INVESTIGATION OF ANTIOXIDANT COMPOUNDS FROM THE BERRIES OF *Teclea simplicifolia* (ENGL.) I. VERD. AND *Ziziphus mucronata* WILLD.

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Master of Science Degree in Chemistry of Egerton University

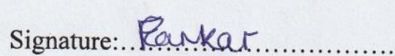
EGERTON UNIVERSITY

JUNE, 2020

DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been submitted or presented, for examination in any institution.

Signature:................ Date:..16/06/2020..

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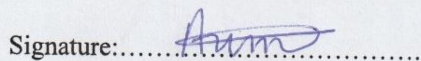
Signature:.......................... Date:...16/06/2020

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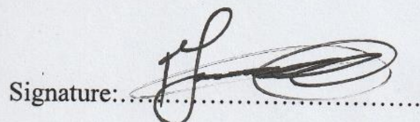
Signature:.......................... Date:..16/06/2020

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DEDICATION

I dedicate this work to my family for their love, support and patience during my studies.

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I am very grateful to the Almighty God for giving me the strength to reach this far in my academic journey. I appreciate Egerton University for giving me the chance to pursue this MSc Degree in Chemistry. I thank the Chemistry Department for letting me use their infrastructure and equipment and the staff for assisting me during my research. I am extremely grateful to my supervisors, Dr Alice Njue and Prof Josiah Omolo, for their advice, guidance and encouragement during my research period. I will forever cherish the dedication they put to ensure that I became an independent scientist. I am grateful to Prof. S. T. Kariuki of Biological Sciences, Egerton University, for identifying the plants I used to obtain my samples from. I thank Dr Moses Langat for his assistance in analyses of the compounds using nuclear magnetic resonance (NMR) spectroscopy at University of Surrey in the United Kingdom. I would also like to thank all those who assisted me and encouraged me throughout my Master's Degree programme.

ABSTRACT

Life threatening diseases like, cancer, diabetes, cardiovascular and neurodegenerative diseases have been linked to oxidative stress in the human body. Oxidative stress results from the uncontrolled generation of reactive oxygen species (ROS) in the body. Naturally, the normal body regulates ROS through *in situ* production of antioxidants. However, the human body is not capable of quenching ROS exceeding a certain level and this can result in oxidative stress. Hence the need of exogenous antioxidants arises and it is in this regard that this study was designed to find novel antioxidant compounds from medicinal plants. The main objective of the study was to investigate the antioxidant compounds present in the berries of *Teclea simplicifolia* and *Ziziphus mucronata* from Kenya. The berries of *T. simplicifolia* and *Z. mucronata* were separately collected, dried, pulverized and extracted using sequential extraction of cyclohexane, ethyl acetate and methanol. The crude extracts were tested for antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) method and ascorbic acid was used as the standard. The responsible compounds were purified via activity guided chromatography. The chemical structures of the purified compounds were determined using nuclear magnetic resonance (NMR) 1-D and 2-D experiments. For *T. simplicifolia* the IC_{50} value for cyclohexane extract was 135,897.6 μg/mL, for ethyl acetate extract it was 32,989.1μg/mL and for methanol extract it was 1,152.1 μg/mL. For *Z. mucronata* the IC_{50} value for cyclohexane extract was 44,466.5 μg/mL, for ethyl acetate extract it was 214.9 μg/mL and for methanol extract it was 8.9 μg/mL. *Z. mucronata* showed stronger antioxidant activity hence it was pursued for antioxidant compounds. A total of 22 compounds were purified but 19 of them were unstable and decomposed on transit to United Kingdom for NMR experiments. Full NMR spectra to allow structure elucidation was gotten for three compounds, namely: ursolic acid, betulin and betulinic acid. The three compounds are known compounds with antioxidant activity reported in the literature. This work has established that the berries of *T. simplicifolia* and *Z. mucronata* possess antioxidant compounds hence verifying their beneficial use as potent sources of antioxidants.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABTS	2-Azobis-3-Ethyl Benzothiazoline-6-Sulphonic Acid
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
COSY	^{1}H-^{1}H Shift-Correlated Spectroscopy
$CDCl_3$	Deuterated Chloroform
CD_3OD	Deuterated Methanol
CV	Coefficient of Variation
DCM	Dichloromethane
DPPH	2,2-Diphenyl-1-PicrylHydrazyl
FRAP	Ferric Reducing Antioxidant Property
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
MDA	Malondialdehyde
NOESY	Nuclear Overhauser Effect Spectroscopy
NMR	Nuclear Magnetic Resonance
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
TBA	Thiobarbituric Acid
TPTZ	2,3,5-Triphenyl-1,3,4-Triaza-2-Azoniacyclopenta-1,4-Diene Chloride

CHAPTER ONE

INTRODUCTION

1.1 Background information

Diseases have been known to be the leading cause of deaths worldwide. It is important that human beings are able to live a good quality of life. Human health being one of the indicators of good quality of life, it is indeed necessary to adapt to a healthy lifestyle in order to lower the risk of acquiring life threatening diseases (Mohit, 2014). One of the factors in the human body that determines good health is the generation of reactive oxygen species (ROS). Reactive oxygen species refers to the free radicals that are derived from molecular oxygen. They are chemically reactive species since they are free radicals and can cause chain reactions. The human body generates ROS during normal cell metabolisms and is also exposed to ROS from external sources including cooking, pollution, radiation and certain medication (Mongalo *et al.*, 2018). The free radicals most commonly associated with human disease include superoxide radical anion ($O_2^{-\bullet}$), hydroxyl radical (HO•), lipid peroxyl radical (ROO•), alkoxyl radical (RO•) and the reactive nitrogen species, such as nitric oxide radical (•NO) (Juranić and Žižak, 2005).

The oxidative damage caused by reactive oxygen species (ROS) includes damage to DNA or RNA, lipid peroxidation, oxidations of amino acids and oxidative deactivation of specific enzymes by oxidation of co-factors, and has been implicated in aging (Bhusari *et al.*, 2012). When the human body is not capable of quenching the free radicals, they accumulate and result in oxidative stress. Oxidative stress is known to be the cause of various diseases including autoimmune disorders, cancer, diabetes, cardiovascular and neurodegenerative diseases (Wu and Ng, 2008).

The human body relies on antioxidants as the greatest means of combating oxidative stress. It produces some antioxidants naturally but also depends on the exogenous ones obtained through foods and/or supplements. Antioxidants are able to terminate the chain reactions caused by free radicals hence can be used to treat and/or prevent diseases caused by free radicals. When the antioxidants produced within the human body are not capable of controlling oxidative stress, it is indeed necessary to provide exogenous antioxidants in the form of nutritional supplements or pharmaceutical products (Pham-Huy *et al.*, 2008).

A large number of the human population suffers from diseases caused by ROS. Cancer, one of the diseases resulting from oxidative stress, is a major health problem worldwide. Studies have revealed that about 14.9 million new cases were reported in the world in 2012. From these studies, it is predicted that there will be up to 22 million new cases in the next twenty years (Ghoncheh *et al.*, 2016). In Kenya, approximately 40,000 new cancer cases and 28,000 cancer deaths occur annually, making cancer the third leading cause of mortality (Topazian *et al.*, 2016).

Various methods are being used to treat diseases caused by oxidative stress, of these medicinal drugs being the most widely used. Most of these drugs are chemical compounds that are synthetically manufactured and/or obtained from natural compounds. Synthetic chemicals have shown a number of limitations including high costs and negative side effects. Natural products have been found to show limited negative side effects hence they are safer than synthetic drugs. However, many potential natural sources have not yet been studied to identify novel compounds possessing medicinal benefits (Sayed-Ahmad *et al.*, 2015; Gül and Pehlivan, 2018). Hence the need for the identification and isolation of antioxidant compounds from natural sources arises.

Plants are a major source of natural compounds, also known as secondary metabolites. Medicinal plants have been used for years in preventing and/or treating diseases (Shyur, 2012). Scientific studies have confirmed the presence of antioxidants in a number of plants and these happen to be secondary metabolites. Medicinal plants provide a starting point for the development of new drugs since they contain a large number of bioactive secondary metabolites that may be possessing antioxidant activity hence being able to quench free radicals (Hassanzadeh and Hassanpour, 2018).

Secondary metabolites can be isolated from plant sources using various techniques including soxhlet extraction, cold extraction, decoction, infusion, and hydro distillation. The secondary metabolites may decompose at high temperatures hence it is better to avoid extraction techniques involving high temperature, such as soxhlet extraction. Activity guided fractionation is the most preferable method for isolation of compounds from plant extracts since it aids in saving of time and laboratory reagents (Bhusari *et al.*, 2012). Chromatographic techniques can aid in the separation of the antioxidant compounds from the plant extracts.

Folk medicine is widely practiced in Kenya due to the availability of a number of medicinal plants in their natural habitats (Kokwaro, 1993). Many Kenyans opt to use both folk and modern

medication when suffering from chronic diseases including hypertension, cancer, HIV/AIDS, infertility and diabetes. Despite this, most of the herbal medications have not been scientifically evaluated to determine their efficacy and dosage. Indeed the need for the identification of these medicinal plants arises so that they can be used for isolation of secondary metabolites possessing therapeutic values that should be exploited in a sustainable manner to benefit the human population (Kigen *et al.*, 2013).

Kenya is blessed with a diverse range of plant species but there is very little literature available showing work that has been done regarding the screening of plants containing antioxidants. *Teclea simplicifolia* (Engl.) I. Verd. and *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. are plants that are used in Kenya for medicinal purposes (Nguta *et al.*, 2010; Kipkore *et al.*, 2014). Studies have shown that certain species of *Teclea* contain antioxidant compounds (Kuete *et al.*, 2008). There is no documented work to show any research carried out to determine the antioxidant activity exhibited by *Teclea simplicifolia*. It has been reported that the extracts from the bark of *Ziziphus mucronata* obtained from South Africa possess antioxidant activity (Olajuyigbe and Afolayan, 2011). The leaves of *Ziziphus mucronata* obtained from Botswana have also shown antioxidant activity (Kwape and Chaturvedi, 2012). However, there are no studies showing work carried out to isolate antioxidant compounds from either of these plants in Kenya. The use of berries promotes a sustainable exploitation of our biodiversity as the berries are seasonal hence they will be replaced by fresh berries. It is in this regard that this study has been designed to investigate the antioxidant compounds present in the berries of Kenyan *Teclea simplicifolia* and *Ziziphus mucronata*.

1.2 Statement of the problem

The accumulation of excessive reactive oxygen species (ROS) in the human body results in oxidative stress which plays a major part in the development of chronic and degenerative illnesses such as cancer, diabetes, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases. Various medical methods are being used to treat diseases caused by oxidative stress, medicinal drugs being the most widely used. Most of these drugs are chemicals that are synthetically manufactured and/or obtained from natural compounds. Synthetic chemicals have shown a number of limitations including high costs and negative side effects. Natural compounds have been found to show limited negative side effects

hence they are safer than synthetic drugs. However, many potential sources have not yet been studied to identify natural compounds possessing medicinal benefits. Indeed, there is need to investigate more sources of natural compounds for management of oxidative stress and the associated diseases. It is in this regard that this study has been designed to investigate the antioxidant compounds present in the berries of *Teclea simplicifolia* and *Ziziphus mucronata.*

1.3 Objectives

1.3.1 General Objective

To investigate the antioxidant compounds present in the berries of *Teclea simplicifolia* and *Ziziphus mucronata.*

1.3.2 Specific Objectives

i) To isolate the antioxidant compounds in the berries of *Teclea simplicifolia* and *Ziziphus mucronata.*
ii) To determine the chemical structures of the isolated antioxidant compounds.
iii) To determine the antioxidant activity and reducing power of the purified compounds.

1.4 Hypotheses

i) There are no antioxidant compounds in the berries of *Teclea simplicifolia* and *Ziziphus mucronata*.
ii) The chemical structures of the isolated antioxidant compounds cannot be determined.
iii) The antioxidant efficacy and reducing power of the purified compounds cannot be established.

1.5 Justification

The oxidative damage caused by reactive oxygen species (ROS) has been known to accumulate in the human body. Since the life expectancy of the world population is increasing, this will lead to an increase in the number of older people acquiring age-related chronic diseases. A large number of the human population is already affected by the diseases linked to oxidative stress. Many Kenyans are also affected by these diseases. A developing nation requires a healthy

workforce to progress in order to improve its standard of living and the increase in the number of people acquiring age-related chronic diseases linked to ROS will be a hindrance. Human beings should adapt to healthy life styles which should include measures to prevent diseases caused by ROS. Currently antioxidants are the most sought compounds to be used in prevention and/or treatment of these diseases. Synthetic antioxidant compounds have been known to exhibit toxicity towards the human body hence the need to resort to natural products to seek for natural compounds that possess antioxidant activity. Antioxidant compounds can be isolated from various sources including plants. The diverse range of plant species in Kenya increases the scope for seeking for natural compounds possessing therapeutic values. Furthermore, traditional medicine practices provide a starting point for the selection of plants containing potential natural compounds with therapeutic benefits. Indeed, ethnomedicine provides a gateway to identifying natural compounds that can aid in treating various diseases. Despite Kenya being blessed with a diverse range of plants, not many studies have been carried out to isolate antioxidant compounds from medicinal plants. Although nature is providing a source for potential antioxidant compounds, it is necessary to use the natural resources in a sustainable manner. The use of berries allows the sustainable exploitation of the natural resources since the plants will not be damaged in any way. *Teclea simplicifolia* (Engl.) I. Verd. and *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. are plants that are used in Kenya for medicinal purposes. Certain species of *Teclea* have been found to contain antioxidant compounds. The bark and leaf extracts of *Ziziphus mucronata* have also been found to contain antioxidant compounds. However, no work has been done in Kenya to isolate antioxidant compounds from either of these plants. Hence this study has been designed to enable investigation of the antioxidant compounds present in the berries of Kenyan *Teclea simplicifolia* and *Ziziphus mucronata*.

1.6 Limitations

The limitations of this study included the following.

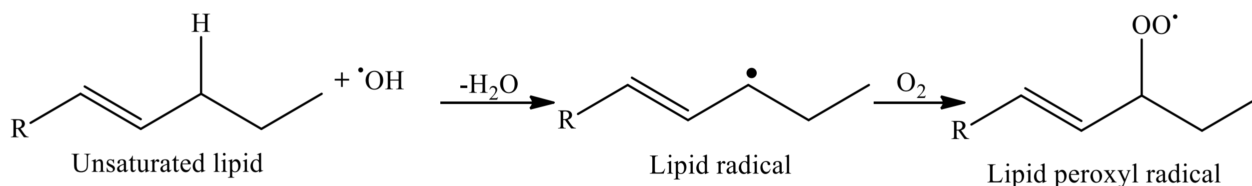
i) Ease of access of equipment.
ii) Stability of isolated compounds.
iii) Low yield of isolated compounds.
iv) Limited funds to support this work fully.

CHAPTER TWO
LITERATURE REVIEW

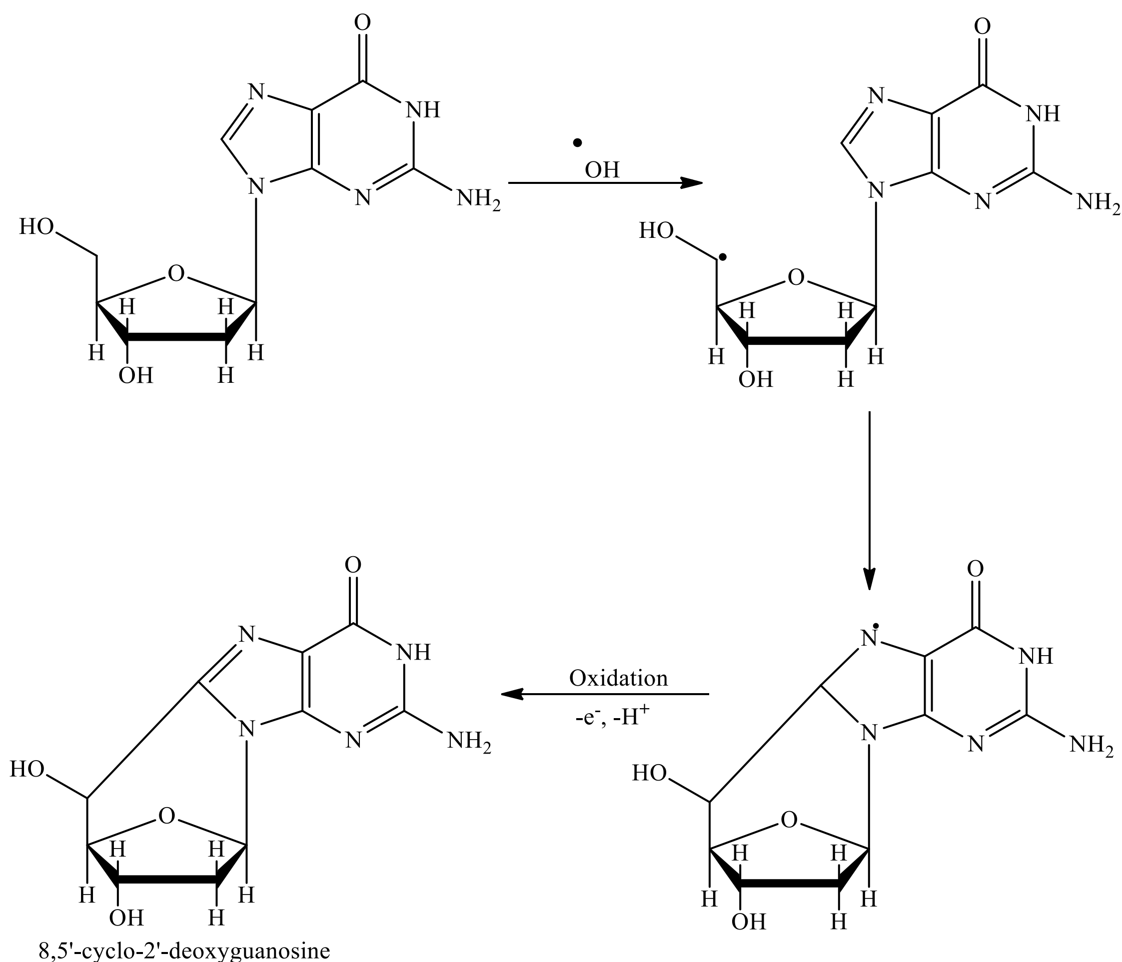
2.1 Reactive oxygen species

Oxygen is essential to human beings but also poses toxicity since it results in the generation of free radicals (Kaur and Kapoor, 2001). Free radicals are very unstable hence they can rapidly react with other atoms or molecules in order to become more stable (Gül and Pehlivan, 2018). The electron structure of oxygen enables it to gain electrons resulting in its reduction, which in turn is responsible for the generation of a number of reactive oxygen species (ROS). Reactive oxygen species are highly oxidizing and so can impair biological molecules (Bhusari *et al.*, 2012).

Many living organisms rely on oxidation for the generation of energy and so the production of endogenous ROS cannot be avoided (Shukla *et al.*, 2009). Reactive oxygen species may result in lipid damage (Scheme 1), DNA damage (Scheme 2), RNA damage, protein damage and oxidation of important enzymes in the human body (Nimse and Pal, 2015). This can give rise to various free radical-related diseases including aging, stroke, inflammation, gastritis, atherosclerosis, diabetes mellitus, autoimmune disorders, cataract, rheumatoid arthritis, cancer, cardiovascular and neurodegenerative diseases (Fattahi *et al.*, 2014; Takaidza *et al.*, 2018).



Scheme 1: Phospholipid peroxidation of unsaturated lipids



Scheme 2: Reaction of hydroxyl radical with sugar moiety of DNA to form impaired dsDNA

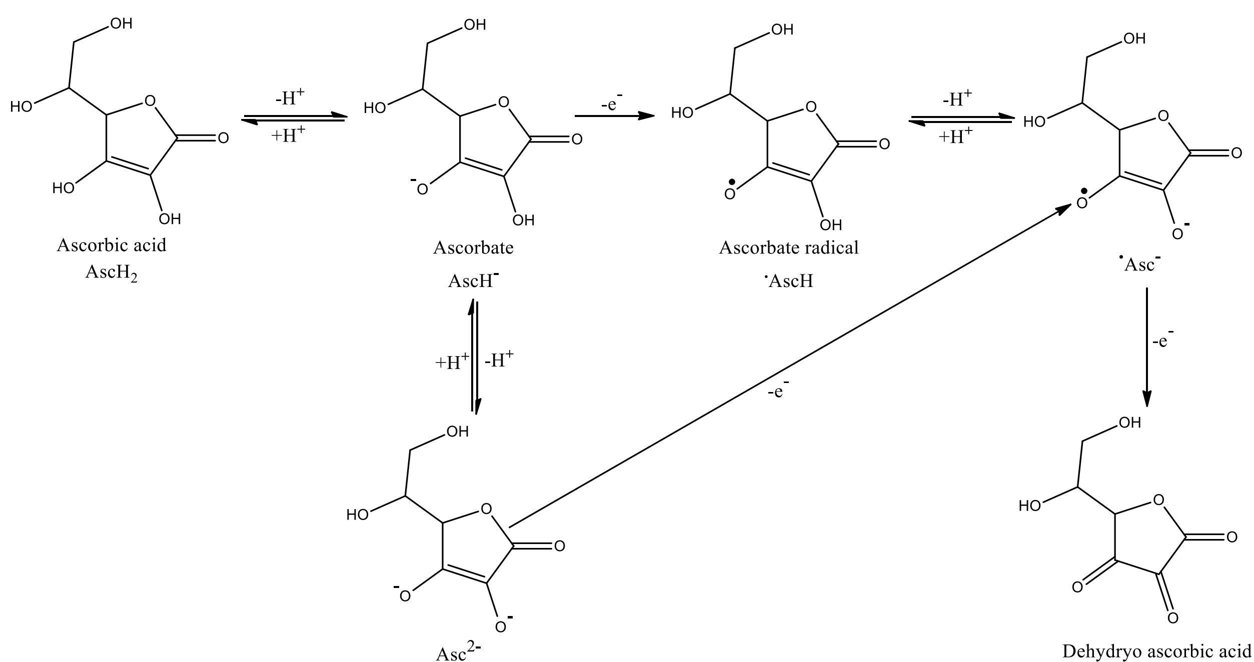
Oxidative damage caused by ROS is known to accumulate throughout the life cycle, resulting in aging and age dependent diseases such as cancer, diabetes and other life threatening diseases. Since the life expectancy of the world population is increasing, more people will be affected by these age-related diseases. Human beings should adapt to healthy life styles which should include measures to prevent diseases caused by ROS (Rahman, 2007). Antioxidants provide the greatest means to combat this issue hence the need for identifying more sources of antioxidants.

2.2 Antioxidants

Antioxidants can be used to safeguard the human body since they can prevent and reverse the harmful effects of free radicals. They can be defined in various ways depending on their

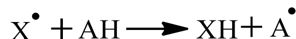
application. In food preservation, an antioxidant can be described as a substance that prevents oxidation of stored food products. They are receiving growing attention because they are known to prevent oxidative damage caused by ROS. In this case an antioxidant can be defined as a substance that quenches free radicals within a living organism (Wu and Ng, 2008). Hence an antioxidant can simply be described as a molecule that prevents the oxidation of other molecules.

Antioxidants can quench free radicals by donating electron(s) to make the free radical more stable by doing away with the unpaired electron (Kaur and Kapoor, 2001). Although they may become new free radicals, they will be less dangerous than the ones they quenched and may get quenched by other antioxidants or mechanisms that can get rid of their lone pair of electrons (Scheme 3) (Lü *et al.*, 2010; Nimse and Pal, 2015).



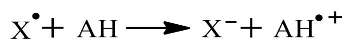
Scheme 3: Mechanism of radical scavenging activity of ascorbic acid (1)

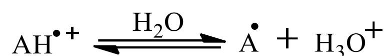
The human body makes use of various defence systems to safeguard the body against free radicals (Alam *et al.*, 2013). These include: preventing oxidants from being formed within the body; quenching and removal of free radicals; repairing the injured tissues and removal of toxic products of oxidation; and adaptive responses (Juranić and Žižak, 2005). Antioxidants are able to quench free radicals by making use of the hydrogen atom transfer (HAT) and single electron transfer (SET) methods. The Hydrogen Atom Transfer mechanism invloves donation of hydrogen to eliminate the unpaired condition of the free radical (Prior *et al.*, 2005).

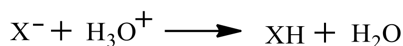


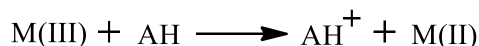
where AH = any hydrogen (H) donor

Single Electron Transfer mechanism involves the reduction of compounds, including free radicals, by the transfer of an electron.









where AH = any hydrogen (H) donor

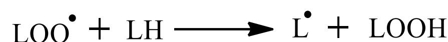
The chain reactions caused by ROS during oxidation involve initiation, propagation, branching and termination steps. The chain reactions may be initiated by radiation, heat, light or chemicals including metal ions (Pisoschi and Negulescu, 2011).

Initiation:

where LH is the substrate molecule and R· is the initiating oxidizing radical.

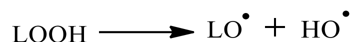
When the substrate is a lipid, it gives rise to an allyl radical (L·) that is able to react with oxygen resulting in a lipid peroxyl radical (LOO·).

Propagation:



The peroxyl radicals are capable of further oxidizing the lipid to give lipid hydroperoxides (LOOH).

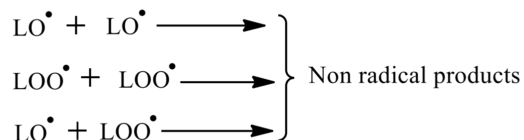
Branching:



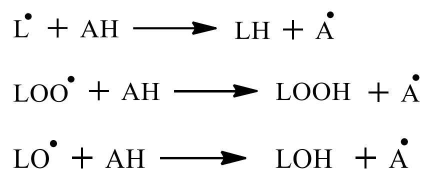
The lipid hydroperoxides (LOOH) branch into various compounds, including lipid peroxyl and lipid alkoxyl radicals.

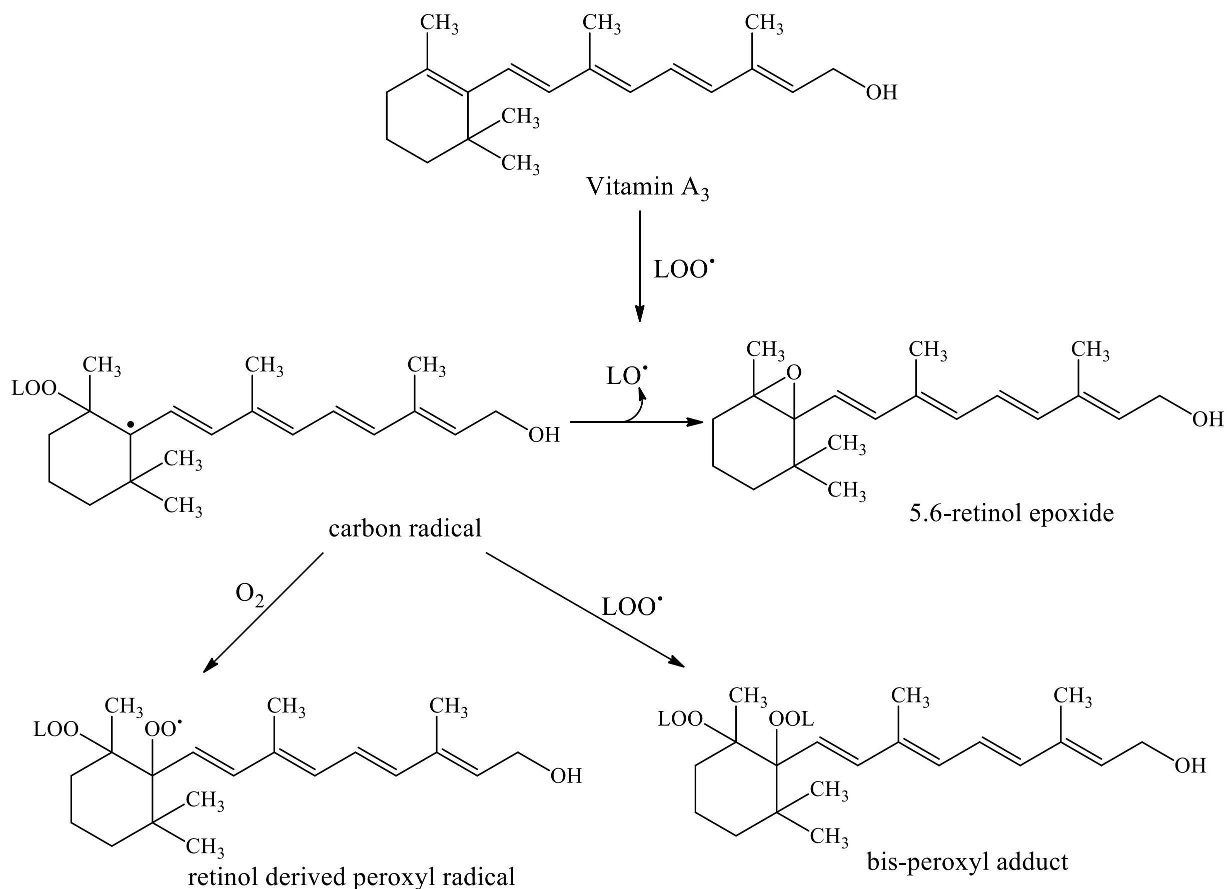
Termination:

During the termination stage, radicals combine to form non-radical products:



Primary antioxidants, AH, protect the human body from free radicals by reacting with peroxyl or alkoxyl radicals, hence preventing or slowing down the chain reactions caused by the oxidising free radicals (Scheme 4) (Pisoschi and Negulescu, 2011; Nimse and Pal, 2015).

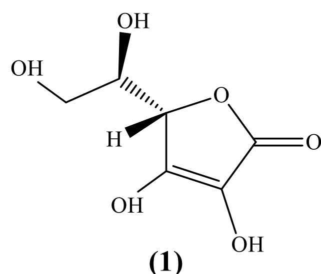




Scheme 4: Mechanism of radical scavenging activity of Vitamin A_3 **(2)**

Various classes of compounds act as antioxidants. These include vitamins, some mineral compounds, polysaccharides, stilbenes, coumarins, diterpenes and polyphenols such as

carotenoids, lignans, proanthocyanidins, flavonoids, phenolic acids, lignins and anthocyanins (Kardošová and Machova, 2006; Ghaima *et al.*, 2013; Rodgers *et al.*, 2013). Antioxidants include both exogenous and endogenous sources (Pham-Huy *et al.*, 2008). The endogenous sources of antioxidants include enzymes, large molecules, small molecules and some hormones (Prior *et al.*, 2005). In certain cases the endogenous antioxidants are not capable of fully quenching the free radicals present within the human body. Hence there is need for exogenous antioxidants. Exogenous antioxidants include both natural as well as synthetic antioxidants (Pisoschi and Negulescu, 2011). Dietary antioxidants are exogenous sources of antioxidants. They include Vitamin C (ascorbic acid) **(1)**, Vitamin A **(2)**, Vitamin E **(3)**, β-carotene **(4)**, lycopene **(5)** and selenium (Nimse and Pal, 2015).



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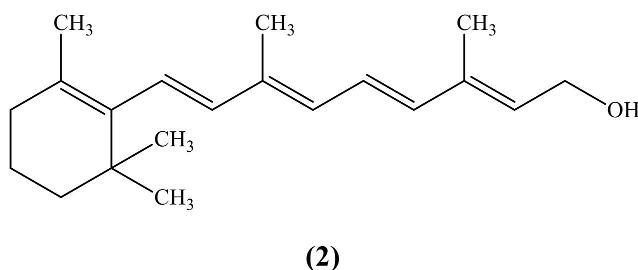

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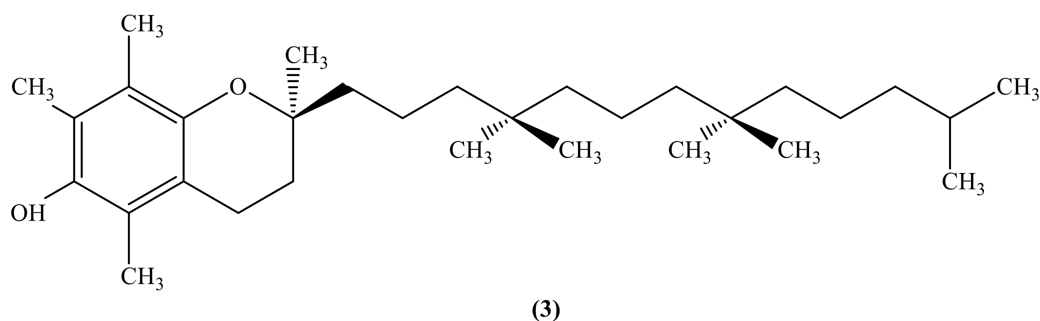

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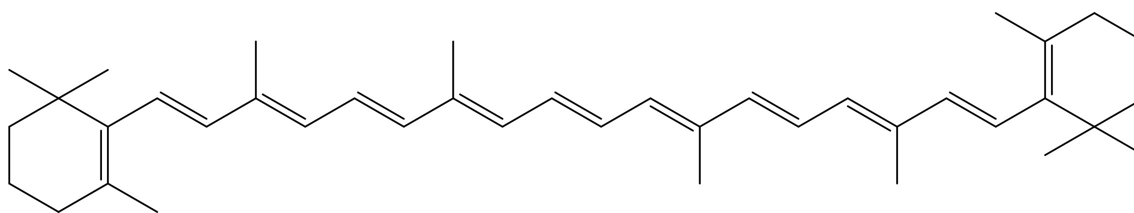

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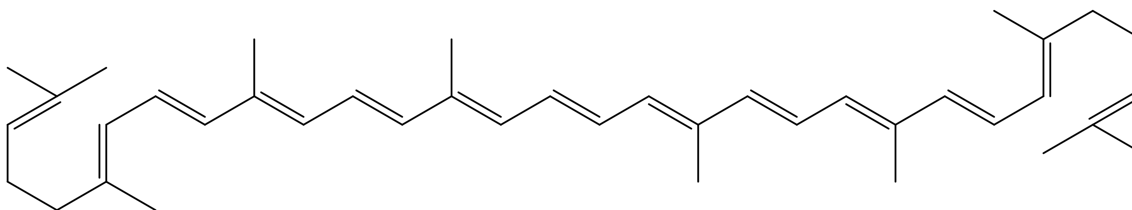
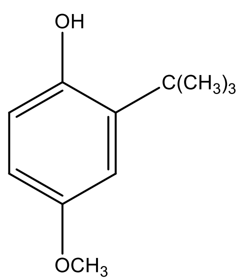


(5)

2.3 Plants as sources of antioxidants

Synthetic antioxidants, including butylated hydroxyl anisole (BHA) **(6)** and butylated hydroxyl toluene (BHT) **(7),** are widely used in industrial processing but are known to exhibit toxicity towards human health. Hence, natural compounds possessing antioxidant activity are preferred in preventing and/or treating diseases caused by ROS (Karamac and Amarowicz, 1997; Shukla *et al.*, 2009).



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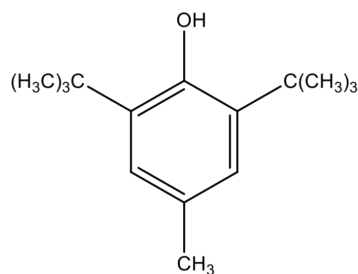
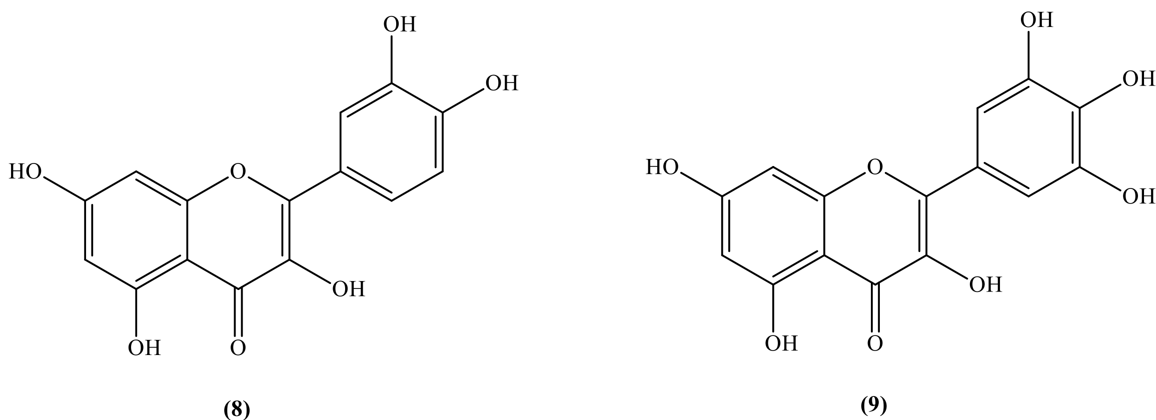


(7)

Various plants have been found to contain antioxidant compounds. Ascorbic acid **(1)**, carotenoids, vitamin E **(3)** and phenolic compounds like flavonoids such as quercetin **(8)** and myricetin **(9)**, are the most commonly found antioxidant compounds present in the plant kingdom (Manganaris *et al.*, 2014). *Urtica dioica* L. (stinging nettle) has various uses in traditional medicine. Scientific studies have shown evidence of antioxidants in extracts obtained from stinging nettle (Khare *et al.*, 2012; Ghaima *et al.*, 2013; Sayed-Ahmad *et al.*, 2015). Leaf

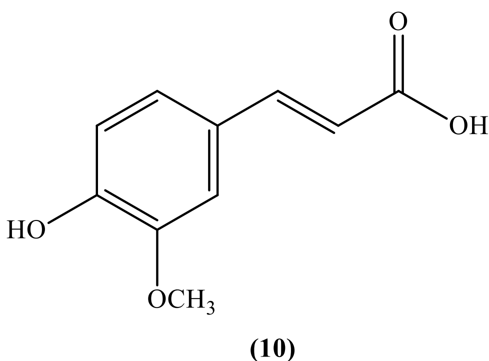
extracts of *Mangifera indica* and extracts of wild bitter melon (*Momordica charantia* Linn. var. *abbreviata* Ser.) have also shown antioxidant activity (Wu and Ng, 2008; Bhusari *et al.*, 2012). The ethanolic extract obtained from the seeds of *Caesalpinia bonducella* have been found to possess strong antioxidant activity (Shukla *et al.*, 2009). The antioxidant activity observed from the leaf extracts of *Turnera ulmifolia* Linn. var. elegans has been attributed to the phenolic compounds present (Brito *et al.*, 2012).



Foods obtained from plants are known to possess various antioxidants. These antioxidants include: phenolic compounds in wines and vegetable oils; flavonoids and phenolic acids contained in fruits and vegetables; hydroxycinnamic acids in coffee; catechins in teas; specific ingredients of common spices such as capsaicin in chillies, gingerol and curcumin; and anthocyanins and carotenoids in berries (Knasmüller *et al.*, 2008). Although the French are known for their unhealthy diet and lifestyle, they have a low incidence of cardiovascular heart diseases. This can be attributed to their high intake of red wine since red grapes are known to be rich in flavonoids (Procházková *et al.*, 2011).

Berries are known to aid in maintaining human health since they are a rich source of natural antioxidants (Forino *et al.*, 2016; Benatrehina *et al.*, 2018). Their dietary intake is known to prevent a number of chronic and degenerative diseases including cancer and diabetes (Manganaris *et al.*, 2014; Muniyandi *et al.*, 2019). The antioxidant activity of berries has been majorly attributed to phenolic compounds. Flavanols, including quercetin **(8)** and myricetin **(9)**, are known to be abundantly found in berries. Proanthocyanidins are the main phenolic compounds in hawthorn (*Crataegus spp.*) berries, and anthocyanins prevail in dark-skinned berries, such as black currant (*Ribes nigrum*) and bilberry (*Vaccinium myrtillus*). Phenolic acids,

including ferulic acid **(10)** and *p*-coumaric acid **(11)**, are also known to be abundant in berries (Tian *et al.*, 2017).



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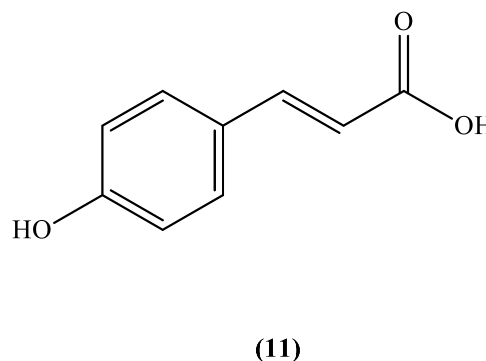
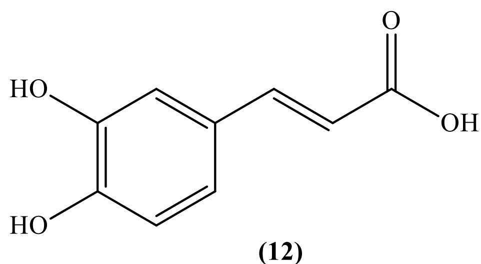


(11)

Various species of berries have been found to be rich in antioxidant compounds. Berries of *Mahonia aquifolium* have been found to possess high antioxidant activity (Coklar and Akbulut, 2017). The Saskatoon berry (*Amelanchier alnifolia Nutt.*) is known to be rich in polyphenols, tocopherols and carotenoids and these attribute to its high antioxidant activity (Lachowicz *et al.*, 2017). The berries of *Rhodomyrtus tomentosa* are known to be a rich source of antioxidants and have been found to possess a wide range of phenolic compounds (Zhao *et al.*, 2017). Thirty eight polyphenolic compounds have been isolated from blue honeysuckle (*Lonicera caerulea L.* var. *kamtschatica*) berries as listed by (Oszmiański *et al.*, 2016). Goji berries (*Lycium barbarum*) have been found to contain various antioxidants including p-coumaric acid **(11)**, caffeic acid **(12)**, scopoletin **(13)**, rutin **(14)**, N-cis-feruloyl tyramine **(15)** and N-trans-feruloyl tyramine **(16)** (Forino *et al.*, 2016; Protti *et al.*, 2017).



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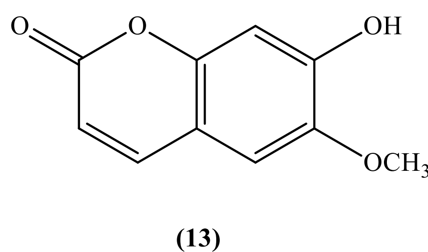

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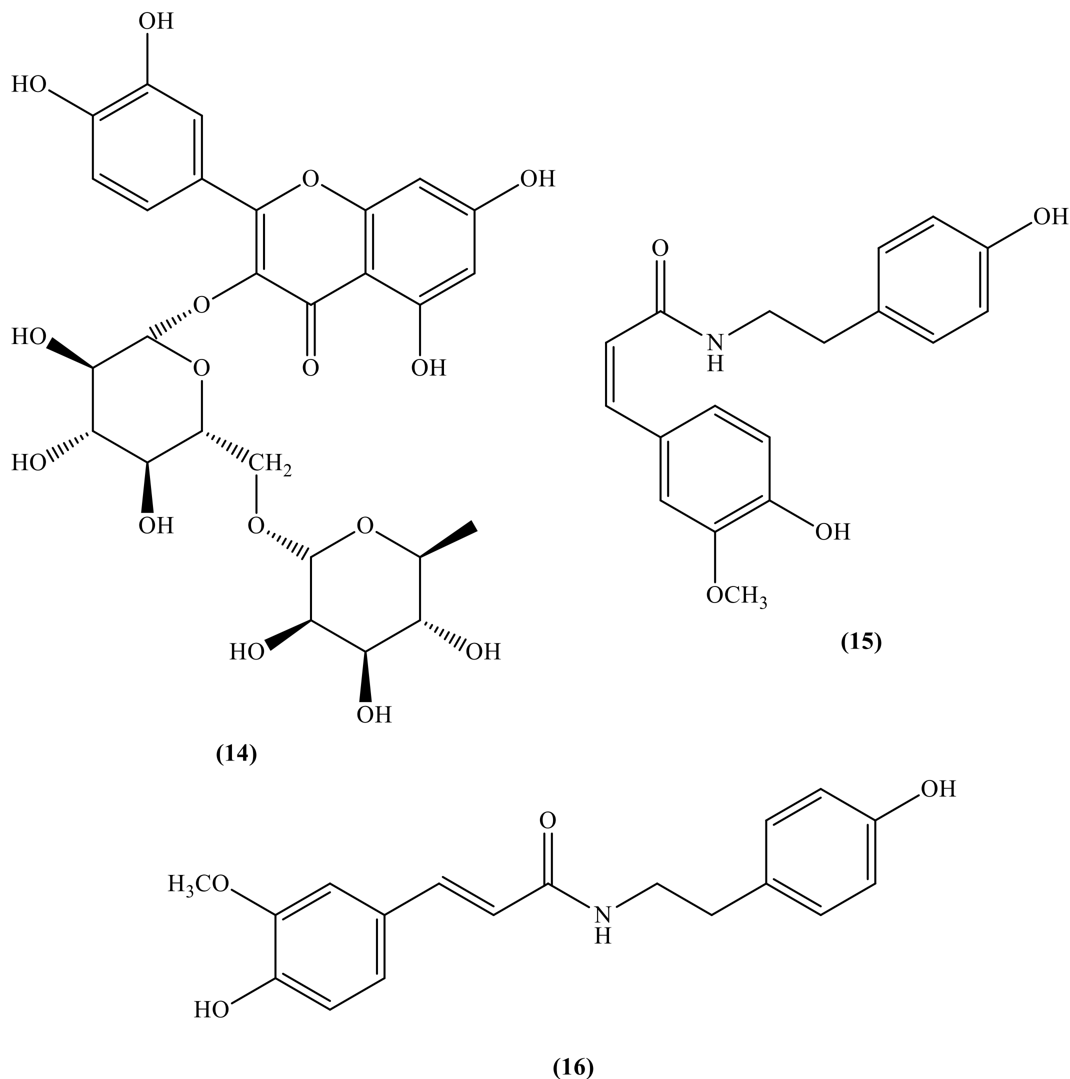
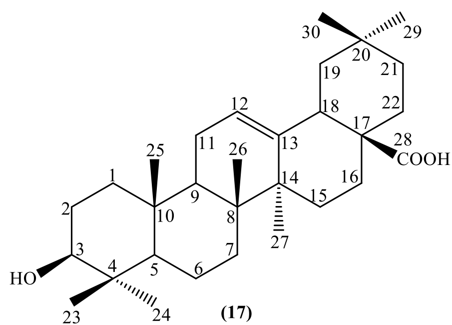


Fifteen triterpenoids have been isolated from hawthorn berries (*Crataegus pinnatifida*) and are known to contribute to their antioxidant activity. These triterpenoids include oleanolic acid **(17)**, 2α,3β-dihydroxy-olean-12-en-28-oic acid **(18)**, 3β,23-dihydroxy-olean-12-en-28-oic acid **(19)** and 2α,3β,23-trihydroxyolean-12-en-28-oic acid **(20)**. Four triterpenoids were isolated for the first time and their structures were elucidated and found to be those of 3β,6β,18β-trihydroxy-olean-12-en-28-oic acid **(21)**, 3β,6β,18β,23-tetrahydroxy-olean-12-en-28-oic acid **(22)**, 2α,3β,6β,18β-tetrahydroxy-olean-12-en-28-oic acid **(23)** and 18β,23-pentahydroxy-olean-12-en-28-oic acid **(24)**. However, compound **(24)** can be named as 2α,3β,6β,18β-pentahydroxy-olean-12-en-28-oic acid on the basis of the structure provided. These four compounds were found to

have the skeleton of an olean-12-ene type triterpene with an additional hydroxyl group attached at C-18 (Qiao *et al.*, 2015).



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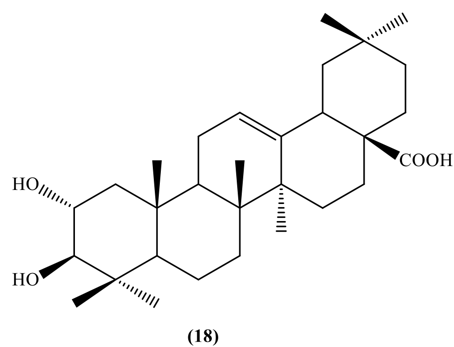

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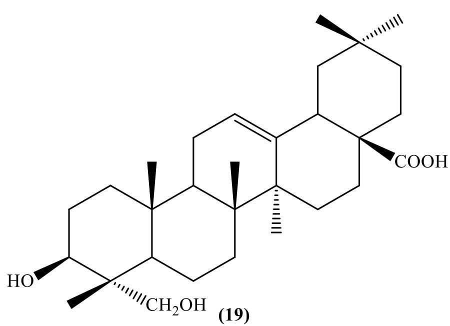

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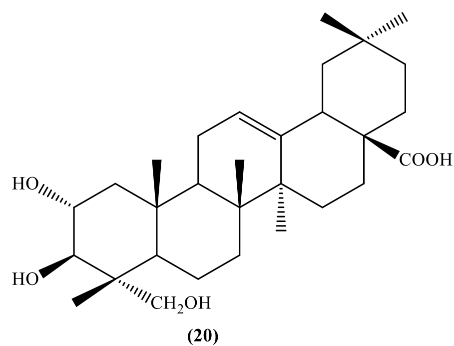

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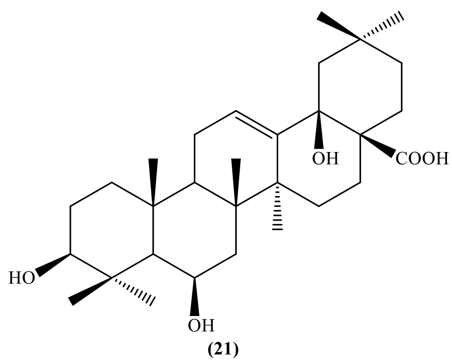

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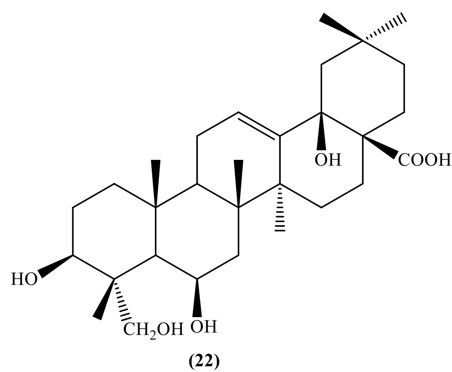

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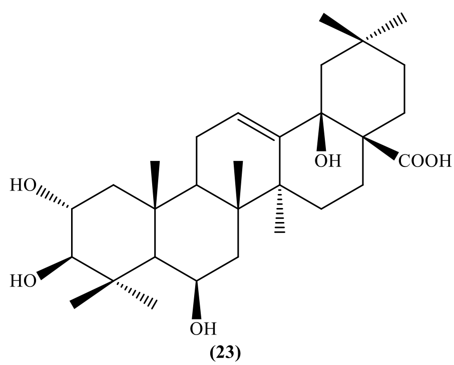

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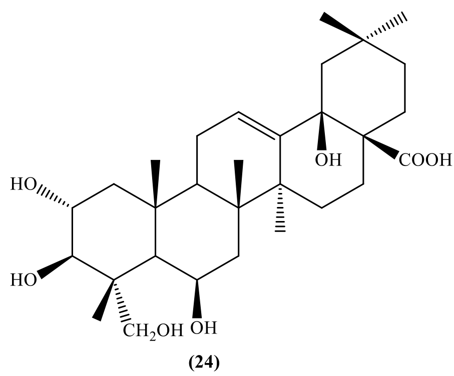
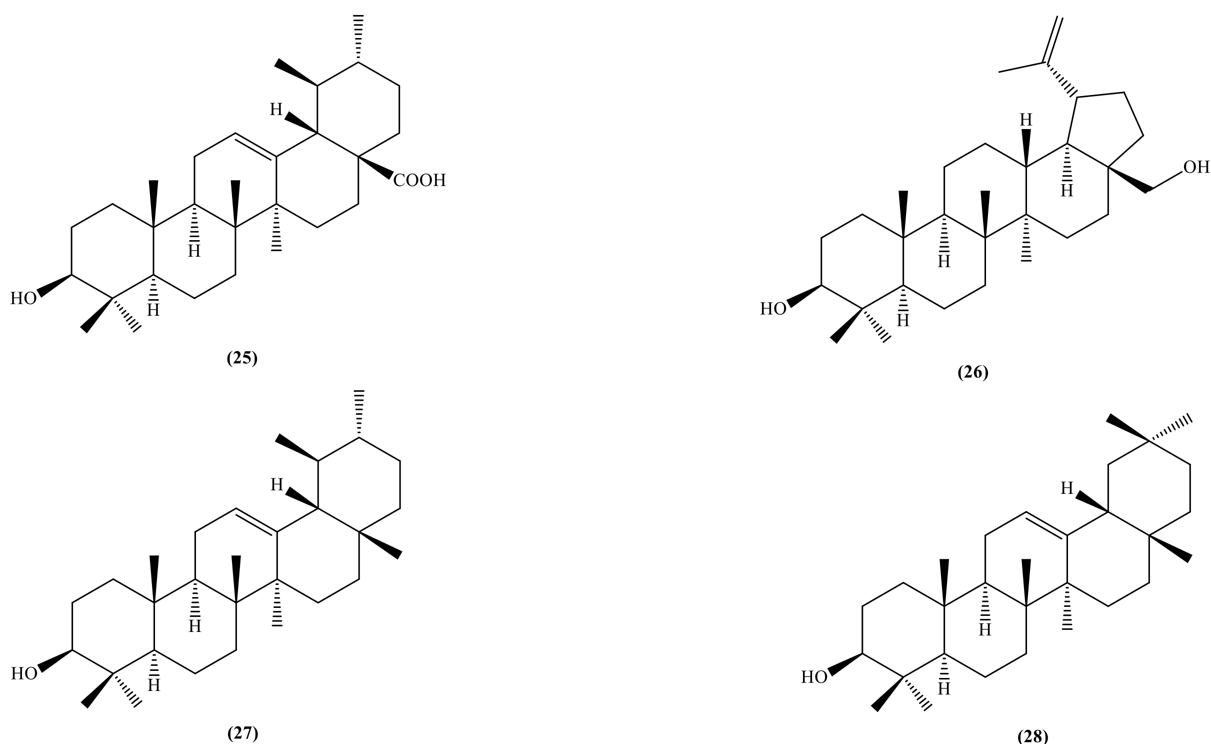
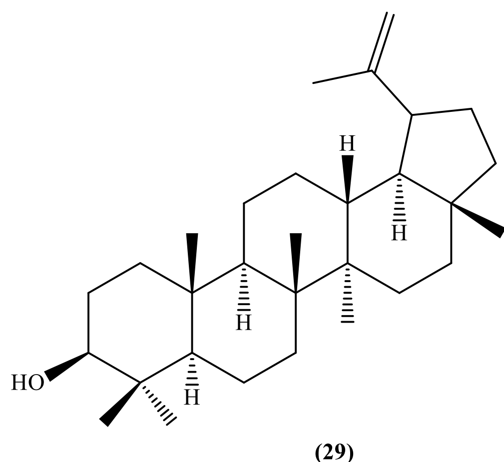


(24)

Anthocyanins, flavanols and phenolic acids have been isolated from berries of maqui (*Aristotelia chilensis* [Mol.] Stuntz) and are known to result in the antioxidant activity of these berries (Rodríguez *et al.*, 2016). Extracts obtained from berries of *Juniperus phoenicea* have also shown antioxidant activity (Laouar *et al.*, 2017). Grape (genus *Vitis*) and bilberries are well known for their high antioxidant activity due to their abundance of phenolic compounds (Fabani *et al.*, 2017; Colak *et al.*, 2017). Scientific studies have confirmed that blueberries (*Vaccinium angustifolium*) are also a rich source of antioxidants (Rodgers *et al.*, 2013). The "rabbiteye" blueberry, *Vaccinium ashei,* has been found to contain the antioxidant active triterpenes ursolic acid **(25)**, betulin **(26),** α-amyrin **(27)** and β-amyrin **(28)** (Neto, 2011).



American persimmons (*Diospyros virginiana* L.) are known to contain various triterpenes which are responsible for their antioxidant activity. These triterpenes include betulin **(26),** lupeol **(29),** and betulinic acid **(30)** (Grygorieva *et al.*, 2018). The wide variety of antioxidants in all these berries confirms berries are a rich source of antioxidants.



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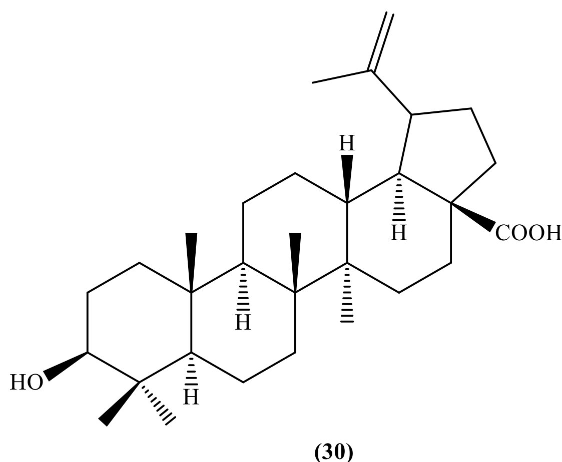

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Many registered drugs have an antioxidant action which may contribute to their pharmacological activity. Aspirin (acetyl salicylic acid) is a derivative of salicylic acid and is used to treat a number of diseases. Aspirin is also known to help prevent heart attacks and may reduce the risk of certain types of cancer. It has been found that aspirin possesses antioxidant activity (Shi *et al.*, 1999).

2.4 Plants selected for this study

2.4.1 *Teclea simplicifolia*

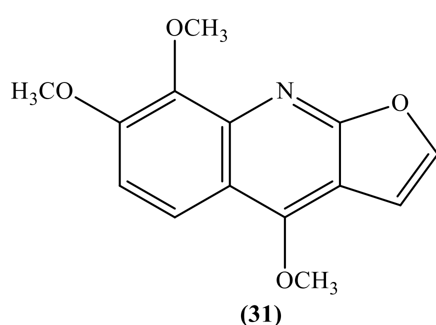
The genus *Teclea* belongs to the Rutaceae family. *Teclea simplicifolia* (Engl.) I. Verd. is a tree that grows up to 10-20 meters tall. It has been found to grow in Kenya, Ethiopia and Tanzania. The berries of *Teclea simplicifolia* are reported to be edible to both human beings and goats. *T. simplicifolia* is known as *munderendu* in Kikuyu, *ikirai* in Dorobo and *nkilaiorok* in Samburu (Kokwaro, 1993; Njoroge and Bussmann, 2006).

In traditional medicine a bark decoction of *Teclea simplicifolia* is drunk to treat chest complaints while a root decoction is drunk to treat stomachache, backache, leprosy, gonorrhoea and brucellosis. Leaves and twigs are used for treating pleurisy. A leaf decoction is taken against pneumonia, leaf ash is applied externally against leprosy and fruits are chewed to relieve toothache (Louppe *et al.*, 2008). In Kenya the root decoction of *T. simplicifolia* is used for treating pneumonia (Muthaura *et al.*, 2007). The Maasai use a root infusion for treating gonorrhoea (Nguta *et al.*, 2010).

Figure 1: *Teclea simplicifolia* tree (Self)

Figure 2: Berries of *Teclea simplicifolia* (Self)

A number of scientific studies have been carried out to investigate the medicinal properties of *Teclea simplicifolia*. In Kenya, extracts obtained from *T. simplicifolia* are used as traditional plant medicine for managing malaria (Njoroge and Bussmann, 2006). Various species of *Teclea*, including *T. simplicifolia* have been found to contain quinolone compounds, including skimmianine **(31)** and montrifoline **(32)**, and this explains their antimalarial properties (Wondimu *et al.*, 1988; Nguta *et al.*, 2010).



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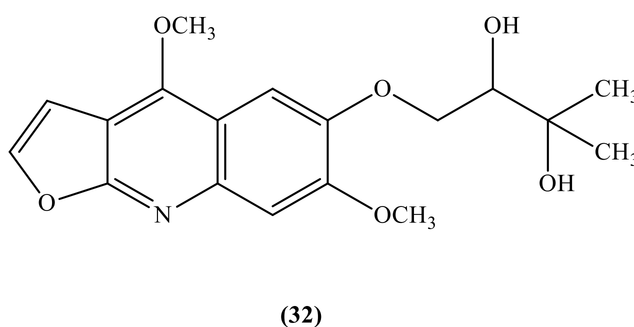

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Flavonoids and triterpenoids, including lupeol **(29)**, are among the type of compounds isolated from various species of *Teclea*. Compounds belonging to these classes are known to possess antioxidant activity hence these species of *Teclea* are a potential source of antioxidant compounds (Kuete *et al.*, 2008; Tesfaye *et al.*, 2018). However, there is no literature available on

scientific studies carried out to determine antioxidant compounds in berries of *Teclea simplicifolia*.

2.4.2 *Ziziphus mucronata*

The genus *Ziziphus* belongs to the Rhamnaceae family (Nyila *et al.*, 2012). Plants belonging to this genus are able to withstand drought since they are heat resistant. *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. is known as buffalo thorn in English and *mkunazi* in Swahili. It is a small-to medium-sized tree with a spreading canopy. It is found to grow in sub-Saharan Africa (Muga *et al.*, 2014). The berries of *Z. mucronata* are edible to human beings (Olajuyigbe and Afolayan, 2011).

Figure 3: *Ziziphus mucronata* tree (Tsammalex, 2005)

Figure 4: Berries of *Ziziphus mucronata* (West African Plants)

In ethnomedicine, the pastes of the roots and leaves of *Ziziphus mucronata* are used to treat boils, swollen glands, wounds and sores while steam baths from the bark are used to purify and improve skin complexion. *Z. mucronata* is also used to treat coughs and chest problems. Its bark and roots are used for the treatment of rheumatism and gastrointestinal complaints. The roots are used for treating snake bites, gonorrhoea, diarrhoea, and dysentery (Olajuyigbe and Afolayan, 2011; Nyila *et al.*, 2012).

Scientific studies carried out to investigate the medicinal properties of *Ziziphus mucronata* have reported *Z. mucronata* to possess antimicrobial and antifungal activity (Mokgolodi *et al.*, 2011).

In vivo studies have revealed that extracts obtained from *Z. mucronata* showed antimalarial properties (Zininga *et al.*, 2017). *Z. mucronata* is suspected to contain antioxidant activity. The bark extracts of *Z. mucronata* have been found to exhibit strong antioxidant property and free radical scavenging capability. The leaf extracts obtained from *Z. mucronata* have also been found to possess antioxidant activity. The antioxidant activity for the leaves and bark extracts was determined using the ABTS, DPPH and FRAP methods (Olajuyigbe and Afolayan, 2011; Kwape and Chaturvedi, 2012).

A number of antioxidant compounds have been isolated from various species of *Ziziphus*. Rutin **(14),** ursolic acid **(25)** and betulinic acid **(30)** have been isolated from *Ziziphus jujuba* (Lingampally *et al.*, 2012; Tahergorabi *et al.*, 2015). Betulin **(26)** has been isolated from *Ziziphus mauritiana* and *Ziziphus vulgaris* (Król *et al.*, 2015). There is barely any literature available on scientific studies carried out in Kenya to investigate antioxidant activity in berries of *Z. mucronata*.

CHAPTER THREE

MATERIALS AND METHODS

3.1 General experimental methods

The solvents used were of analytical grade and were purchased from Kobian Kenya Limited, Nairobi. Silica gel of 0.063-0.2 mm from Machery-Nagel was used for column chromatography. Thin layer chromatography was carried out using aluminium-backed TLC plates (Merck, 20 x 20 cm, silica gel 60 F_{254}-coated). Visualisation of the spots on the TLC plates was aided by viewing the developed plates under UV lamp pre-set at fixed wavelengths of 254 nm and 365 nm. The TLC plates were then sprayed with a freshly prepared *p*-anisaldehyde solution before heating at 115ºC to observe coloured spots. The solution of *p*-anisaldehyde was prepared by adding 2.5% (v/v) concentrated sulphuric acid and 1.5% (v/v) p-anisaldehyde to 96% (v/v) cold methanol. The compounds were identified using NMR spectroscopy. Both 1-D NMR and 2-D NMR spectra were obtained using a 500 MHz Bruker AVANCE nuclear magnetic resonance spectrometer.

3.2 Sample collection and preliminary preparations

Berries of *Teclea simplicifolia* (Engl.) I. Verd. were collected from Egerton University in Njoro (0º22'11"S 35º55'55"E) while berries of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. were collected from Mogotio in Baringo County (0º00'20"S 35º57'11"E). The plants were identified by a taxonomist, Prof. S. T. Kariuki of Biological Sciences, Egerton University.

Fresh berries of *Teclea simplicifolia* and *Ziziphus mucronata* were collected and brought to the Chemistry laboratory at Egerton University. The berries collected for *T. simplicifolia* weighed 3 kilogrammes while those for *Z. mucronata* weighed 1.5 kilogrammes. The berries were air dried at room temperature inside the laboratory. When they were completely dry, they were ground into fine powder using a Waring Commercial Heavy Duty Blender (Model Number 24CB10C, serial number 563972). The powdered material was weighed, dried to constant weight, packaged in paper bags, then sealed and stored under dry conditions awaiting further processing and analysis.

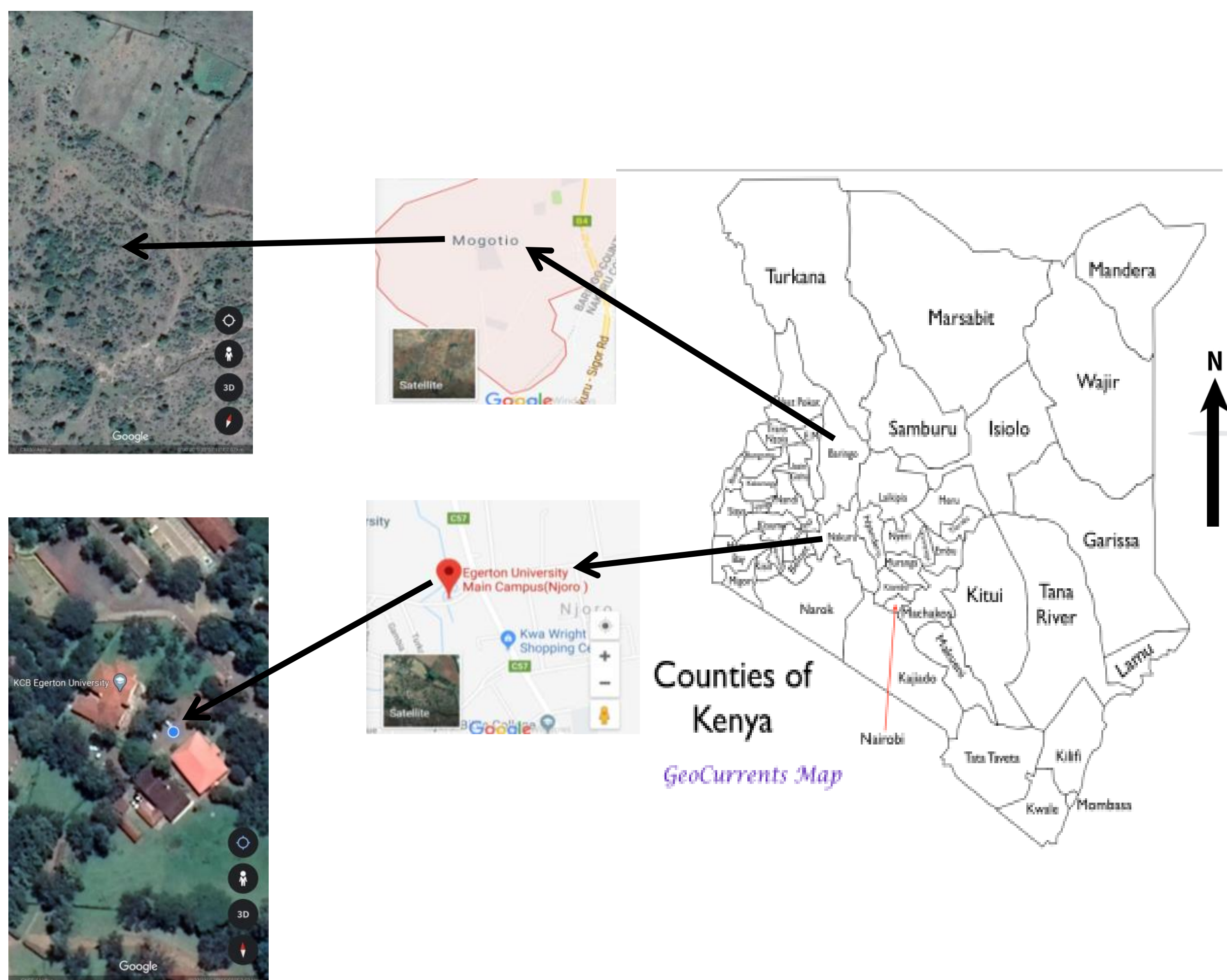


Figure 5: Sampling areas (GeoCurrents Map, 2018)

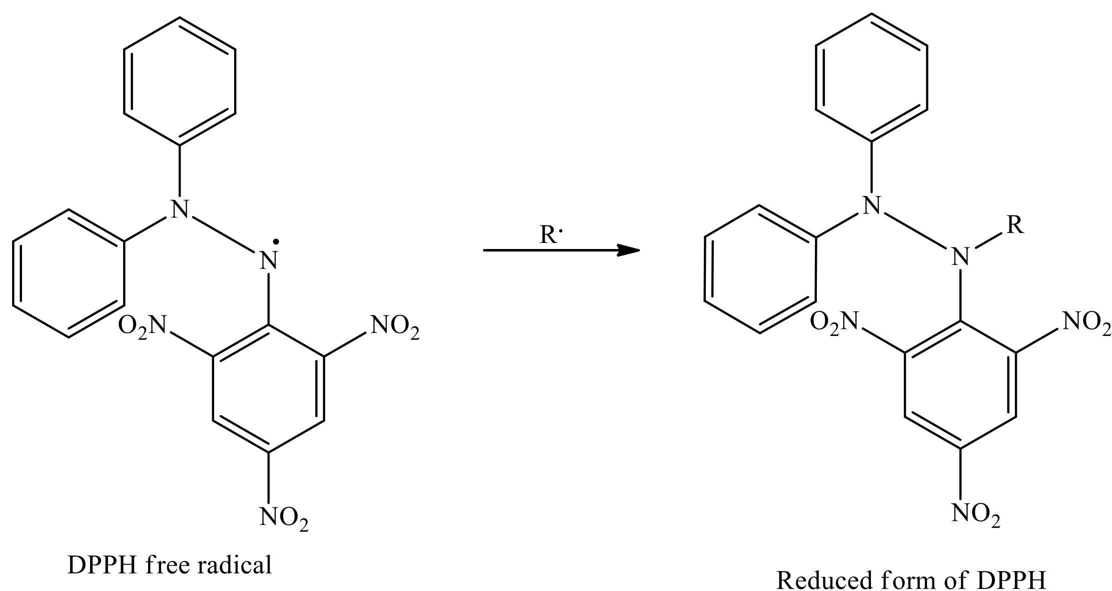
3.3 Preparation of crude extracts

Cold extraction was carried out by soaking the powdered materials in the solvents at room temperature. Pulverized samples of the berries of *Teclea simplicifolia* and *Ziziphus mucronata* of known weight (800 grams of *Ziziphus mucronata* and 2 kilograms of *Teclea simplicifolia)* were equally divided into 4x2000 mL conical flasks each. They were soaked and extracted using cyclohexane followed by ethyl acetate and then methanol for 48 hours with regular shaking using the procedure described by (Kwape and Chaturvedi, 2012). The solvent and the pulverized sample in each flask were in the ratio 3:1. Extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (Elisters, Serial Number 14585). Extraction was first carried out using cyclohexane to de-fat the crude extract.

Extraction using each solvent was carried out thrice. Weights of the crude extracts collected were noted.

3.4 Testing the antioxidant activity of the crude extracts

The DPPH method was used to check if the crude extracts exhibit antioxidant activity. The DPPH solution has a deep violet colour but when an antioxidant is added to it, it gets reduced and loses its violet colour (Scheme 5) (Alam *et al.*, 2013). The absorbance of DPPH solution is measured spectrophotometrically at 517 nm, the λ_{max} for DPPH radical, after which crude extracts are added to the DPPH solution. The reaction is allowed for 30 minutes and absorbance is again measured at 517 nm (Fattahi *et al.*, 2014). A decrease in intensity of absorbance confirms antioxidant activity of the crude extracts.



Scheme 5: Reaction of DPPH free radical with other radicals (R· = ·H, alkyl radical etc.)

The DPPH free radical scavenging activity was determined for the standard and the crude extracts using the method described by (Sayed-Ahmad *et al.*, 2015). Ascorbic acid **(1)** was used as the standard hence its DPPH activity was first established. A stock solution of ascorbic acid **(1)** was prepared by dissolving 781.25 µg of ascorbic acid **(1)** in 25 mL of methanol in a 25 mL volumetric flask. The next serial dilution was prepared by pipetting out 12.5 mL of ascorbic acid **(1)** from the previous solution into another 25 mL volumetric flask and this was diluted to the

mark. This was repeated until 6 solutions of ascorbic acid **(1)** with varying concentrations were obtained. The volumetric flasks were labelled 1 to 6, 1 being the most concentrated solution.

A solution of DPPH was prepared by dissolving 0.01 grams of DPPH in methanol in a 250 mL volumetric flask. This volumetric flask was covered using aluminium foil and kept in the dark. The control was prepared by adding 2 mL of methanol to 2 mL of DPPH solution. The absorbance of the control was measured using a Shimadzu UV-1800 240V UV-VIS spectrophotometer at 517 nm and methanol was used as the blank. The absorbance for the control had to be measured every time a fresh stock solution of DPPH was prepared.

The antioxidant activity of ascorbic acid **(1)** was determined by adding 2 mL of DPPH solution to 2 mL of each of the serial dilutions prepared for ascorbic acid **(1)**. This was done in triplicates for each serial dilution. The test tubes were kept in the dark for 30 minutes after which the absorbance was measured at 517 nm using methanol as a blank. The antioxidant activity of the crude extracts obtained from the berries of both *Teclea simplicifolia* and *Ziziphus mucronata* was determined in a similar manner as that of the ascorbic acid **(1)**.

The DPPH free radical scavenging activity was determined using the formula

$$\%\ scavenging\ activity = \left[\frac{(Abs\ control - Abs\ sample)}{Abs\ control}\right] \times 100$$

The IC_{50} value was the parameter used to compare the antioxidant activity of the crude extracts with that of ascorbic acid **(1)**. The IC_{50} value is the concentration required for 50% scavenging of DPPH radicals in the specified time period. Hence in this case the IC_{50} value is the concentration required for 50% scavenging of DPPH radicals in 30 minutes.

3.5 Fractionation of the crude extracts to obtain pure active compounds

The extracts obtained from the berries of *Teclea simplicifolia* exhibited less antioxidant activity in comparison to ascorbic acid **(1)**. Furthermore, they exhibited less antioxidant activity than that shown by the extracts obtained from the berries of *Ziziphus mucronata* hence they were not further pursued for seeking antioxidant compounds. The ethyl acetate and methanol extracts obtained from the berries of *Ziziphus mucronata* showed good antioxidant activity in comparison to ascorbic acid **(1)** hence they were pursued for antioxidant compounds. Column

chromatography was used to separate the crude extracts into enriched fractions while thin layer chromatography was used to determine the purity of the eluent fractions.

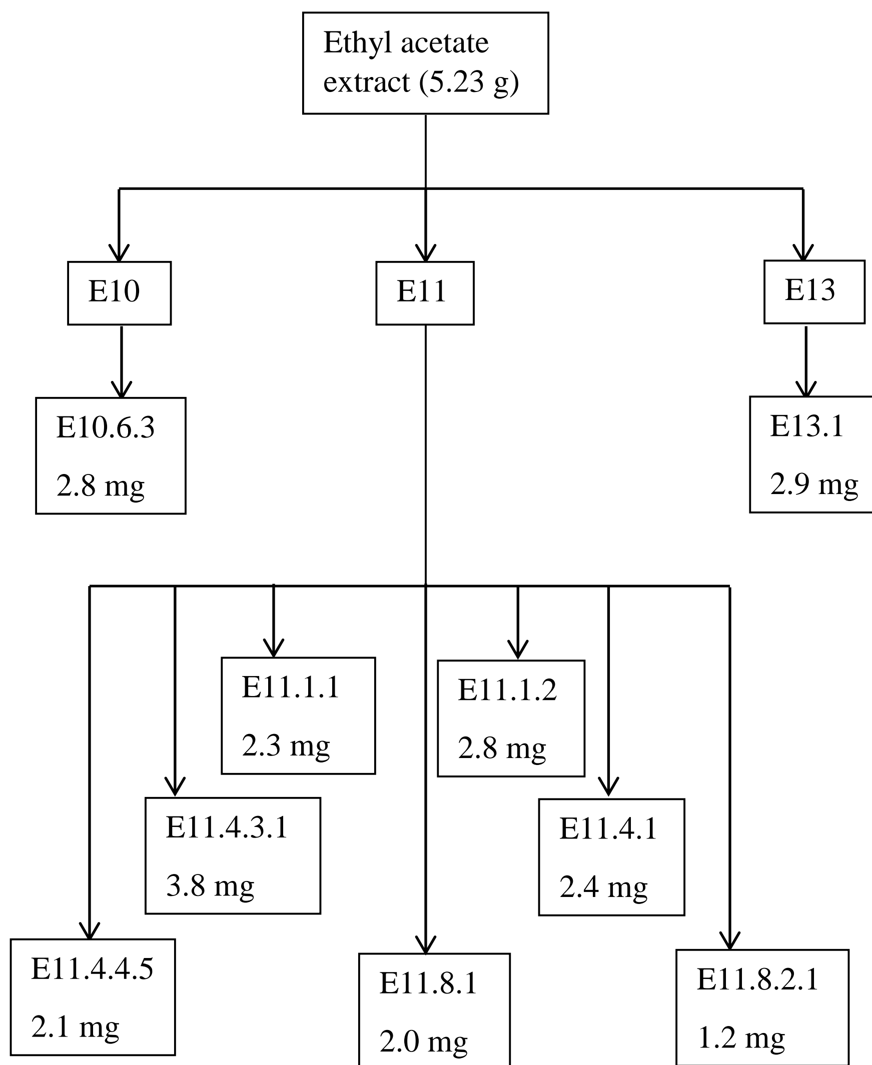


Figure 6: Flow chart showing isolation of compounds from ethyl acetate extract

The flow chart in Figure 6 shows the compounds isolated from the ethyl acetate extract obtained from the berries of *Ziziphus mucronata*. The ethyl acetate extract was dissolved in minimum quantity of ethyl acetate and adsorbed onto silica gel. Silica gel slurry was prepared using silica gel and 50% cyclohexane - 50% DCM (dichloromethane). The slurry was carefully introduced into a vertically mounted glass column to ensure that no air bubbles were trapped. The silica gel adsorbed ethyl acetate extract was introduced into the column. The column was eluted using

discrete solvent gradient mobile phase of varying polarities, starting from 50% cyclohexane - 50% DCM and increasing proportions of DCM, ethyl acetate and methanol. The eluent fractions were collected differently for each solvent gradient system as much as possible. Thin layer chromatography was used to determine the purity of the eluent fractions. A sample of each of the fractions was loaded onto a TLC plate using a capillary tube. The TLC plates were then placed in chromatographic tanks and left to develop in the selected solvent systems. Visualisation of the spots on the TLC plates was aided by viewing the developed plates under UV lamp and then spraying with a freshly prepared *p*-anisaldehyde solution before heating at 115ºC to observe coloured spots. Similar fractions were combined and further separated using column chromatography to obtain purified compounds. Eventually 8 possibly pure compounds were isolated from the ethyl acetate extract. The possibly pure compounds were each transferred into a screw-capped vial and stored at 4ºC for further analysis.

The methanol extract obtained from the berries of *Ziziphus mucronata* showed the best antioxidant activity in comparison to ascorbic acid **(1)** out of all of the 6 extracts. However, it could not be packed into a column due to its high polarity. Hence it was further extracted by carrying out solvent-solvent extraction using ethyl acetate and water in the ratio 2:1. A 500 mL separating funnel was used for the fractionation. Ethyl acetate and water were added to the methanol extract in a ratio of 3:1. The separating funnel was shaken vigorously and left to stand. The upper layer containing the ethyl acetate extract was collected in a conical flask so that it could be concentrated under reduced pressure using the rotary evaporator. Fresh ethyl acetate was added to the separating funnel and the extraction procedure carried out two more times. The weight of the ethyl acetate extract obtained from the methanol extract was noted down. The ethyl acetate extract obtained from the methanol extract was then separated into enriched fractions using column chromatography. Similar fractions were combined and further separated using column chromatography to obtain purified compounds. Eventually 14 possibly pure compounds were isolated from the ethyl acetate extract obtained from the methanol extract as shown in Figure 7. The possibly pure compounds were each transferred into a screw-capped vial and stored at 4ºC for further analysis.

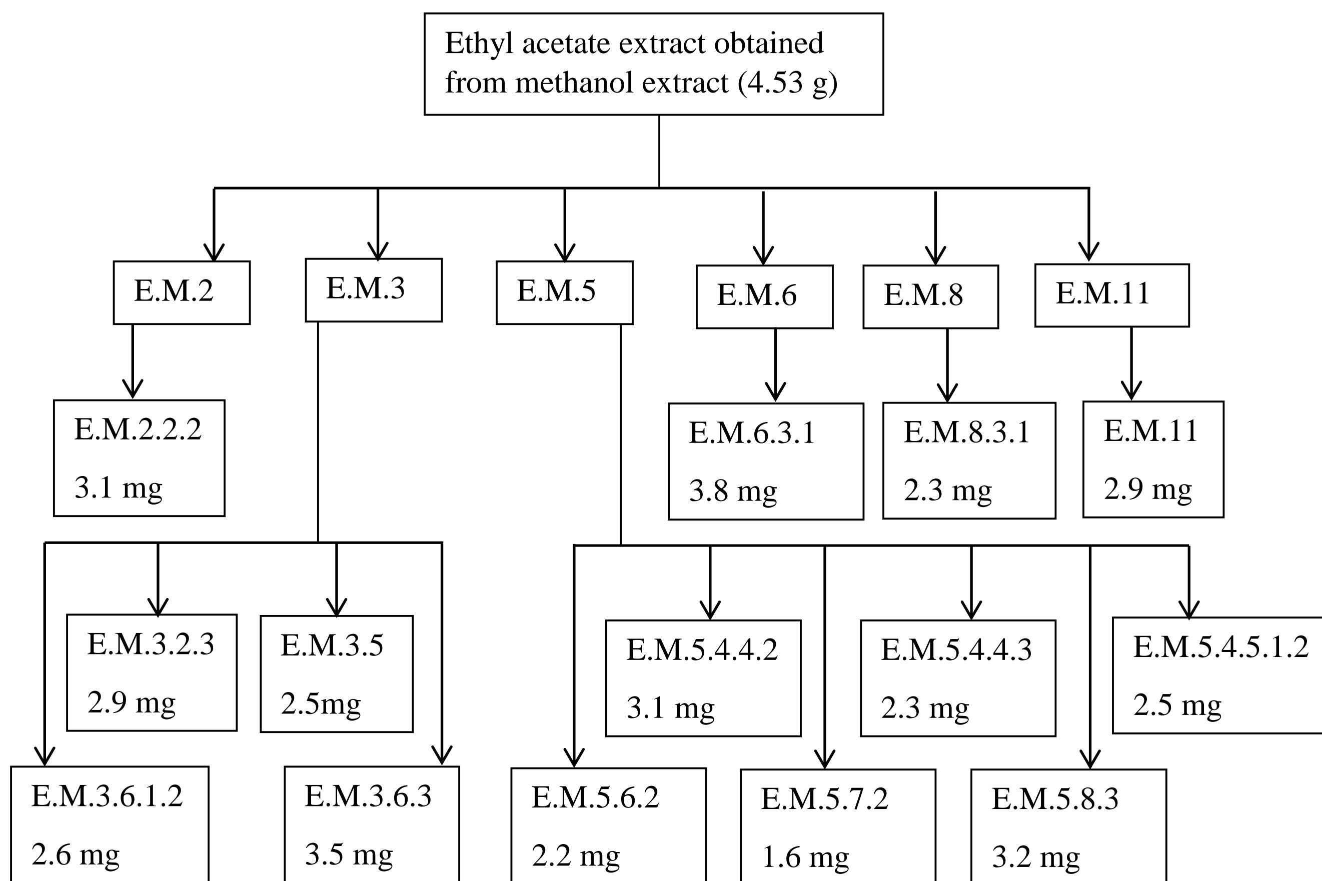


Figure 7: Flow chart showing isolation of compounds from berries of *Ziziphus mucronata*

3.6 Determination of the chemical structures of the isolated antioxidant compounds

3.6.1 Spectroscopic analysis of the purified compounds

Nuclear magnetic resonance spectroscopy was used to deduce the most probable structures of the purified compounds. The NMR analysis was carried out at University of Surrey in the United Kingdom. Both 1-D NMR and 2-D NMR spectra were obtained using a 500 MHz Bruker AVANCE nuclear magnetic resonance spectrometer. The compounds were dissolved in deuterated solvents and spectra recorded at ambient temperature. The solvents used were deuterated chloroform ($CDCl_3$) with the solvent signals δ_H = 7.26 and δ_C = 77.23 as reference and deuterated methanol (CD_3OD) with the solvent signals δ_H = 3.35, δ_H = 4.87 and δ_C = 49.1 as reference. The compounds were dissolved in 5 mL of deuterated solvent in a 5 mm NMR tube. The coupling constants were reported in Hertz (Hz) and the chemical shifts (δ) in ppm using tetramethylsilane (TMS) as the internal standard.

3.6.2 Structure elucidation of the compounds from the spectral data

The data obtained from the NMR spectra was processed using TOPSPIN software (Bruker, Version 4.0.6). The chemical shifts in ppm and coupling constants observed in NMR spectra were used to deduce the most probable structures of the compounds. HSQC showed which hydrogen is attached to which carbon, HMBC showed which carbons are two or three bonds away from which protons, COSY showed which protons are adjacent and NOESY located protons having the same orientation. Although 22 compounds were sent for NMR analysis, structure elucidation was only possible for 3 compounds since the full set of spectra could not be obtained for the remaining compounds as the compounds had decomposed.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Crude extract yield

The respective crude extract yields from the berries of *Teclea simplicifolia* and *Ziziphus mucronata* were expressed as percentage weight of the dried pulverized sample.

4.1.1 Crude extract yield obtained from *Teclea simplicifolia*

Two (2) kilogrammes of dried pulverised berries of *Teclea simplicifolia* were weighed and extracted successively using cyclohexene, ethyl acetate and methanol as shown in Table 1. The corresponding percentage yields are also presented in the table.

Table 1: Crude extract yield for extracts from *Teclea simplicifolia*

Order of extraction	Solvent used (mL)	Crude extract yield (grammes)	Crude extract yield (%w/w)
1	Cyclohexane (6000)	138.24	6.91
2	Ethyl acetate (6000)	193.67	9.68
3	Methanol (6000)	595.92	29.80

4.1.2 Crude extract yield obtained from *Ziziphus mucronata*

Starting with 800 grammes of dried pulverised berries of *Ziziphus mucronata*, the corresponding yields of crude extracts from successive extractions using cyclohexane, ethyl acetate and methanol are presented in Table 2.

Table 2: Crude extract yield for extracts from *Ziziphus mucronata*

Order of extraction	Solvent used (mL)	Crude extract yield (grammes)	Crude extract yield (%w/w)
1	Cyclohexane (2400)	4.45	0.56
2	Ethyl acetate (2400)	5.23	0.65
3	Methanol (2400)	224.45	28.06

4.2 Antioxidant activity of crude extracts

The DPPH free radical scavenging activity was determined for the crude extracts and ascorbic acid **(1)** using the formula

$$\% \, scavenging \, activity = \left[\frac{(Abs \, control - Abs \, sample)}{Abs \, control} \right] \times 100$$

Ascorbic acid **(1)** was used as the standard hence its DPPH free radical scavenging activity was first determined. Each of the concentrations given in the second column gave an absorbance value for each of the replicate experiments, from which the percentage scavenging capacity was calculated as shown in Table 3.

Table 3: Free radical scavenging activity of ascorbic acid (1)

		Exp. 1		Exp. 2		Exp. 3	
	Conc. (μg/mL)	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.
1	31.25	0.008	98.28	0.008	98.28	0.008	98.28
2	15.63	0.034	92.70	0.024	94.85	0.008	98.28
3	7.81	0.035	92.48	0.035	92.49	0.026	94.42
4	3.91	0.028	93.99	0.037	92.06	0.036	92.28
5	1.95	0.171	63.31	0.205	56.01	0.244	47.64
6	0.98	0.376	19.31	0.350	24.89	0.349	25.11

*Absorbance of control: 0.466

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

The IC_{50} value was calculated using the IC_{50} Calculation Software (Bio Soft, Version 1.00). In order for the software to calculate the IC_{50} value, it required the input of the concentrations of the sample and their corresponding average percentage scavenging capacities. The IC_{50} value for ascorbic acid **(1)** was found to be 1.5 μg/mL.

4.2.1 Antioxidant activity of extracts from berries of *Teclea simplicifolia*

The DPPH free radical scavenging activity was determined for the cyclohexane, ethyl acetate and methanol crude extracts (Table 1) obtained from berries of *Teclea simplicifolia*. Each of the concentrations given in the first column gave an absorbance value for each of the replicate experiments, from which the percentage scavenging capacity was calculated as shown in Tables 4, 5 and 6.

Table 4: Free radical scavenging activity of cyclohexane extract from berries of *Teclea simplicifolia*

		Exp. 1		Exp. 2		Exp. 3	
	Conc. (μg/mL)	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.
1	31.25	0.435	18.54	0.435	18.54	0.443	17.04
2	15.63	0.468	12.36	0.484	9.36	0.454	14.98
3	7.81	0.469	12.17	0.429	19.66	0.488	8.61
4	3.91	0.468	12.36	0.462	13.48	0.471	11.80
5	1.95	0.451	15.54	0.456	14.61	0.503	5.81
6	0.98	0.451	15.54	0.501	6.18	0.519	2.81

*Absorbance of control: 0.534

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

Table 5: Free radical scavenging activity of ethyl acetate extract from berries of *Teclea simplicifolia*

		Exp. 1		Exp.2		Exp. 3	
	Conc. (µg/mL)	Abs SAMPLE	% Sc. cap.	Abs SAMPLE	% Sc. cap.	Abs SAMPLE	% Sc. cap.
1	31.25	0.415	25.09	0.421	24.01	0.442	20.22
2	15.63	0.440	20.58	0.463	16.43	0.477	13.90
3	7.81	0.454	18.05	0.463	16.43	0.471	14.98
4	3.91	0.479	13.54	0.479	13.54	0.479	13.54
5	1.95	0.467	15.70	0.474	14.44	0.475	14.26
6	0.98	0.482	13.00	0.487	12.09	0.493	11.01

*Absorbance of control: 0.554

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

Table 6: Free radical scavenging activity of methanol extract from berries of *Teclea simplicifolia*

		Exp. 1		Exp. 2		Exp. 3	
	Conc. (µg/mL)	Abs SAMPLE	% Sc. cap.	Abs SAMPLE	% Sc. cap.	Abs SAMPLE	% Sc. cap.
1	31.25	0.399	30.37	0.381	33.51	0.369	35.60
2	15.63	0.423	26.18	0.428	25.31	0.431	24.78
3	7.81	0.432	24.61	0.452	21.12	0.438	23.56
4	3.91	0.451	21.29	0.456	20.42	0.456	20.42
5	1.95	0.478	16.58	0.478	16.58	0.493	13.96
6	0.98	0.483	15.71	0.448	21.82	0.458	20.07

*Absorbance of control: 0.573

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

The IC_{50} value was used to compare the antioxidant activity of the extracts with that of the standard. The IC_{50} value was calculated using the IC_{50} Calculation Software. Ascorbic acid **(1)** was used as the standard for the DPPH assay. Thus the antioxidant activity of the extracts was compared to that of ascorbic **(1)** acid which was found to have an IC_{50} value of 1.5 µg/mL.

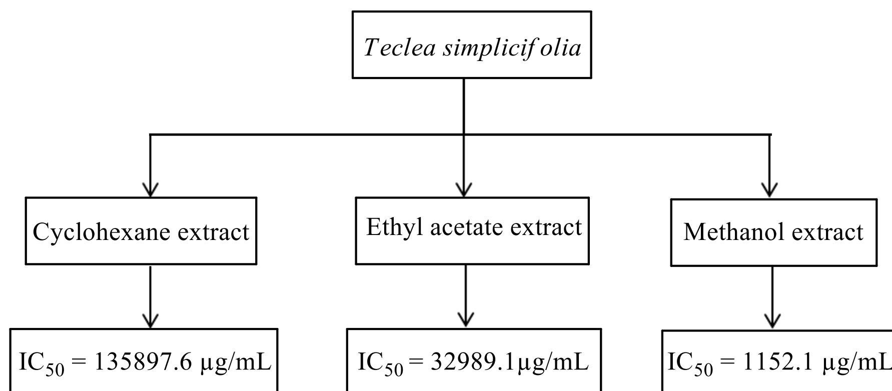


Figure 8: Antioxidant activity of extracts obtained from berries of *Teclea simplicifolia*

The extracts obtained from the berries of *Teclea simplicifolia* showed very low antioxidant activity since their IC_{50} values were quite high compared to that of ascorbic acid **(1)** which was found to have an IC_{50} value of 1.5 µg/mL. The methanol extract obtained from the berries of *Teclea simplicifolia* exhibited the highest antioxidant activity compared to the other extracts obtained from the berries of *Teclea simplicifolia* with an IC_{50} value of 1,152.1 µg/mL, followed by ethyl acetate extract with an IC_{50} value of 32,989.1µg/mL and the cyclohexane extract exhibited the least antioxidant activity with an IC_{50} value of 135,897.6 µg/mL.

4.2.2 Antioxidant activity of extracts from berries of *Ziziphus mucronata*

The DPPH free radical scavenging activity was determined for the cyclohexane, ethyl acetate and methanol crude extracts (Table 2) obtained from berries of *Ziziphus mucronata*. Each of the concentrations given in the first column gave an absorbance value for each of the replicate experiments, from which the percentage scavenging capacity was calculated as shown in Tables 7, 8 and 9.

Table 7: Free radical scavenging activity of cyclohexane extract from berries of *Ziziphus mucronata*

		Exp. 1		Exp. 2		Exp. 3	
	Conc. (µg/mL)	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.
1	31.25	0.398	28.16	0.411	25.81	0.441	20.40
2	15.63	0.449	18.95	0.477	13.90	0.483	12.82
3	7.81	0.475	14.26	0.467	15.70	0.488	11.91
4	3.91	0.494	10.83	0.460	16.97	0.474	14.44
5	1.95	0.522	5.78	0.485	12.46	0.471	14.98
6	0.98	0.477	13.90	0.472	14.80	0.482	13.00

*Absorbance of control: 0.554

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

Table 8: Free radical scavenging activity of ethyl acetate extract from berries of *Ziziphus mucronata*

	Conc.	Exp. 1		Exp 2		Exp. 3	
	(µg/mL)	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.	Abs $_{SAMPLE}$	% Sc. cap.
1	31.25	0.353	38.50	0.320	44.25	0.353	38.50
2	15.63	0.399	30.49	0.421	26.66	0.369	35.71
3	7.81	0.423	26.31	0.438	23.69	0.431	24.91
4	3.91	0.432	24.74	0.462	19.51	0.438	23.69
5	1.95	0.452	21.25	0.452	21.25	0.452	21.25
6	0.98	0.461	19.69	0.466	18.82	0.466	18.82

*Absorbance of control: 0.574

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

Table 9: Free radical scavenging activity of methanol extract from berries of *Ziziphus mucronata*

		Exp. 1		Exp. 2		Exp. 3	
	Conc.(μg/mL)	Abs SAMPLE	% Sc. cap.	Abs SAMPLE	% Sc. cap.	Abs SAMPLE	% Sc. cap.
1	31.25	0.064	86.58	0.065	86.37	0.057	88.05
2	15.63	0.195	59.12	0.150	68.55	0.177	62.89
3	7.81	0.299	37.32	0.309	35.22	0.320	32.91
4	3.91	0.403	15.51	0.373	21.80	0.373	21.80
5	1.95	0.371	22.22	0.405	15.09	0.410	14.05
6	0.98	0.423	11.32	0.427	10.48	0.423	11.32

*Absorbance of control: 0.477

Key:

Conc.: Concentration

Exp.: Experiment

Sc. cap.: Scavenging capacity

The IC_{50} value was used to compare the antioxidant activity of the extracts with that of the standard. The IC_{50} value was calculated using the IC_{50} Calculation Software. Ascorbic acid **(1)** was used as the standard for the DPPH assay. Thus the antioxidant activity of the extracts was compared to that of ascorbic **(1)** acid which was found to have an IC_{50} value of 1.5 μg/mL.

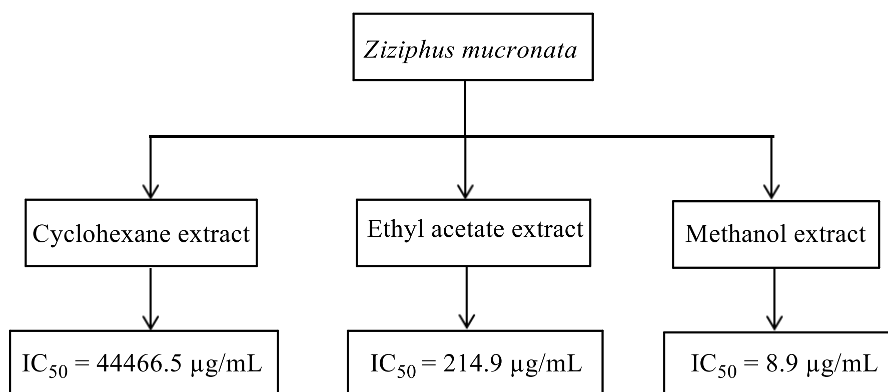


Figure 9: Antioxidant activity of extracts obtained from berries of *Ziziphus mucronata*

The extracts obtained from the berries of *Ziziphus mucronata* showed higher antioxidant activity than those obtained from *Teclea simplicifolia* since their IC_{50} values were lower than those for the extracts obtained from *Teclea simplicifolia*. The methanol extract obtained from the berries of *Ziziphus mucronata* exhibited the highest antioxidant activity compared to the other extracts obtained from the berries of *Ziziphus mucronata* with an IC_{50} value of 8.9 µg/mL, followed by ethyl acetate extract with an IC_{50} value of 214.9 µg/mL and the cyclohexane extract exhibited the least antioxidant activity with an IC_{50} value of 44,466.5 µg/mL.

4.2.3 Comparison of antioxidant activity of extracts obtained from berries of *Teclea simplicifolia*

A graph of concentration in µg/ml vs mean of % scavenging capacity was plotted to compare the DPPH scavenging activity of the crude extracts obtained from the berries of *Teclea simplicifolia* with that of ascorbic acid **(1)**.

Table 10: Scavenging capacity of extracts from *Teclea simplicifolia*

Conc.	Vit. C Mean of % sc. cap ± S. D.	CV	Cycl. Mean of % sc. cap. ± S. D.	CV	Eth. acet. Mean of % sc. cap. ± S. D.	CV	Meth. Mean of % sc. cap. ± S. D.	CV
0.98	23.10 ± 3.29	14.24	8.18 ± 6.60	80.68	12.03 ± 0.99	8.23	19.20 ± 3.15	16.41
1.95	55.65 ± 7.84	14.09	11.99 ± 5.37	44.78	14.80 ± 0.79	5.34	15.71 ± 1.51	9.61
3.91	92.78 ± 1.06	1.14	12.55 ± 0.86	6.85	13.54 ± 0.00	0.00	20.71 ± 0.50	2.41
7.81	93.13 ± 1.12	1.20	13.48 ± 5.64	41.84	16.49 ± 1.54	9.38	23.10 ± 1.79	7.74
15.63	95.28 ± 2.81	2.95	12.24 ± 2.81	22.96	16.97 ± 3.37	19.89	25.42 ± 0.71	2.79
31.25	98.28 ± 0.00	0.00	18.04 ± 0.87	4.82	23.11 ± 2.56	11.08	33.16 ± 2.64	7.96

Key:

Conc.: Concentration

Vit. C: Vitamin C

Sc. cap.: Scavenging capacity

Cycl.: Cyclohexane

Eth. acet.: Ethyl acetate

Meth: Methanol

From Table 10, the mean % scavenging capacity was calculated from the values reported in Tables 4, 5 and 6 and the standard deviations were also calculated for each concentration. It is evident that the coefficient of variations (CVs) for each of the determinations were not quite precise i.e. several CVs were beyond the acceptable limits (> 5%). However, this was more pronounced in the case of cyclohexane extract. This can be attributed to the types of compounds that are extracted by the nonpolar cyclohexane solvent. Given that they were re-dissolved in methanol for this determination, the solutions obtained were not always homogenous. Hence during dilutions, it is possible not to have an accurate distribution of the dissolving compounds in the final solution for the experiments. Equally the ethyl acetate and methanol extracts showed varied values for the CV, indicating that the nature of crude extracts in solutions for the experiment is affected by the dissolution. This was confirmed by the fact that solubility had been

enhanced by warming and constantly mixing the solution mixture when preparing the extracts for the DPPH assay. Nonetheless the values for the mean obtained provided sufficient grounds to be used to calculate the IC_{50} values and make comparisons with the ascorbic acid (**1**) to be able to track the antioxidant activity in the crude extracts.

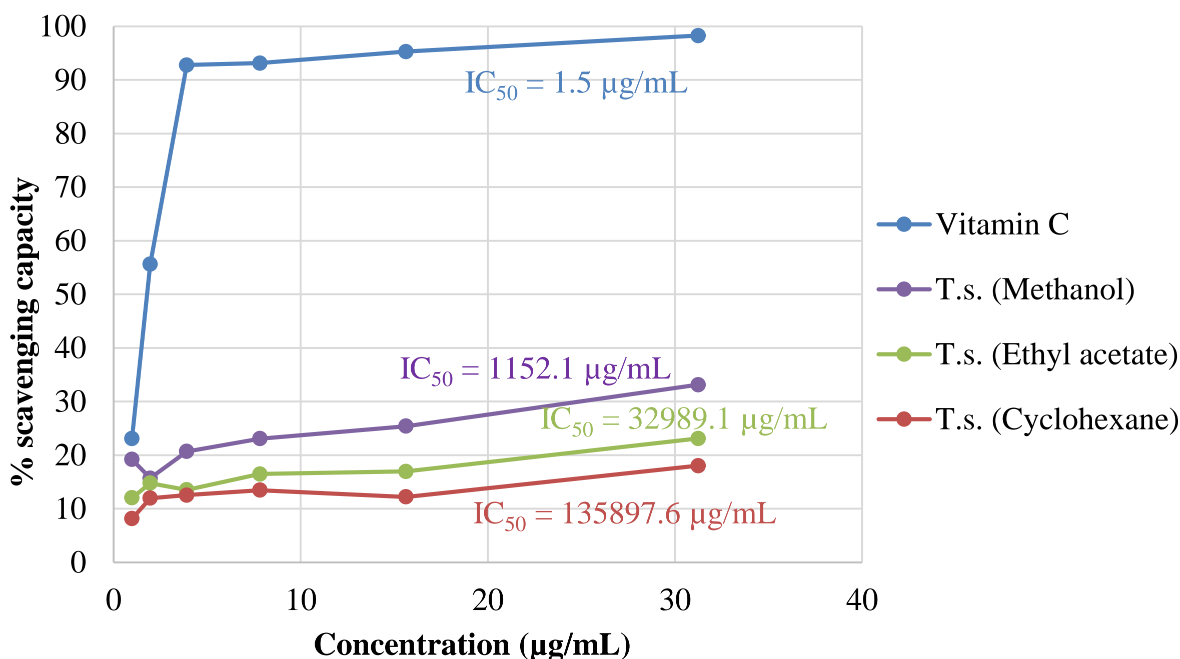


Figure 10: Graph showing antioxidant activity of extracts obtained from *Teclea simplicifolia*

The graph in Figure 8 clearly shows that the methanol extract obtained from the berries of *Teclea simplicifolia* exhibited the highest antioxidant activity compared to the ethyl acetate and the cyclohexane extracts. However, all of the three extracts demonstrated very low DPPH scavenging capacity in comparison to ascorbic acid (**1**) hence the berries of *T. simplicifolia* may be containing potential antioxidants but are not a rich source of antioxidants. It is in this regard that the extracts obtained from the berries of *Teclea simplicifolia* were not further pursued for antioxidant compounds.

4.2.4 Comparison of antioxidant activity of extracts obtained from berries of *Ziziphus mucronata*

A graph of concentration in µg/ml vs mean of % scavenging capacity was plotted to compare the DPPH scavenging activity of the crude extracts obtained from the berries of *Ziziphus mucronata* with that of ascorbic acid **(1)**.

Table 11: Scavenging capacity of extracts from *Ziziphus mucronata*

Conc.	Vit. C Mean of % sc. cap. ± S. D.	CV	Cycl. Mean of % sc. cap. ± S. D.	CV	Eth. acet. Mean of % sc. cap. ± S. D.	CV	Meth. Mean of % sc. cap. ± S. D.	CV
0.98	23.10 ± 3.29	14.24	13.90 ± 0.90	6.48	19.11 ± 0.50	2.62	11.04 ± 0.48	4.35
1.95	55.65 ± 7.84	14.09	11.07 ± 4.76	43.00	21.25 ± 0.00	0.00	17.12 ± 4.45	26.00
3.91	92.78 ± 1.06	1.14	14.08 ± 3.08	21.88	22.65 ± 2.77	12.23	19.71 ± 3.63	18.42
7.81	93.13 ± 1.12	1.20	13.96 ± 1.91	13.68	24.97 ± 1.31	5.25	35.15 ± 2.20	6.26
15.63	95.28 ± 2.81	2.95	15.22 ± 3.28	21.55	30.95 ± 4.55	14.70	63.52 ± 4.75	7.51
31.25	98.28 ± 0.00	0.00	24.79 ± 3.98	16.05	40.42 ± 3.32	8.21	87.00 ± 0.91	1.05

Key:

Conc.: Concentration

Vit. C: Vitamin C

Sc. cap.: Scavenging capacity

Cycl.: Cyclohexane

Eth. acet.: Ethyl acetate

Meth: Methanol

From Table 11, the mean % scavenging capacity was calculated from the values reported in Tables 7, 8 and 9 and the standard deviations were also calculated for each concentration. The coefficient of variations (CVs) for each of the determinations were not quite precise, just as for the extracts obtained from *Teclea simplicifolia*. However, the values for the mean obtained

provided sufficient grounds to be used to calculate the IC_{50} values and make comparisons with the ascorbic acid (**1**) to be able to establish the antioxidant activity in the crude extracts.

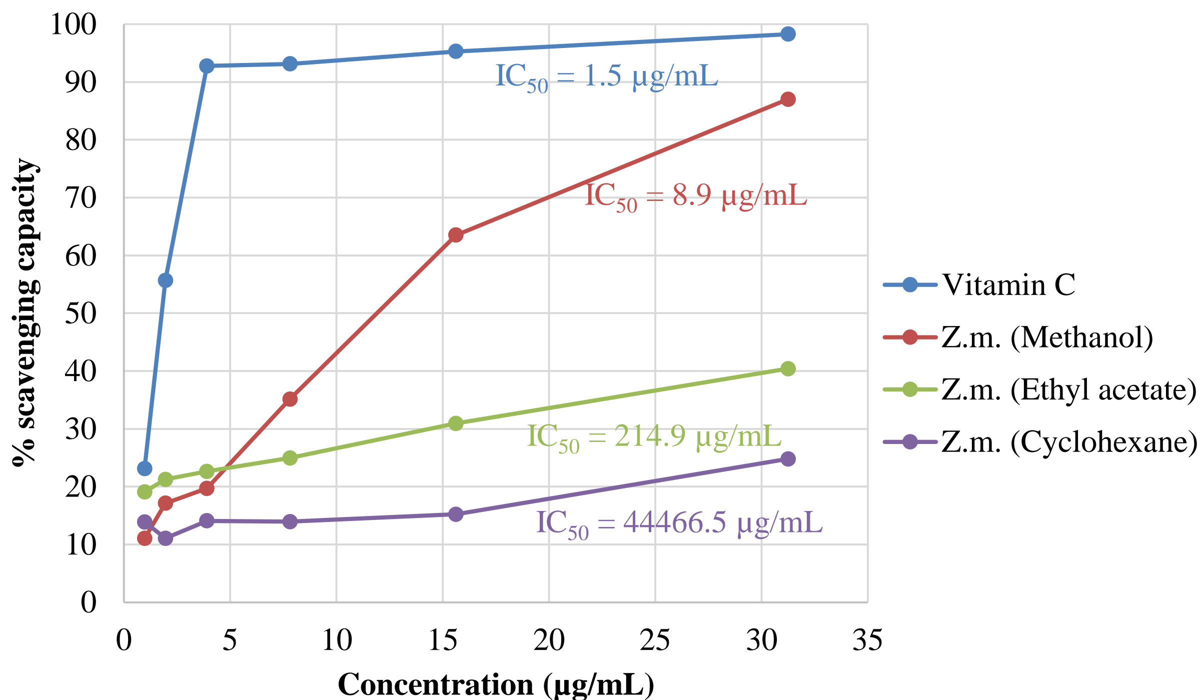


Figure 11: Graph showing antioxidant activity of extracts obtained from *Ziziphus mucronata*

The graph in figure 9 clearly shows that the methanol extract obtained from the berries of *Ziziphus mucronata* exhibited the highest antioxidant activity compared to the ethyl acetate and the cyclohexane extracts. Furthermore, the methanol extract obtained from the berries of *Ziziphus mucronata* exhibited the highest antioxidant activity compared to all of the other extracts. The methanol extract's IC_{50} value of 8.9 µg/mL is quite close to the IC_{50} value of ascorbic acid **(1)** which was found to be 1.5 µg/mL. The ethyl acetate extract obtained from the berries of *Ziziphus mucronata* also showed good antioxidant activity since its IC_{50} value of 214.9 µg/mL was relatively closer to that of ascorbic acid **(1)** compared to those of the remaining extracts. Hence the ethyl acetate and methanol extracts obtained from the berries of *Ziziphus mucronata* were further pursued for antioxidant compounds.

4.3 Isolation of compounds from berries of *Ziziphus mucronata*

The ethyl acetate and methanol extracts obtained from the berries of *Ziziphus mucronata* showed good antioxidant activity in comparison to ascorbic acid **(1)** hence they were pursued for antioxidant compounds. Column chromatography was used to isolate the compounds from both the ethyl acetate extract and the ethyl acetate extract obtained from the methanol extract. The solvent systems used for elution comprised of cyclohexane, DCM (dichloromethane), ethyl acetate, diethyl ether and methanol in various combinations and ratios. This afforded several fractions, including fractions E10.6, E11.4 and E.M.2, which were further separated to obtain purified compounds.

From fraction E10.6, compound **(26)** was obtained and was eluted from the initial column using the solvent system ethyl acetate and DCM (dichloromethane) (1:1). Fraction E10.6 was further separated by packing in a smaller column and compound **(26)** was obtained by elution using the solvent system DCM and ethyl acetate (9:1). Compound **(26)** was labelled as E10.6.3 and its purity was checked by subjecting it to thin layer chromatography. It was found to be UV inactive at wavelengths of 254 and 365 nm and reacted with *p*-anisaldehyde to develop purple spots. The fraction E10.6.3 was repacked into a small column to remove any remaining impurities and this column was also eluted using the solvent system DCM and ethyl acetate (9:1). Its purity was again checked by subjecting it to thin layer chromatography using the same solvent system of DCM and ethyl acetate (9:1). The TLC plate showed only single spots confirming the presence of a purified compound.

The fraction containing compound **(25)**, fraction E11.4 (Appendix A1), was eluted from the initial column using the solvent system ethyl acetate and DCM (6:4) which was further separated by packing in a smaller column and compound **(25)** was obtained by elution using the solvent system DCM and ethyl acetate (8:2). Compound **(25)** was labelled as E11.4.1 and its purity was checked by subjecting it to thin layer chromatography. It was found to be UV inactive at wavelengths of 254 and 365 nm and reacted with *p*-anisaldehyde to develop purple spots. It was also found to have an R_f of 0.63 in the solvent system DCM and ethyl acetate (8:2). The fraction E11.4.1 was repacked into a small column to remove any remaining impurities and this column was also eluted using the solvent system DCM and ethyl acetate (8:2). Its purity was again checked by subjecting it to thin layer chromatography using the same solvent system of DCM

and ethyl acetate (8:2). The TLC plate showed only single spots confirming the presence of a purified compound which had an R_f of 0.63.

The fraction containing compound **(30)**, fraction E.M.2 (Appendix A2), was eluted from the initial column using DCM which was further separated by packing in a smaller column that was eluted using the solvent system cyclohexane and DCM (1:1). The second fraction obtained, fraction E.M.2.2, was further separated by packing in a small column that was eluted using the solvent system cyclohexane, DCM and diethyl ether (5:4:1). Compound **(30)** was the second fraction obtained from this column and was labelled as fraction E.M.2.2.2. Its purity was checked by subjecting it to thin layer chromatography using the solvent system of cyclohexane, DCM and diethyl ether (8.5:0.5:1). It was found to be UV inactive at wavelengths of 254 and 365 nm and reacted with *p*-anisaldehyde to develop pink spots. The TLC plate showed only single spots confirming the presence of a purified compound which had an R_f of 0.11.

Eventually 8 possibly pure compounds were isolated from the ethyl acetate extract (Appendix A3) and 14 possibly pure compounds were isolated from the ethyl acetate extract obtained from the methanol extract (Appendices A4 and A5).

4.4 Structure elucidation of compounds isolated from berries of *Ziziphus mucronata*

Nuclear magnetic resonance (NMR) spectroscopy was used to deduce the most probable structures of the purified compounds. The data obtained from the NMR spectra was processed using TOPSPIN Software (Bruker, Version 4.0.6). From the 22 compounds that were sent for NMR analysis, structure elucidation was only possible for 3 compounds [**(25)**, **(26)** and **(30)**]. The full set of NMR spectra could not be obtained for the remaining compounds as the compounds had decomposed.

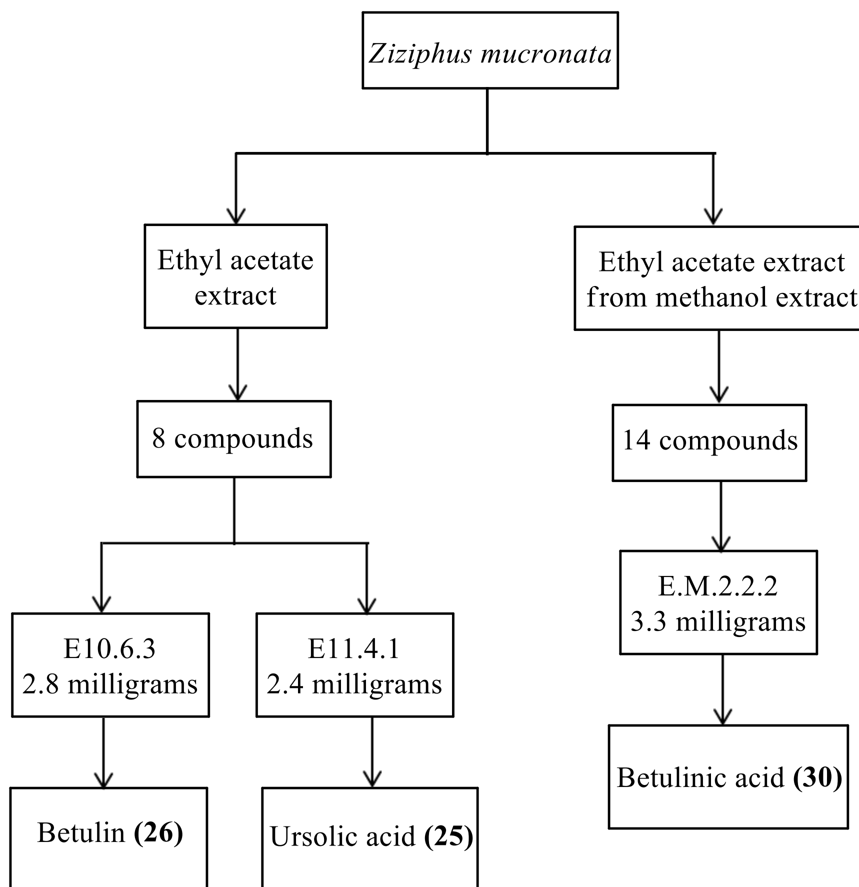
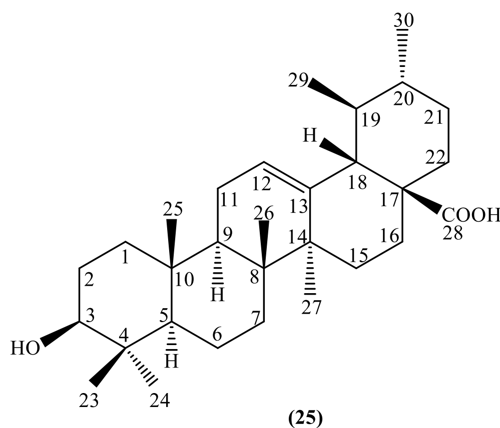


Figure 12: Compounds isolated from berries of *Ziziphus mucronata*

4.4.1 Structural elucidation of ursolic acid (25)

Compound **(25)** was isolated as a white crystalline solid and was identified as ursolic acid **(25)**.



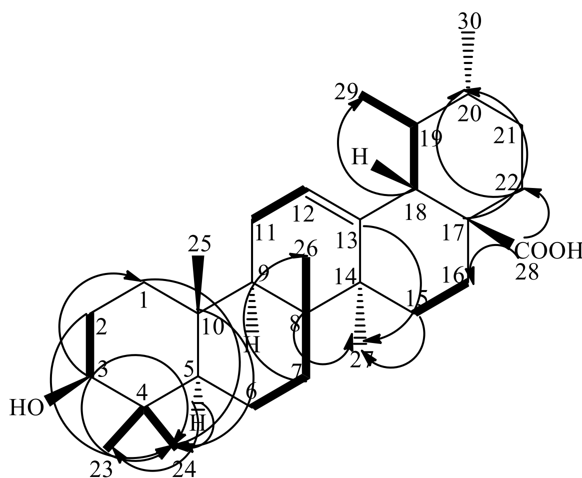
The structure of ursolic acid **(25)** was determined based on its NMR spectra. The NMR spectra of compound **(25)** displayed thirty carbon resonances, including seven methyl, nine methylene, seven methine and seven quaternary carbon resonances which indicated that this compound is a pentacyclic triterpenoid derivative [Table 12; appendices A6 to A12]. The ^{1}H NMR (500 MHz, $CDCl_3$) showed the presence of seven methyl group resonances at δ_H 0.99 (s, 3H-23), 0.78 (s, 3H-24), 0.93 (s, 3H-25), 0.80 (s, 3H-26), 1.09 (s, 3H-27), 0.86 (d, 3H-29) and 0.96 (d, 3H-30). The ^{13}C NMR spectrum confirmed the presence of double bond signals at δ_C 126.1 and 138.2 ppm which were assigned to C-12 and C-13 respectively, typical of an olean-12-ene type skeleton (Hatem and Najah, 2016). The resonances δ_C 126.1 and 138.2 ppm compared well with literature values (Martins *et al.*, 2013).

The olean-12-ene type skeleton has the hydroxyl group at C-3 hence the oxygenated carbon at resonance δ_C 79.3 was assigned as C-3. The resonances at δ_H 1.26 and 1.60 corresponding to the methylene carbon resonance at δ_C 27.5 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methine carbon with carbon resonance δ_C 79.3 (C-3) hence the methylene carbon was assigned as C-2. The resonance at δ_H 3.23 (C-3) showed correlations in the HMBC spectrum with the C-23 resonance (δ_C 28.4) and the C-24 resonance (δ_C 15.8). The resonance at δ_H 0.99 corresponding to the methyl carbon resonance at δ_C 28.4 in the HSQC spectrum showed correlations in the HMBC spectrum with the methyl carbon resonance δ_C 15.8 (C-24) hence the methyl carbon was assigned as C-23. The resonances at δ_H 1.63 and 1.42 corresponding to the methylene carbon resonance at δ_C 38.9 in the HSQC spectrum showed correlations in the HMBC spectrum with the methine carbon resonance δ_C 79.3 (C-3) hence the methylene carbon was assigned as C-1.

The resonance at δ_H 1.09 corresponding to the methyl carbon resonance at δ_C 23.8 in the HSQC spectrum showed correlations in the HMBC spectrum with the olefinic carbon resonance δ_C 138.2 at C-13 hence the methyl carbon was assigned as C-27. The resonances at δ_H 2.00 and 1.65 corresponding to the methylene carbon resonance at δ_C 24.4 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methylene carbon with resonance δ_C 28.3 (C-15) hence the methylene carbon was assigned as C-16. The methine carbon resonance at δ_C 42.3 showed correlations in the HMBC spectrum with the methyl carbon resonance δ_C 17.2 (C-26) and the methylene carbon resonance δ_C 28.3 (C-15) hence the methine carbon was

assigned as C-14. The resonance at δ_H 2.20 corresponding to the methine carbon resonance at δ_C 53.0 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methine carbon with resonance δ_C 39.3 (C-19) hence the methine carbon was assigned as C-18.

The resonances at δ_H 1.51 and 1.70 corresponding to the methylene carbon resonance at δ_C 37.0 in the HSQC spectrum showed correlations in the HMBC spectrum with the methine carbon resonance δ_C 39.1 (C-20) hence the methylene carbon was assigned as C-20. The resonance at δ_H 0.86 corresponding to the methyl carbon resonance at δ_C 17.3 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methine carbon with resonance δ_C 39.3 (C-19) hence the methyl carbon was assigned as C-29. The resonance at δ_H 2.20 corresponding to the methine carbon resonance at δ_C 53.0 in the HSQC spectrum showed correlations in the HMBC spectrum with the methyl carbon resonance δ_C 17.3 (C-29) hence the methine carbon was assigned as C-18. The carbon resonance at δ_C 173.4 showed correlations in the HMBC spectrum with the methylene carbon resonances δ_C 24.4 (C-16) and δ_C 37.0 (C-22) hence the quaternary carbon was assigned as C-28. The NMR data compared well with literature values as per (Martins *et al.*, 2013) and compound **(25)** was identified as ursolic acid **(25)**.



Key: HMBC **H ⟶ C** (curved arrows) and **^{1}H-^{1}H** COSY (bold lines) correlations

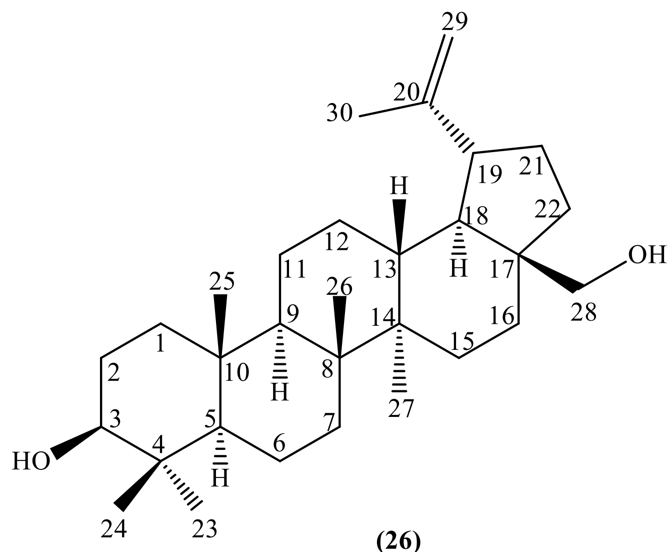
Table 12: NMR data for ursolic acid (25) in $CDCl_3$

No	DEPT	^{13}C NMR (125 MHz)	^{13}C NMR (125 MHz) (Martins *et al.*, 2013)	^{1}H NMR (500 MHz)	^{1}H NMR (400 MHz) (Martins *et al.*, 2013)
1α	CH_2	38.9	38.6	1.63 (m)	1.72 (m)
1β				1.42 (m)	
2α	CH_2	27.5	28.2	1.60 (m)	1.60 (m)
2β				1.26 (m)	
3	CHOH	79.3	78.7	3.23 (dd; *J*=10.7; 4.9 Hz)	3.22 (dd; *J*=10.8; 4.9 Hz)
4	C	39.7	38.5	-	-
5	CH	55.8	55.5	0.71 (m)	1.34 (m)
6α	CH_2	18.5	18.3	1.52 (m)	1.60 (m)
6β				1.34 (m)	
7α	CH_2	33.2	32.9	1.46 (m)	1.72 (m)
7β				1.32 (m)	
8	C	39.0	39.5	-	-
9	CH	47.8	47.3	1.51 (m)	1.60 (m)
10	C	37.2	37.0	-	-
11α	CH_2	23.5	23.7	1.93 (m)	1.91 (m)
11β				1.22 (m)	
12	CH	126.1	125.9	5.26 (dd; *J* = 3.6; 3.4Hz)	5.27 (dd; *J* = 3.6; 3.5Hz)
13	C	138.2	137.9	-	-
14	C	42.3	42.0	-	-
15α	CH_2	28.3	28.1	1.86 (m)	1.60 (m)
15β				1.09 (m)	
16α	CH_2	24.4	25.0	1.65 (m)	1.34 (m)
16β				2.00 (m)	
17	C	48.1	48.1	-	-
18	CH	53.0	53.8	2.20 (m)	2.20 (m)
19	CH	39.3	38.5	1.31 (m)	1.00 (m)
20	CH	39.1	38.5	0.94 (m)	0.95 (m)
21α	CH_2	30.9	30.3	1.29 (m)	1.27 (m)
21β				1.50 (m)	
22α	CH_2	37.0	37.4	1.70 (m)	1.72 (m)
22β				1.51 (m)	
23	CH_3	28.4	28.9	0.99 (s)	1.00 (s)
24	CH_3	15.8	15.6	0.78 (s)	0.79 (s)
25	CH_3	15.7	15.4	0.93 (s)	0.94 (s)
26	CH_3	17.2	17.1	0.80 (s)	0.82 (s)
27	CH_3	23.8	23.5	1.09 (s)	1.10 (s)
28	COOH	173.4	179.6	-	-
29	CH_3	17.3	17.0	0.86 (d; *J* = 6.6 Hz)	0.87 (d; *J* = 6.4 Hz)
30	CH_3	21.4	21.4	0.96 (d; *J* = 6.3 Hz)	0.97 (d; *J* = 6.3 Hz)

Ursolic acid **(25)** is a naturally occurring pentacyclic triterpene found in many plants and is known to have various pharmacological activities. It is known to possess anti-tumour activity, anti-inflammatory activity, cardio protective activity, hepatoprotective activity, anti-ulcer activity and decreases muscle atrophy (Jamal *et al.*, 2018; Ali *et al.*, 2019). Ursolic acid **(25)** is not known to have previously been isolated from *Ziziphus mucronata* but has been reported to be isolated from *Ziziphus jujuba* (Tahergorabi *et al.*, 2015). Several studies have shown that ursolic acid **(25)** does possess significant antioxidant activity (Santiago *et al.*, 2014; Nascimento *et al.*, 2014; Chang *et al.*, 2017).

4.4.2 Structural elucidation of betulin (26)

Compound **(26)** was isolated as a white crystalline solid and was identified as betulin **(26)**.



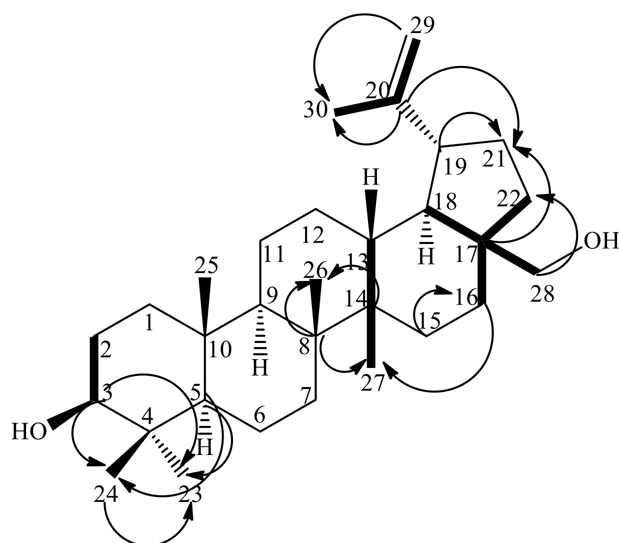
(26)

The structure of betulin **(26)** was determined based on its NMR spectra. The NMR spectra of compound **(26)** displayed thirty carbon resonances, including six methyl, twelve methylene, six methine and six quaternary carbon resonances which was consistent with a pentacyclic triterpenoid derivative [Table 13; appendices A13 to A19]. The ^{1}H NMR (500 MHz, CD_3OD) showed the presence of six methyl group resonances at δ_H 0.77 (s, 3H-23), 0.97 (s, 3H-24), 0.87 (s, 3H-25), 0.99 (s, 3H-26), 1.02 (s, 3H-27) and 1.71 (s, 3H-30). The ^{13}C NMR spectrum confirmed the presence of double bond signals at δ_C 151.9 and 110.3 ppm which were assigned to C-20 and C-29 respectively, corresponding to the typical olefinic carbons of a lupane triterpene skeleton (Li *et al.*, 2018).

The lupane type skeleton has the hydroxyl group at C-3 hence the oxygenated carbon at resonance δ_C 79.7 was assigned at C-3. The resonances at δ_H 1.64 and 1.07 corresponding to the methylene carbon resonance at δ_C 26.9 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methine carbon with resonance δ_C 79.7 (C-3) hence the methylene carbon was assigned as C-2. The resonance at δ_H 3.15 (C-3) showed correlations in the HMBC spectrum with the C-23 resonance (δ_C 16.2) and the C-24 resonance (δ_C 28.6). The resonance at δ_H 0.97 corresponding to the methyl carbon resonance at δ_C 28.6 in the HSQC spectrum showed correlations in the HMBC spectrum with the methyl carbon resonance δ_C 0.77 (C-23) hence the methyl carbon was assigned as C-24. The resonance at δ_H 0.73 corresponding to the methine carbon resonance at δ_C 56.9 in the HSQC spectrum showed correlations in the HMBC spectrum with the methyl carbon resonances δ_C 16.2 (C-23) and δ_C 28.6 (C-24) hence the methine carbon was assigned as C-5.

The resonance at δ_H 1.02 corresponding to the methyl carbon resonance at δ_C 15.2 in the HSQC spectrum showed correlations in the HMBC spectrum with the carbon resonances δ_C 41.9 (C-8) and 31.7 (C-16) hence the methyl carbon was assigned as C-27. The resonance at δ_H 0.99 corresponding to the methyl carbon resonance at δ_C 16.7 in the HSQC spectrum showed correlations in the HMBC spectrum with the methine carbon resonance δ_C 43.6 (C-14) hence the methyl carbon was assigned as C-26.

Betulin **(26)** has been run in $CDCl_3$ in the available literature. However, for this structural elucidation betulin **(26)** was run in CD_3OD. This accounted for the disparities between the δ_C and δ_H resonances as shown in Table 13. Furthermore, the NMR correlations confirmed the structure of compound **(26)** hence it was identified as betulin **(26).**



Key: HMBC **H⟶C** (curved arrows) and **^{1}H-^{1}H** COSY (bold lines) correlations

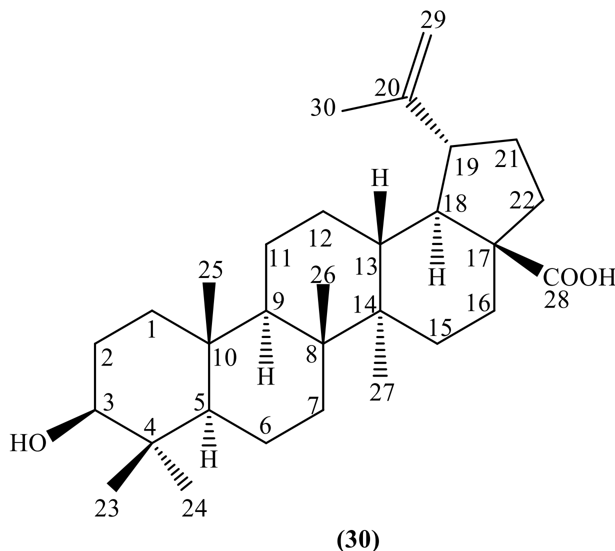
Betulin **(26)** is a naturally occurring pentacyclic triterpenoid of lupane structure. It has been previously isolated from a number of plants and is known to have a broad range of biological and pharmacological properties, including antibacterial, antiviral and anti-inflammatory activities (Ci *et al.*, 2017). Betulin **(26)** is not known to have been previously isolated from *Ziziphus mucronata* but has been isolated from other species of *Ziziphus* including *Ziziphus mauritiana* and *Ziziphus vulgaris* (Król *et al.*, 2015). Scientific studies have revealed that betulin **(26)** exhibits antioxidant activity (Chong *et al.*, 2018).

Table 13: NMR data for betulin (26) in CD_3OD and betulin (26) in $CDCl_3$

No	DEPT	^{13}C NMR (125 MHz)	^{13}C NMR (125 MHz) (Tijjani *et al.*, 2012)	^1H NMR (500 MHz)	^1H NMR (500 MHz) (Tijjani *et al.*, 2012)
1α	CH_2	40.1	38.9	1.71 (m)	-
1β				0.96 (m)	-
2α	CH_2	26.9	27.5	1.64 (m)	1.55 (m)
2β				1.07 (m)	-
3	CHOH	79.7	79.2	3.15 (dd; *J*=9.8; 6.6 Hz)	3.18 (dd; *J*=10.1; 5.3 Hz)
4	C	40	38.8	-	-
5	CH	56.9	55.4	0.73 (m)	-
6α	CH_2	19.5	18.4	1.55 (m)	-
6β				1.44 (m)	-
7α	CH_2	33.4	34.3	2.26 (m)	-
7β				2.09 (m)	-
8	C	41.9	40.3	-	-
9	CH	50.4	50.5	1.63 (m)	-
10	C	38.2	37.4	-	-
11α	CH_2	22.1	20.9	1.46 (m)	-
11β				1.28 (m)	-
12α	CH_2	26.2	25.3	1.63 (m)	-
12β				1.07 (m)	-
13	CH	39.6	37.2	2.32 (m)	-
14	C	43.6	42.1	-	-
15α	CH_2	30.8	27.1	1.56 (m)	-
15β				1.38 (m)	-
16α	CH_2	31.7	29.2	1.96 (m)	-
16β				1.39 (m)	-
17	C	57.5	55.9	-	-
18	CH	48.5	46.7	3.04 (m)	-
19	CH	50	48.8	3.37 (m)	-
20	C	151.9	150.6	-	-
21α	CH_2	28.1	29.8	2.08 (m)	-
21β				1.62 (m)	-
22α	CH_2	35.6	34.1	1.53 (m)	-
22β				1.43 (m)	-
23	CH_3	16.2	15.4	0.77 (s)	0.75 (s)
24	CH_3	28.6	28.1	0.97 (s)	0.96 (s)
25	CH_3	16.8	16.2	0.87 (s)	0.80 (s)
26	CH_3	16.7	16.1	0.99 (s)	0.97 (s)
27	CH_3	15.2	14.8	1.02 (s)	0.99 (s)
28α	CH_2OH	63.4	60.6	4.38 (d)	3.79 (d; J=10.8 Hz)
28β				4.18 (d)	3.33 (d; J=10.8 Hz)
29α	CH_2	110.3	109.8	4.73 (s)	4.70 (s)
29β				4.61 (s)	4.58 (s)
30	CH_3	19.7	19.2	1.71 (s)	1.67 (s)

4.4.3 Structural elucidation of betulinic acid (30)

Compound **(30)** was isolated as a white crystalline solid and was identified as betulinic acid **(30)**.



(30)

The structure of betulinic acid **(30)** was determined based on its NMR spectra. The NMR spectra of compound **(30)** displayed thirty carbon resonances, including six methyl, eleven methylene, six methine and seven quaternary carbon resonances which indicated that this compound is a pentacyclic triterpenoid derivative [Table 14; appendices A20 to A26]. The ^{1}H NMR (500 MHz, CD_3OD) showed the presence of six methyl group resonances at δ_H 0.77 (s, 3H-23), 0.97 (s, 3H-24), 0.87 (s, 3H-25), 0.98 (s, 3H-26), 1.01 (s, 3H-27) and 1.70 (s, 3H-30). The ^{13}C NMR spectrum confirmed the presence of double bond signals at δ_C 150.6 and 109.8 ppm which were assigned to C-20 and C-29 respectively, corresponding to the typical olefinic carbons of a lupane triterpene skeleton (Li *et al.*, 2018).

The lupane type skeleton has the hydroxyl group at C-3 hence the oxygenated carbon at resonance δ_C 78.1 was assigned as C-3. The resonances at δ_H 1.65 and 1.08 corresponding to the methylene carbon resonance at δ_C 26.8 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methine carbon with resonance δ_C 78.1 (C-3) hence the methylene carbon was assigned as C-2. The resonances at δ_H 1.72 and 0.95 corresponding to the methylene carbon resonance at δ_C 38.6 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methylene carbon with resonance δ_C 26.8 (C-2) hence the methylene carbon was assigned as C-1. The resonance at δ_H 3.15 (C-3) showed correlations in

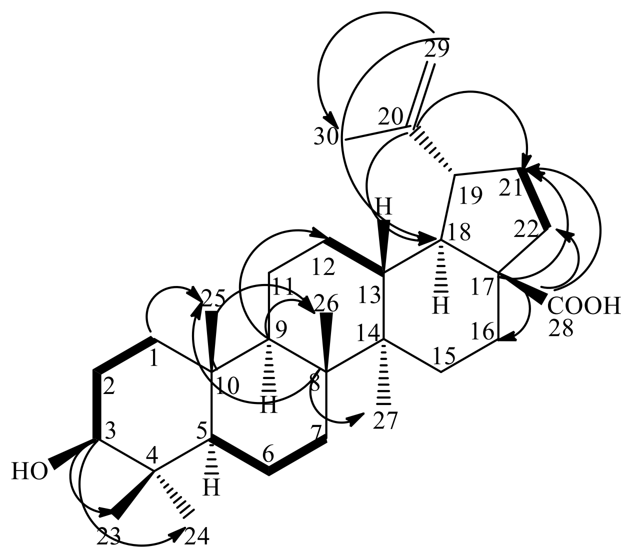
the HMBC spectrum with the methyl resonances δ_H 0.77 and δ_H 0.97 hence the methyl carbons were assigned C-23 and C-24 respectively.

The resonances at δ_H 1.56 and 1.46 corresponding to the methylene carbon resonance at δ_C 18.2 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methine carbon with resonance δ_C 55.2 (C-5) hence the methylene carbon was assigned as C-6. The resonance at δ_H 2.28 corresponding to the methylene carbon resonance at δ_C 34.2 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methylene carbon with resonance δ_C 18.2 (C-6) hence the methylene carbon was assigned as C-7. The resonances at δ_H 1.09 and 1.68 corresponding to the methylene carbon resonance at δ_C 25.4 in the HSQC spectrum showed correlations in the HMBC spectrum with the methine carbon resonance δ_C 50.4 (C-9) hence the methylene carbon was assigned as C-12. The resonance at δ_H 2.38 corresponding to the methine carbon resonance at δ_C 37.6 in the HSQC spectrum showed correlations in the COSY spectrum with the methylene carbon resonance δ_C 25.4 (C-12) hence the methylene carbon was assigned as C-13.

The resonance at δ_H 3.12 corresponding to the methine carbon resonance at δ_C 46.5 in the HSQC spectrum showed correlations in the HMBC spectrum with the quaternary carbon resonance δ_C 150.6 (C-20) hence the methine carbon was assigned as C-18. The resonances at δ_H 1.51 and 1.43 corresponding to the methylene carbon resonance at δ_C 36.4 in the HSQC spectrum showed correlations in the HMBC spectrum with the quaternary carbon resonance δ_C 180.1 (C-28) hence the methylene carbon was assigned as C-22. The resonances at δ_H 2.11 and 1.61 corresponding to the methylene carbon resonance at δ_C 29.5 in the HSQC spectrum showed correlations in the COSY spectrum with the proton of the methylene carbon with resonance δ_C 36.4 (C-22) hence the methylene carbon was assigned as C-21.

Table 14: NMR data for betulinic acid (30) in CD_3OD

No	DEPT	^{13}C NMR (125 MHz)	^{13}C NMR (125 MHz) (Kim *et al.*, 2009)	^1H NMR (500 MHz)	^1H NMR (500 MHz) (Rosa *et al.*, 2017)
1α	CH_2	38.6	38.4	1.72 (m)	-
1β				0.95 (m)	-
2α	CH_2	26.8	26.5	1.65 (m)	1.55 (m)
2β				1.08 (m)	-
3	CHOH	78.1	78.3	3.15 (dd; J=10.8; 5.3 Hz)	3.12 (dd; J=11.0; 5.0 Hz)
4	C	38.5	38.4	-	-
5	CH	55.2	55.0	0.73 (m)	-
6α	CH_2	18.2	17.9	1.56 (m)	-
6β				1.46 (m)	-
7α	CH_2	34.2	34.0	2.28 (m)	-
7β				-	-
8	C	40.3	40.3	-	-
9	CH	50.4	50.2	1.64 (m)	-
10	C	36.8	36.8	-	-
11α	CH_2	21.1	20.5	1.48 (m)	-
11β				1.30 (m)	-
12α	CH_2	25.4	25.1	1.68 (m)	-
12β				1.09 (m)	-
13	CH	37.6	37.9	2.38 (m)	-
14	C	42.3	42.1	-	-
15α	CH_2	30.4	30.2	1.57 (m)	-
15β				1.40 (m)	-
16α	CH_2	32.0	31.9	1.41 (m)	-
16β				-	-
17	C	55.6	55.9	-	-
18	CH	46.5	46.7	3.12 (m)	-
19	CH	48.6	48.8	3.39 (m)	-
20	C	150.6	150.4	-	-
21α	CH_2	29.5	29.3	2.11 (m)	-
21β				1.61 (m)	-
22α	CH_2	36.4	36.8	1.51 (m)	-
22β				1.43 (m)	-
23	CH_3	15.0	14.9	0.77 (s)	0.75 (s)
24	CH_3	27.6	27.4	0.97 (s)	0.95 (s)
25	CH_3	15.7	15.6	0.87 (s)	0.86 (s)
26	CH_3	15.6	15.4	0.98 (s)	0.97 (s)
27	CH_3	14.4	14.2	1.01 (s)	1.00 (s)
28	COOH	180.1	178.9	-	-
29α	CH_2	109.8	109.9	4.75 (s)	4.70 (s)
29β				4.59 (s)	4.58 (s)
30	CH_3	19.0	18.8	1.70 (s)	1.69 (s)



Key: HMBC **H⟶C** (curved arrows) and **^{1}H-[1]H** COSY (bold lines) correlations

The resonance at δ_H 1.70 corresponding to the methyl carbon resonance at δ_C 19.0 in the HSQC spectrum showed correlations in the HMBC spectrum with the olefinic methylene carbon resonance δ_C 109.8 (C-29) hence the methyl carbon was assigned as C-30. The resonance at δ_H 1.01 corresponding to the methyl carbon resonance at δ_C 14.4 in the HSQC spectrum showed correlations in the HMBC spectrum with the quaternary carbon resonance δ_C 40.3 (C-8) hence the methyl carbon was assigned as C-27. The resonance at δ_H 0.98 corresponding to the methyl carbon resonance at δ_C 15.6 in the HSQC spectrum showed correlations in the HMBC spectrum with the methine carbon resonance δ_C 50.4 (C-9) hence the methyl carbon was assigned as C-26. The resonance at δ_H 0.87 corresponding to the methyl carbon resonance at δ_C 15.7 in the HSQC spectrum showed correlations in the HMBC spectrum with the methylene carbon resonance δ_C 38.6 (C-1) hence the methyl carbon was assigned as C-25. The NMR data compared well with literature values as per (Kim *et al.*, 2009; Rosa *et al.*, 2017) hence compound **(30)** was identified as betulinic acid **(30)**.

Betulinic acid **(30)** is a naturally occurring pentacyclic triterpenoid of lupane structure. It has been previously isolated from a number of plants and is known to have a broad range of biological and pharmacological properties, including anti-inflammatory, antiretroviral, antimalarial and anti-cancer activities. Betulinic acid **(30)** is not known to have been previously isolated from *Ziziphus mucronata* but has been isolated from *Ziziphus jujuba* (Lingampally *et al.*,

2012; Tahergorabi *et al.*, 2015). Betulinic acid **(30)** is known to exhibit antioxidant activity (Souza *et al.*, 2011).

Betulin **(26)** and betulinic acid **(30)** are both pentacyclic triterpenoids of lupane structure and are derived from lupeol **(29)** (Zhou *et al.*, 2016; Alonso- Serra *et al.*, 2019). Their structures differ at C-28 where betulin **(26)** has CH_2OH group while betulinic acid **(30)** has a COOH group hence the slight differences in their δ_C and δ_H resonances.

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The crude extracts obtained for *Teclea simplicifolia* and *Ziziphus mucronata* showed antioxidant activities, but lower than that of the standard, ascorbic acid **(1)**, which was found to have an IC_{50} value of 1.5 µg/mL. For *Teclea simplicifolia* the IC_{50} value for the cyclohexane extract was 135,897.6 µg/mL, for the ethyl acetate extract it was 32,989.1µg/mL and for the methanol extract it was 1,152.1 µg/mL. The corresponding IC_{50} values for the extracts obtained from *Ziziphus mucronata* were 44,466.5 µg/mL for the cyclohexane extract, 214.9 µg/mL for the ethyl acetate extract and 8.9 µg/mL for the methanol extract. The berries of *Teclea simplicifolia* did not show good antioxidant activity in comparison to that of the standard, ascorbic acid **(1)**, hence they were not further pursued for antioxidant compounds. The berries of *Ziziphus mucronata* showed good antioxidant activity in comparison to that of the standard, ascorbic acid **(1)**, hence they were further pursued for antioxidant compounds.

Column chromatography was used to isolate the purified compounds. Although 22 compounds were sent for NMR analysis, structure elucidation was only possible for 3 compounds. The full set of NMR spectra could not be obtained for the remaining compounds as the compounds had decomposed. It is apparent that these compounds are unstable when exposed for a long time during the experiments. The three compounds, whose structures are reported in this work, are ursolic acid **(25)**, betulin **(26)** and betulinic acid **(30)**.

The yield of the compounds isolated was too low to enable the determination of the antioxidant activity and reducing power of the purified compounds. However, ursolic acid **(25)**, betulin **(26)** and betulinic acid **(30)** have significant antioxidant activity which is corroborated by earlier literature reports. This work has established that the berries of *Teclea simplicifolia* and *Ziziphus mucronata* possess antioxidant compounds hence verifying their beneficial use as potent sources of antioxidants.

5.2 Recommendations

i) One of the limitations of this work is that the compounds decomposed in transit to the location of the nuclear magnetic resonance (NMR) spectrometer. Indeed it is necessary to minimise the time interval between the processes, particularly chromatography and structural elucidation, and also control the conditions of exposure. It is therefore recommended that the compounds should be isolated closer to the spectroscopic equipment.

ii) An attempt should be made to derivatise the isolated compounds in order to enhance their stability.

iii) Future studies carried out on this work should make use of more refined techniques to purify compounds in order to obtain them in larger yields to enable the determination of the reducing power of the purified compounds.

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Reference for Figure 3

(Tsammalex, 2005) http://tsammalex.clld.org/parameters/ziziphusmucronata as on 4/12/2018 at 4:15p.m.

Reference for Figure 4

(West African Plants) http://www.westafricanplants.senckenberg.de/root/index.php?page_id=14&id=1687 as on 4/12/2018 at 4:17p.m.

Reference for Figure 5

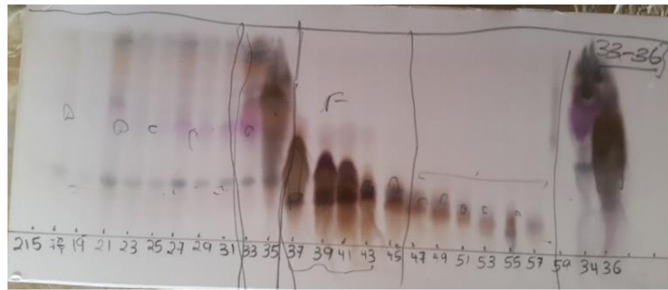
(GeoCurrents Map, 2018) https://www.google.com/search?client=firefox-b-ab&tbm=isch&q=compass+direction+map+symbol&chips=q:compass+direction+map+symbol,online_chips:north&usg=AI4_-kQbLMjifw2OE2JPcGLldgt9gx5wPw&sa=X&ved=0ahUKEwjboJGaopzfAhWuzIUKHZDuBR0Q4lYIKCgC&biw=1366&bih=635&dpr=1

APPENDICES

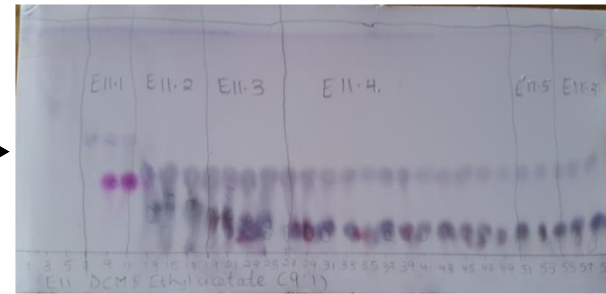
Appendix Ⅰ: Key Data Analysis Outputs

A1

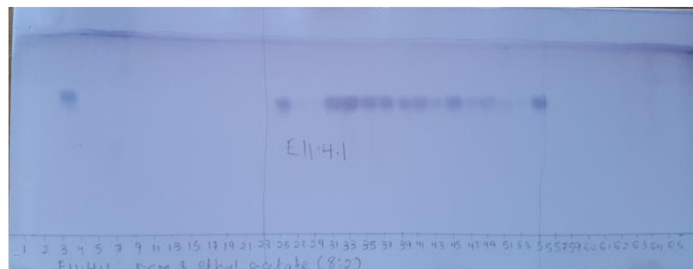
TLC chromatograms for Compound (25)



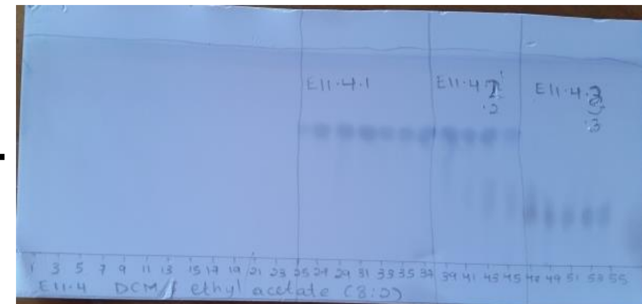
Fraction E11=37-45 (ethyl acetate and DCM 6:4)



Fraction E11.4 (DCM and ethyl acetate 8:2)



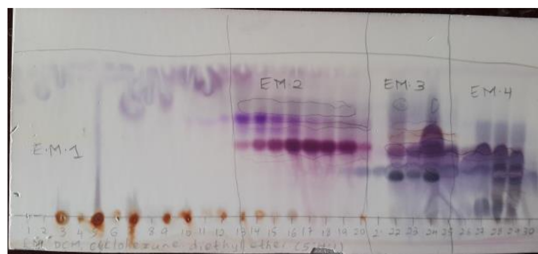
Compound E11.4.1 (DCM and ethyl acetate 8:2)



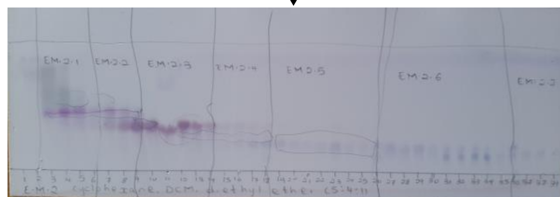
Fraction E11.4.1 (DCM and ethyl acetate 8:2)

A2

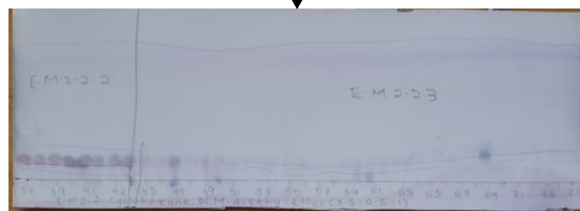
TLC chromatograms for Compound (30)



Fraction E.M.2 (DCM)



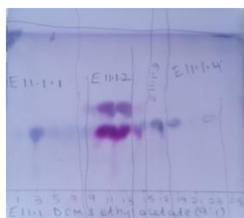
Fraction E.M.2.2 (cyclohexane, DCM and diethyl ether 5:4:1)



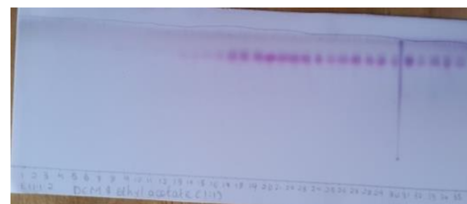
Compound E.M.2.2.2 (cyclohexane, DCM and diethyl ether 5:4:1)

A3

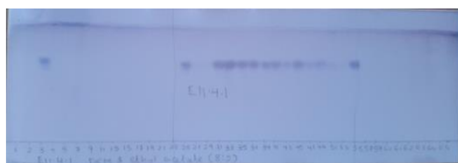
TLC chromatograms for compounds isolated from ethyl acetate extract



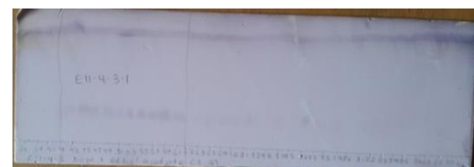
Compound E.11.1.1 (DCM and ethyl acetate 9:1)



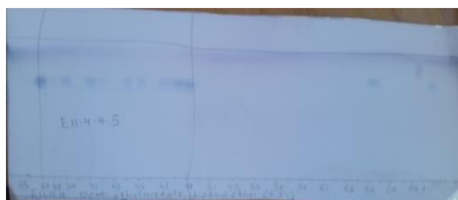
Compound E.11.1.2 (DCM and ethyl acetate 1:1)



Compound E.11.4.1 (DCM and ethyl acetate 8:2)

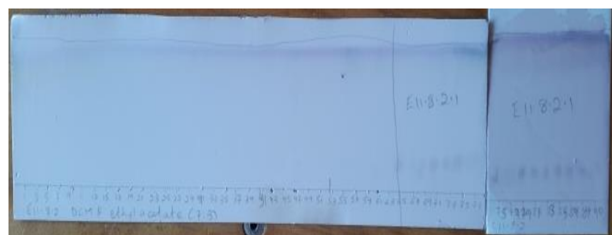


Compound E.11.4.3.1 (DCM and ethyl acetate 7:3)

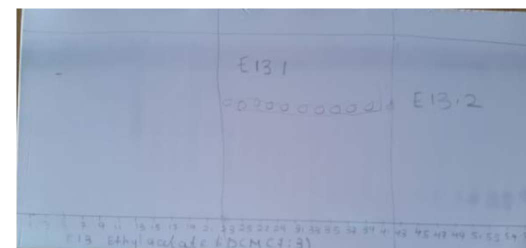


Compound E.11.4.4.5 (DCM, ethyl acetate and diethyl ether

Compound E.11.8.1 (DCM and ethyl acetate 7:3)



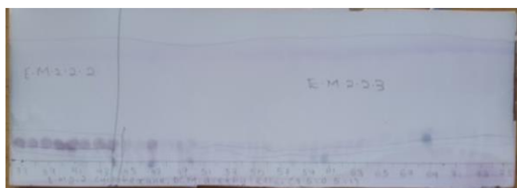
Compound E.11.8.2.1 (DCM and ethyl acetate



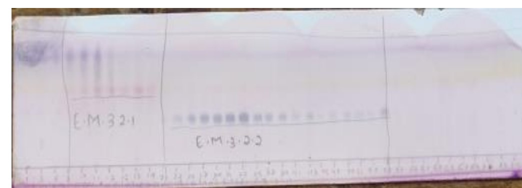
Compound E.13.1 (DCM and ethyl acetate 7:3)

A4

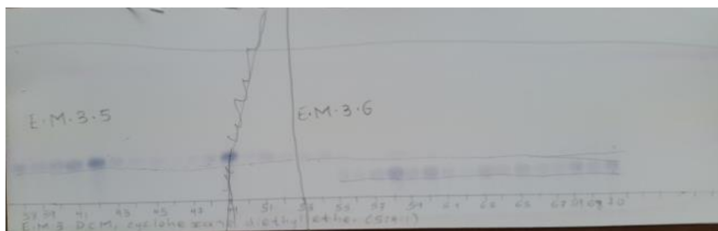
TLC chromatograms for compounds isolated from ethyl acetate extract obtained from methanol extract



Compound E.M.2.2.2 (cyclohexane, DCM and diethyl ether

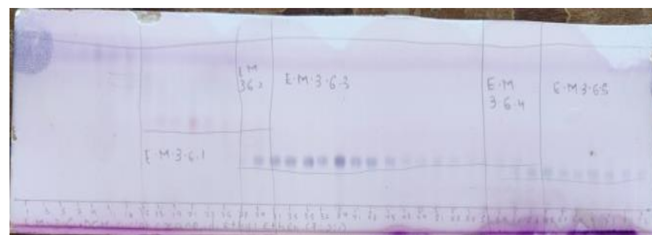


Compound E.M.3.2.3 (DCM, cyclohexane and diethyl ether

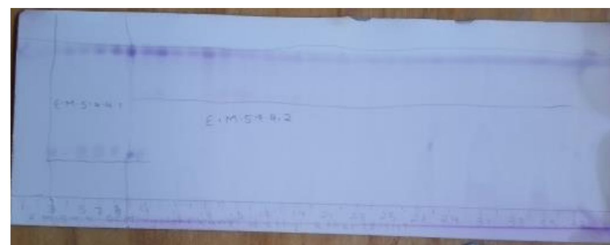


Compound E.M.3.5 (DCM, cyclohexane and diethyl ether 5:4:1)

Compound E.M.3.6.1.2 (DCM, cyclohexane and diethyl ether 7:2:1)

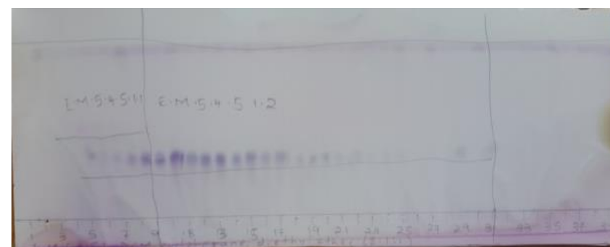


Compound E.M.3.6.3 (DCM, cyclohexane and diethyl ether 7:2:1)



Compound E.M.5.4.4.2 (DCM, cyclohexane and diethyl ether 8:1:1)

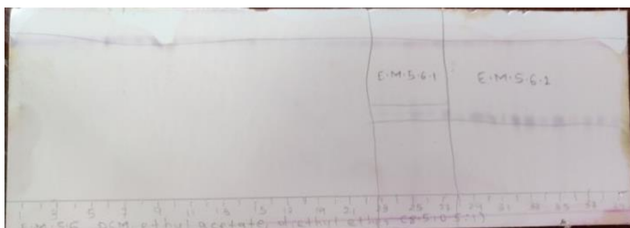
Compound E.M.5.4.4.3 (DCM, cyclohexane and diethyl ether 8:1:1)



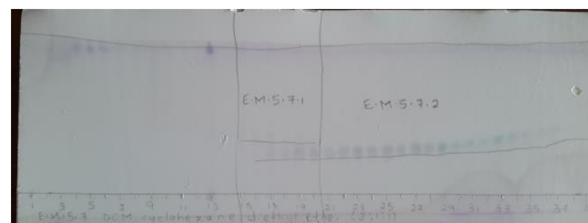
Compound E.M.5.4.5.1.2 (DCM, cyclohexane and diethyl ether 8:1:1)

A5

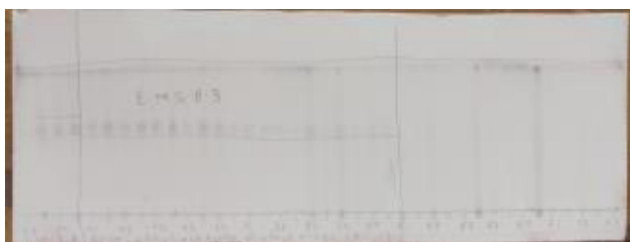
TLC chromatograms for compounds isolated from ethyl acetate extract obtained from methanol extract



Compound E.M.5.6.2 (DCM, ethyl acetate and diethyl ether 8.5:0.5:1)

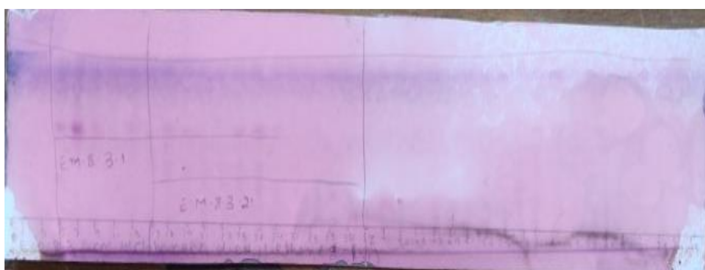


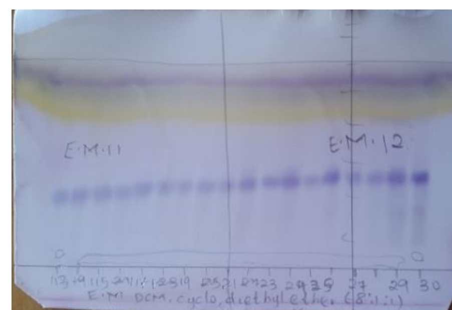
Compound E.M.5.7.2 (DCM, cyclohexane and diethyl ether 8:1:1)



Compound E.M.5.8.3 (DCM, ethyl acetate and diethyl ether 8:1:1)

Compound E.M.6.3.1 (DCM, cyclohexane and diethyl ether 8.5:0.5:1)

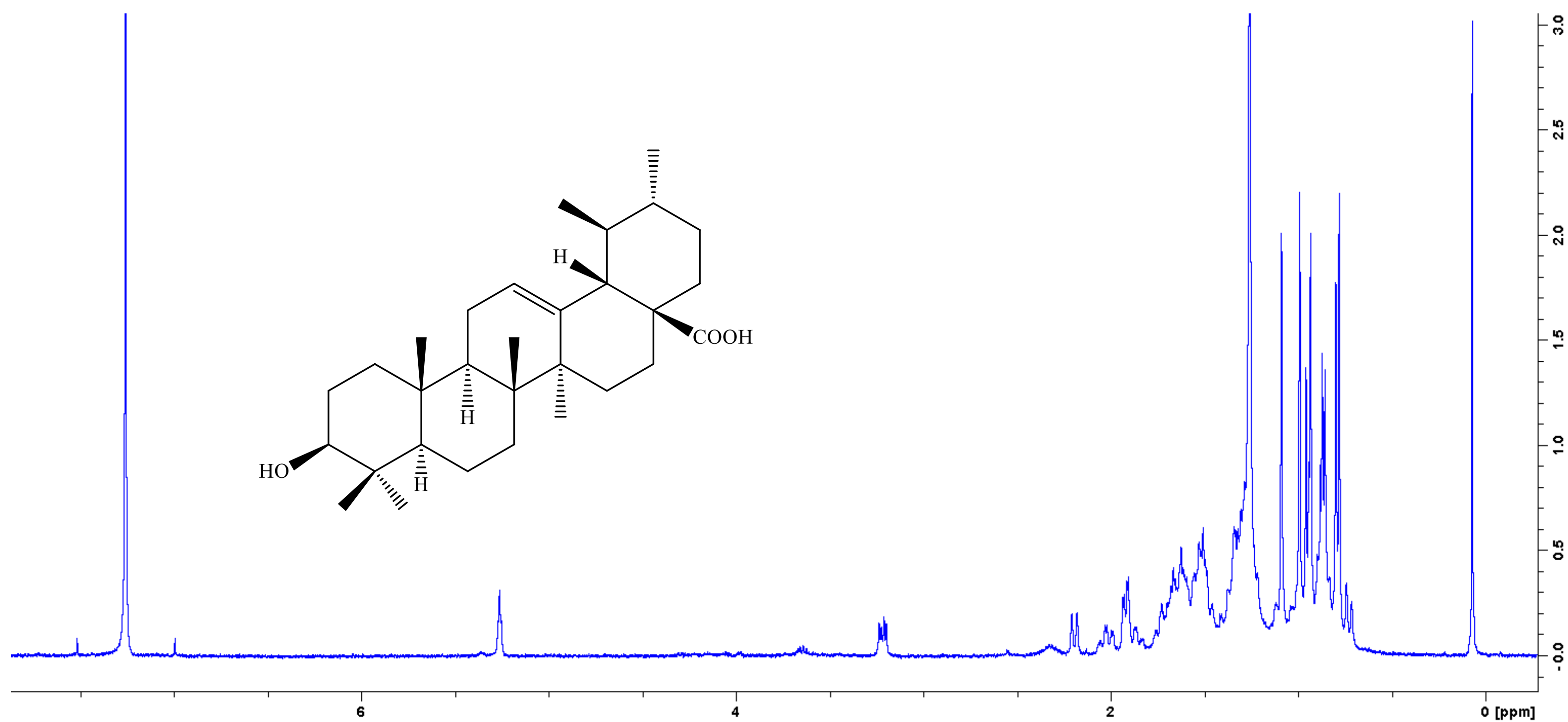

Compound E.M.8.3.1 (DCM, cyclohexane and diethyl ether



Compound E.M.11 (DCM, cyclohexane and diethyl ether 8:1:1)

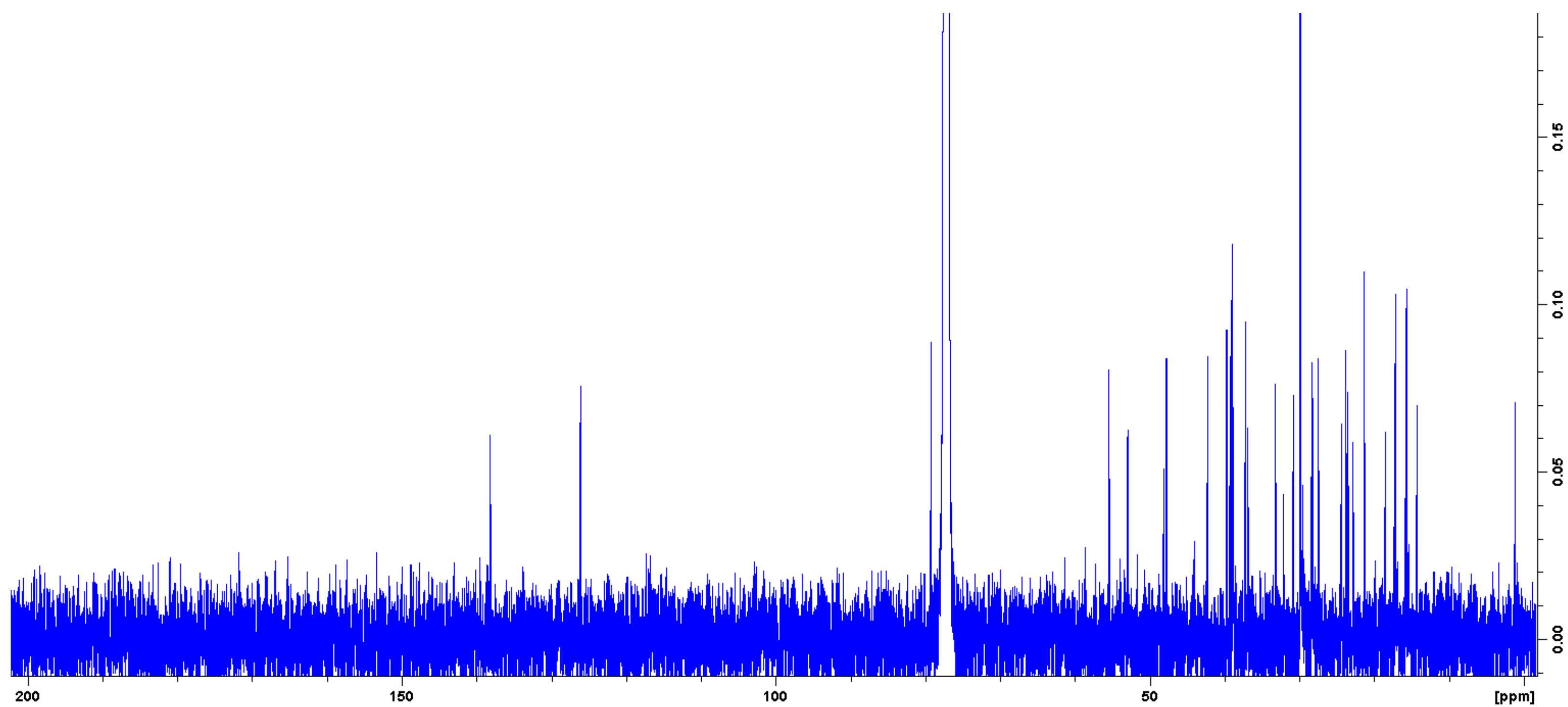
A6

^{1}H NMR spectrum for compound (25) in $CDCl_3$



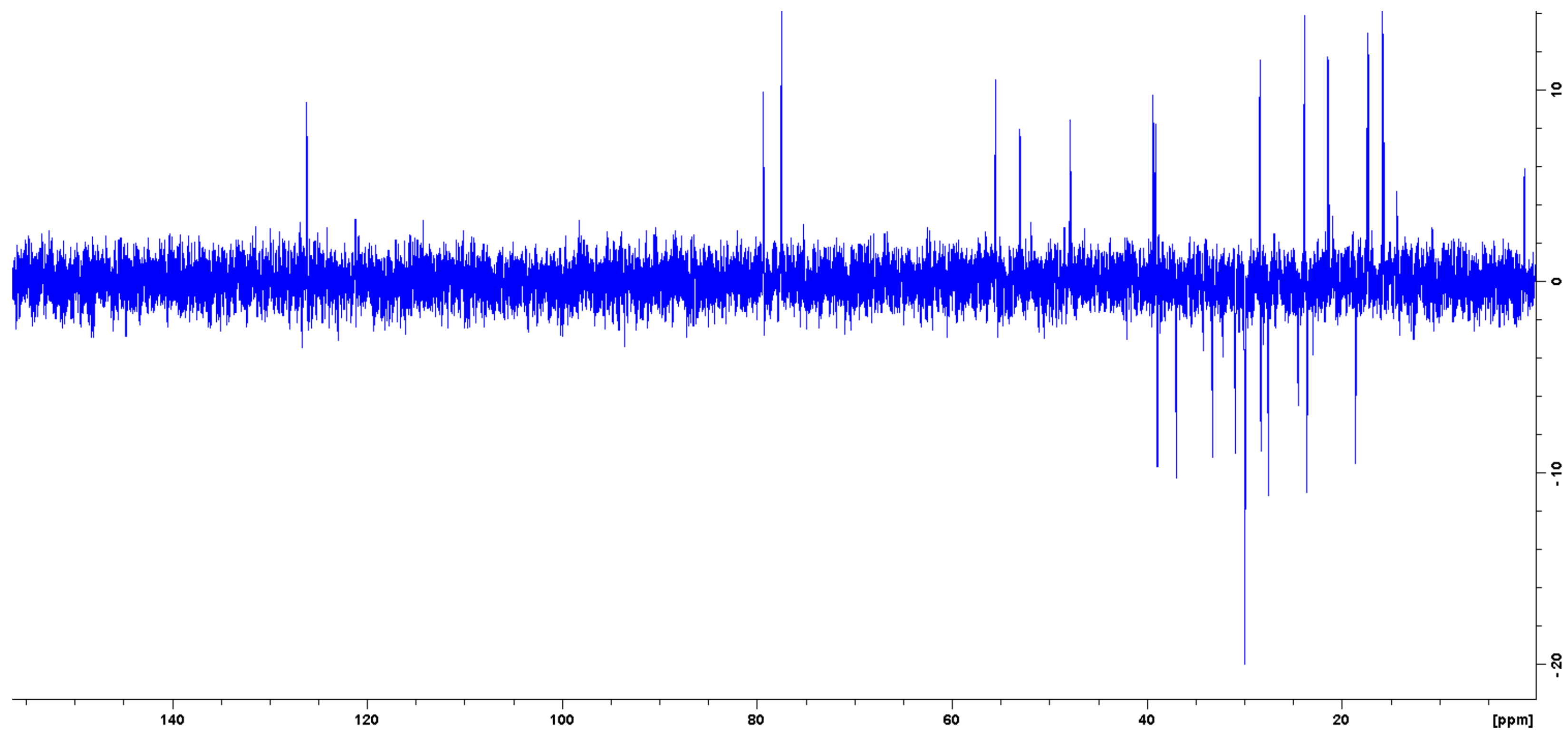
A7

^{13}C NMR spectrum for compound (25) in $CDCl_3$



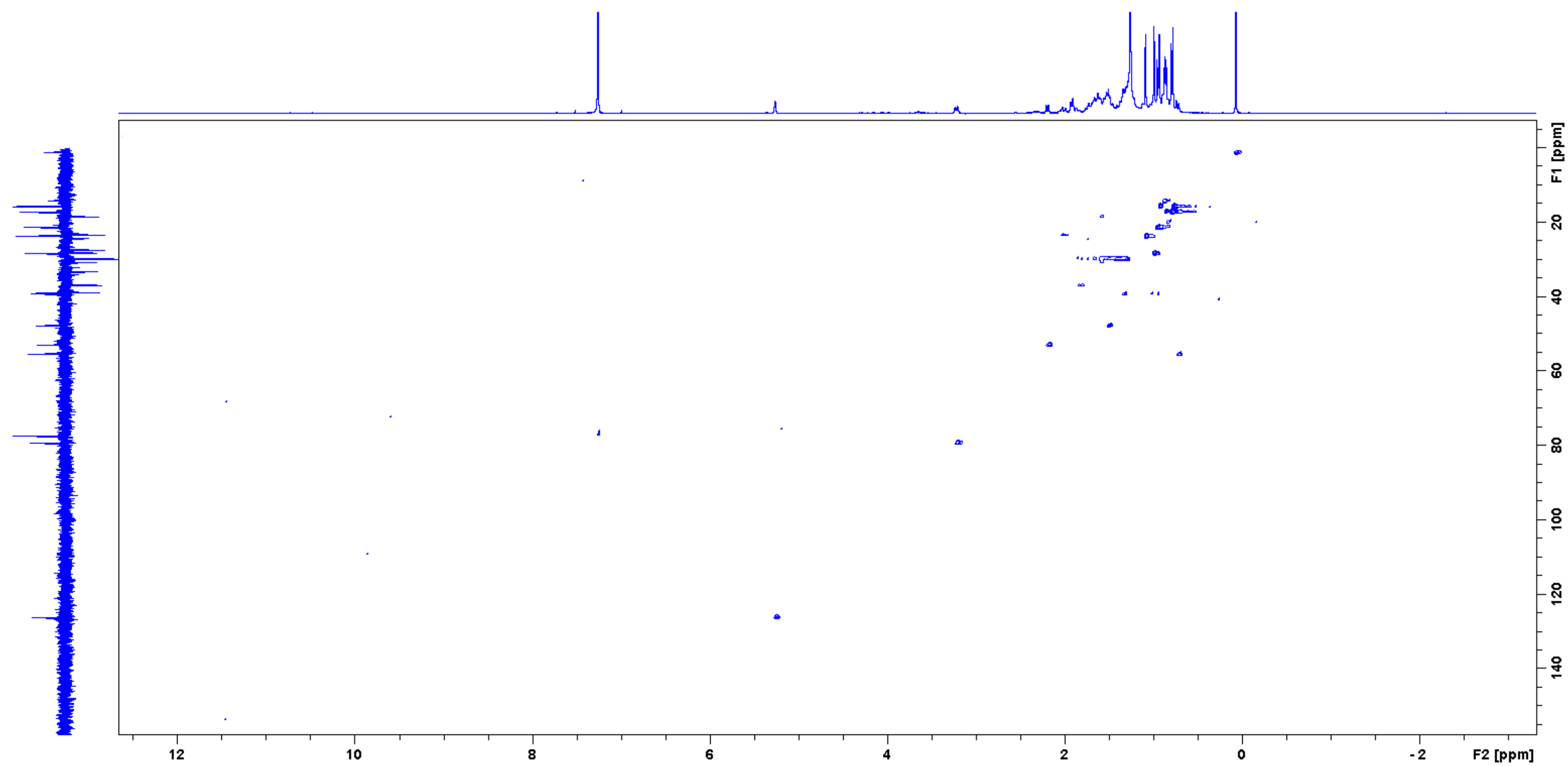
A8

DEPT spectrum for compound (25) in $CDCl_3$



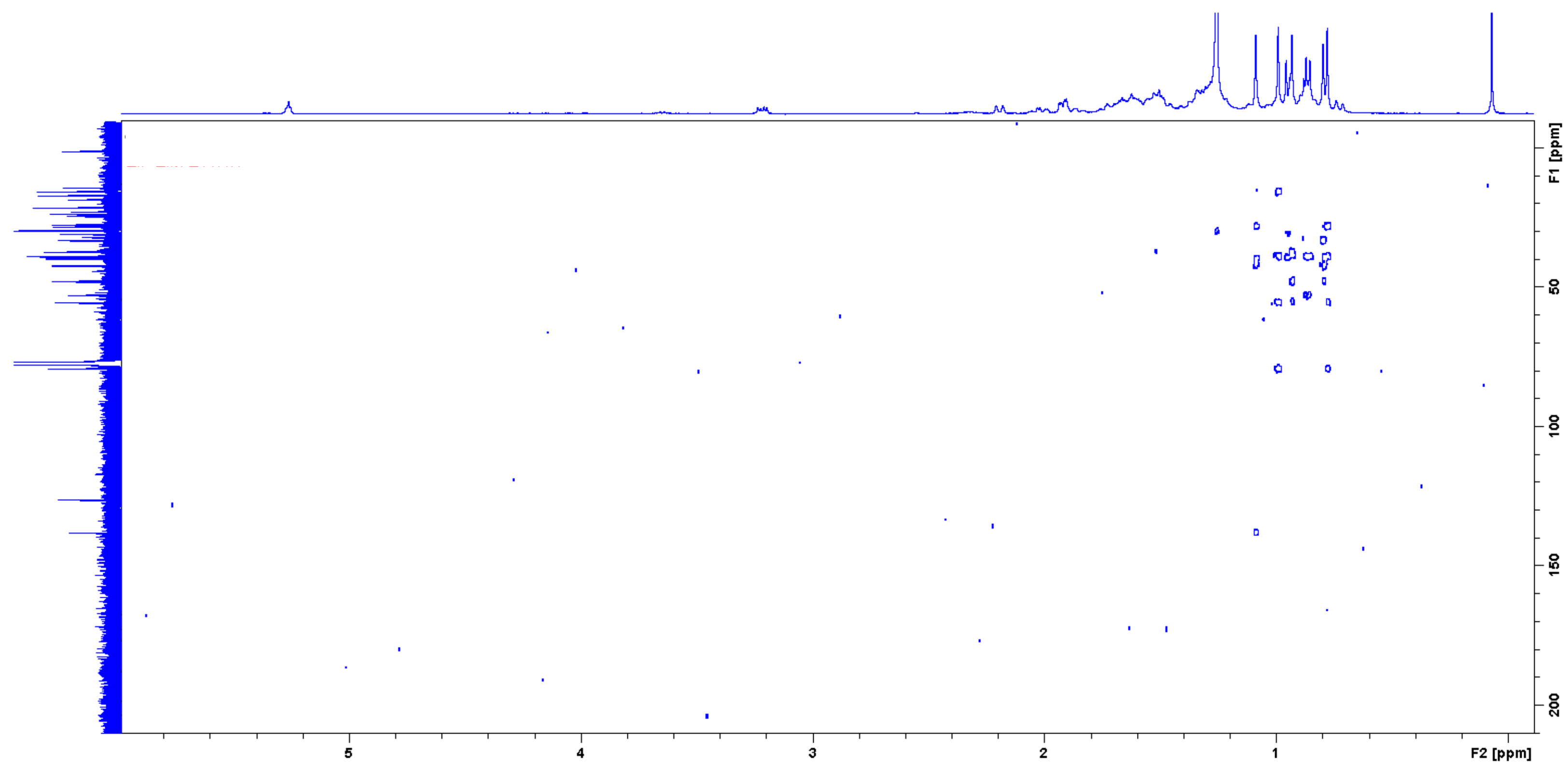
A9

HSQCDEPT spectrum for compound (25) in $CDCl_3$



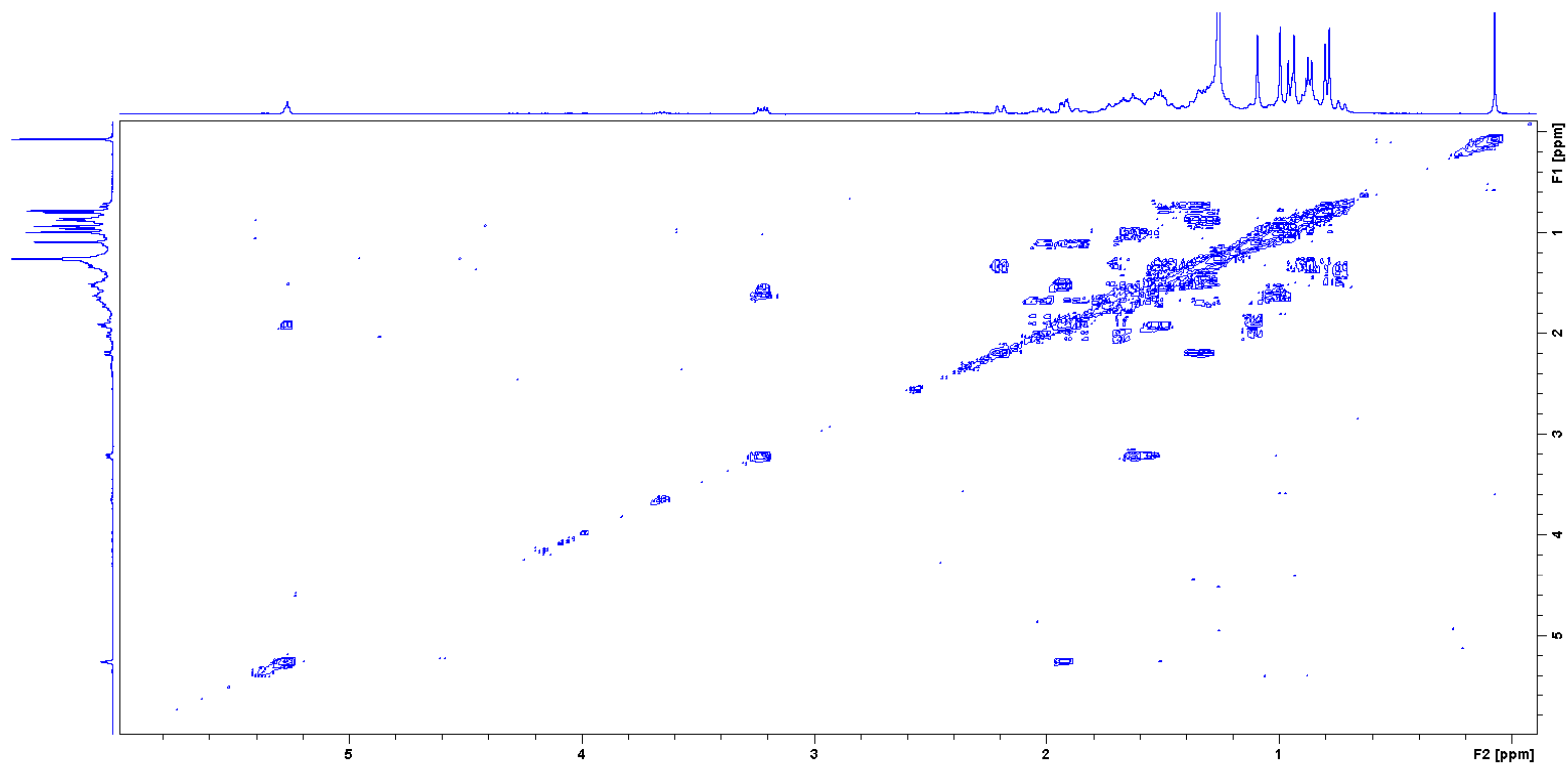
A10

HMBC spectrum for compound (25) in $CDCl_3$



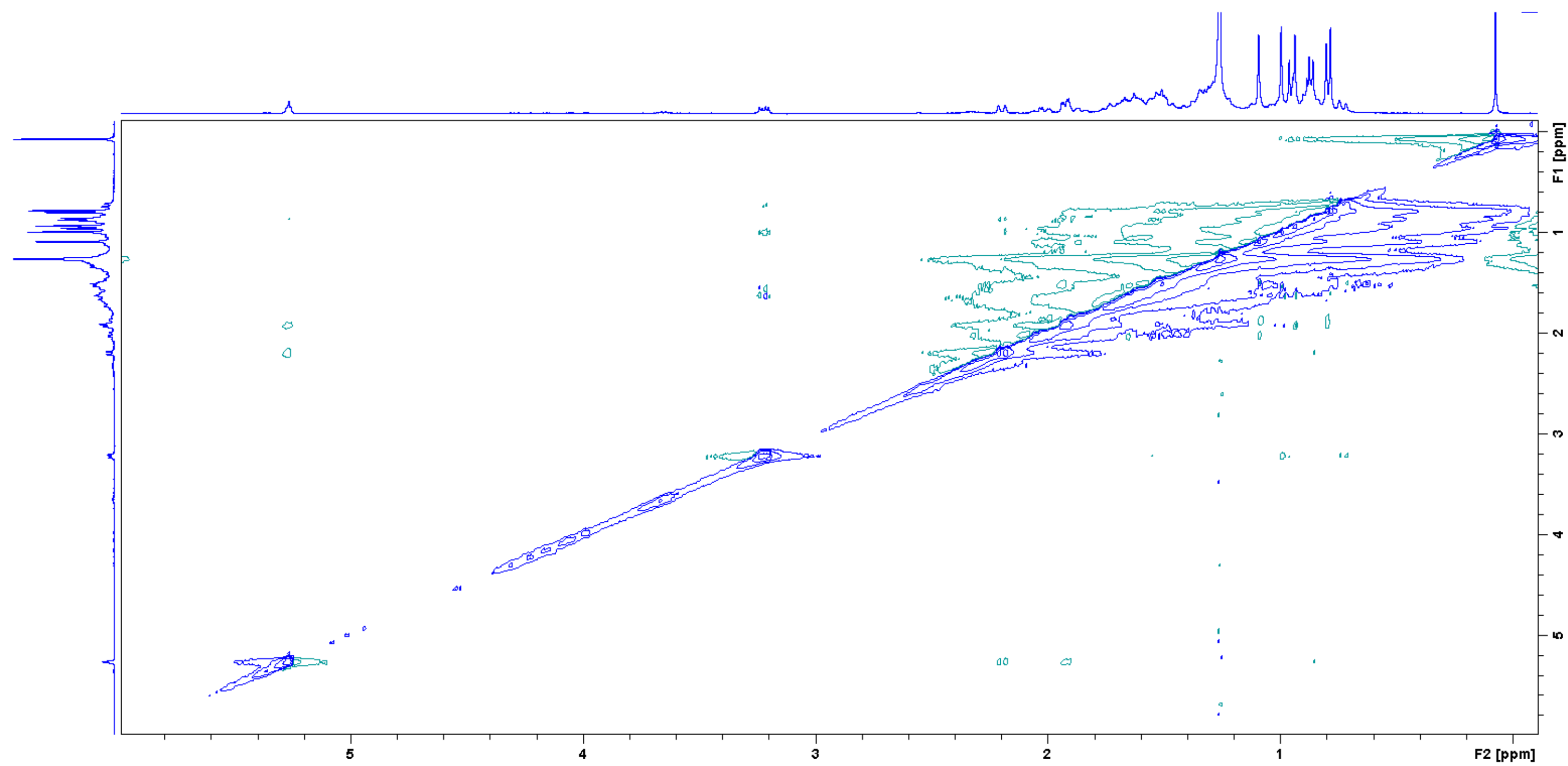
A11

COSY spectrum for compound (25) in $CDCl_3$



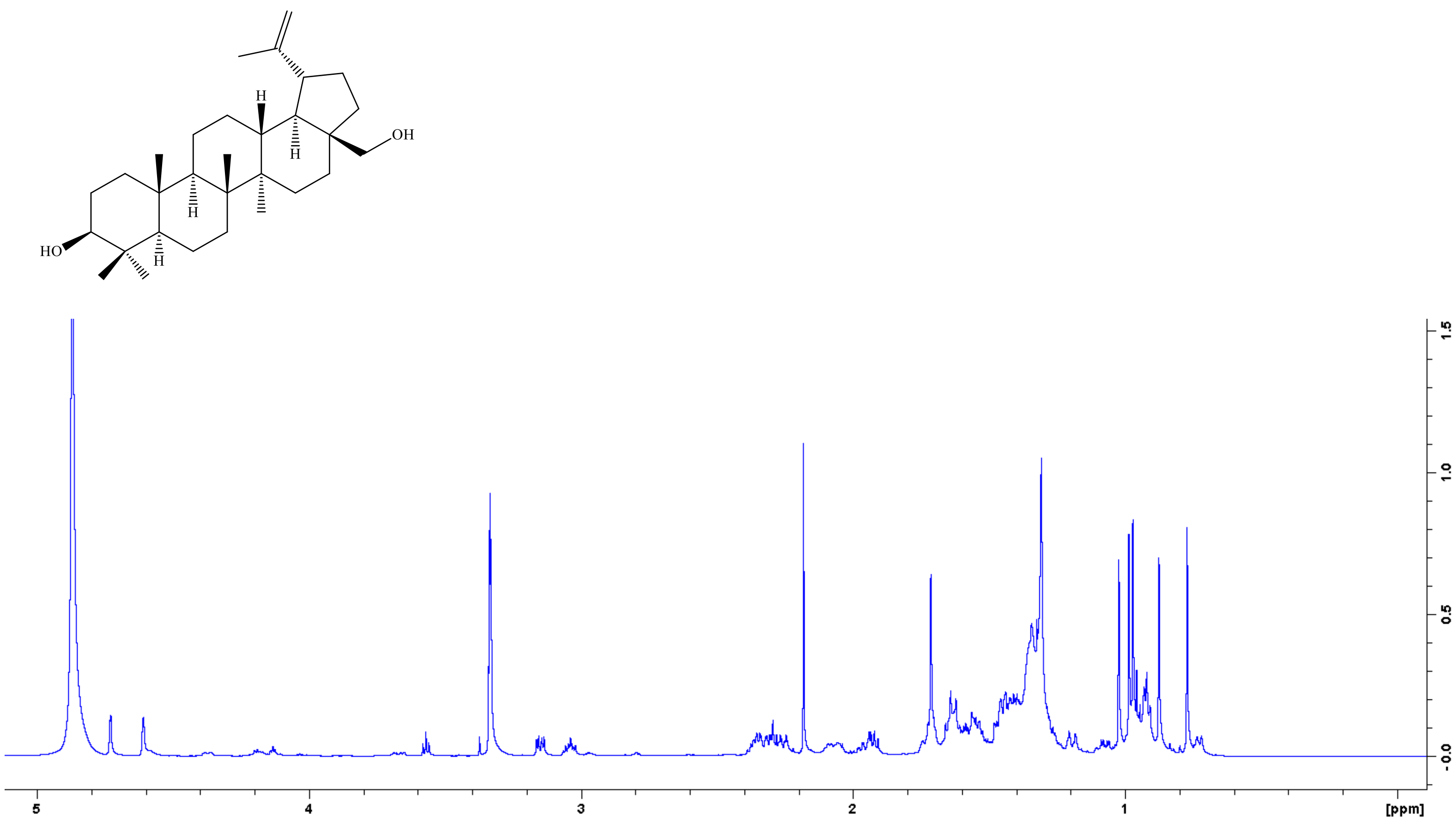
A12

NOESY spectrum for compound (25) in $CDCl_3$



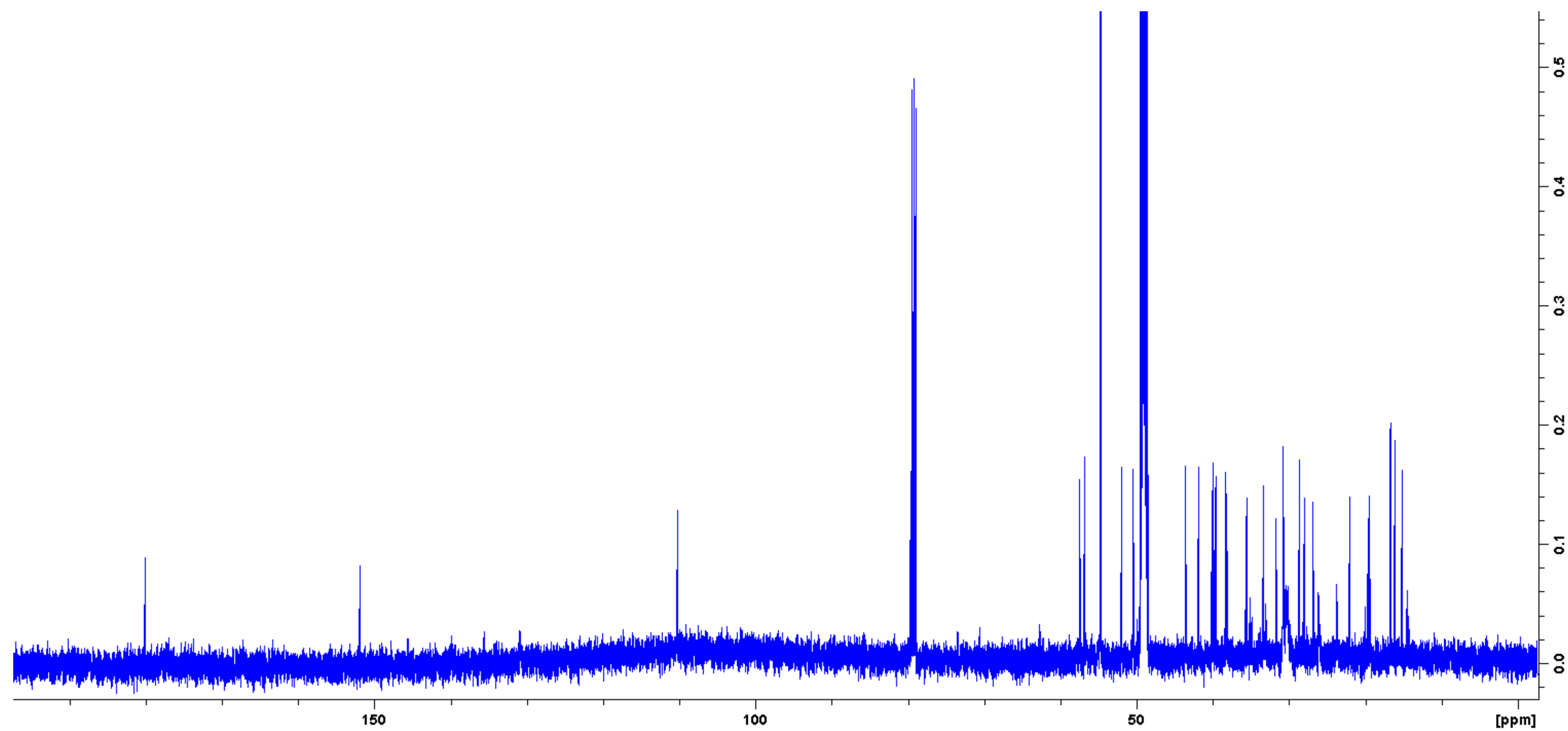
A13

^{1}H NMR spectrum for compound (26) in CD_3OD



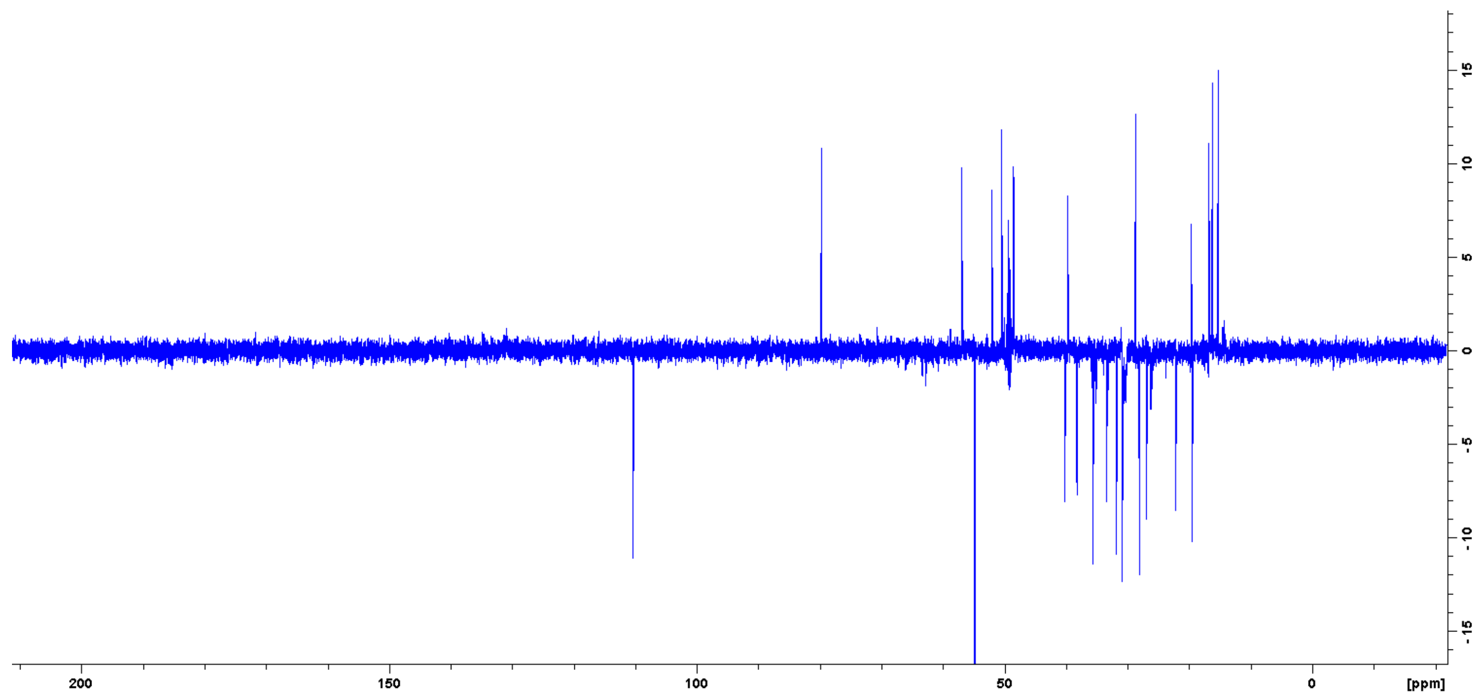
A14

^{13}C NMR spectrum for compound (26) in CD_3OD



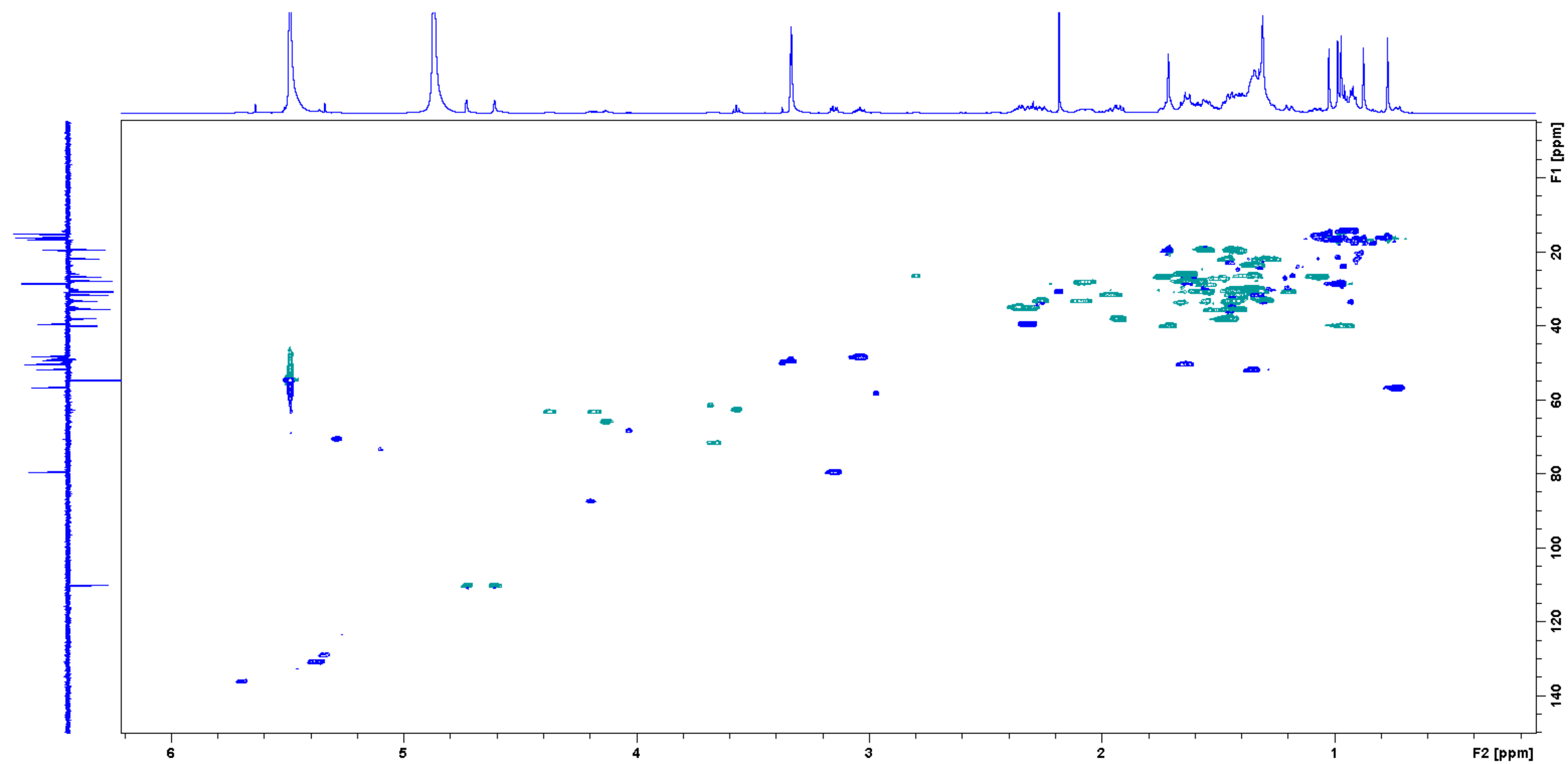
A15

DEPT spectrum for compound (26) in CD_3OD



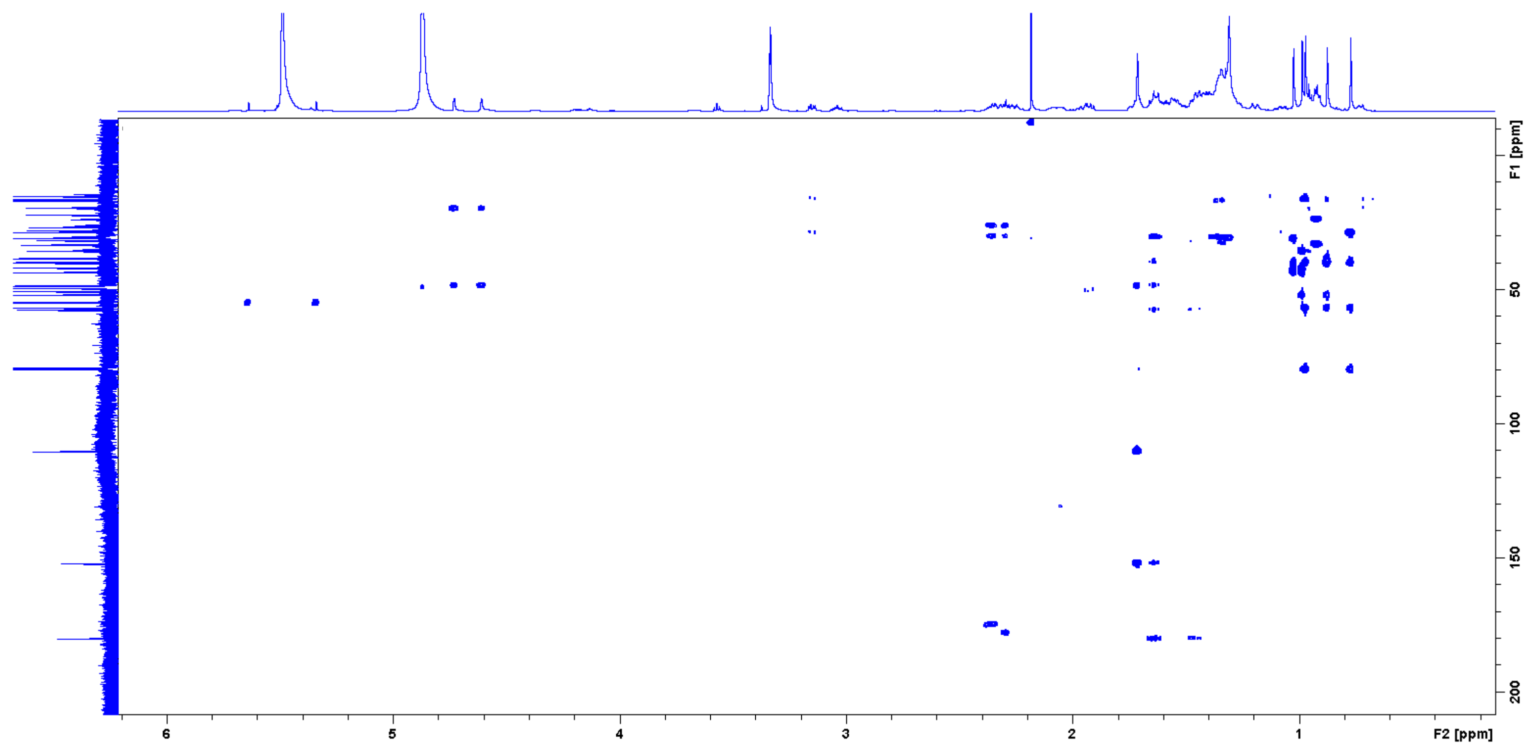
A16

HSQCDEPT spectrum for compound (26) in CD_3OD



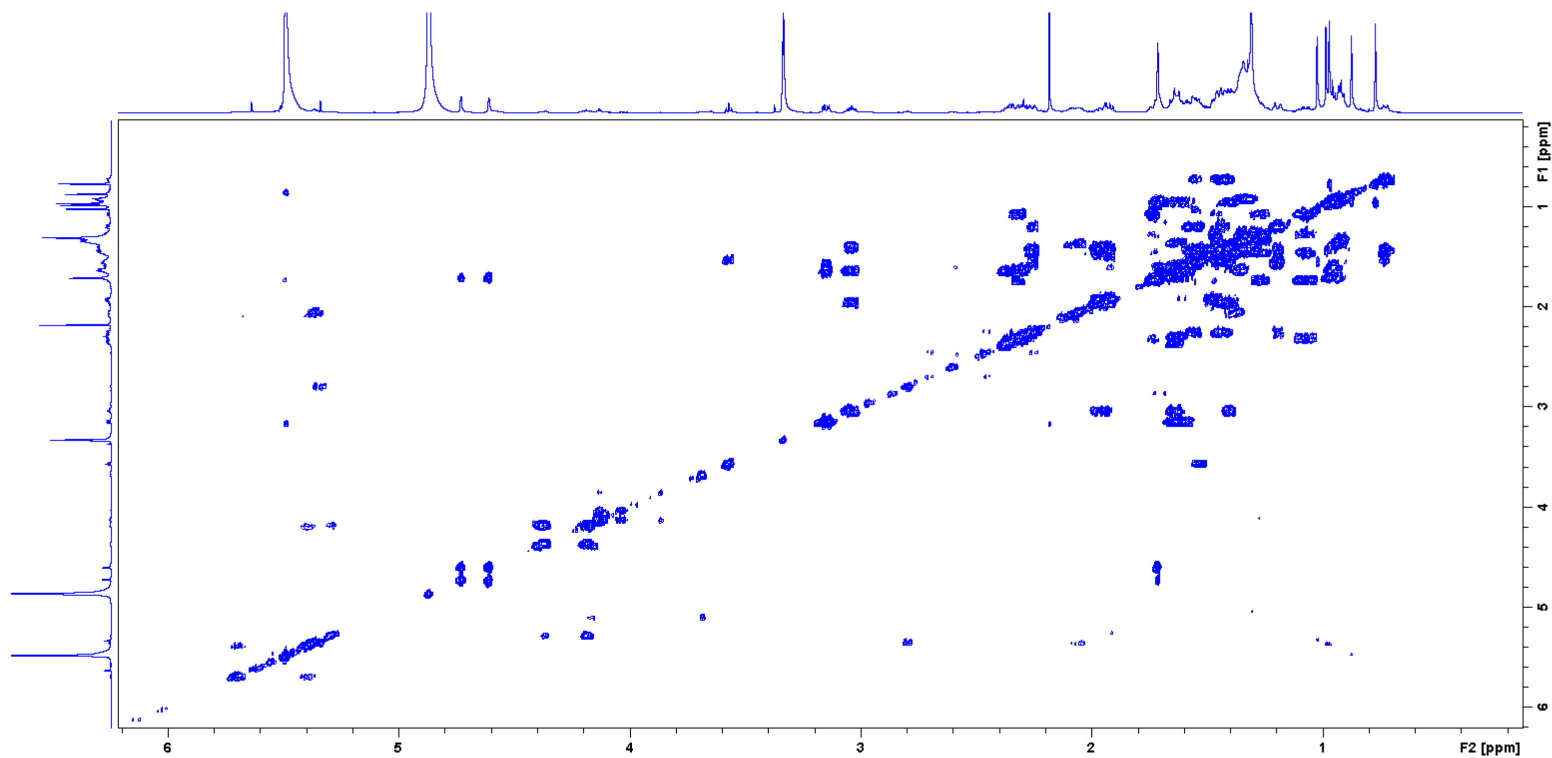
A17

HMBC spectrum for compound (26) in CD_3OD



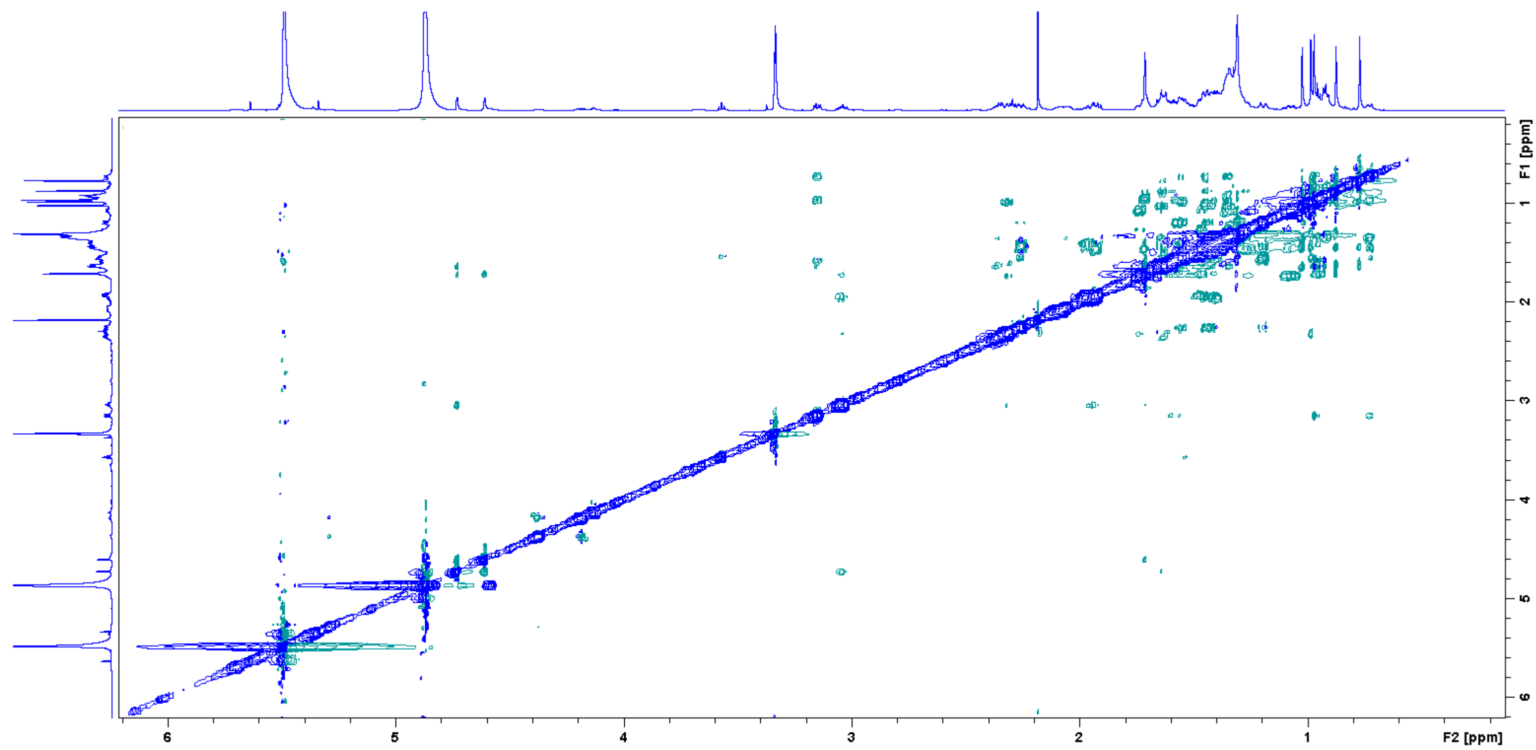
A18

COSY spectrum for compound (26) in CD_3OD



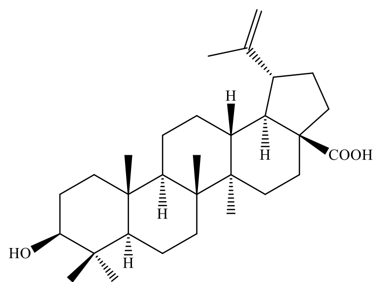
A19

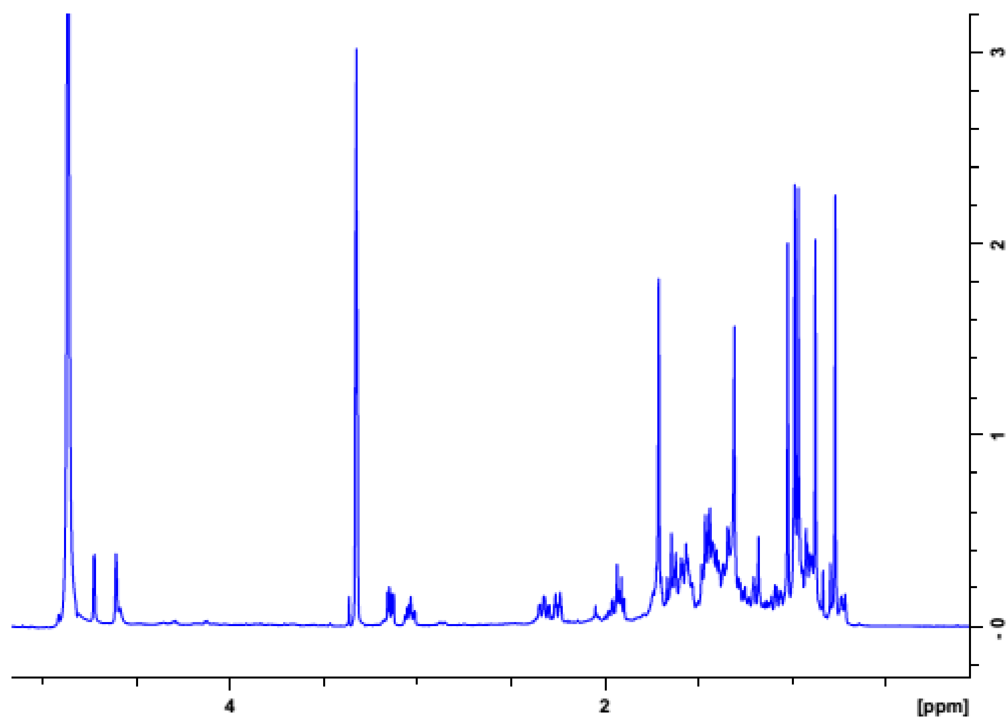
NOESY spectrum for compound (26) in CD_3OD



A20

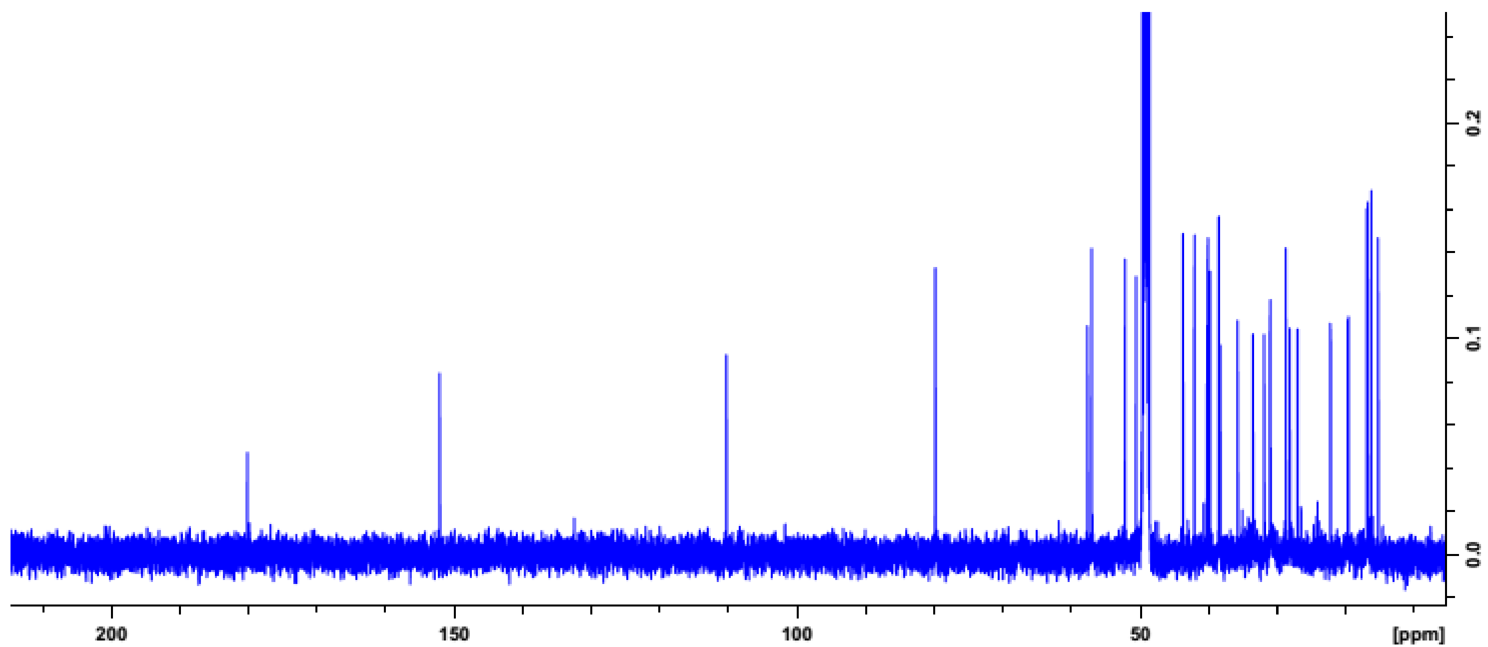
^{1}H NMR spectrum for compound (30) in CD_3OD





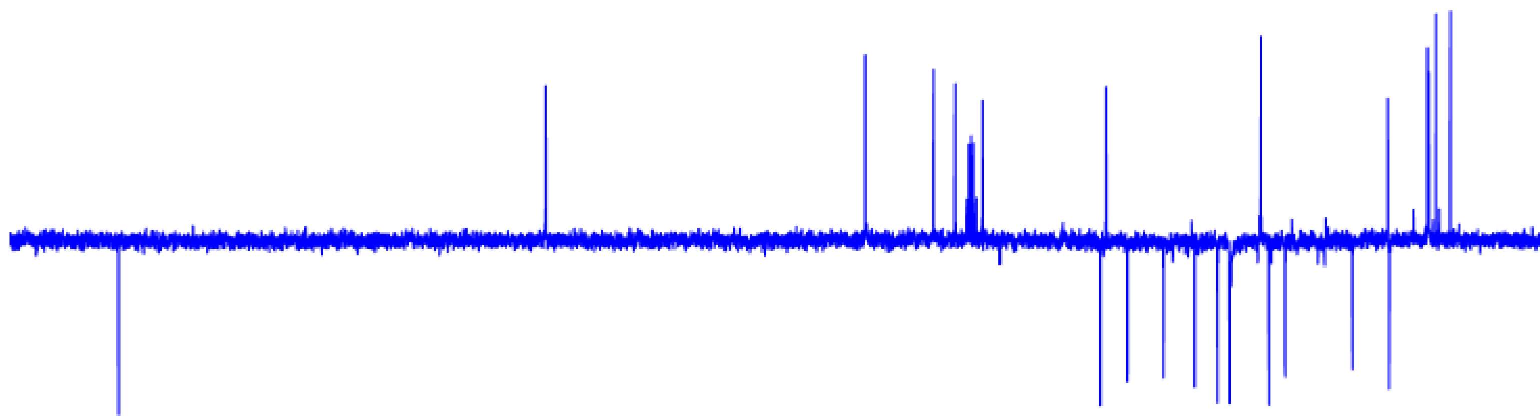
A21

^{13}C NMR spectrum for compound (30) in CD_3OD



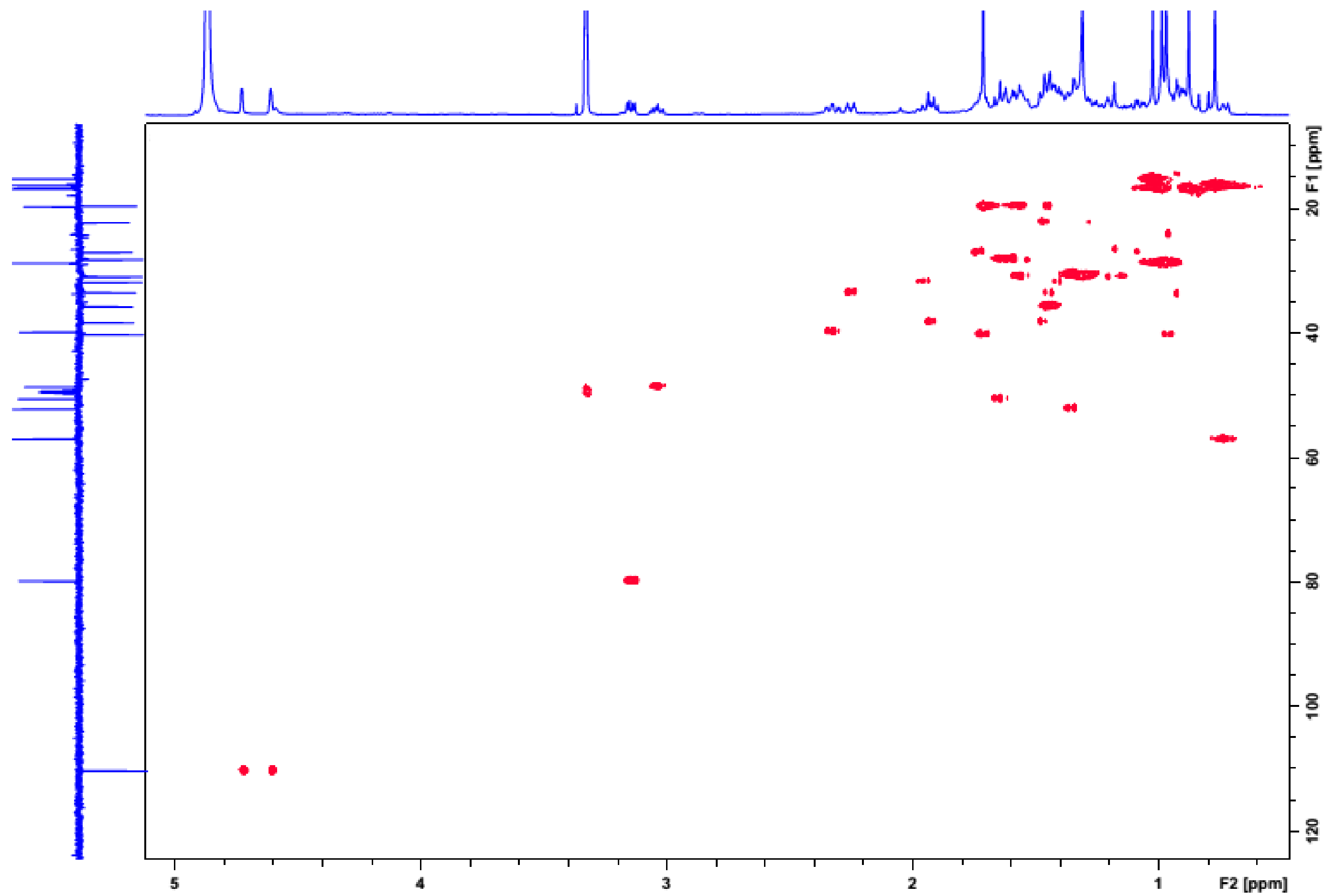
A22

DEPT spectrum for compound (30) in CD_3OD



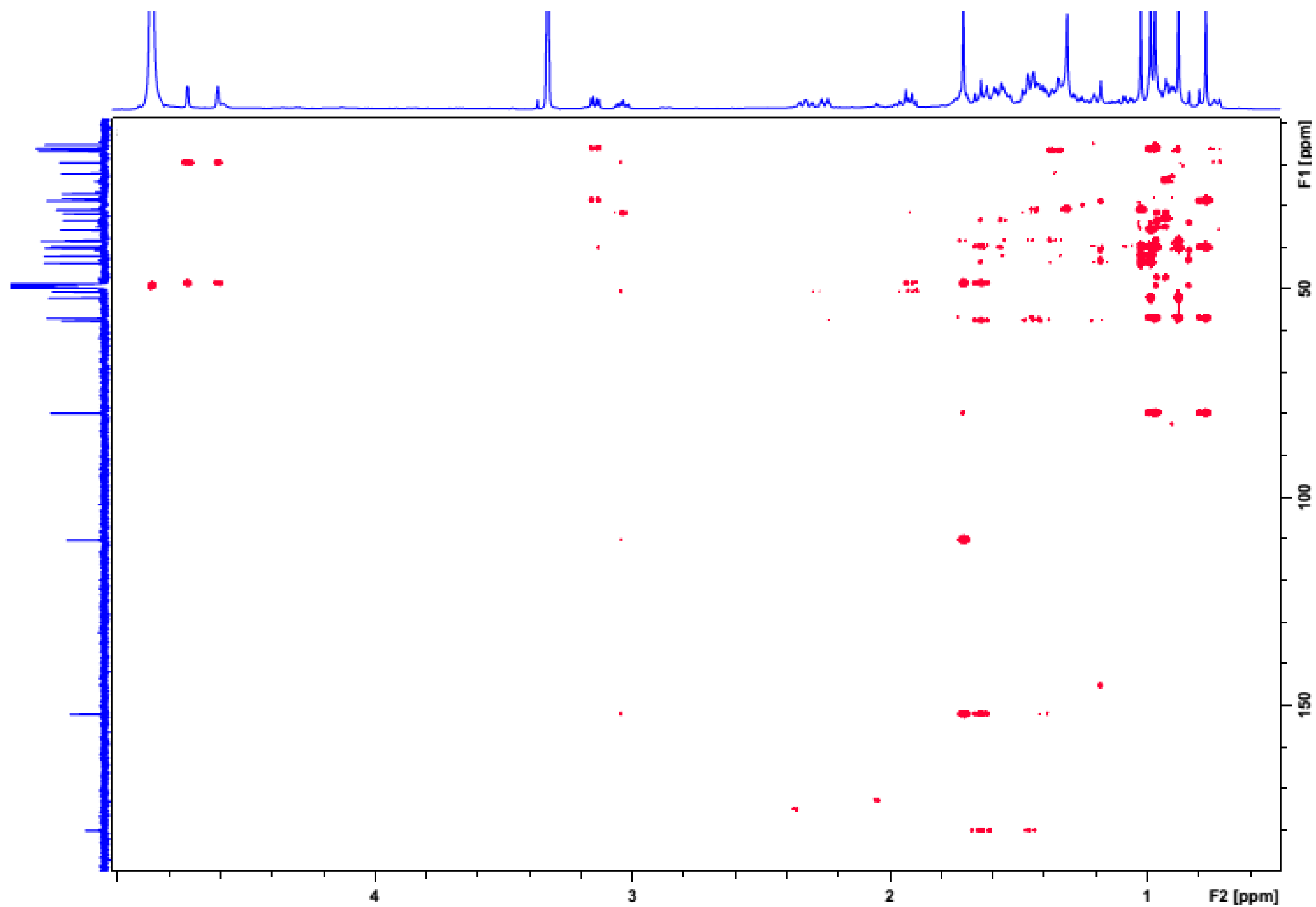
A23

HSQCDEPT spectrum for compound (30) in CD_3OD



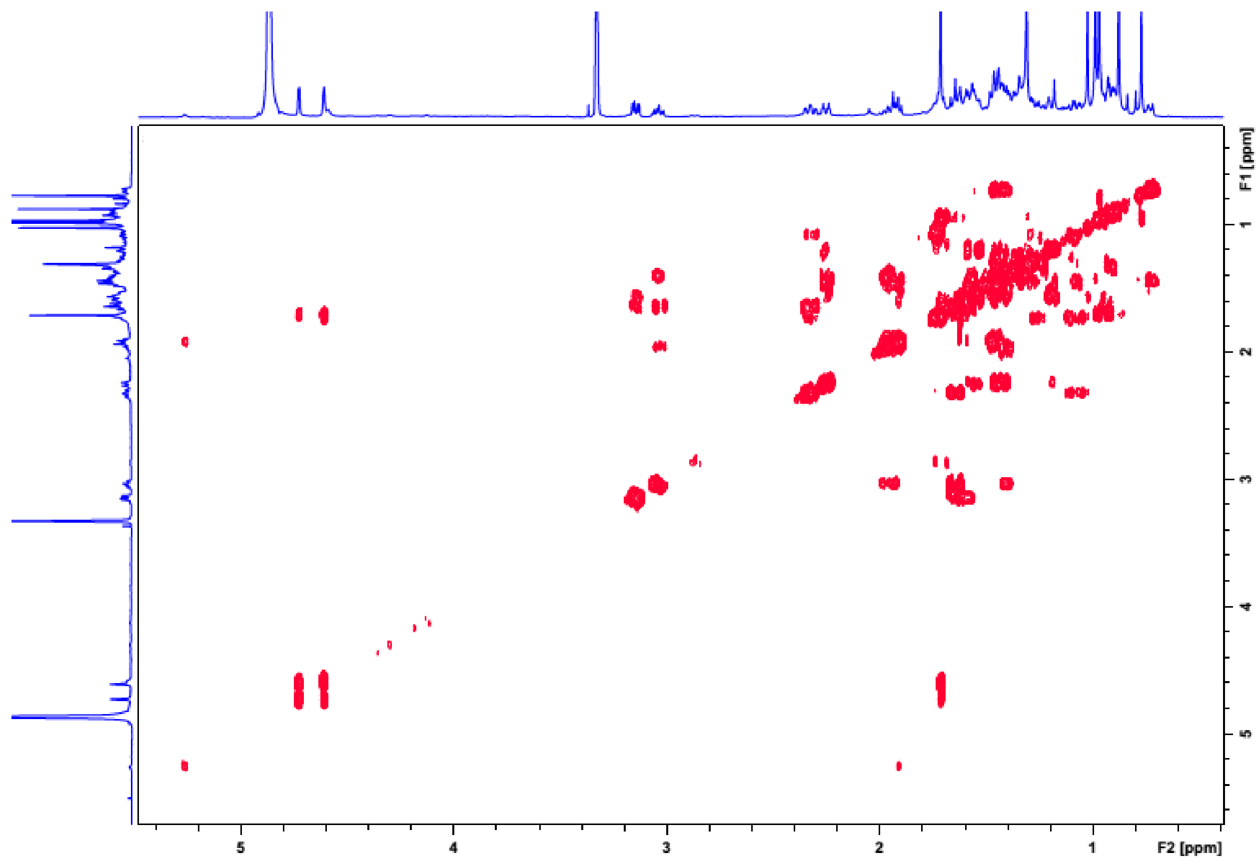
A24

HMBC spectrum for compound (30) in CD_3OD



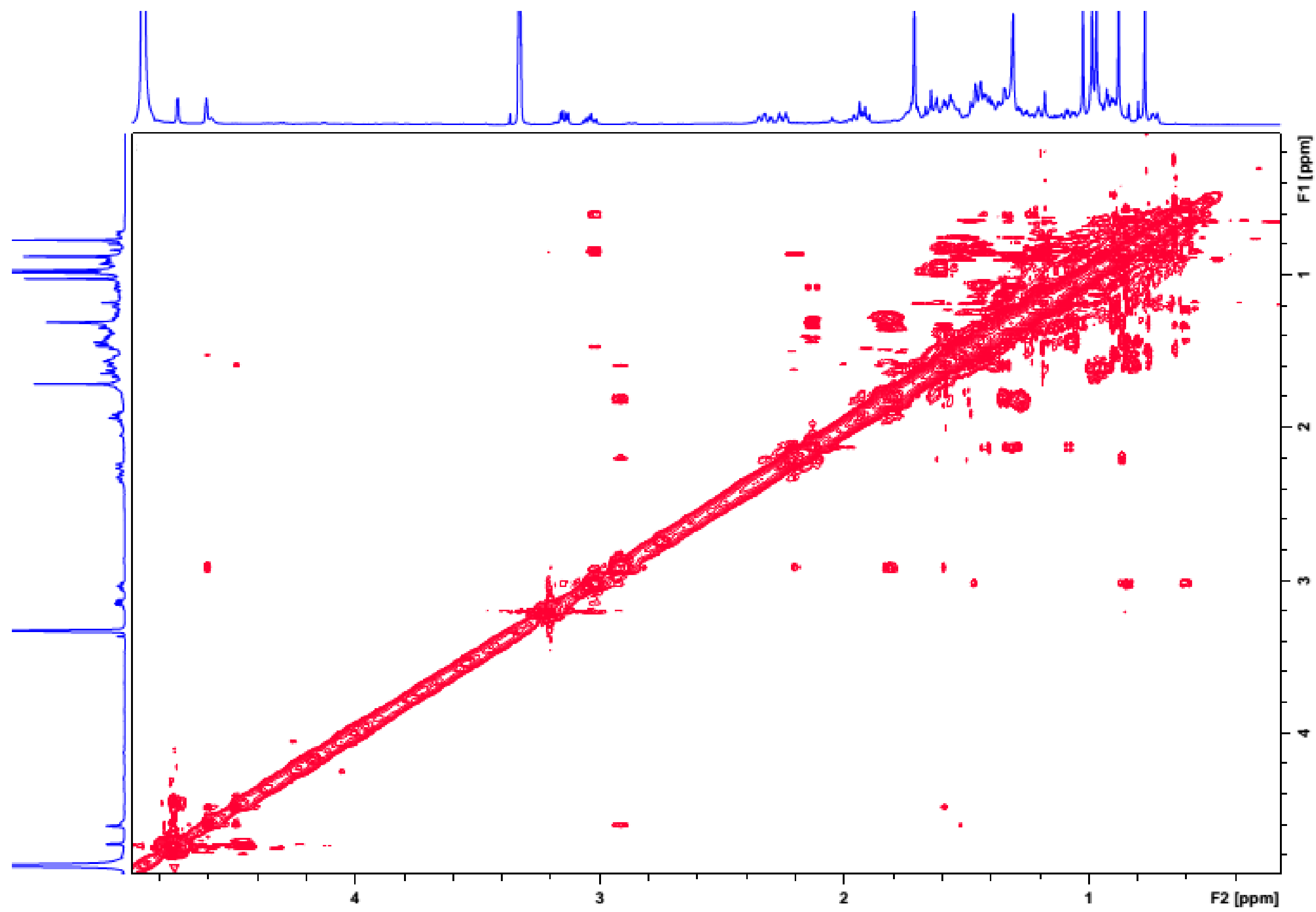
A25

COSY spectrum for compound (30) in CD_3OD



A26

NOESY spectrum for compound (30) in CD_3OD



Appendix Ⅱ: Abstract Page of Publication

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2019.1705816

SHORT COMMUNICATION

Check for updates

Characterization of secondary metabolites from the berries of *Ziziphus mucronata* and their antioxidant properties

Fasahat Parkar[a], Alice Wanjiku Njue[a], Moses Kiprotich Langat[b] and Josiah Ouma Omolo[a]

[a]Department of Chemistry, Egerton University, Njoro, Kenya; [b]Department of Natural Capital and Plant Health, Kew Royal Botanic Gardens, Richmond, UK

ABSTRACT

Ziziphus mucronata is a medicinal plant used traditionally for treating various diseases. The antioxidant assay for the crude extracts obtained from the berries of *Ziziphus mucronata* was carried out using DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Purified antioxidant compounds were obtained by chromatographic methods and were subjected to structure elucidation using nuclear magnetic resonance (NMR). Berries of *Ziziphus mucronata* were found to have high antioxidant activity since their ethyl acetate extract had an IC_{50} value of 214.9 µg/mL and methanol extract had an IC_{50} value of 8.9 µg/mL showing significant antioxidant activity in comparison to that of the standard ascorbic acid, which was found to have an IC_{50} value of 1.5 µg/mL. Betulin, betulinic acid and ursolic acid were among the compounds isolated from the berries of *Ziziphus mucronata* and are known to possess significant antioxidant activity. They have not been known to be previously isolated from *Ziziphus mucronata*.

ARTICLE HISTORY
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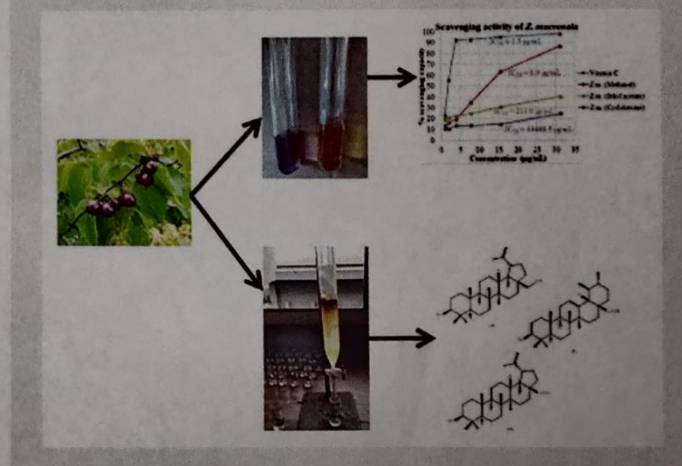
KEYWORDS
Antioxidant; DPPH; ROS (reactive oxygen species)



CONTACT Josiah Ouma Omolo jomolo@egerton.ac.ke

Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2019.1705816.

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Appendix III: Research Permit

THIS IS TO CERTIFY THAT:
MISS. FASAHAT ABDUL PARKAR
of EGERTON UNIVERSITY, 0-20100
NAKURU,has been permitted to conduct
research in *Baringo , Nakuru Counties*

on the topic: *INVESTIGATION OF ANTIOXIDANT COMPOUNDS FROM THE BERRIES OF TWO MEDICINAL PLANTS: TECLEA SIMPLICIFOLIA (ENGL.) I. VERD. AND ZIZIPHUS MUCRONATA WILLD.*

for the period ending:
30th April,2020

Permit No : NACOSTI/P/19/61063/29124
Date Of Issue : 30th April,2019
Fee Recieved :Ksh 1000

Applicant's Signature

Director General
National Commission for Science, Technology & Innovation