoxide
Tetracosane
Tetracosane, 11-decyl-
Tetradecane
Tetradecane, 1-iodo-
Tetradecane, 2,6,10-trimethyl-
Tetradecane, 4-ethyl-
Tetrahydropyran Z-10-dodecenoate
Thiazole-5-carboxylic acid, 2-tert-butylthio-4-methyl-
Thymol, TBDMS derivative
trans-2-Dodecen-1-ol
Triacontane
Tridecane
Tridecane, 2-methyl-
Tridecane, 3-methyl-
Tridecane, 4,8-dimethyl-
Tridecane, 4-methyl-
Tridecane, 6-methyl-
Tridecanoic acid, 2-ethyl-2-methyl-, ethyl ester
Tridecanoic acid, methyl ester
Tridecanol, 2-ethyl-2-methyl-
Trimethylacetic Acid, 2,2,2-trifluoroethyl ester
Trimethylsilyl-di(timethylsiloxy)-silane
Tris(tert-butyldimethylsilyloxy)arsane
Undecane
Undecane, 2,4-dimethyl-
Undecane, 2,5-dimethyl-
Undecane, 2,6-dimethyl-
Undecane, 2,7-dimethyl-
Undecane, 2-methyl-
Undecane, 3,7-dimethyl-
Undecane, 3,8-dimethyl-
Undecane, 4,6-dimethyl-
Undecane, 4,7-dimethyl-
Undecane, 4,8-dimethyl-
Undecane, 4-methyl-
Undecane, 5,7-dimethyl-
Undecane, 6-methyl-
Undecanoic acid, methyl ester
Vitamin E

Tridecane, 7-methyl-	
Tridecanoic acid, methyl ester	
Tridecanol, 2-ethyl-2-methyl-	
Tris(tert-butyldimethylsilyloxy)arsane	
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	
Undecane	
Undecane, 2,4-dimethyl-	
Undecane, 2,5-dimethyl-	
Undecane, 2,6-dimethyl-	
Undecane, 2-methyl-	
Undecane, 3,7-dimethyl-	
Undecane, 3,8-dimethyl-	
Undecane, 3,9-dimethyl-	
Undecane, 4,6-dimethyl-	
Undecane, 4,8-dimethyl-	
Undecane, 4-methyl-	
Undecane, 5-ethyl-	
Undecane, 6-methyl-	
Undecanoic acid, methyl ester	
Vitamin E	
Xylo-hexos-5-ulose, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, bis(O-methyloxime)	
504 chemical compounds	**468 chemical compounds**

APPENDIX N

Table 6.2: Comparison of chemical compounds in the stem bark of all 12 the *C. dentata* trees from the southern Cape and all 12 the *C. dentata* trees from KZN, collected November 2015 and November 2018 respectively. The highlighted compounds occur in trees at both sites but not necessarily in every tree at each of the respective sites.

Southern Cape	KZN
(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	(1R,4aS,8aR)-1-Isopropyl-4,7-dimethyl-1,2,4a,5,6,8a-hexahydronaphthalene
(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane	(1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone	(2S,6R)-2-Methyl-6-nonylpiperidin-4-one
1-(4-tert-Butylphenyl)propan-2-one	(Z)-2-Hydroxyimino-3-oxobutyric acid, 1,1-dimethylethyl ester
1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline	.alfa.-Copaene
1,2-Bis(tert-butylimino)ethane	1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	1-(3,5-Di-tert-butyl-4-hydroxyphenyl)-2-bromethanone
1,3-Butadiyne	1-(4-tert-Butylphenyl)propan-2-one
1,3-di-iso-propylnaphthalene	1,1,1,3,5,5,5-Heptamethyltrisiloxane
1,3-Dioxolane, 4-methyl-2-pentadecyl-	1,2,3,4-Tetrahydro-1-naphthylamine, N-heptafluorobutyryl-
1,3-Dioxolane, 4-methyl-2-pentyl-	**1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline**
1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate]	1,2,3-Butatriene, 1-chloro-
1,5-Pentanediol, O,O'-di(3-methylbut-2-enoyl)-	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester
1,7-di-iso-propylnaphthalene	1,2-Benzenedicarboxylic acid, decyl octyl ester
11-Hexadecen-1-ol, (Z)-	1,2-Benzenedicarboxylic acid, mono(1-methylheptyl) ester, (ñ)-
11-Methyldodecanol	1,2-Benzisothiazol-3-amine, TBDMS derivative
1-Bromo-8-tetrahydropyranyloxyoctane	1**,3-Butadiyne**
1-Butanol, 2,2-dimethyl-	**1,3-di-iso-propylnaphthalene**
1-Buten-3-yne, 1-chloro-, ®-	1,3-Dioxolane, 2-(3-bromo-5,5,5-trichloro-2,2-dimethylpentyl)-
1-Decene, 3,3,4-trimethyl-	1,3-Dioxolane, 2-heptyl-4-methyl-
1-Decene, 4-methyl-	1,3-Dioxolane, 2-pentadecyl-
1-Docosene	1,3-Dioxolane, 4-methyl-2-pentadecyl-
1-Dodecanol, 2-hexyl-	1,4-Cyclohexanedimethanamine
1-Dodecanol, 2-hexyl-	1,4-di-iso-propylnaphthalene
1-Dodecanol, 3,7,11-trimethyl-	**1,7-di-iso-propylnaphthalene**
1-Heptadecyne	10-Undecynoic acid, methyl ester
1-Heptanol, 2-propyl-	**11-Methyldodecanol**
1-Hexadecanol	11-Tricosene
1-Hexanol, 6-amino-	1-Acetoxynonadecane
1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)-	1-Butanamine
1H-Indene, 1-hexadecyl-2,3-dihydro-	1-Butanamine, 3-methyl-
1H-Pyrazole, 4,5-dihydro-5,5-dimethyl-4-isopropylidene-	1-Butanol, 2,2-dimethyl-
1-Hydroxy-3-methyl-2-butanone	**1-Buten-3-yne, 1-chloro-, (Z)-**
1-Iodo-2-methylundecane	1-Decanol, 2-hexyl-
1-Nonene	1-Decanol, 2-hexyl-
1-Octanesulfonyl chloride	1-Decene, 3,3,4-trimethyl-
1-Octanol, 2-butyl-	1-Decene, 4-methyl-
1-Octanol, 3,7-dimethyl-, (S)-	**1-Docosene**
1-Octene, 3,7-dimethyl-	**1-Heptanol, 2-propyl-**
1-Octyn-3-ol, 4-ethyl-	1-Hexanol, 2-ethyl-
1-Pentanol, 2,4-dimethyl-, (ñ)-	1-Hexene, 3,5,5-trimethyl-
1-Penten-3-ol, 3-methyl-	
1-Undecene, 4-methyl-	
1-Undecene, 7-methyl-	

1-Undecyn-4-ol
2(2-Bromoethyl)-1,3-dioxane
2(3H)-Furanone, 5-hexyldihydro-
2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid
2,2-Dimethyl-3-heptene trans
2,2'-Isopropylidenebis(5-methylfuran)
2,3,4,5,6-Pentamethyl acetophenone
2,3-Dihydroxypropyl icosanoate, 2TMS derivative
2,3-Dimethyldecane
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
2,4,6-Trimethyl-1-nonene
2,4-Dimethyl-1-heptene
2,4-Dimethylhept-1-ene
2,4-Disilapentane, 2,4-dimethyl-
2,4-Di-tert-butylphenol
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
2,5-Hexanediol, 2,5-dimethyl-
2,6,10-Trimethyltridecane
2,9-Decanedione
2-Bromo dodecane
2-Butanone, 1-bromo-3,3-dimethyl-
2-Cyclohexylpiperidine
2-Cyclopenten-1-one, 3-(acetyloxy)-
2-Decene, 5-methyl-, (Z)-
2-Diethylamino-N-naphthalen-1-yl-acetamide
2-Hexanol
2-Imidazolidinone
2-Methoxybenzoylformic acid, TMS
2-Methyl-à-pyrrolidinobutirophenone
2-Methyltetracosane
2-Nonanol
2-Nonanone
2-Octene, 3,7-dimethyl-, (Z)-
2-Pentanol, 4-methyl-
2-Penten-1-ol, (Z)-, TBDMS derivative
2-Piperidinemethanamine
2-Propanone, 1,1-dibutoxy-
2-Propyl-1-Pentanol, trifluoroacetate
2-sec-Butyl-3-methyl-1-pentene
2-Tridecen-1-ol, ®-
2-Trifluoroacetoxypentadecane
2-Undecanethiol, 2-methyl-
2-Undecanol
2-Undecene, 5-methyl-
3,5-Dimethyl-4-heptanone
3,5-Dimethyldodecane
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
3,6,6-Trimethyl-cyclohex-2-enol
3,7,11,15,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-
3-Acetoxydodecane
3-Carene
3-Eicosene, ®-
3-Methoxyhex-1-ene
3-Methyl-2-butenoic acid, tridec-2-ynyl ester
3-Pentanone, 2,2,4,4-tetramethyl-
4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one
1-Hexene, 4,5-dimethyl-
1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)-
1H-Indene, 1-hexadecyl-2,3-dihydro-
1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl-
1H-Indene-4-acetic acid, 6-(1,1-dimethylethyl)-2,3-dihydro-1,1-dimethyl-
1H-Indole, 1-methyl-
1-Iodo-2-methylundecane
1-Methoxy-3-methyl-3-butene
1-Naphthalenol, 5,6,7,8-tetrahydro-2,5-dimethyl-8-(1-methylethyl)-
1-Octanesulfonyl chloride
1-Octanol, 2-butyl-
1-Octanol, 3,7-dimethyl-, (S)-
1-Pentanol, 2,4-dimethyl-, (ñ)-
1-Pentanol, 3-methyl-2-propyl-
1-Undecene, 4-methyl-
2-((Pent-4-enyloxy)carbonyl)benzoic acid
2(2-Bromoethyl)-1,3-dioxane
2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid
2,2,4,4-Tetramethyloctane
2,2,4-Trimethyl-3-hexene
2,2-Dimethyl-3-heptene trans
2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone
2,3,4,5,6-Pentamethyl acetophenone
2,4-Dimethyl-1-heptene
2,4-Dimethylhept-1-ene
2,4-Disilapentane, 2,4-dimethyl-
2,4-Di-tert-butylphenol
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
2,5-di-tert-Butyl-1,4-benzoquinone
2,5-Hexanediol, 2,5-dimethyl-
2,5-Monoformal-l-rhamnitol triacetate
2,6,10-Trimethyltridecane
2,6-Diisopropylnaphthalene
2,6-Dimethyldecane
2,6-Nonadienal, (E,Z)-
2,9-Decanedione
2-Bromo dodecane
2-Cyano-2-O-fluorosulfatofluoropropane
2-Decene, 5-methyl-, (Z)-
2-Hexanol
2-Hexanol, 2,5-dimethyl-, (S)-
2-Hexen-1-ol, ®-
2-Hexene, 5,5-dimethyl-, (Z)-
2-Hydroxy-2,4-dimethyl-3-pentanone
2-n-Hexylcyclopentanone
2-Nitro-1,3-bis-octyloxy-benzene
2-Nonanone
2-Octene, 2,6-dimethyl-
2-Octene, 3,7-dimethyl-, (Z)-
2-Pentanol, 4-methyl-
2-Pentanone, 4,4-dimethyl-
2-Penten-1-ol, (Z)-, TBDMS derivative
2-Propanone, 1,1-dibutoxy-
2-sec-Butyl-3-methyl-1-pentene
2-Tridecen-1-ol, ®-

4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone
4-Amino-1-butanol
4-Methyl-2-heptene
4-Nonene, 2,3,3-trimethyl-, (Z)-
4-Nonene, 3-methyl-, (Z)-
5(4H)-Oxazolone, 2-(1,1-dimethylethyl)-
5-[2-Tert-butyldiazen-1-yl]-2-methyl-1,2,3-triazol-4-amine
5-Hexen-3-ol, 2,3-dimethyl-
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
6-(1'-Hydroxyethyl)-2,2-dimethylchromene
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
7-Chloro-1-[[2-[diethylamino]ethyl]imino]-1,3,4,10-tetrahydro-10-hydroxy-3-[3,4,5-(trimethoxy)phenyl]-9(2H)-acridinone
8-Hexadecanol
9-Hexadecen-1-ol, (Z)-
Acetic acid, chloro-
à-Cubebene
à-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-O-(trimethylsilyl)-, cyclic methylboronate
á-Hydroxypyruvic acid, trimethylsilyl ether, trimethylsilyl ester
à-Muurolene
à-Pinene
Argon
à-Tocopheryl acetate
Benzamide, 2-[3-(diethylamino)propanamido]-
Benzene, 1,3-bis(1,1-dimethylethyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-
Benzene, 1,4-bis(1,1-dimethylethyl)-
Benzene, tert-butyl-
Benzoic acid, 3-(3-hydroxy-3-methyl-1-butynyl)-
Benzyl Benzoate
Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-
Binapacryl
Boron trifluoride
Butanoic acid, 2-methylbutyl ester
Butanoic acid, 3-oxo-, 1,1-dimethylethyl ester
Butanoic acid, 4-pentenyl ester
Butylated Hydroxytoluene
Carbonic acid, heptyl vinyl ester
Chlorfenapyr
Chlorine
Chloromethane
Chloromethanesulfonyl chloride
cis-9-Tetradecen-1-ol
Cyclohexane, (1,1-dimethylpropyl)-
Cyclohexane, 1,1'-(2-ethyl-1,3-propanediyl)bis-
Cyclohexane, 1-ethyl-2-propyl-
Cyclohexane, octyl-
Cyclohexane, propyl-
Cyclooctane, 1,4-dimethyl-, cis-
2-Undecanethiol, 2-methyl-
3,3-Diethylpentadecane
3,5-Dimethyldodecane
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
3,6,10,14,17-Pentaoxa-2,18-disilanonadecane, 2,2,18,18-tetramethyl-
3-Chlorohexane
3-Ethyl-2-pentanol
3-Heptene, 4-methyl-
3-Heptene, 4-methyl-
3H-Naphtho[2,3-b]furan-2-one, 5,8a-dimethyl-3-(345ydrazine345e-1-ylmethyl)-3a,5,6,7,8,8a,9,9a-octahydro-
3-Pentanol, 2,2-dimethyl-
3-Undecene, ®-
4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone
4-Amino-3-methoxypyrazolo[3,4-d]pyrimidine
4-Aminofurazan-3-carboxylic acid, 3,3-dimethyl-2-oxobutyl ester
4-Heptyn-2-ol
4-Methoxycarbonyl-4-butanolide
4-Nonene, 3-methyl-, (Z)-
4-Propionyloxytridecane
4-tert-Butyl-2,6-diisopropylphenol
4'-tert-Butyl-2',6'-dimethylacetophenone
4-tert-Octylphenol, TMS derivative
5-Cyano-1,2,3-thiadiazole
5-Ethyl-5-methylnonadecane
5-Ethyldecane
5-Hexen-3-one
5-Methoxy-2,3-dimethylindole
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
5-Undecene, 7-methyl-, (Z)-
7,7,9,9,11,11-Hexamethyl-3,6,8,10,12,15-hexaoxa-7,9,11-trisilaheptadecane
7-Acetyl-1,7-diazabicyclo[2.2.0]heptane
8,9,9,10,10,11-Hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo[5.4.1.0(2,6).0(8,11)]dodecane
8-Dodecenol
9-Hexadecen-1-ol, (Z)-
Acetamide, 2-(1-pyrrolidinyl)-N-(3-chlorophenyl)-
Acetic acid, 1-cyano-1-[7-(2-methoxyethoxymethoxy)-6-methylbicyclo[4.3.0]non-3-ylidene]-, ethyl ester
á-Hydroxypyruvic acid, trimethylsilyl ether, trimethylsilyl ester
à-Muurolene
à-Pinene
Argon
Arsenous acid, tris(trimethylsilyl) ester
à-Tocopheryl acetate
Behenyl chloride
Benzene, 1,1'-(2-butene-1,4-diyl)bis-
Benzene, 1,3-bis(1,1-dimethylethyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-
Benzene, 1,4-dimethoxy-2-methyl-
Benzene, 1-methyl-3-(1-methylethyl)-
Benzene, tert-butyl-
Benzoic acid, 2-(1-oxopropyl)-

Cyclopentane, 1,2-dipropyl-
Cyclopentane, 1-butyl-2-propyl-
Cyclopentane, 1-pentyl-2-propyl-
Cyclopentane, 2-ethyl-1,1-dimethyl-
Cyclopentane, hexyl-
Cyclopentanol O-tert-butyldimethylsilyl ether
Cyclopentene, 1,2,3,3,4-pentamethyl-
Cyclopropanecarboxylic acid, 1-(phenylmethyl)-, 2,6-bis(1,1-dimethylethyl)-4-methylphenyl ester
Cyclotetradecane
Decane
Decane, 1-fluoro-
Decane, 2,3,5,8-tetramethyl-
Decane, 2,4,6-trimethyl-
Decane, 2,5-dimethyl-
Decane, 2,6,7-trimethyl-
Decane, 4-methyl-
Decane, 5-ethyl-5-methyl-
Decane, 5-methyl-
Decanoic acid, methyl ester
Decyl pentyl ether
Desmethyldeprenyl
Dibutyl phthalate
Dichloroacetaldehyde
Dichloroacetic acid, 4-methylpentyl ester
Dicyclohexyl phthalate
Didodecyl phthalate
Dimethyl(1-cyclopentylethoxy)silane
dl-à-Tocopherol
Docosane
Docosane, 11-butyl-
Docosanoic acid, ethyl ester
Dodecane, 1-iodo-
Dodecane, 2,6,11-trimethyl-
Dodecane, 4,6-dimethyl-
Dodecane, 4-methyl-
Dodecane, 6-methyl-
Dotriacontane
E,Z-2,13-Octadecadien-1-ol
Eicosane
Eicosane, 2,4-dimethyl-
Eicosane, 2-methyl-
Ethane, 1,1'-oxybis[2-methoxy-
Ethane-1,1-diol dipropanoate
Ethanol, 2,2-dichloro-
Ethanone, 1-(2,4,5-triethylphenyl)-
Ether, 6-methylheptyl vinyl
Ether, hexyl pentyl
Ether, tert-butyl isopropylidenecyclopropyl
Ethyl 3-hydroxydocosanoate
Ethyl 3-hydroxytetracosanoate
Ethyl 3-methylbut-3-enyl carbonate
Etoloxamine
Glycine
Glycolic acid, 2TMS derivative
Glycyl-L-tryptophylglycine
Heneicosane
Heneicosane, 11-(1-ethylpropyl)-
Hentriacontane
Heptacosane
Benzoic acid, 2-hydroxy-, 2-methylbutyl ester
Benzoic acid, 4-ethoxy-, ethyl ester
Benzonitrile, 4-[[(2.5-dioxo-1-pyrrolidinyl)oxy]carbonyl]-
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
Binapacryl
Bis(tridecyl) phthalate
Boron trifluoride
Butane, 1-chloro-2-methyl-
Butanoic acid, 2-methyl-5-oxo-1-cyclopenten-1-yl ester
Butanoic acid, 2-methylbutyl ester
Butylated Hydroxytoluene
Butyric acid, neopentyl ester
Carbamic acid, (cyanomethyl)-, 1,1-dimethylethyl ester
Carbonic acid, di(decyl) ester
Carbonic acid, heptyl vinyl ester
Chlorfenapyr
Chlorine
Chloromethane
Chloromethanesulfonyl chloride
Cobalt, (ü5-2,4-cyclopentadien-1-yl)[[[(2,3,4,5-ü)-2,4-cyclopentadien-1-yl]methyl]benzene]-
Cyclohexane, 1,2,4,5-tetraethyl-, (1à,2à,4à,5à)-
Cyclohexane, 1-ethyl-2-propyl-
Cyclohexane, octyl-
Cyclohexanone, 2-hydroxy-
Cyclooctane, 1,4-dimethyl-, cis-
Cyclopentane, 1-(S)-(2-methyl-1,3-dioxolan-2-yl)-2-®-formylmethyl-4-®-(2-methoxyethoxymethoxy)-
Cyclopentane, 1,1,3,4-tetramethyl-, cis-
Cyclopentane, 1-butyl-2-propyl-
Cyclopentane, hexyl-
Cyclopentene, 1,2,3,3,4-pentamethyl-
Cyclopropanecarboxylic acid, 1-(phenylmethyl)-, 2,6-bis(1,1-dimethylethyl)-4-methylphenyl ester
Cyclotetradecane
Cyclotrisiloxane, hexamethyl-
Decane
Decane, 1-(ethenyloxy)-
Decane, 1-iodo-
Decane, 2,3,5,8-tetramethyl-
Decane, 2,3,7-trimethyl-
Decane, 2,4,6-trimethyl-
Decane, 2,5,9-trimethyl-
Decane, 2,5-dimethyl-
Decane, 2,6,7-trimethyl-
Decane, 2,6,8-trimethyl-
Decane, 2-methyl-
Decane, 3,8-dimethyl-
Decane, 5-methyl-
Decanoic acid, methyl ester
Desmethyldeprenyl

Heptacosane, 1-chloro-
Heptacosanoic acid, methyl ester
Heptadecane
Heptadecane, 2,6,10,14-tetramethyl-
Heptadecane, 2,6,10,15-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
heptadecanoic acid, 2-methoxyethyl ester
Heptane, 2,2,3,3,5,6,6-heptamethyl-
Heptane, 2,3,5-trimethyl-
Heptane, 2,4,6-trimethyl-
Heptane, 2,4-dimethyl-
Heptane, 3,3-dimethyl-
Heptane, 3-ethyl-
Heptane, 4,4-dimethyl-
Hexacosane
Hexadecane
Hexadecane, 2,6,10,14-tetramethyl-
Hexadecen-1-ol, trans-9-
Hexane, 2,2,3,3-tetramethyl-
Hexane, 2,3,3-trimethyl-
Hexane, 2,3,4-trimethyl-
Hexane, 2,4-dimethyl-
Hexane, 3,3-dimethyl-
Hexane, 3-ethyl-
Hexane, 3-ethyl-2-methyl-
Hexane, 3-methyl-
Hexane, 4-ethyl-2-methyl-
Hexasiloxane, tetradecamethyl-
Hexatriacontane
Hexyl octyl ether
Hydroperoxide, 1-methylpentyl
Hydroxylamine, O-decyl-
Indole, 3-methyl-
Isodecyl methacrylate
Isophthalic acid, isohexyl 1-isopropyl-2-methylpropyl ester
Isopropyl myristate
Kaur-16-ene
m-Aminophenylacetylene
Mercaptamine
Mercaptoacetic acid, 2TMS derivative
Methane-d, trichloro-
Methyl 3-butynoate
Methylene chloride – solvent
N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide
Nonadecane
Nonadecane, 2-methyl-
Nonane
Nonane, 2,5-dimethyl-
Nonane, 2-methyl-
Nonane, 4-methyl-
Norfenfluramine
n-Tridecan-1-ol
Octa-3,5-diene-2,7-dione, 4,5-dihydroxy-
Octacosane
Octadecane
Octadecane, 1-iodo-
Octadecane, 4-methyl-
Diallyl carbonate
Diazene, bis(1,1-dimethylethyl)-
Diaziridine, tetrafluoro-
Dibutyl phthalate
Dicyclohexyl phthalate
Difluoromethane
Difluoromethane
Dimethyl 2-hydroxy-2-methylbutane-1,4-dioate
Dimethyl tetradecanedioate
dl-3,4-Dehydroproline methyl ester
Docosane, 11-butyl-
Dodecane
Dodecane, 1-iodo-
Dodecane, 2,6,11-trimethyl-
Dodecane, 4,6-dimethyl-
Dodecane, 5,8-diethyl-
Dodecane, 6-methyl-
Dodecanoic acid, methyl ester
Dotriacontane
d-Ribo-tetrofuranose, 4-c-cyclopropyl-1,2-O-isopropylidene-, à-
d-Xylitol, 1,3,5-trideoxy-3-nitro-, 4-(2,2-dimethylpropanoate)
E-10-Pentadecenol
Eicosane
Eicosane, 2,4-dimethyl-
Eicosane, 2-methyl-
Eicosane, 7-hexyl-
Erythro-2-methyl-3,4-dibromo-2-butanol
Ethanol, 1-(methylenecyclopropyl)-1-(methylene-1-trimethylsilylcyclopropyl)-
Ethanol, 2,2-dichloro-
Ether, hexyl pentyl
Ethyl 3-hydroxyhexadecanoate
Ethyl 3-methylbut-3-enyl carbonate
Fenpipramide
Formic acid, 2,2-dimethylpent-3-yl ester
Furan, 2,5-dihydro-
Glycine
Glycolic acid, 2TMS derivative
Glycyl-L-tryptophylglycine
Heneicosane
Heneicosanoic acid, isopropyl ester
Hentriacontane
Heptacosane
Heptacosane, 1-chloro-
Heptadecane
Heptadecane, 2,6,10,14-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
heptadecanoic acid, 2-methoxyethyl ester
Heptane, 2,3,4-trimethyl-
Heptane, 2,3,5-trimethyl-
Heptane, 2,4,6-trimethyl-
Heptane, 2,4-dimethyl-
Heptane, 3,3,5-trimethyl-
Heptane, 3,3-dimethyl-
Heptane, 3,4-dimethyl-
Heptane, 3,5-dimethyl-
Heptane, 3-ethyl-

Octadecane, 6-methyl-
Octan-2-one, 3,6-dimethyl-
Octane
Octane, 2,3,6,7-tetramethyl-
Octane, 2,6-dimethyl-
Octane, 2,7-dimethyl-
Octane, 3,3-dimethyl-
Octane, 3-ethyl-
Octane, 3-ethyl-2,7-dimethyl-
Octane, 3-methyl-
Octane, 4,5-dimethyl-
Octane, 4-methyl-
Oxacyclotetradeca-4,11-diyne
Oxalic acid, 2-ethylhexyl pentyl ester
Oxalic acid, 3,5-difluorophenyl tetradecyl ester
Oxalic acid, 3,5-difluorophenyl undecyl ester
Oxalic acid, decyl 3,5-difluorophenyl ester
Oxalic acid, hexyl isohexyl ester
Oxalic acid, hexyl neopentyl ester
Oxalic acid, isobutyl hexyl ester
Oxalic acid, isobutyl octyl ester
Oxalic acid, isobutyl pentyl ester
Oxalic acid, isohexyl pentyl ester
Oxalic acid, neopentyl pentyl ester
Oxeladin
Oxirane, tetradecyl-
Pentaborane(11)
Pentadecane
Pentadecane, 2,6,10-trimethyl-
Pentadecane, 2-methyl-
Pentadecanoic acid, 14-methyl-, methyl ester
Pentane, 1-butoxy-
Pentane, 2,2,4,4-tetramethyl-
Pentane, 2,4-dimethyl-
Pentane, 2-bromo-4-methyl-
Pentane, 3-ethyl-2,4-dimethyl-
Pentane, 3-ethyl-3-methyl-
Phenol, 2,4,6-tris(1-methylethyl)-
Phenol, 2,5-bis(1,1-dimethylethyl)-
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-, methylcarbamate
Phthalic acid, di(hept-2-yl) ester
Phthalic acid, di(hept-4-yl) ester
Phthalimide, N-isopropyl-
Piperazin-1,4-diium, 1,4-di(2-chloroethyl)-1,4-dimethyl-, dichloride
Pivalic acid vinyl ester
Pregn-5-ene-3,20-diol, (3á,20S)-, 2TMS derivative
Pregnan-20-one, 3-(acetyloxy)-5,6-epoxy-6-methyl-, cyclic 20-(1,2-ethanediyl acetal), (3á,5á,6á)-
Pregnane-3,20-diol, (3à,5á,20S)-, 2TMS derivative
Propane, 1-nitro-
Propanoic acid, 2,2-dimethyl-, methyl ester
Propiolonitrile
Pyridine, 1,2,3,6-tetrahydro-1-nitroso-
Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester
Salicylic acid, tert.-butyl ester
Scyllo-Inositol
Heptane, 3-ethyl-2-methyl-
Heptane, 4-(1-methylethyl)-
Heptane, 4,4-dimethyl-
Heptane, 4-ethyl-
Heptane, 4-methyl-
Heptane, 4-propyl-
Hexacosane
Hexadecane
Hexadecane, 2,6,11,15-tetramethyl-
Hexadecane, 2-methyl-
Hexadecanoic acid, 15-methyl-, methyl ester
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Hexadecanoic acid, methyl ester
Hexadecen-1-ol, trans-9-
Hexane, 2,2,3,3-tetramethyl-
Hexane, 2,3,4-trimethyl-
Hexane, 2,4-dimethyl-
Hexane, 3,3,4,4-tetramethyl-
Hexane, 3-ethyl-
Hexane, 3-ethyl-2,5-dimethyl-
Hexane, 3-ethyl-4-methyl-
Hexane, 3-methyl-
Hexanoic acid, hexadecyl ester
Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-
Hexasiloxane, tetradecamethyl-
Hexyl isobutyl carbonate
Hydroxylamine, O-(2-methylpropyl)-
Indole, 3-methyl-
Isophthalic acid, isohexyl 1-isopropyl-2-methylpropyl ester
Isopropyl myristate
Isopropyl palmitate
L-Serine, 3TBDMS derivative
m-Aminophenylacetylene
Mercaptoacetic acid, 2TMS derivative
Methane-d, trichloro-
Methanol, TMS derivative
Methyl 12,13-tetradecadienoate
Methyl 1-acetylpyrrolidine-2-carboxylate
Methyl 3-butynoate
Methylene chloride – solvent
N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide
Nonadecane
Nonadecane, 2-methyl-
Nonane
Nonane, 2,5-dimethyl-
Nonane, 2-methyl-
Nonane, 3-methyl-
Nonane, 4-ethyl-5-methyl-
Nonane, 4-methyl-
Nonane, 4-methyl-5-propyl-
Nonane, 5-methyl-
Norfenfluramine
n-Tetracosanol-1
n-Tridecan-1-ol
o-Anisic acid, tridec-2-ynyl ester
Octa-3,5-diene-2,7-dione, 4,5-dihydroxy-

sec-Butyl nitrite
Silane, cyclohexyldimethoxymethyl-
Silane, trichlorooctadecyl-
Silicic acid, diethyl bis(trimethylsilyl) ester
Silicon tetrafluoride
Sulfone, 2-hydroxyoctyl t-butyl
Sulfurous acid, 2-ethylhexyl hexyl ester
Sulfurous acid, 2-pentyl pentyl ester
Sulfurous acid, butyl hexyl ester
Sulfurous acid, butyl octyl ester
Sulfurous acid, butyl pentyl ester
Sulfurous acid, dodecyl 2-propyl ester
Sulfurous acid, hexyl heptyl ester
Sulfurous acid, hexyl pentadecyl ester
Sulfurous acid, hexyl pentyl ester
Sulfurous acid, hexyl tridecyl ester
Sulfurous acid, isobutyl pentyl ester
Sulfurous acid, isohexyl 2-pentyl ester
Sulfurous acid, nonyl pentyl ester
Sulfurous acid, octyl 2-pentyl ester
Sulfurous acid, pentyl tridecyl ester
Terephthalic acid, allyl 4-fluoro-2-methoxyphenyl ester
Terephthalic acid, isohexyl phenyl ester
tert-Butyl Hydroperoxide
tert-Butyl N-hydroxycarbamate
Tetracosane
Tetradecane
Tetradecane, 4-ethyl-
Tetradecane, 5-methyl-
Tetrahydrofuran, 2-propyl-
Tetratriacontane
Thiophene, 2-ethynyl-5-[(trimethylsilyl)ethynyl]-
Triacontane
Tricyclo[4.3.1.1(3,8)]undecane, 1-bromo-
Tridecane
Tridecane, 2-methyl-
Tridecane, 3-methyl-
Tridecane, 4,8-dimethyl-
Tridecane, 4-methyl-
Tridecanoic acid, methyl ester
Trimethylsilyl O,O'-bis(trimethylsilyl)vanilpyruvate
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-
Undecane
Undecane, 2,10-dimethyl-
Undecane, 2,4-dimethyl-
Undecane, 2,5-dimethyl-
Undecane, 2,6-dimethyl-
Undecane, 2-methyl-
Undecane, 3,7-dimethyl-
Undecane, 3,8-dimethyl-
Undecane, 4,6-dimethyl-
Undecane, 4,8-dimethyl-
Undecane, 4-methyl-
Undecane, 6-methyl-
Undecanoic acid, methyl ester
Octacosane
Octadecane
Octadecane, 1-iodo-
Octadecane, 2-methyl-
Octan-2-one, 3,6-dimethyl-
Octane
Octane, 2,3,6,7-tetramethyl-
Octane, 2,3,6-trimethyl-
Octane, 2,7-dimethyl-
Octane, 3,3-dimethyl-
Octane, 3,5-dimethyl-
Octane, 3-ethyl-
Octane, 3-ethyl-2,7-dimethyl-
Octane, 4,5-dimethyl-
Octane, 4-methyl-
Oleic Acid
Oxalic acid, butyl isohexyl ester
Oxalic acid, decyl 3,5-difluorophenyl ester
Oxalic acid, isobutyl hexyl ester
Oxalic acid, isobutyl nonyl ester
Oxalic acid, isobutyl octyl ester
Oxalic acid, isobutyl pentyl ester
Oxalic acid, octyl propyl ester
Oxanilic acid, O,O'-bis(trimethylsilyl)
Oxirane, [(tetradecyloxy)methyl]-
Oxirane, tetradecyl-
Pentaborane(11)
Pentadecane
Pentadecane, 2,6,10-trimethyl-
Pentadecane, 2-methyl-
Pentadecanoic acid, 14-methyl-, methyl ester
Pentadecanoic acid, methyl ester
Pentanal, 2,4-dimethyl-
Pentane, 1-butoxy-
Pentane, 2-bromo-2-methyl-
Pentane, 3-ethyl-3-methyl-
Pentanol, 5-amino-
Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,1,4a,7-tetramethyl-, [4aS-(4aà,4bá,7á,10aá)]-
Phenol, 2,4,6-tris(1-methylethyl)-
Phenol, 2,5-bis(1,1-dimethylethyl)-
Phenol, 2,6-bis(1,1-dimethylethyl)-4-ethyl-
Phthalic acid, butyl 2-chloropropyl ester
Phthalic acid, butyl hexyl ester
Phthalic acid, butyl undecyl ester
Phthalic acid, di(hept-2-yl) ester
Phthalic acid, di(hept-4-yl) ester
Phthalic acid, isohexyl tridec-2-yn-1-yl ester
Pivalic acid vinyl ester
Precocene I
Pregn-5-ene-3,20-diol, (3á,20S)-, 2TMS derivative
Pregnan-20-one, 3-(acetyloxy)-5,6-epoxy-6-methyl-, cyclic 20-(1,2-ethanediyl acetal), (3á,5á,6á)-
Pregnane-3,20-diol, (3à,5á,20S)-, 2TMS derivative
Propane, 1-(chloromethoxy)-2-methyl-
Propanoic acid, 1,1-dimethylethyl ester
Propanoic acid, 2,2-dimethyl-, 2,4-dinitrophenyl ester

Propanoic acid, 2,2-dimethyl-, propyl ester
Propanoic acid, pentyl ester
Propene
Propiolonitrile
Pyrrolidine, 1-[(3,4-dichlorophenyl)acetyl]-2á-[(1-pyrrolidyl)methyl
Pyrrolidine, 1-[2-(4-bromophenoxy)ethyl]-
Salicylic acid, 1-methylpropyl ester
Silane, trichlorocyclohexyl-
Silane, trichlorooctadecyl-
Silicic acid, diethyl bis(trimethylsilyl) ester
s-Tetrazine, 3,6-bis(diisopropylamino)-
Succinic acid, 3-methylbut-2-yl tetrahydrofurfuryl ester
Succinic acid, di(2-chloro-4-methylphenyl) ester
Sulfone, 2-hydroxypropyl t-butyl
Sulfurous acid, butyl hexyl ester
Sulfurous acid, butyl isohexyl ester
Sulfurous acid, butyl nonyl ester
Sulfurous acid, butyl pentyl ester
Sulfurous acid, butyl tridecyl ester
Sulfurous acid, hexyl 2-pentyl ester
Sulfurous acid, hexyl heptyl ester
Sulfurous acid, hexyl pentadecyl ester
Sulfurous acid, hexyl pentyl ester
Sulfurous acid, hexyl tridecyl ester
Sulfurous acid, nonyl pentyl ester
Sulfurous acid, octadecyl pentyl ester
Sulfurous acid, octyl 2-pentyl ester
Terephthalic acid, allyl 4-fluoro-2-methoxyphenyl ester
Terephthalic acid, dodec-2-enyl hexyl ester
tert-Butyl Hydroperoxide
Tetracosane
Tetradecane
Tetradecane, 1-fluoro-
Tetradecane, 1-iodo-
Tetradecane, 4-ethyl-
Tetrahydropyran Z-10-dodecenoate
Thiocyanic acid, ethyl ester
trans-2,3,4-Trichloro-2-butene nitrile
Tridecane
Tridecane, 2-methyl-
Tridecane, 3-methyl-
Tridecane, 4,8-dimethyl-
Tridecane, 4-methyl-
Tridecanoic acid, methyl ester
Tridecanol, 2-ethyl-2-methyl-
Tris(tert-butyldimethylsilyloxy)arsane
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-
Undecane
Undecane, 2,4-dimethyl-
Undecane, 2,5-dimethyl-
Undecane, 2,6-dimethyl-
Undecane, 2,7-dimethyl-
Undecane, 2-methyl-
Undecane, 3,5-dimethyl-
Undecane, 3,7-dimethyl-
Undecane, 3,8-dimethyl-

	Undecane, 4,6-dimethyl- **Undecane, 4,8-dimethyl-** **Undecane, 4-methyl-** **Undecanoic acid, methyl ester** Vitamin E Zinc Chloride
378 chemical compounds	**427 chemical compounds**

APPENDIX O

Table 6.3: Comparison of chemical compounds in the stem bark of all 12 the *C. dentata* trees from the southern Cape and all 12 the *C. dentata* trees from KZN, collected January 2016 and January 2019 respectively. The compounds highlighted occur in trees at both sites but not necessarily in every tree at each of the respective sites.

Southern Cape	KZN
(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	**(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane**
(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane	(2-Methyl[1,3]dioxolan-2-yl)thioacetic acid, S-[3-(2-methyl[1,3]dioxolan-2-yl)-2-oxopropyl] ester
1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone	(Z)-2-Hydroxyimino-3-oxobutyric acid, 1,1-dimethylethyl ester
1-(2-Hydroxymethylpyrrolidin-1-yl)ethanone	**1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone**
1-(2-Methoxy-1-methylethoxy)-2-propanol, TMS derivative	**1-(2-Hydroxymethylpyrrolidin-1-yl)ethanone**
1-(4-tert-Butylphenyl)propan-2-one	**1-(4-tert-Butylphenyl)propan-2-one**
1,1,1',1'-Tetrafluorodimethyl ether	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane
1,12-Tridecadiene	**1,12-Tridecadiene**
1,13-Tetradecadiene	**1,2,3,4-Tetrahydro-1-naphthylamine, N-heptafluorobutyryl-**
1,15-Pentadecanediol	**1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline**
1,2,3,4-Tetrahydro-1-naphthylamine, N-heptafluorobutyryl-	1,2,3-Butatriene, 1-chloro-
1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline	1,2-Benzenedicarboxylic acid, bis(1-methylethyl) ester
1,2,5-Oxadiazole-3-carboxamide, 4-amino-N-[2-[[(2,3-dihydro-1,3-dimethyl-2-oxo-1H-1,3-benzimidazol-5-yl)methyl]amino]ethyl]-	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester
1,2-Benzenedicarboxylic acid, decyl octyl ester	**1,3,2-Dioxaborinane, 2,4-diethyl-5-methyl-6-propyl-**
1,2-Benzenediol, o-(1-adamantancarbonyl)-o'-(cyclobutanecarbonyl)-	**1,3-Butadiyne**
1,2-Octadecanediol	**1,3-Dioxolane, 4-methyl-2-pentadecyl-**
1,3,2-Dioxaborinane, 2,4-diethyl-5-methyl-6-propyl-	1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-1,4-di-iso-propylnaphthalene
1,3,2-Dioxaborolane, 4,4-dimethyl-5-oxo-, 2-ethyl	1,4-S,S-2,5-Bis[carbethoxy]phenylene bis[N,N-dimethyldithiocarbamate]
1,3,5,7=Tetrazaadamantane borane	**11-Hexadecen-1-ol, (Z)-**
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	**11-Methyldodecanol**
1,3-Butadiyne	1-Bromoeicosane
1,3-di-iso-propylnaphthalene	**1-Butanamine, 3-methyl-**
1,3-Dioxolane, 4-methyl-2-pentadecyl-	**1-Butanol, 2,2-dimethyl-**
1,3-Dioxolane, 4-methyl-2-pentyl-	1-Butanol, 3-methoxy-, acetate
1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)-	**1-Buten-3-yne, 1-chloro-, ®-**
1,4-Dioxane-2,5-diol, 2TBDMS derivative	**1-Buten-3-yne, 1-chloro-, (Z)-**
1,5-Pentanediol, O,O'-di(3-methylbut-2-enoyl)-	1-Buten-3-yne, 2-chloro-
1,7-di-iso-propylnaphthalene	1-Cyclopentyleicosane
1,7-Octadiene, 3-methoxy-	1-Decanol, 5,9-dimethyl-
11-Hexadecen-1-ol, (Z)-	1-Decene, 3,3,4-trimethyl-
11-Methyldodecanol	**1-Docosene**
11-Tricosene	1-Heptene, 4-methyl-
1-Butanamine, 3-methyl-	1-Hexadecanol
1-Butanol, 2,2-dimethyl-	**1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)-**
1-Butanol, 3-methyl-, carbonate (2:1)	**1H-Indene, 1-hexadecyl-2,3-dihydro-**
1-Buten-3-yne, 1-chloro-, ®-	1H-Indene-4-acetic acid, 6-(1,1-dimethylethyl)-2,3-dihydro-1,1-dimethyl-
1-Buten-3-yne, 1-chloro-, (Z)-	
1-Docosene	
1-Dodecanol, 2-hexyl-	

1-Heptanol, 2-propyl-
1-Hexacosanol
1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)-
1H-Indene, 1-hexadecyl-2,3-dihydro-
1H-Indole, 1-methyl-
1-Iodo-2-methylundecane
1-Octanesulfonyl chloride
1-Octanol, 2-butyl-
1-Octanol, 3,7-dimethyl-, (S)-
1-Octene, 3,7-dimethyl-
1-Pentanol, 2,4-dimethyl-, (ñ)-
1-Trifluorosilyltridecane
1-Undecene, 4-methyl-
1-Undecene, 7-methyl-
2(2-Bromoethyl)-1,3-dioxane
2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid
2(5H)-Furanone, 5-(acetyloxy)-
2,2-Bithiazolidine
2,2-Dimethyl-3-heptene trans
2,2'-Isopropylidenebis(5-methylfuran)
2,3,4,5,6-Pentamethyl acetophenone
2,4-Dimethyl-1-heptene
2,4-Dimethyldodecane
2,4-Disilapentane, 2,4-dimethyl-
2,4-Di-tert-butylphenol
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
2,5-di-tert-Butyl-1,4-benzoquinone
2,5-Hexanediol, 2,5-dimethyl-
2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene) pentadecane
2,6,10-Trimethyltridecane
2,6-Difluoro-3-methylbenzoic acid, tridecyl ester
2,6-Diisopropylnaphthalene
2,6-Dimethyldecane
2,9-Decanedione
2-[(2-Cyano-3,3-di-methylcyclopropyl)methyl]-2-methyl-1,3-dioxolane
2-Bromo dodecane
2-Butenoic acid, 4-hydroxy-, methyl ester
2-Cyano-2-O-fluorosulfatofluoropropane
2-Decene, 5-methyl-, (Z)-
2-Heptanol, 3-methyl-
2-Heptene, 4-methyl-, ®-
2-Hexanol
2-Methyl-2-chloro-3-nitroso-4-cyclohexyloxy-butane
2-Methyltetracosane
2-Octene, 2,6-dimethyl-
2-Octene, 3,7-dimethyl-, (Z)-
2-Pentanol, 3-chloro-4-methyl-, (R*,R*)-(ñ)-
2-Pentanol, 4-methyl-
2-Penten-1-ol, (Z)-, TBDMS derivative
2-Propanol, 1-amino-
2-Propanone, 1,1-dibutoxy-
2-Propyn-1-amine, N,N-dimethyl-
2-Pyrrolidinone, 5-(ethoxymethyl)-
2-sec-Butyl-3-methyl-1-pentene
2-Undecen-4-ol
3,5-Dimethyl-4-heptanone
1H-Indole, 1-methyl-
1-Iodo-2-methylundecane
1-Octanesulfonyl chloride
1-Octanol, 2-butyl-
1-Octanol, 3,7-dimethyl-, (S)-
1-Pentanol, 2,4-dimethyl-, (ñ)-
1-Penten-3-ol
1-Propylheptyl methylphosphonofluoridate
1-Undecene, 4-methyl-
2(2-Bromoethyl)-1,3-dioxane
2-(4-Hydroxy-4-methyl-tetrahydro-pyran-3-ylamino)-3-(1H-indol-2-yl)-propionic acid
2,2,4,4-Tetramethyloctane
2,2-Bithiazolidine
2,2-Dichloronorbornane
2,2-Dimethyl-3-heptene trans
2,2-Dimethylpropionic acid, 4-methylpentyl ester
2,3,4,5,6-Pentamethyl acetophenone
2,4-Dimethyl-1-heptene
2,4-Disilapentane, 2,4-dimethyl-
2,4-Di-tert-butylphenol
2,4-Pentanedione, 3-methyl-
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
2,5-di-tert-Butyl-1,4-benzoquinone
2,5-Hexanediol, 2,5-dimethyl-
2,6-Dimethyldecane
2,9-Decanedione
2-Bromo dodecane
2-Decene, 5-methyl-, (Z)-
2-Diethylamino-N-naphthalen-1-yl-acetamide
2H-1,2-Oxazine, 6-(4-chlorophenyl)tetrahydro-2-methyl-
2-Hexanol, 3,4-dimethyl-
2-Methyltetracosane
2-Nonanone
2-Octene, 2,6-dimethyl-
2-Pentanol, 3-chloro-4-methyl-, (R*,S*)-(ñ)-
2-Penten-1-ol, (Z)-, TBDMS derivative
2-Propanol, 1-cyclohexyloxy-(1-piperidinyl)-
2-Propanone, 1,1-dibutoxy-
2-Propyl-1-Pentanol, trifluoroacetate
3,3-Diethylpentadecane
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
3,7,11,15,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-
3-Ethyl-3-methylheptadecane
3-Methyl-1-tripropylsilyloxybut-2-ene
3-Methyl-2-butenoic acid, tridec-2-ynyl ester
3-Methyloctacosane
3-Octanone
3-Pentanone, 2,2,4,4-tetramethyl-
4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone
4-Heptyn-2-ol
4-Nonene, 2,3,3-trimethyl-, (Z)-
4-Nonene, 3-methyl-, (Z)-
4-Octene, 2,6-dimethyl-, [S-(Z)]-
5-Butyl-5-ethylpentadecane
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran

3,5-Dimethyldodecane
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
3,7,11,15,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-
3,7-Dimethyl-2,3,3a,4,5,6-hexahydro-1-benzofuran
3-Buten-2-ol
3-Chloro-N,N-diethyl-11H-indolo[3,2-c]354ydrazine-11-ethanamine
3-Eicosanone
3-Ethyl-3-methylheptane
3-Heptanone, 2,4-dimethyl-
3-Hexanone, 2,4-dimethyl-
3-Methyl-1-tripropylsilyloxybut-2-ene
3-Methyl-2-butenoic acid, tridec-2-ynyl ester
3-Pentanone, 2,2,4,4-tetramethyl-
4-(2-Amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)-3,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester
4-[1,3]Dioxan-2-yl-3,4-dimethylcyclohex-2-enone
4-Amino-1-butanol
4-Heptafluorobutyryloxyhexadecane
4-Hepten-2-one, ®-
4-Hexen-2-one, 3,3-diethyl-4,5-dimethyl-
4-Methyl-1-heptyn-3-ol
4-Methyl-2-heptene
4-Nonene, 3-methyl-, (Z)-
4-Octene, 2,3,6-trimethyl-
4-Octene, 2,6-dimethyl-, [S-(Z)]-
4-Propionyloxypentadecane
4-Tetradecene, (Z)-
5-Butyl-5-ethylpentadecane
5-Cyano-1,2,3-thiadiazole
5-Ethyldecane
5-Hexenoic acid, 2,4-dioxo-6-phenyl-, ethyl ester
5-Iodo-nonane
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
5-Undecene, 7-methyl-, (Z)-
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
7-Chloro-1-[[3-[diethylamino]-2,2-dimethylpropyl]imino]-1,3,4,10-tetrahydro-3[4-(trifluoromethyl)phenyl]-9(2H)-acridinone
9,12-Octadecadienoic acid (Z,Z)-
9,17-Octadecadienal, (Z)-
9-Hexadecen-1-ol, (Z)-
9-Octadecenoic acid (Z)-, methyl ester
9-octadecenoic acid, 2,2,2-trifluoroethyl ester
Acetic acid, 2,2,3,4,4,4-hexafluorobutyl ester
Acetic acid, chloro-
Argon
à-Tocopheryl acetate
Behenyl chloride
Benzene, 1,3-bis(1,1-dimethylethyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-
Benzene, 1,4-bis(1,1-dimethylethyl)-
Benzene, 1,4-dimethoxy-2-methyl-
Benzene, tert-butyl-
Benzenecarbothioic acid, 2,4,6-triethyl-, S-(2-phenylethyl) ester
5-Tetradecene, (Z)-
5-Undecene, 7-methyl-, (Z)-
6-(1'-Hydroxyethyl)-2,2-dimethylchromene
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
7-Acetyl-1,7-diazabicyclo[2.2.0]heptane
9,12-Octadecadienoic acid, methyl ester, (E,E)-
9,12-Octadecadienoyl chloride, (Z,Z)-
9-Hexadecen-1-ol, (Z)-
Acetamide, N-(2,5-dichlorophenyl)-2-oxo-2-[N2-(1-phenylpropyliden)hydrazino]-
Acetic acid, (3-allyloxy-1,1-dimethylbutyl) ester
á-Pinene
Argon
à-Tocopheryl acetate
Benzene, 1,3-bis(1,1-dimethylethyl)-
Benzene, 1,3-bis(1,1-dimethylethyl)-5-methyl-
Benzene, 1,4-bis(1,1-dimethylethyl)-
Benzene, 1-tert-butyl-4-cyclopropylmethyl-
Benzene, tert-butyl-
Benzoic acid, 2-(1-oxopropyl)-
Benzyl alcohol, à-(1-(dimethylamino)ethyl)-
Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-
Binapacryl
Boron trifluoride
Bromoacetone
Butane, 2,2-dimethyl-
Butane, 2-bromo-2-methyl-
Butanoic acid, 2-methylbutyl ester
Butylated Hydroxytoluene
Carbonic acid, octyl vinyl ester
Carbonic acid, undecyl vinyl ester
Chlorfenapyr
Chloromethane
Chloromethanesulfonyl chloride
Cyclohexane, 1-ethyl-2-propyl-
Cyclohexane, decyl-
Cyclohexane, octyl-
Cyclohexasiloxane, dodecamethyl-
Cyclooctane, 1,4-dimethyl-, cis-
Cyclopentane, 1-butyl-2-propyl-
Cyclopentane, hexyl-
Cyclopentene, 1,2,3,3,4-pentamethyl-
Cyclopropanecarboxylic acid, 1-(phenylmethyl)-, 2,6-bis(1,1-dimethylethyl)-4-methylphenyl ester
Cyclotetradecane
Decane
Decane, 1-fluoro-
Decane, 1-iodo-
Decane, 2,3,5,8-tetramethyl-
Decane, 2,4,6-trimethyl-
Decane, 2,5-dimethyl-
Decane, 2,6,7-trimethyl-
Decane, 2,6,8-trimethyl-
Decane, 4-methyl-
Decane, 5-methyl-
Decane, 6-ethyl-2-methyl-

Benzo[b]dihydropyran, 6-hydroxy-4,4,5,7,8-pentamethyl-
Benzoic acid, 3-(3-hydroxy-3-methyl-1-butynyl)-
Bicyclo[2.2.1]heptane, 2-chloro-, exo-
Bicyclo[2.2.2]octane, 1-methyl-4-(methylsulfonyl)-
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-
Binapacryl
Bis(tridecyl) phthalate
Boron trifluoride
Butanoic acid, 2-methylbutyl ester
Carbonic acid, 2-flurophenyl 2-methoxyethyl ester
Carbonic acid, heptyl vinyl ester
Carbonic acid, octadecyl vinyl ester
Chlorfenapyr
Chlorine
Chloromethane
Chloromethanesulfonyl chloride
Clorgiline
Cyclohexane, 1-ethyl-2-propyl-
Cyclohexane, decyl-
Cyclohexane, ethyl-
Cyclohexane, octadecyl-
Cyclohexane, octyl-
Cyclohexane, propyl-
Cyclooctane, (1-methylpropyl)-
Cyclooctane, 1,4-dimethyl-, cis-
Cyclopentane, 1,2-dipropyl-
Cyclopentane, 1-butyl-2-propyl-
Cyclopentane, hexyl-
Cyclopentanecarboxamide, N-(2-fluorophenyl)-
Cyclopentene, 1,2,3,4,5-pentamethyl-
Cyclopropanecarboxylic acid, 1-(phenylmethyl)-, 2,6-bis(1,1-dimethylethyl)-4-methylphenyl ester
Cyclopropanedodecanoic acid, 2-octyl-, methyl ester
Cyclotetradecane
Decane
Decane, 2,3,5,8-tetramethyl-
Decane, 2,4,6-trimethyl-
Decane, 2,5-dimethyl-
Decane, 2,6,7-trimethyl-
Decane, 2,6,8-trimethyl-
Decane, 4-methyl-
Decane, 5-ethyl-5-methyl-
Decane, 5-methyl-
Decanoic acid, methyl ester
Decyl 2-(N,N-dimethylthiocarbamoylthio)acetate
Diazene, bis(1,1-dimethylethyl)-
Dibutyl phthalate
Dichloroacetaldehyde
Dicyclohexyl phthalate
Didodecyl phthalate
Difluorochloromethane
Difluoromethane
Dithiocarbamate, S-methyl-,N-(2-methyl-3-oxobutyl)-
dl-à-Tocopherol
Decanoic acid, methyl ester
Desmethyldeprenyl
Dichloroacetaldehyde
Dichloroacetic acid, 2-pentadecyl ester
Dicyclohexyl phthalate
Didodecyl phthalate
di-t-Butylhydrazodicarboxylate
dl-à-Tocopherol
Docosane
Docosane, 11-butyl-
Dodecane, 2,6,10-trimethyl-
Dodecane, 4,6-dimethyl-
Dodecane, 4-methyl-
Dodecane, 6-methyl-
Eicosane
Eicosane, 2,4-dimethyl-
Eicosane, 2-methyl-
Ethanol, 2,2-dichloro-
Ethanone, 1-(2,4,5-triethylphenyl)-
Ether, 6-methylheptyl vinyl
Ether, hexyl pentyl
Ethyl 3-hydroxyicosanoate
Ethyl 3-hydroxytetracosanoate
Fumaric acid, ethyl tetrahydrofurfuryl ester
Furan, tetrahydro-2,2,5,5-tetramethyl-
Gibberellic acid
Glutamic acid, N-pivaloyl-, dimethyl ester
Glycine
Glycyl-L-tryptophylglycine
Heneicosane
Heneicosanoic acid, methyl ester
Heptacosane
Heptacosane, 1-chloro-
Heptadecane
Heptadecane, 2,6,10,14-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
heptadecanoic acid, 2-methoxyethyl ester
Heptane, 2,2,3,3,5,6,6-heptamethyl-
Heptane, 2,4,6-trimethyl-
Heptane, 2,4-dimethyl-
Heptane, 2-bromo-
Heptane, 3,3,5-trimethyl-
Heptane, 3,3-dimethyl-
Heptane, 3-ethyl-
Heptane, 3-ethyl-2-methyl-
Heptane, 4-(1-methylethyl)-
Heptane, 4,4-dimethyl-
Heptane, 4-ethyl-
Hexacosane
Hexadecane
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Hexadecen-1-ol, trans-9-
Hexane, 1-(hexyloxy)-2-methyl-
Hexane, 2,2,3,3-tetramethyl-
Hexane, 2,3,4-trimethyl-
Hexane, 2,4-dimethyl-
Hexane, 3,3,4,4-tetramethyl-
Hexane, 3-ethyl-

DL-Norvaline
Docosane
Docosane, 11-butyl-
Dodecane, 2,6,11-trimethyl-
Dodecane, 4,6-dimethyl-
Dodecane, 4-methyl-
Dodecane, 6-methyl-
Dodecanoic acid, methyl ester
Dotriacontane
d-Ribo-tetrofuranose, 4-c-cyclopropyl-1,2-O-isopropylidene-, à-
Eicosane
Eicosane, 2,4-dimethyl-
Eicosane, 2-methyl-
Eicosyl isopropyl ether
Ethanone, 1-(2,4,5-triethylphenyl)-
Ethyl 2-chloro-3,3,3-trifluoro-2-propionamidopropionate
Ethyl 3-hydroxyicosanoate
Ethyl 3-hydroxytetracosanoate
Ethyl 3-methylbut-3-enyl carbonate
Fenpipramide
Formamide, N-methyl-
Ginsenol
Glycine
Glycyl-L-tryptophylglycine
Heneicosane
Heneicosane, 11-(1-ethylpropyl)-
Heneicosanoic acid, methyl ester
Heptacosane
Heptacosane, 1-chloro-
Heptadecane
Heptadecane, 2,6,10,14-tetramethyl-
Heptadecane, 2,6,10,15-tetramethyl-
Heptadecane, 2,6-dimethyl-
Heptadecane, 2-methyl-
heptadecanoic acid, 2-methoxyethyl ester
Heptane, 2,2,3,3,5,6,6-heptamethyl-
Heptane, 2,3,5-trimethyl-
Heptane, 2,4,6-trimethyl-
Heptane, 2,4-dimethyl-
Heptane, 3,3-dimethyl-
Heptane, 3-ethyl-
Heptane, 3-ethyl-2-methyl-
Heptane, 4-(1-methylethyl)-
Heptane, 4,4-dimethyl-
Heptane, 4-ethyl-
Heptane, 4-propyl-
Heptasiloxane, hexadecamethyl-
Hexacosane
Hexadecane
Hexadecane, 2,6,11,15-tetramethyl-
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Hexadecen-1-ol, trans-9-
Hexane, 2,2,3,3-tetramethyl-
Hexane, 2,3,4-trimethyl-
Hexane, 2,4-dimethyl-
Hexane, 3,3,4,4-tetramethyl-
Hexane, 3-ethyl-
Hexane, 3-ethyl-4-methyl-
Hexasiloxane, tetradecamethyl-
Indole, 3-methyl-
Isobutylamine
Isophthalic acid, hexyl 2-isopropylphenyl ester
Mercaptamine
Mercaptoacetic acid, 2TMS derivative
Methane-d, trichloro-
Methyl 2-methoxypropenoate
Methyl 3-butynoate
Methylene chloride - solvent
Methyl isobutyrate
Morpholine, 4-[[(dimethylamino)thioxomethyl]thio]-
N-(2-Octyl)heptafluorobutyramide
N,N,3-Trimethyl-5-oxo-1-phenyl-2-pyrazolinyl-4-dithiocarbamate
N,N-Diethyl-11H-indolo[3,2-c]quinoline-11-ethanamine
n-Heptadecanol-1
Nitrous acid
N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide
Nonadecane
Nonadecane, 1-chloro-
Nonadecane, 2-methyl-
Nonane, 2,5-dimethyl-
Nonane, 2-methyl-
Norepinephrine, (R)-, 4TMS derivative
Norfenfluramine
n-Propyl fluoride
n-Tetradecyltrichlorosilane
n-Tridecan-1-ol
Octacosane
Octadecane, 1-iodo-
Octadecane, 4-methyl-
Octadecane, 5,14-dibutyl-
Octadecane, 6-methyl-
Octadecanoic acid, 3-hydroxypropyl ester
Octan-2-one, 3,6-dimethyl-
Octane
Octane, 2,3,6,7-tetramethyl-
Octane, 2,7-dimethyl-
Octane, 3,5-dimethyl-
Octane, 3-ethyl-
Octane, 3-methyl-
Octane, 4,5-dimethyl-
Octane, 4-methyl-
Oleic Acid
Oxalic acid, 2-ethylhexyl nonyl ester
Oxalic acid, allyl tridecyl ester
Oxalic acid, butyl 2-ethylhexyl ester
Oxalic acid, dineopentyl ester
Oxalic acid, heptyl isohexyl ester
Oxalic acid, hexyl isohexyl ester
Oxalic acid, isobutyl hexyl ester
Oxalic acid, isobutyl octyl ester
Oxalic acid, isobutyl undecyl ester
Oxalic acid, monoamide, N-allyl-, hexadecyl ester
Oxalic acid, neopentyl pentyl ester
Oxirane, [(hexadecyloxy)methyl]-

Hexane, 3-ethyl-4-methyl-
Hexane, 4-ethyl-2-methyl-
Hexasiloxane, tetradecamethyl-
Hexyl octyl ether
Hydroxylamine, O-decyl-
Indole, 3-methyl-
Isoamyl laurate
Isophthalic acid, hexyl tridec-2-ynyl ester
Isophthalic acid, isohexyl 1-isopropyl-2-methylpropyl ester
Isovaline, 3-methyl-
Kaur-16-en-18-oic acid, methyl ester, (4á)-
Kaur-16-ene
Malonic acid, bis(2-trimethylsilylethyl ester
Malonic acid, di(2-methylpent-3-yl) ester
Methane-d, trichloro-
Methoxyacetic acid, 3-tridecyl ester
Methyl 1-acetylpyrrolidine-2-carboxylate
Methyl 3-butynoate
Methylene chloride – solvent
n-Butyric acid 2-ethylhexyl ester
N-Isopropoxy-2-carbomethoxyaziridine
N-Methyl-N-methoxy-5,6,7,8-tetrahydro-1-naphtamide
Nonacosane
Nonadecane
Nonadecane, 2-methyl-
Nonane, 2,5-dimethyl-
Nonane, 2-methyl-
Nonane, 3-methyl-5-propyl-
Nonane, 4-methyl-
Norfenfluramine
n-Tridecan-1-ol
Octa-3,5-diene-2,7-dione, 4,5-dihydroxy-
Octacosane
Octadecane
Octadecane, 1-iodo-
Octadecane, 3-methyl-
Octadecane, 4-methyl-
Octadecane, 6-methyl-
Octadecanoic acid, 2,3-dihydroxypropyl ester
Octane
Octane, 2,3,6,7-tetramethyl-
Octane, 2,6-dimethyl-
Octane, 2,7-dimethyl-
Octane, 3,3-dimethyl-
Octane, 3,5-dimethyl-
Octane, 3-ethyl-
Octane, 3-methyl-
Octane, 4-methyl-
Oxalic acid, 3,5-difluorophenyl tetradecyl ester
Oxalic acid, butyl isobutyl ester
Oxalic acid, dineopentyl ester
Oxalic acid, heptyl isohexyl ester
Oxalic acid, hexyl octadecyl ester
Oxalic acid, isobutyl octyl ester
Oxalic acid, isohexyl neopentyl ester
Oxeladin
Oxirane, [(hexadecyloxy)methyl]-
Oxirane, tetradecyl-
p-Anisic acid, 4-nitrophenyl ester
Pentaborane(11)
Pentacosane
Pentadecane, 2,6,10-trimethyl-
Pentadecane, 2-methyl-
Pentane, 1-bromo-5-methoxy-
Pentane, 2,3,4-trimethyl-
Pentane, 3,3-dimethyl-
Pentane, 3-ethyl-3-methyl-
Phenol, 2,5-bis(1,1-dimethylethyl)-
Phthalic acid, 2-chloropropyl isobutyl ester
Phthalic acid, 7-methyloct-3-yn-5-yl pentyl ester
Phthalic acid, bis(2-pentyl) ester
Phthalic acid, butyl 8-chlorooctyl ester
Phthalic acid, di(hept-2-yl) ester
Phthalic acid, di(hept-3-yl) ester
Phthalic acid, hexyl tridec-2-yn-1-yl ester
Phthalic acid, isohexyl nonyl ester
Piperonyl alcohol, TBDMS derivative
Pivalic acid vinyl ester
Pivalyl chloride
Pregnan-20-one, 3-(acetyloxy)-5,6-epoxy-6-methyl-, cyclic 20-(1,2-ethanediyl acetal), (3á,5á,6á)-
Propanoic acid, 2,2,2-trichloro-1-methylethyl ester
Propanoic acid, 2,2-dimethyl-, 2,4-dinitrophenyl ester
Propanoic acid, 2,2-dimethyl-, chloromethyl ester
Propanoic acid, 2,2-dimethyl-, methyl ester
Propanoic acid, 2-methyl-, 3-methylbutyl ester
Propene
Propylamine
Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino)ethyl-3-methyl-, 2-ethyl ester
Pyrrolidine, 1-(1-oxo-2,5-octadecadienyl)-
sec-Butyl nitrite
Silane, trichlorodecyl-
Silane, trichlorooctadecyl-
Silicon tetrafluoride
Succinic acid, di(2-tert-butylphenyl) ester
Sulfurous acid, 2-propyl tridecyl ester
Sulfurous acid, bis(1-methylethyl) ester
Sulfurous acid, butyl dodecyl ester
Sulfurous acid, butyl hexadecyl ester
Sulfurous acid, butyl octyl ester
Sulfurous acid, butyl pentyl ester
Sulfurous acid, hexadecyl pentyl ester
Sulfurous acid, hexyl 2-pentyl ester
Sulfurous acid, hexyl heptyl ester
Sulfurous acid, hexyl pentadecyl ester
Sulfurous acid, hexyl pentyl ester
Sulfurous acid, hexyl tridecyl ester
Sulfurous acid, nonyl 2-pentyl ester
Sulfurous acid, nonyl pentyl ester
Sulfurous acid, pentadecyl pentyl ester
t-Butyldichlorophosphine
Terephthalic acid, 4-bromophenyl hexyl ester
tert-Butyl Hydroperoxide
tert-Butyl isopropyl carbonate
Tetracosane

Palmitic acid vinyl ester
p-Anisic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester
Pentacosane
Pentadecane
Pentadecane, 2,6,10-trimethyl-
Pentadecane, 2-methyl-
Pentane, 1,3-epoxy-4-methyl-
Pentane, 1-bromo-5-methoxy-
Pentane, 1-butoxy-
Pentane, 2,2,3,3-tetramethyl-
pentanoic acid, 2-bromo-4,4-dimethyl-3-oxo-, methyl ester
Pentyl tetradecyl ether
Phenol, 2,4,6-tris(1-methylethyl)-
Phenol, 2,5-bis(1,1-dimethylethyl)-
p-Hexylacetophenone
Phthalic acid, 2-chloropropyl propyl ester
Phthalic acid, 2-propylphenyl tetradecyl ester
Phthalic acid, 8-chlorooctyl nonyl ester
Phthalic acid, butyl hexyl ester
Phthalic acid, cyclobutyl isobutyl ester
Phthalic acid, di(hept-2-yl) ester
Phthalic acid, di(hept-3-yl) ester
Phthalic acid, di(oct-3-yl) ester
Phthalic acid, hex-2-yn-4-yl nonyl ester
Phthalic acid, pentadecyl 2-propylphenyl ester
Pivalic acid vinyl ester
Plastoquinone 3
Precocene I
Pregna-3,5-dien-20á-ol, O-trimethylsilyl
Pregnan-20-one, 3-(acetyloxy)-5,6-epoxy-6-methyl-, cyclic 20-(1,2-ethanediyl acetal), (3á,5á,6á)-
Propanoic acid, 1,1-dimethylethyl ester
Propanoic acid, 2,2-dimethyl-, 2,4-dinitrophenyl ester
Propanoic acid, 2,2-dimethyl-, anhydride with diethylborinic acid
Propanoic acid, 2,2-dimethyl-, methyl ester
Propiolonitrile
Propionic acid, thio-, S-isopentyl ester
Quinoline-2,2-dicarbonitrile, 1,2,3,4,5,6,7,8-octahydro-1-dimethylamino-3-phenyl-
Scyllo-Inositol
Silane, trichlorooctadecyl-
Silicon tetrafluoride
Succinic acid, 3-chlorophenyl 3-phenylprop-2-en-1-yl ester
Succinic acid, 3-methylbut-2-yl tetrahydrofurfuryl ester
Succinic acid, phenethyl 2,3-dichlorophenyl ester
Sulfurous acid, butyl hexadecyl ester
Sulfurous acid, butyl octadecyl ester
Sulfurous acid, butyl octyl ester
Sulfurous acid, dodecyl 2-propyl ester
Sulfurous acid, hexyl 2-pentyl ester
Sulfurous acid, hexyl heptyl ester
Sulfurous acid, hexyl nonyl ester
Sulfurous acid, hexyl pentyl ester
Sulfurous acid, hexyl undecyl ester
Tetracosane, 11-decyl-
Tetradecane
Tetradecane, 1-iodo-
Tetradecane, 4-ethyl-
Tetrahydropyran Z-10-dodecenoate
Thiophene, 2-ethynyl-5-[(trimethylsilyl)ethynyl]-
Triacontane
Triallylsilane
Tridecane
Tridecane, 2-methyl-
Tridecane, 3-methyl-
Tridecane, 4,8-dimethyl-
Tridecane, 4-methyl-
Tridecanoic acid, methyl ester
Trifluoroacetic acid
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-
Undecane
Undecane, 2,4-dimethyl-
Undecane, 2,5-dimethyl-
Undecane, 2,6-dimethyl-
Undecane, 2,7-dimethyl-
Undecane, 2-methyl-
Undecane, 3,7-dimethyl-
Undecane, 3,8-dimethyl-
Undecane, 4,6-dimethyl-
Undecane, 4,8-dimethyl-
Undecane, 4-methyl-
Undecane, 5-ethyl-
Undecane, 6-methyl-
Undecanoic acid, methyl ester

Sulfurous acid, isohexyl 2-pentyl ester	
Sulfurous acid, nonyl 2-pentyl ester	
Sulfurous acid, nonyl pentyl ester	
Sulfurous acid, pentadecyl pentyl ester	
Sulfurous acid, pentyl tridecyl ester	
Terephthalic acid, hexyl 2-methylphenyl ester	
tert-Butyl Hydroperoxide	
Tetracosane	
Tetracosane, 11-decyl-	
Tetradecane	
Tetradecane, 2,6,10-trimethyl-	
Tetradecane, 4-ethyl-	
Tetrahydrocyclopenta[1,3]dioxin-4-one	
Thiophene, 2-ethynyl-5-[(trimethylsilyl)ethynyl]-	
Triacontane	
Tridecane	
Tridecane, 2-methyl-	
Tridecane, 3-methyl-	
Tridecane, 4,8-dimethyl-	
Tridecane, 4-methyl-	
Tridecane, 5-methyl-	
Tridecanoic acid, methyl ester	
Tridecanol, 2-ethyl-2-methyl-	
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	
Undecane	
Undecane, 2,4-dimethyl-	
Undecane, 2,6-dimethyl-	
Undecane, 2-methyl-	
Undecane, 3,7-dimethyl-	
Undecane, 4,6-dimethyl-	
Undecane, 4,8-dimethyl-	
Undecane, 4-methyl-	
Undecane, 5-ethyl-	
Undecane, 6-methyl-	
Vitamin E	
402 chemical compounds	**349 chemical compounds**

APPENDIX P

Submission confirmation for the manuscript "The chemical composition of *Curtisia dentata* (Burm.f.)C.A.Sm. stem bark" submitted to the South African Journal of Botany

Confirming submission to South African Journal of Botany

South African Journal of Botany <em@editorialmanager.com>

Tue 28/07/2020 18:02

To: Anna van Wyk <anne.vanwyk@hotmail.com>

This is an automated message.

The chemical composition of Curtisia dentata (Burm.f.)C.A.Sm. stem bark

Dear Ms van Wyk,

We have received the above referenced manuscript you submitted to South African Journal of Botany.

To track the status of your manuscript, please log in as an author at https://www.editorialmanager.com/sajb/, and navigate to the "Submissions Being Processed" folder.

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