Phytochemistry of *Dais cotinifolia* L.

Thesis submitted in fulfilment of the requirements for the degree

Master of Science

By

Busisiwe Buyisile Danca

School of Chemistry and Physics

University of KwaZulu-Natal

Pietermaritzburg

Supervisor: Professor Fanie R. van Heerden

May 2018

Abstract

Many plant-derived secondary metabolites have interesting biological activities and some plant compounds such as artemisinin (antimalarial) and vincristine (anticancer agent) are the active principles of main-stream pharmaceuticals. South Africa has an immense biodiversity and there are in the order of 25 000 indigenous plant species in the country. The phytochemistry of many of these plants has never been investigated. Many bioactive compounds have been isolated from the Thymelaeaceae, such as prostatin (potent anti-HIV agent) and antitumour diterpenoids. Although a large number of Thymelaeaceae species are indigenous to South Africa, only a limited number of species have been subjected to phytochemical investigations. *Dais cotinifolia* L. is one species on which phytochemical results have not been published and this tree was the subject of this investigation.

Four compounds were isolated from *D. cotinifolia*. Three of these compounds are known metabolites, two furofuran lignans, kobusin and eudesmin, and the flavonoid catechin. A fourth compound is novel norlignan, 2-hydroxy-5-(3-methoxyphenyl)-1-(4-methoxyphenyl)pentan-1-one.

The compounds were isolated by applying different chromatographic procedures, such as column chromatography, preparative centrifugal thin-layer chromatography and semi-preparative high-performance liquid chromatography. The structural elucidation of the compounds was based on mass spectrometry and nuclear magnetic spectroscopy.

The antioxidant activities of the crude extracts of the different plant parts were determined by the DPPH assay. In comparison with ascorbic acid, the crude extracts only had mild antioxidant activities. The mild activities of the extracts can be explained by the fact that three of the compounds isolated are aromatic compounds, but have methoxy substituents and not free phenolic groups, which are often associated with high-antioxidant activity.

Declaration

The experimental work described in this dissertation was carried out in the School of Chemistry, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Fanie R. van Heerden.

I hereby declare that these studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where the use of published information from other authors has been made and it is duly acknowledged in the text.

Signed …….

Busisiwe Buyisile Danca

Signed ……..

Professor Fanie R. van Heerden (Supervisor)

Acknowledgements

I would first like to thank the Lord Almighty for giving me the strength to persevere through thick and thin, it was never easy but through His grace I endured every single challenge. I would also like to appreciate my Supervisor Prof FR van Heerden for believing in me and encouraging me to work tirelessly towards my goal, she believed in me even when I did not believe in myself. The greatest appreciation goes to my support system (my two sisters) Xolisile Danca and Thandekile Shinga “my people” this wouldn’t have been possible if you guys were not by my side throughout, and I would also like to extend my gratitude to my entire family. Not forgetting my friend Zethu Zulu you’ve been my go-to friend since day one. A special thanks to Babalwa Mazingisa your support does not go unnoticed. Great appreciations to my church family, all this would not have been possible without your prayers. Also extend my gratitude to the Natural Products Research Group, a special thanks to Mahama Alhasan, Nasir Tajuddeen, and the School of Chemistry and Physics for letting me do this study in their labs. Special thanks go to all my friends if I were to mention you one by one I wouldn’t finish, but your presence in my life means everything and I appreciate you. How can I forget my dear friend and house mate Sifikelwe Dumalisile, thank you for assisting me with the antioxidants studies.

I cannot end my list without appreciating the ff:

- Mr. C. Grimmer for his assistance with NMR experiments
- Mrs. C. Janse Van Rensburg for helping with mass spectrometry
- Mr. F. Shaik, Dr Avela, Miss Prudence Lubanyana, Miss Zimbini, Miss Sai, Mrs Irene Ngubane and Mr S Ball.
- The admin staff (CTO)

“With man this is impossible, but with God all things are possible.”

Matt 19:26

Table of Contents

List of Abbreviations

ACN	Acetonitrile
Ar	Aromatic
CC	Column chromatography
$CDCl_3$	Deuterochloroform
^{13}C NMR	Carbon-13 nuclear magnetic resonance
COSY	COrrelation SpectroscopY
DCM	Dichloromethane
1D	One-dimensional
2D	Two-dimensional
d	Doublet
EtOAc	Ethyl acetate
ESI	Electrospray ionisation
FA	Formic acid
Figure	Figure
^{1}H	Proton
HPLC	High-performance liquid chromatography
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
^{1}H NMR	Proton nuclear magnetic resonance
Hz	Hertz
J	Coupling constant
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
CD_3OD	Deuteromethanol
m/*z*	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
ppm	Parts per million
s	Singlet
t	Triplet
TCM	Traditional Chinese medicine
TLC	Thin-layer chromatography
TOF-MS	Time-of-flight mass spectrometry

List of Tables

List of Figures

Chapter 1: Introduction and Aim

1.1 Traditional Medicine/Medicinal Plants

The field of traditional medicine stimulated research on natural products (NPs) and drug discovery. Traditional medicine arises from the knowledge, skills, and practices based on beliefs and experiences that are native to various cultures and it is utilised for prevention, diagnosis, and treatment of multiple ailments (Benzie and Wachtel-Galor, 2011). Plants, medicinal plants in particular, have played a significant role in advancing traditional medicine systems (Koleva et al., 2002). Medicinal plants are a class of plants used in herbalism and they contain compounds that are usually extracted for therapeutic purposes, and these compounds can be located in various parts of the plant (Mehta et al., 2010). Traditional medicine mostly comprises of herbs, herbal preparations, and finished herbal products, which have either active plant ingredients or plant materials or both (Crouch et al., 2006). Herbs include chopped or powdered plant material such as leaves, bark, roots, seeds, flowers, stems, and rhizomes (Crouch et al., 2006). Herbal preparations can be either extracts or essential oils from plant material, and these are produced by extraction, fractionation, purification and other procedures. Finished herbal products are simple herbal preparations made from one or more herbs (Scartezzini and Speroni, 2000). Traditional medicine helps mend the medical gap between developed and developing countries since it is more affordable to most people. The only downfall of traditional medicine is that information is based solely on empirical grounds, and there are often no scientific validations (Peteros and Uy, 2010).

In China, traditional medicine is referred to as "Traditional Chinese Medicine (TCM)" and has existed for over 3000 years. The first herbal text in the world was compiled in China about 2000 years ago (Benzie and Wachtel-Galor, 2011). TCM produced the three top-selling botanical products, *Ginkgo biloba* L.*, Allium sativum* (garlic), and *Panax ginseng*, which are used to treat various diseases (Benzie and Wachtel-Galor, 2011). In the Philippines, medicinal plants are regarded as one of their 'living treasures' (Peteros and Uy, 2010). Out of 13 500 plant species that are found in the Philippines, 1 500 are medicinal, but only 120 of them have been studied for safety and efficacy (Peteros and Uy, 2010). About 90% of the African population and 70% of the people in India depend solely on traditional medicine for primary health care needs (Benzie and Wachtel-Galor, 2011). In Africa, traditional medicine is the oldest and most diverse medicine system (Watt and Breyer-Brandwijk, 1962). Africa is considered as one of the continents with

biological and cultural diversity with marked regional differences in healing practices. Nonetheless, African medicinal plants are threatened due to deforestation (Koleva et al., 2002). In early years, people in the United States of America adopted traditional remedies, but during the 20th century the safety and efficacy of herbs and botanicals were questionable and due to that herbal medicine was rejected (Yadav and Agarwala, 2011). However, plants are still regarded as a source of pure chemical compounds in the development of medicine (Yadav and Agarwala, 2011). In Europe, a growing trend has been observed towards the use of traditional medicine along with pharmaceutical drugs. In Germany alone, it is estimated that 90% of the people use herbal medicines (RBGK, 2017).

Medicinal plants contain a diverse collection of secondary metabolites with various functions like defence against herbivores, diseases, and parasites (Peteros and Uy, 2010). These chemicals often have complex chemical structures that are not available in synthetic compound libraries. Botanic Gardens Conservation International estimates that there are about 400 000 plants worldwide (BGCI, 2017) and only about 28 000 are considered as medicinal plants (RBGK, 2017). Furthermore, just 5-20% of these species have been investigated for general usefulness or biologically active compounds (Peteros and Uy, 2010). The potential of using plants as a source of compounds that are used for drugs production has been proven, for example, by paclitaxel, isolated from *Taxus brevifolia* Nutt., which is used for lung, ovarian and breast cancer (Gurib-Fakim, 2006). This has prompted researchers worldwide to investigate different species and their therapeutic principles (Richards and Sharma, 1991). In some countries, traditional medicine is now being integrated into mainstream healthcare systems. In December 2016, the Chinese Government announced that by 2020 TCM would be incorporated into their healthcare system (RBGK, 2017).

1.2 Traditional Medicine in South Africa

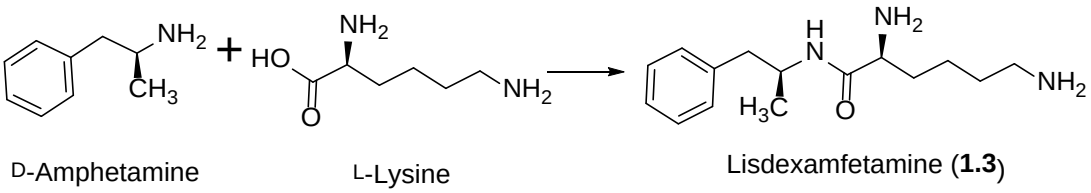
The use of plants in traditional medicine in South Africa is well documented. A treatise on southern Africa was published by Watt and Breyer-Brandwijk in 1962. Hutchings and co-workers compiled an inventory of nearly 1000 Zulu medicinal plants (Hutchings et al., 1996) and Van Wyk et al. (2009) described the ethnobotany and pharmacology of more than 150 plant species used in traditional medicine.

Medicinal plants are marketed in two separate ways in Southern Africa, i.e. a formal and an informal system. The formal market system includes herbal remedies, nutraceuticals,

phytomedicines, and cosmeceuticals, while the informal market system focuses entirely on traditional medicines and herbal remedies (Crouch et al., 2006).

Southern Africa is one of the most diverse floristic regions in the world. It is estimated to have over 30 000 plant species and 1 000 of these are traded in informal markets, but only 5% are traded formally. Below (Table 1.1) is a list of some popular medicinal plants used in South Africa (Crouch et al., 2006).

1.3 Natural Products

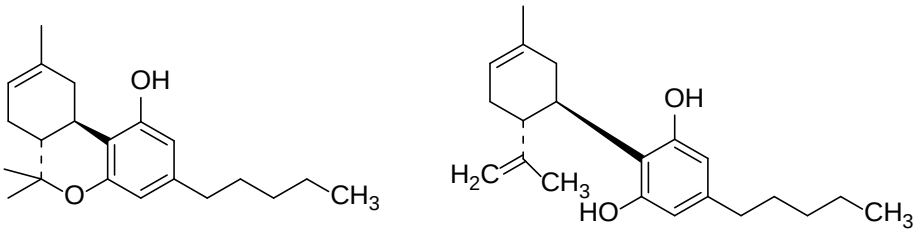


D-Amphetamine L-Lysine Lisdexamfetamine (**1.3**)

Scheme 1.1. Lisdexamfetamine a hybrid molecule of dextroamphetamine and the amino acid L-lysine.

Natural products (NPs) are secondary metabolites derived from natural sources such as plants, animals, and microorganisms (Cragg et al., 1997). Secondary metabolites are compounds that are not essential for the growth, development, or reproduction of an organism. NPs have been a significant source of chemical diversity for starting materials in organic synthesis and have also been influential in the discovery of pharmaceuticals for over a 100 years (Firn and Jones, 2003). Back in history, pharmaceutical companies have been using plant extracts to form relatively crude therapeutic formulations. However, formulation of pure compounds became the norm with the advancement of antibiotics in the mid-twentieth century (Mishra and Tiwari, 2011). There are 1562 new drugs that came on the market in the United States from 1981 – 2014, 67 (4%) are unaltered natural products, 9 (1%) are botanical drugs (herbal extracts), 320 (21%) are natural product derivatives, 61 (4%) are synthetic compounds with NP pharmacophors and 334 (21%) are synthetic compounds that mimic natural products, i.e. in total 51% of these drugs are NPs or have their origin in NPs (Newman and Cragg, 2016). To mention one, Sativex® is a mixture of dronabinol (**1.1**) and cannabinol (**1.2**) and this drug is an analgesic for severe pain used by advanced cancer patients. Lisdexamfetamine (Scheme 1.1), a hybrid molecule of dextroamphetamine and the amino acid L-lysine, is used as a prodrug of dextroamphetamine used for the treatment of attention deficit

hyperactivity disorder (ADHD) and binge eating disorder (Luqman and Pezzuto, 2010) (Figure 1.1).

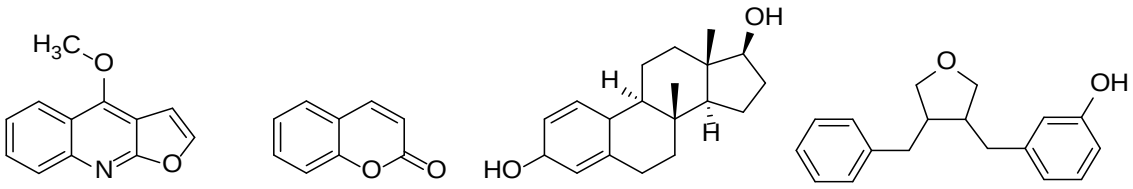


Dronabinol (**1.1**) Cannabinol (**1.2**)

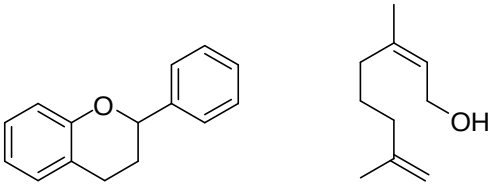
Figure 1.1. Some natural product-derived drugs.

1.4 Phytochemicals

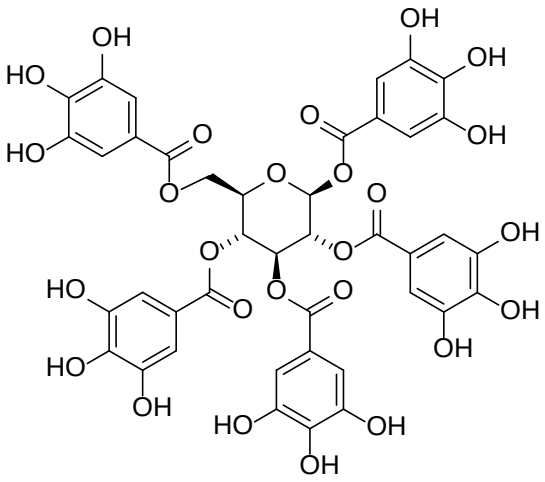
Plants produce a large number of chemical compounds (phytochemicals) (Crouch et al., 2006). These compounds are produced to ensure the survival of the plants by providing preventive properties against parasites and herbivores (Crouch et al., 2006). In higher plants, these phytochemicals are classified as alkaloids, coumarins, steroids, lignans, flavonoids, terpenoids and tannins, amongst others (Peteros and Uy, 2010). Almost 50% of drugs derived from NPs are plants based. However, only a tiny fraction of plants have been studied for their chemical and pharmacological properties (Richards and Sharma, 1991). Figure 1.2 shows examples of some of the classes of secondary metabolites that are frequently found in plants and they are discussed in detail below.



Alkaloid Coumarin Steroid Lignan



Flavonoid Nerol a terpenoid

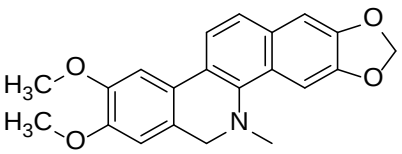


Pentagalloylglucose, a hydrolysable tannin

Figure 1.2. Some classes of natural products that are frequently found in plants.

1.4.1 Alkaloids

Alkaloids were first introduced in the 19th century by Carl Friedrich Wilhelm Meisner (Bruneton, 1999). They are defined as natural substances that react like bases, but there is no exact definition for alkaloids, and it is also difficult to distinguish them from other natural nitrogen-containing metabolites (Bruneton, 1999). However, the simplest way of identifying them is that the nitrogen is part of the heterocyclic structure and that they are derived from amino acids (Bruneton, 1999). Even though alkaloids are regarded as nitrogen-containing organic compounds, simple amines and peptides are not considered as alkaloids (Roeder and Wiedenfeld, 2011). Nonetheless, from a different perspective, alkaloids are regarded as a cyclic organic compound that possesses a nitrogen atom in its negative oxidation state (Bruneton, 1999). According to this definition amines, amides and quaternary ammonium are included but nitrate derivatives are excluded (Bruneton, 1999). Alkaloids consist of complex structures and due to the presence of nitrogen atoms they often have significant pharmacological activity (Bruneton, 1999). One example of an alkaloid is nitidine (**1.4**) (Figure 1.3), which is known to have antimalarial (antiplasmodial) activity (Bouquet et al., 2012).



Nitidine (**1.4**)

Figure 1.3. Structure of nitidine, an alkaloid.

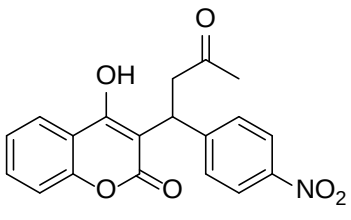
Table 1.1. Some popular medicinal plants of southern Africa.

Scientific name	Common name	Zulu name	Traditional/Medicinal uses
Alepidea amatymbica Eckl. & Zeyh. (Apiaceae)	Giant Alepidea	iKhathazo/ iQwili	The plant is extensively used for the treatment of tuberculosis. The rhizome is carried as a charm and is also used for colds, asthma, chest pain and influenza (Crouch et al., 2006)
Artemisia afra Jacq. ex Willd. (Asteraceae)	African wormwood	uMhlonyane	The plants are used for the treatment of respiratory tract infections, indigestion, fever, cold, influenza, sore throats, asthma, pneumonia, gout, and malaria (Crouch et al., 2006).
Bersama tysoniana Oliv. (Melianthaceae)	Common white ash	isiNdiyandiya/ uNdiyaza	The bark of the plant is used as the sniffing powder for the treatment of headaches and sinus congestion. The plant is also used in the treatment of snakebites (Crouch et al., 2006).
Bowiea volubilis Harv. ex Hook.f. (Hyacinthaceae)	Climbing lily	iGibisila/ iGuleni	The plant is used to treat bladder problems, sterility in women and tissue swelling which is caused by the accumulation of fluid (Crouch et al., 2006).
Combretum caffrum (Eckl. & Zeyh.) Kuntze (Combretaceae)	Cape bushwillow	uMdubu	The Xhosa people use the roots of the plant to soothe body pain, while Zulu people use root bark as a malicious charm. The plant contains a compound called combretastatin, which has strong anticancer properties. This compound is under development as an anticancer drug (Crouch et al., 2006).
Cryptocarya latifolia Sond. (Lauraceae)	Broad-leaved quince/ Bastard stinkwood	uMkhondweni/ uNdlangwenya	The bark of the plant is used to treat chest pains, headaches, stomach ailments and bladder diseases (Crouch et al., 2006).
Dioscorea dregeana (Kunth) T.Durand & Schinz (Dioscoreaceae)	Wild yam	isiDakwa	The tuber of the plant is used as a sedative for treating insane people. Adding small pieces of tuber to beer is claimed to increase the strength of the beer. The plant is also used to treat sores, fits, wounds and also to facilitate childbirth (Crouch et al., 2006).
Drimia robusta Baker (Hyacinthaceae)	Satin squill	isiKlenama/ iNdongana-zimbomvana	The bulb is used for relieving pain, feverish cold, coughs, and as a heart tonic (Crouch et al., 2006).
Elaeodendron transvaalense (Burtt Davy) R.H.Archer (Celastraceae)	Transvaal saffron	uMgodudo/ iNgwavuma	The bark of the plant is used as an emetic to strengthen the blood of men to improve their sexual ability. Bark infusions are used in the treatment of stomach cramps and diarrhoea (Crouch et al., 2006).

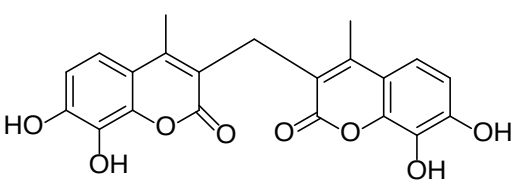
Eriospermum mackenii (Eriospermaceae)	Yellow fluffy seed	iNsulansula/ iNcamashela/ uMathintha	The tuber is used in the treatment of diarrhoea by means of an enema (Crouch et al., 2006).
Gasteria croucheri (Hook.f.) Baker (Asphodelaceae)	Gasteria	iMpundu	The plant is used in the treatment of rheumatoid pains by either application as an enema or by washing the painful area. The leaves of the plant are used to treat paralysis. The plant can also be sprinkled around the household to prevent conflict (Crouch et al., 2006).
Hypoxis hemerocallidea Fisch., C.A.Mey. & Avé-Lall. (Hypoxidaceae)	African potato/ Star flower	iLabatheka/ iNkomfe	The tuberous rootstock is used for the treatment of headaches, mental disorders, dizziness, cancers, inflammation, and HIV-AIDS (Crouch et al., 2006).

1.4.2 Coumarins

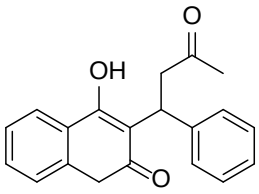
Coumarins are heterocyclic molecules that have been intensely studied due to their biological activities (Zhang et al., 2017). Apart from that, coumarins also have favourable photophysical properties (Zhang et al., 2017). Coumarins have been used as potential fluorescence materials and emissive dopants in organic light-emitting diode (OLEDs) applications (Yu et al., 2010). Their derivatives are regarded as a new class of antituberculosis candidates, and also possess other pharmacological properties like anticancer (Sashidhara et al., 2010), antiproliferative, anti-HCV, anti-HIV, antimalaria (Kadhum et al., 2011), antioxidant, antibacterial, antifungal, and anti-Alzheimer activities (Hu et al., 2017). Some coumarins-based derivatives are acenocoumarol (**1.5**), dicoumarolum (**1.6**), warfarin (**1.7**), hymecromone (**1.8**) and carbocromen (**1.9**) (Figure 1.4) (Hu et al., 2017).



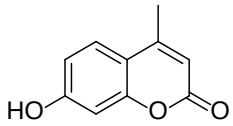
Acenocoumarol (**1.5**)



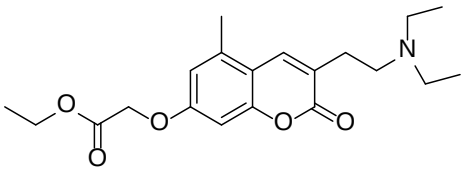
Dicoumarolum (**1.6**)



Warfarin (**1.7**)



Hymecromone (**1.8**)

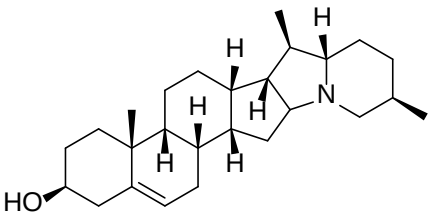


Carbocromen (**1.9**)

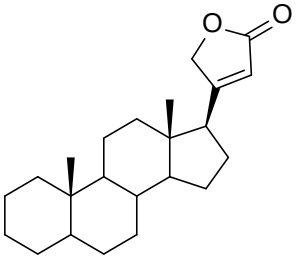
Figure 1.4. Some examples of coumarins.

1.4.3 Steroids

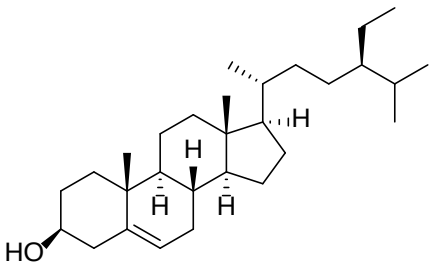
Steroids are one of the largest group of secondary metabolites (Bruneton, 1999). Steroids, in general, arise from the mevalonate pathway and plant steroids arise from squalene-like triterpenes (Bruneton, 1999). The plant steroids have a similar mechanism (act as architectural components of membranes) to that of triterpenes except that theirs is specific to plants (Bruneton, 1999). This applies to steroidal alkaloids (**1.10**)**,** cardenolides (**1.11**), phytosterols (**1.12**), and saponins (**1.13**) (Figure 1.5) (Bruneton, 1999) (Figure 1.5). Steroids exhibit biological activities, especially the nitrogen-containing steroidal compounds (Martínez-Pascual et al., 2017). These compounds have several biological activities such as anti-inflammatory (Huang et al., 2012), antiparasitic, antifungal, and antitumour activity (Krojer et al., 2013). Steroidal lactams have antiproliferative, antileukemic, and antifungal activities (Martínez-Pascual et al., 2017).



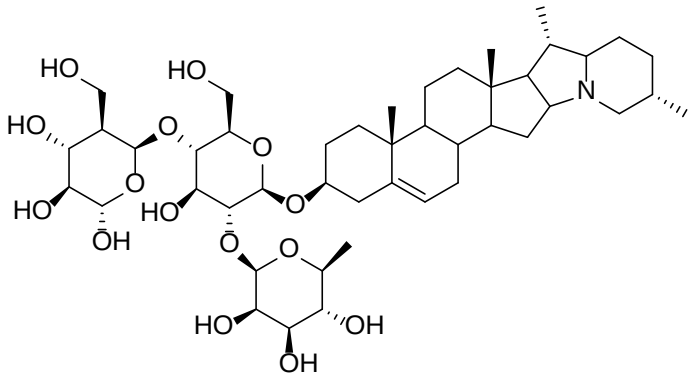
Steroidal alkaloid - solanidine (**1.10**)



Cardenolide (**1.11**)



Phytosterols - beta-sitosterol (**1.12**)



Saponin (**1.13**)

Figure 1.5. Some examples of steroids.

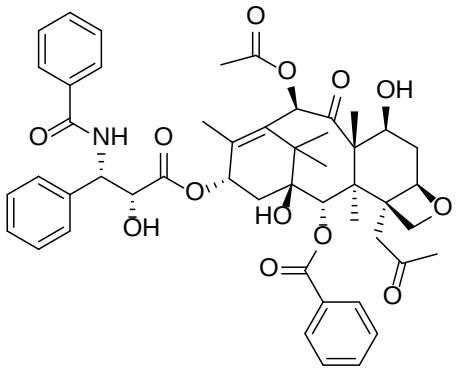
1.4.4 Flavonoids

Flavonoids are phenolic compounds that are found in plants and fungus, and some beverages such as tea and wine (Gonyela, 2016). They consist of different skeletons, either flavone, isoflavone or neoflavone based and are commonly polyphenolic (Mouri et al., 2014). There are about 4000 flavonoids that have been identified so far (Dengada, 2014). They possess a variety of biological activities including antifungal, antibacterial, antiviral, antioxidants, anti-inflammatory, antiproliferative and anti-allergenic properties (Feyen et al., 2007). Due to their diverse structural patterns, flavonoids are regarded as a rich source of compounds with good anticancer potential. Some flavonoids are also known to inhibit angiogenesis by blocking cell cycle-induced apoptosis and disrupting mitotic spindle formation (Mouri et al., 2014).

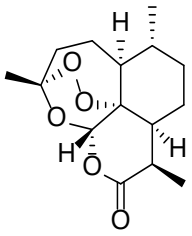
1.4.5 Terpenoids

Terpenoids are one of the largest groups of natural secondary metabolites (Bruneton, 1999), and they are produced by nearly all life forms (Xie et al., 2017). There are over 15 000 structures of terpenoids that have been elucidated, however, the exact number of terpenoids that exist in nature is still unclear (Bian et al., 2017). Some examples of terpenoids (Figure 1.6) are paclitaxel (**1.15**), an important anticancer drug, artemisinin (**1.16**), an antimalarial drug, and ginsenoside (**1.17**) (Figure 1.6), a major component of the Chinese traditional medicine ginseng (Bian et al., 2017). In the search for antidiabetic compounds, three triterpenoid glycosides were isolated from *Centipeda minima*; 3′-

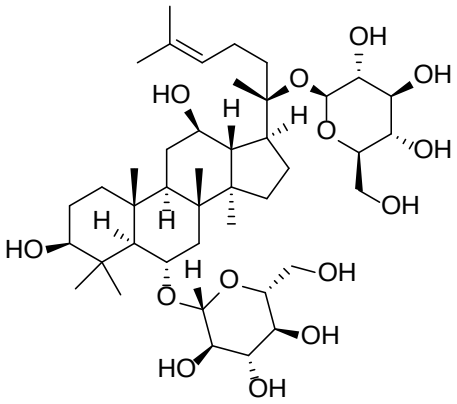
desulfated atractyloside, 15-*O*-[α-L-rhamnosyl-(1→2)-β-D-glucosyl]grandiflorolic acid, and 2α-hydroxylemmonin, which show α-glucosidase inhibitory activity (Nguyen et al., 2017).



Paclitaxel (**1.15**)



Artemisinin (**1.16**)



Ginsenoside (**1.17**)

Figure 1.6. Some examples of terpenoids.

1.4.6 Tannins

Tannins are secondary metabolites that are widespread in "woody and herbaceous plants" (p.1072) with predominant availability in legumes and twigs (Widsten et al., 2010). Primarily, they are divided into two categories, condensed tannins and hydrolysable tannins (Theodoridou et al., 2010). The hydrolysable tannins consist of a carbohydrate as the central core (Figure 1.7). Hydrolysable tannins are polyols, glucose usually, which are esterified with phenolic acids (Akiyama et al., 1998). These phenolic acids are usually gallic acid in gallotannins or hexahydroxydiphenic acid in ellagitannins. Hydrolysable tannins are commonly found in fruit pods and in contrast to condensed tannins, their degradation products are absorbed in the small intestines of animals (Theodoridou et al., 2010). Condensed tannins are mostly found in forage legumes,

trees and shrubs (Theodoridou et al., 2010), and are complexes of oligomers or polymers of flavonoid units that are linked by carbon-carbon bonds (Theodoridou et al., 2010). Both hydrolysable (Figure 1.8) and condensed tannins have bactericidal activity against *Staphylococcus aureus* (Chung et al., 1993).

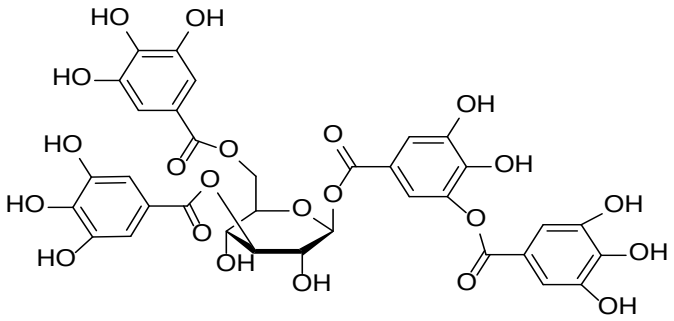


Figure 1.7. Structure of tannic acid, a hydrolysable tannin.

1.4.7 Lignans

Lignans are diaryl compounds that are widely distributed to different plants (Milder et al., 2005). The richest source of lignans is flaxseed; other sources are grains, vegetables, fruits and beverages (Shyu and Hwang, 2002). Lignans are a class of NPs that are derived from cinnamic acid and they exhibit a variety of biological activities, which include antioxidant and anti-oestrogenic properties (Davin and Lewis, 2000). These activities are the source of the ability of lignans to reduce the risk of some cancers and cardiovascular diseases (Davin and Lewis, 2000). They reduce these diseases by interfering with cell division in both animals and humans (Moss, 2000). They also play a vital role in the most famous and old traditional medicine; TCM. In TCM lignans are used to treat viral hepatitis and as liver protection (Moss, 2000).

1.5 Problem statement

Cancer is a group of diseases that are characterised by an uncontrollable growth of cells and the spread of abnormal cells (Luqman and Pezzuto, 2010). It is a major public health problem in the United States and other countries around the globe (Luqman and Pezzuto, 2010). According to statistics of the World Health Organization (WHO) published February 2017, cancer is one of the leading causes of morbidity and mortality worldwide; it had approximately 14 million new cases in 2014, and the number is expected to increase by 70% in 20 years' time. It is estimated to cause 8 million deaths each year, and the number is continuously increasing (Jemal et al., 2008). If the existing

trend continues, 1 in 4 people will die of cancer (Luqman and Pezzuto, 2010). The economic impact of cancer is significantly rising. According to WHO about 70% of deaths from cancer occur in low and middle-income countries (WHO, 2014). In 2010, the sum of the annual economic cost of cancer was estimated to be US$ 1.16 trillion.

Literature shows that there are a number of natural products (NPs) that are available as chemoprotective agents against the most commonly occurring cancers around the globe (Reddy et al., 2003). Antioxidants, phenols and reactive groups that show protective properties are marked as the major groups of these products, these natural products are found in fruits, vegetables, plant extracts and herbs (Table 1.2).

Table 1.2. Chemoprotective products found in fruits, vegetables, plant extracts, and herbs.

Source	**Active component**	**Mechanism of action**	**Cancer inhibited**	**References**
Olives	Polyphenols	Antioxidants	Various cancers	(Langseth, 1995)
Citrus fruits	β–Cryptoxanthin, bioflavonoids, chalcones, Vitamin C	Antioxidants stimulate expression of RB genes and p73 gene (a p53 related gene)	Rat tumour, various cancers	(Nishino et al., 2000)
Garlic, onions, leeks, chives	Allicin, flavonoids, vitamin C, selenium, sulfur	Detoxifies carcinogen, inhibits *Helicobacter pylori*, cell cycle arrest from S to G2M boundary phase	Stomach cancer	(Barch et al., 1996)
Gymnosporia rothiana Laws	GCE: chloroform ether extract	DNA/RNA and protein synthesis inhibited after treatment for 12-36 hr	Leukaemia in mice	(Chapekar and Sahasrabudhe, 1981)
Scutellaria radix, S. indica	Flavonoids	Prostaglandin E_2 production	Rat C6 glioma cells	(Nakahata et al., 1998)

The mechanism of the protective effect is still unclear. However, there is undeniable evidence that the consumption of fruits and vegetables decreases the incidence of carcinogenesis (Reddy et al., 2003). Diet and diseases have been associated since the early history of medicine (Mehta et al., 2010). “Let food be thy medicine and medicine be thy food," said Hippocrates the father of modern medicine 2 500 years ago (Mehta et

al., 2010). Later Galen of Pergamon (129–199 A.D.) who was a Greek physician, prescribed different types of food, including barley, and various vegetables for the treatment of cancer (Karpozilos and Pavlidis, 2004). Some anticancer NPs can be directly isolated from plants, i.e. *Daphne genkwa* Sieb.et Zucc, a species of the Thymelaeaceae family that is known to have a number of compounds, for example flavonoids, lignans, coumarins and daphnane-type diterpene esters. All these compounds are known to have moderate anticancer activity but the diterpene esters are known to have the highest anticancer capacity (Li et al., 2013).

Human Immunodeficiency Virus (HIV) is a lentivirus that can escalate to acquired immune deficiency syndrome (AIDS). HIV was discovered in 1981, and it already caused the deaths of 25 million people globally. It is a major threat to humankind to date (Kurapati et al., 2016). Nonetheless, the introduction of highly active antiretroviral therapy (HAART) has reduced the HIV incidence drastically (Kurapati et al., 2016). The natural products that commonly show activity against HIV are prenylated benzophenones, guttiferones that are extracted from different genera namely; *Garcinia*, *Symphonia* and *Clusia* (Mzozoyana, 2015). In the Thymelaeaceae family, there is *Stellera chamaejasme* L. that was found to possess anti-HIV compounds (Asada et al., 2013). The plant has been used in TCM as dermatological and anthelmintic agent (Asada et al., 2013). When investigation on HIV agents was conducted on the plant, eight compounds were isolated and only two compounds; stelleralide A and gnidimacrin which were found to have a very potent anti-HIV activity (Asada et al., 2013).

Dais L. is one of the smallest genera of the Thymelaeaceae family with only 2 species. The phytochemistry of both species in this genus has not been published apart from a conference abstract on *Dais cotinifolia* L., which reported that a number of lignans had been isolated from this species (Crabtree and Belofsky, 2010). No structures are given in this references and no follow-up papers have been published. Nevertheless, there are other genera of this family with species with known phytochemistry, like *Daphne* L. and *Gnidia* L. and these genera are rich in anticancer and anti-HIV compounds (Meruelo et al., 1988). The limitation in drug discovery from this family is that some genera are toxic, i.e. *Gnidia* species have diterpene esters which are PKC activators for adjuvant therapy towards the eradication of HIV-1 but the downfall is the toxicity of these compounds (Meruelo et al., 1988). This family has a potential of producing antiviral and anticancer compounds, therefore, it deserves attention since most species of this family that are found in South Africa have not been studied. The family is reviewed in depth in Chapter 2.

1.6 Aim and objectives

Aim

The aim of this study is to investigate the phytochemistry of the medicinal plant species *Dais cotinifolia* L.

The research objectives were as follows:

- Identify and collect the tree material.
- Extract different parts of the plant with various solvents.
- Isolate pure compounds from different parts of the tree using DIOL column, vacuum liquid chromatography (VLC) column, column chromatography, centrifugal thin-layer chromatography (CTLC), HPLC and TLC.
- Identification and characterisation of the isolated compounds following various spectroscopic techniques; NMR (1D & 2D), LC-MS, TOF-MS, and UV.
- Identification of appropriate bioassays based on classes of the isolated compounds.
- Assaying the compounds for biological activity thereby enhancing further use of the plants or validating their ethnomedicinal use.

1.7 Structure of the thesis

This thesis is divided into five chapters. Chapter 2 provides an overview of the literature of the Thymalaeaceae family and *Dais cotinifolia* L., it also gives a brief overview of the instrumentation that was used during this study. Chapter 3 focuses on the results obtained in the investigation and Chapter 4 gave the experimental details of the project. Chapter 5 is a general conclusion of the study, limitations are discussed and future work is proposed.

Chapter 2: Literature review

The plant under investigation in this study, *Dais cotinifolia* L., is part of the family Thymelaeaceae. This Chapter focuses on the literature of the Thymelaeaceae, some genera and species of this family that are used in traditional medicine, and genera that exhibit biological activity. In the second part of the Chapter, methods used to isolate pure compounds are discussed and spectroscopic methods essential for the structure elucidation of pure compounds are briefly reviewed.

2.1 Thymelaeaceae

Thymelaeaceae is a medium-sized family of flowering plants that comprises about 900 species that are divided into around 55 genera, which are widespread in tropical and sub-tropical areas (Beyers and Marais, 1998). Thymelaeaceae is classified into four subfamilies; Aquilarioideae, Gonystyloideae, Synandrodaphnoideae and Thymelaeoideae (Beaumont et al., 2009). Aquilarioideae, Gonystyloideae, and Synandrodaphnoideae are small subfamilies and contain seven, three and one genera, respectively. The majority of the genera are classified under Thymelaeoideae, including the largest genus *Gnidia* (140 species).

In Africa alone, there are more than 40 genera of Thymelaeaceae (Ferrari et al., 2000), including *Dais, Dicranolepis, Craterosiphon, Englerodaphne, Gnidia, Lachnaea, Lasiosiphon, Octolepis, Peddiea, Struthiola, Synandrodaphne*, and *Synaptolepis* (Oladipo and Oyaniran, 2013). However, some of the genera in this family, such as *Gnidia and Pimelea*, need revision because of uncertain generic circumscription (Maurin et al., 2013). The key problem is the classification within the subfamily Thymelaeoideae (Maurin et al., 2013). The largest genera in this family are discussed in detail below. *Dais* has only two species, but it is included since it is the plant of interest in this study. In Figure 2.1 a graph listing some genera of the Thymelaeaceae and the number of species in each genus, is given.

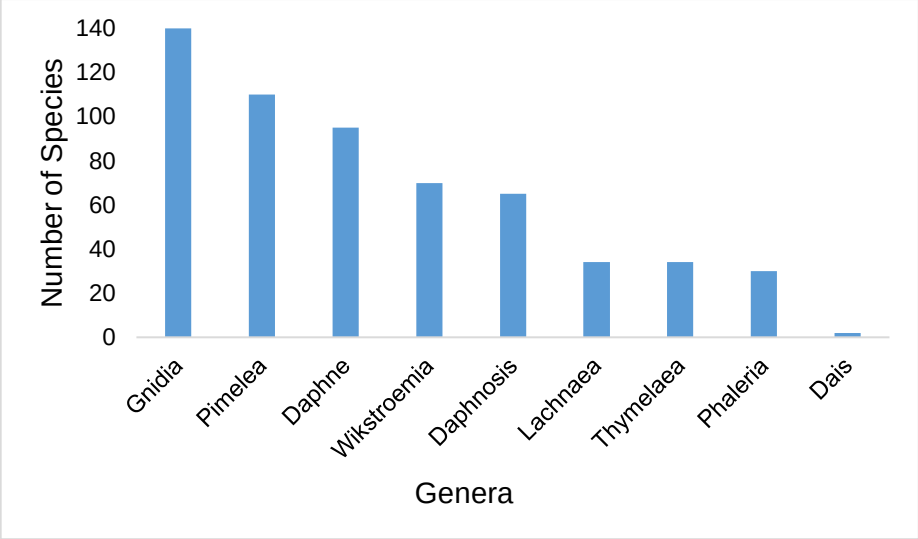


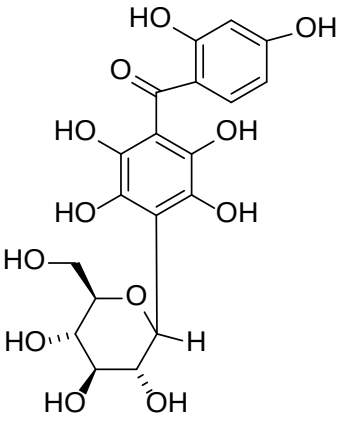
Figure 2.1. Some genera of the Thymelaeaceae.

2.1.1 *Gnidia* L.

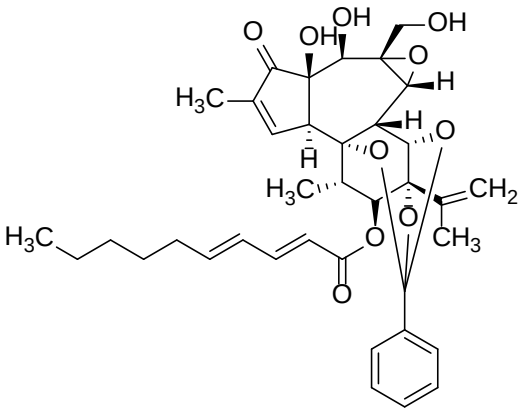
The genus *Gnidia* L. was established by Linnaeus in 1753, when the genus had only three species (Beaumont et al., 2009). *Gnidia* is one of the genera of the subfamily Thymelaeoideae of Thymelaeaceae (Franke et al., 2002) and comprises about 140 species (Beaumont et al., 2009). This genus is also known to be polyphyletic and, therefore, needs to be reclassified (Boatwright et al., 2017). Many species in this genus are used in African traditional medicine (ATM) to treat ailments such as a sore throat, abdominal pain, wounds, and burns (Franke et al., 2002). *Gnidia* species are known to contain daphnane-type diterpene esters that possess antineoplastic activity (Mothana and Lindequist, 2005). Phytochemical investigations that were conducted on species of this genus identified coumarin, lignans, flavonoids and benzophenone glycosides in this genus (Franke et al., 2002).

Gnidia glauca Fresen. is used to treat sore throats, abdominal pain, wounds, burns, snake bites, contusions, swellings, backache and joint ache (Ghosh et al., 2012). *G. glauca* has been recently reported to have compounds with antidiabetic properties (Ghosh et al., 2012). The stem, leaf and flower extract of *G. glauca* were assayed for inhibition of porcine pancreatic α-amylase and glucosidases from pancreas, liver, and small intestine of Swiss mice, extracts were found to have a high inhibition of α-amylase (Ghosh et al., 2012). The results provided evidence that *G. glauca* has anti-diabetic activity (Ghosh et al., 2012). The compounds that have been isolated from this plant are gnidilatin 20-palmitate (**2.4**), gnidilatidin 20-palmitate (**2.7**), gnidilatin (**2.5**) and gnidilatidin (**2.6**) (Kiptoon et al., 1982) (Figure 2.2).

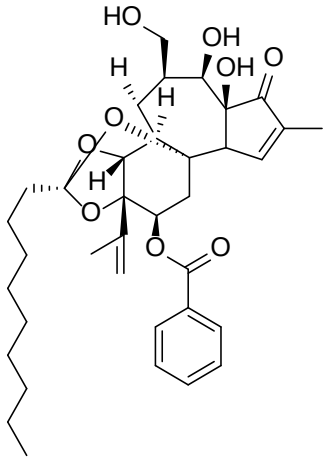
Gnidia latifolia and *Gnidia glaucus* have activity against leukemia (Kupchan et al., 1976). *G. latifolia* also has piscicidal activity (Kupchan et al., 1976). Another species in this genus is *Gnidia socotrana*, which is endemic to Socotra Island (Franke et al., 2002). When phytochemical studies were conducted on the leaves and twigs of the plant, six novel compounds were identified, i.e. a biscoumarin (**2.3**), two novel umbelliferyl-flavonoids and three other compounds with a spiro-bis-γ-lactone structure (Franke et al., 2002). *Gnidia lamprantha* contains predominantly diterpenoid daphnetoxins with antitumour activity (Ferrari et al., 2000). Compounds isolated from this plant include gnididin (**2.2**) and huratoxin (Kupchan et al., 1976). The compounds that were isolated from *Gnidia latifolia* were similar to that of *G. lamprantha* except for some sesquiterpenoids (Kiptoon et al., 1982). The roots of *Gnidia involucrata* Steud. ex A. Rich, along with other Thymelaeaceae species, are used in Zimbabwe to reduce the virginal orifice (Ferrari et al., 2000). When phytochemical studies were conducted on *G. involucrate,* six compounds were obtained and identified as 2,3,4,5,6-pentahydroxybenzophenone-4'-*C*-glucoside (**2.1**), 2,4',6-trihydroxy-4-methoxybenzophenone-2-*O*-glucoside, mangiferin, kaempferol-3-*O*-glucoside, yuankanin and manniflavanone (Ferrari et al., 2000). Furthermore, the roots of the plant are also internally used in Ethiopia as laxatives and vermifuge (Borris et al., 1988). In some African countries like Nigeria, Congo, Zimbabwe, Malawi and Sudan they *Gnidia kraussiana* Meissn. is used as a source of hunting and fish poisons (McGaw and Eloff, 2008).



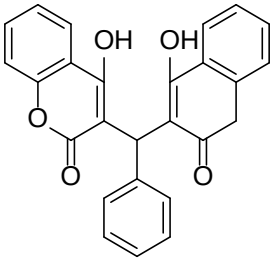
2,3,4,5,6-Pentahydroxybenzophenone-4-*C*-glucoside (**2.1**)



Gnididin (**2.2**)

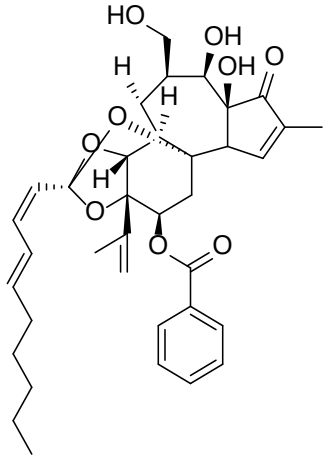


Gnidilatin (**2.4**)

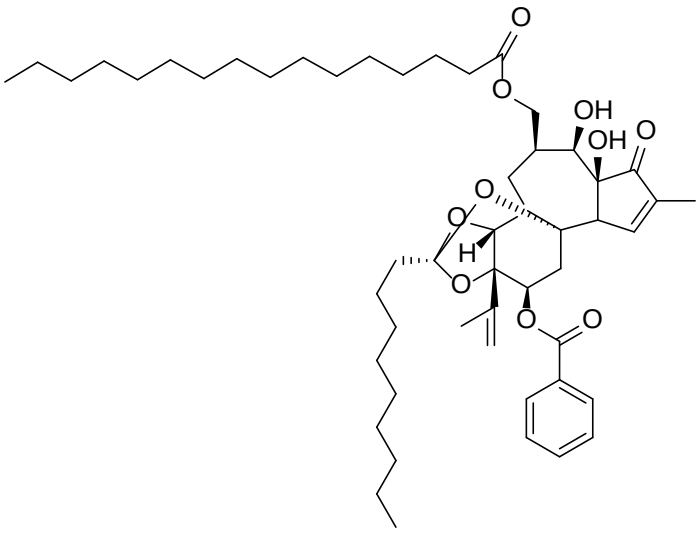


3,3'-Benzylidene-bis-(4-hydroxycoumarin) (**2.3**)

Gnidilatidin 20-palmitate (**2.7**)



Gnidilatidin (**2.6**)



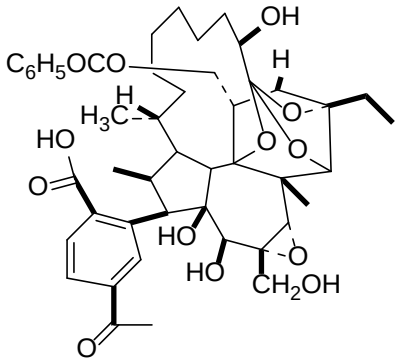
Gnidilatin 20-palmitate (**2.5**)

Figure 2.2. Some of the compounds isolated from *Gnidia* species.

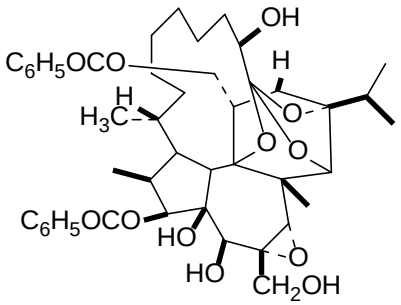
2.1.2 *Pimelea* Banks & Sol. ex Gaertn.

Pimelea (Thymelaeoideae subfamily) originates in Australia and consists of about 110 species. It is now widespread in Lord Howe Island, New Zealand, and Timor, with about 90 species endemic to inland Australia (Silcock et al., 2012). *Pimelea* is closely related to *Thecanthes* (a small genus with about five species in total) (Motsi et al., 2008).

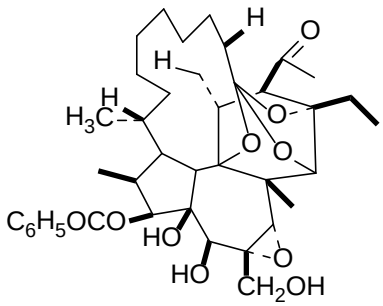
Pimelea linofolia (P. linofolia) and *Pimelea ligustrina (P. ligustrina)* are native to the east coast of Australia (Tyler and Howden, 1981). Extracts from these two species have anticancer activity. Nevertheless, the compounds associated with this activity were not successfully identified from these species but two compounds (linifolins A and B) with piscicidal activity were identified (Tyler and Howden, 1981). The name of linifolin A was later changed to linimacrin A and linifolin B to pimelea factor P_3 (Tyler and Howden, 1985). Many species in this genus are reported to be poisonous when ingested, for example, *P. altior* F. Muell., *P. decora* Domin, *P. elongata* Threlfall, *P. flava* R. Br., *P. glauca* R. Br., *P. haematostachya* F. Muell., *P. linifolia* Sm., *P. microcephala* R. Br., *P. pauciflora* R. Br., *P. simplex* F. Muell., *P. trichostachya* Lindl, *P. neo-anglica* Threlfall and *P. prostrata* Willd. (Silcock et al., 2012). Among the species above, three species cause most of the problems and these are *P. trichostachya*, *P. simplex* and *P. elongate*. However, the mechanism of poisoning is still unclear (Silcock et al., 2012). Although most species in this genus are poisonous, there are species that possess anti-neoplastic activity, and these are *P. linifolia* (Smith and Towers, 1985), *P. ligustrina*, and *P. simplex* (Tyler and Howden, 1985). The compounds that are responsible for the antineoplastic activity are gnidimacrin (**2.9**) from *P. ligustrina,* simpleximacrin (**2.8**) from *P. simplex,* linimacrin D (**2.10**) and pimelea factor P_3 (**2.11**) from *P. linifolia* (Tyler and Howden, 1985). Figure 2.3 below shows some of the compounds that were isolated from different *Pimelea* species.



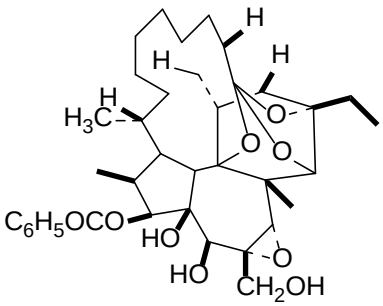
Simpleximacrin (**2.8**)



Gnidimacrin (**2.9**)



Linimacrin (**2.10**)



Pimelea factor P_3 (**2.11**)

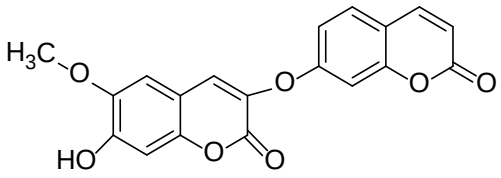
Figure 2.3. Compounds present in *Pimelea* species.

2.1.3 *Daphne* L.

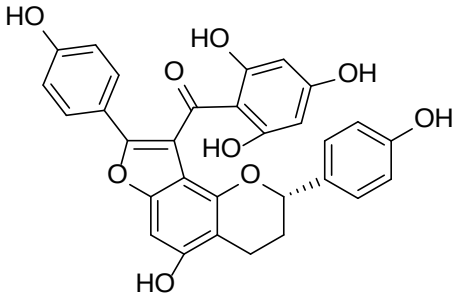
The genus *Daphne* comprises about 100 species (Khodadadian et al., 2016), widespread across Europe, Asia, Africa, and Australia (Noshad et al., 2009). Forty-four of these species occur in China (Zhang et al., 2008) and seven in Turkey (Sanda et al., 2015). The seven species that are found in Turkey are *D. gnidioides* Jaub. & Spach., *D. mezereum* L., *D. mucronata* Royle., *D. oleoides* Schreb., *D. pontica* L. and *D. serica* (Sanda et al., 2015). In various countries, species of *Daphne* are used as herbal folk medicine to treat different ailments (Sanda et al., 2015). The most-used species are *Daphne oleoides*, which is used to treat rheumatic pain, lumbago, and to reduce fever (Kupeli et al., 2007), *D. genkwa*, which is used to treat inflammatory disorders (Zheng et al., 2006), *D. acutiloba*, used for wounds and bruises, *D. tangutica*, which is used for the treatment of rheumatoid arthritis, *D. mezerium*, which is used for chronic rheumatism, skin diseases, gout, and inflammations in the lymph tissue (Chen et al., 2004), and *D. giraldii* Nitshe, which is used for the treatment of aches and rheumatism (Zhang et al., 2008). Various compounds have been isolated from *D. giraldii*, to name a few, monomeric coumarins (Sun et al., 2006), biscoumarins, flavonoids (Liao et al., 2005), lignans, phenolic glycosides (**2.9**), daphnodorin A (**2.13**), daphnodorin B, daphnoretin (**2.12**), glucogenkwanin (**2.14**), yuankanin, daphneside (**2.15**), and syringaresinol (**2.16**) (Zhang et al., 2008).

The genus *Daphne* is prominent for having species used for treatment of cancer since the time of Aphodisias (AD 2nd century) (Kupeli et al., 2007). One of the species that is used to treat cancer is *D. mucronate* Royle., which is indigenous to Iran (Khodadadian et al., 2016). Daphnodorins (flavonoids), which were isolated from the roots of *D. genkwa*, have antitumour activity (Zheng et al., 2007). The flower buds of this species were used against mammary cancers (Zheng et al., 2006). *D. mezereum* was also reported to have anticancer activity (Kupeli et al., 2007). The extracts of *D. gnidioides*

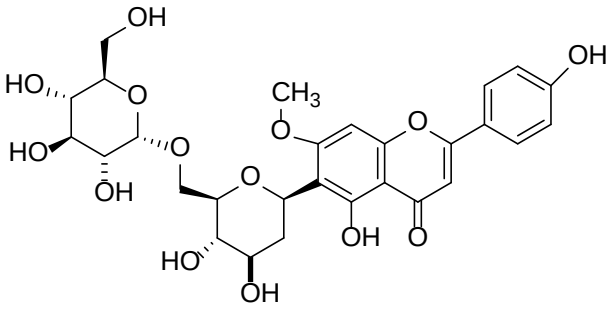
and *D. pontica* were found to be an excellent source of natural antioxidants for both medicinal and food applications (Sanda et al., 2015). The secondary metabolites that are usually isolated from *Daphne* species are flavonoids, coumarins and diterpenoids (Sanda et al., 2015). Figure 2.4 shows some compounds that were isolated from *Daphne* species.



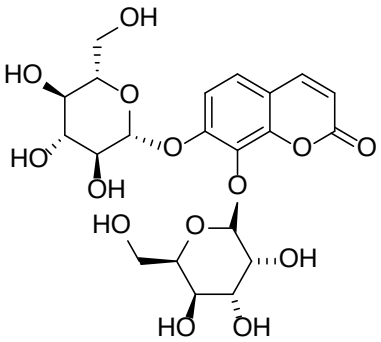
Daphnoretin (**2.12**)



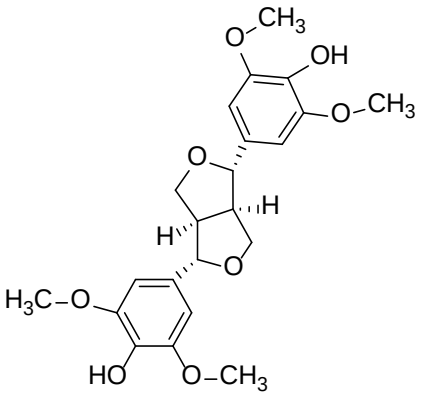
Daphnodorin A (**2.13**)



Glucogenkwanin (**2.14**)



Daphneside (**2.15**)



Syringaresinol (**2.16**)

Figure 2.4. Some organic compounds isolated from the genus *Daphne*.

2.1.4 *Wikstroemia* Endl.

The genus *Wikstroemia* comprises about 70 species (Guo et al., 2015). It consist of tropical and subtropical shrubs or trees that are distributed in southeast Asia to Malaysia, northeast Australia and in the Pacific islands (Gupta and Gillett, 1969). Phytochemical investigations conducted on species in this genus have revealed that it has daphnane-type diterpenoids. These compounds possess a number of biological activities such as antitumour, cytotoxic, anti-HIV, pesticide and skin irritant activities (Guo et al., 2015). Some compounds that have been isolated from species in this genus are active daphnane-type diterpene esters, tiglianes, lignans, bioflavonoids and bicoumarins (**2.21**) (Liao et al., 2006). *Wikstroemia hainanensis* Merr., is a shrub that shows anticancer activity, and the active compounds were three lignans, wikstrone (**2.17**), wikstroemol (**2.18**), and wikstroemone (**2.19**). The three lignans were isolated from the aerial parts of the shrub along with twelve known compounds, *erythro*-guaiacylglycerol-*O*-4'-sinapyl ether, *trans*-4-hydroxycinnamic acid (**2.20**), pinoresinol, medioresinol, syringaresinol, nortrachelogenin, lariciresinol, ficusesquilignan A, sitosterol, hederagenin, umbelliferone, and daphnoretin (Liao et al., 2006). The aforementioned compounds are illustrated in Figure 2.5.

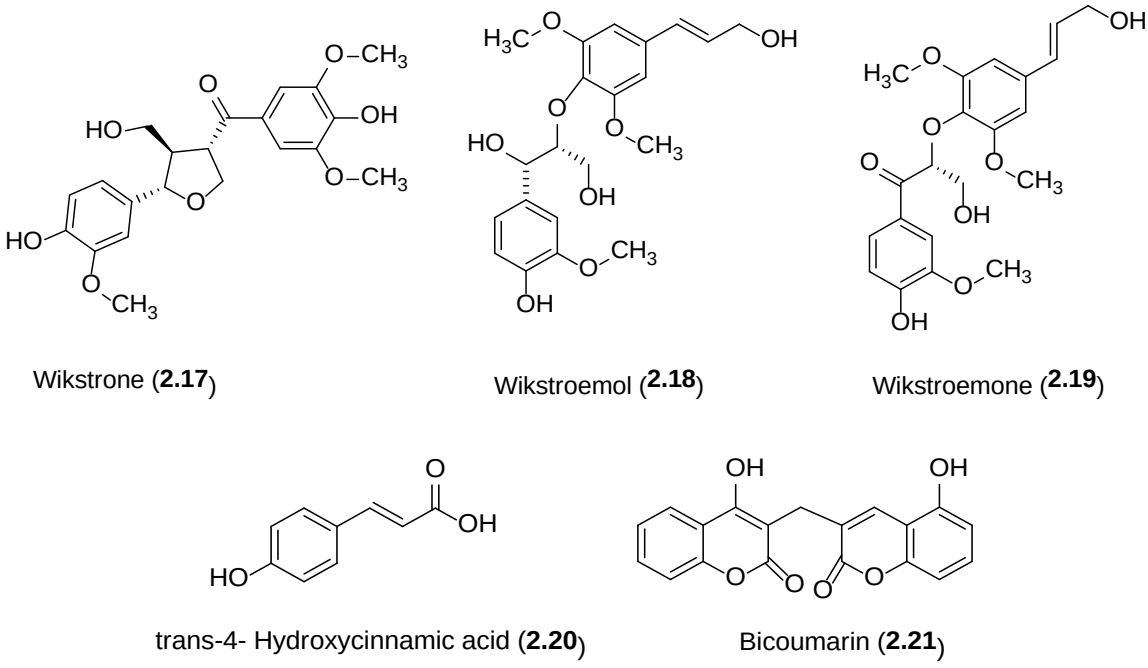
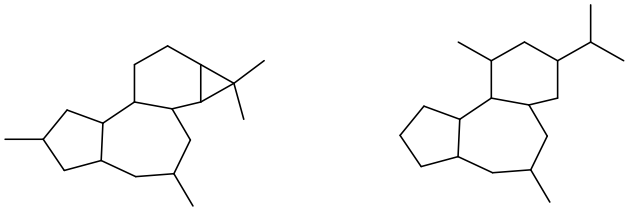


Figure 2.5. Structures of compounds from the genus *Wikstroemia*.

2.1.5 *Daphnopsis* Mart.

The genus *Daphnopsis*t has 65 species. *Daphnopsis* was first described by Martius and Zuccarinil in 1824 and is native to Central America, Antilles and South America (Chang et al., 2017). Although different *Daphnopsis* species have been reported to have different medicinal and practical properties, limited phytochemical studies have been reported on this genus (Blaskó et al., 1988). *Daphnopsis brasiliensis* Mart. et Zucc. is known to be used to treat psoriasis and also as a purgative (Blaskó et al., 1988). *Daphnopsis schwartzii* Meisn is used as a stimulant, sialagogue and diuretic (Blaskó et al., 1988). Apart from medicinal uses, *Daphnopsis brasiliensis* is used to manufacture paper (Adolf and Hecker, 1982) and *Daphnopsis ramnosa* Griseb fibrous bark is used to make rope. *D. ramnosa* was the first species in this genus on which phytochemical studies were conducted and tigliane (**2.22**), daphnane (**2.23**), and alpha-alkyldaphnane esters were identified (Adolf and Hecker, 1982). Some compounds isolated from species in this genus are shown in Figure 2.6 below.



Tigliane (**2.22**) Daphnane (**2.23**)

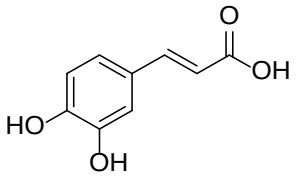
Figure 2.6. A few compounds isolated from the genus *Daphnopsis*.

2.1.6 *Lachnaea* L.

The genus *Lachnaea* is native to the Cape Floral Region (Beyers and Van Wyk, 1998) and was first established by Linnaeus in 1753. New species were later discovered, but the taxonomy for the genus was only established in 1840 by Meisner based on Drege's collection (Beyers and van der Walt, 1995). Nineteen species and eight infra-specific taxa were discovered in 1915 (Beyers and van der Walt, 1995). However, there are 34 species and eight infra-specific taxa known to date (Beyers and Van Wyk, 1998). Most species of this genus are found on mountain ranges (Beyers and Van Wyk, 1998). No phytochemical results have been reported on this genus.

2.1.7 *Thymelaea* Mill.

Thymelaea comprises about 30 species of evergreen shrubs (Vincent and Thieret, 1987). This genus consists of woody and herbaceous species that are native to the northern hemisphere of the Old World, especially to the Mediterranean region (Vincent and Thieret, 1987). The genus *Thymelaea* is only represented by one species in North America, *Thymelaea passerine* L (Vincent and Thieret, 1987). Some species in this genus are known to have activities against some ailments, for example, *Thymelaea hirsuta* L a medicinal plant that grows in Tunisia (Yahyaoui et al., 2018), is used as a decoction in the treatment of diabetes (Djeridane et al., 2006). Apart from being used as a decoction in the treatment of diabetes, *T. hirsuta* has been used in folk medicine as a source of antioxidant (Djeridane et al., 2006), and compounds that possess anti-melanogenic activity (Akrout et al., 2011). The phytochemical investigation revealed that the plant is rich in phenolics (**2.24**) (Figure 2.7) and tannins (Yahyaoui et al., 2018).

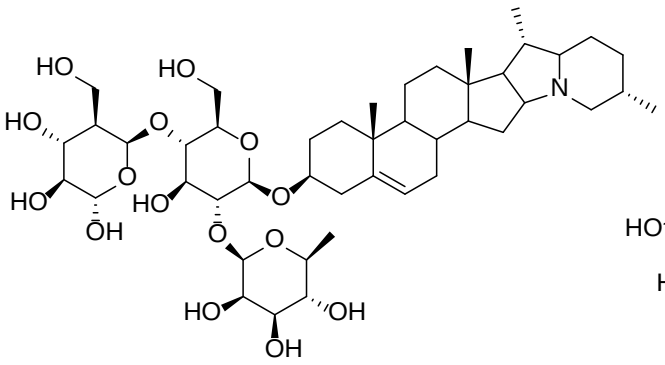


Phenolic-caffeic acid (**2.24**)

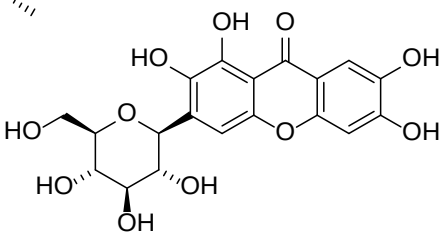
Figure 2.7. Structure of caffeic acid (**2.24**), a phenolic present in *T. hirsuta*.

2.1.8 *Phaleria* Jack.

Phaleria comprises about 30 species. However, the predominant species is *Phaleria macrocarpa* (Scheff.) Boerl. because of its anticancer activity (Altaf et al., 2013). *P. macrocarpa* is a medicinal plant that is native to Indonesia and Malaysia. The leaves and fruits of this plant have been used for the treatment of several types of cancers since the ancient times (Altaf et al., 2013). The extracts of this plant have been evaluated and reported to have biological activities like anticancer, antioxidant, antifungal, anti-inflammatory, antibacterial, antidiabetic and vasorelaxant effects (Hendra et al., 2011). Compounds isolated from the various parts of the plant include lignans, flavonoids, gallic acid, alkaloids, saponins (**1.13**), and mangiferin (**2.26**) (Figure 2.8) (Altaf et al., 2013)



Saponin (**1.13**)



Mangiferin (**2.26**)

Figure 2.8. Structure of compounds from the genus *Phaleria*.

2.1.9 *Dais* L.

Dais L. is a small genus in Thymelaeaceae. There are two species, *Dais cotinifolia* L. (SANBI, 2017) (Figure 2.9) and *Dais madagascariensis* Lam, found in Madagascar (Zavada and Lowrey, 1995). *D. cotinifolia* is the plant of interest in this study. The phytochemistry and bioactivity of neither of the two species have been investigated.

Dais cotinifolia (D. cotinifolia)

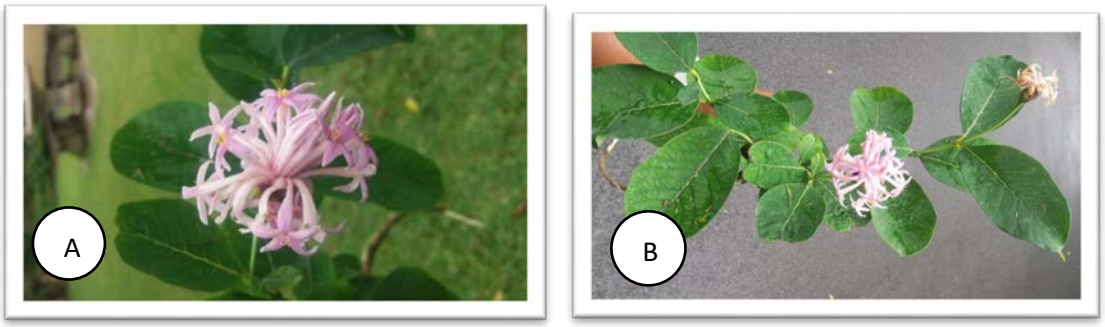


Figure 2.9. *Dais cotinifolia* L. (photographed by B. Danca).

Dais cotinifolia L. (kannabas, pompom tree – *intozwane emnyama*) is a flowering tree that belongs to the Thymelaeaceae family. This plant is one of the two species of *Dais*, and is indigenous to South Africa with an isolated population in the eastern highlands of Zimbabwe. However it is now widely distributed as a garden tree in a number of countries in Africa and Europe . In South Africa the plant is widespread in the eastern part of the country, i.e. KwaZulu-Natal, Eastern Cape, Gauteng, Mpumalanga, Limpopo and Free State provinces (SANBI, 2017). *D. cotinifolia* is a small tree that can grow up to six meters in height, with flowers that can grow up to three centimeters (Zavada and Lowrey, 1995). The flowering times for this plant varies according to the climate

conditions of the area. In warm places like Cape Town, the plant flowers in the beginning of summer and in cold places, it flowers towards the end of December (SANBI, 2017). The plant has a strong bark and as a result, it is used for tying and weaving. The fibrous bark are also used for plaiting strong ropes. This plant is not important in traditional medicine, but a decoction of the leaves is ingested to treat stomach ache (SANBI, 2017). In some countries, the plant is planted for ornamental purposes. The only phytochemical report on this is a conference proceeding which mentioned the isolation of lignans from the plant extracts, with no indication of the structures of the isolated compounds (Crabtree and Belofsky, 2010).

2.2 Separation of compounds

Chromatographic techniques are widely used for the analysis of chemical substances and natural products, it is used for the separation of complex mixtures and the isolation of pure compounds.The mobile phase is the gas or liquid (solvent) that carries the components of a sample through the stationary phase, whereas the stationary phase is the solid material to which a sample is added. Stationary phases can be kaolin, alumina, silica, and activated charcoal. Substances that are added to a stationary phase move according to the rate at which they are adsorbed by both the stationary and mobile phases (Skoog et al., 2004).

2.2.1 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is a simple, affordable, and fast procedure that determines how many components are in a mixture (Meyers and Meyers, 2008). TLC can also be used in the identification of unknown compounds by comparing it with a known compound using the retention factor (R_f). To accomplish this, the compound or the components of the mixture can be either viewed under ultraviolet (UV) light or sprayed by a phytochemical screening reagent that will cause colour change according to the class of phytochemicals that are present on the plate. Stains can also be used to view the components that are not visible under UV light (Sasidharan et al., 2011). TLC can also be used to determine the purity of isolated compounds. In TLC, the alumina or any powdered adsorbent is fixed on the glass, plastic or metal as a stationary phase. The mobile phase is then allowed to travel up the stationary phase with the components that were spotted just above the solvent (Tesso, 2005).

2.2.2 Column chromatography (CC)

In column chromatography (Figure 2.10), the solutes of a solution are allowed to travel down the column, while the separate components are adsorbed by the stationary phase. A common stationary phase in natural products is silica gel or alumina. The component that is strongly adsorbed by the stationary phase will remain near the top of the column while other components travel down the column along with the mobile phase (solvent). In column chromatography, there are two different kinds of packing, wet packing and dry packing. With wet packing, the silica gel is suspended in a suitable solvent before it is introduced onto the column. With dry packing, the silica gel is added dry to the column, and then the solvent is added (Tesso, 2005). The solvent system is usually chosen according to how the individual components of a solution respond to it on a TLC plate. The fractions that are eluted from the column are again monitored by TLC (Tesso, 2005).

Figure 2.10. Column chromatography (photographed by B. Danca).

2.2.3 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an advanced column chromatographic method, whereby the columns are packed with very tiny particles. The flow rate is altered to decrease band broadening and also to keep the baseline straight (Yamaguchi et al., 1998). The pump is the pressure supplier. HPLC can be performed on a normal or a reversed phase column. With a normal column, polar silica gel and non-polar solvents are used, whereas with reversed phase, polar solvents are usually used with a non-polar surface of the packing material (Engelhardt, 2012). The HPLC system detector determines the solvents that can be used, and also limits compounds that can be absorbed (Tesso, 2005).

2.2.4 Preparative Centrifugal Thin-layer Chromatography (PCTLC)

PCTLC (Figure 2.11) is performed on a thin layer of absorbent on a rotor that is covered by a teflon lid that is transparent to ultraviolet (UV) light, to allow the detection of UV absorbing bands. A motor rotates the rotor at a constant speed by a shaft that passes through a hole in the centre of the main vessel. A solution of compounds that is to be separated is applied to the absorbent through the inlet and wick. Elution by solvent forms concentric bands of separated substances which leave the edge of the rotor together with the solvent. A channel collects the eluate and transfers it to the output tube. The absorbent layer can be prepared in sizes of 1 mm, 2 mm or 4 mm (Desai et al., 1985).

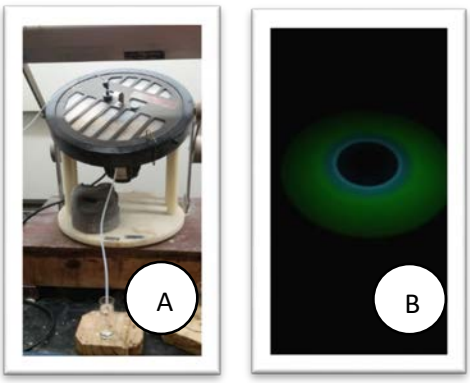


Figure 2.11. Normal PCTLC (A), PCTLC plate under UV showing concentric bands of separated substances (B) (photographed by B. Danca).

2.3 Structural elucidation

2.3.1 Nuclear magnetic resonance spectroscopy (NMR)

NMR is a technique that relies on the ability of unpaired atomic nuclei to spin when it interacts with a radio wave at a specific frequency in an external magnetic field. When molecules are exposed to a strong magnetic field, the magnetic moment of the nuclei align with the magnetic field and when the radio frequency is applied the nuclei gain energy (excited state). When the nuclei lose energy (revert to ground state), a radio frequency is emitted (Clayden et al., 2001), which is recorded and transformed into a NMR spectrum This method helps with the structural elucidation of different chemical compounds. One-dimension (1D) NMR (^{1}H NMR) assists with the determination of proton environments while ^{13}C NMR determines the carbon atom environments (Tesso, 2005). Distortionless Enhancement by Polarization Transfer (DEPT) 135 is used for the identification of methylene carbons which appear as negative peaks in the spectrum.

Two-dimension (2D) NMR Correlation Spectroscopy (COSY) relies on $^{1}H,^{1}H$ correlations, whereas Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bonding Correlation (HMBC) spectra give information on the $^{1}H,^{13}C$ correlations (Bruice, 2014).

2.3.2 One-Dimensional NMR

In 1D pulsed Fourier transform NMR the signal is recorded as a function of a single time variable followed by the Fourier transformed to give a spectrum which is a function of a single frequency variable, whereas in 2D NMR the signal is noted as a function of two time variables followed by a double Fourier transformed to give a spectrum which is a function of a double frequency variables (Noda et al., 2000).

Proton NMR (^{1}H NMR) is used to detect the number of protons in a compound. The number of signals designates the different proton environments in a spectrum. ^{1}H is the most abundant isotope of hydrogen in nature, 99.985% to be precise. In ^{1}H NMR protons couple to show connectivity of the structure (Clayden et al., 2001). A spectrum is divided into six regions (Bruice, 2014). The chemical shift range of a spectrum is between 0-12 parts per million (ppm). The first region (0-1.5 ppm) is the saturated region followed by allylic region (1.5-2.5 ppm), Z= O, N, halogen (2.5-4.5 ppm), vinylic region (4.5-6.5 ppm), aromatic region (6.5-8.0 ppm), and a carbonyl region (9.0-12 ppm) (Bruice, 2014).

The number of signals in a ^{13}C NMR spectrum designates the number of various kinds of carbons a compound consists of, just like the number of signals in a ^{1}H NMR spectrum tell us the number of various kinds of hydrogens in a compound. The signals in this experiment appear as singlets because the ^{13}C NMR are recorded based on the proton decoupling and all the ^{13}C-^{1}H coupling are decoupled by irradiation of all the protons. This kind of analytical procedure is only carried out through computers that carry out a Fourier transform (FT) (Clayden et al., 2001). In ^{13}C NMR, FT is required because carbon has a poor sensitivity and this results in obtaining weak signals that are not easily differentiated from the background electronic noise and this is because of the the poor sensitivity of carbon. ^{13}C FT-NMR has to be rapidly repeated to achieve visible signals (Clayden et al., 2001). Visible signals are achieved by adding together many scans that are recorded. Hundreds of scans are added together to achieve visible ^{13}C signals. Due to the randomness of the electric noise, its sum is close to zero. The separate signals of ^{13}C are weak, one of the reasons is the isotope of carbon (^{13}C) that produces ^{13}C NMR signals. It makes only 1.11% of carbon in nature and also ^{13}C has a very low magnetogyric ratio of about one-fourth as compared to that of a proton. The most

abundant carbon in nature is ^{12}C, nonetheless, this carbon cannot produce an NMR signal because it does not have a nuclear spin. However, ^{13}C NMR spectroscopy has a chemical shift of carbon atom range of over 220 ppm, in contrast to 10-12 ppm for hydrogens. This enables carbons in different environments to be easily distinguished from one another (Bruice, 2014).

2.3.3 1D DEPT ^{13}C NMR

Distortionless Enhancement by Polarization Transfer (DEPT), is a technique that was created to differentiate between CH_3, CH_2, CH and C group (Clayden et al., 2001). This technique is now usually used in place of proton-coupled ^{13}C spectra to detect the number of hydrogens that are attached to each carbon in a compound (Bruice, 2014).

2.3.4 Two-dimensional NMR

This spectroscopy is used to examine complex molecules like proteins and nucleic acids because the signals in their spectra overlap. ^{1}H NMR and ^{13}C NMR have one frequency axis and one intensity axis, whereas 2D NMR spectra consist of two frequency axes and one intensity axis. The common 2D NMR in natural products are COSY, NOESY, HMQC and HMBC (Tesso, 2005).

2.3.5 2D ^{1}H, ^{1}H-COSY (Correlated Spectroscopy)

Correlated Spectroscopy (COSY) is a 2D NMR spectroscopy that is used in the characterization of small molecules but also of larger biomolecules such as proteins. In COSY spectroscopy, the spectra show coupling between neighbouring protons in a ^{2}J, ^{3}J and ^{4}J range (Tesso, 2005). A ^{1}H NMR spectrum is plotted on both axes (x and y) to determine the correlation. In analyzing the spectrum, a diagonal line is drawn through the dots that intersect the spectrum (Ernst et al., 1987). Cross peaks that are found on the sides of the diagonal line are used to determine proton coupling. The COSY spectrum is symmetrical with regards to the diagonal line (Friebolin and Becconsall, 1993).

2.3.6 HMBC (Heteronuclear Multiple Bond Correlation)

The heteronuclear multiple bond correlation (HMBC) experiment is used to determine the long-range coupling between hydrogen and carbon that are two or three bonds apart. It is used to identify a correlation between ^{1}H and ^{13}C nuclei in different spectra (Clayden

et al., 2001). This experiment is sensitive to the extent that it can detect large coupling constraints, i.e. 4-10 Hz (Tesso, 2005).

2.3.7 HSQC (Heteronuclear Single Quantum Correlation)

The heteronuclear single quantum correlation experiment is used to identify one-bond ^{1}H and ^{13}C or ^{15}N correlations of two different spectra of a single compound (Tesso, 2005). In biochemistry, HSQC is used as a correlating experiment between ^{1}H and ^{15}N chemical shifts. In chemistry, it is mostly ^{1}H,^{13}C correlations that are important. This experiment proved to be useful in elucidating larger molecule (Yuan et al., 2011).

2.3.8 LCMS (Liquid Chromatography-Mass Spectrometry)

Liquid chromatography-mass spectrometry (LC-MS) experiments are a combination of chromatography and mass spectrometry. As all different chromatography methods, LC has a stationary and a mobile phase. The stationary phase is a solid and a mobile phase is a gas. Mass spectrometry is used for the mass measurement. LCMS as a combined method deals with separation and measurement. In this technique, a sample is injected onto a column, eluted with a selected solvent system (Lindsey et al., 2001). The mass of an LC eluent is measured by a MS detector, the commonly used MS detector is a triple quadrupole MS (QQQ). In a MS QQQ a high voltage is applied into an LC eluent which creates a spray of ionized molecules, the ionized molecule is transferred to the first quadrupole where they are separated and selected according to specific ions (Taylor, 2005). In the second quadrupole the specific ion is fragmented into product ions, and the product ions are transferred to the third quadrupole. In the third quadrupole, a product ion is isolated and sent to a detector for measurement. However, in this study, the MS used was a single quadrupole which only deals with separation and selection of a specific ion. The results are displayed as molecular ion adducts (Niessen, 2006).

2.3.9 Time-Of-Flight Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that helps with structural elucidation. This technique measure mass (*m*) to charge (*z*) ratio (*m/z*) of the ions in an analyte or sample (Breitkopf et al., 2017). The ratios that are obtained from the results provide atomic signatures for the different components of the sample. Usually $z = 1$, therefore, the ratio is the mass of the ion. MS gives a mass as an integer (Breitkopf et al., 2017). The disadvantage of this technique is that a molecular mass does not give concrete evidence of the exact mass of a compound. High-resolution mass spectrometry (HRMS),

is used to amend that disadvantage by means of determination of the exact mass of a fragment to a 0.0001 precision by separating the analyte into fragments prior to analysis. This technique gives the molecular weight in five decimal places, and this eliminates 99% of the error, hence making it easy to differentiate between the compounds with a similar molecular mass to the nearest integer (Clayden et al., 2001). Time-of-flight is an HRMS technique that uses time measurement to determine *m/z* of an ion. This technique is used for both qualitative and quantitative analyses. Accurate results can be obtained from the spectral data of this instrument. This technique provides an accurate mass and isotopic reliability (Issaq et al., 2002).

2.3.10 Ultraviolet (UV) spectroscopy

The ultraviolet (UV) spectroscopy gives details of the organic compounds with conjugated double bonds. The part of the molecule that absorbs UV light is called a chromophore. UV light consists of relevant energy that causes an electronic transition in a molecule. In other words, it causes an electron to move from one molecular orbital to a molecular orbital with higher energy (excited state) (Förster, 2004). The molecules that absorb UV light have a UV spectrum that ranges between 180-400 nm, in contrast to visible light which ranges between 400-780 nm. A UV spectrum consists of broad absorption bands due to electronic state that has vibration sublevels. Therefore, the electronic transitions occur within these various sublevels (Bruice, 2014).

2.4 Conclusion

To conclude, though a number of plants have been studied and found to have bioactive compounds, there is still a need to explore the phytochemistry of many other South African indigenous species. No literature is available on the phytochemistry of the genus *Dais.* Exploring the phytochemistry of this genus could possible results in obtaining new bioactive compounds with interesting properties.

Chapter 3: Results and Discussion

3.1 Introduction

As discussed in Chapter 2, many bioactive compounds have been isolated from plants of the Thymelaeaceae family. The compounds are active against several diseases that claim lives in South Africa, i.e. cancer, hypertension, malaria, diabetes and HIV. This plant family is well represented in most provinces in South Africa. The focus of this thesis was to investigate the phytochemistry of a Thymelaeaceae species that has not been studied previously and *Dais cotinifolia* was selected.

D. cotinifolia was collected from the University of Kwazulu-Natal Pietermaritzburg campus (UKZN-PMB) Botanical Garden. The different parts of the plant, i.e. leaves, branches, roots and root bark, were dried and extracted using different solvent systems. The structures of the compounds were elucidated by interpretation of their ^{1}H and ^{13}C NMR, LC-MS, TOF-MS and UV/Vis experimental data and by comparison with literature data. The antioxidant activity of the various crude extracts was determined.

3.1.1. Isolation and structural elucidation of kobusin (3.1)

The crude DCM extract of the leaves was fractionated by VLC. TLC was used to monitor the fractions and one fraction (fraction C) was taken for further fractionation by VLC and preparative centrifugal thin-layer chromatography. A single compound (**3.1**) was isolated and the purity of the compound was assessed by HPLC (Figure 3.1).

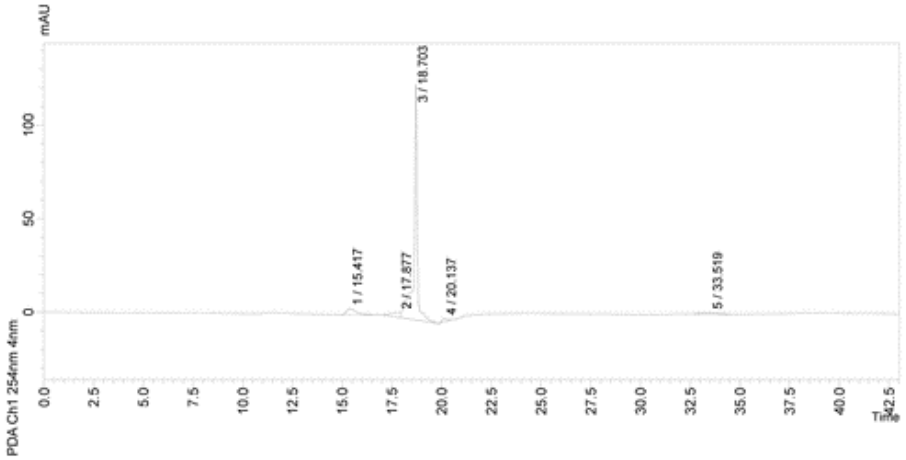


Figure 3.1. HPLC chromatogram of kobusin (**3.1**).

The structural elucidation of **3.1** was based on NMR, MS and UV analysis. In the low-resolution ESI-(+) mass spectrum of **3.1**, a pseudo-molecular ion was observed at *m/z*

393.1 $[M+Na]^+$, which corresponds to a molecular formula of $C_{21}H_{22}O_6$ (calculated for $C_{21}H_{22}O_6Na$, 393.4).

The NMR data of compound **3.1** are collated in Table 3.1. The proton NMR analysis was based on the ^1H NMR (Figure 3.2) and COSY NMR (Figure 3.3) spectra. There was evidence of six aromatic protons that were allocated to two aromatic rings. A careful analysis of the splitting patterns of the aromatic proton signals indicated that there were two 1,3,4-trisubstituted phenyl rings (arbitrarily designated as ring A and ring B). In ring A, a proton signal was observed at δ_H 6.84 (1H, *d*, *J* = 8.3 Hz, H-5) and this proton was *ortho*-coupled to the proton resonating at δ_H 6.87 (1H, *dd*, *J* = 1.8 Hz; 9.9 Hz, H-6). The proton resonating at δ_H 6.87 also has a *meta*-coupling to the proton resonating at δ_H 6.90 (1H, *d*, *J* = 1.7 Hz, H-2). On the other aromatic ring (ring B), the proton resonating at δ_H 6.85 (1H, *d*, *J* = 1.7 Hz, H-2') was *meta*-coupled to a proton resonating at δ_H 6.81 (1H, *dd*, *J* = 1.3 Hz; 8.2 Hz, H-6'). The proton resonating at δ_H 6.81 was *ortho*-coupled to the proton resonating at δ_H 6.78 (1H, *d*, *J* = 8.0 Hz, H-5') and *meta*-coupled to the proton resonating at δ_H 6.85.

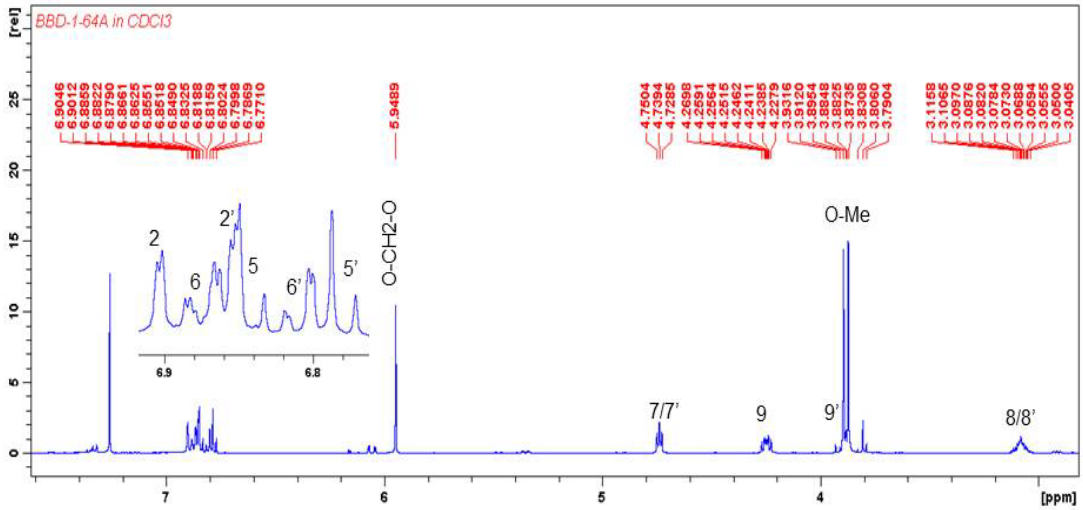


Figure 3.2. ^1H NMR spectrum of kobusin (**3.1**) in $CDCl_3$ at 400 MHz.

At δ_H 5.95, a two-proton singlet resonance was observed and the chemical shift indicated that these protons were attached to a carbon atom bonded to two oxygen atoms. These protons were assigned as a $-OCH_2O-$ (methylenedioxy) group. More upfield, there was an indication of two closely-related sets of protons resonating at δ_H 4.75 (*d*, *J* 5.5 Hz) and 4.73 (*d*, *J* 5.4 Hz) ppm, a two-proton multiplet at δ_H 4.26 ppm, a 2-proton multiplet at δ_H 3.87 ppm and a two-proton multiplet resonating at δ_H 3.12 ppm. In the COSY spectrum (Figure 3.3), a correlation was observed between the proton resonance at δ_H 4.74 and the resonance at δ_H 3.12 ppm. The latter resonance also showed a correlation with both the resonances at δ_H 4.26 ppm and δ_H 3.87. These two resonances also

showed a correlation with each other. The remaining two signals in the ^{1}H NMR spectrum were two three-proton singlets resonating at δ_H 3.90 and δ_H 3.87, consistent with two methoxy groups attached to aromatic rings.

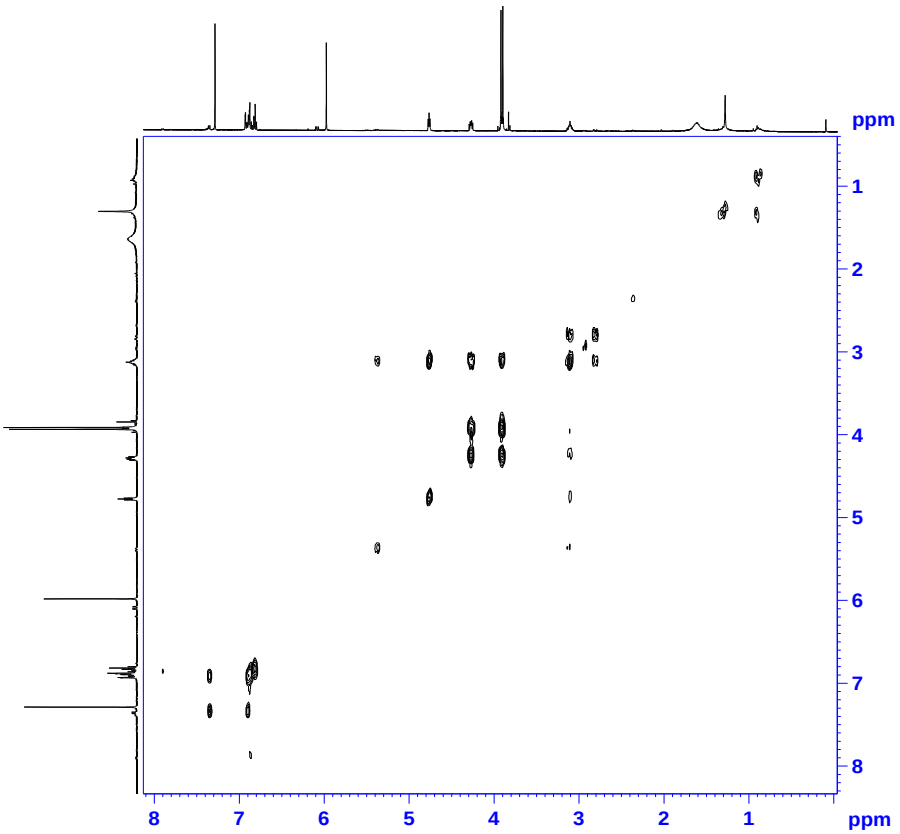


Figure 3.3. COSY NMR spectrum of kobusin (**3.1**) in $CDCl_3$.

In the ^{13}C NMR spectrum (Figure 3.4), there were 21 signals indicating that there were 21 carbons in different chemical environments. A DEPT spectrum (Figure 3.5) was used to differentiate between methyl, methylene, methine and quaternary carbon atom resonances. The HSQC spectrum (Figure 3.6) enabled us to establish one-bond correlations between proton and carbon atoms, whereas the HMBC spectrum (Figure 3.7) allowed us to connect different fragments.

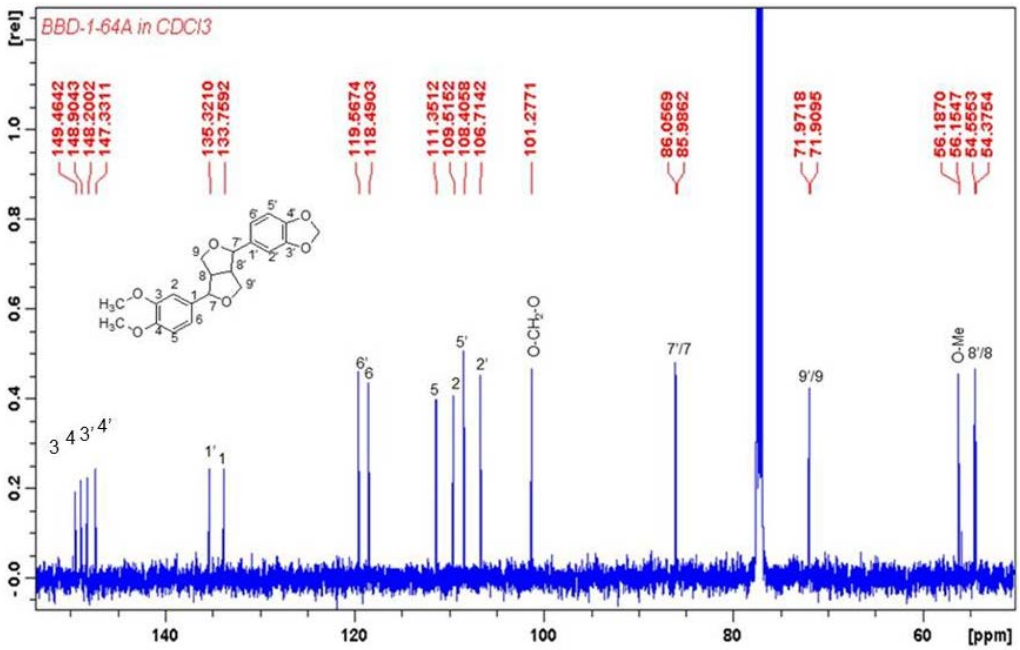


Figure 3.4. ^{13}C NMR spectrum of kobusin (**3.1**) in $CDCl_3$ at 100 MHz.

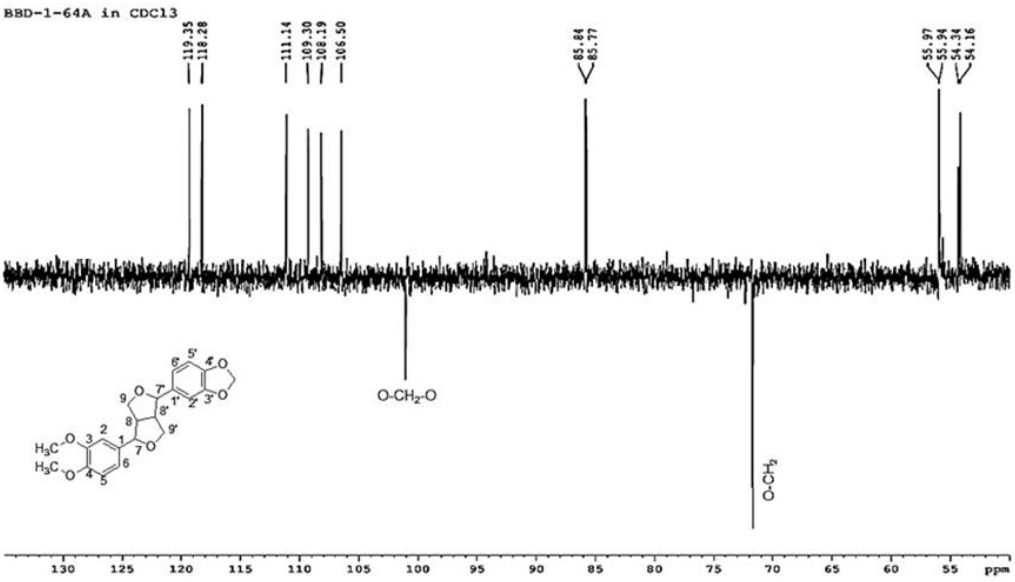


Figure 3.5. DEPT 135 NMR spectrum of kobusin (**3.1**) in $CDCl_3$ at 100 MHz.

In the aromatic region, six quaternary carbons resonated between δ_C 133 and 149 ppm. Among the six quaternary carbons, four were more downfield, indicating that they were directly bonded to oxygen atoms. These four resonances were at δ_C 147.3, 148.2, 148.9 and 149.5 ppm, while the other two quaternary carbons resonated at 133.8 and 135.3 ppm. Six additional aromatic carbon signals ranging from δ_C 106.7 to 119.6 ppm were identified as methine carbons. In the HSQC spectrum, it was observed that the three proton resonances on aromatic ring A, δ_H 6.84 (*s*, *J* = 8.3 Hz, H-5), δ_H 6.87 (*dd*, *J* = 1.8 Hz; 9.9 Hz, H-6) and δ_H 6.90 (*d*, *J* = 1.7 Hz, H-2), correlated to the carbon resonances

at δ_C 109.5, 118.5 and 111.4, respectively. The three B-ring proton resonances at δ_H 6.85 (*d*, *J* = 1.7 Hz, H-2'), δ_H 6.81 (*dd*, *J* = 1.3 Hz; 8.2 Hz, H-6'), and δ_H 6.78 (*d*, *J* = 8.0 Hz, H-5') correlated to the carbon resonances at δ_C 106.7, 119.6, and 108.4, respectively.

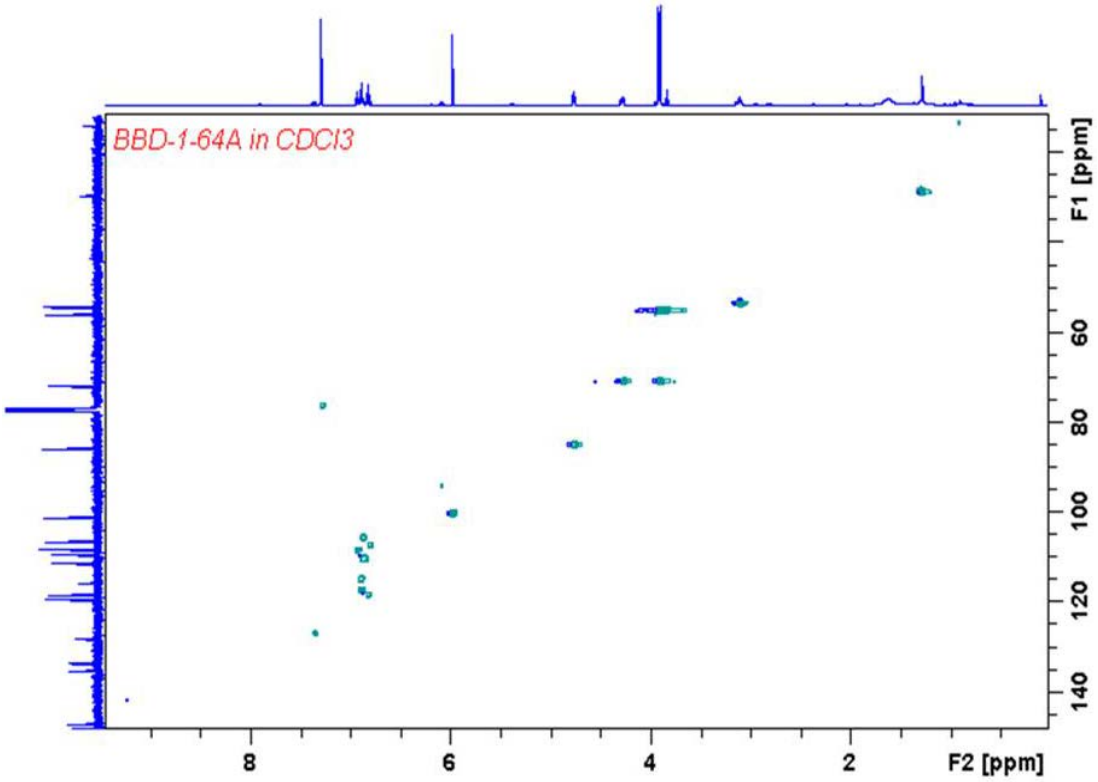


Figure 3.6. HSQC NMR spectrum of kobusin (**3.1**) in $CDCl_3$.

In the HMBC spectrum (Figure 3.7), a correlation between the proton resonance at δ_H 6.84 (*J* = 8.3 Hz, H-5, ring A) and the carbon resonance at δ_C 149.5, and the proton resonance at δ_H 6.90 (H-2, ring A) and the carbon resonance at δ_C 149.5, were observed. Therefore, the signals at δ_C 149.5 and δ_C 149.5 were assigned to C-3 and C-4, respectively, of ring A. In the HMBC spectrum, a correlation between the signals at δ_C 149.5 and δ_C 148.9 and the methoxy proton resonances at δ_H 3.90 and δ_H 3.87, respectively, were observed. The methoxy proton resonances at δ_H 3.87 and 3.90 correlated to the carbon resonances at δ_C 56.2 and 56.1, respectively, in the HSQC spectrum (Figure 3.8). The correlations discussed above enabled us to assign a part structure to ring A (Figure 3.8).

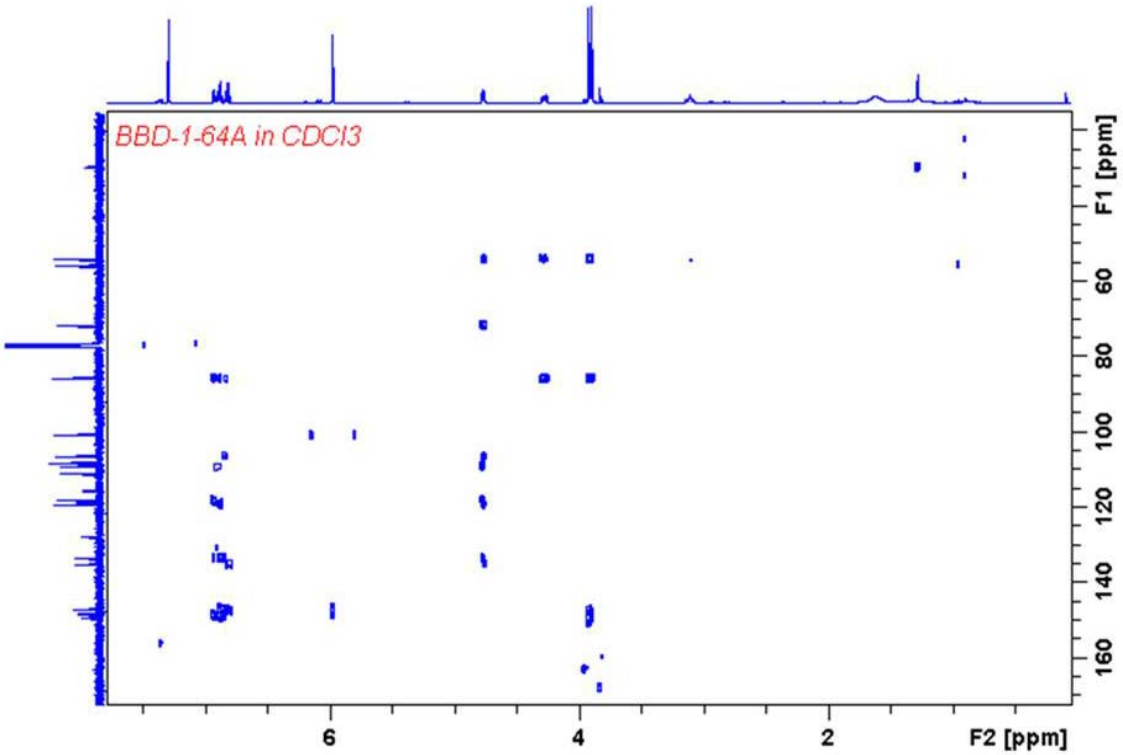


Figure 3.7. HMBC NMR spectrum of kobusin (**3.1**) in $CDCl_3$.

A methylene carbon resonating at δ_C 101.3 was observed in the DEPT spectrum (Figure 3.5) and in the HSQC spectrum (Figure 3.6) and this carbon resonance correlated to the proton resonance at δ_H 5.95, confirming the presence of a $–OCH_2O-$ fragment. In the HMBC spectrum (Figure 3.7), the methylene proton resonance (δ_H 5.95) correlated to the two B-ring carbon resonances at δ_C 148.2 and 147.3. These two resonances also correlated to proton resonances at δ_H 6.81 (H-5') and δ_H 6.85 (H-2'), respectively, and was assigned to C-3' and C-4', respectively. These correlations allowed us to construct ring B as indicated in Figure 3.8.

Ring A Ring B

Figure 3.8. Part-structures proposed for ring A and ring B of **3.1**.

The remaining signals in the ^{13}C NMR spectrum consisted of three pairs of resonances at δ_C 86.1/86.0 (oxymethine), δ_C 72.0/71.9 (oxymethylene) and δ_C 54.6/54.4 (methine carbon). These carbons correlated to the proton resonances at δ_H 4.75/4.73 ppm, the two-proton multiplet at δ_H 4.26 ppm and the two-proton multiplet at δ_H 3.87 ppm, and the two-proton multiplet resonating at δ_H 3.12 ppm, respectively. This observation allowed us to propose the presence of two $-OCH_2-CH-CHO-$ fragments in compound **3.1**. By

combining these two fragments with ring A and ring B, a furofuran lignan structure was proposed for **3.1**, with the signals at δ_H 4.75/4.73 ppm assigned to H-7/7', δ_H 3.12 ppm to H-8/8', and signals at δ_H 4.26 ppm and δ_H 3.87 ppm to the two diastereotopic protons at C-9/9'.

There are 4 stereocentres in the proposed furofuran lignan; therefore, there are eight possible diastereomers. Geometric constraints restrict the ring junction between the two five-membered rings as *cis* and only four possible diastereomers remain, the 7-H/8-H *trans*, 7'-H,8'-H *trans* isomer (A), the 7-H/8-H *trans*, 7'-H,8'-H *cis* isomer (B), the 7-H/8-H *cis*, 7'-H,8'-H *trans* isomer (C), and the 7-H/8-H *cis*, 7'-H,8'-H *cis* isomer (D) (Figure 3.9).

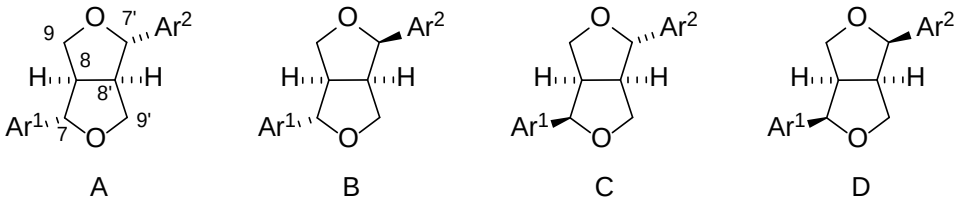


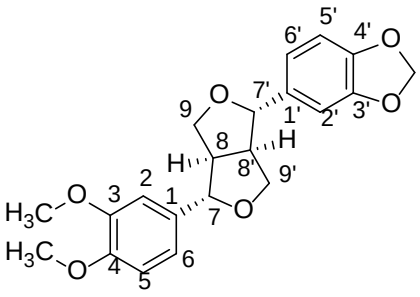
Figure 3.9. Possible diasteromers for furofuran lignans.

The determination of the relative configuration of the furofuran lignans is not always easy to determine by NMR due to conformational changes that can occur with different substituents on C-7 and C-7'. Takahashi and Nakagawa (1966) reported that a small coupling constant ($J \approx 4$ Hz) between 7-H/8-H or 7′-H/8′-H corresponds to a *trans* configuration and a large coupling constant ($J \approx 7$ Hz) corresponds to a *cis* configuration. Kamikado et al. (1975) reported that for the symmetric diastereomer A (Figure 3.9), the chemicals shift of H-7, 8 and 9 are almost the same as those of H-7', 8' and 9', whereas for the asymmetric diastereomer C, substantial chemical shift differences for these protons were observed. By considering the NMR data of a number of furofuran lignans, it was proposed that the relative configuration of these compounds could be derived by considering the chemical shift differences between the two diastereotopic protons on C-9 and C-9' (Xu et al., 2018). These authors proposed that for type A (Figure 3.9), ΔδH-9 and ΔδH-9′ = 0.30−0.40, for type B and type C ΔδH-9 = 0.25−0.36, ΔδH-9′ > 0.50, and for type D ΔδH-9 and ΔδH-9′ < 0.20.

Considering the discussion above, **3.1** was identified as a class A lignan (Figure 3.9). The small differences in chemical shifts between H-7 and H-7′, H-8 and H-8′, and H-9 and H-9′, indicated that the furofuran moiety is symmetrical and that class B and class C could be eliminated. The coupling constant between H-7 and H-8 and also between H-7' and H-8' in **3.1** are both $J \sim 5.5$ Hz, which is in agreement with a *trans* relationship between the protons. Finally, the chemical shift difference between the two

diastereotopic C-9 protons is δH = 4.26 – 3.87 = 0.39, which is characteristic of a 7-H/8-H *trans*, 7'-H,8'-H *trans* isomer (Xu et al., 2018).

Based on the NMR data discussed above, the structure of the isolated compound **3.1** was identified as kobusin (Figure 3.10), a lignan that was first reported by Kamikado et al. (1975), who isolated the compound from *Magnolia kobus* DC. The NMR data of kobusin (**3.1**) is in agreement with the published data by Kamikado et al., 1975.



Kobusin (**3.1**)

Figure 3.10. Structure of kobusin (**3.1**).

The UV/Vis spectrum of compound **3.1** showed two absorption maxima at λ_{max} = 230 nm and λ_{max} = 285 nm (Figure 3.11). This spectrum is in agreement with the chromophore of the proposed structure, i.e. two benzene rings.

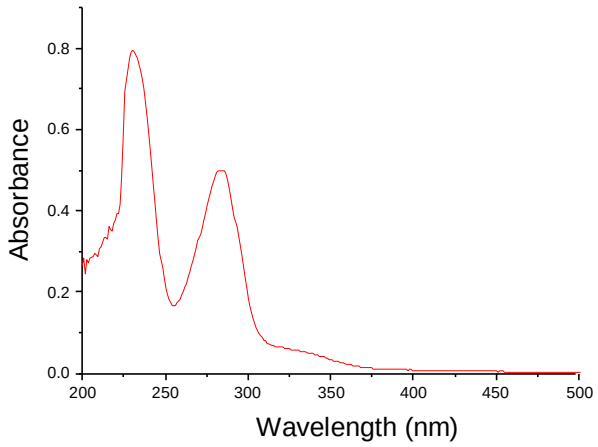


Figure 3.11. The UV/Vis spectrum of kobusin (**3.1**).

As stated already above, the furofuran lignan kobusin (**3.1**) was first isolated from the methanol crude extract of the leaves and seeds of *Magnolia kobus* DC. and this lignan was found to be a growth inhibitor of silkworm larvae (Iida et al., 1982, Kamikado et al., 1975). The same lignan was also isolated from the plant *Melicope hayesii* T.G.Hartley in 1999 (Latip et al., 1999).

Table 3.1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of kobusin (**3.1**) in $CDCl_3$.

Position	**δ_C (ppm)**	**δ_H (ppm)**	**δ_H (ppm) (Kamikado et al., 1975)**	**δ_C (Latip et al., 1999)**
1 1’	133.8 135.3	-		135.7
2 2’	109.5 106.7	6.90 (1H, d, *J* = 1.7 Hz) 6.85 (1H, d, *J* = 1.7 Hz)	*	106.4
3 3’	148.9 147.3	-		147.8
4 4’	149.5 148.2	-		148.8
5 5’	111.4 108.4	6.84 (1H, d, *J* = 8.3 Hz) 6.78 (1H, d, *J* = 8.0 Hz)	*	109.3
6 6’	118.5 119.6	6.87(1H, dd, *J* = 1.8 Hz; 9.9 Hz) 6.81 (1H, dd, *J* = 1.3 Hz; 8.2 Hz)	*	119.3
7 7’	86.0 86.1	4.74 (2H, d, *J* = 5.5 Hz)	4.75 (2H, d, *J* = 4)	86.6
8 8’	54.4 54.6	3.08 (2H, m)		54.9
9 9’	71.9 72.0	4.26 (1H, m) 3.87 (1H, m)	4.16 – 4.40 (2H, m)	71.9
O-Me	56.2	3.87 (3H, s)	3.87 (3H, s)	56.1
O-Me	56.1	3.90 (3H, s)	3.90 (3H, s)	
O-CH_2-O	101.3	5.96 (2H, s)	5.95 (2H, s)	101.2

*Given as δ_H 6.8-7.8 (6H, Ar-H) by Kamikado et al. (1975).

3.1.2 Isolation and structural elucidation of eudesmin (3.2)

The crude MeOH extract of the leaves was fractionated by VLC. TLC was used to monitor the fractions and fraction C was further fractionated by preparative centrifugal thin-layer chromatography. The purity of a single compound that was isolated was assessed by HPLC (Figure 3.12).

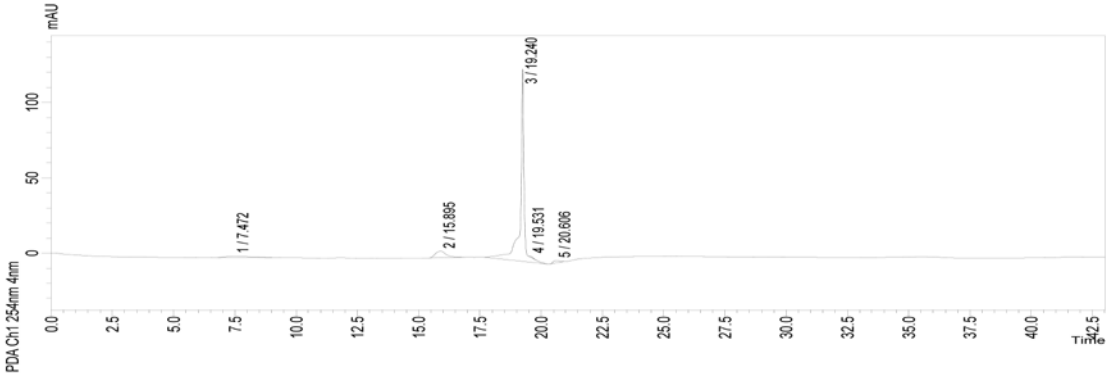


Figure 3.12. A HPLC chromatogram of eudesmin (**3.2**).

The structural elucidation of **3.2** was performed on the basis of NMR, MS and UV analysis. The low-resolution ESI-(+)-mass spectrum of **3.2**, a pseudo-molecular ion was observed at *m/z* 409.3 $[M+Na]^+$, which corresponds to a molecular formula of $C_{22}H_{26}O_6$ (calculated for $C_{22}H_{26}O_6Na$, 409.4).

The NMR spectroscopic data of compound **3.2** are summarised in Table 3.2. Both the ^1H and ^{13}C NMR spectra of **3.2** were much simpler than those of **3.1**. In the ^1H NMR spectrum (Figure 3.13) nine different signals, including two methoxy resonances were observed, whereas in the ^{13}C NMR spectrum (Figure 3.15), eleven carbon resonances could be observed. Taking the molecular mass into account, it was clear that **3.2** was a symmetrical molecule.

The proton environments of the proposed structure were determined using ^1H NMR (Figure 3.13) and COSY NMR (Figure 3.14) spectra. Since **3.2** was identified as a symmetrical molecule, only one half of the molecule will be discussed. There was an indication of 3 aromatic protons that were attributed to an aromatic ring. The splitting patterns indicated that this was a 1,3,4-trisubstituted phenyl ring. A proton that resonated at δ_H 6.91 (*d*, *J* = 2.3 Hz) was observed, and this proton was *meta*-coupled to the proton resonating at δ_H 6.87. A proton resonating at δ_H 6.85 (*d*, *J* = 2.35 Hz) was observed, and this proton was *ortho*-coupled to the proton resonating at δ_H 6.87 (2H, *d*, *J* = 2.3 Hz; 7.0 Hz).

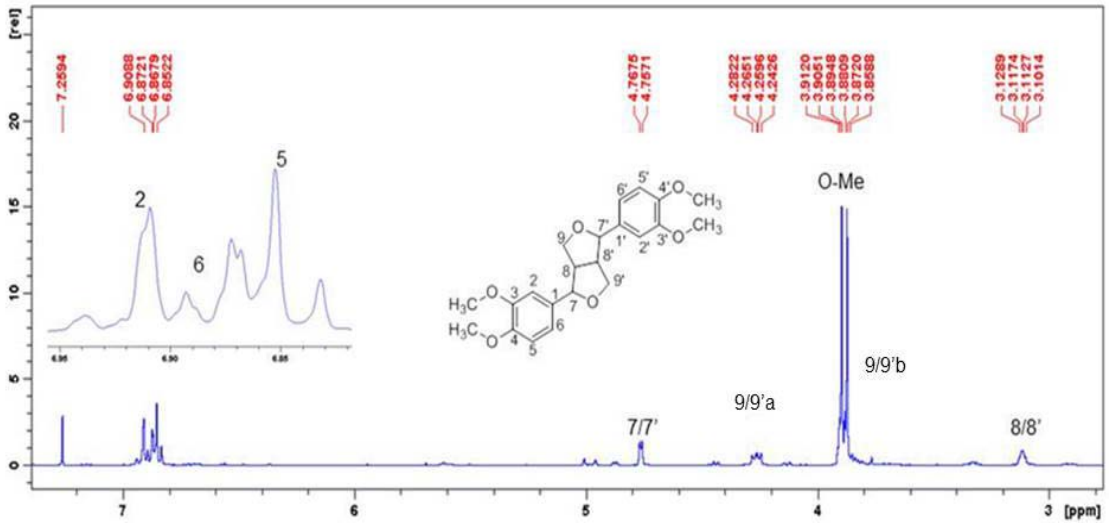


Figure 3.13. ^{1}H NMR spectrum of eudesmin (**3.2**) in $CDCl_3$ at 400 MHz.

At δ_H 4.76 a one-proton doublet resonance was observed, and the chemical shift indicated that this proton was attached to a carbon bonded to an oxygen. The proton was assigned as a -OCHPh- (oxymethine) group. A proton resonating at δ_H 4.29 was split by a proton resonating at δ_H 3.89. This suggested the presence of a methylene group and the chemical shift indicated that the protons were attached to a carbon bonded to an oxygen. There were two singlets resonating at δ_H 3.89 and δ_H 3.87, consistent with methoxy groups attached to an aromatic ring. A one-proton multiplet resonance at δ_H 3.12 was observed and this proton was assigned as a -CH- (methine) group.

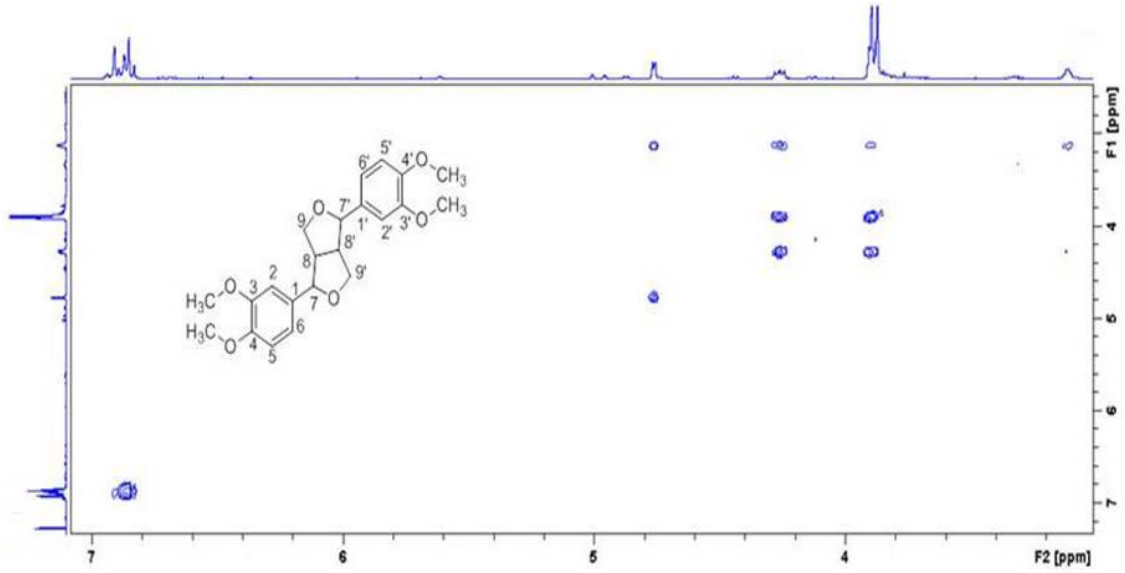


Figure 3.14. COSY NMR spectrum of eudesmin (**3.2**) in $CDCl_3$.

The ^{13}C NMR spectrum (Figure 3.15) showed signals equivalent to 11 carbons and this was due to the plane of symmetry in the proposed structure. A DEPT spectrum (Figure 3.16) was used to differentiate between methyl, methylene, methine and quaternary carbon atom resonances. The HSQC spectrum (Figure 3.17) enabled us to establish one-bond correlations between proton and carbon atoms, whereas the HMBC spectrum (Figure 3.18) allowed us to connect different fragments.

There were six aromatic carbon resonances between δ_C 109.5-149.5. Three of these carbon resonances were attributed to quaternary carbons. The chemical shifts of the quaternary carbon resonances at δ_C 148.9 and 149.5 indicated that they were directly bonded to oxygen and the carbon at δ_C 133.8 was C-C bonded. In the DEPT NMR and HSQC NMR, three other aromatic carbons ranging between δ_C 109.5-118.5 ppm were identified as methine carbon signals. The rest of the signals were observed at δ_C 86.0 (oxymethine), 71.9 (oxymethylene) and 54.4 (methine). The last two signals at δ_C 56.1 and 56.2 were assigned as two methoxy carbons.

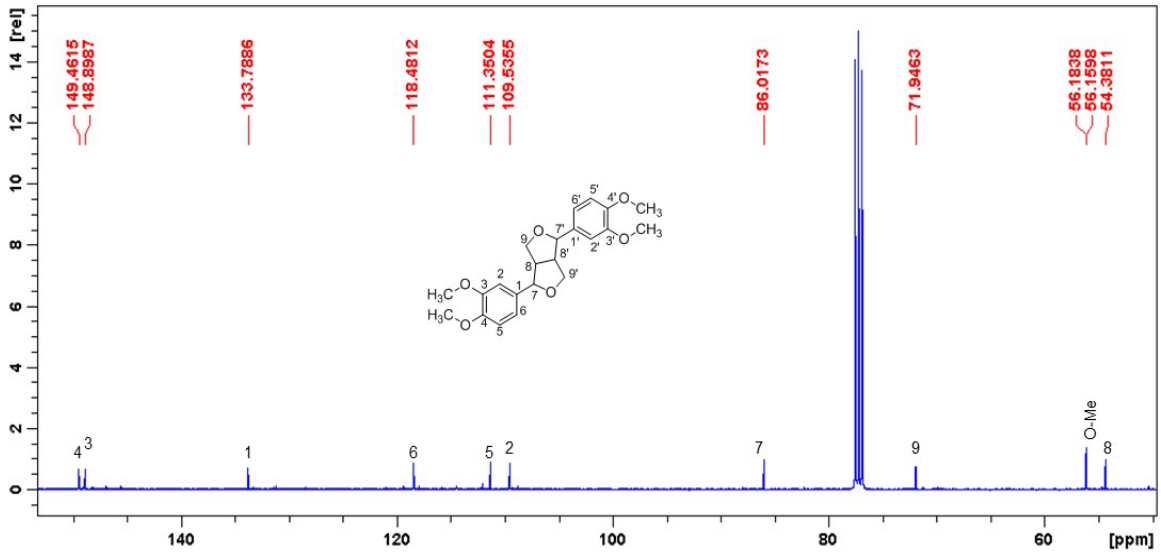


Figure 3.15. ^{13}C NMR spectrum of eudesmin (**3.2**) in $CDCl_3$ at 100 MHz.

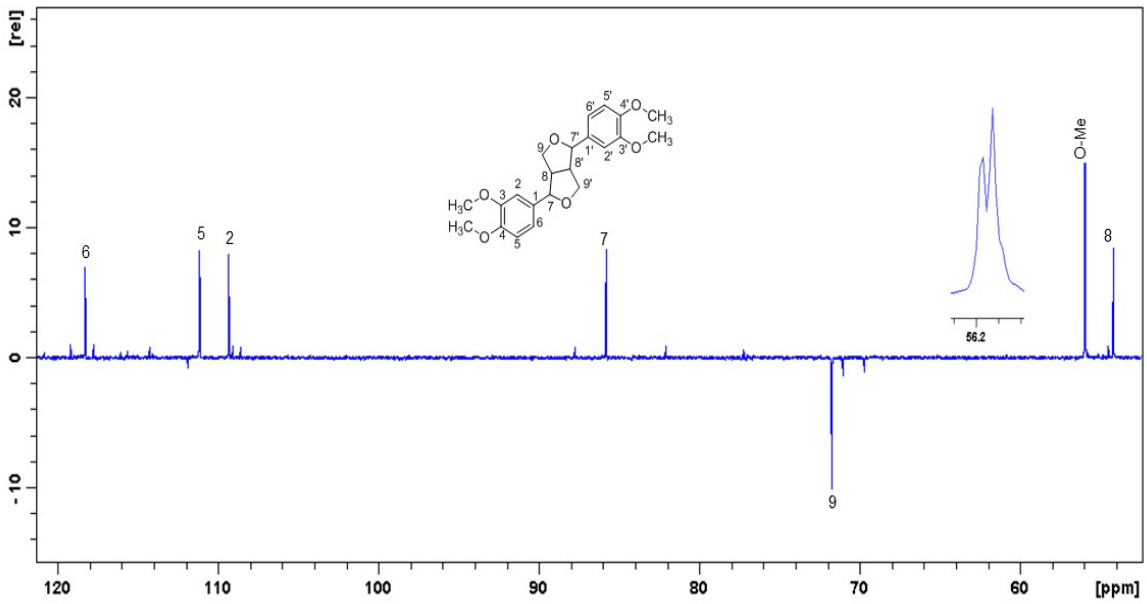


Figure 3.16. DEPT 135 NMR spectrum of eudesmin (**3.2**) in $CDCl_3$ at 100 MHz.

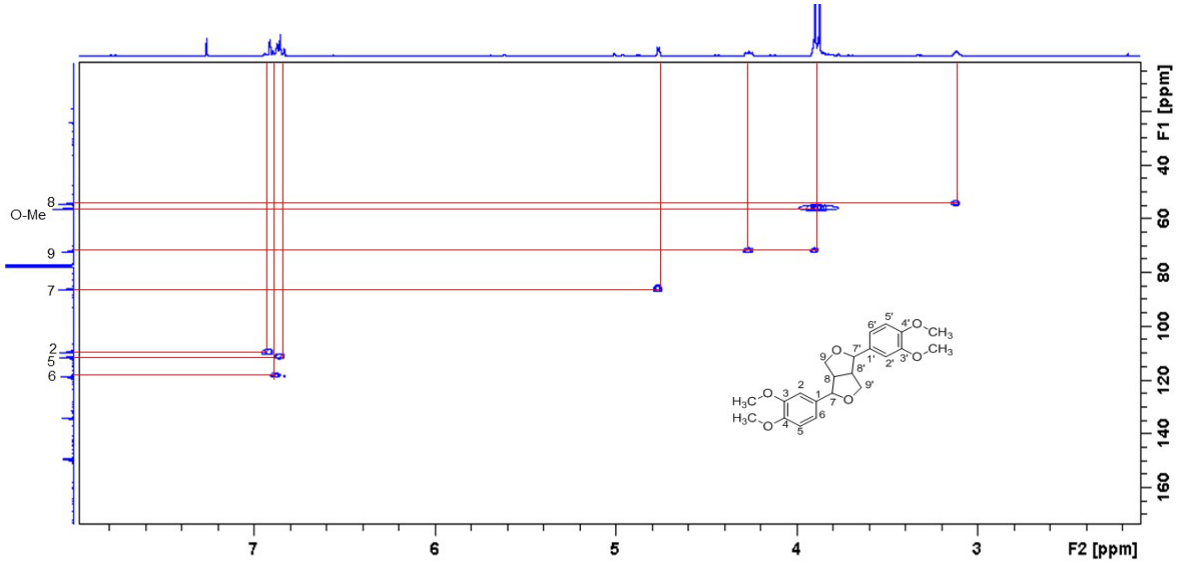


Figure 3.17. HSQC NMR spectrum of eudesmin (**3.2**) in $CDCl_3$.

The HSQC spectrum (Fig 3.15) confirmed that the proton resonating at δ_H 4.26 was attached to the same carbon as the proton at 3.89, confirming that this was an oxymethylene group.

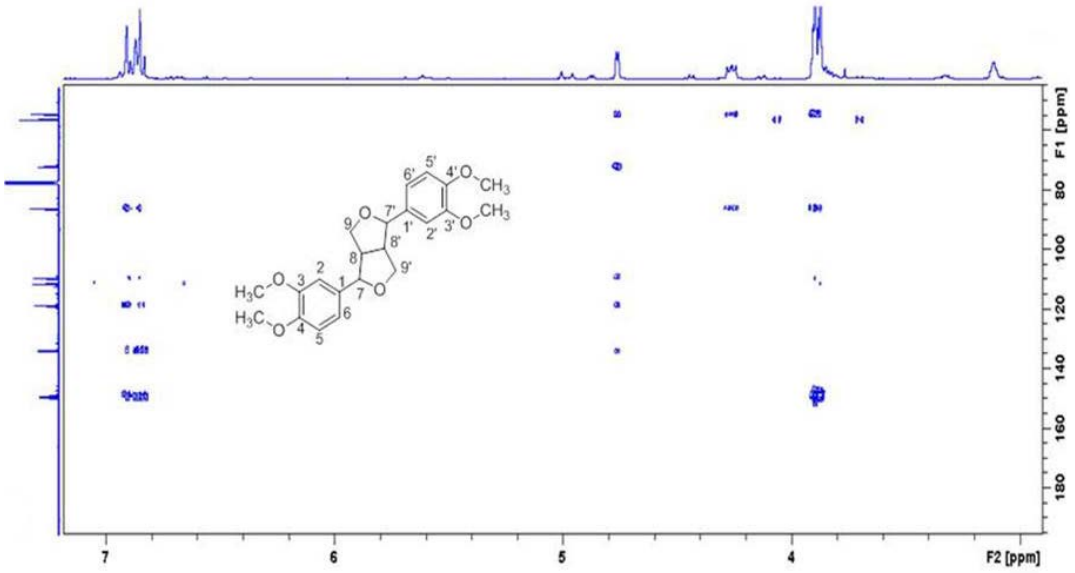
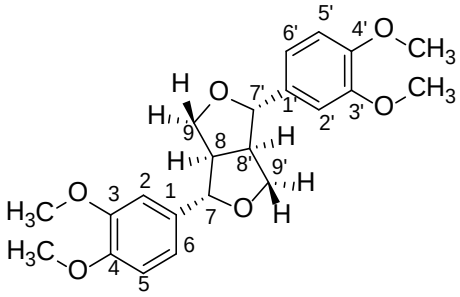


Figure 3.18. HMBC NMR spectrum of eudesmin (**3.2**) in $CDCl_3$.

The HMBC spectrum (Figure 3.18) showed a ^3J correlation between δ_C 148.7 and the methoxy proton protons. A ^3J connectivity was between a proton resonating at δ_H 4.76 and carbons resonating at δ_C 85.8, δ_C 133.6 and δ_C 109.3. This confirms that in the proposed structure, a furofuran ring system is connected to a benzene ring. This was further confirmed by the ^2J correlation of a proton resonating at δ_H 4.76 and carbons resonating at δ_C 85.8 and δ_C 133.6. A ^2J connectivity was observed between the carbon resonating at δ_C 54.7 and the proton resonating at δ_H 4.76 and also the proton resonating at δ_H 4.27. Similar to compound **3.1**, compound **3.2** was a lignan with signals at δ_H 4.73 ppm assigned to H-7/7', δ_H 3.12 to H-8/8', and signals at δ_H 4.25 and 3.85 attributed to the two diastereotopic protons at H-9/9'.

The structure of the isolated compound was assigned as eudesmin (**3.2**) (Figure 3.19). The isolation and NMR structural elucidation of eudesmin were first reported in 1975 when it was isolated from *Magnolia kobus* DC (Kamikado et al., 1975). The isolation of this compound from *D. cotinifolia* has not been reported previously. The NMR data was consistent with data reported previously (Yang et al., 2006). Kobusin (**3.1**) and eudesmin (**3.2**) have the same relative stereochemistry (Yang et al., 2006).



Compound (**3.2**)

Figure 3.19. Structure of eudesmin (**3.2**).

The UV/Vis spectrum of compound **3.2** showed two absorption wavelengths (λ_{max} = 230 nm and λ_{max} = 280 nm) (Figure 3.20). The chromophore of the proposed structure was the benzene ring.

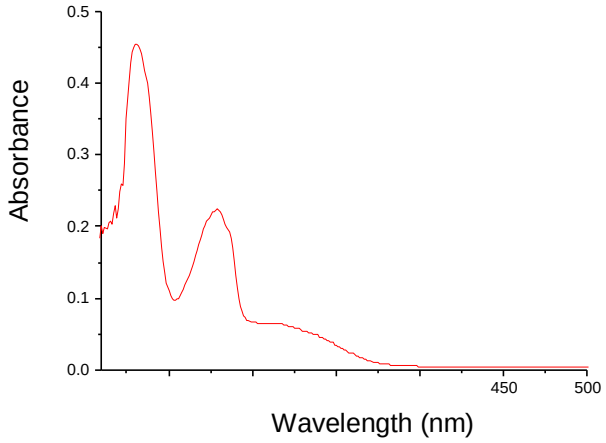


Figure 3.20. The UV/Vis spectrum of eudesmin (**3.2**).

Similar to kobusin (**3.1**), eudesmin (**3.2**) was first isolated from the methanol crude extract of *Magnolia kobus* as a growth inhibitor of silkworm larvae. They were also both isolated from *Melicope hayesii* (Latip et al., 1999). The furofuran lignans are known to possess a number of biological activities, including anti-inflammatory, cytotoxic and antioxidant activity (Xu et al., 2018). The antioxidant free radical scavenging activity enables these lignans to prevent cancer. There have been a number of studies that have been conducted to investigate the anticancer activity of lignans, and the literature states that lignans have a protective effect against cancer, hormone-related cancers in particular, i.e. breast cancer (Thompson, 1998).

Table 3.2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of eudesmin (**3.2**) in $CDCl_3$.

H and C positions	δ_C	δ_H	δ_H (Yang et al., 2006)	δ_C (Yang et al., 2006)
1/1’	133.6	-		134.8
2/2’	109.3	6.91 (2H, d, *J* = 1.6 Hz)	7.02 (2H, d, *J* = 1.0 Hz)	110.5
3/3’	148.7	-		149.9
4/4’	149.3	-		149.2
5/5’	111.2	6.85 (2H, d, *J* = 8.2 Hz)	6.92 (2H, d, *J* = 8.5 Hz)	112.1
6/6’	118.3	6.87 (2H, dd, *J* = 8.2, 1.6 Hz)	6.94 (2H, *dd, J* = 2.0, 6.0 Hz)	118.5
7/7’	85.8	4.76 (2H, d, *J* = 4.2 Hz)	4.73 (2H, d, *J* = 4.5 Hz)	85.9
8/8’	54.2	3.12 (2H, m)	3.10-3.14 (2H, m, H-8, H-8')	54.6
9/9’a 9/9’b	71.7	4.27 (1H, dd, J = 9.0, 6.8 Hz) 3.89 (1H, m)	4.25 (2H, *dd, J* = 5.5, 8.0 Hz) 3.85 (2H, *dd, J* = 4.0, 11.0 Hz)	71.7
O-Me	56.0	3.87 (3H, s) 3.89 (3H, s)	3.82 (6H, s, 4- and 4') 3.84 (6H, s, 3- and 3')	55.6

3.1.3 Isolation and structural elucidation of catechin (3.3)

The crude MeOH extract of the root bark was fractionated by VLC. TLC was used to monitor the fractions. Fraction E was further purified by column chromatography and preparative TLC.

The structural elucidation of **3.3** was based on NMR, MS and LC-MS analysis. In the low-resolution ESI-(+)-mass spectrum of **3.3** obtained from a LC-MS run (Figure 3.21), a molecular ion was observed at *m/z* 291 $[M+Na]^+$, which corresponds to a molecular formula of $C_{15}H_{13}O_6$ (calculated for $C_{15}H_{13}O_6Na$, 291).

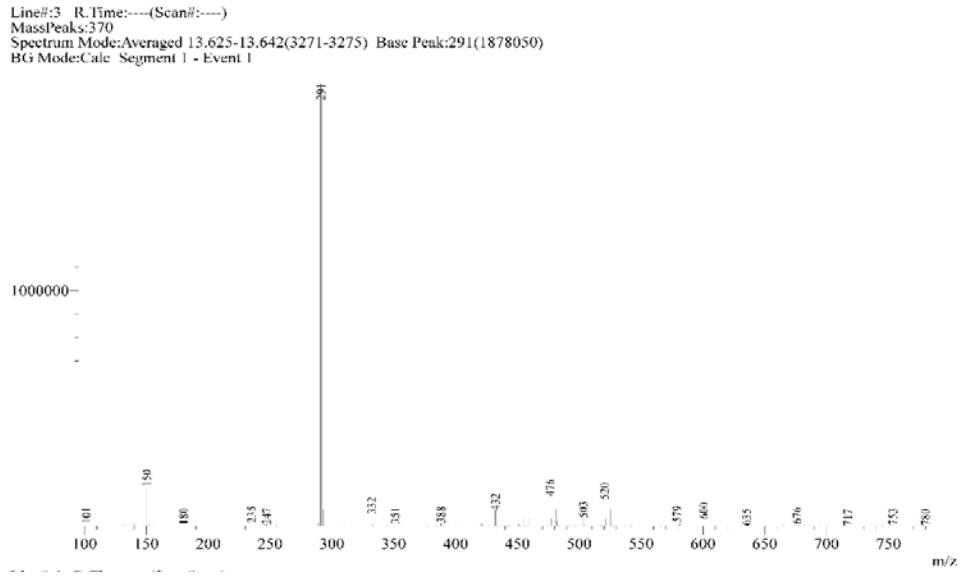


Figure 3.21. The LC-MS spectrum of catechin (**3.3**).

The NMR data of **3.3** are collated in Table 3.3. The proton NMR analysis was based on ^{1}H NMR (Figure 3.22) and COSY NMR (Figure 3.23) spectra. There was evidence of 5 aromatic protons that were allocated to two aromatic rings. A careful analysis of the splitting patterns of the aromatic protons revealed that one ring was a 1,2,3,5-tetrasubstituted phenyl ring (benzopyran ring system) and the second one was a 1,3,4-trisubstituted phenyl ring. A proton resonance was observed at δ_H 5.93 (1H, *d*, *J* = 2.3 Hz). This proton was *meta*-coupled to the proton resonating at δ_H 5.86 (1H, *d*, *J* = 2.3 Hz). A proton resonance was observed at δ_H 6.72 (1H, *dd*, 1.9, 8.2 Hz) and this proton was *meta*-coupled to the proton resonating at δ_H 6.84 (1H, *d*, *J* = 1.9 Hz) and *ortho*-coupled to the proton resonating at δ_H 6.77 (1H, *d*, *J* = 8.1 Hz). A one-proton signal resonating at δ_H 4.57 was observed and the chemical shift indicated that the proton was attached to both an oxygen and a phenyl group and was assigned as a -CH- (methine) group. A one-proton multiplet resonating at δ_H 3.98 was observed, the chemical shift indicated that the proton was attached to a carbon bonded to a hydroxy group and was assigned as a -HCOH- (hydroxymethylene). At δ_H 2.85 a one-proton doublet of doublets (*dd*) was observed and this proton was split by a one-proton signal resonating at δ_H 2.51. The proton was assigned as a -CH_2- (methylene) group.

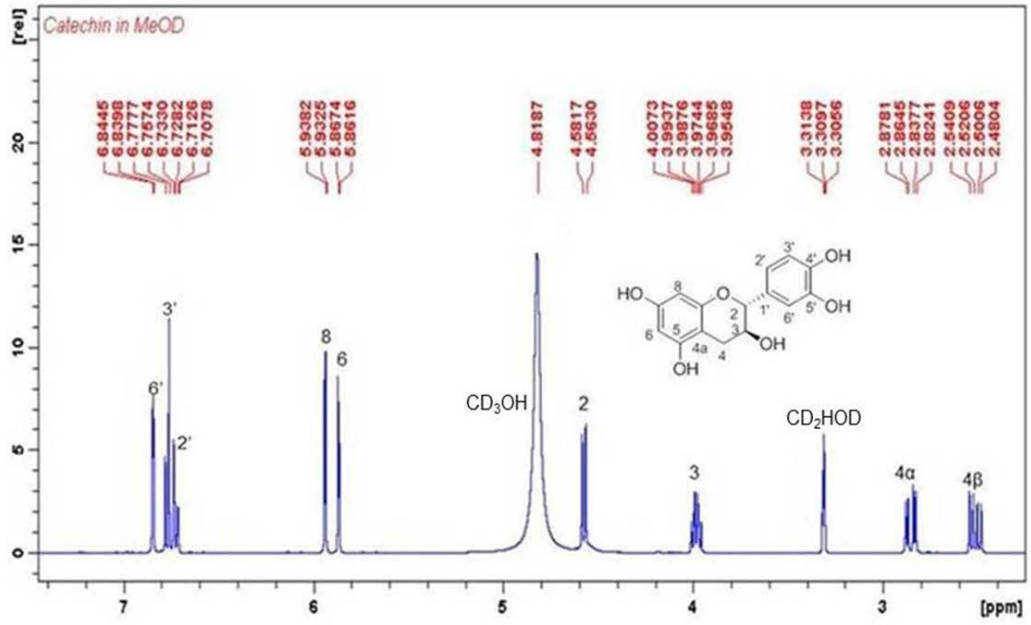


Figure 3.22. ^{1}H NMR spectrum catechin (**3.3**) in CD_3OD at 400 MHz.

A COSY correlation confirmed that the protons resonating at δ_H 2.85 and δ_H 2.51 were attached to the same carbon and that the multiplicity of these protons was a *dd*. A proton resonating at δ_H 4.57 showed a correlation to a proton resonating at δ_H 3.98, and was assigned as a doublet. A proton resonation at δ_H 3.98 had a correlation with the proton resonating at δ_H 2.85 and δ_H 2.51 and was assigned as a multiplet.

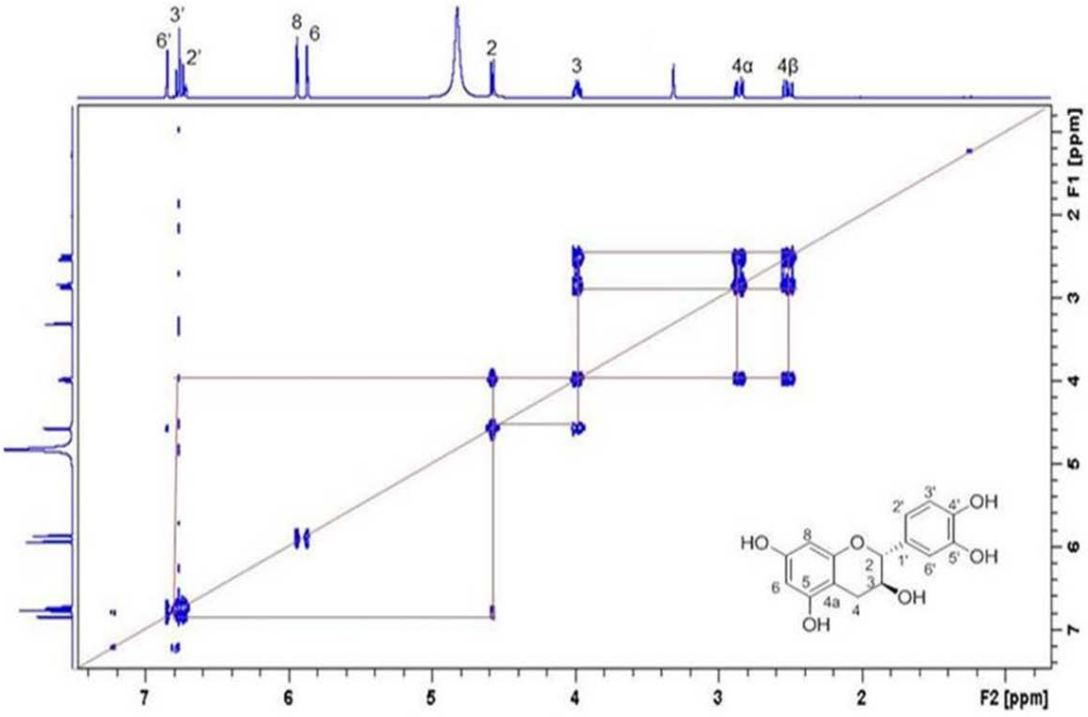


Figure 3.23. COSY NMR spectrum of catechin (**3.3**).

In the ^{13}C NMR spectrum (Figure 3.24), fifteen carbon signals were observed. There were twelve aromatic carbons resonating at δ_C 95.7-158.0 ppm and seven of these were quaternary carbons with five carbons directly bonded to oxygen. The five C-O carbons resonated at δ_C 158.0, 157.7, 157.0, 146.4 and 146.3 ppm. The other two quaternary carbons resonated at δ_C 132.4 and 101.0 ppm. In the DEPT NMR (Figure 3.25) and HSQC NMR (Figure 3.26) spectra, the other aromatic carbons were identified as methine carbons. The rest of the signals were observed at δ_C 83.0 (methine), 68.9 (hydroxymethylene) and 28.6 (methylene).

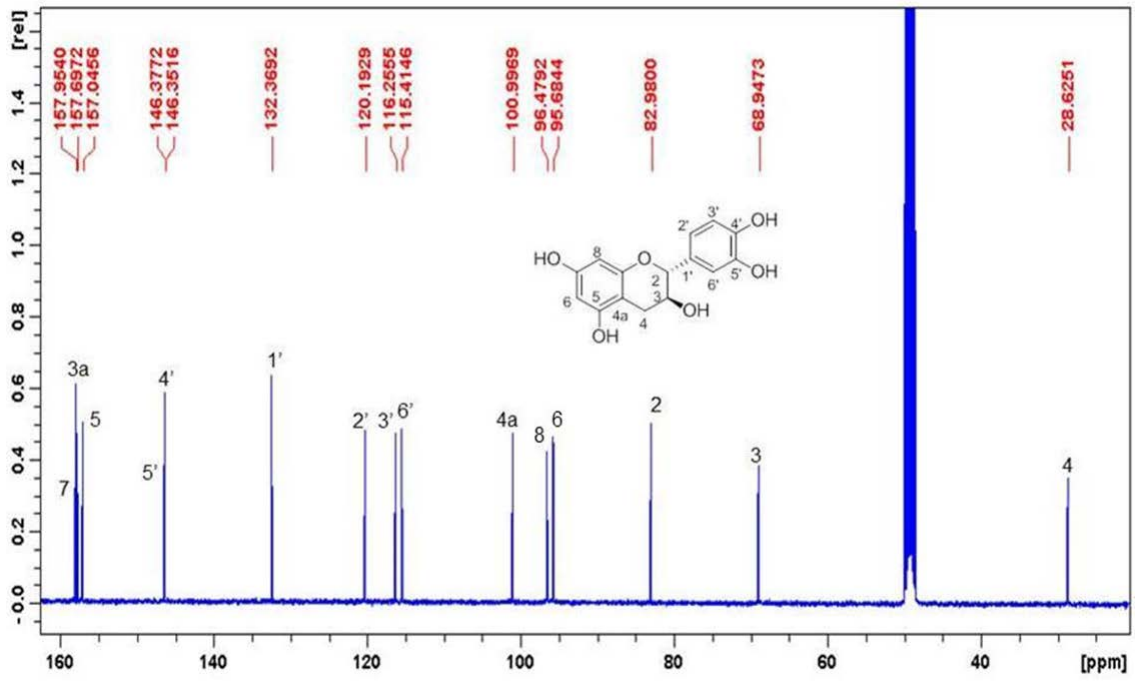


Figure 3.24. ^{13}C NMR spectrum of catechin (**3.3**) in CD_3OD at 100 MHz.

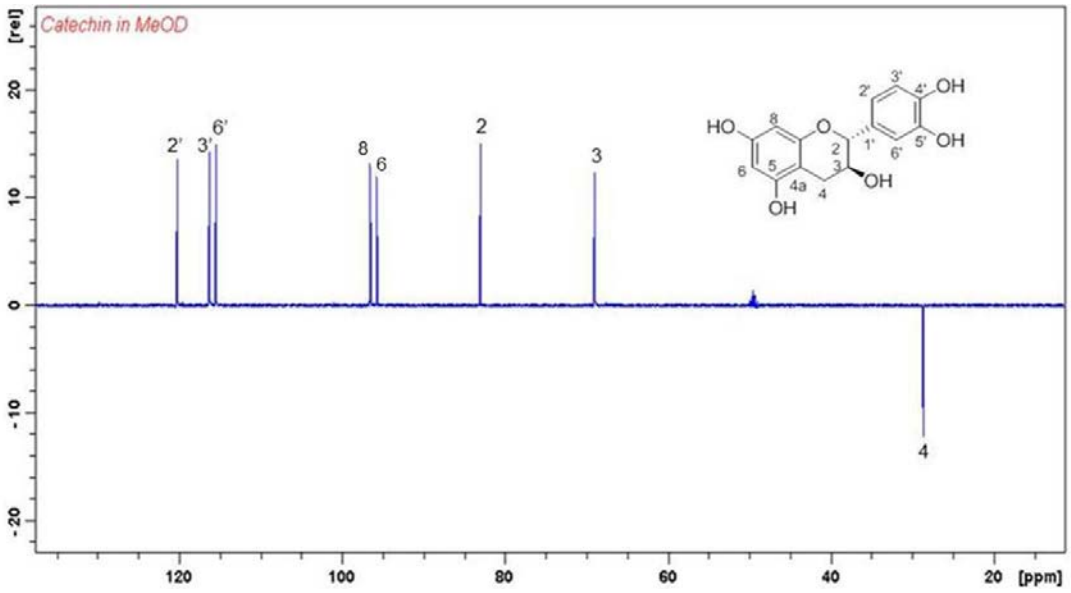


Figure 3.25. DEPT 135 NMR spectrum of catechin (**3.3**) in CD_3OD at 100 MHz.

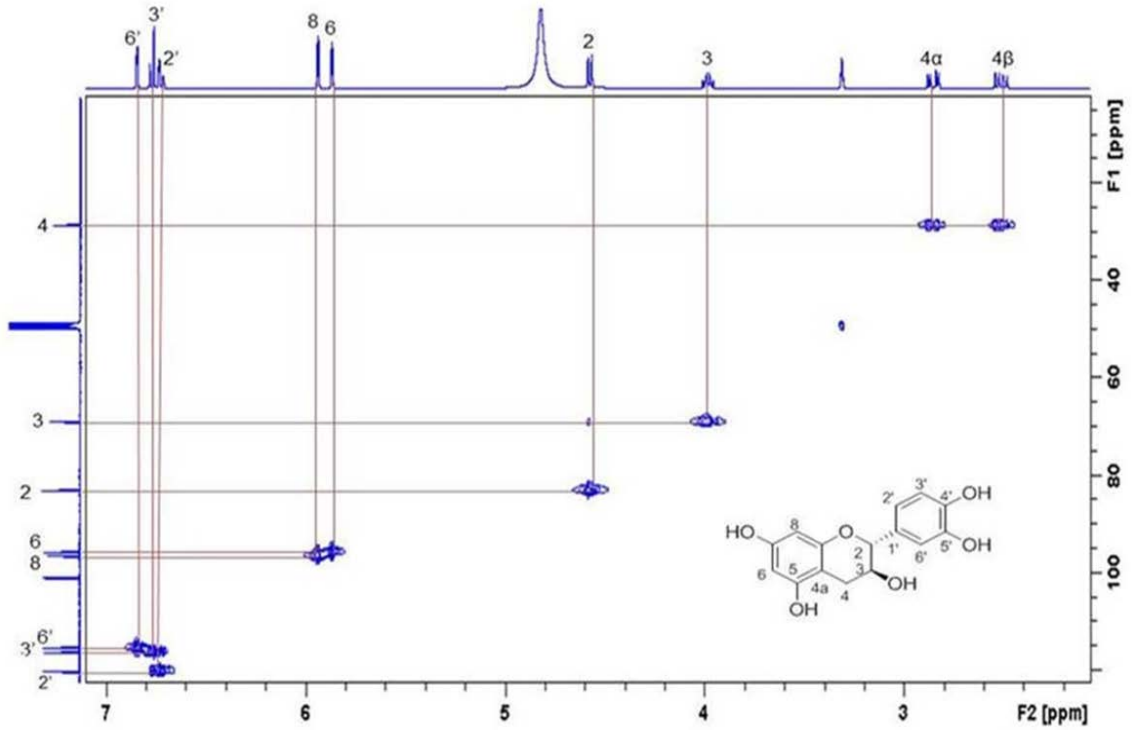


Figure 3.26. HSQC NMR spectrum of catechin (**3.3**) in MeOH.

The HMBC NMR spectrum (Figure 3.27) and Figure 3.29 were used to confirm connectivities between the atoms in the proposed structure. A ^{2}J correlation was observed between the carbon resonating at δ_C 158.0 and a proton resonating at δ_H 5.93. Another ^{2}J connectivity was observed between proton resonating at δ_H 5.86 and a carbon at δ_C 95.7. There was a ^{2}J correlation between a carbon resonating at δ_C 132.4 and a proton at δ_H 4.57, confirming the connectivity between a benzene ring and a pyran ring system. ^{3}J correlations were observed between a carbon resonating at δ_C 101.0 and proton resonances at δ_H 3.98, 2.85 and 2.51. These correlations confirmed the presence of a benzopyran ring system and were supported by the correlation of carbons resonating at δ_C 101.0 and 157.7 and protons at δ_H 5.93 and 5.86. The NMR data suggested that the isolated compound was catechin (**3.3**) (Figure 3.28). The long-range ^{1}H,^{13}C-correlations derived from the HMBC spectrum (Figure 3.27) for **3.3** are indicated in Figure 3.29.

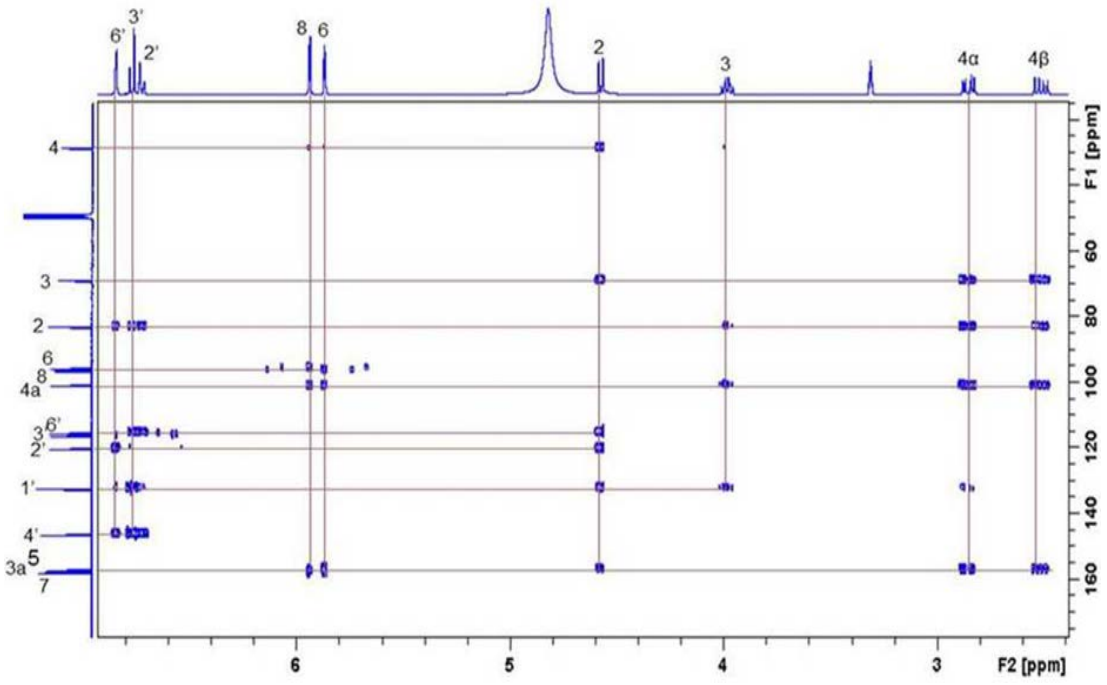
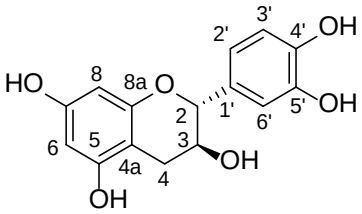


Figure 3.27. HMBC NMR spectrum of catechin (**3.3**) in CD_3OD.



Catechin (**3.3**)

Figure 3.28. Structure of catechin (**3.3**).

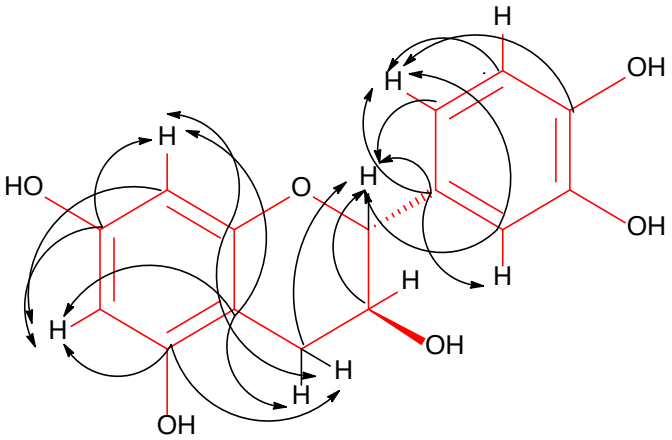


Figure 3.29. HMBC correlations observed for catechin (**3.3**).

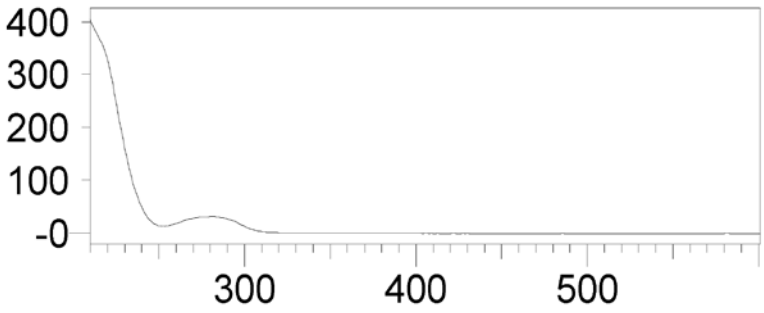


Figure 3.30. HPLC UV spectrum of catechin (**3.3**).

The UV spectrum of compound (**3.3**), obtained from the diode-array detector of the HPLC, showed a maxima absorption at λ_{max} = 280 nm (Figure 3.30). This spectrum is in agreement with the chromophore of the proposed structure, which is a benzene ring.

Table 3.3. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of catechin (**3.3**) in CD_3OD.

Carbon (C) and Proton (H) Positions	**δ_H (ppm)**	**δ_C (ppm)**	**DEPT-135**
1’	-	132.4	
2’	6.72 (1H, dd, 1.92, 8.16 Hz)	120.2	CH
3’	6.77 (1H, d, *J* = 8.12 Hz)	116.3	CH
4’	-	146.3	-
5’	-	146.4	-
6’	6.84 (1H, d, *J* = 1.90 Hz)	115.4	CH
2	4.57 (1H, d, *J* = 7.48 Hz)	83.0	CH
3	3.98 (1H, m, Hz)	68.9	CH
4α 4β	2.85 (1H, dd, *J* = 5.44, 10.72 Hz) 2.51 (1H, dd, *J* = 8.12, 8.00 Hz)	28.6	CH_2
4a	-	101.0	-
5	-	157.0	-
6	5.86 (1H, d, *J* = 2.32 Hz)	95.7	CH
7	-	158.0	-
8	5.93 (1H, d, *J* = 2.28 Hz)	96.5	CH
8a	-	157.7	-

The most common type of catechins is tea catechin, which is a major constituent of fresh tea (Okushio et al., 1996). Catechins that are abundant in green tea are epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (Okushio et al., 1996). The configurations at C-2 and C-3 are used to describe the compounds.If the relative configuration is *trans*, the compound is called *catechin* and if it a *cis*, the compound is called *epicatechin.* Catechin, like most flavonoids, possesses antioxidant activity, therefore, preventing the effects of cancer.

3.1.4 Structural elucidation of 3.4

The crude DCM extract of the leaves was fractionated by VLC. HPLC analysis indicated the presence of two compounds in fraction B of the VLC. The compounds that were labelled by the chromatogram (Figure 3.31) were then isolated by semipreparative HPLC. The mass of the compound correlating with peak A was very small and spectroscopic data could not be obtained. Compound **3.4** was obtained from peak B. Further HPLC analysis (Figure 3.32) confirmed the purity of **3.4**.

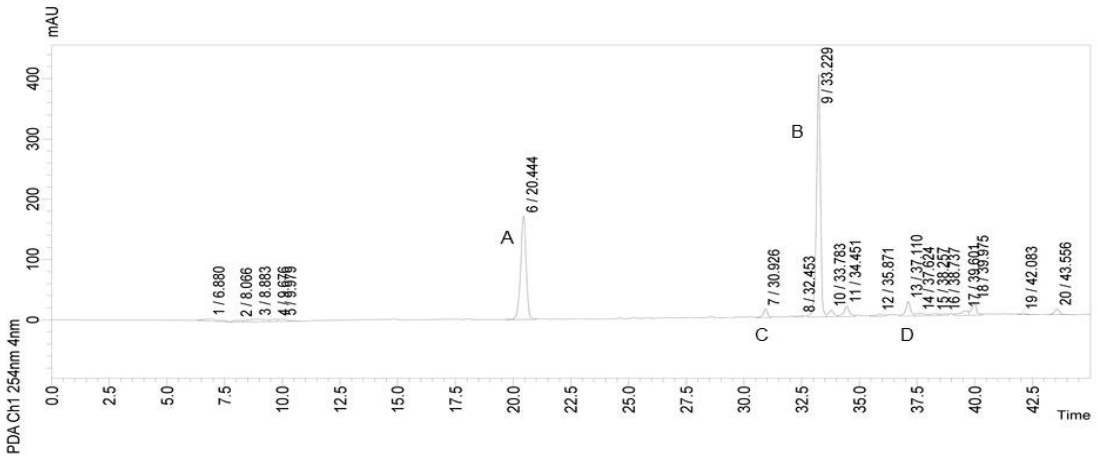


Figure 3.31. HPLC chromatogram showing the separation of a fraction of the crude DCM extracts of the leaves.

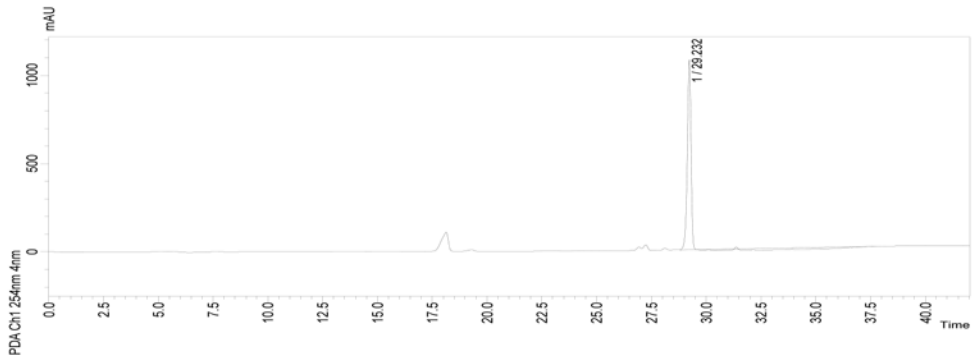


Figure 3.32. HPLC chromatogram of **3.4**.

The structural determination of **3.4** was based on NMR, MS and UV analysis. In the high-resolution ESI-(+)-mass spectrum of **3.4** (Figure 3.33), a pseudo-molecular ion was observed at *m/z* 337.1403 $[M+Na]^+$, corresponding to a molecular formula of $C_{19}H_{22}O_4$ (calculated for $C_{19}H_{22}O_4Na$, 337.1416).

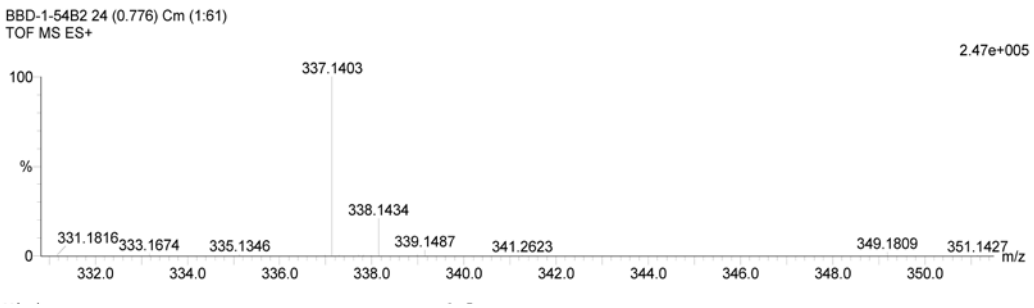


Figure 3.33. The ESI-(+)-TOF-MS spectrum of **3.4**.

The NMR spectroscopic data of compound **3.4** are summarised in Table 3.4. The ^{1}H NMR spectrum Figure 3.34 suggested that there were eight aromatic protons distributed around two aromatic rings and resonating δ_H 6.70 – 7.84. Two two-proton resonances at δ_H 7.84 (2H, *d*, *J* = 8.9 Hz) and 6.94 (2H, *d*, *J* = 8.9 Hz) were characteristic of a 1,4-disubstituted phenyl ring and a correlation between them could be observed in the COSY spectrum (Figure 3.35). The triplet resonance at δ_H 7.16 (1H, *t*, *J* = 7.8 Hz), two broad doublets at δ_H 6.70 (1H, d, *J* = 7.2 Hz) and 6.73 (1H, d, *J* = 8.5 Hz) and a broad singlet at δ_H 6.94 in the ^{1}H NMR spectrum suggest the presence of a second disubstituted benzene ring. The triplet signal at δ_H 7.16 (1H, *t*, *J* = 7.8 Hz) indicated the presence of a proton adjacent to two other *ortho*-protons and thus the presence of a 1,3-disubstituted phenyl ring.

A signal for a methine proton was observed at δ_H 5.01 (1H, dd, *J* = 2.8, 7.5 Hz). The chemical shift of this proton suggested that it is not only on a carbon that contains an oxygen substituent, but that there is also another electron-withdrawing group attached to the carbon. In the COSY spectrum (Figure 3.35), a correlation with a signal at δ_H 1.57 (overlapping with water peak), which was assigned to an aliphatic methylene. In the aliphatic region, a total of six protons were observed as complex multiplets at δ_H 2.64 (1H, *m*), 2.57 (1H, *m*), 1.87 (2H, *m*), 1.75 (1H, *m*), 1.57 (1H, *m*). A COSY correlation was observed between the protons resonating at δ_H 2.61 and 2.57 and this observation suggested that these protons were attached to one carbon. The COSY correlation also suggested that the proton resonances at δ_H 1.87 and 1.57 were attached to the same carbon and lastly the proton resonances at δ_H 1.87 and 1.75 ppm were also assigned to two methylene protons. The aliphatic signals were assigned to three adjacent methylene groups. Therefore, the following fragment could be constructed: -CHOH-$CH_2CH_2CH_2$-.

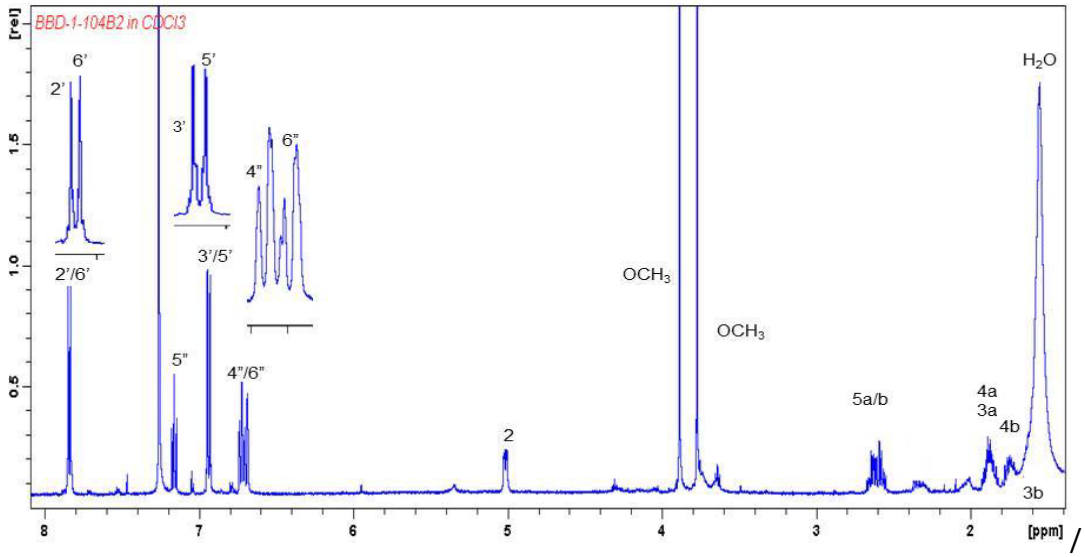


Figure 3.34. ^{1}H NMR for compound **3.4** in $CDCl_3$ at 500 MHZ.

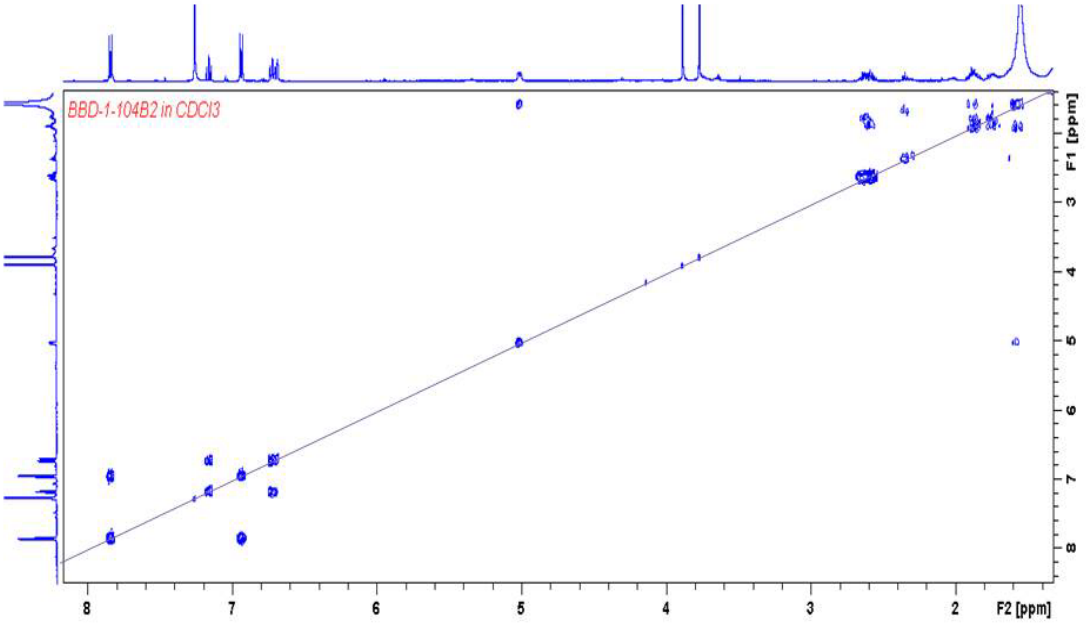


Figure 3.35. COSY NMR spectrum of compound **3.4**.

The remaining two signals were observed as two three-proton singlets resonating at δ_H 3.89 and δ_H 3.77, consistent with methoxy groups attached to an aromatic ring.

Only a small amount of **3.4** could be isolated and not enough material was available for a ^{13}C NMR spectrum. However, by using the ^{13}C projections of the HSQC (Figure 3.36) and HMBC (Figure 3.37) spectra, the ^{13}C NMR shifts of all 19 carbons could be obtained (Table 3.4).

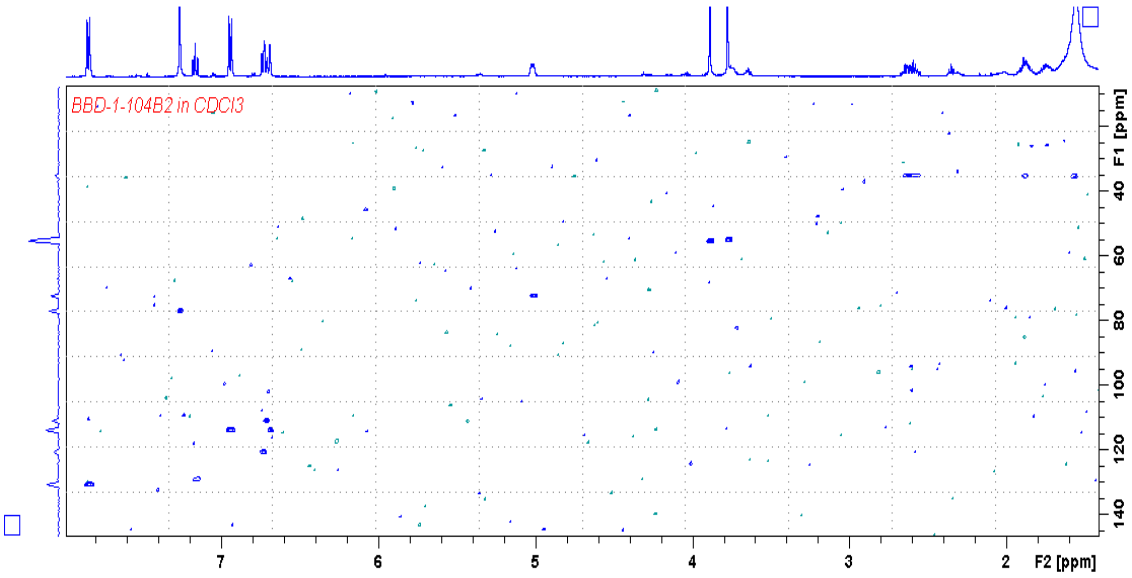


Figure 3.36. HSQC NMR spectrum of **3.4** in $CDCl_3$.

The ^{13}C NMR data confirmed the presence of a 1,4-disubstituted phenyl ring in **3.4**. In the HMBC spectrum of **3.4**, the doublet resonance at δ_H 7.84 showed a correlation with a carbonyl carbon resonating at δ_C 200.5 and also with a sp^2- carbon resonating at δ_C 164.0. The latter carbon also showed a correlation with one of the methoxy proton signals. These observations enabled us to propose the presence of a 4-methoxybenzoyl moiety in **3.4**. The HMBC correlations also confirm that the second aromatic ring also had a methoxy substituent.

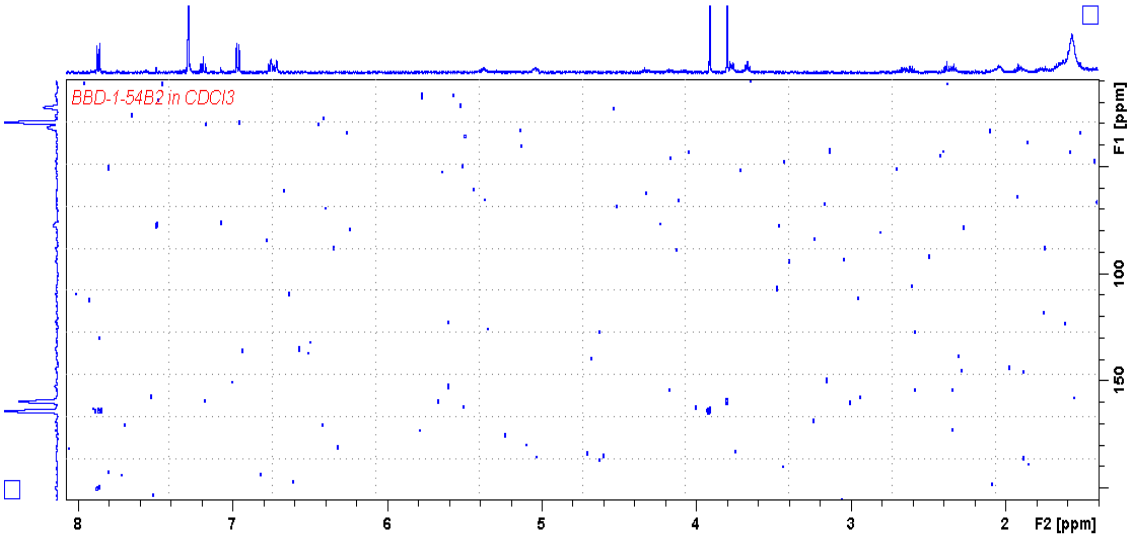
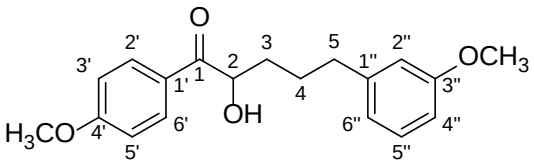


Figure 3.37. HMBC NMR spectrum of **3.4** in $CDCl_3$.

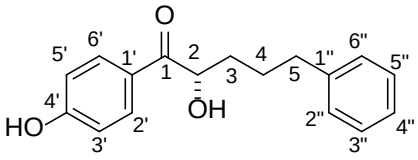
The NMR data suggested that compound **3.4** might be a diarylpentanoid derivative (Celebioglu et al., 2017). Based on the spectroscopic evidence, the structure of **3.4** was assigned as 2-hydroxy-5-(3-methoxyphenyl)-1-(4-methoxyphenyl)pentan-1-one, a novel diarylpentanoid (Figure 3.38). In order to confirm the structure of **3.4**, the NMR

data was compared with those reported for diplomorphanone (**3.5**) (Figure 3.39) (Devkota et al., 2012) (for the 'left-hand' side of the molecule) and for 3-butylanisole (Tietze et al., 2009) for the 'right-hand side' of the molecule (Table 3.37). The data obtained for **3.4** showed an excellent fit with the published data.



Compound **3.4**

Figure 3.38. Structure of compound (**3.4**).



Diplomorphanone (**3.5**)

Figure 3.39. Structure of diplomorphanone.

The UV spectrum of compound **3.4** (Figure 3.40), showed a maximum absorption at λ_{max} = 270 nm and this absorption was attributed to the benzene rings in the structure.

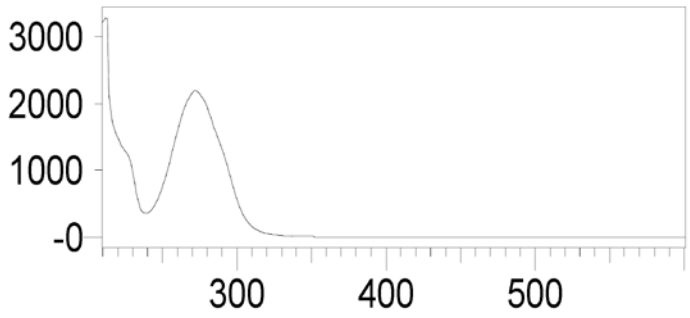


Figure 3.40. HPLC UV spectrum of **3.4**.

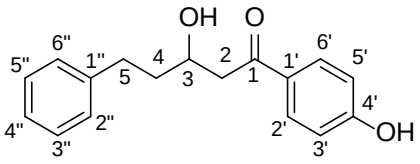
Diarylheptanoids (two aryl groups connected by a seven-carbon chain) are well known compounds and over 400 different derivatives have been isolated, mostly from the Zingiberaceae. The most well-known member of this family is curcumin. In contrast, only twenty diarylpentanoids have been reported (Celebioglu et al., 2017). From these 20 compounds, 15 were isolated from the Thymelaeaceae family (Celebioglu et al., 2017). The structure of **3.4** is closely related to daphneolone (**3.6**) (Figure 3.41), the first diarylpentanoid to be isolated (Suzuki and Umezawa, 2007). It has been reported that the diarylpentanoids isolated from Thymelaeaceae plants have anti-HIV, cytotoxic and

insecticidal effects. Considering the interesting biological effects of closely related compounds, it would be of interest to investigate the biological activity of **3.4** in a future study.

Table 3.4. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound (**3.4**) in $CDCl_3$.

Position	**δ_C**	**δ_H**	**δ_H** (Devkota et al., 2012)	**δ_C** (Devkota et al., 2012)[1] (Tietze et al., 2009)[2]
C-1	200.5	-		200.1[1]
C-4'	164.0	-		161.3[1]
C-3''	159.8	-		159.5[2]
C-1''	143.8	-		144.6[2]
C-2'.6'	130.6	7.84 (2H, *d*, *J* = 8.9 Hz)	7.78 (*d*, *J* 8.8 Hz)	131.3[1]
C-5''	129.0	7.16 (1H, *t*, *J* = 7.8 Hz)	6.87 (*d*, *J* 8.8. Hz)	129.1[2]
C-1'	126.4	-		126.1[1]
C-6''	120.6	6.70 (1H, *dd*, *J* = 1.7, 7.2 Hz)		120.8[2]
C-2''	113.9	6.94 (1H, *dd*, *J* = 1.9, 7.0 Hz)		114.2[2]
C-3',5'	113.8	6.94 (1H, *dd*, *J* = 1.9, 7.0 Hz)		115.9[1]
C-4''	110.9	6.73 (1H, *ddd*, *J* = 2.4, 8.5 Hz)		110.6[2]
C-2	72.3	5.01 (1H, *dd*, *J* = 2.8, 7.5 Hz)	5.02 (*m*)	72.5[1]
4'-OCH_3	55.3	3.89 (3H, *s*)		
3''-OCH_3	54.9	3.77 (3H, *s*)		55.1[2]
C-4	35.3	1.87 (1H, *m*) 1.75 (1H, *m*)	1.89 (*m*) 1.76 (*m*)	33.5[2]
C-5	35.0	2.64 (1H, *m*) 2.57 (1H, *m*)	2.63 (*m*)	35.5[1] 35.7[2]
C-3	26.0	1.87 (1H, *m*) 1.57 (1H, *m*)	1.89 (*m)* 1.58 (*m)*	35.5[1]

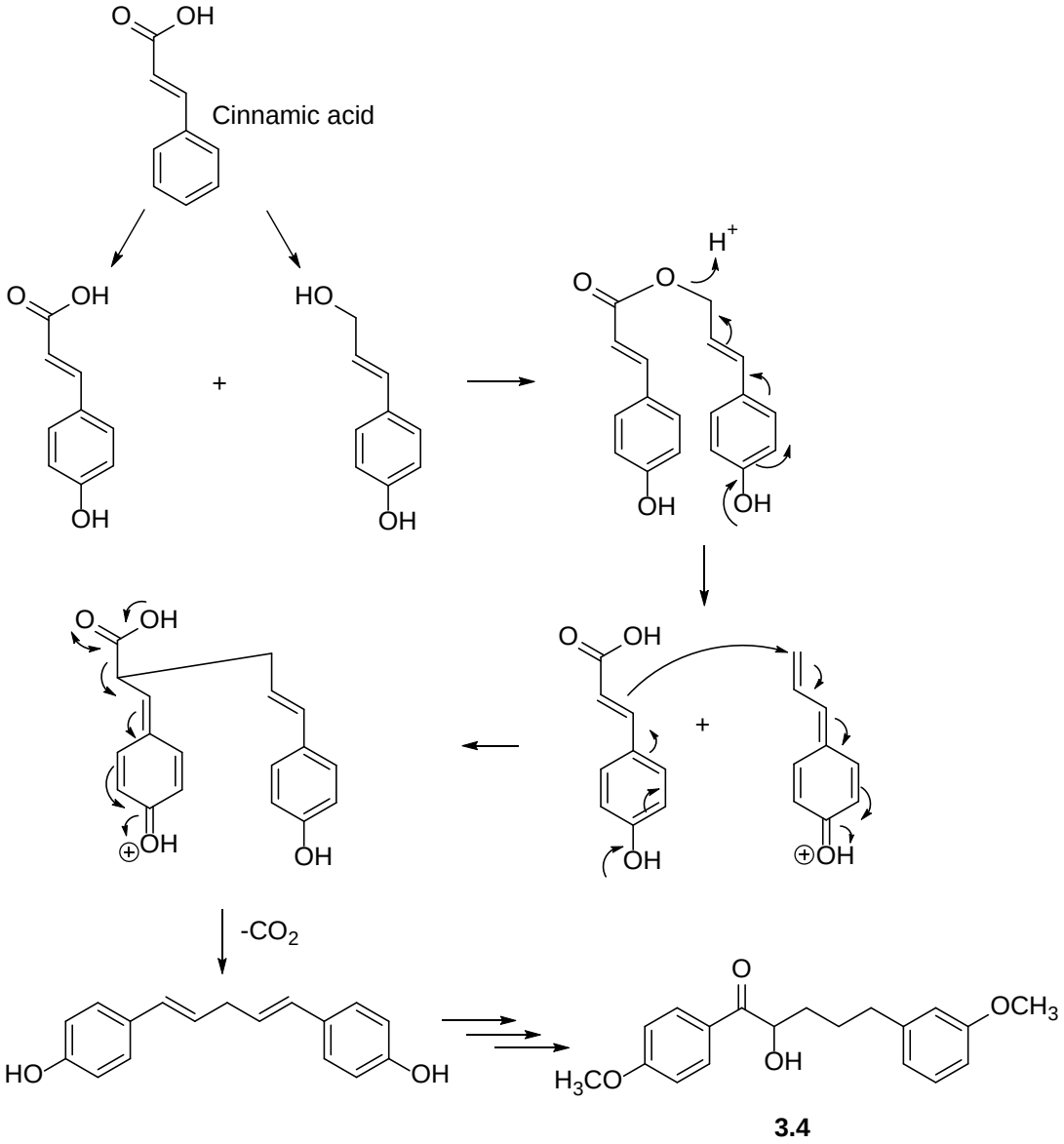
[1]Chemical shifts reported for 2-hydroxy-1-(4-hydroxyphenyl)-5-phenyl-1-pentanone (dilpomorphanone, **3.5**) by Devkota et al. (2009) (Figure 3.38).
[2]Chemical shifts reported for 3-butylanisole by Tietze et al. (2009).



Daphneolone (**3.6**)

Figure 3.41. Structure of diplomorphanone.

Lignans are phenylpropanoid (C_6C_3) dimers. Based on biosynthetic considerations, 1,5-diarylpentanoids can be considered as norlignans, i.e. they have one carbon less than a lignan. Compound **3.1** can be considered as a C7-C9'-linked norlignan. Suzuki and Umezawa (2007) proposed a biosynthetic pathway for norlignans, which is shown in Scheme 3.1.

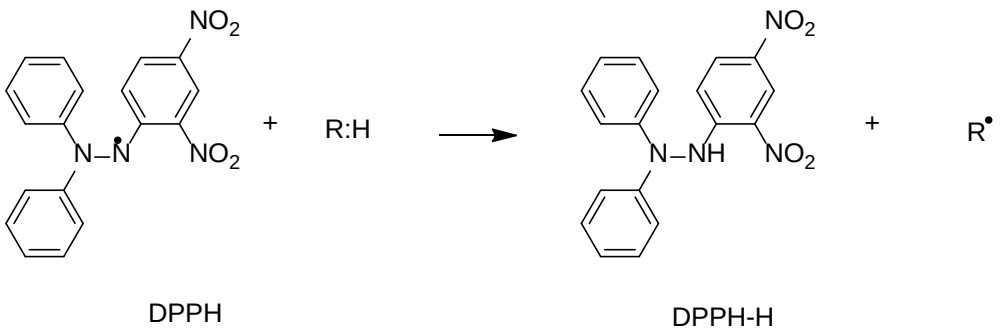


Scheme 3.1. Biosynthetic pathway for norlignans.

3.2 Antioxidant assay: DPPH radical scavenging activity

Recent studies found that free radicals cause oxidative damage to biomolecules. The damage caused by the free radical induce atherosclerosis, ageing, cancer and other diseases (Yamaguchi et al., 1998). Antioxidants which scavenge free radicals play a vital role in preventing the free radical-induced diseases (Yamaguchi et al., 1998). The common secondary metabolites with antioxidant activity are flavonoids, and anti-oxidative vitamins such as ascorbic acid and α-tocopherol (Yamaguchi et al., 1998). The DPPH assay was used to evaluate the free radical-scavenging activity of an extract of the plant. The 2,2-diphenyl-1-picryhydrazyl (DPPH) assay is the most common assay in plant biochemistry and it is used for the evaluation of plant constituents for the scavenging of free radicals (Gupta et al., 2016).

In the DPPH assay, the antioxidant activity of a sample is determined by the reduction of a stable radical DPPH (λ_{max} 517 nm) to a yellow-coloured diphenyl-picryl hydrazine (Scheme 3.1). The results are usually expressed as the IC_{50} value, which is the amount of antioxidant required to decrease the initial DPPH concentration by 50%. In this study the different concentration of each plant extract (5, 10, 15, 20 and 25 mg/ml) and ascorbic acid(AA) (0.07, 0.14, 0.21, 0.28, and 0.35 mg/ml) were plotted against the radical scavenging activity (RSA) percentage to generate the standard curve for each plant extract and AA. The IC_{50} values were calculated from the straight line equation from each curve (Gupta et al., 2016). The results of antioxidant scavenging ability of AA along with the leaves, root bark, and root extracts of the plant are shown in Table 3.1, Table 3.2, Table 3.5 and Table 3.8, respectively. The IC_{50} values were 0.20 mg/ml (AA), 14.57 mg/ml (leave extract), 15.47 mg/ml (root bark extract), and 16.59 mg/ml (root extract). The results for each research sample show that the %RSA of each sample was directly proportional to the concentration (Thaipong et al., 2006).



Scheme 3.2. Scavenging the DPPH Radical by an antioxidant.

Table 3.5. The radical scavenging activity of ascorbic acid at different concentrations against the DPPH stable free radical

Sample volume (µL)	Blank Absorbance	Extract Absorbance	Extract concentration (mg/mL)	%RSA	IC_{50} (mg/ml)
200	0.99264	0.74606	0.07	24.84	0.20
400	0.99264	0.64171	0.14	35.093	
600	0.99264	0.50312	0.21	48.952	
800	0.99264	0.32576	0.28	66.688	
1000	0.99264	0.10752	0.35	88.512	

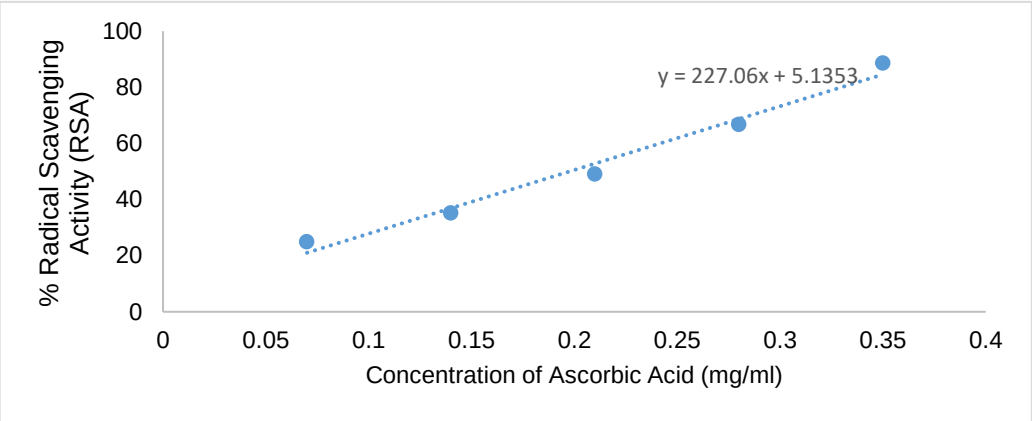


Figure 3.42. The relationship between the radical scavenging activity of ascorbic acid in different concentrations by DPPH assay.

Table 3.6. The radical scavenging activity of leave extract of *Dais cotinifolia* at different concentrations against the DPPH stable free radical.

Sample volume (µL)	Blank Absorbance	Extract Absorbance	Extract concentration (mg/mL)	%RSA	IC_{50} (mg/ml)
200	0.99264	0.82462	5	16.9	14.57
400	0.99264	0.61859	10	37.7	
600	0.99264	0.50786	15	48.8	
800	0.99264	0.32291	20	67.5	
1000	0.99264	0.13723	25	86.2	

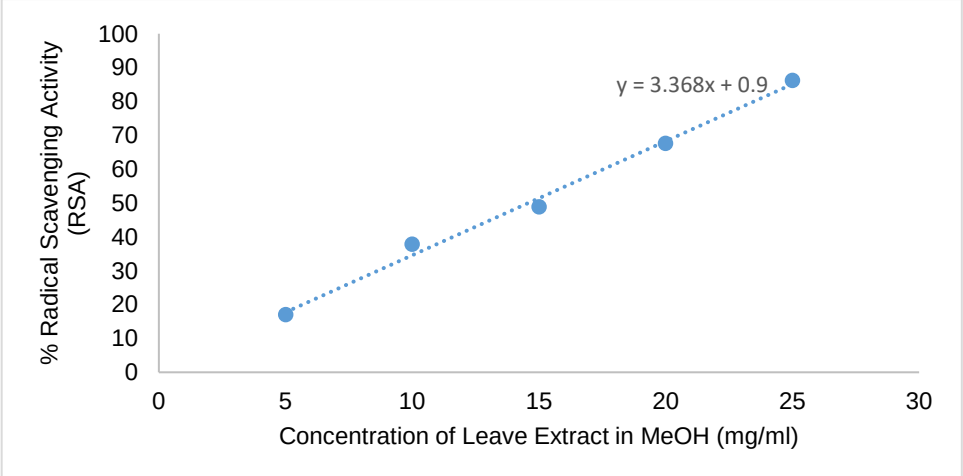


Figure 3.43. The radical scavenging activity MeOH leave extract of *D. cotinifolia* in different concentrations by means of DPPH assay.

Table 3.7. The radical scavenging activity of root bark extract of *Dais cotinifolia* at different concentrations against the DPPH stable free radical.

Sample volume (µL)	**Blank Absorbance**	**Extract Absorbance**	**Extract concentration (mg/mL)**	**%RSA**	**IC_{50} (mg/ml)**
200	0.99264	0.85606	5	13.6	15.47
400	0.99264	0.69171	10	30.3	
600	0.99264	0.52312	15	47.3	
800	0.99264	0.32676	20	67.1	
1000	0.99264	0.16752	25	83.1	

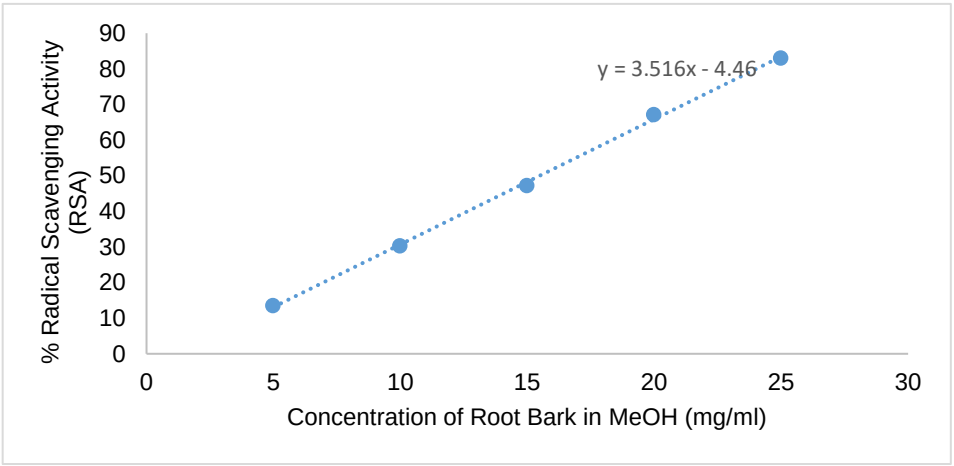


Figure 3.44. The relationship between the radical scavenging activity MeOH root bark extract of *D. cotinifolia* in different concentrations by means of DPPH assay.

Table 3.8. The radical scavenging activity of root extract of *Dais cotinifolia* at different concentrations against the DPPH stable free radical.

Sample volume (µL)	Blank Absorbance	Extract Absorbance	Extract concentration (mg/mL)	%RSA	IC_{50} (mg/ml)
200	0.99264	0.85606	5	8.5	16.59
400	0.99264	0.69171	10	20.6	
600	0.99264	0.52312	15	42.2	
800	0.99264	0.32676	20	68.9	
1000	0.99264	0.16752	25	79.6	

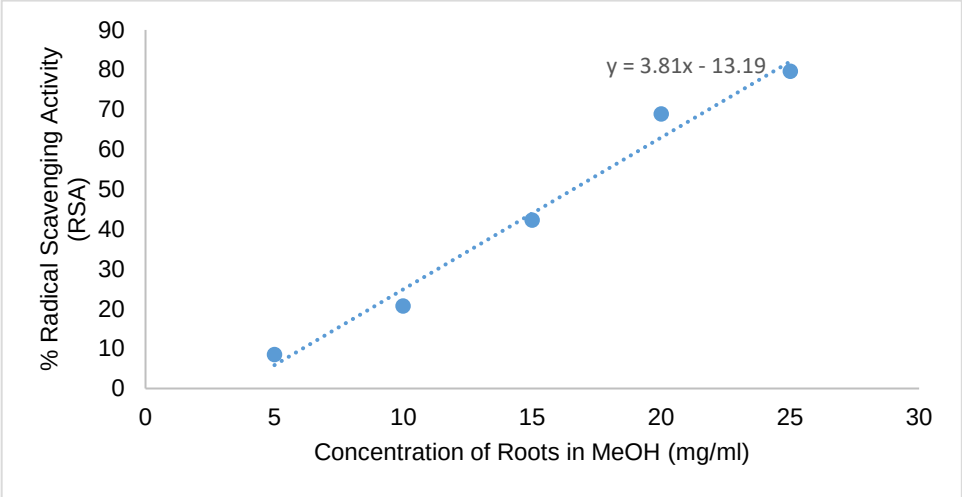


Figure 3.45. The relationship between the radical scavenging activity MeOH root extract *of D. cotinifolia* in different concentrations by means of DPPH assay.

The ascorbic acid had a higher radical scavenging ability (Figure 3.42) as compared to the crude extract followed by the leaves crude extract, root bark extract and then the roots, the evidence is exhibited by the IC_{50} values (listed above). This means that the plant extracts are required at a higher concentration as compared to the ascorbic acid to scavenge the DPPH stable free radical. Therefore, ascorbic acid was observed to be a better antioxidant than the plant extract. When comparing the plant extracts, the evidence pointed out that the leaf extract had a better antioxidant activity than the other parts of the plant. Nonetheless, there was no much difference in the antioxidant activity between the plant extracts. The differences between the leaves and root bark were 0.9 mg/ml and 2.02 mg/ml between the leaves and the roots. Furthermore, when looking at the plant extracts at a concentration of 5 mg/ml, the leaf extracts could scavenge 16.9% of DPPH, root bark extract scavenged 13.6% and the root extract scavenged 8.5%. At

a concentration of 15 mg/ml, the leaves scavenged 48.8%, the root bark scavenged 47.8% and the roots scavenged 42.2%. From this observation it was noticed that the leaves had a better antioxidant activity as compared to the other parts of the plant, followed by the root bark and then the roots. Nevertheless, all the different parts of the plant had antioxidant activity (Gupta et al., 2016) (Figure 3.43-3.45).

Chapter 4: Experimental

4.1 General Experimental Procedures

Extraction and chromatography were performed with analytical grade dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH). Hexanes (Hex) (a mixture of hexane isomers) were distilled before use. Water used always refers to distilled water.

4.1.1 Thin-Layer Chromatography (TLC)

Aluminum-backed thin-layer chromatography (TLC) plates (Silica gel 60 F_{254}, 0.25 mm, Merck) were used for the detection of different components in extracts and to select solvent systems for column chromatography and preparative centrifugal thin-layer chromatography (PCTLC). A TLC plates (stationary phase) would be introduced into a particular solvent system (mobile phase) for the detection of various components in an extract. The separation of the components was then examined under ultraviolet (UV) light at two different wavelengths, a short wavelength (254 nm) and a long wavelength (365 nm). To detect the components in a sample that do not absorb UV, the plate was sprayed with a reactive stain reagent, i.e. anisaldehyde stain reagent. The anisaldehyde stain reagent was prepared by introducing 84 mL of MeOH in a 250 mL volumetric flask in an ice bath. While continuously shaking and maintaining a low temperature, the following reagents were added sequentially: acetic acid (100 mL), sulfuric acid (40 mL, dropwise) and anisaldehyde (5 mL, dropwise). The homogenous mixture was stored in a fridge.

4.1.2 DIOL SPE Chromatography

Applied Separations SPE Diol column (2 g/6 mL) were used for analytical fractionation of crude extracts. A crude extract was fractionated into five different fractions by eluting the sample components with solvents of increasing polarity. The C_{18} DIOL served as a stationary phase in which the silica is derivatised with 1,2-dihydroxypropane. To run a DIOL column, 100 mg of extract dissolved in 1 mL of a DCM-MeOH (1:1) mixture was deposited onto the DIOL column. The solution was allowed to percolate down the column and was allowed to dry for 24 h. The following day a slight vacuum was applied to the column for at least 15 minutes ensuring that the residual solvent was removed. Sequential elution was then performed with 12 mL of each of the following solvents with increasing polarity, Hex-DCM (9:1), DCM-EtOAc (20:1), EtOAc, EtOAc-MeOH (5:1), and MeOH.

4.1.3 Vacuum Liquid Chromatography (VLC)

Vacuum Liquid Chromatography (VLC) is similar to DIOL SPE chromatography regarding analytical fractionation, but this method was used for fractionation of larger amounts of crude extracts. The crude extract was divided into five fractions. The solvent systems that were used for fractionation were similar to the solvent systems that were used for the DIOL fractionation, but sequential elution was performed with 200 mL of each solvent system. The stationary phase in the VLC was silica gel 60 (40-63 µm, Merck). With a VLC column, 30 g of silica gel was used for every 1 g of the sample.

4.1.4 Liquid-Liquid Extraction

Liquid-Liquid extraction was performed to isolate the extract components according to polarity. A summary of the procedure is given in Figure 4.1 below.

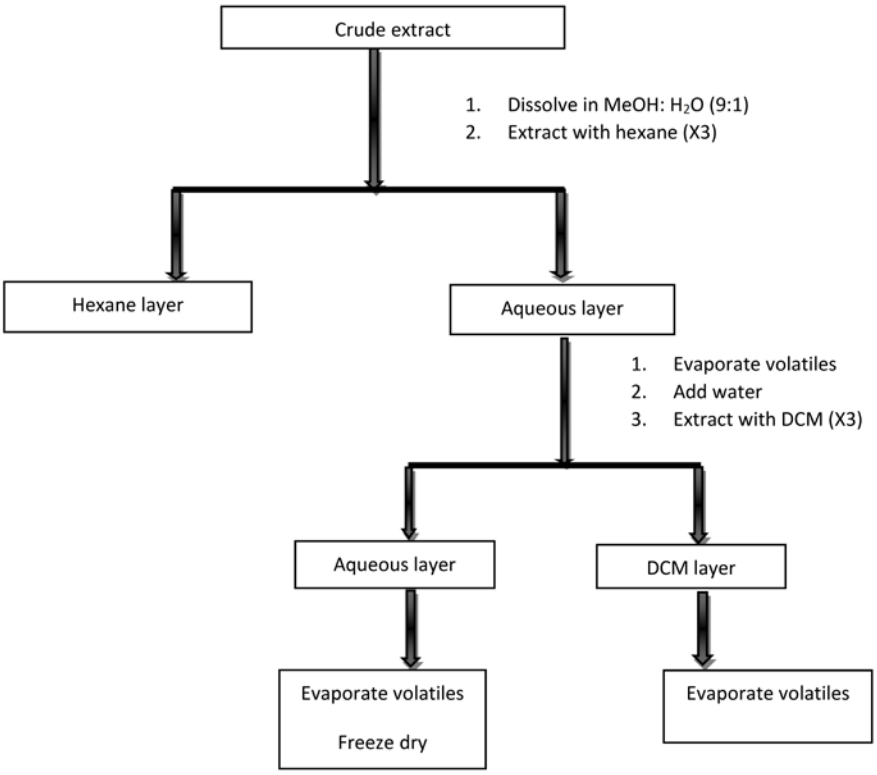


Figure 4.1. Summary of liquid-liquid extraction.

4.1.5 Column Chromatography (CC)

Column Chromatography (CC) is an analytical method that was used for the isolation of a mixture of compounds. CC was performed on a glass column packed with silica gel 60 (40-63 µm, Merck) as the stationary phase. To pack the column, a silica gel slurry was loaded into the glass column, followed by the sea sand to separate the sample from the silica gel, sample (pre-adsorbed in silica gel 60), and then the sea sand to prevent the solvent from disturbing the sample layer. Sequential isolation based on the polarity of solvents was performed to separate the components of a sample or extracts. Figure 4.2 is the summary of a general procedure for column chromatography that was followed in the isolation of compounds from different plant extracts.

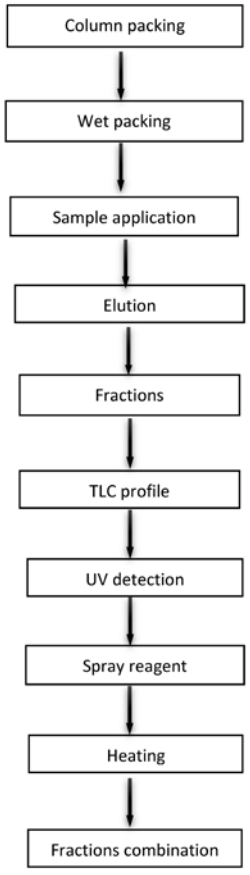


Figure 4.2. The general procedure for column chromatography.

4.1.6 Preparative Centrifugal Thin-layer Chromatography (PCTLC)

Preparative Centrifugal Thin-Layer Chromatography (PCTLC) combines the advantages of both TLC and CC, and this method was used to isolate compounds from smaller

amounts of mixtures. PCTLC was performed on a Harrison Research Chromatotron™, model ENF-280 C/F. The rotor was coated with a mixture of silica gel 60 PF_{254} containing gypsum (Merck) and dried in an oven at 50 ºC before use. For 1 mm thickness, 45 g of silica was mixed with 90 mL of distilled water, for 2 mm thickness 65 g of silica was mixed with 130 mL of distilled water, and for 4 mm thickness, 115 g of silica was mixed with 230 mL of distilled water.

The sample was dissolved in a small volume of eluting solvent before it was applied to the spinning plate. The eluting solvent was delivered by gravity from a container that was lifted about 70 cm above the rotor and flow through an inlet stopper marked 1, 2 or 4, following the thickness of the silica layer. Separation was achieved through gradient or isocratic elution based on the TLC profile. The moving bands were visualised using UV light at λ 254 or λ 365 nm. Small fractions were collected (Desai et al., 1985).

4.1.7 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is an analytical method that was used for analysis of small quantities of compound mixtures. Analyses were performed on a SHIMADZU HPLC, CBM-20A communication bus module, LC-20AB prominence liquid chromatography, SIL-20A prominence autosampler, and SPD-M20A prominence diode-array detector. A 200 ppm sample was prepared with an HPLC analytical solvent. The solution was injected onto a LUNA 5µ C18 (2) (250 x 4.60 mm, 5µ) column. The sample was eluted using the following a standard gradient method, as shown in Table 4.1 below.

Table 4.1. Standard gradient method used for HPLC**.**

Time (minutes)	**% MeOH: ACN: FA (4: 3: 0.1)**	**% Ultrapure water with 0.1 % formic acid**
0.01	40	60
28	100	0
35	100	0
38	40	60
42	Stop	stop

4.1.8 Liquid Chromatography-Mass Spectrometry (LC-MS)

A SHIMADZU LCMS-2020 with a quadrupole mass detector was used for the characterisation of compounds in terms of spectrum and mass determination. A 200-ppm solution of crude samples was injected onto a Shim-pack GIST (3 µm C_{18}-HP, 4.6 x 150 mm) column and eluted with solvents of different polarity. For a pure compound, a 10 ppm solution was injected.

4.1.9 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy was used for the characterisation of pure compounds. The NMR spectra were recorded on a Bruker Avance III 500 (operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C) or a Bruker Avance III 400 (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometers. The NMR experiments that were used for structural elucidation were ^{1}H, ^{13}C, DEPT-135, COSY, NOESY, HSQC and HMBC. All spectra were recorded in deuterated chloroform and methanol at 30 ºC using a 5 mm BBOZ probe or 5 mm TBIZ probe. Chemical shifts (δ) are given in part per million (ppm) relative to tetramethylsilane, TMS (δ = 0) and are referenced to residual protonated solvent peaks, for $CDCl_3$: ^{1}H = δ 7.26, ^{13}C = δ 77.0 and CD_3OD: ^{1}H δ 3.31, ^{13}C δ 49.1. The multiplicities of peaks were abbreviated as follows; *s* = singlet, *d* = doublet, *dd* = doublet of doublets, *ddd* = doublets of doublets of doublets, *t* = triplet, *dt* = doublet of triplet and *m* = multiplet. Coupling constant (*J*) are given in Hz. A 10 mg/ 0.5 mL sample was prepared for ^{1}H NMR, and a 20 mg/0.5 mL sample was prepared for ^{13}C NMR.

4.1.10 Time-of-Flight Mass Spectrometry (TOFMS)

An LCT Premier Time-of-flight Mass Spectrometer (TOF-MS) from MICROMASS TECHNOLOGIES was used for the determination of the accurate mass of a compound. TOF-MS (time-of-flight mass spectrometry) is a type of a high-resolution mass spectrometry. The ionisation used was electrospray ionisation (ESI) either in the positive mode or negative (Breitkopf et al., 2017).

4.1.11 Ultraviolet (UV)-Vis Spectroscopy

A Cary 100 UV-Vis spectrometer from Agilent Technology was used to determine the spectrum of a pure compound. The ultraviolet (UV) spectrometer characterises organic compounds with conjugated double bonds (Bruice, 2014). The absorption of the

compound was recorded at different concentrations. All the spectra were recorded at a UV range of 180-400 nm.

4.2 Extraction and Isolation

4.2.1 Plant Material

Plant material of *D. cotinifolia* was collected in the UKZN Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, on the 19th of April 2016. The plant was identified by Ms Alison Young, curator of the UKZN Botanical Garden. A voucher specimen (B. Danca1 (NU)) was prepared and deposited in the Bews Herbarium, School of Life Sciences, UKZN.

The various parts of the plant (leaves, branches, roots and root barks) were separated, any soil particles removed from the plant material, and plant parts dried at room temperature for two to three weeks. The dry material was ground with a hammer mill. The masses of the dry materials are shown in Table 4.2.

Table 4.2. Mass of dry plant material.

Plant material	**Dry powdered Mass (g)**	**Description**
Leaves	529	Green powder
Branches	544	Light-brown powder
Roots	616	Light-brown powder
Root barks	950	Brown powder

4.2.2 Preparation of Crude Extracts

The powdered plant materials [leaves (529 g), branches (544 g), roots (616 g) and root bark (950 g)] were extracted sequentially with DCM, DCM-MeOH (1:1) and MeOH. The plant material was first submerged in DCM and then left on an orbital shaker (120 rpm) at 23 ºC for 24 h. The plant material was filtered, and the solvent was removed under vacuum with a rotavapor to yield a crude DCM extract; this process was repeated, and the two DCM extracts were combined. The recovered plant material was left to dry at room temperature, DCM-MeOH (1:1) mixture was added to the plant material and the

mixture left on an orbital shaker (120 rpm) at 23 ºC for 24 h. The material was filtered, and the solvents removed under vacuum using a rotavapor to yield a crude DCM-MeOH extract. This process was repeated, and the extract combined with the first DCM-MeOH extract. The recovered plant material was dried at room temperature, MeOH was added, and the mixture was left on an orbital shaker (120 rpm) at 23 ºC for 24 h. The contents were filtered, and the solvent was evaporated using a rotavapor to yield the MeOH crude extract. This extraction was also repeated a second time, and the two MeOH extracts were combined. The masses of the crude extracts are shown in Table 4.3.

Table 4.3. Mass of crude extracts of different plant parts.

Plant material	Solvents	Weight of dry material (g)	Weight of extracts (g)	Description	Fraction
Leaves	DCM	10	0.399		BBD-1-5A
	DCM-MeOH	10 50	0.650 5.874		BBD-1-7B BBD-1-11A
	MeOH	10 50	0.603 2.718		BBD-1-8B BBD-1-11B
Leaves mass extraction	DCM DCM-MeOH MeOH	450	42.315 14.493 14.810	Dark green Dark green Dark green	BBD-1-15A BBD-1-15B BBD-1-15C
Branches	DCM-MeOH	50	3.744		BBD-1-15D
	MeOH	50	1.693		BBD-1-15E
Branches mass extraction	DCM-MeOH MeOH	470	23.234 13.934	Brown Dark brown	BBD-1-27A BBD-1-27B
Roots	DCM	10	0.079		BBD-1-5A
	DCM-MeOH	10 50	0.293 2.024		BBD-1-7A BBD-1-21A
	MeOH	10 50	0.603 1.997		BBD-1-8A BBD-1-21B

Roots mass extraction	DCM-MeOH MeOH	550	7.933 6.878	Brown Dark brown	BBD-1-40A BBD-1-40B
Root barks	DCM	10	0.209		BBD-1-5C
	DCM-MeOH	10 50	0.852 6.516		BBD-1-7C BBD-1-17A
	MeOH	10 50	0.603 4.905		BBD-1-8C BBD-1-17B
Root barks mass extraction	DCM-MeOH MeOH	885	42.296 25.124	Light brown Dark brown	BBD-1-47A BBD-1-47B

4.2.3 Isolation of Pure Compounds

A DCM leave extract (BBD-1-50A) of *D. cotinifolia* (4.128 g) was subjected to VLC on 120 g silica gel. Five fractions, BBD-1-54A to BBD-1-54E, were obtained after performing sequential elution with the solvent systems described in Section 4.1.3. The yields of the fractions were BBD-1-54 A (54 mg), B (1.162 g), C (1.581 g), D (43 mg) and E (245 mg). A TLC analysis was performed on the fractions and fraction B and C were investigated further. Fraction BBD-1-54 C was subjected to VLC on 30 g of silica gel eluting with solvents of increasing polarity, Hex-EtOAc (8:2), (7:3), (6:4), (1:1), (4:6), (7:3), (2:8), EtOAc, and MeOH. A TLC profile was performed, and fractions with the same compounds according to the R_f values were combined. Further isolation on 2 mm CTLC plate was performed for BBD-1-57G (73 mg), with an isocratic solvent system of hexane: Hex: EtOAc (6:4), which yielded 14 mg of kobusin (**3.1**).

Similar to the above: A MeOH leaf extract (BBD-1-50C) of *D. cotinifolia* (12.114 g) was subjected to VLC on 200 g silica gel. Five fractions were obtained with the solvent systems described in Section 4.1.3. The yields of the fractions were BBD-1-67A (11 mg), B (513 mg), C (648 mg), D (537 mg) and E (9.448 g). After performing TLC analysis, fractions with the same R_f values were combined, and BBD-1-67C was further purified on a 4 mm CTLC plate. A TLC analysis was performed on the fractions, and the fractions were combined based on the R_f values. Fraction BBD-1-70B (combination of the BBD-1-67C fractions) was subjected to PCTLC on a 1 mm Chromatotron™ plate and eluted

with an isocratic mixture of hex: EtOAc (6:4), yielding BBD-1-70B2 (17 mg), which both contain eudesmin (**3.2**).

A DCM-MeOH root bark extract (BBD-1-71A) of *D. cotinifolia* (12.046 g) was subjected to VLC on 200 g of silica gel. Five fractions, BBD-1-73A-E were obtained with Hex-DCM (9:1), DCM-EtOAc (20:1), EtOAc, EtOAc-MeOH (5:1) and MeOH as solvent systems. The yields of the fractions were A (11 mg), B (251 mg), C (469 mg), D (752 mg) and E (8.755 g). BBD-1-73 B (125 mg) was subjected to PCTLC on a 4 mm plate with an isocratic solution, hex-EtOAc (7:3), which yielded BBD-1-75I (3 mg), which was also identified as eudesmin (**3.2**).

A MeOH root bark extract (BBD-1-71B) of *D. cotinifolia* (12.001 g) subjected to VLC on 200 g silica gel. Five fractions were obtained from the following solvent systems, Hex-DCM (9:1), DCM-EtOAc (20:1), EtOAc, EtOAc-MeOH (5:1) and MeOH. The yields of the fractions were BBD-1-84C (8 mg), D (313 mg), E (367 mg), F (197 mg) and G (6.164 g), respectively. After considering the TLC profile, fractions with the same R_f values were combined, and BBD-1-84E was fractionated further on a small column with 3 g of silica gel. TLC analysis was performed on the fractions, and the fractions were mixed based on the R_f values. The sample was further isolated on a preparative plate (TLC) using DCM-MeOH (9:1) as a solvent system yielding BBD-1-92A (5mg), which was characterised as catechin (**3.3**).

The crude DCM extract of leaves of *D. cotinifolia* BBD-1-50A (4.128 g) was fractionated on a VLC column. Five fractions were obtained from the following solvent systems, Hex-DCM (9:1), DCM-EtOAc (20:1), EtOAc, EtOAc-MeOH (5:1) and MeOH. The yield of the fraction was as follows; BBD-1-54A (54 mg), B (1.162 g), C (1.581 g), D (43 mg), and E (245 mg) respectively. TLC analysis was performed, and a further fractionation was performed on fraction B. 100 mg of BBD-1-54B was dissolved into a DCM-MeOH (1:1) solvent and left to percolate down the DIOL SPE column for 24 h. The extract was then fractionated with the solvent systems that were used on VLC. The yields of the fractions were BBD-1-104A (30.1 mg), B (40.6 mg), C (9.6 mg), D (3.9 mg) and E (4.2 mg). After TLC analysis, fraction BBD-1-104B was taken for assessment on HPLC. A 40.6 mg of BBD-1-104B fraction was dissolved in HPLC grade MeOH (1 mL), sonicated for 5 min in a SCIENTECH ultrasonic bath. The solution was then filtered through a C_8 SPE cartridge under vacuum. Multiple injections of 200 µL of the extract were performed in HPLC using the standard gradient method (in the experimental section) yielding BBD-1-104B2 (2.5 mg) which was compound (**3.4**). The extraction summary of all the compounds is shown in Figure 4.3.

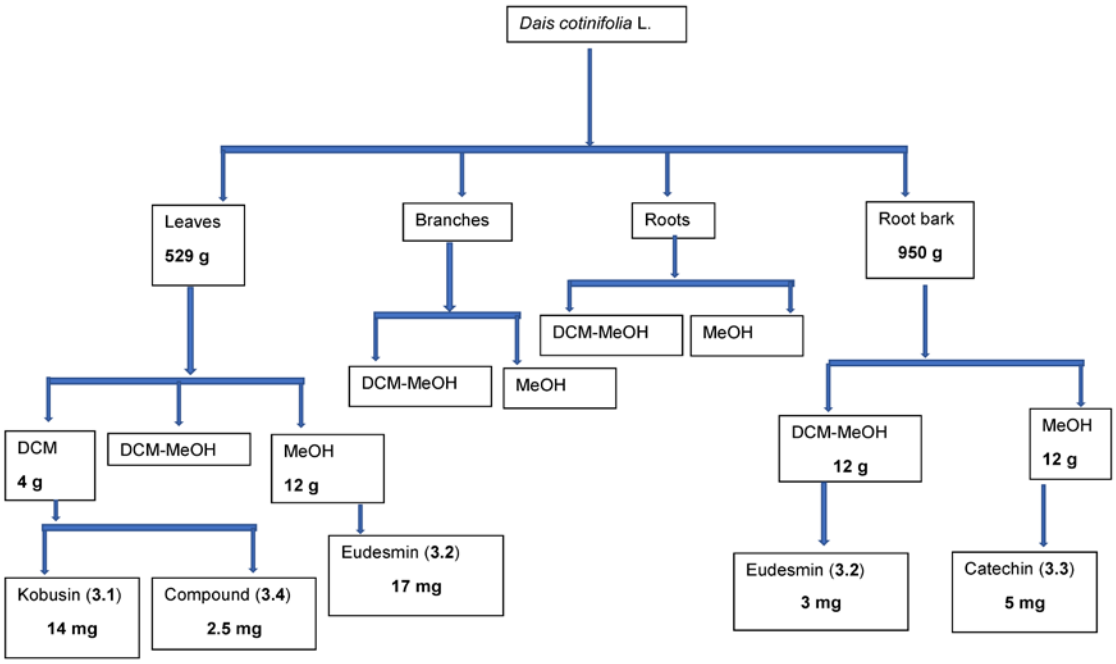


Figure 4.3. Extraction summary.

The physical data for the pure compounds:

Physical data of kobusin (3.1)

Physical description: White powder

Yield: 14 mg

TOF-MS: 393.1

Molecular formula: $C_{22}H_{22}O_6$

NMR data:

^{1}H NMR: Plate 1A

^{13}C NMR: Plate 1B

DEPT: Plate 1C

COSY: Plate 1D

HSQC: Plate 1E

HSQC: Plate 1F (Ar-HSQC)

HMBC: Plate 1G

HMBC: Plate 1H (Ar-HMBC)

Physical data of eudesmin (3.2)

Physical description: White powder

Yield: 17 mg

TOF-MS: 409.3

Molecular formula: $C_{22}H_{26}O_6$

NMR data:

^{1}H NMR: Plate 2A

^{13}C NMR: Plate 2B

DEPT: Plate 2C

COSY: Plate 2D

HSQC: Plate 2E

HMBC: Plate 2F

Physical data of catechin (3.3)

Physical description: Yellow powder

Yield: 5 mg

MS: 291

Molecular formula: $C_{15}H_{14}O_6$

NMR data:

^{1}H NMR: Plate 3A

^{13}C NMR: Plate 3B

DEPT: Plate 3C

COSY: Plate 3D

HSQC: Plate 3E

HMBC: Plate 3F

Physical data of compound 3.4

Physical description: White powder

Yield: 2.5 mg

TOF-MS: 337.1

Molecular formula:

NMR data:

^{1}H NMR: Plate 4A

COSY: Plate 4B

HSQC: Plate 4C

HMBC: Plate 4D

4.3 Biological Assay

4.3.1 DPPH Radical Scavenging Assay

This experiment was carried out according to Shivashankara *et* al., 2004.

Preparation of stock solutions

1 mM DPPH methanolic stock solution: 0.0049 g of DPPH was dissolved in 12.5 mL of methanol in the dark and stored at -20 ºC before use.

0.1 mM DPPH working solution (10x dilution): 10 mL of the stock solution was mixed with 90 mL of methanol and was used immediately.

2 mM ascorbic acid (AA) standard solution: 0.0035 g of AA was dissolved in 10 mL of methanol. Dilutions of AA in a 2 mL centrifuge tube was prepared as indicated in Table 4.4 below.

Table 4.4. Prepared dilutions of ascorbic acid (AA).

	Concentration (mg/mL)	**2 mM AA (µL)**	**Methanol (µL)**	**Final volume (µL)**
Std1	0	0	1000	1000
Std2	0.07	200	800	1000
Std3	0.14	400	600	1000
Std4	0.21	600	400	1000
Std5	0.28	800	200	1000
Std6	0.35	1000	0	1000

Sample preparation: For each crude extract (leaves, root bark and roots MeOH extracts) of *D. cotinifolia*, the following procedure was followed. The extract (0.5 g) was dissolved in 10 mL of methanol using an Ultra Turrax ® T25 basic homogeniser (IKA Works, Willmington NC). The mixture was then sonicated for 30 minutes in a CHECK (Bransonic Ultrasonic Co. Danbury, CT) and centrifuged at 10 000 rpm for 10 min at 4 °C. The aliquot was collected and stored at -26 °C before analysis with 2,2-diphenyl-1-picrylhydrazyl (DPPH).

200 µL of each standard extract was added into a clean cuvette, 800 µL of methanol was added to each tube, and then 1 mL of 0.1 mM DPPH solution was added to each tube in the dark, covered with aluminium foil and allowed to stand for 60 min at room temperature. The absorbance was measured at 517 nm against the blank (methanol) under dim light. Table 4.5 below, shows the dilutions of different plant extracts.

Table 4.5. The standard stock solutions of different parts of the plant extract (leaves, root bark, and roots) were diluted into 2 mL centrifuge tubes as follows:

	Extracts concentration (mg/mL)	**Extract (µL)**	**Methanol (µL)**	**Final volume (µL)**
Std1	0	0	1000	1000
Std2	5	200	800	1000
Std3	10	400	600	1000
Std4	15	600	400	1000
Std5	20	800	200	1000
Std6	25	1000	0	1000

A 0.1 mM DPPH solution in methanol was prepared and 1 mL of the solution was added to 1 mL of the sample. After shaking the mixture vigorously, it was allowed to remain at room temperature in the dark for 30 minutes. The absorbance of each mixture was measured in triplicates at 517 nm using a UV Shimadzu UV-1800 spectrometer. Ascorbic acid (AA) was used as the standard because of its intense reducing power and a weak metal chelating ability. The absorbance was measured at different concentrations of AA as depicted in Table 4.4. The IC_{50} was calculated using different concentrations of the extracts/ascorbic acid versus radical scavenging capacity curve. The IC_{50} value is the concentration of an analyte or sample that is needed to inhibit 50% of the DPPH free radicals. The free radical scavenging activity was observed by the discolouration of the DPPH solution. The percentage DPPH scavenging effect of each extract was calculated by the following equation:

$$\%\ Radical\ Scavenging\ Activity = \frac{Abs\ (blank) - Abs(extract)}{Abs\ (blank)} \times 100$$

The blank was the absorbance without the extract or AA. Extract indicated the absorbance that was taken in the presence of an extract or AA. Abs denoted the absorbance.

Chapter 5: Conclusion

In this thesis, a phytochemical investigation of the tree *Dais cotinifolia* (Thymelaeaceae) is described in detail. Many bioactive compounds have been isolated from the Thymelaeaceae, such as prostratin (potent anti-HIV agent) and antitumour diterpenoids. Although a large number of Thymelaeaceae species are indigenous to South Africa, only a limited number of species have been subjected to phytochemical investigations. *Dais cotinifolia* L. is one species on which phytochemical results have not been published and this tree was the subject of this investigation.

Four compounds were isolated from the different parts of the plant, two furofuran lignans, kobusin (**3.1**) and eudesmin (**3.2**), a flavonoid, catechin (**3.3**), and a novel norlignan (**3.4**). The antioxidants activities in plants extracts are often associated with the presence of phenolic compounds. Catechin (**3.1**) is a known antioxidant. The two lignans and the norlignan only have methoxy substituents on the phenyl rings and there are no free hydroxy groups. The lack of free hydroxy groups on the major compounds may explain the relatively low antioxidant activity observed for the crude extracts of the different plant parts of *D. cotinifolia*.

Lignans are known to have anticancer and anti-HIV activity. The norlignans are associated with a variety of biological activities. Advanced biological assays were not part of this investigation but the compounds isolated, especially the novel norlignan need to be assessed for their biological acvtivities.

Future work should include

- The isolation of larger amounts of compounds
- The reinvestigation of the extracts for the presence of the interesting norlignans
- Determination of the biological activity of the compounds
- The relatively simple structure of the norlignan may lead to the synthesis of this compound and synthetic analogues for bioactivity assays.

References

ADOLF, W. & HECKER, E. 1982. On the active principles of the Thymelaeaceae. *Planta Medica,* 45**,** 177-182.

AGGARWAL, S., THAREJA, S., VERMA, A., BHARDWAJ, T. R. & KUMAR, M. 2010. An overview on 5α-reductase inhibitors. *Steroids,* 75**,** 109-153.

AKIYAMA, H., YAMASAKI, O., KANZAKI, H., TADA, J. & ARATA, J. 1998. Effects of sucrose and silver on *Staphylococcus aureus* biofilms. *The Journal of Antimicrobial Chemotherapy,* 42**,** 629-634.

AKROUT, A., GONZALEZ, L. A., EL JANI, H. & MADRID, P. C. 2011. Antioxidant and antitumor activities of *Artemisia campestris* and *Thymelaea hirsuta* from southern Tunisia. *Food and Chemical Toxicology,* 49**,** 342-347.

ALTAF, R., ASMAWI, M. Z. B., DEWA, A., SADIKUN, A. & UMAR, M. I. 2013. Phytochemistry and medicinal properties of *Phaleria macrocarpa* (Scheff.) Boerl. extracts. *Pharmacognosy Reviews,* 7**,** 73.

ASADA, Y., SUKEMORI, A., WATANABE, T., MALLA, K. J., YOSHIKAWA, T., LI, W., KUANG, X., KOIKE, K., CHEN, C.-H. & AKIYAMA, T. 2013. Isolation, structure determination, and anti-HIV evaluation of tigliane-type diterpenes and biflavonoid from *Stellera chamaejasme*. *Journal of Natural Products,* 76**,** 852-857.

BADAWI, M. M., HANDA, S. S., KINGHORN, A. D., CORDELL, G. A. & FARNSWORTH, N. R. 1983. Plant anticancer agents XXVII: Antileukemic and cytotoxic constituents of *Dirca occidentalis* (Thymelaeaceae). *Journal of Pharmaceutical Sciences,* 72**,** 1285-1287.

BAKOTA, E. L., WINKLER-MOSER, J. K., HWANG, H. S., BOWMAN, M. J., PALMQUIST, D. E. & LIU, S. X. 2013. Solvent fractionation of rice bran oil to produce a spreadable rice bran product. *European Journal of Lipid Science and Technology,* 115**,** 847-857.

BARCH, D. H., RUNDHAUGEN, L. M., STONER, G. D., PILLAY, N. S. & ROSCHE, W. A. 1996. Structure-function relationships of the dietary anticarcinogen ellagic acid. *Carcinogenesis,* 17**,** 265-269.

BEAUMONT, A. J., EDWARDS, T. J., MANNING, J., MAURIN, O., RAUTENBACH, M., MOTSI, M. C., FAY, M. F., CHASE, M. W. & VAN DER BANK, M. 2009. *Gnidia* (Thymelaeaceae) is not monophyletic: taxonomic implications for Thymelaeoideae and a partial new generic taxonomy for *Gnidia*. *Botanical Journal of the Linnean Society,* 160**,** 402-417.

BENZIE, I. F. & WACHTEL-GALOR, S. 2011. *Herbal Medicine: Biomolecular and Clinical Aspects,* Boca Raton, CRC Press.

BEYERS, J. B. & MARAIS, E. M. 1998. Palynological studies of the Thymelaeaceae of the Cape Flora. *Grana,* 37**,** 193-202.

BEYERS, J. B. & VAN WYK, A. 1998. A new species of *Lachnaea* (Thymelaeaceae) from the Western Cape. *South African Journal of Botany,* 64**,** 66-69.

BEYERS, J. B. P. & VAN DER WALT, J. J. A. 1995. The generic delimitation of Lachnaea and Cryptadenia (Thymelaeaceae). *Bothalia,* 25**,** 65-85.

BGCI. 2017. *Plant Species Number* [Online]. Botanical Gardens Conservation International. Available: https://www.bgci.org/policy/1521/ [Accessed 28 November].

BIAN, G., DENG, Z. & LIU, T. 2017. Strategies for terpenoid overproduction and new terpenoid discovery. *Current Opinion in Biotechnology,* 48**,** 234-241.

BLASKÓ, G., XUN, L. & CORDELL, G. A. 1988. Studies in the Thymelaeaceae, V. 2'-Hydroxyflavone from *Daphnopsis sellowiana*: Isolation and Synthesis. *Journal of Natural Products,* 51**,** 60-65.

BOATWRIGHT, J., VAN DER BANK, M., MAGEE, A. & MANNING, J. 2017. Transfer of Madagascan species of *Gnidia* L. to *Lasiosiphon* Fresen.(Thymelaeaceae: Thymelaeoideae). *South African Journal of Botany,* 112**,** 336-337.

BOND, T. C. & BERGSTROM, R. W. 2006. Light absorption by carbonaceous particles: An investigative review. *Aerosol Science and Technology,* 40**,** 27-67.

BORRIS, R. P., BLASKÓ, G. & CORDELL, G. A. 1988. Ethnopharmacologic and phytochemical studies of the Thymelaeaceae. *Journal of Ethnopharmacology,* 24**,** 41-91.

BOUQUET, J., RIVAUD, M., CHEVALLEY, S., DEHARO, E., JULLIAN, V. & VALENTIN, A. 2012. Biological activities of nitidine, a potential anti-malarial lead compound. *Malaria Journal,* 11**,** 67.

BREITKOPF, S. B., RICOULT, S. J., YUAN, M., XU, Y., PEAKE, D. A., MANNING, B. D. & ASARA, J. M. 2017. A relative quantitative positive/negative ion switching method for untargeted lipidomics via high resolution LC-MS/MS from any biological source. *Metabolomics,* 13**,** 1-21.

BRUICE, P. Y. 2014. *Organic Chemistry,* Upper Saddler River, Pearson Prentice Hall.

BRUNETON, J. 1999. *Pharmacognosy, Phytochemistry, Medicinal Plants,* Paris, Lavoisier publishing.

CANÁRIO, C., SILVESTRE, S., FALCÃO, A. & ALVES, G. 2017. Steroidal oximes: useful compounds with antitumor activities. *Current Medicinal Chemistry,* 25**,** 660-686.

CELEBIOGLU, N., OZGEN, U. & SECEN, H. 2017. Naturally Occurring 1, 5-Diarylpentanoids: A Review. *Organic Communications,* 10**,** 250.

CHANG, H., WANG, Y., GAO, X., SONG, Z., AWALE, S., HAN, N., LIU, Z. & YIN, J. 2017. Lignans from the root of *Wikstroemia indica* and their cytotoxic activity against PANC-1 human pancreatic cancer cells. *Fitoterapia,* 121**,** 31-37.

CHAPEKAR, M. & SAHASRABUDHE, M. 1981. Mode of action of GCE: an active anticancer principle isolated from an indigenous plant *Gymnosporia rothiana* Laws. *Indian Journal of Experimental Biology,* 19**,** 333.

CHEN, J., LIU, X. & SHI, Y.-P. 2004. Determination of daphnetin in *Daphne tangutica* and its medicinal preparation by liquid chromatography. *Analytica Chimica Acta,* 523**,** 29-33.

CHEN, Y., FU, W. W., SUN, L. X., WANG, Q., QI, W. & YU, H. 2009. A new coumarin from *Wikstroemia indica* (L.) CA Mey. *Chinese Chemical Letters,* 20**,** 592-594.

CHUNG, K. T., SE JR, S., LIN, W. F. & WEI, C. 1993. Growth inhibition of selected food-borne bacteria by tannic acid, propyl gallate and related compounds. *Letters in Applied Microbiology,* 17**,** 29-32.

CLAYDEN, J., GREEVES, N., WARREN, S. & WOTHERS, P. 2001. *Organic Chemistry,* Oxford Oxford University Press.

CRABTREE, W. M. & BELOFSKY, G. 2010. Isolation and characterization of compounds from organic extracts of *Dais cotinifolia* (Thymelaeaceae). *Joint 65th Northwest and 22nd Rocky Mountain Regional Meeting of the American Chemical Society, Pullman, WA, United States.* American Chemical Society.

CRAGG, G. M., GROTHAUS, P. G. & NEWMAN, D. J. 2009. Impact of natural products on developing new anti-cancer agents. *Chemical Reviews,* 109**,** 3012-3043.

CRAGG, G. M., NEWMAN, D. J. & SNADER, K. M. 1997. Natural products in drug discovery and development. *Journal of Natural Products,* 60**,** 52-60.

CROUCH, N., DIEDERICHS, N., DREWES, S., FEITER, U., GELDENHUYS, C., MANDER, M., MCKEAN, S., MITCHELL, D., SPRING, W. & SYMMONDS, R. W., R 2006. *Commercialising Medicinal Plants A Southern African Guide,* Stellenbosch, SUN PRESS.

DAVIN, L. B. & LEWIS, N. G. 2000. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. *Plant Physiology,* 123**,** 453-462.

DENGADA, A. H. 2014. *Isolation and Structural Elucidation of Compounds from Natural Products.* MSc, Virginia Tech.

DESAI, H. K., JOSHI, B. S., PANU, A. M. & PELLETIER, S. W. 1985. Separation of diterpenoid alkaloid mixtures using the Chromatotron. *Journal of Chromatography A,* 322**,** 223-227.

DEVKOTA, H. P., WATANABE, M., WATANABE, T. & YAHARA, S. 2012. Diarylpentanoids from Diplomorpha canescens and Diplomorpha ganpi. *Phytochemistry Letters,* 5**,** 284-286.

DHARMADASA, R., SIRIWARDANA, A., SAMARASINGHE, K. & ADHIHETTY, P. 2013. Standardization of *Gyrinops walla* Gaertn (Thymalaeaceae): Newly discovered, fragrant industrial potential, indemic plant from Sri Lanka. *World Journal of Agricultural Research,* 1**,** 101-103.

DJERIDANE, A., YOUSFI, M., NADJEMI, B., BOUTASSOUNA, D., STOCKER, P. & VIDAL, N. 2006. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry,* 97**,** 654-660.

EL AMRANI, F., RHALLAB, A., ALAOUI, T., EL BADAOUI, K. & CHAKIR, S. 2009. Hypoglycaemic effect of *Thymelaea hirsuta* in normal and streptozotocin-induced diabetic rats. *Journal of Medicinal Plants Research,* 3**,** 625-629.

ENGELHARDT, H. 2012. *High Performance Liquid Lhromatography*, Springer Science & Business Media.

ERNST, R. R., BODENHAUSEN, G. & WOKAUN, A. 1987. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions,* Oxford Clarendon Press.

FERRARI, J., TERREAUX, C., SAHPAZ, S., MSONTHI, J. D., WOLFENDER, J.-L. & HOSTETTMANN, K. 2000. Benzophenone glycosides from *Gnidia involucrata*. *Phytochemistry,* 54**,** 883-889.

FIELD, L. D., KALMAN, J. R. & STERNHELL, S. S. 2006. *Organic Structure from Spectra,* England, John Wiley and Sons LTD.

FIRN, R. D. & JONES, C. G. 2003. Natural products: A simple model to explain chemical diversity. *Natural Product Reports,* 20**,** 382-391.

FÖRSTER, H. 2004. UV/Vis spectroscopy. *Characterization I***,** 2734-2734.

FRANKE, K., PORZEL, A. & SCHMIDT, J. 2002. Flavone-coumarin hybrids from *Gnidia socotrana*. *Phytochemistry,* 61**,** 873-878.

FRIEBOLIN, H. & BECCONSALL, J. K. 1993. *Basic One-and Two-Dimensional NMR Spectroscopy,* University of Heidelberg, American Society of Pharmacognosy.

GHOSH, S., AHIRE, M., PATIL, S., JABGUNDE, A., BHAT DUSANE, M., JOSHI, B. N., PARDESI, K., JACHAK, S., DHAVALE, D. D. & CHOPADE, B. A. 2012. Antidiabetic activity of *Gnidia glauca* and *Dioscorea bulbifera:* Potent amylase and glucosidase inhibitors. *Evidence-Based Complementary and Alternative Medicine,* 2012.

GONYELA, O. 2016. *A scientific investigation of the immunomodulatory properties of an indigenous plant, Sutherlandia frutescens.* MSc, University of South Africa

GUO, J., TIAN, J., YAO, G., ZHU, H., XUE, Y., LUO, Z., ZHANG, J., ZHANG, Y. & ZHANG, Y. 2015. Three new 1α-alkyldaphnane-type diterpenoids from the flower buds of *Wikstroemia chamaedaphne*. *Fitoterapia,* 106**,** 242-246.

GUO, T., DENG, Y.-X., XIE, H., YAO, C.-Y., CAI, C.-C., PAN, S.-L. & WANG, Y.-L. 2011. Antinociceptive and anti-inflammatory activities of ethyl acetate fraction from Zanthoxylum armatum in mice. *Fitoterapia,* 82**,** 347-351.

GUPTA, M., KARMAKAR, N., SASMAL, S., CHOWDHURY, S. & BISWAS, S. 2016. Free radical scavenging activity of aqueous and alcoholic extracts of *Glycyrrhiza glabra* Linn. measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical antioxidant assay. *International Journal of Pharmacology and Toxicology,* 4**,** 235-240.

GUPTA, S. & GILLETT, G. 1969. Observations on Hawaiian species of *Wikstroemia* (Angiospermae: Thymelaeaceae).

HARMATHA, J. & NAWROT, J. Insect feeding deterrent activity of lignans and related phenylpropanoids with a methylenedioxyphenyl (piperonyl) structure moiety. Proceedings of the 11th International Symposium on Insect-Plant Relationships, 2002. Springer, 51-60.

HENDRA, R., AHMAD, S., SUKARI, A., SHUKOR, M. Y. & OSKOUEIAN, E. 2011. Flavonoid analyses and antimicrobial activity of various parts of *Phaleria macrocarpa* (Scheff.) Boerl fruit. *International Journal of Molecular Sciences,* 12**,** 3422-3431.

HU, Y.-Q., XU, Z., ZHANG, S., WU, X., DING, J.-W., LV, Z.-S. & FENG, L.-S. 2017. Recent developments of coumarin-containing derivatives and their anti-tubercular activity. *European Journal of Medicinal Chemistry,* 136**,** 122-130.

HUANG, L.-H., ZHENG, Y.-F., LU, Y.-Z., SONG, C.-J., WANG, Y.-G., YU, B. & LIU, H.-M. 2012. Synthesis and biological evaluation of novel steroidal[17,16-d][1,2,4] triazolo[1,5-a] pyrimidines. *Steroids,* 77**,** 710-715.

IIDA, T., NAKANO, M. & ITO, K. 1982. Hydroperoxysesquiterpene and lignan constituents of Magnolia kobus. *Phytochemistry,* 21**,** 673-675.

ISSAQ, H. J., VEENSTRA, T. D., CONRADS, T. P. & FELSCHOW, D. 2002. The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. *Biochemical and Biophysical Research Communications,* 292**,** 587-592.

JEMAL, A., SIEGEL, R., WARD, E., HAO, Y., XU, J., MURRAY, T. & THUN, M. J. 2008. Cancer statistics, 2008. *CA: A Cancer Journal for Clinicians,* 58**,** 71-96.

KADHUM, A. A. H., AL-AMIERY, A. A., MUSA, A. Y. & MOHAMAD, A. B. 2011. The antioxidant activity of new coumarin derivatives. *International Journal of Molecular Sciences,* 12**,** 5747-5761.

KAMIKADO, T., CHANG, C.-F., MURAKOSHI, S., SAKURAI, A. & TAMURA, S. 1975. Isolation and structure elucidation of growth inhibitors on silkworm larvae from *Magnolia kobus* DC. *Agricultural and Biological Chemistry,* 39**,** 833-836.

KARPOZILOS, A. & PAVLIDIS, N. 2004. The treatment of cancer in Greek antiquity. *European Journal of Cancer,* 40**,** 2033-2040.

KHODADADIAN, Z., HASSANPOUR-EZATTI, M., MOUSAVI, S. Z. & ASGARPANAH, J. 2016. Analgesic and anti-inflammatory potential of aerial parts of the *Daphne mucronata* Royle extract in mice: Opioid-independent action. *Asian Pacific Journal of Tropical Biomedicine,* 6**,** 198-201.

KIPTOON, J., MUGERA, G. & WAIYAKI, P. 1982. Haematological and biomedical changes in cattle poisoned by *Gnidia latifolia* (Oliv.) Gilg synonym of *Lasiosiphon latifolius* (Oliv.) Gill (thymelaeaceae). *Toxicology,* 25**,** 129-139.

KOGISO, S., HOSOZAWA, S., WADA, K. & MUNAKATA, K. 1974. Daphneolone in roots of *Daphne odora*. *Phytochemistry,* 13**,** 2332-2334.

KOLEVA, I. I., VAN BEEK, T. A., LINSSEN, J. P., GROOT, A. D. & EVSTATIEVA, L. N. 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis,* 13**,** 8-17.

KROJER, M., KELLER, M. & BRACHER, F. 2013. 7-Aza-des-A-steroids with antimicrobial and cytotoxic activity. *Scientia Pharmaceutica,* 81**,** 329-338.

KUPCHAN, S. M., SHIZURI, Y., SUMNER JR, W. C., HAYNES, H. R., LEIGHTON, A. P. & SICKLES, B. R. 1976. Tumor inhibitors. 117. The isolation and structural elucidation of new potent antileukemic diterpenoid esters from *Gnidia* species. *The Journal of Organic Chemistry,* 41**,** 3850-3853.

KUPELI, E., TOSUN, A. & YESILADA, E. 2007. Assessment of anti-inflammatory and antinociceptive activities of *Daphne pontica* L (Thymelaeaceae). *Journal of Ethnopharmacology,* 113**,** 332-337.

KURAPATI, K. R. V., ATLURI, V. S., SAMIKKANNU, T., GARCIA, G. & NAIR, M. P. 2016. Natural products as anti-HIV agents and role in HIV-associated neurocognitive disorders (HAND): a brief overview. *Frontiers in Microbiology,* 6**,** 1444.

LANGSETH, L. 1995. *Oxidants, antioxidants, and disease prevention*, ILSI Europe Brussels.

LATIP, J., HARTLEY, T. G. & WATERMAN, P. G. 1999. Lignans and coumarins metabolites from Melicopehayesii. *Phytochemistry,* 51**,** 107-110.

LI, S., CHOU, G., HSEU, Y., YANG, H., KWAN, H. & YU, Z. 2013. Isolation of anticancer constituents from flos genkwa (*Daphne genkwa* Sieb. et Zucc.) through bioassay-guided procedures. *Chemistry Central Journal,* 7**,** 159.

LIAO, S. G., WU, Y. & YUE, J. M. 2006. Lignans from *Wikstroemia hainanensis*. *Helvetica Chimica Acta,* 89**,** 73-80.

LIAO, S. G., ZHANG, B. L., WU, Y. & YUE, J. M. 2005. New phenolic components from *Daphne giraldii*. *Helvetica Chimica Acta,* 88**,** 2873-2878.

LINDSEY, M. E., MEYER, M. & THURMAN, E. 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Analytical Chemistry,* 73**,** 4640-4646.

LUQMAN, S. & PEZZUTO, J. M. 2010. NFκB: A promising target for natural products in cancer chemoprevention. *Phytotherapy Research,* 24**,** 949-963.

MADIKIZELA, B., NDHLALA, A., FINNIE, J. & VAN STADEN, J. 2012. Ethnopharmacological study of plants from Pondoland used against diarrhoea. *Journal of Ethnopharmacology,* 141**,** 61-71.

MARTÍNEZ-PASCUAL, R., MEZA-REYES, S., VEGA-BAEZ, J. L., MERINO-MONTIEL, P., PADRÓN, J. M., MENDOZA, Á. & MONTIEL-SMITH, S. 2017. Novel synthesis of steroidal oximes and lactams and their biological evaluation as antiproliferative agents. *Steroids,* 122**,** 24-33.

MAURIN, O., BOATWRIGHT, J., MANNING, J. & VAN DER BANK, M. 2013. Phylogenetic and evolutionary studies of the family Thymelaeaceae in southern Africa. *South African Journal of Botany***,** 153.

MCGAW, L. & ELOFF, J. 2008. Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties. *Journal of Ethnopharmacology,* 119**,** 559-574.

MEHTA, R. G., MURILLO, G., NAITHANI, R. & PENG, X. 2010. Cancer chemoprevention by natural products: how far have we come? *Pharmaceutical Research,* 27**,** 950-961.

MERUELO, D., LAVIE, G. & LAVIE, D. 1988. Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. *Proceedings of the National Academy of Sciences,* 85**,** 5230-5234.

MEYERS, C. & MEYERS, D. 2008. Thin-layer chromatography. *Current Protocols in Nucleic Acid Chemistry***,** 1-13.

MILDER, I. E., ARTS, I. C., VAN DE PUTTE, B., VENEMA, D. P. & HOLLMAN, P. C. 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. *British Journal of Nutrition,* 93**,** 393-402.

MISHRA, B. B. & TIWARI, V. K. 2011. Natural products: an evolving role in future drug discovery. *European Journal of Medicinal Chemistry,* 46**,** 4769-4807.

MOSS, G. 2000. Nomenclature of lignans and neolignans (IUPAC Recommendations 2000). *Pure and Applied Chemistry,* 72**,** 1493-1523.

MOTHANA, R. A. & LINDEQUIST, U. 2005. Antimicrobial activity of some medicinal plants of the island Soqotra. *Journal of Ethnopharmacology,* 96**,** 177-181.

MOTSI, M., RYE, B. & VAN DER BANK, M. 2008. Molecular phylogenetics of the genera *Pimelea* and *Thecanthes* (Thymelaeaceae). *South African Journal of Botany,* 74**,** 373-381.

MOURI, C., MOZAFFARIAN, V., ZHANG, X. & LAURSEN, R. 2014. Characterization of flavonols in plants used for textile dyeing and the significance of flavonol conjugates. *Dyes and Pigments,* 100**,** 135-141.

MZOZOYANA, V. 2015. *Synthesis of Fluorinated Benzophenones and Phenylcoumarins.* PhD, University of KwaZulu-Natal, Pietermaritzburg.

NAKAHATA, N., KUTSUWA, M., KYO, R., KUBO, M., HAYASHI, K. & OHIZUMI, Y. 1998. Analysis of inhibitory effects of scutellariae radix and baicalein on prostaglandin E2 production in rat C6 glioma cells. *The American Journal of Chinese Medicine,* 26**,** 311-323.

NEVLING, L. I. 1959. A revision of the genus Daphnopsis. *Annals of the Missouri Botanical Garden,* 46**,** 257-358.

NEWMAN, D. J. & CRAGG, G. M. 2016. Natural products as sources of new drugs from 1981 to 2014. *Journal of Natural Products,* 79**,** 629-661.

NGUYEN, N. Y. T., NGUYEN, T. H., DANG, P. H. & LE TRAN, Q. 2017. Three terpenoid glycosides of *Centipeda minima*. *Phytochemistry Letters,* 21**,** 21-24.

NIESSEN, W. M. 2006. *Liquid chromatography-mass spectrometry,* London, Taylor & Fransis Group.

NIMSE, S. B. & PAL, D. 2015. Free radicals, natural antioxidants, and their reaction mechanisms. *Royal Society of Chemistry Advances,* 5**,** 27986-28006.

NISHINO, H., TOKUDA, H., MURAKOSHI, M., SATOMI, Y., MASUDA, M., ONOZUKA, M., YAMAGUCHI, S., TAKAYASU, J., TSURUTA, J. & OKUDA, M. 2000. Cancer prevention by natural carotenoids. *Biofactors,* 13**,** 89-94.

NODA, I., DOWREY, A., MARCOTT, C., STORY, G. & OZAKI, Y. 2000. Generalized two-dimensional correlation spectroscopy. *Applied Spectroscopy,* 54**,** 236A-248A.

NOSHAD, D., MIRESMAILI, S., RISEMAN, A. & EKRAMODDOULLAH, A. 2009. In vitro propagation of seven *Daphne* L. species. *Plant Cell, Tissue and Organ Culture (PCTOC),* 96**,** 201-209.

OKUSHIO, K., MATSUMOTO, N., KOHRI, T., SUZUKI, M., NANJO, F. & HARA, Y. 1996. Absorption of tea catechins into rat portal vein. *Biological and Pharmaceutical Bulletin,* 19**,** 326-329.

OLADIPO, O. & OYANIRAN, A. 2013. Wood anatomy and taxonomy of members of the genus *Ocimum* L.(Lamiaceae) and a variety in Nigeria. *South African Journal of Botany***,** 152.

PETEROS, N. P. & UY, M. M. 2010. Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants. *Journal of Medicinal Plants Research,* 4**,** 407-414.

RBGK. 2017. *Useful plants medicines* [Online]. Royal Botanical Gardens Kew. Available: https://stateoftheworldsplants.com/2017/useful-plants.html [Accessed 28 November 2017].

REDDY, L., ODHAV, B. & BHOOLA, K. 2003. Natural products for cancer prevention: a global perspective. *Pharmacology & Therapeutics,* 99**,** 1-13.

RICHARDS, R. & SHARMA, H. 1991. Free radicals in health and disease. *Indian Journal of Clinical Practice,* 2**,** 15-26.

ROBINSON, T. 1974. Metabolism and function of alkaloids in plants. *Science,* 184**,** 430-435.

ROEDER, E. & WIEDENFELD, H. 2011. Pyrrolizidine alkaloids in plants used in the traditional medicine of Madagascar and the Mascarene islands. *Die Pharmazie-An International Journal of Pharmaceutical Sciences,* 66**,** 637-647.

SANBI. 2017. *Dais cotinifolia L.* [Online]. Available: <http://pza.sanbi.org/dais-cotinifolia> [Accessed 2017].

SANDA, M. A., ZENGIN, G., AKTUMSEK, A. & CAKMAK, Y. S. 2015. Evaluation of antioxidant potential of two *Daphne* species (*D. gnidioides and D. pontica*) from Turkey. *Emirates Journal of Food and Agriculture,* 27**,** 488.

SASHIDHARA, K. V., KUMAR, A., KUMAR, M., SARKAR, J. & SINHA, S. 2010. Synthesis and in vitro evaluation of novel coumarin–chalcone hybrids as potential anticancer agents. *Bioorganic & Medicinal Chemistry Letters,* 20**,** 7205-7211.

SCARTEZZINI, P. & SPERONI, E. 2000. Review on some plants of Indian traditional medicine with antioxidant activity. *Journal of Ethnopharmacology,* 71**,** 23-43.

SCHANEBERG, B. & KHAN, I. 2004. Quantitative and qualitative HPLC analysis of thermogenic weight loss products. *Die Pharmazie-An International Journal of Pharmaceutical Sciences,* 59**,** 819-823.

SHARMA, O. P. & BHAT, T. K. 2009. DPPH antioxidant assay revisited. *Food Chemistry,* 113**,** 1202-1205.

SHELEMBE, B. G. 2014. *Phytochemical and Elemental Studies of Two Indigenous Medicinal Plants of South Africa, Bridelia micrantha and Sideroxylon inerme.* MSc, University of KwaZulu-Natal, Durban.

SHIVASHANKARA, K., ISOBE, S., AL-HAQ, M. I., TAKENAKA, M. & SHIINA, T. 2004. Fruit antioxidant activity, ascorbic acid, total phenol, quercetin, and carotene of Irwin mango fruits stored at low temperature after high electric field pretreatment. *Journal of Agricultural and Food Chemistry,* 52**,** 1281-1286.

SHYU, Y.-S. & HWANG, L. S. 2002. Antioxidative activity of the crude extract of lignan glycosides from unroasted Burma black sesame meal. *Food Research International,* 35**,** 357-365.

SILCOCK, R., MANN, M., CHOW, S. & VITELLI, J. 2012. Herbicides to control poisonous *Pimelea* species (Thymelaeaceae). *Crop Protection,* 31**,** 99-106.

SKOOG, D., WEST, D., HOLLER, F. & CROUCH, S. 2004. *Fundamentals of Analytical Chemistry.,* Brooks/Cole, Mary Finch.

SMITH, B. & TOWERS, N. 1985. Pithomycotoxicosis (facial eczema) in New Zealand and the use of zinc salts for its prevention. *Plant Toxicology***,** 70-79.

SOFOWORA, A. 1982. Medicinal plants and traditional medicine in Africa.

SUN, W. X., ZHANG, Q. & JIANG, J. Q. 2006. Chemical constituents of *Daphne giraldii* Nitsche. *Journal of Integrative Plant Biology,* 48**,** 1498-1501.

SUZUKI, S. & UMEZAWA, T. 2007. Biosynthesis of lignans and norlignans. *Journal of Wood Science,* 53**,** 273.

TAKAHASHI, K. & NAKAGAWA, T. 1966. Studies on constituents of medicinal plants. VIII. The stereochemistry of paulownin and isopaulownin. *Chemical and Pharmaceutical Bulletin,* 14**,** 641-647.

TAYLOR, P. J. 2005. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry,* 38**,** 328-334.

TESSO, H. 2005. *Isolation and structure elucidation of natural products from plants.* PhD, Alemaya University.

THAIPONG, K., BOONPRAKOB, U., CROSBY, K., CISNEROS-ZEVALLOS, L. & BYRNE, D. H. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis,* 19**,** 669-675.

THEODORIDOU, K., AUFRÈRE, J., ANDUEZA, D., POURRAT, J., LE MORVAN, A., STRINGANO, E., MUELLER-HARVEY, I. & BAUMONT, R. 2010. Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on in vivo and in situ digestion in sheep. *Animal Feed Science and Technology,* 160**,** 23-38.

THOMPSON, L. U. 1998. 9Experimental studies on lignans and cancer. *Bailliere Clinical Endocrinology and Metabolism,* 12**,** 691-705.

TIETZE, L. F., VOCK, C. A., KRIMMELBEIN, I. K. & NACKE, L. 2009. Synthesis of novel structurally simplified estrogen analogues with electron-donating groups in ring A. *Synthesis,* 2009**,** 2040-2060.

TYLER, M. I. & HOWDEN, M. E. 1981. Piscicidal constituents of *Pimelea* species. *Tetrahedron Letters,* 22**,** 689-690.

TYLER, M. I. & HOWDEN, M. E. 1985. Antineoplastic and piscicidal 1-alkyldaphnane orthoesters from *Pimelea* species. *Journal of Natural Products,* 48**,** 440-445.

VEERESHAM, C. 2012. Natural products derived from plants as a source of drugs. Wolters Kluwer--Medknow Publications.

VINCENT, M. A. & THIERET, J. W. 1987. *Thymelaea passerina* (Thymelaeaceae) in Ohio. *Sida Contributions to Botany*, 75-78.

WATT, J. M. & BREYER-BRANDWIJK, M. G. 1962. *The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal,* Edinburgh and London, E and S Livingstone Ltd.

WHO. 2014. *World Cancer Report 2014* [Online]. International Agency for Research on Cancer. Available: http://www.who.int/cancer/prevention/en/ [Accessed 29 November 2017].

WIDSTEN, P., HEATHCOTE, C., KANDELBAUER, A., GUEBITZ, G., NYANHONGO, G. S., PRASETYO, E. N. & KUDANGA, T. 2010. Enzymatic surface functionalisation of lignocellulosic materials with tannins for enhancing antibacterial properties. *Process Biochemistry,* 45, 1072-1081.

XIE, Y., SEN, B. & WANG, G. 2017. Mining terpenoids production and biosynthetic pathway in thraustochytrids. *Bioresource Technology*.

XU, W.-H., ZHAO, P., WANG, M. & LIANG, Q. 2018. Naturally occurring furofuran lignans: structural diversity and biological activities. *Natural Product Research*, 1-17.

YADAV, R. & AGARWALA, M. 2011. Phytochemical analysis of some medicinal plants. *Journal of Phytology,* 3, 10-14.

YAHYAOUI, M., BOUAJILA, J., CAZAUX, S. & ABDERRABBA, M. 2018. The impact of regional locality on chemical composition, anti-oxidant and biological activities of *Thymelaea hirsuta* L. extracts. *Phytomedicine*, 13-23.

YAMAGUCHI, T., TAKAMURA, H., MATOBA, T. & TERAO, J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Bioscience, Biotechnology, and Biochemistry,* 62, 1201-1204.

YANG, Y. J., PARK, J. I., LEE, H.-J., SEO, S.-M., LEE, O.-K., CHOI, D.-H., PAIK, K.-H. & LEE, M. K. 2006. Effects of (+)-eudesmin from the stem bark ofMagnolia kobus DC. var. borealis Sarg. on neurite outgrowth in PC12 cells. *Archives of Pharmacal Research,* 29, 1114-1118.

YU, T., ZHANG, P., ZHAO, Y., ZHANG, H., MENG, J., FAN, D., CHEN, L. & QIU, Y. 2010. Synthesis, crystal structure and photo- and electro-luminescence of the coumarin derivatives with benzotriazole moiety. *Organic Electronics,* 11, 41-49.

YUAN, T.-Q., SUN, S.-N., XU, F. & SUN, R.-C. 2011. Characterization of lignin structures and lignin–carbohydrate complex (LCC) linkages by quantitative 1D carbon 13 and 2D HSQC NMR spectroscopy. *Journal of Agricultural and Food Chemistry,* 59, 10604-10614.

ZAVADA, M. & LOWREY, T. 1995. Floral heteromorphism in *Dais cotinifolia* L.(Thymelaeaceae): a possible case of heterostyly. *Bulletin du Museum National D'histoire Naturelle*, 1-2.

ZHANG, H., LUO, Q., MAO, Y., ZHAO, Y. & YU, T. 2017a. Synthesis and characterization of coumarin-biphenyl derivatives as organic luminescent materials. *Journal of Photochemistry and Photobiology A: Chemistry*, 363-374.

ZHANG, H., LUO, Q., MAO, Y., ZHAO, Y. & YU, T. 2017b. Synthesis and characterization of coumarin-biphenyl derivatives as organic luminescent materials. *Journal of Photochemistry and Photobiology A: Chemistry,* 346, 10-16.

ZHANG, Q., YE, N., SUN, W.-X., ZHANG, K.-M. & JIANG, J.-Q. 2008. Phytochemical investigation of *Daphne giraldii* Nitsche (Thymelaeaceae). *Biochemical Systematics and Ecology,* 36, 63-67.

ZHENG, W.-F., SHI, F. & WANG, L. 2006. Analgesic activity of ethanol extracts from root of *Daphne genkwa*. *Chinese Traditional and Herbal Drugs,* 37, 398.

ZHENG, W., GAO, X., GU, Q., CHEN, C., WEI, Z. & SHI, F. 2007. Antitumor activity of daphnodorins from *Daphne genkwa* roots. *International Immunopharmacology,* 7, 128-134.

Appendix

Plate 1A. ^{1}H NMR spectrum of kobusin (**3.1**) in $CDCl_3$ at 400 MHz.

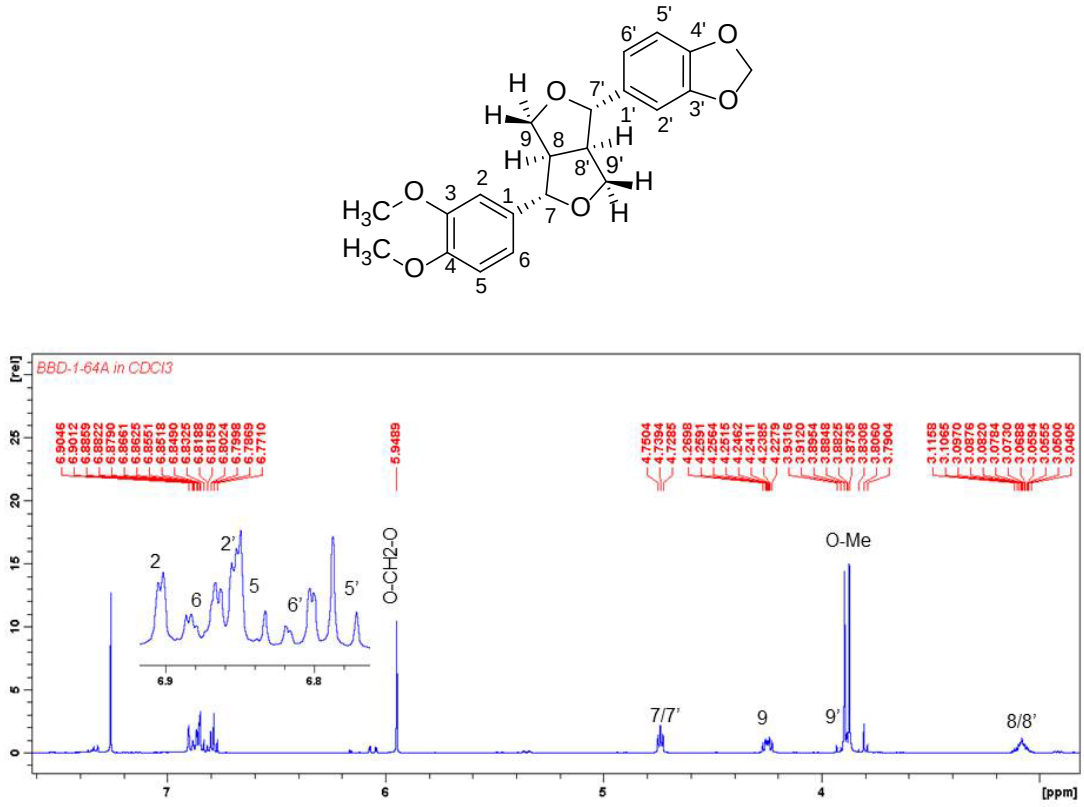


Plate 1B. ^{13}C NMR spectrum of kobusin (**3.1**) in $CDCl_3$ at 100 MHz.

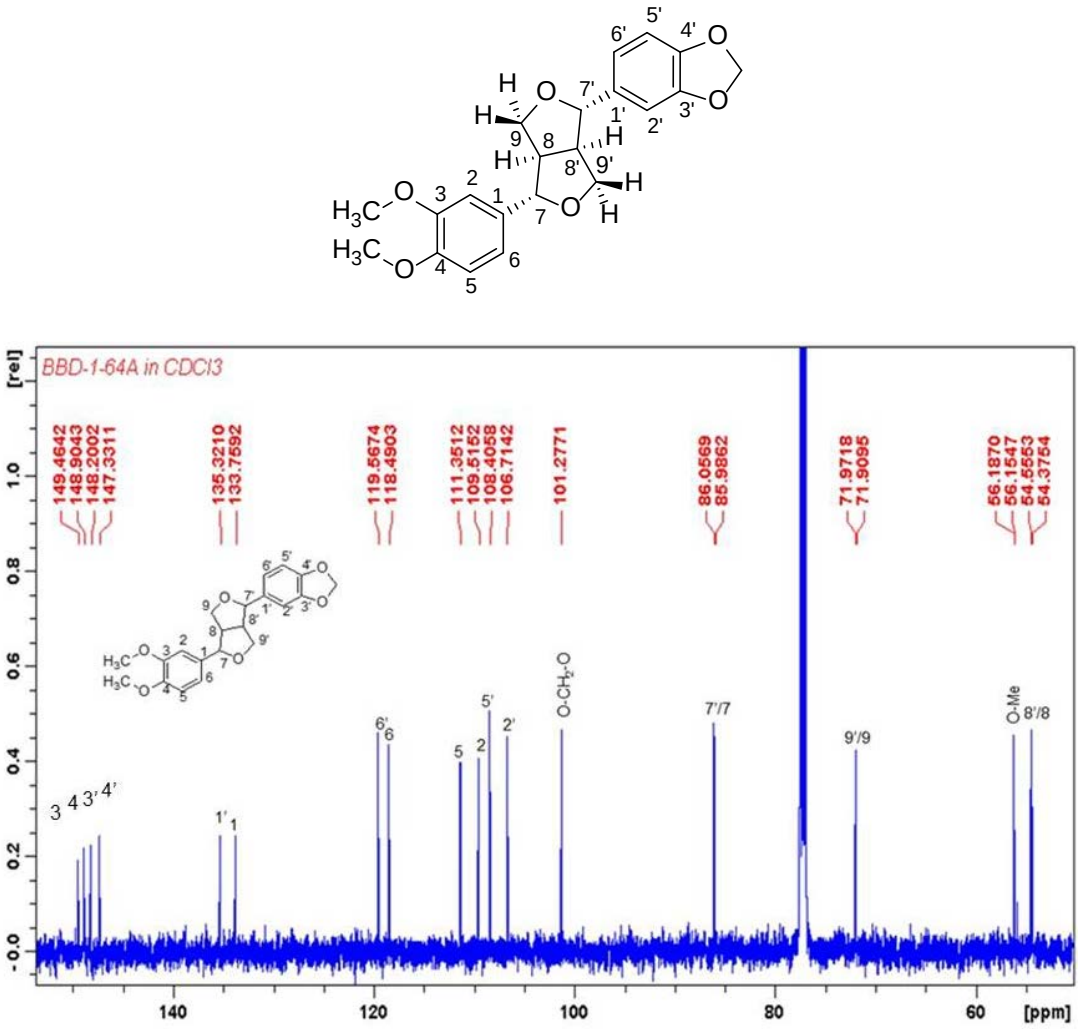


Plate 1C. DEPT 135 NMR spectrum of kobusin (**3.1**) in $CDCl_3$ at 100 MHz.

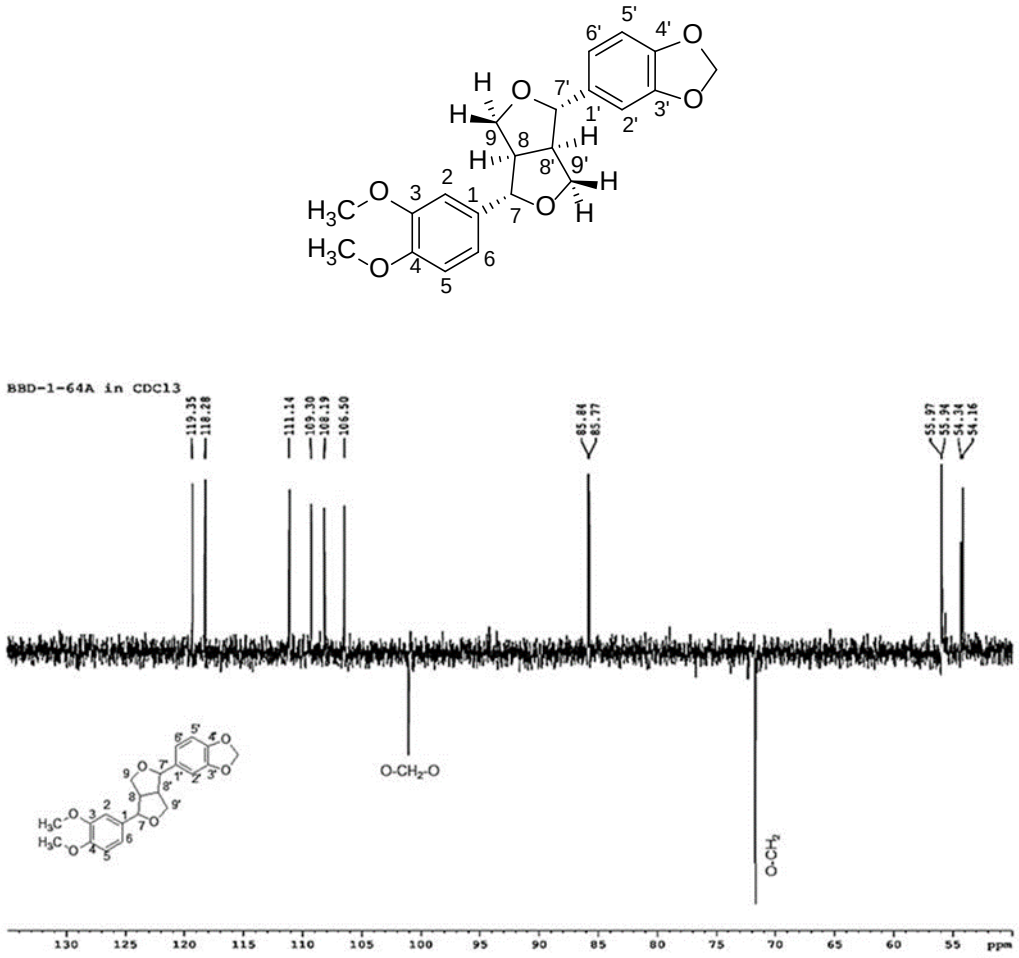


Plate 1D. COSY NMR spectrum of kobusin (**3.1**) in $CDCl_3$.

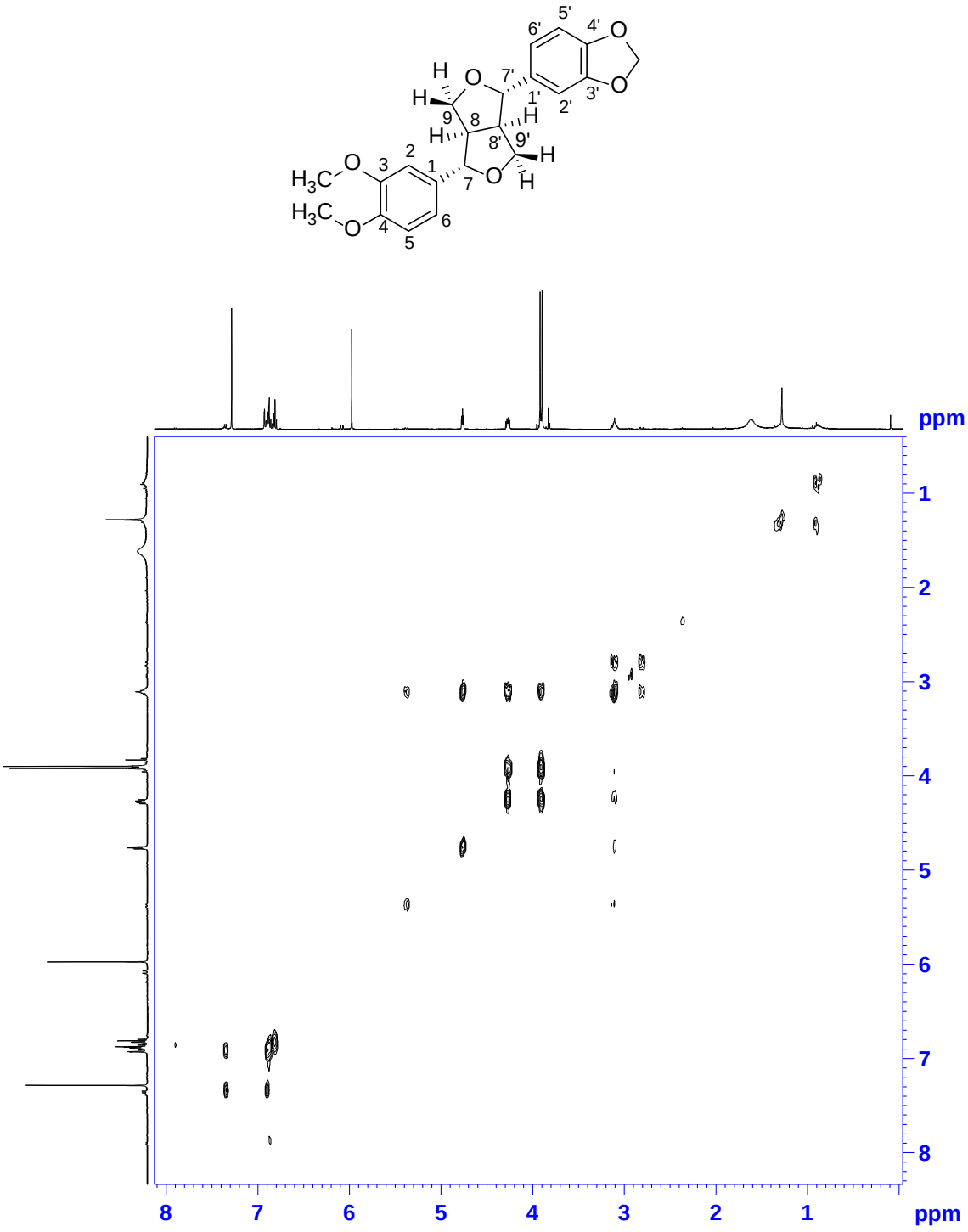


Plate 1E. HSQC NMR spectrum of kobusin (**3.1**) in $CDCl_3$.

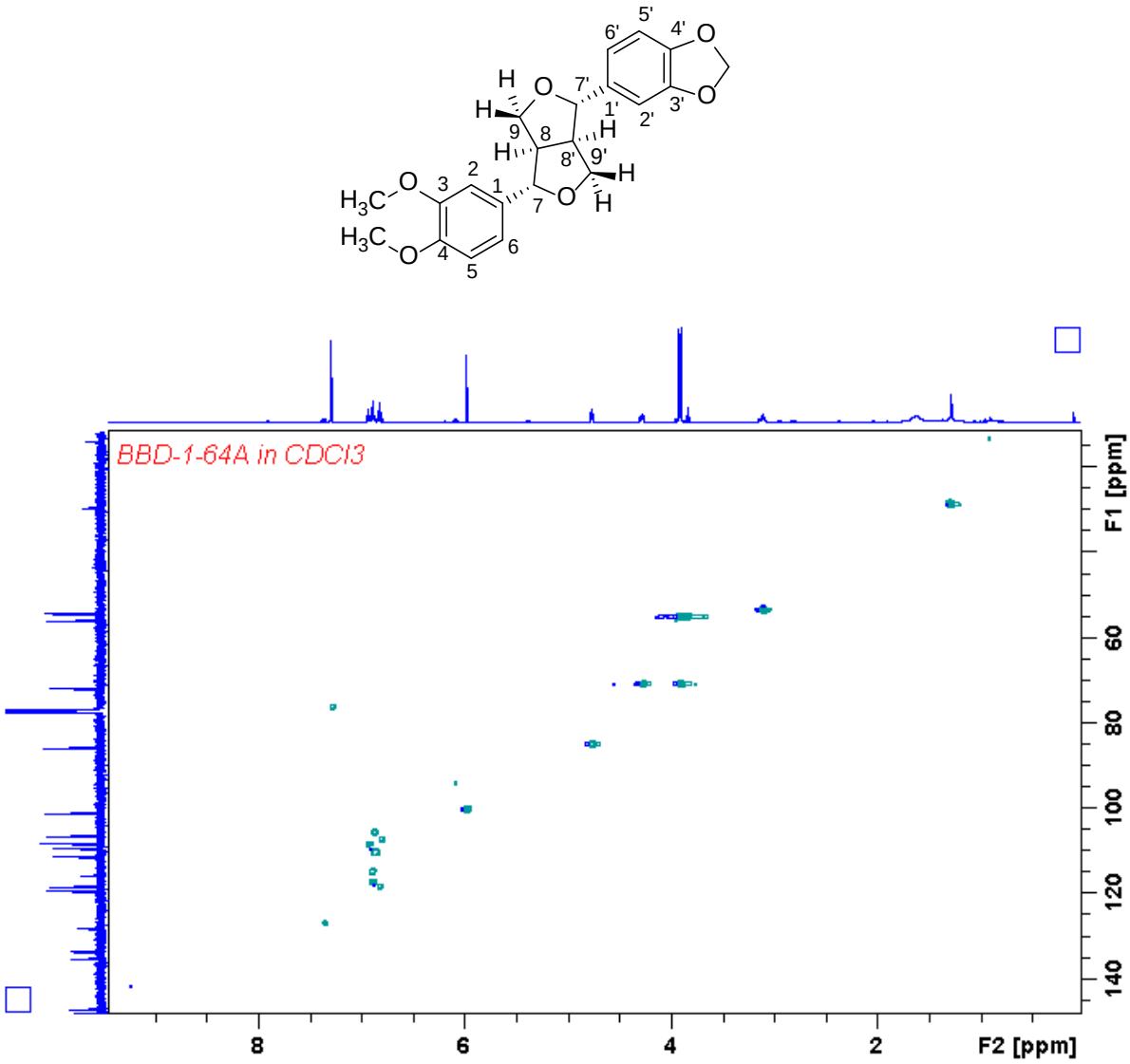


Plate 1F. HSQC NMR aromatic region spectrum of kobusin (**3.1**) in $CDCl_3$.

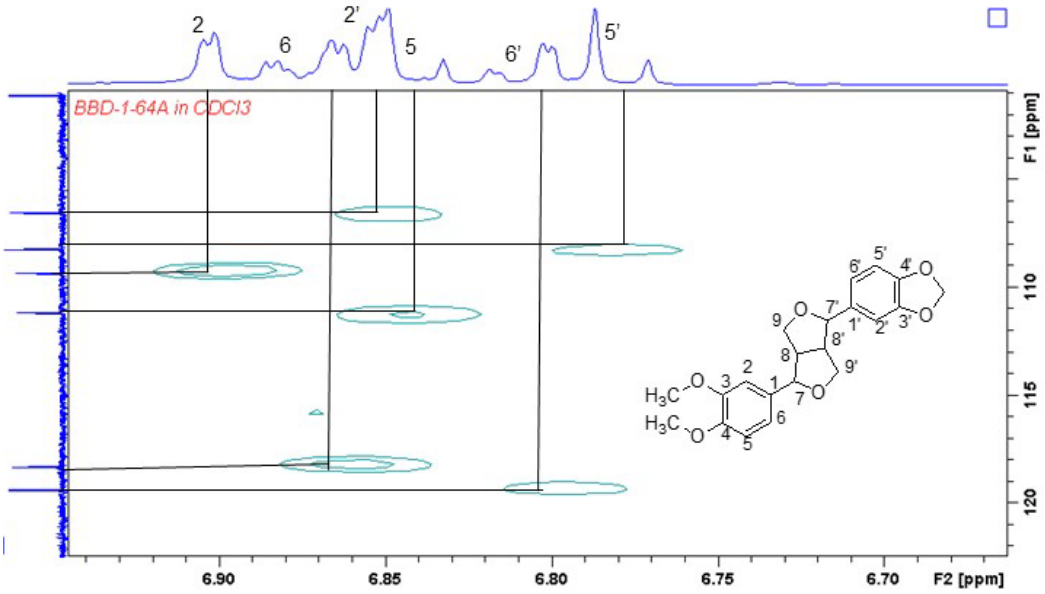


Plate 1G. HMBC NMR spectrum of kobusin (**3.1**) in $CDCl_3$.

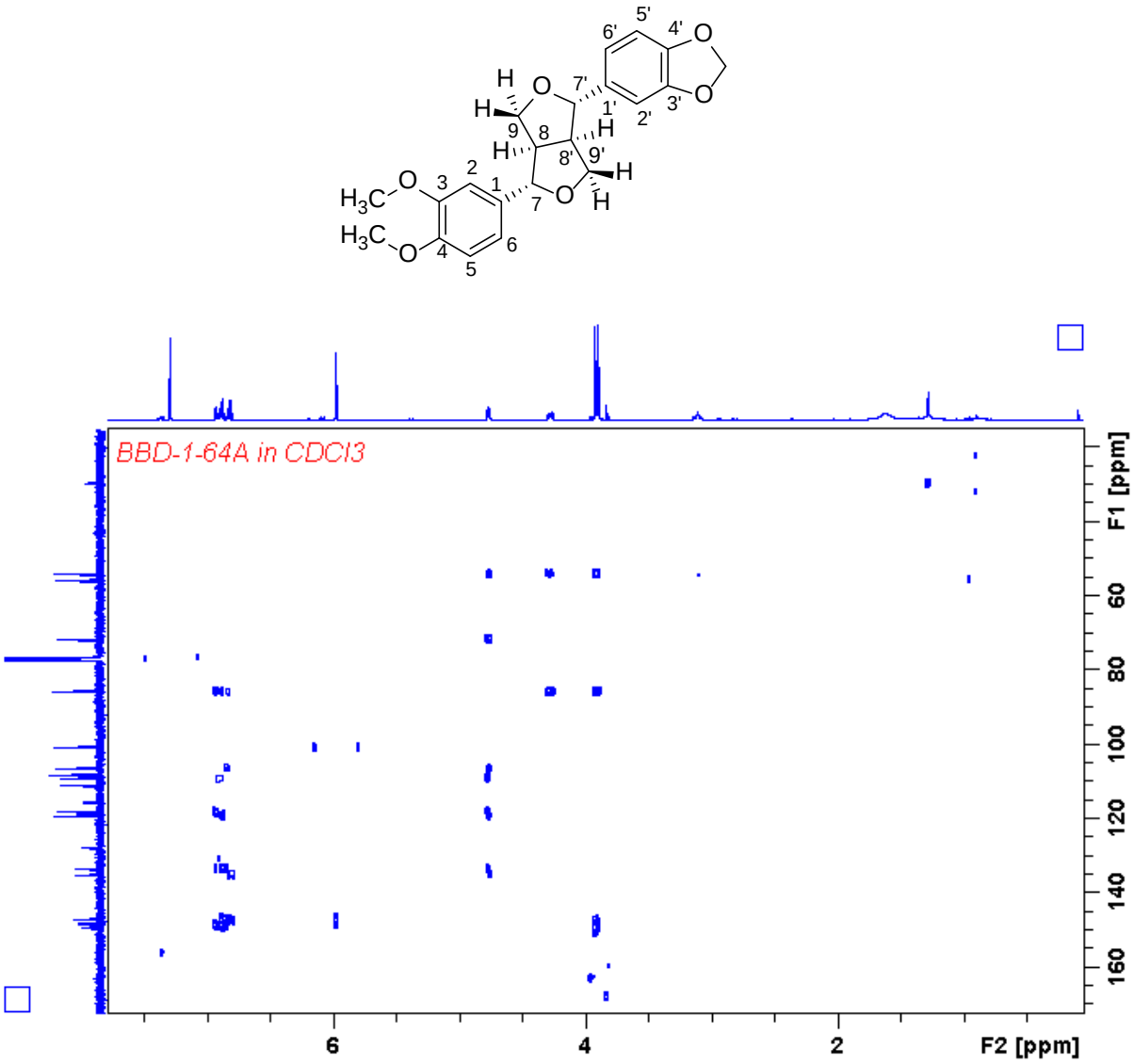


Plate 1H. HMBC NMR aromatic region spectrum of kobusin (**3.1**) in $CDCl_3$.

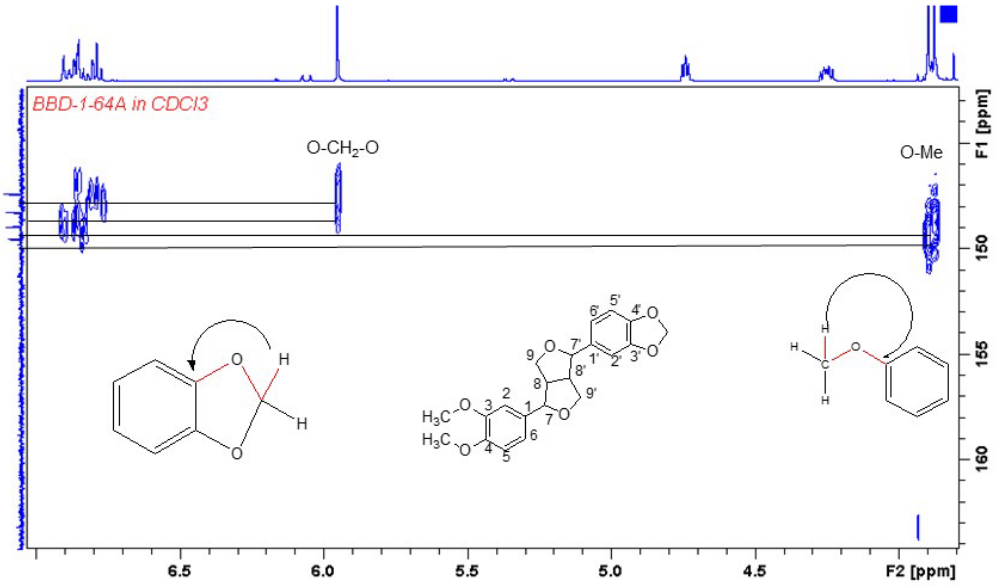


Plate 2A. ^{1}H NMR spectrum of eudesmin (**3.2**) $CDCl_3$ at 400 MHz.

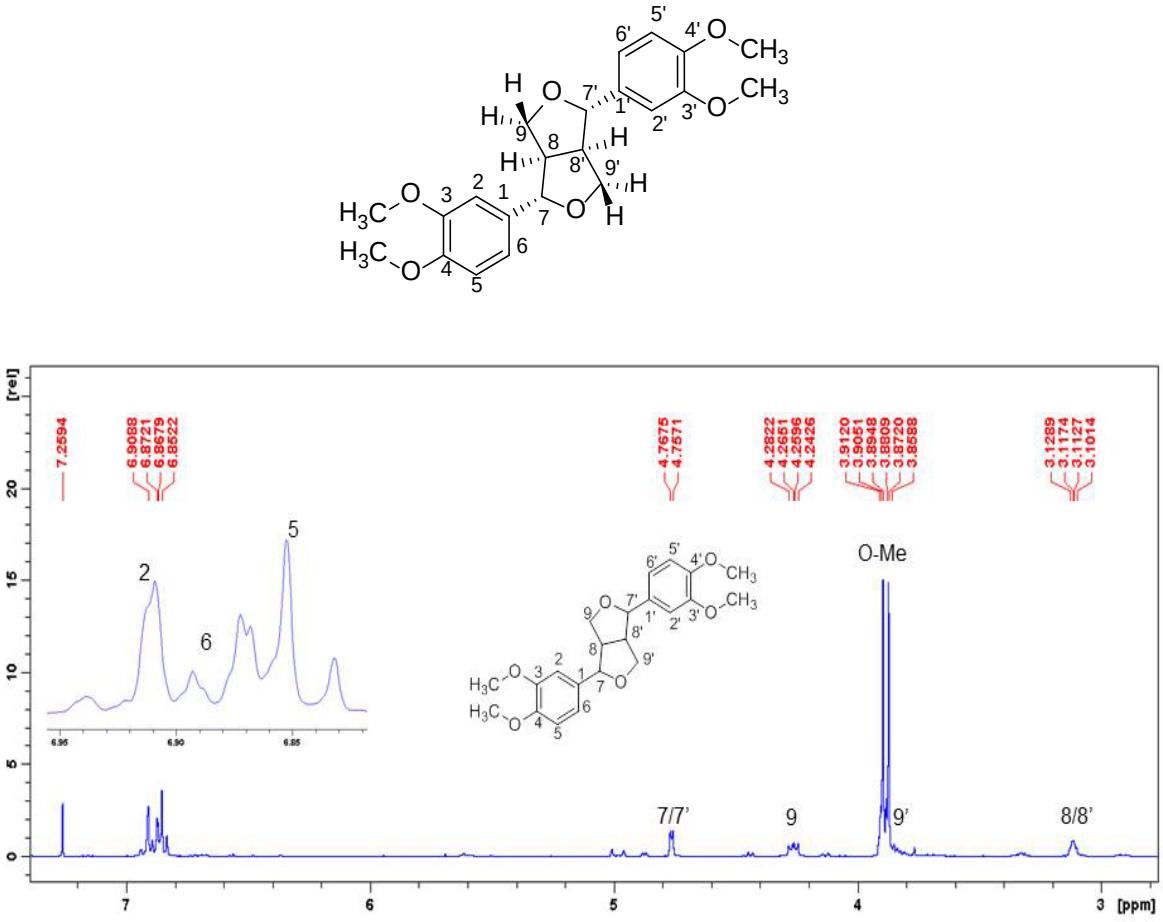


Plate 2B. ^{13}C NMR spectrum of eudesmin (**3.2**) in $CDCl_3$ at 100 MHz.

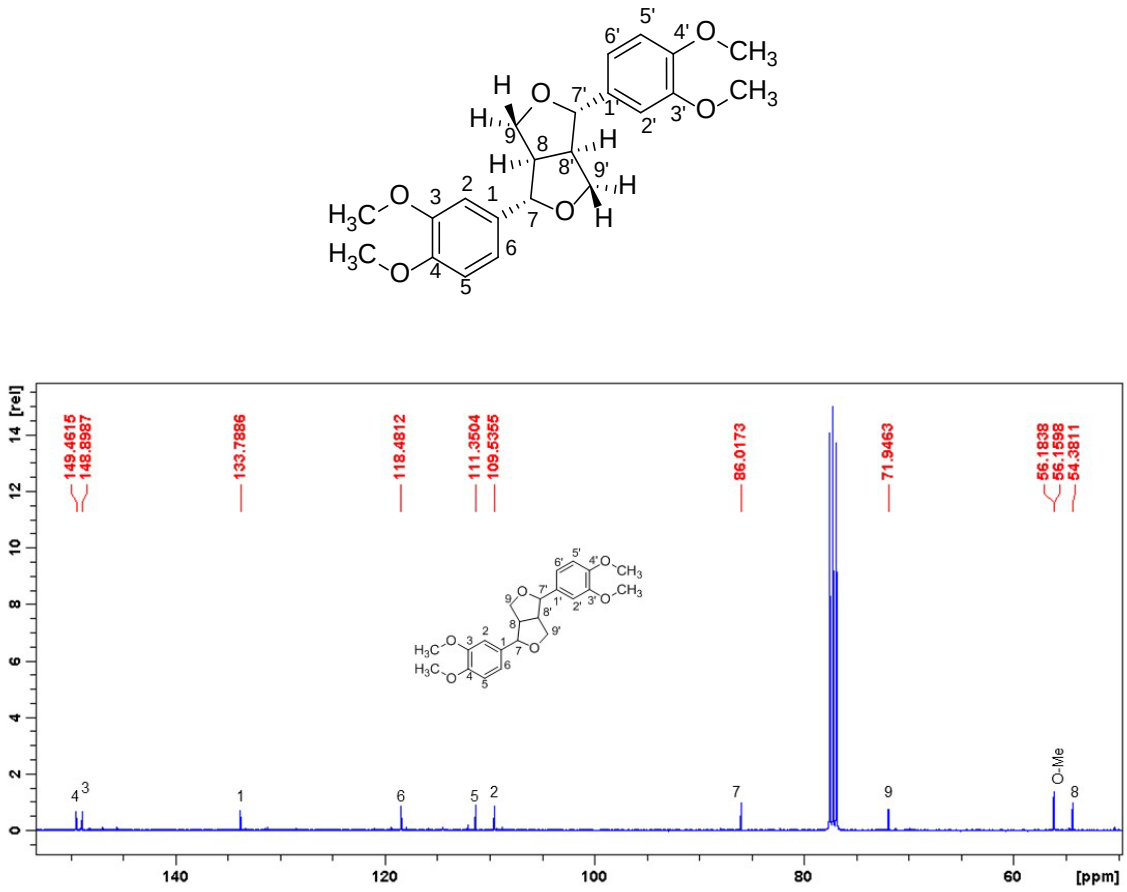


Plate 2C. DEPT NMR spectrum of eudesmin (**3.2**) in $CDCl_3$ at 100 MHz.

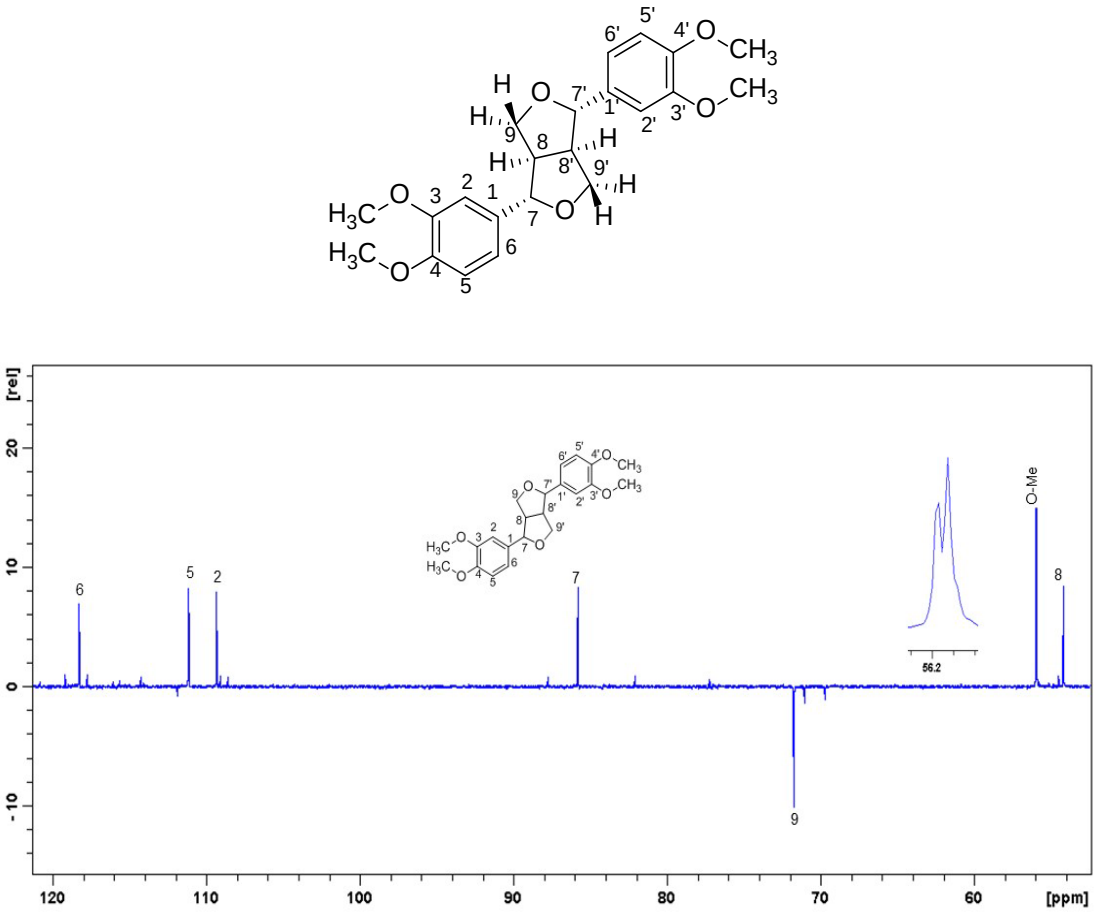


Plate 2D. COSY NMR spectrum of eudesmin (**3.2**) in $CDCl_3$.

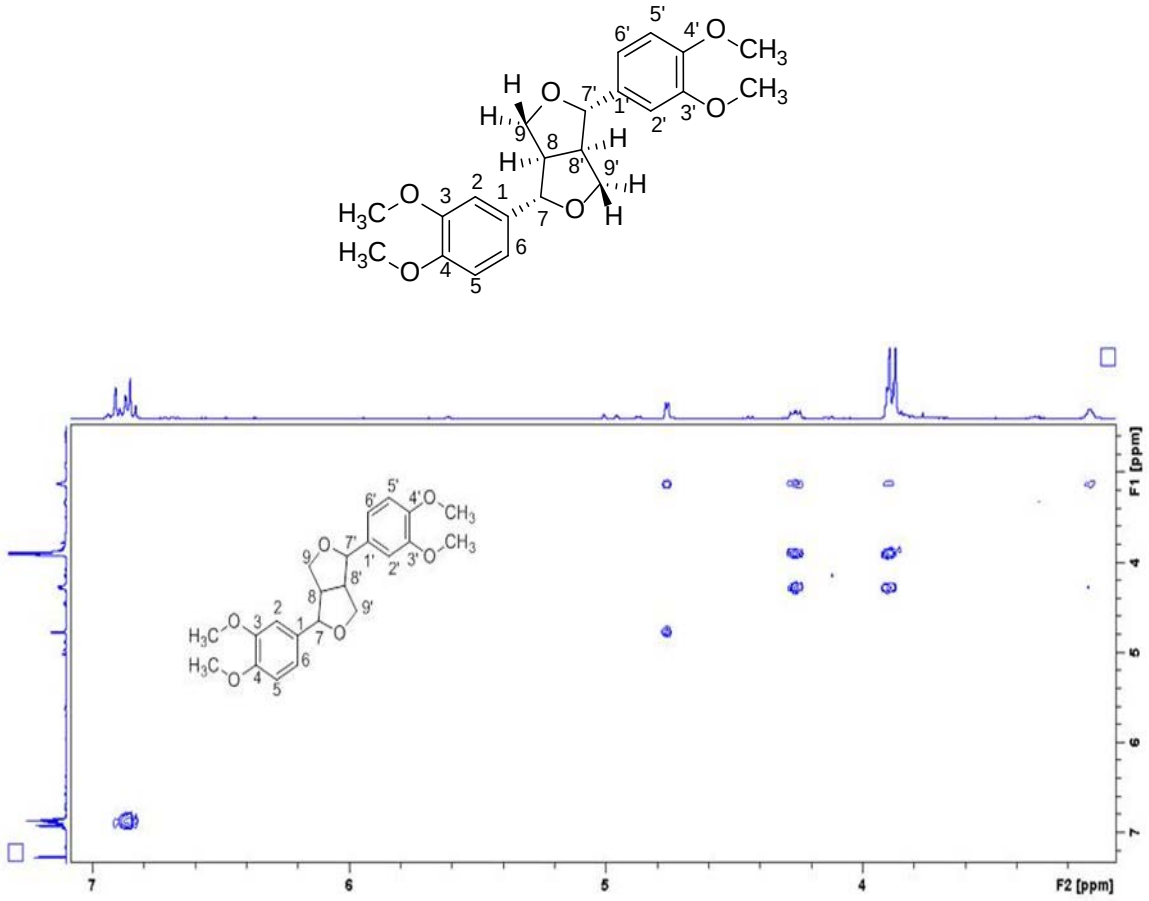


Plate 2E. HSQC NMR spectrum of eudesmin (**3.2**) in $CDCl_3$.

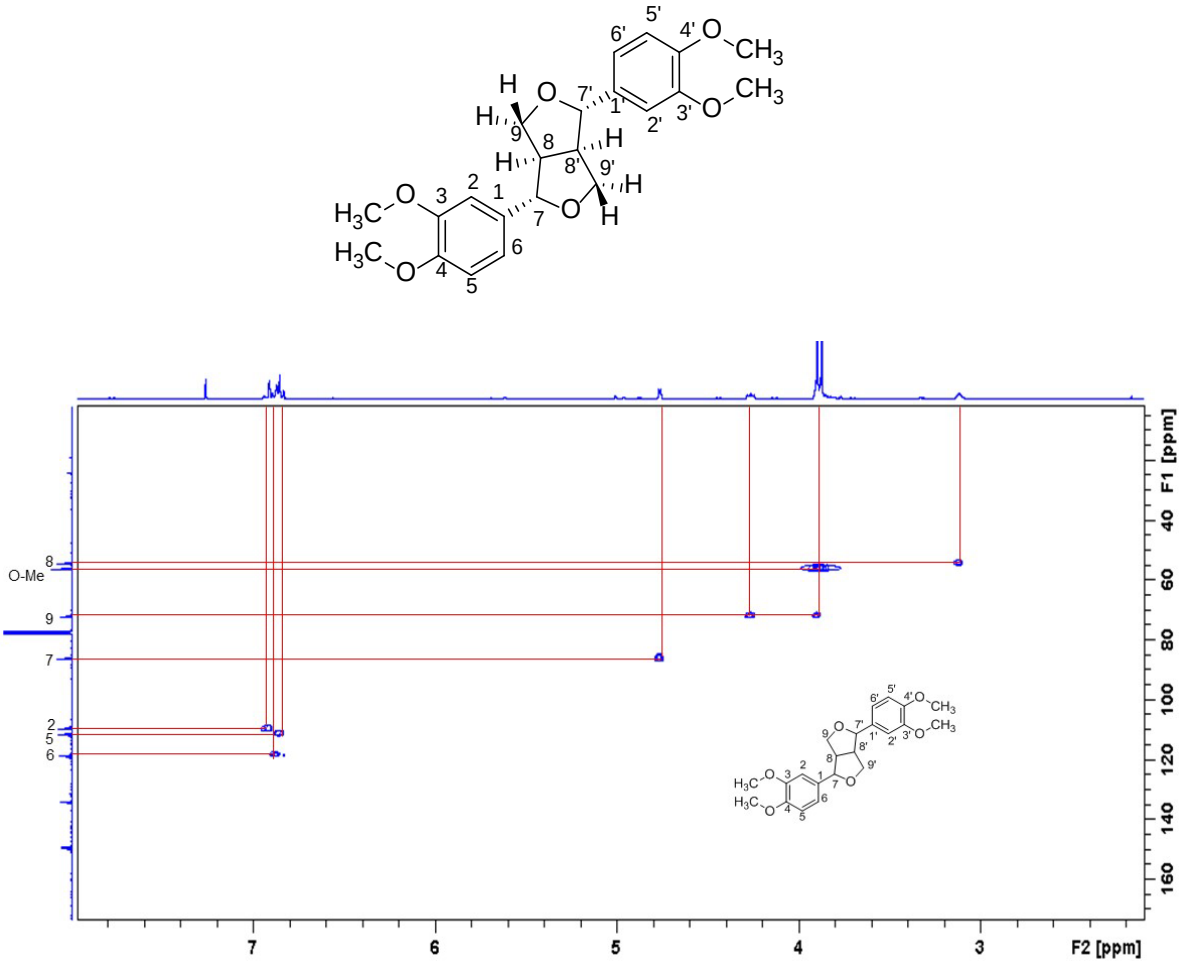


Plate 2F. HMBC NMR spectrum of eudesmin (**3.2**) in $CDCl_3$.

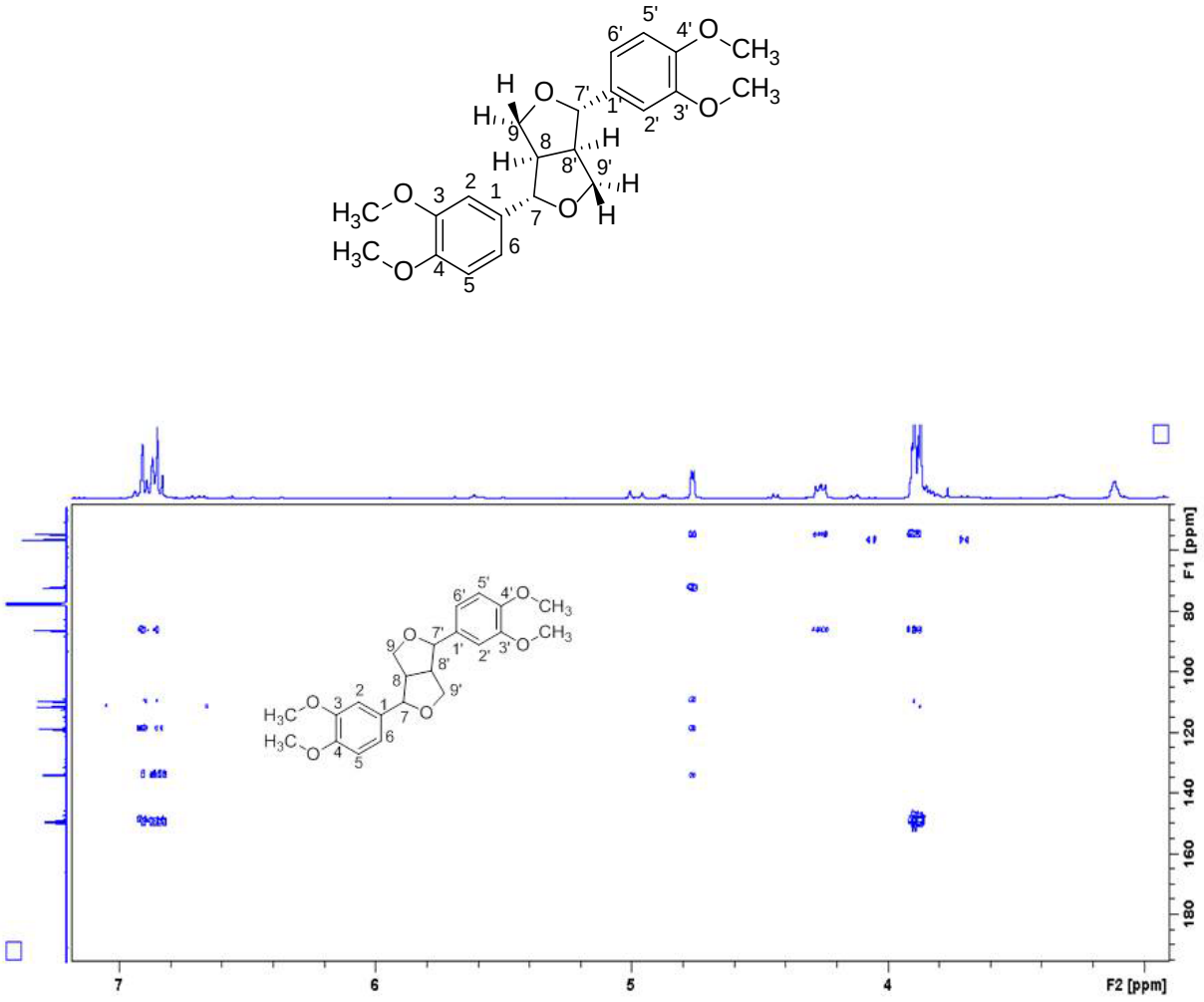
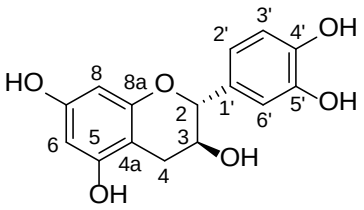


Plate 3A . ^{1}H NMR spectrum of catechin (**3.3**) in CD_3OD at 400 MHz.



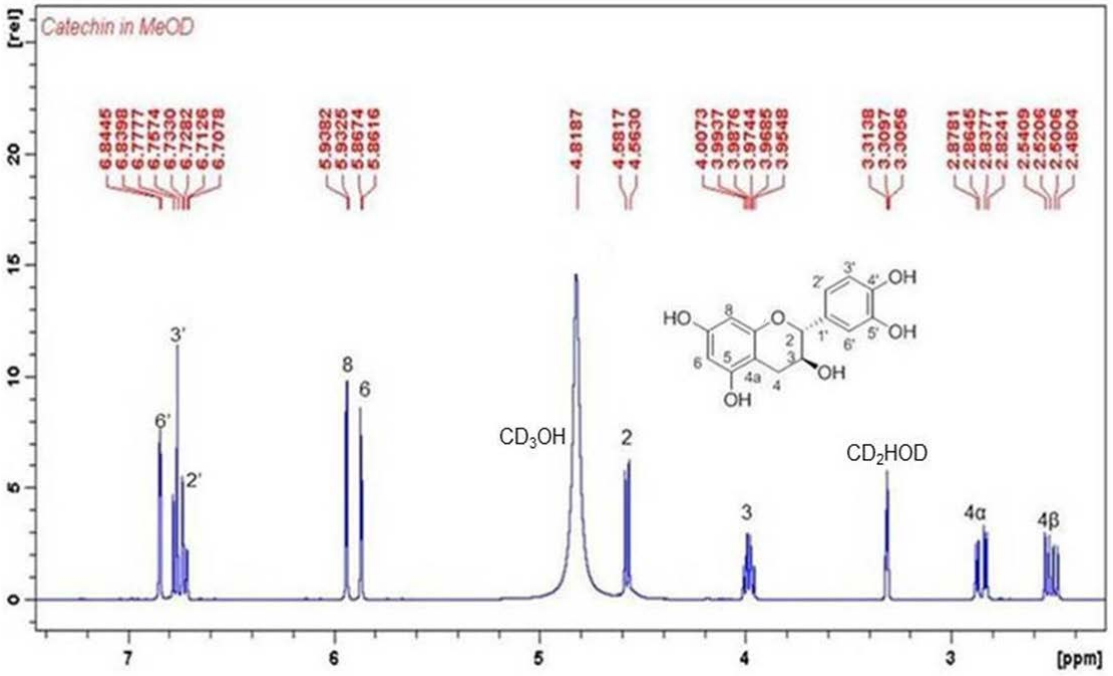
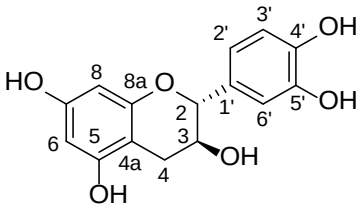


Plate 3B. ^{13}C NMR spectrum of catechin (**3.3**) in CD_3OD at 100 MHz.



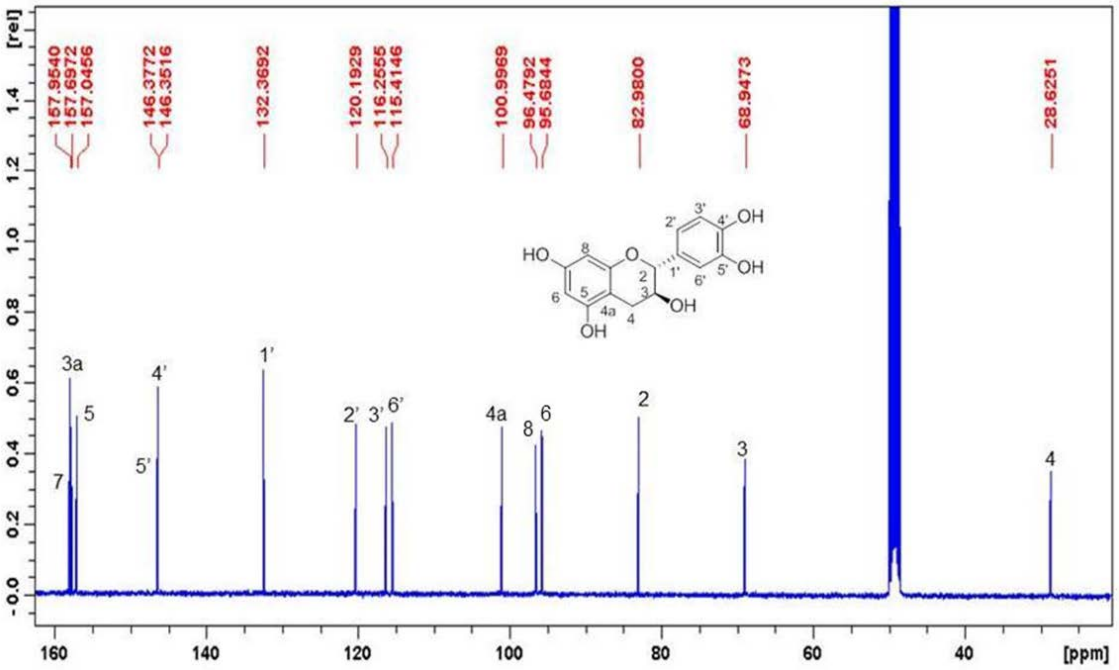
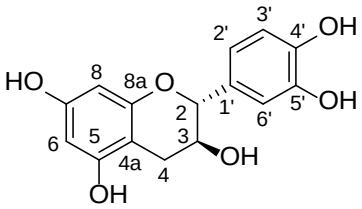


Plate 3C. DEPT NMR spectrum of catechin (**3.3**) in CD_3OD at 100 MHz.



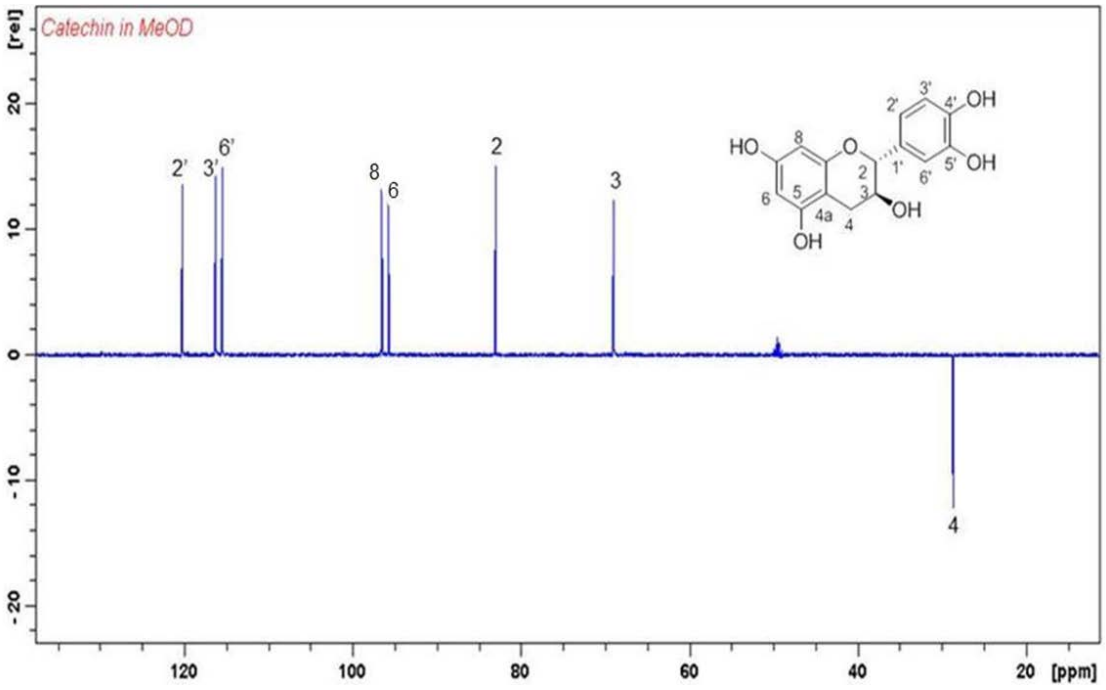


Plate 3D. COSY NMR spectrum of catechin (**3.3**) in CD_3OD.

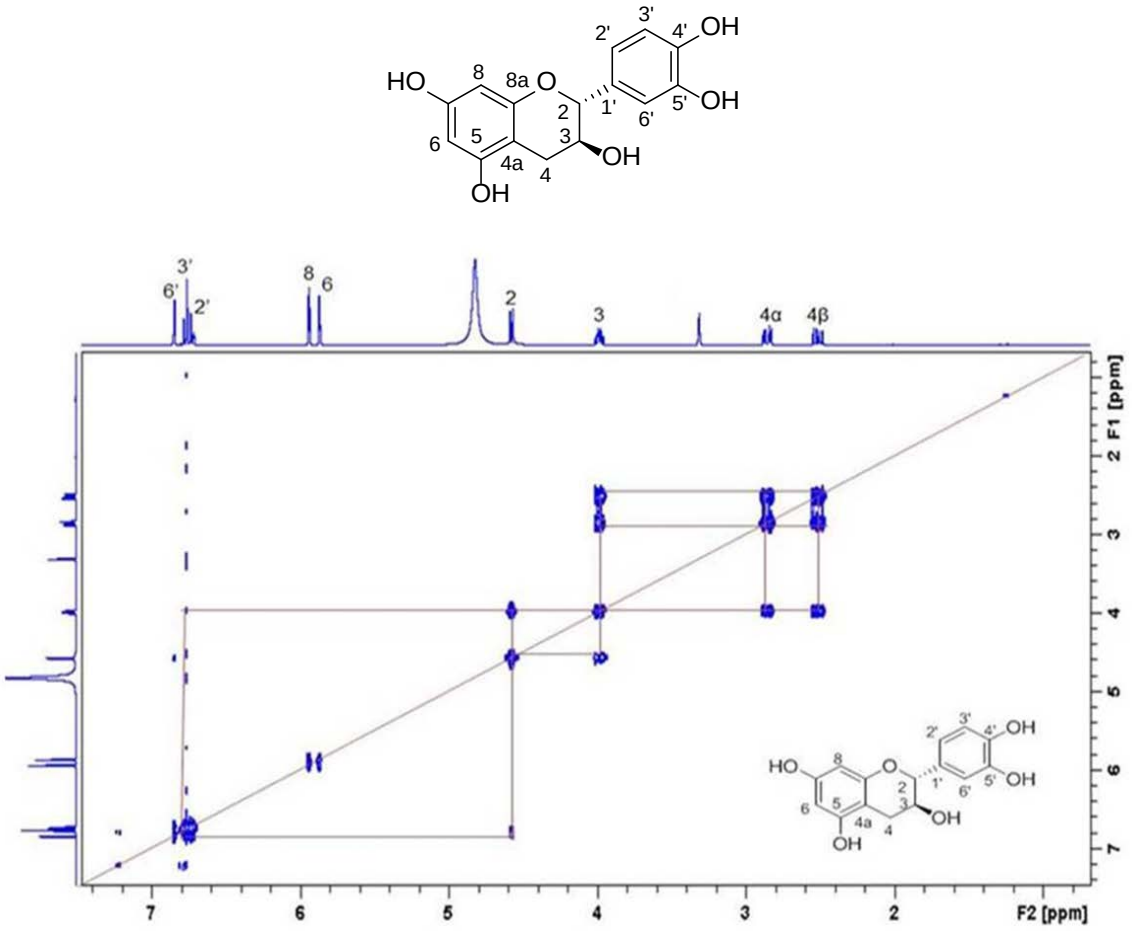
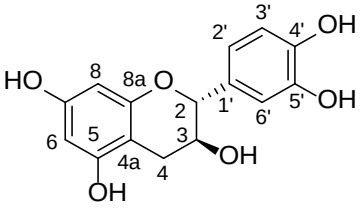


Plate 3E. HSQC NMR spectrum of catechin (**3.3**) in CD_3OD.



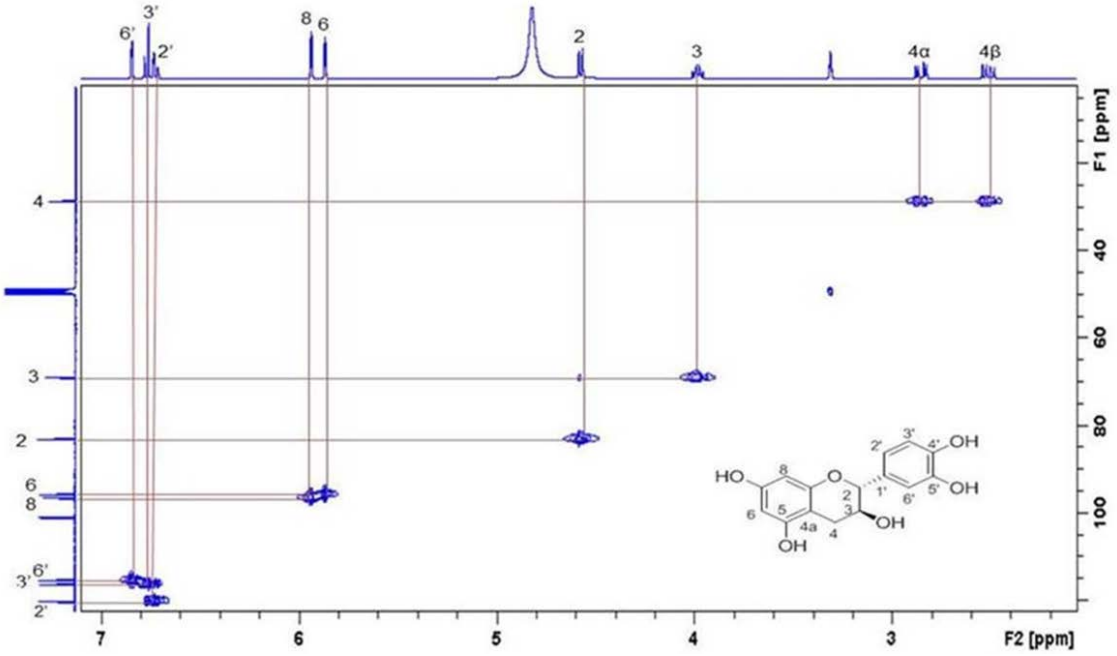


Plate 3F. HMBC NMR spectrum of catechin (**3.3**) in CD_3OD.

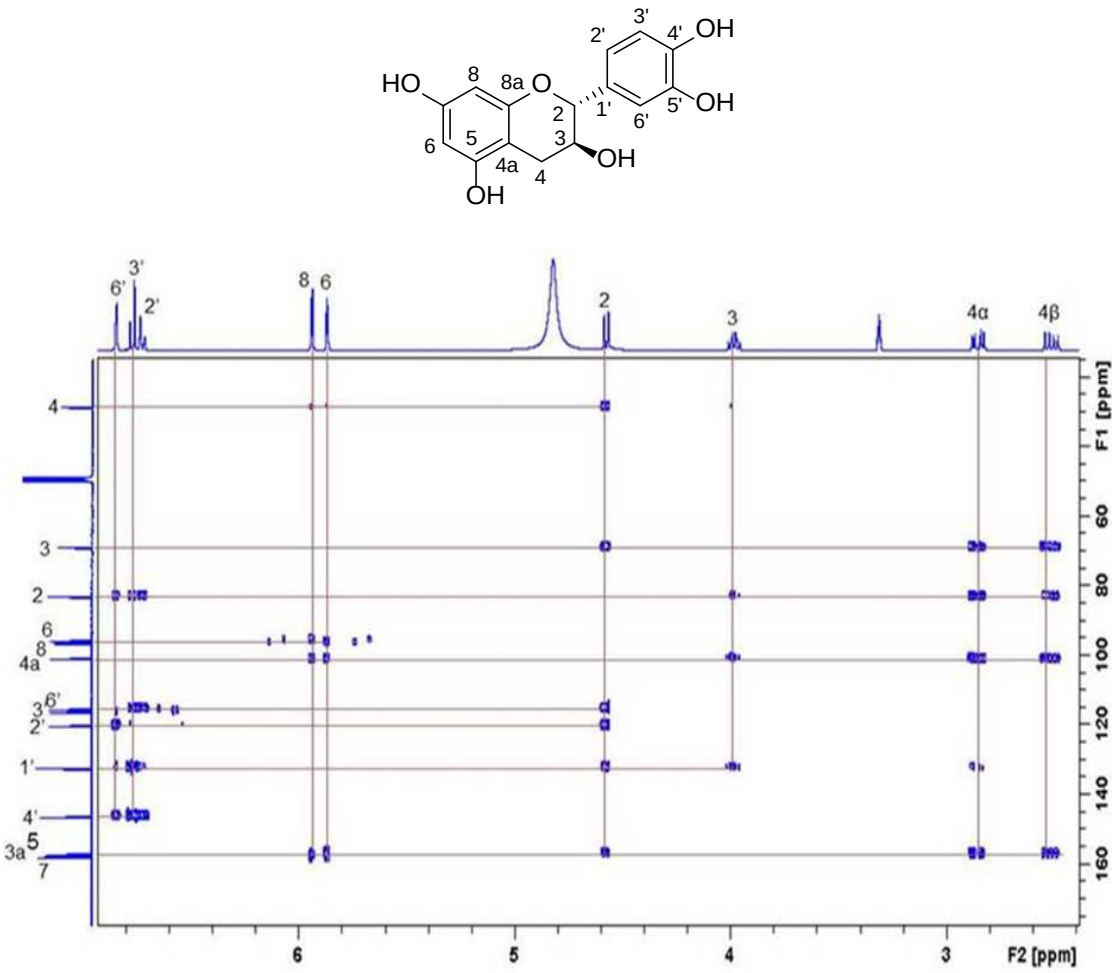


Plate 4A. ^{1}H NMR spectrum of compound **3.4** in $CDCl_3$ at 500 MHz.

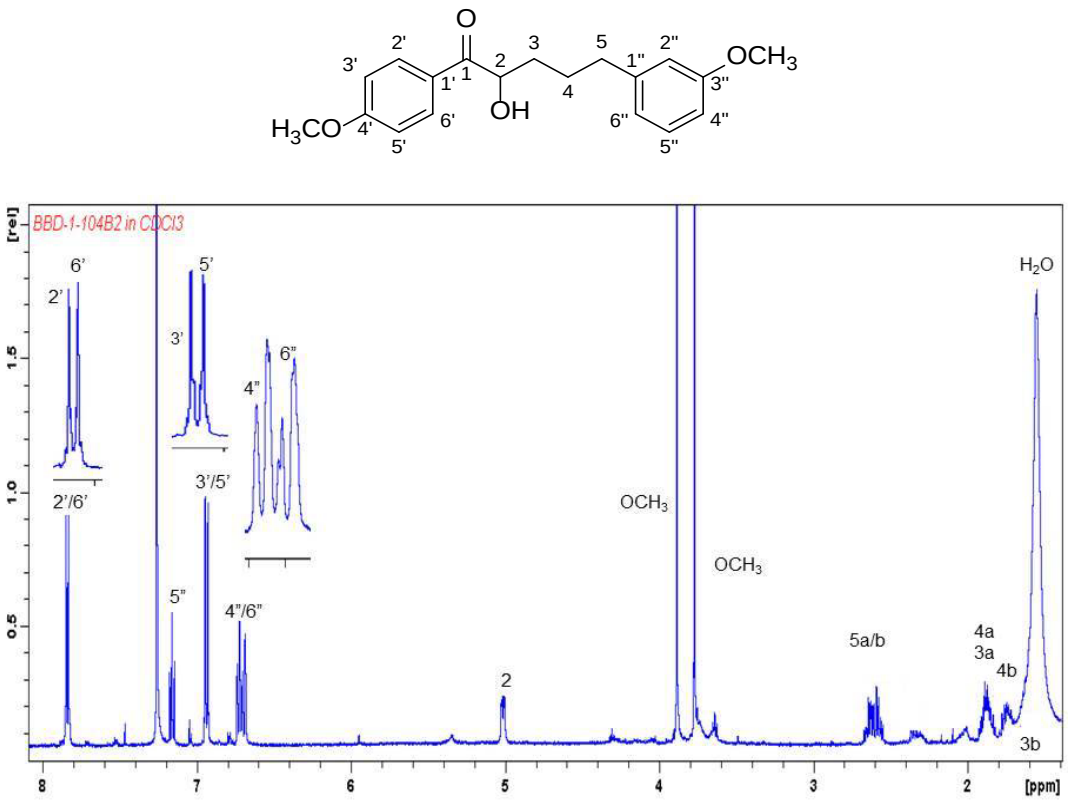
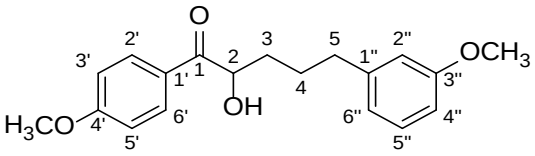


Plate 4B. COSY spectrum of compound **3.4** in $CDCl_3$ at 500 MHz.



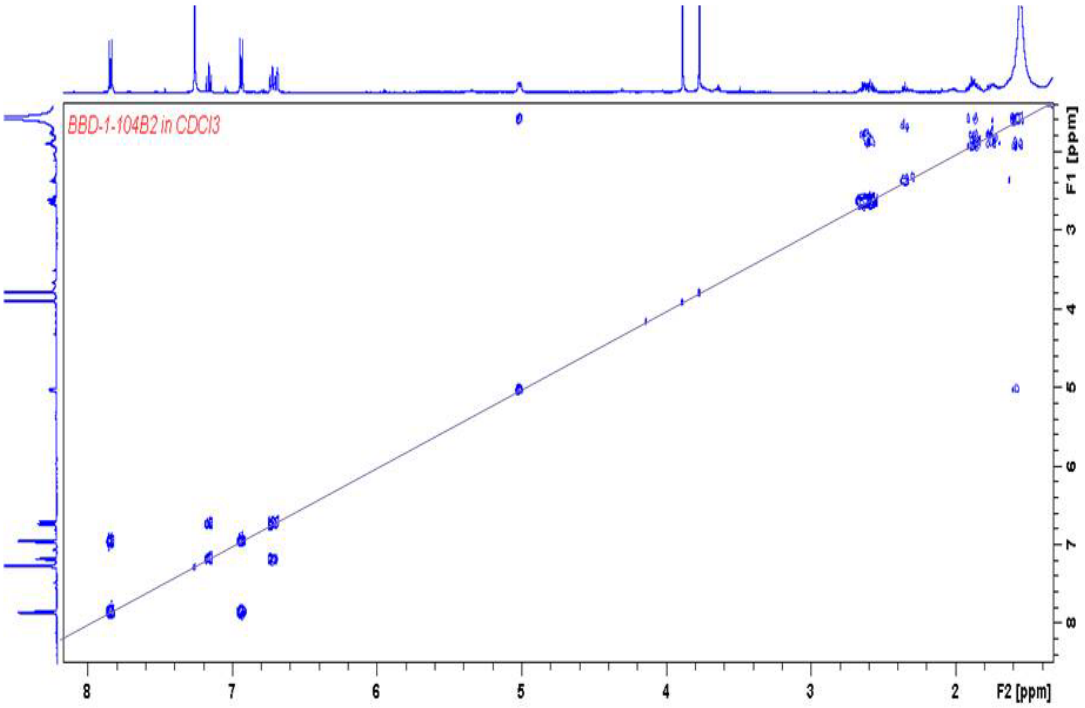


Plate 4C. HSQC spectrum of compound **3.4** in $CDCl_3$.

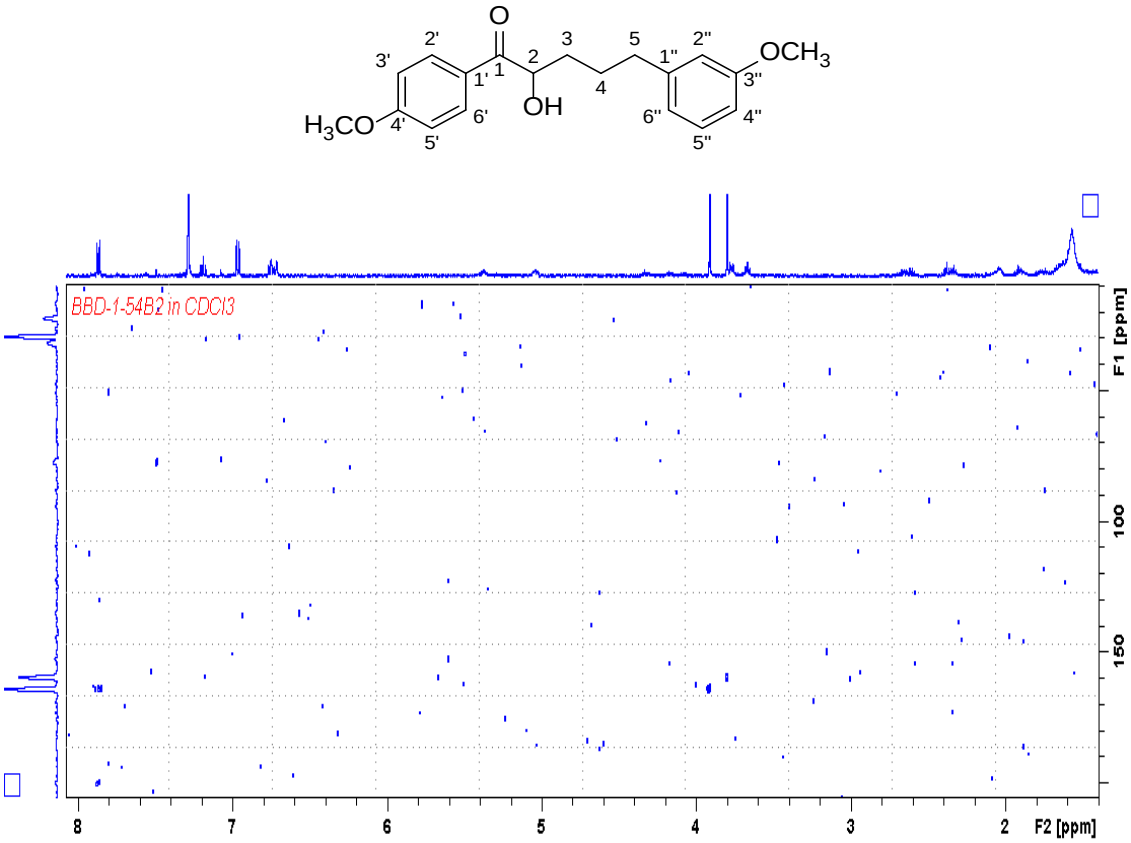


Plate 4D. HMBC spectrum of compound **3.4** in $CDCl_3$.