SECONDARY METABOLITES FROM *Dovyalis abyssinica* (A. Rich.) Warb FRUITS AND THEIR ANTIOXIDANT EFFECTS

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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

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented at this university or any other for the award of a degree.

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SM11/17507/17

Recommendation

This thesis has been submitted with our approval as University supervisors.

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DEDICATION

To the quiet giants who nurtured my curiosity – Mom and Dad, your unwavering belief in me became the wind beneath my wings. Your financial and emotional support allowed me to chase this dream, even when doubt clouded my vision.

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ABSTRACT

One of the main underlying causes of modern diseases is oxidative stress, an imbalance between radicals and antioxidants in living organisms. Its causative agents, unquenched radicals that outnumber antioxidants, remain challenging due to industrialization and modern lifestyles. In humans, these free radicals continue to harm fatty tissues, DNA, and proteins, which results in a multitude of diseases, including cancer, neurological, and cardiovascular diseases. There is, therefore, a need to document and add sources of natural antioxidants. Traditional medicine has long used extracts from *D. abyssinica* to treat certain human pathogens. Scientific research has demonstrated the efficacy of several secondary metabolites isolated from its leaves, roots, and bark against human infections. However, no investigation on the fruits of this plant has yet been reported. This research, therefore, studied the antioxidant capacities and total phenolic content of fruit extracts of *D. abyssinica* and isolated three known secondary metabolites from its fruits. Ripe fruits were collected from Egerton University botanic garden, freeze-dried, and ground into fine powder. Solvent extraction was done to obtain hexane, methylene chloride, ethyl acetate, and methanol crude extracts. Samples of these four crude extracts were subjected to the folin-ciocalteu method and DPPH assay to determine their total phenolic content and antioxidant capacities, respectively. The methanol extract gave 921.79 ± 1.63×10^{-3} mg GAE/100g (mg of gallic acid equivalents per 100g of extract), whereas the ethyl acetate extract gave 517.95 ± 1.4×10^{-3} mg GAE/100g phenolic content. In contrast, the methylene chloride and hexane extracts gave 261.54 ± 1.0×10^{-4} mg GAE/100g and 24.36 ± 8.2×10^{-4} mg GAE/100g respectively. From these extracts, data on IC_{50} showed a significant radical scavenging capacity in the methanol extract (4.4 μg/mL) than those of ethyl acetate, methylene chloride, and hexane extracts (8.4 μg/mL, 28.8 μg/mL, and 55.8 μg/mL respectively). Subsequently, methanol and ethyl acetate crude extracts were subjected to column, thin layer, and ultimately preparative thin layer chromatographic techniques for separation and purification. 1-D and 2-D NMR spectroscopic analysis revealed three known compounds: betulinic acid (**31**), sitosterol (**32**), and *3*,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol (**41**), and the compounds are new reports from the fruits of *D. abyssinica*. This research showed that the studied fruits of *D. abyssinica* exhibited a substantial phenolic content and antioxidant capacity, providing a source of natural antioxidants and hence contributing to SDGs 2.1 and 3.7 on improved nutrition and well-being promotion.

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AAAA	Amino acid aminotransferase
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACA	Aldehyde/carboxylic acid
AD	Alzheimer's disease
ADC	Arginine decarboxylase
ADH	Arogenate dehydrogenase
ADT	Arogenate dehydratase
AF	1,5-anhydro-D-fructose
AG	1,5-anhydro-D-glucitol
AGM	Agmatine
ALS	Amyotrophic lateral sclerosis disease
ANOVA	Analysis of variance
ARG	Arginine
BDE	Bond dissociation enthalpy
CANSDH	Carboxynorspermidine dehydrogenase
CAS	Cycloartenol synthase
CBA	Crocin bleaching assay
CD	Circular dichroism
CM	Chorismite mutase
C-Nspd	Carboxynorspermidine
COSY	Correlation spectroscopy
CUPRAC	Cupric reducing antioxidant capacity
CYPs	Cytochrome P450 enzymes
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH-H	2,2-diphenyl-1-picrylhydrazine
dSAM	decarboxylated *S*-adenosylmethionine
ET	Electron transfer
FCR	Folin-ciocalteu reagent

FPP	Farnesyl diphosphate
FPS	Farnesyl diphosphate synthase
FRAP	Ferric reducing antioxidant power
FTC	Ferric thiocyanate
GAE	Gallic acid equivalents
GLOBOCAN	Global Cancer Observatory
GTs	Glycosyltransferases
HAT	Hydrogen atom transfer
HAT	Hydrogen atom transfer
HMBC	Heteronuclear multiple bond correlation
HMWA	High molecular weight antioxidants
HSCCC	High speed counter current chromatography
HSQC	Heteronuclear single quantum correlation
IPP	Isopentenyl diphosphate
IR	Infrared
LMWA	Low molecular weight antioxidants
LUS	Lupeol synthase
MCE	Multi-stage counter current extraction
MVA	Mevalonic acid pathway
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
Nspd	Norspermidine
ODC	Ornithine decarboxylase
ORAC	Oxygen radical absorbance capacity
OSCs	Oxidosqualene cyclases
PAT	Prephenate aminotransferase
PD	Parkinson’s disease
PDH	Prephenate dehydratase
PG	Phenol glucoside
PUT	Putrescine
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
SAM	*S*-adenosylmethionine
SAMDC	*S*-adenosylmethionine decarboxylase
SDGs	Sustainable development goals
SET	Single electron transfer
SMT	Sitosterol C24-methyltransferases
SPDS	Spermidine synthase
SQE	Squalene epoxidase
SQS	Squalene synthase
SuSy	Sucrose synthase
TAC	Total antioxidant capacity
TLC	Thin layer chromatography
UDP-Glucose	Uridine diphosphate glucose
UV	Ultraviolet
UV-Vis	Ultraviolet visible
VERO	Cell lineages used in cell cultures
WHO	World Health Organisation
6-HCH	6-hydroxy-2-cyclohexene-on-oyl

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Oxidative stress, which is labelled as the existence of more unquenched radicals within a biological system, is a precursor of many cell dysfunctions leading to associated diseases. As identified by Vina *et al*. (2013), it is implicated in cell death and later, in cell death after free radicals were proven to exist in living organisms. In the context of biological systems, reactive oxygen species (ROS) form the majority of free radicals with negative impacts (Greenman *et al.*, 2007). The sources of these free radicals range from internal metabolic processes to external environmental stressors.

Since the postulation of Harman's free radical theory concerning oxidative stress, research studies on developing diseases related to oxidative stress have been expanded. A theoretical and empirical background regarding the implications of their damage is necessary to highlight the milestones of the advancements in this area. Since its postulation in the 20th century, the theory of free radicals has been followed by a number of empirical studies to ascertain its claims of interfering with the normal functioning of cells (Chatterjee *et al*., 2011; Fang *et al*., 2009). When this was finally established, researchers saw it necessary to build on these findings by further probing the physical diseases associated with this implication. The 21st century witnessed accelerated research that aimed at bridging the gap between free radical damage and diseases. Indeed, it was evidently proven that they are linked to, especially non-communicable diseases like cardiovascular diseases (Stallings-Smith *et al.*, 2013), neurodegenerative diseases (Zhuo *et al.*, 2011), and cancer (Halliwell, 2007; Members *et al.*, 2010). This school of thought and research dynamics was perhaps the most significant in this area.

Later on, Harman advanced his theory on free radicals and aging (Harman, 2009). In an effort to expound more on his advanced theory, he suggested that these species are implicated in, more specifically, the interference with biologically important molecules. Stemming from the mitochondria, their detrimental effect is felt in genetically controlling molecules and important primary metabolites. This damage leads to a cascading production of other ROS, thus causing more damage over time. Their accumulation and ultimate outnumbering of their control species causes oxidative stress. These unquenched radicals have been proven to be one of the initiators and propagators of fatty tissue damage, the main reason they cause rapid and irreversible cell

damage by compromising the integrity of the protective layer holding the cell contents in place. The fatty acid membrane containing enol moieties is subjected to self-propagating chain reactions with the production of peroxides (Delgado *et al.*, 2019). Due to this self-propagating nature, the initiation requires the destruction of a few molecules, which in turn spreads rapidly to neighbouring cells if no intervention is done (Huang *et al.*, 2005).

The advancement of oxidative stress as a cell disruptor has been associated with parallel research in a number of diseases. For instance, cancer initiation and progression are due to the damage of organelles hosting DNA and RNA by ROS (Visconti & Grieco, 2009). A key contributor to cancer is inflammation, which has also been associated with ROS damage (Bartsch & Nair, 2006; Reuter *et al.,* 2010; Virgilio, 2004). Traditionally, it was established that chronic irritation without proper interventions could lead to cancerous growths. These irritations stem from the impairment of organelles, accelerating abnormal apoptosis, due to the overproduction of ROS (Garodia *et al.*, 2007).

Life was far more perilous and mysterious for our forefathers than it is for us now. Everyone sought natural explanations, from academics to local practitioners, and plants were a logical choice. Like early scholars and practitioners of science, “wise women,” herbalists, and apothecaries relied on empirical evidence to conclude the effects of plants, particularly in medicinal contexts. The “cunning folk” infused the story with a mystical quality by elaborating on plants' symbolic and practical functions. Through thousands of years, intricate folklore traditions emerged, which at times developed separately in various regions of the globe. For instance, even during the Christian era, astrology was a significant factor in medical practice. A plethora of romanticized versions of folktales emerged with the abundance of folklore. That glitz and glitter is still with us today. Local and universal superstitions and folklore are both resilient and subject to change. A plant’s characteristics can take on two completely different meanings from one place, culture, or person. Herbs have a complicated and often perplexing past, and no dissertation, review, report, book, or research article of any size could ever hope to do them justice. Plant lore is incredibly fascinating, and all it can do is touch on the fundamental notions (Lawrence, 2020).

Plants have a long history of human consumption due to their high nutritional density and abundance of beneficial vitamins. Traditional medicine also made use of a wide range of plant remedies. Due to the widespread belief that oxidative stress is a key component of the majority of human diseases and the fact that our antioxidant defense system is inadequate to counteract the

abundance of free radicals in the body, the idea of plants as a source of antioxidants has gained increasing attention. Therefore, there has been much research to find plant compounds with antioxidant properties (Asao & Asaduzzaman, 2018). To control free radicals, enzymatic and non-enzymatic antioxidant defence systems are present in plants, like humans. Non-enzymatic antioxidants are species with low molecular weights (LMWA), such as ascorbic acid and proline, as well as high molecular weights (HMWA), such as tannins. Because plants do not have an immune system, animals may rely on their antioxidant defence system to ward off microbes and herbivores. This lack of an immune system could explain why plants contain antioxidants. These phytochemicals also protect plants from harmful environmental factors.

Several plants have antioxidant characteristics because they contain phytochemicals demonstrating antioxidant activity in human and animal studies. Vegetables and fruit intake that contain high levels of antioxidant phytochemicals have been shown to enhance immunity (Mazza *et al.*, 2002). In addition, the polyphenols found in fruits, which are abundant in flavonoids and phenolics, may be responsible for the serum's overall antioxidant activity. The phytochemicals found in fruits and vegetables may have antioxidant properties, but they may also interact with one another to increase those benefits (Liu, 2003). Studies show that inflammatory cells emit free radicals, which contribute to the pathophysiology of some chronic diseases such as cancer, cardiovascular disease, and type 2 diabetes (Dahlén *et al.*, 2014; Qiao & Li, 2014). Research has shown that curcumin, resveratrol, and anthocyanins are only a few of the antioxidant phytochemicals that can reduce inflammation by increasing cytokine production, inhibiting nuclear factor-kB activity, and enzyme inhibition (Steinberg & Schertzer, 2014). Table 1 summarise the plants discussed here, and this part focuses on those plants' antioxidant phytochemicals and *in-vitro* and *in-vivo* antioxidant properties against certain oxidative stress illnesses.

Table 1: Selected plants and their phytochemicals oxidative stress-related diseases and their proposed mode of action

Oxidative stress-related diseases	Name of plant and part investigated	Phytochemicals or extract type	Mechanism of action
Diabetes	*Chrysobalanus icaco* (leaves)	Polyphenolics	Intense antioxidant action and reduction of glycemia in rats
	Ascophyllum nodosum (fronds)	Phenolics	Antioxidant activity and anti-diabetic effect
	Zingiber officinale (root tuber)	Curcumin	Anti-inflammatory and anti-oxidant activities
		Butein	Inhibit the formation of nitric oxide *in-vitro* and protect pancreatic B-cells against cytokine-induced toxicity.
Cancer	*Camellia sinensis* (leaves), *Vitis vinifera* L (grapes)	Polyphenols and proanthocyanidins	Protect the skin from the adverse effects of UV radiation, preventing the risk of skin cancers.
Alzheimer's disease	*Crataegus pinnatifida* (fruits)	Crude extract	Potential neuroprotective activity for preventing oxidative-related disorders *in vitro*
Cardiovascular disease	*Gnetum macrostachyum* (fruits and seeds)	Stilbenoids	Antioxidant and anti-inflammation activities
	Euterpe oleracea (berries)	Flavonoids	*In-vitro* atheroprotective effects
	Flos chrysanthemi (flowers)	Flavonoids	Vasodilating effects and protected vasodilator reactivity
Anti-obesity	*Vaccinium floribundum* (berries) *Aristotelia chilensis* (berries)	Anthocyanins, proanthocyanidins	Limits adipogenesis and inflammatory pathways *in vitro*
	Edible grapes	Polyphenol	Antioxidant action, blocking proinflammatory cytokines
Aging	*Elaeis guineensis* (fruits and	Methanol extract	High antioxidant activities and potential ability as an anti-

Oxidative stress-related diseases	Name of plant and part investigated	Phytochemicals or extract type	Mechanism of action
	seeds)		aging agent
	Epigallocatechin gallate		Extended lifespan of healthy rats by reducing the damage to the liver and kidney and improving age-associated inflammation and oxidative stress through inhibiting NF-B signaling

1.2 Statement of the Problem

Having established the theoretical and empirical framework of oxidative stress and its association with some diseases, it is imperative to deduce that its existence is one of the major causes of diseases. It is brought about by free radicals not being quenched by antioxidants. The sources of these species range from industrialization to modern lifestyles. These sources include living and working in or near ecosystems prone to environmental pollution, such as air and water, continuous consumption of processed foods, and exposure to some industrial chemical substances. These unquenched free radicals damage fatty tissues, DNA, and proteins in the body, leading to several diseases, including cardiovascular diseases, neurodegenerative diseases, and cancer. According to the World Health Organisation (WHO), cancer is a leading cause of many deaths occurring worldwide, accounting for nearly 10 million deaths in 2020, or almost one in six deaths. It is estimated that there will be 27.5 million new cases of cancer worldwide each year by 2040. Reports from the Global Cancer Observatory (GLOBOCAN) in Eastern Africa indicated annual mortality of 236,904, new cases reported were 349,500, and prevalent cases over 5 years were 708,956. Without looking into other diseases' reported instances, mortality rates, and prevalence cases in our region, these cancer statistics are already grim. The scientific strides in dealing with oxidative stress to help curb these deadly statistics have not yielded significant achievements, requiring more to be done.

1.3 Objectives

1.3.1 General Objective

The broader scope was centred around antioxidants from natural products. A general objective was therefore formulated to contribute to this scope. The general objective of the research study was to investigate antioxidant secondary metabolites from the fruits of *D. abyssinica*.

1.3.2 Specific Objectives

To achieve this general objective, the study had to be broken down into time-bound, accurate, measurable, and realistic specific objectives. These specific objectives were:

i) To evaluate the total phenolic content of fruit extracts of *D. abyssinica* using the Folin-Ciocalteu method.

ii) To determine the *in vitro* antioxidant capacity of fruit extracts of *D. abyssinica* using the DPPH assay.
iii) To isolate and identify the active secondary metabolites from fruit extracts of *D. abyssinica* using chromatographic techniques.
iv) To elucidate the structures of the isolated secondary metabolites from fruit extracts of *D. abyssinica* using spectroscopic techniques.

1.4 Hypotheses

In an effort to explain the antioxidant effects of these fruits, four hypotheses for an in-depth evaluation were formulated.

i) The total phenolic content of fruit extracts of *D. abyssinica* will be significantly low.
ii) The *in-vitro* antioxidant capacity of fruit extracts of *D. abyssinica* cannot be determined.
iii) Secondary metabolites from fruit extracts of *D. abyssinica* cannot be isolated and identified.
iv) Structures of the isolated secondary metabolites from fruit extracts of *D. abyssinica* cannot be elucidated.

1.5 Justification

Kenya has more than 42 distinct ethnic communities that have different cultures and beliefs, leading to rich indigenous knowledge systems, which, among others, include the use of medicinal plants. Reports on the use of *D. abyssinica* as a medicinal plant among the East African community are scarce, just as its phytochemical information. This plant is mainly related to its role as a food source for both humans and wildlife.

The uses of *D. abyssinica* by most, if not all, of the 42 distinct ethnic communities in Kenya are vast. The diversity of these communities makes the use of this plant vary from one ethnic group to another. Some use the plant as food or a food additive for their meals, while others use it for its medicinal value. However, literature on using the plant as medicine for various diseases is scarce. This study will, therefore, aim to enrich the literature associated with *D. abyssinica* by investigating the medicinal properties of this plant, precisely, its antioxidant properties. This might provide alternatives for health scientists for a more natural source of antioxidants that are cost-effective, freely occur in nature, are environmentally friendly, and support sustainable

development in the drug industry in Kenya. All these are in line with the Sustainable Development Goals (SDGs) 2.1 and 3.7.

CHAPTER TWO
LITERATURE REVIEW

2.1 Morphology of *D. abyssinica*

Dovyalis abyssinica, commonly known as the Abyssinian Gooseberry, is an evergreen, rounded, crown-spiny shrub about 3 metres tall. It has a grey bark bearing spines of about 1.5 centimetres long. Protruding from its branches are branchlets with *lenticels*. It has vibrant green leaves with a curved apex. The edges of the leaves are unevenly rounded. The leaves also bear a stalk and visible veins that appear red-brown. Its flowers lack petals. Female flowers sprout independently, but males appear in clusters of up to 65 stamens. Its edible fruit is a round red-orange berry about 2 centimetres in diameter (Figure 1: Photos courtesy of Botanic Garden, Egerton University 2021). A calyx surrounds the berry. When unripe, the berries are green and hairy at first, then smooth red-orange when ripe.

Figure 1: *D. abyssinica* whole plant, unripe and ripe berries

2.2 Taxonomy of *D. abyssinica*

Dovyalis is a genus of shrubs and small trees. Based on phylogenetic studies, *D. abyssinica* is largely considered and accepted to be in the family Salicaceae. Its former classification in the

family Flacourtiaceae was disregarded following the controversial limitations of its polyphyletic nature (Chase *et al*., 2002; Stanstrup *et al*., 2010).

2.3 Geographical Distribution of *D. abyssinica*

The 15 species in the genus are native to Africa (Eastern to Southern Africa), as depicted in Figure 2. *D. abyssinica* mainly grows in tropical regions of Ethiopia, Uganda, Sudan, South Sudan, Eritrea, Kenya, Somalia, Tanzania, Mozambique, Malawi, the DRC Congo, and South Africa. Kenya's habitat ranges from rainforests to riparian forests. It also does well in scrub vegetation, sometimes in open wooded grassland in Kenya. It is abundantly distributed in Ethiopia's vast Nile basin, while in Tanzania, it grows in semi-evergreen or deciduous bushland and on rocky slopes of Mount Kilimanjaro at elevations from 600 to 3,050 metres. Its Malawian habitat comprises rain forests and riparian forests in highlands of over 1800 metres (Barwick, 2005).

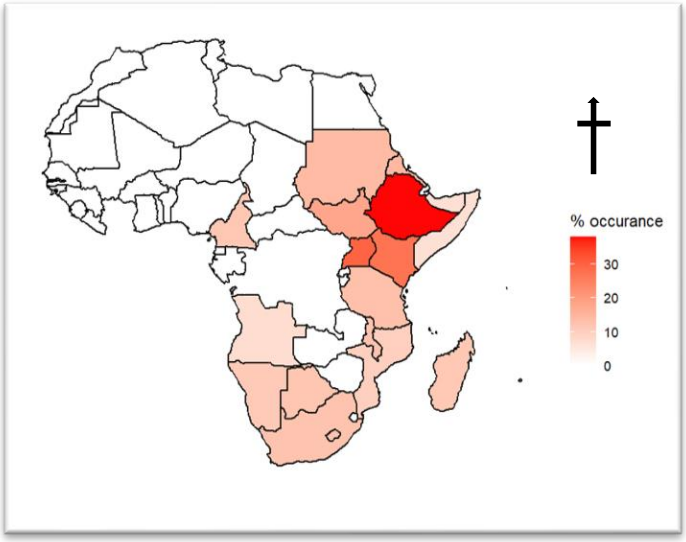


Figure 2: Distribution of *D. abyssinica* along Eastern Africa

2.4 Agronomic Factors of *D. abyssinica*

Dovyalis abyssinica thrives well in hot and dry climatic conditions, precisely in the tropics. However, it also thrives in areas of higher rainfall (Galan Saúco, 2006). It grows best in fertile, humus-rich, well-drained, non-calcareous soils (Rani *et al.*, 2010). It can do well in three types of soils: light (sandy), medium (loamy), and heavy (clay) soils. The soil's pH should be either acidic or neutral for optimum productivity. It can only be grown under open sunlight but not under shade.

As explained by Griffiths (1994), this species can be propagated by directly planting its seed or through layering or grafting buds of desirable varieties onto seedling rootstocks. It requires pruning on a biannual basis. The plant begins bearing berries when about 4 to 5 years old from the time of planting. It is a dioecious plant with reproductive organs in separate plants. For this reason, if the plant is planted for its fruits, there has to be a blend of both sexes within the plantation. Its green, unripe berries take about 8 weeks to mature and ripen when they become orange-red. The ripe berries are plucked with their calyx to increase their shelf life.

2.5 The Phytochemistry of *D. abyssinica*

Various studies have reported the purification and isolation of natural bioactive compounds from different parts of *D. abyssinica* apart from its fruit. This section seeks to highlight these key reports while highlighting plausible biosynthetic pathways for these compounds proposed in the literature. This has been done with a keen interest in understanding the chemistry behind the plant.

2.5.1 Spermidine Alkaloids from *D. abyssinica*

Isolation and purification studies by Rasmussen *et al*. (2006) revealed four dovyalicin-type spermidine alkaloids (Figure 3) from the leaves as well as the twigs of *D abyssinica*; dovyalicin A [(S)-1-(4-benzoylaminobutyl)hexahydro-5-methyl-4-phenyl-1,5-diazocin-2(1H)-one], compound **1**; dovyalicin B [(S)-1-(4-acetylaminobutyl)hexahydro-5-methyl-4-phenyl-1,5-diazocin-2(1H)-one], compound **2**; dovyalicin E [(S)-1-(4-benzoylaminobutyl)hexahydro-4-phenyl-1,5-diazocin-2(1H)-one], compound **3**; and dovyalicin F [N-(4-benzoylaminobutyl)-N-(3-dimethylaminopropyl)-3-phenylpropenamide], compound **4**.

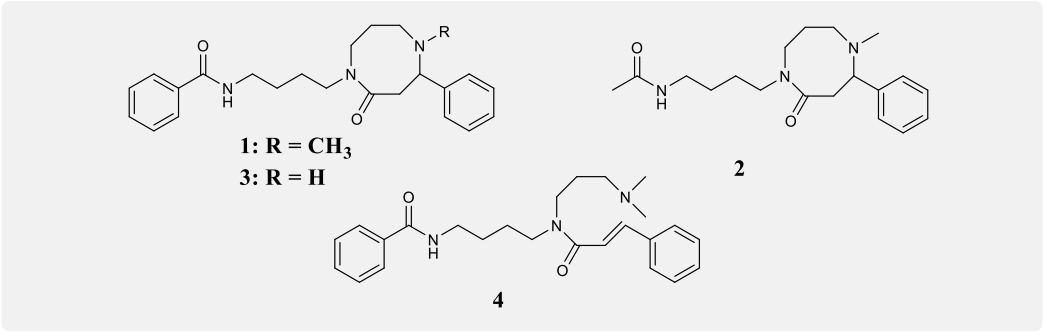
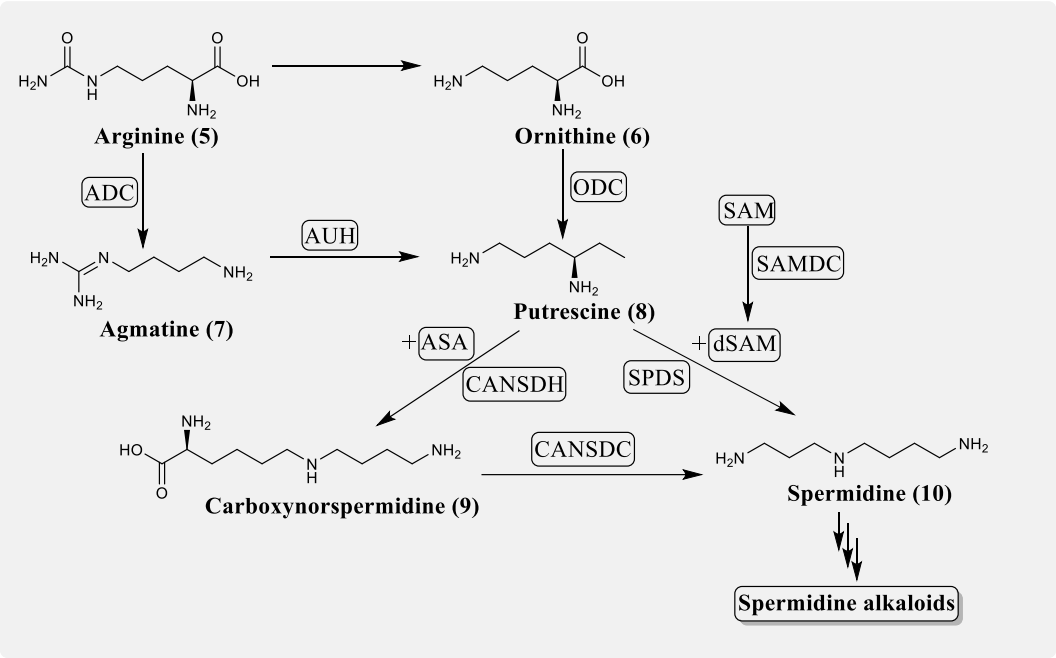


Figure 3: Spermidine alkaloids from leaves and twigs of *D. abyssinica*

2.5.2 Biosynthesis of Spermidine Alkaloids

Two key precursors of spermidine alkaloid biosynthesis in organisms that produce them are SAM and PUT. These two undergo enzymatic conversions with ODC, SAMDC, and ADC through a bifurcated pathway as shown in Scheme 1 (Graser & Hartmann, 2000). The main processes involve decarboxylation and dehydrogenation to generate intermediates, which are later reduced and oxidised to spermidine alkaloids (Shi *et al*., 2022).

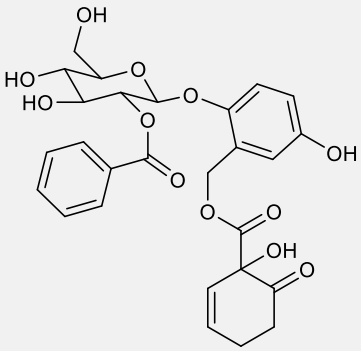


Scheme 1: A proposed biosynthetic pathway of spermidine alkaloids

Source: Shi *et al*. (2022)

2.5.3 A 4-hydroxytremulacin from *D. abyssinica*

Studies by Rasmussen *et al*. (2006) reported the isolation of 4-hydroxytremulacin [4-hydroxy-2-(1-hydroxy-6-oxocyclohex-2-enecarbonyloxymethyl)-phenyl-2-*O*-benzoyl-β-D-glucopyranoside], compound **11**, from the twigs of *D. abyssinica* (Figure 4). They reported it as a new phenol glucoside (PG) which was also isolated from the twigs of *D. hebecarpa* in the same report. Two years later, (Chirchir *et al.*, 2018) isolated this compound from the roots of the plant.



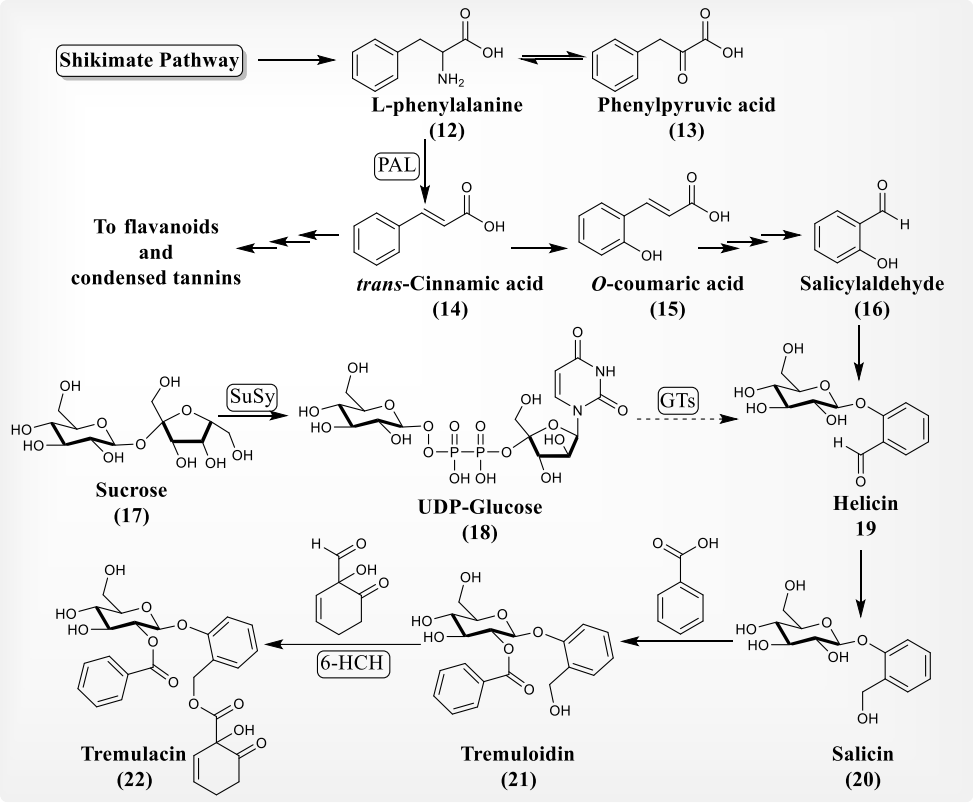
4-Hydroxytremulacin (11)

Figure 4: 4-Hydroxytremulacin (**11**), from the twigs of *D. abyssinica*
Source: Rasmussen *et al*. (2006)

2.5.4 Biosynthesis of Tremulacin

Chedgy (2015) offered a detailed method that shows the biosynthetic pathway to tremulacin. He highlighted that the main distinction between his model and the earlier proposed one, Babst et al. (2010) model, was the use of salicylaldehyde from *O*-coumaric to synthesize Helicin (Scheme 2) from Uridine diphosphate glucose (UDP-Glucose).

It is apparent that the involvement of GTs in this pathway is due to the influence of carbohydrates, which form a better part of primary metabolites in organisms. Thus, the more available carbohydrates are, the more PGs are produced. According to Koch (2004), the hydrolysis of a simple sugar like sucrose yields an important intermediate that influences the production of various forms of PGs before ultimately yielding tremulacin. Chedgy (2015) saw it plausible that tremuloidin (**4**) is generated by adding a benzoic acid group to salicin (**1**). Tremulacin (**4**) is the product of tremuloidin (**3**) plus a 6-hydroxy-2-cyclohexen-on-oyl (6-HCH) moiety. It was important to revisit the biosynthesis of **4** as a significant similarity exists in the benzoyl moieties of **4**, **23**, and **41**.



Scheme 2: A proposed biosynthesis of tremulacin

Source: Babs *et al*. (2010) and Chedgy (2015)

2.5.5 A Phenylpropanoid from *D. abyssinica*

The isolation of trans-2-[3-*O*-Acetyl-4-*O*-[(*E*)-4-hydroxycinnamoyl]-β-D-glucopyranosyloxy]cyclohexanol, compound **23**, from the leaves of *D. abyssinica* was realised by Rasmussen et al. (2006) as a novel compound (Figure 5). Its identification was aided by a spectroscopic comparison of data with related structures bearing the *p*-hydroxycinnamoyl or caffeoyl moiety in the 2-position of the glucoside and with a *cis*-1,2-cyclohexanediol moiety.

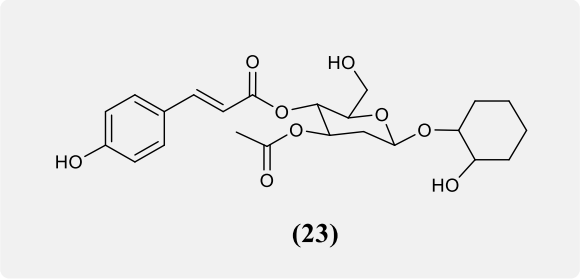


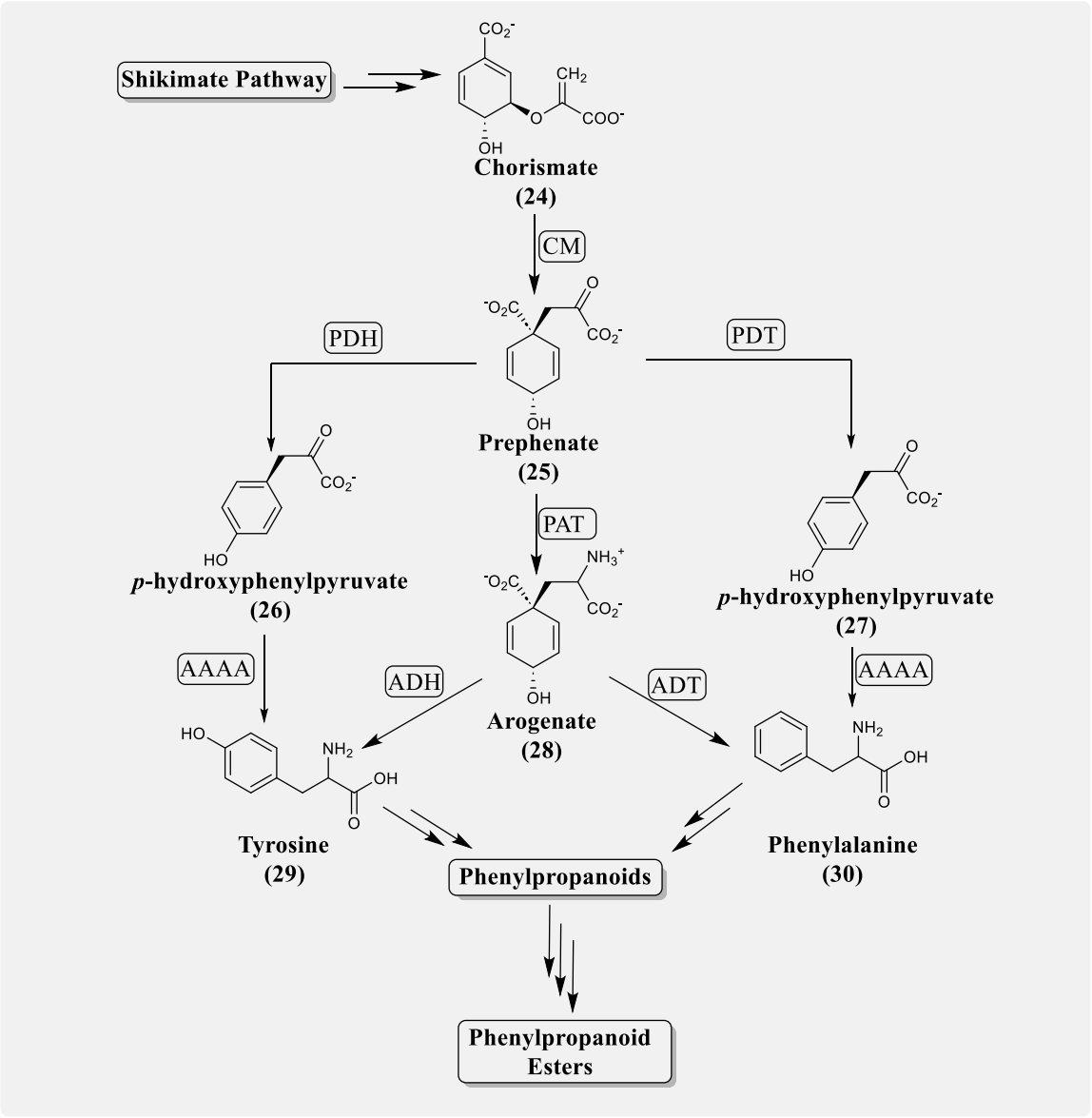
Figure 5: A phenylpropanoid glucoside from *D. abyssinica*
Source: Rasmussen *et al*. (2006)

2.5.6 Biosynthesis of Phenylpropanoids

Phenylpropanoid biosynthesis, as proposed by Tzin *et al*. (2012), is initiated in the shikimate pathway with the formation of chorismate as the core skeletal unit prior to its conversion to prephenate via the enzyme chorismite mutase (CM). Most pathways then pass through the p-Coumaroyl-CoA directly to phenylpropanoid esters or through the prephenate route by forming more diverse intermediates. Prephenate dehydratase (PDH) catalyzes the conversion of prephenate to phenylpyruvate and, ultimately, phenylalanine via aromatic amino acid aminotransferase (AAAA).

The route from prephenate to phenylalanine could also be achieved via arogenate catalysed by prephenate aminotransferase (PAT) and arogenate dehydratase (ADT). Tyrosine is also included as a key intermediate in the biosynthesis of phenylpropanoids (Tzin *et al.*, 2012). It is realised via the arogenate route catalysed by arogenate dehydrogenase (ADH) or via the *p*-hydroxyphenylpyruvate catalysed by prephenate dehydrogenase (PDH) and subsequently AAAA (Scheme 3).

The phenylpropanoid biosynthetic pathway shown here only briefly illustrates the diversified metabolism for this group of compounds. The intermediates of the shikimate pathway serve as the fundamental building block for the vast array of secondary metabolites produced by the phenylpropanoid metabolism. A combination of reductases, oxygenases, and transferases amplifies the resulting esters in multiple cascades to create a pattern of metabolites unique to plant species and specific to their organs and developmental stages.

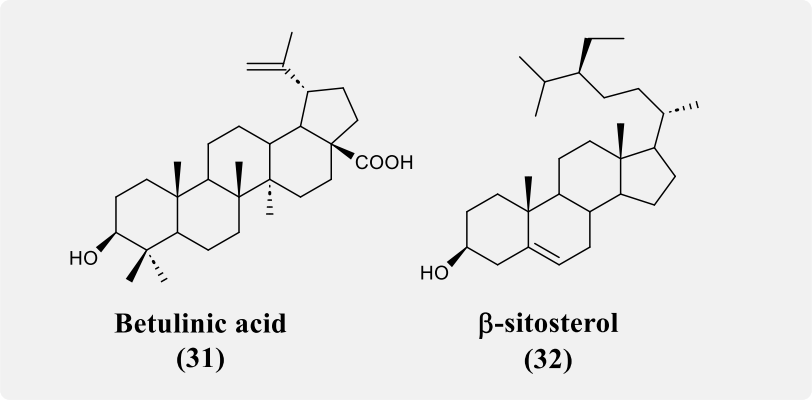


Scheme 3: A proposed biosynthetic pathway of phenylpropanoid esters

Source: Tzin *et al*. (2012)

2.5.7 Triterpenes from *D. abyssinica*

Fruits of *D. abyssinica* yielded betulinic acid and sitosterol (Figure 6) upon purification from repetitive column chromatography. Prior to the current study, only one research study had documented the isolation of betulinic acid from *D. abyssinica*. Chirchir *et al*. (2018) isolated three known compounds from *D. abyssinica*: betulinic acid, benzoic acid, and tremulacin (**22**). In their report, compounds were isolated and explicitly purified from the roots of the plant.

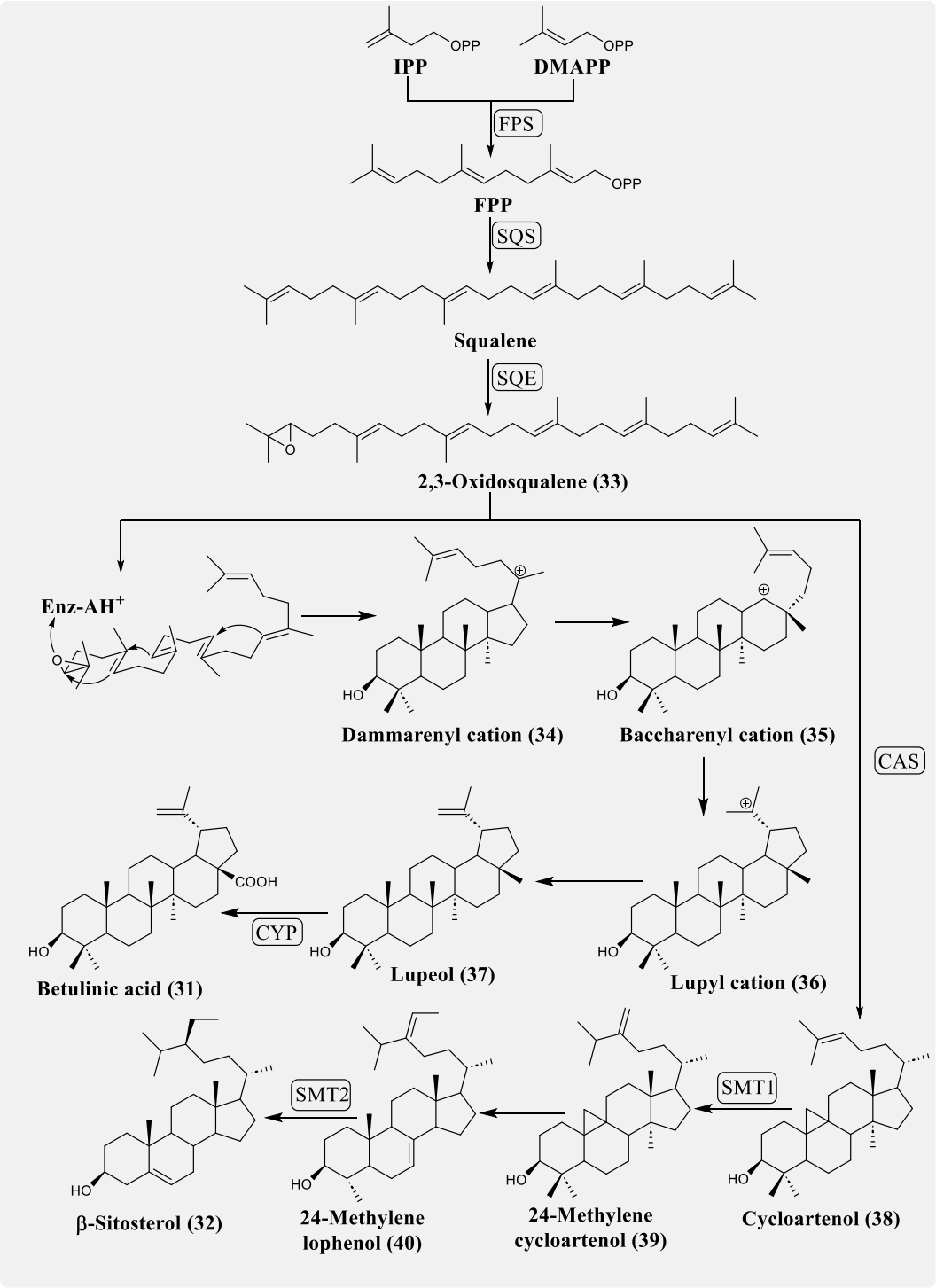


Betulinic acid (31) **β-sitosterol (32)**

Figure 6: **31** and **32** isolated from roots and fruits of *D. abyssinica*
Source: Chirchir *et al*. (2018)

2.5.8 Biosynthesis of Triterpenes

The biosynthesis of triterpenes follows the conventional mevalonic acid (MVA) pathway. The pathway starts with the condensation of DMAPP and IPP by FPS to form FPP. A skeleton structure that forms the framework for high molecular weight terpenoids. Squalene synthase (SQS) then attaches two FPP molecules to squalene (Scheme 4). The introduction of squalene to two units of FPP through oxidation yields a species that if key in forming desired terpenes (Thimmappa *et al.*, 2014). Oxidosqualene cyclases (OSCs) are responsible for catalysing its cyclization into different ring-system skeletons (Thimmappa *et al.*, 2014). As a result, OSCs have significant functions in the biosynthesis of triterpenoids. The triterpenoid precursor 2,3-oxidosqualene undergoes a cyclization and rearrangement process catalysed by lupeol synthase (LUS) to produce lupeol. Betulinic acid is achieved by the conversion of a methyl group into a carboxylic acid. By CYPs (Dewick, 2002; Thimmappa *et al.*, 2014). This conversion is informed by three critical cationic species as illustrated in Scheme 4. For the case of sitosterol, it uniquely takes a distinct route from other triterpenes through the oxidised squalene. The precursor is converted to cycloartenol via cycloartenol synthase (CAS), which is then converted to β-sitosterol via 24-methylene cycloartenol and 24-methylene lophenol aided by C24-methyltransferases 1 and 2 respectively (SMT1 and SMT2) as illustrated in Scheme 4.



Scheme 4: Biosynthesis of betulinic acid and β-sitosterol

Source: An *et al*. (2020) and Suo *et al*. (2019)

2.6 Biological Activities of *D. abyssinica*

Various medicinal purposes, such as antibacterial, antifungal, antitrypanosomal, antidiabetic, and wound healing properties of D. abyssinica, have been established from different reports. Antibacterial findings by Legesse *et al*. (2019) provided rational arguments for the use of *D. abyssinica* as folk medicine in African communities to treat bacterial-related infections. These findings concluded that the leaf extracts of *D. abyssinica* had substantial efficacy against some highly lethal bacteria. According to Geyid *et al*. (2005), the fungicidal activity of the plant was first realised when its polar leaf extracts were tested against six fungal strains. Additionally, Nibret and Wink (2011) confirmed its antitrypanosomal activity in selected leaf extracts of the plant. Here, it showed some potent ability to inhibit trypanosomal cell proliferation with significant selectivity indices for methylene chloride and methanol extracts. Kamau (2018) realized the hypoglycaemic activity of the aqueous root extracts of the plant and inferred that they are significantly vital in lowering blood glucose levels in mice through a dose-dependent design, thus proving antidiabetic properties. Less prominently, Abdissa (2011) provided some empirical arguments for the plant's wound healing properties. Findings from this study showed that the methanol fruit extract of the plant was capable of significantly accelerating contraction that fastened the wound healing process.

2.7 Free Radicals

Due to the extensive nature of free radicals, this study focused on ROS and RNS owing to their significant association with living organisms. These ROS and RNS with significant contributions to biological systems are shown in Table 2. Their detrimental effect goes beyond not only the destruction of cell organelles but also crippling important molecules (Nimse & Pal, 2015; Wu & Ng, 2008). The thermodynamic stability of radicals is based on their half-lives ($t_{½}$). A radical is described as stable when $t_{½}$ is greater than 10^{-3} seconds. On the other hand, a radical is considered unstable when $t_{½}$ is less than 10^{-3} seconds. Since the hydroxyl anion radical $(OH)^{-\cdot}$ has a half-life of 10^{-10} seconds (Table 2), it will be, for this study be, considered as one of the most potent radicals (Fossey *et al.*, 1995).

There is a complicated web of interactions between peroxisomes, enzyme reactions, and mitochondria that leads to the in vivo generation of free radicals. Although these ROS generators are crucial for cellular signaling, metabolism, and defense mechanisms, oxidative diseases, and

stress can result from their dysregulation. Research studies have been conducted to create targeted therapeutic strategies to reduce oxidative damage and keep the body's redox balance in check. This is made possible by understanding the mechanisms that govern free radical production and regulation.

Table 2: Half-life of selected radicals and non-radicals

Reactive oxygen species		Reactive nitrogen species	
Name (symbol))	Half-life (seconds)	Name (symbol)	Half-life (seconds)
Radicals		Radicals	
Superoxide anion radical ($O_2^{-\bullet}$)	10^{-6}	Nitric oxide ($NO^{\bullet}$)	1
Hydroxyl anion radical ($OH^{-\bullet}$)	10^{-10}	Nitrogen dioxide ($NO_2^{\bullet}$)	1
Alkoxyl radical ($RO^{\bullet}$)	10^{-6}	Non-radicals	
Peroxyl Radical ($ROO^{\bullet}$)	17	Peroxynitrite anion ($ONOO^-$)	10^{-3}
Non-radicals		Nitrosyl cation (NO^+)	1
Singlet oxygen ($^1O_2$)	10^{-6}	Nitroxyl anion (NO^-)	1
Ozone (O_3)	1	Dinitrogen trioxide (N_2O_3)	1
Hypochlorous acid (HOCl)	60	Dinitrogen tetraoxide (N_2O_4)	1
Hypobromous acid (HOBr)	60	Nitrous acid (HNO_2)	1
		Peroxynitrous acid (ONOOH)	1.7
		Nitryl chloride $ClNO_2$	1

2.7.1 Mitochondrial Electron Transport Chain and Free Radical Production

For cells to generate energy, the mitochondria must carry out oxidative phosphorylation. Unfortunately, ROS are undesirable by-products of this process. ROS can serve as signaling molecules or exacerbate oxidative stress in unhealthy states (Matsuzaki *et al.*, 2009; Nolfi-Donegan *et al.*, 2020). Leaks of electrons from the energy conveyer belt in the mitochondria are also implicated in the production of species that can react with oxygen to create these highly reactive species (Matsuzaki *et al.*, 2009; Sandalio & Romero-Puertas, 2015). The bifurcated role of mitochondria in cellular homeostasis and oxidative damage, and understanding the relationship between oxidative phosphorylation and ROS production, is crucial for this theme.

Empirical research has revealed that the electrons transferred in the mitochondria have been implicated as the leading factor involved in the complicated process of regulating ROS. The intricacy of the functioning of the mitochondria and the dynamic nature of their associated pathways and cycles are key. Both pathological and physiological processes involve the electron transport chain-controlled production of free radicals; for example, in ischemic preconditioning, controlled ROS production can protect against subsequent ischemic injury (Matsuzaki *et al.*, 2009).

2.7.2 Peroxisomes: ROS Production and Regulation

A shift in perspective has revealed peroxisomes as active organelles contributing to the synthesis of these damaging species. Rather than static H_2O_2 sinks. These organelles produce a lot of unstable species, which can signal at low concentrations or cause oxidative stress when they build up too much. Metabolic pathways like photorespiration are associated with ROS production in peroxisomes, and disruptions to these pathways trigger cellular defense responses. To keep cellular redox balance, peroxisomal homeostasis is essential since ROS and redox changes in peroxisomes set off particular reactions to environmental signals. Research has shown that peroxisomes have enzymatic defenses that neutralise ROS, such as superoxide dismutases. It is crucial to keep the redox balance within peroxisomes because scavenging systems tightly control the regulation of ROS levels. In addition to maintaining cellular redox balance, peroxisomes are critical decision-making platforms where ROS and RNS impact signalling networks (Sandalio & Romero-Puertas, 2015).

2.7.3 Enzymatic Reactions and Free Radical Production

Enzymatic reactions form a major part of ROS production due to their association with rapid redox reactions. Various enzymatic activities within the anabolic and catabolic processes produce these species (Abd-Elmaksoud *et al.,* 2015). Previously, Lobo *et al*. (2010) had argued that these highly reactive species have the potential to harm vital biomolecules such as primary and secondary metabolites, which can then disturb the balance and function of cells.

2.8 Natural Antioxidants

Antioxidants from natural sources provide a sustainable and healthy alternative in combating ROS. Their intake is maximized from the consumption of the Mediterranean diet, which maximizes the consumption of vegetables, fish, whole grains, and fruits. In addition, medicinal herbs complement the effect of this diet when physical illness sets in. In a bid to fill the gap in understanding the fate of ROS, it was essential to study the literature explaining the mechanisms around them and natural antioxidants. This was done by exploring literature that proposes plausible mechanisms of isolated compounds in scavenging free radicals. This is in pursuit of their potential health benefits and developing effective antioxidant remedies.

2.8.1 Ascorbic Acid

As a foundational species in the antioxidant world, it serves as both a template for many mechanisms of action in quenching ROS. Through its unique chemical structure, as shown in Figure 7, ascorbic acid (**41**) can effectively neutralize free radicals. It exhibits an α-β unsaturation that stabilizes the unpaired electron after donating one to an unstable ROS (Charlton *et al.*, 2023). Its sp^2 conjugation forms a basis of explanation of many mechanisms involving antioxidants and free radicals preventing the formation of more reactive species and breaking the radical propagation chain (Charlton *et al.*, 2023; Lobo *et al.*, 2010).

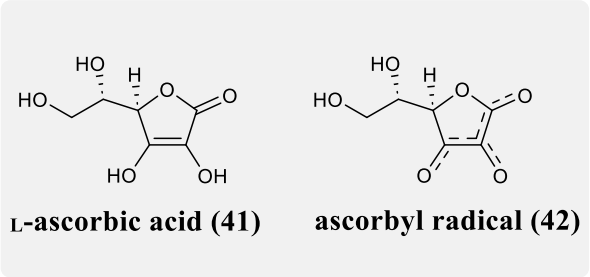
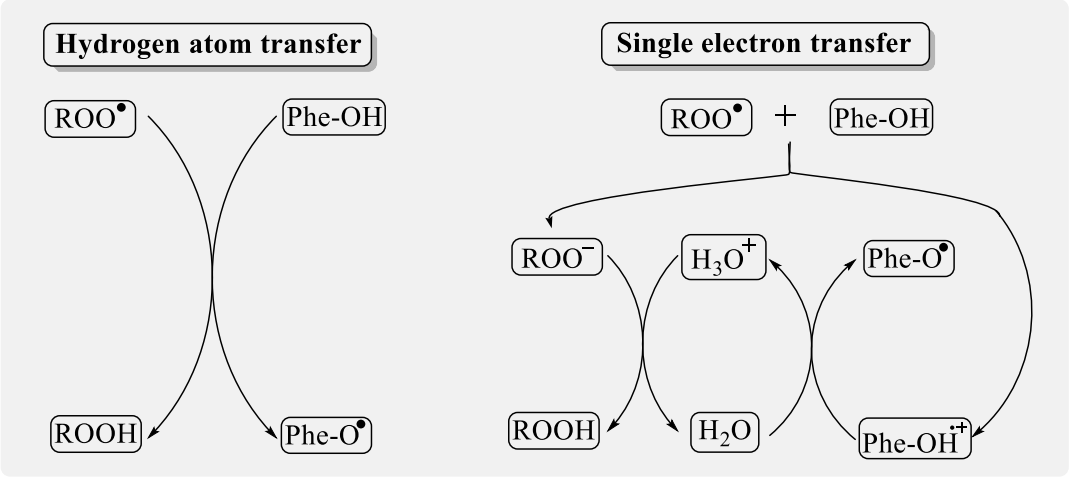


Figure 7: Structures of ascorbic acid (**41**) and the ascorbyl radical (**42**)

2.8.2 Phenolic Antioxidants: Hydrogen Atom Transfer and Electron Transfer

Building on the mechanistic concept of ascorbic acid, other species like the phenols arise mimicking this same mechanism of donating a proton and stabilizing their unpaired electron through conjugation. Their mechanisms have, however, been advanced through two bifurcated pathways as shown in Scheme 5.



Scheme 5: Hydrogen atom transfer and/or single electron transfer quenching mechanisms

On one hand, HAT mechanism has phenolic antioxidants (Phe-OH) oxidizing to a stable phenoxyl radical while reducing a ROS. The stability of the new species is determined by the conjugation of the phenyl ring system, preventing the formation of more reactive species. The SET mechanism involves the transfer of an electron from the phenolic antioxidant to the free radical, forming a radical cation. This process is influenced by the ionization potential of the phenolic

compound and the reduction potential of the free radical. The resulting phenoxyl radical can be further stabilized through resonance delocalization within the aromatic ring (Jaganjac *et al.*, 2021).

Natural antioxidants, such as the ones described in sections **2.5.1** and **2.5.2**, play a crucial role in scavenging free radicals and reactive oxygen species. These antioxidants can neutralize free radicals through various mechanisms, including hydrogen atom transfer and single electron transfer. One of the isolated compounds, 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol (compound **41**), is an anydroglucitol with two phenol substituents at positions 3 and 4 of the D-glucitol skeleton. These substituents might be responsible for the portrayed radical scavenging capacity by the methanol extract from fruits of *D. abyssinica*. Its mechanism of scavenging the DPPH radical has been proposed under Chapter 5.

2.9 Antioxidant Assays

Assays for total antioxidant capacity (TAC) quantify the combined impact of chemical components in a sample. Most laboratory probes are in the form of processed or organic foods or samples extracted from plants (during assay-guided separations). For this reason, the most commonly used chemical antioxidant assays to quantify TAC are the cupric reducing antioxidant capacity (CUPRAC) assay (Apak *et al.*, 2008), the trolox equivalent antioxidant capacity based on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (TEAC/ABTS) (Re *et al.*, 1999), the oxygen radical antioxidant capacity (ORAC) (Huang *et al.*, 2005), the ferric reducing antioxidant power (FRAP) test (Benzie & Strain, 1996), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand-Williams *et al.*, 1995). There are other tests, like the ferrous oxidation-xylenol orange (FOX) assay, the ferric thiocyanate (FTC) assay, and the aldehyde/carboxylic acid (ACA) assay (Huang *et al.*, 2005), that are similar to the TAC test but not used as often. The list continues to expand as existing methodologies undergo enhancements to align with their associated hypotheses, ensuring sustainability relevance, reducing experimental time, and satisfying financial limitations.

There is a wealth of up-to-date published research on TAC evaluation that compares in vitro and simulated in vivo studies. The study on the chemistry behind antioxidant assays by Huang *et al*. (2005) thoroughly assessed the mechanisms and dynamics of the processes under investigation. They argued that in vitro TAC analysis bears no similarity to biological systems and emphasised the need to incorporate in vivo TAC testing in future research studies. López-Alarcón and Denicola (2013) also emphasised a similar opinion, pointing out that exaggerating, unscientific, and out-of-

context conclusions that the antioxidant potency of samples should not be based on these in vitro assays. The option of cell culture assays (López-Alarcón & Denicola, 2013) was presented and labeled "very attractive" as intermediates for testing methods to replace in vitro laboratory practices. A similar review (Karadag *et al.*, 2009) was done and explained that when choosing the TAC method, the analysis conditions, substrate concentration, and antioxidant concentration should be as close as possible to real food or biological systems. The nature of antioxidants is sometimes multifaceted, and so are these TAC assays. We should not rely solely on in vitro quantification to confirm its universal applicability. Additional in vivo tests should consolidate their validation.

A number of studies have provided commentary on the development of TAC assays, as well as their applications, advantages, and limitations based on their different chemical mechanisms. A study on the reaction mechanisms behind the ABTS, ORAC, FRAP, and DPPH antioxidant tests (Gulcin, 2020) was critically evaluated. In addition to these mechanistic principles, Gulcin (2020) highlighted that the reaction medium was of great influence in determining the potency of a sample and recommended standardised protocols for TAC of common laboratory samples. There are different ways to test the chelating power of antioxidants (Ivanova *et al.*, 2020). Ivanova *et al*. (2020) didn't include methods that use electron and hydrogen atom transfer, which is something that most current research doesn't go into much detail about. In the same report by Apak (2019), they discussed more than just the applicability and limitations of standard TAC. They also discussed the reaction kinetics and thermodynamics of the currently used analytical methods, the physicochemical aspects of antioxidant action, and measuring techniques.

Furthermore, we conducted a comprehensive examination of the mechanisms, benefits, and drawbacks of several TAC assays, as supported by references (Moon & Shibamoto, 2009). This report by Moon and Shibamoto (2009) discussed an in-depth analysis of DPPH, FRAP, ABTS, FOC, ACA, and FTC, as well as the underlying mechanisms and their use in different plant and food samples. Journals associated with food sciences (Schaich *et al.*, 2015) lead the line in extensively evaluating ABTS, DPPH, and ORAC assays. Schaich *et al*. (2015) carefully looked at the conceptual and technical flaws in the three commonly used assays that make them less useful and less accurate. They identified two significant modifications required for all three assays. Schaich *et al*. (2015) first proposed redirecting ABTS and DPPH assays to distinguish electron transfer reaction mechanisms and discontinuing their use in measuring radical quenching,

secondly, by refocusing the ORAC test on differentiating substances that neutralise radicals by the transfer of hydrogen atoms.

Several previously published studies have examined the TAC of a variety of samples. This has been the subject of many previously published studies in this field. However, more work remains to refine the methodologies underlying these assays. Perhaps we should strive to establish a shared method that integrates comparable chemical mechanistic techniques, both *in-vitro* and *in-vivo*, and suitable terminology that aligns with the assay's methodology.

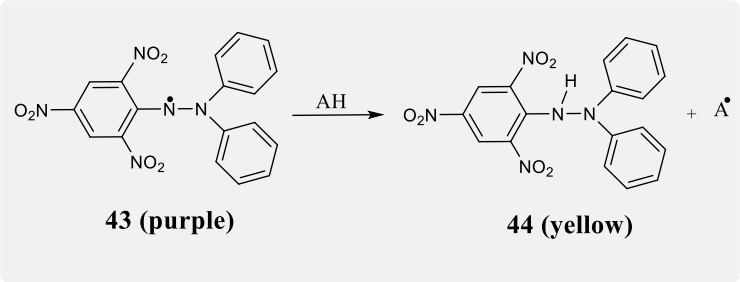
2.10 Antioxidant Assays and Efficacies

It is important to bear in mind the underlying factors when settling for an assay to use in the laboratory. The context and resource constraints should be a key consideration in selecting assays. For these reasons, different research probes settle for different assays. As seen from the findings by Shah and Modi (2015), the results from multiple assays may not necessarily be the same. This difference is due to the different chemical compositions of different antioxidants used, the reaction medium, and the physical parameters used. Apart from the DPPH assay, the other widely used radicals include the Oxygen Radical Absorbance Capacity (ORAC), Crocin Bleaching Assay (CBA), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay (ABTS), and Ferric Reducing Antioxidant Power (FRAP) assay.

Despite the ORAC assay showing more advantages over the ABTS and DPPH assays, it poses a critical limitation in the assumption of temperature control, oxygen concentration, and concentration of reagents (Prior *et al.*, 2003), a fall-back which is immensely covered up in the DPPH assay. Procedural limitations engulf the crocin assay, as many researchers report a dilemma when trying to integrate the preheating phase in their reactions. Additionally, the crocin assay presents its results with the assumption of linearity in reaction modes, an assumption that is quite erroneous (Prieto *et al.*, 2015). The only significant drawback of the ABTS assay is its inefficiency in rapidly getting to the initial result after initiating the experiment. Its preparation is relatively time-intensive compared to other assays. However, it is suitable for use in both polar and non-polar samples (Magalhães *et al.*, 2008).

2.11 DPPH Assay (2, 2-diphenyl-1-picrylhydrazyl assay)

This assay achieves its objective through the use of a mimic radical, DPPH, and the ability of test samples to stabilize it. The mimic radical features an unpaired electron that is stabilized in the conventional conjugated ring system. The fluorescence of the molecule is such that the unpaired electron is responsible for the absorption and re-emission of photons to give a characteristic purple colour as depicted in Scheme 6. When the electron is paired with another electron during stabilization, a characteristic yellow colour is observed. The intensity of the colour change depends on how many electrons are donated by the test sample to the DPPH molecules. Spectrophotometrically, the colour changes occur at a wavelength of about 520 nanometres.



Scheme 6: DPPH radical being scavenged by an antioxidant

It is a peculiar stable free radical that has found a wide variety of applications in the food industry due to its compatibility with the organic nature of many food samples (Huang *et al.*, 2005). Its usage in testing natural products for their antioxidant activity remains vast (Mau *et al.*, 2004; Shah & Modi, 2015; Sreeramulu & Raghunath, 2010). Its general simplicity in usage makes it suitable for most researchers who opt for a less resource-intensive and rapid assay. Additionally, as described by Shah (2015), this DPPH assay has reported high reproducibility, giving it an edge in perfection. However, it is unreliable for testing protein-based samples as its reaction media, either methanol or ethanol, precipitates them (Magalhães *et al.*, 2008). For these reasons, the scavenging capacity assay was chosen for this research study.

2.12 Separation Techniques of Natural Products

Regardless of the routes taken in separating metabolites embedded in the plant parts, there must be extraction and subsequent purification of fractions. As initial separation involves

extraction, there have been advancements in incorporating efficiency while minimising resources used. Studies in the subsequent purification of fractions are likewise focusing on achieving efficiency. (Qilong *et al.*, 2013).

Extraction of natural substances using conventional solvents such as water has been a popular method for more than 2000 years. Maceration (batch single pot extraction) (Gasik *et al.*, 2008), percolation (Blumberg *et al.*, 2010), and counter-current extraction are commonly used solvent extraction methods in natural products (Kassing *et al.*, 2010). The multi-stage counter-current extraction (MCE) technique has attracted much attention due to its high efficiency. This study focused on maceration as the primary extraction technique. The separation and purification of crude extracts and their consequent fractions in this research study will be centered on column and thin-layer chromatography.

2.13 Structure Elucidation

Both chemical and spectroscopic information are needed when elucidating a chemical structure. There has been a continued adoption of various spectroscopic tools in the determination of purified compounds. However, most research studies employ the use of an integrated approach by combining the inferences of various outcomes in spectroscopy (Stroobant, 2007). Furthermore, novel compounds require the need for absolute configuration for full characterisation. Linington *et al*. (2019) expressed this opinion of the need for absolute configuration in unknown compounds through the use of CD and X-ray crystallography. Nuclear magnetic resonance was mainly employed for the structure elucidation of purified fractions from *D. abyssinica*.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Sample Collection and Preparation

About 10 kg of ripe fruits of an identified *D. abyssinica* plant were collected from Egerton University - Njoro Botanical Garden in Nakuru County at 0° 22' 16.104" S, 35° 56' 15.612" E (Figure 8, Scribble Maps, 2024). The fruits were then transported to the Chemistry Laboratory, Egerton University, where they were stored at room temperature, awaiting further analysis.

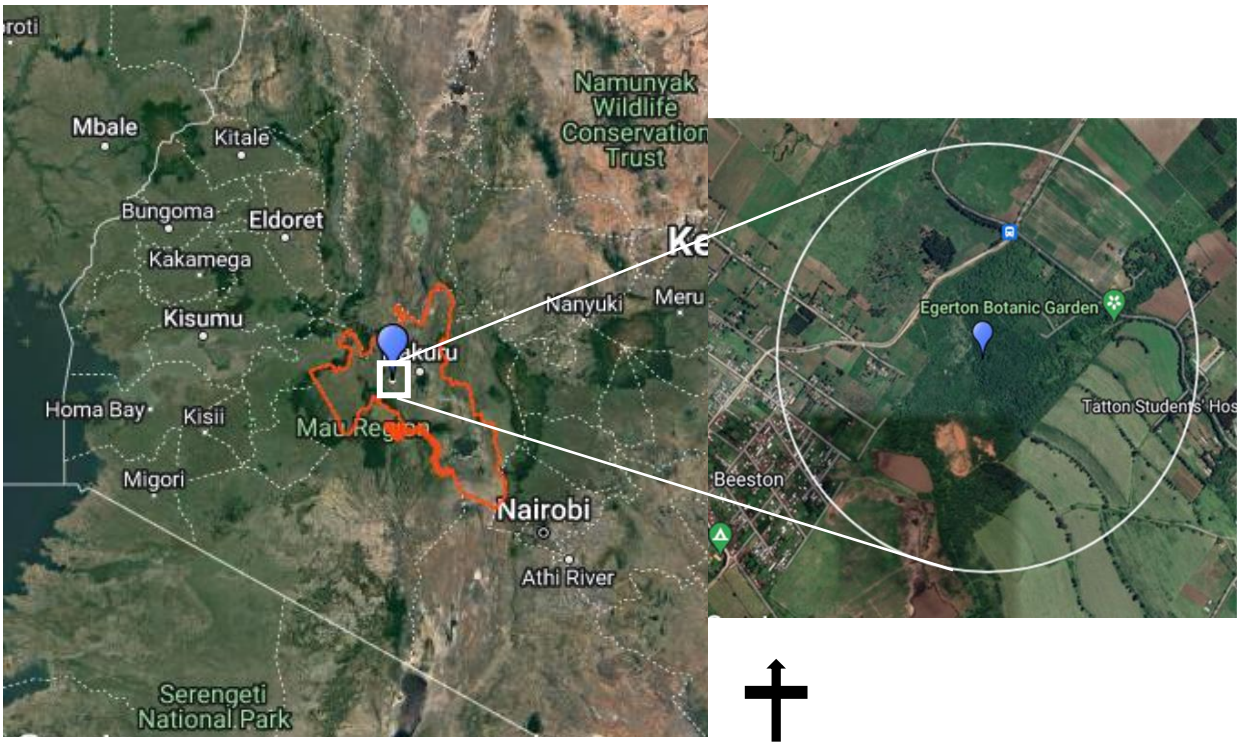


Figure 8: Sampling area in Nakuru County, Kenya

A modified method was adopted for the fruit selection process. This method entailed the identification of mature branches before flowering and marking using identifiable ropes. The marked branches were observed closely before, during, and after flowering. Fruits that developed on the identified branches were then selected in terms of size (2.0 to 2.5 centimeters), weight (7.0 to 7.5 grams), and color (red-brown) for further processing (Cavalcante & Martins, 2005). The selected fruits were washed to remove adhering particles and debris. The cleaned fruits were weighed using an analytical balance to obtain the gross weight and blended into a slurry mixture

using a blender (Waring commercial heavy-duty blender, Model Number 24CB10C, Serial Number 563972).

3.2 Preparation of Crude Extracts

The fruit pulp was freeze-dried, ground into a fine powder before being dried under shade, and placed into a constant weight. Maceration was carried out on the powdered fruit material using a slightly modified Kupchan partitioning method (Emran *et al.*, 2015). It was first soaked in hexane for 72 hours at room temperature with frequent shaking. The solvent-containing extract was then decanted and filtered through cotton wool to remove coarse particles before filtering through a filter paper (Whatman No. 1) to obtain crude hexane extract. The solute (in this case, fruit pulp powder) was left to stand in a fume hood to dry out any remaining solvent after decantation and filtration. Serial extraction on the dry powder was followed by methylene chloride, ethyl acetate, and methanol to increase the polarity of the solvents, each for 72 hours, with frequent shaking and filtration at every step. This process was done three times on each solvent to ensure maximum extraction. Using a rotary evaporator, the solutions containing crude extracts were then consolidated and concentrated under reduced pressure to a constant weight. The concentrated crude extracts were allowed to dry to a constant weight at room temperature.

3.3 Total Phenolic Content of the Crude Extracts

The quantification of phenolic compounds in each crude extract was determined using the Folin-Ciocalteu assay (Tibuhwa, 2014). Every 0.1 g of the four crude extracts was mixed with 5 mL of methanol. Subsequently, 200 µL of the diluted extract was transferred into a test tube and thoroughly mixed with 1 mL of Folin-Ciocalteu reagent (FCR; its preparation is explicitly described in section 3.4).

After 3 minutes, a volume of 0.8 mL of sodium carbonate with a concentration of 7.5% (w/v) was introduced into the mixtures. The solutions were stirred for 30 minutes in the dark and then centrifuged at 3300 revolutions per minute for 5 minutes. The absorbance of the supernatants and the prepared blank (section 3.4) were measured using a real-time probed λ_{max} of FCR in section 3.5.

This procedure was repeated four times for each extract, and the mean absorbances were used to determine the phenolic content present in each case. A calibration curve was generated

using OriginPro 2019 software (version 9.6.5.169) by utilising the concentrations of gallic acid as the standard. The results were quantified as Gallic Acid Equivalent (GAE) per 100 grammes of crude extract by calculations from the generated curve.

3.4 Supplementary Reagents for Total Phenolic Content

A 2 mL volume of Folin-Ciocalteu was measured into a beaker, and distilled water was added for a 10-times dilution. A blank consisted of 5 mL FCR, 1 mL methanol, and 4 mL Na_2CO_3 solution. Subsequently, preparation of 7.5% Na_2CO_3 solution was prepared by dissolving 7.5 g of Na_2CO_3 in a 100 mL volumetric flask, and a small amount of distilled water was added to it and shaken to dissolve Na_2CO_3; the volume was made up to the mark by adding distilled water.

The standard gallic acid solution was prepared by dissolving 1 mg of gallic acid into 1 mL of distilled water, so the solution concentration was 1 mg/mL (1000 μg/mL). This stock solution was diluted serially to obtain differently concentrated solutions (125.0 μg/mL, 62.5 μg/mL, 31.3 μg/mL, 15.6 μg/mL, 7.8 μg/mL, and 3.9 μg/mL) of gallic acid.

3.5 Wavelength Scans of FCR and DPPH Solution

The wavelength of maximum absorbances for FCR and DPPH solutions prepared were determined during this study. These scans were done to avoid reliance on literature values and use λ_{max} values relating to the instrument's calibration conditions and ambient temperatures in an effort to minimise analytical measurement errors.

Their wavelength scans were performed to their equivalent analogues (FCR and DPPH in methanol) with methanol as a blank using a Shimadzu UV-1800 UV-Vis-spectrophotometer with a wavelength range of 200 nm to 1100 nm. The scan results were represented as graphs of absorbance units versus wavelength in nanometres in OriginPro 2019 software (version 9.6.5.169).

3.6 Radical Scavenging Capacity of the Crude Extracts

The DPPH assay was performed according to the procedures described previously in the literature (Pal *et al.*, 2010; Tibuhwa, 2014). A solution of DPPH radical was prepared by dissolving 0.01 grams of DPPH in 250 mL of methanol. This solution was covered and kept dark after its λ_{max} was determined, as elaborated in section 3.5. A series of dilutions of the crude extracts were prepared to obtain 7 aliquots (0.39 μg/mL, 0.78 μg/mL, 1.56 μg/mL. 3.13 μg/mL, 6.25 μg/mL,

12.25 μg/mL, and 25 μg/mL). A 5 mL volume of each aliquot from a crude extract was mixed with an equal volume of the freshly prepared DPPH-methanol solution. The mixtures were shaken vigorously and left to stand for 30 min in the dark at ambient temperatures. The absorbance of the resultant solution was measured at the earlier probed DPPH solution λ_{max} under section 3.5. This procedure was repeated four times for each extract. The percentage of DPPH radical scavenging capacity of each extract was calculated using equation (1) (Hussein *et al.*, 2015). L-ascorbic acid was used as a standard, and its scavenging activity was determined prior to those of the extracts.

$$Scavenging\ Capacity\ (\%) = \frac{A_o-(A_1-A_s)}{A_o} \times 100\% \qquad (1)$$

Scavenging capacities versus concentrations were visualised using dose-response curves. The scavenging capacities of the crude extracts were compared to that of L-ascorbic acid using the IC_{50} parameter. The IC_{50} values were calculated using regression equations of mean scavenging capacities and extracts' concentrations.

3.7 Separation and Purification of the Crude Extracts

In order to separate the crude extracts into enriched fractions and purified compounds, the crude extracts were subjected to selected chromatographic techniques. Column chromatography was used to separate the crude extracts into enriched fractions. Out of the four extracts probed for their antioxidant capacities, only ethyl acetate and methanol extract were selected for further separation and purification based on their relatively high phenolic content and antioxidant capacities. A silica gel adsorbed methanol extract was introduced into a vertically mounted glass column. The column was eluted using gradient elution starting from 70:30 hexane : methylene chloride and increasing proportions of methylene chloride until 100% while reducing proportions of hexane until 0%. The methylene chloride/ethyl acetate (80:20%) mobile phase was introduced thereafter, up to 100% ethyl acetate. Lastly, 90:10% ethyl acetate : methanol up to 60% methanol. Fractions were collected at least in 10 mL intervals. The same procedure was repeated for the ethyl acetate extract.

Identical fractions informed by TLC elution patterns were pooled together and underwent further column chromatography separation. Isocratic elution was used on subsequent collected fractions. Thin layer chromatography TLC was used to determine the elution pattern or purity of the eluent fractions. The development of TLC plates was done inside a chromatographic tank. Pure fractions were subjected to preparative thin-layer chromatography, and the silica gel-adhered

compounds were scraped off and rinsed off with highly polar solvent systems. They were then left to dry at room temperature and stored between 0 ℃ and 4 ℃, awaiting NMR analysis.

3.8 Data Analyses

There was a total of four replicates of the procedures measuring total phenolic content and DPPH radical scavenging capacities. The data were processed using RStudio version 2023.06.0 build 421 (Posit software, 2022); Posit Software, PBC open-source software company (Boston, MA, USA), and the results were presented as means ± standard deviations. Tukey's honest significant difference (Tukey'sHSD) tests were used to determine the significance of the difference between means at a 95% confidence level, precisely by knowing exactly where those differences lie after subjecting the mean values of each extract to a one-way analysis of variance (ANOVA). Bartlett's, Levene's, and Fligner-Killeen's tests were carried out to determine the homogeneity of variances of the means statistically. The normality of populations was also factored in and tested using the Shapiro-Wilk normality test. The means scavenging capacities of the four extracts and the control were subjected to a two-way ANOVA to determine their level of interaction.

Two assumptions of ANOVA needed clarification before conducting the analysis. Firstly, the assumption is that population variances must be equal (i.e., homoscedastic). Or, there is homogeneity of variance – the deviation of means (measured by the range or standard deviation in this study) was similar between populations. This assumption was statistically tested using the p-values of Barlett's, Levene's, or Fligner-Killeen's tests. Levene's and Fligner-Killeen's tests analysed means of scavenging capacities from methanol and hexane extracts due to their deviation from a normal distribution.

Interpolating from linear regression plot lines of mean scavenging capacities versus concentration of the extracts allowed us to obtain the IC_{50} values, which are the effective concentrations at which DPPH radicals were scavenged by 50%. Pearson correlation coefficients determined the correlation of phenolic content in crude extracts to radical scavenging capacity.

3.9 Structure Determination of the Isolated Antioxidant Compounds

The purified compounds had their most likely structures inferred using nuclear magnetic resonance (NMR) spectroscopy. We used a Bruker AVANCE nuclear magnetic resonance spectrometer operated at 400 MHz to acquire 1-D and 2-D NMR spectra. We recorded spectra at

room temperature after dissolving the compounds in deuterated solvents. Two compounds were dissolved in deuterated chloroform with solvent signals δ_H = 7.26 ppm and δ_C = 77.23 ppm as the reference peaks, and one polar compound was dissolved in deuterated methanol with solvent signals δ_H = 3.35 ppm, δ_H = 4.87 ppm, and δ_C = 49.1 ppm as the reference peaks.

Structure elucidation was followed systematically to propose the purified compounds' chemical structures. Both Proton and Carbon NMR and their correlations were used. HSQC was used to determine which hydrogen atoms are bonded to which carbon atoms, correlation in terms of which hydrogen atoms are adjacent to each other was determined by COSY, and HMBC was used to explore the distance in terms of bonds between hydrogen and carbon atoms (De Graaf, 2019).

CHAPTER FOUR

RESULTS

4.1 Yield of Crude Extracts

A total weight of 5,751.33 g of fruits was harvested and freeze-dried to a constant weight of 2,058.60 g (Figure 9).

Figure 9: Freeze-dried *D. abyssinica* fruits ground into fine powder

Four crude extracts were successfully prepared from the freeze-dried *D. abyssinica*; hexane, methylene chloride, ethyl acetate, and methanol extracts. Their respective weights and percentage extracts are shown in Table 3. The methanol extract was the highest among all extracts' weights, at 6.01 %. A percentage that was calculated from the gross weight of the harvested fruits.

Table 3: Crude extract yield for extracts from *D. abyssinica*

Extract	Yield (g)	Percentage yield (%)
Hexane	88.36	1.5
Methylene chloride	105.31	1.83
Ethyl acetate	237.68	4.13
Methanol	345.81	6.01

4.2 Total Phenolic Content Results

4.2.1 Gallic Acid Calibration Curve

Firstly, a calibration curve of absorbance versus gallic acid concentration was drawn from the mean absorbance data of four replicates Table 4. Each mean was presented with its respective standard deviation.

Table 4: Absorbance data for the five concentrations of gallic acid

Concentration	Absorbance				
	1st Run	2nd Run	3rd Run	4th Run	Mean ± SD
3.907 µg/mL	0.007	0.006	0.005	0.005	$0.00575 \pm 8.291 \times 10^{-4}$ **a**
7.813 µg/mL	0.023	0.021	0.023	0.022	$0.0222 \pm 8.291 \times 10^{-4}$ **b**
15.625 µg/mL	0.092	0.093	0.091	0.095	$0.0928 \pm 1.479 \times 10^{-3}$ **c**
31.250 µg/mL	0.155	0.157	0.156	0.155	$0.1558 \pm 8.291 \times 10^{-4}$ **d**
62.500 µg/mL	0.269	0.265	0.263	0.264	$0.2653 \pm 2.278 \times 10^{-3}$ **e**
125.000 µg/mL	0.538	0.541	0.569	0.529	$0.5443 \pm 1.496 \times 10^{-2}$ **f**

Notes: SD = standard deviation (n=4), means in columns with the same superscript letter are not significantly different (at 95% confidence level, Tukey's test).

In the calibration curve of Figure 10, each data point was fitted with standard error bars calculated from standard deviations of the means from Table 4.

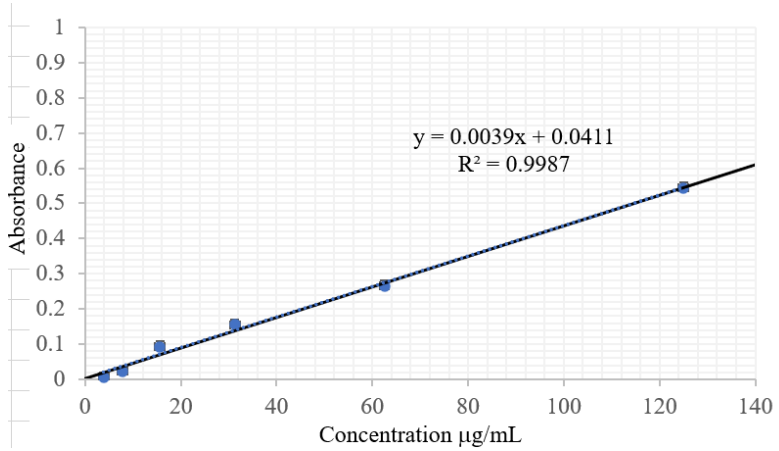


Figure 10: Absorbance of gallic acid versus concentration

4.2.2 Total Phenolic Content of the Extracts

The total phenolic content of the extracts was expressed as gallic acid equivalent (GAE) per 100 grammes of crude extract. This calibration curve was used to calculate TPC for the four extracts. The quadruplicate results for the TPC of the four extracts are shown in Table 5, accompanied by their means with standard deviation.

Table 5: Total phenolic content of crude extracts from *D. abyssinica* fruit pulp

	Total phenolic content (mg GAE/100g)				
Extract	1st Run	2nd Run	3rd Run	4th Run	Means ± SD
Methanol	908.97	921.79	921.79	934.62	$921.79 \pm 1.63 \times 10^{-3}$ **a**
Ethyl acetate	537.18	537.18	498.72	498.72	$517.95 \pm 1.41 \times 10^{-3}$ **b**
Methylene chloride	280.77	280.77	242.31	242.31	$261.54 \pm 1.00 \times 10^{-4}$ **c**
Hexane	24.36	24.36	24.36	24.36	$24.36 \pm 8.20 \times 10^{-4}$ **d**

Notes: SD = standard deviation (n=4), means in columns with the same superscript letter are not significantly different (at 95% confidence level, Tukey's test).

A bar plot was also developed to visualise the total phenolic content of the four extracts as illustrated in Figure 11. Methanol extract reported the highest TPC compared to the other three extracts which satisfies the rationale that most phenolic compounds are polar.

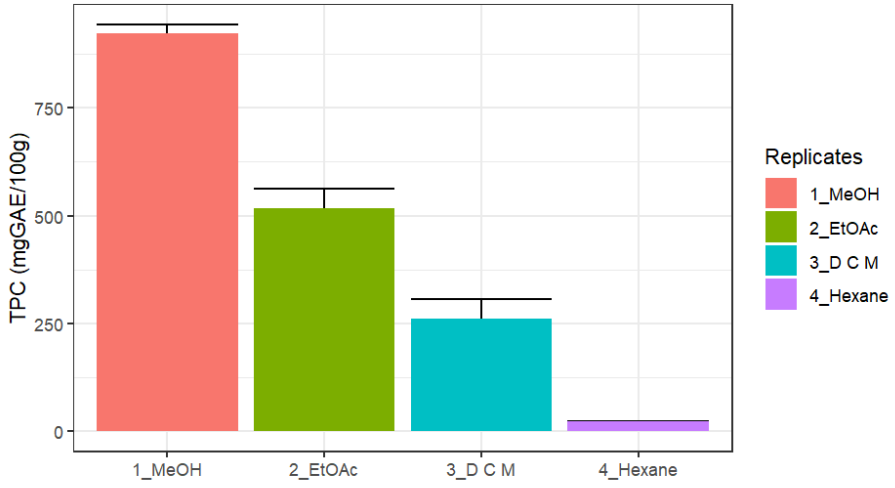


Figure 11: A bar plot of the total phenolic content of the four extracts

4.3 Wavelength Scans of FCR and DPPH Solution Results

After immediate preparations of FCR and DPPH solutions, their wavelength scans were run, and their λ_{max} was determined as 740 nm and 516 nm, respectively, as shown in Figure 12. This was done to avoid reliance on literature values and use independent λ_{max} values relating to the instrument's calibration conditions and ambient temperatures to minimise instrumental errors (a determinate type of error).

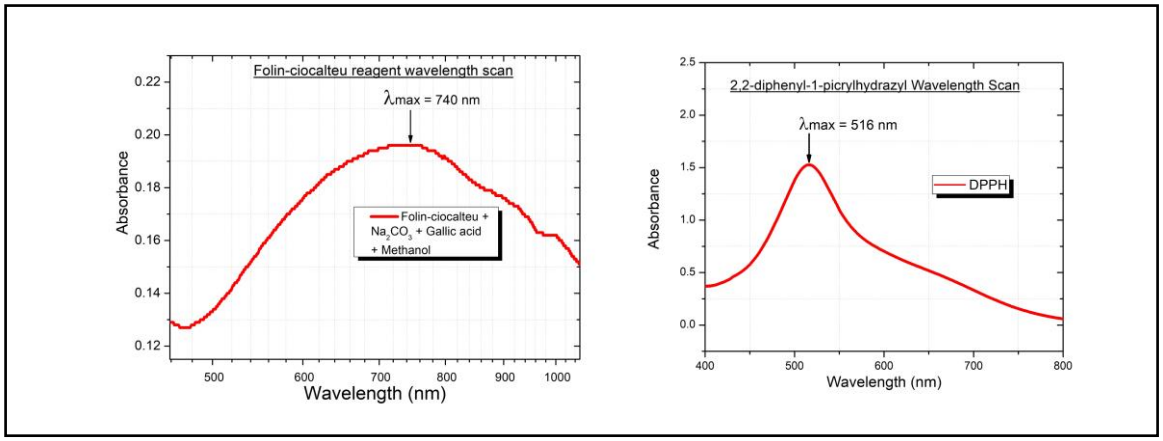


Figure 12: Wavelength scans of FCR and DPPH solution

4.4 DPPH Radical Scavenging Capacity of the Crude Extracts

Dose-response curves were used to visualise the relationship between scavenging capacities and concentrations (Figure 13). The scavenging capacities of the crude extracts were evaluated and compared to that of L-ascorbic acid using the IC_{50} parameter. The IC_{50} values (Table 6) were calculated using regression equations of mean scavenging capacities and extracts' concentrations.

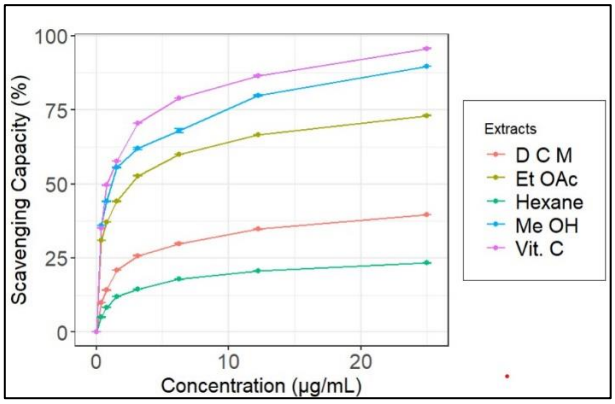


Figure 13: Dose-response curve of extracts from *D. abyssinica* fruit pulp

From these extracts, data on IC_{50} showed a significant antioxidant capacity in the methanol and ethyl acetate extracts (4.4 μg/mL and 8.4 μg/mL, respectively) than those of methylene chloride and hexane extracts (28.8μg/mL and 55.8 μg/mL respectively). For this reason, the two later extracts were dropped in pursuit of isolation and purification of compounds (a bioassay-guided separation and purification approach).

Table 6: Scavenging capacity (%) and IC_{50} (μg/mL) experimental data

Concentration (μg/mL)	(A). L-ascorbic acid percentage scavenging capacity (positive control)					
	1st Run	2nd Run	3rd Run	4th Run	Means ± SD	IC_{50} (μg/mL)
0.39	35.17	35.36	35.17	34.84	35.13 ± 0.2172	
0.78	49.57	49.44	49.71	49.57	49.57 ± 0.1069	
1.56	57.69	57.56	57.76	57.76	57.69 ± 0.0926	
3.13	70.46	70.46	70.53	70.40	70.46 ± 0.0535	1.8492
6.25	79.04	78.78	78.91	78.98	78.93 ± 0.1118	
12.25	86.57	86.51	86.51	86.64	86.56 ± 0.0627	
25.00	95.68	95.74	95.81	95.61	95.71 ± 0.0845	
Concentration (μg/mL)	(B). Methanol extract percentage scavenging capacity					
	1st Run	2nd Run	3rd Run	4th Run	Means ± SD	IC_{50} (μg/mL)
0.39	35.82	35.82	36.08	35.95	35.92 ± 0.1254	
0.78	44.07	44.07	44.20	44.01	44.09 ± 0.0824	
1.56	55.47	55.66	55.60	55.40	55.53 ± 0.1196	
3.13	60.77	62.28	62.41	62.21	61.92 ± 0.7684	4.4037
6.25	70.01	67.39	67.26	67.26	67.98 ± 1.3548	
12.25	80.62	79.57	79.44	79.57	79.80 ± 0.5492	
25.00	89.59	89.59	89.65	89.59	89.60 ± 0.0327	
Concentration (μg/mL)	(C). ethyl acetate extract percentage scavenging capacity					
	1st Run	2nd Run	3rd Run	4th Run	Means ± SD	IC_{50} (μg/mL)

0.39	30.84	30.84	30.78	30.91	30.84 ± 0.0535	
0.78	37.00	37.07	37.00	37.00	37.02 ± 0.0327	
1.56	44.01	43.94	44.34	44.34	44.16 ± 0.2097	
3.13	52.78	52.78	52.72	52.59	52.72 ± 0.0926	8.4000
6.25	59.99	59.99	59.99	59.99	59.99 ± 0.0001	
12.25	66.54	66.54	66.60	66.60	66.57 ± 0.0378	
25.00	73.08	73.08	72.95	73.15	73.07 ± 0.0824	

Concentration	(D). methylene chloride extract percentage scavenging capacity					
(µg/mL)	1st Run	2nd Run	3rd Run	4th Run	Means ± SD	IC_{50} (µg/mL)
0.39	9.89	9.82	9.69	9.89	9.82 ± 0.0926	
0.78	14.15	14.15	14.08	14.15	14.13 ± 0.0327	
1.56	20.83	20.83	21.02	20.83	20.87 ± 0.0982	
3.13	25.74	25.93	25.67	25.74	25.77 ± 0.1134	28.7858
6.25	29.93	29.67	29.80	29.93	29.83 ± 0.1254	
12.25	34.84	34.51	34.71	34.77	34.71 ± 0.1415	
25.00	39.55	39.49	39.62	39.55	39.55 ± 0.0535	

Concentration	(E). hexane extract percentage scavenging capacity					
(µg/mL)	1st Run	2nd Run	3rd Run	4th Run	Means ± SD	IC_{50} (µg/mL)
0.39	5.17	5.04	5.17	4.72	5.03 ± 0.2164	
0.78	8.38	8.25	8.38	8.25	8.32 ± 0.0756	
1.56	11.92	11.85	12.05	11.98	11.95 ± 0.0845	
3.13	14.47	14.47	14.41	14.54	14.47 ± 0.0535	55.8263
6.25	17.88	17.62	17.88	17.81	17.80 ± 0.1240	
12.25	20.63	20.63	20.69	20.63	20.65 ± 0.0327	
25.00	23.38	23.18	23.25	23.38	23.30 ± 0.0982	

For better visualisation of data in Table 6, a bar plot (Figure 15) was developed to demonstrate the different concentrations for scavenging capacities for each extract.

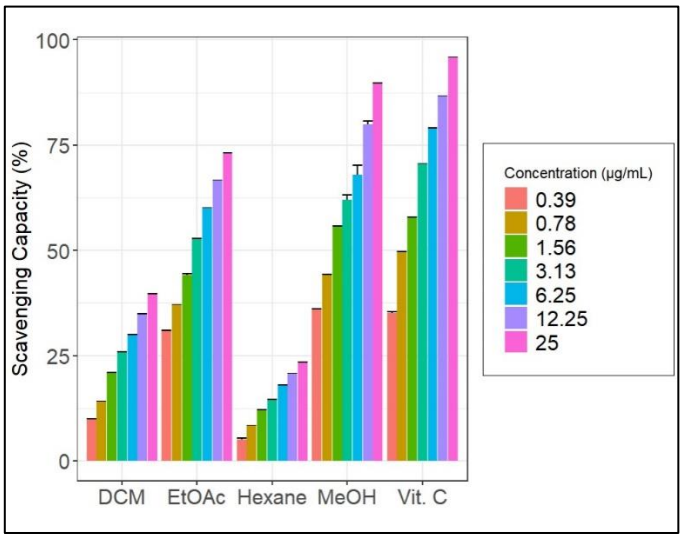
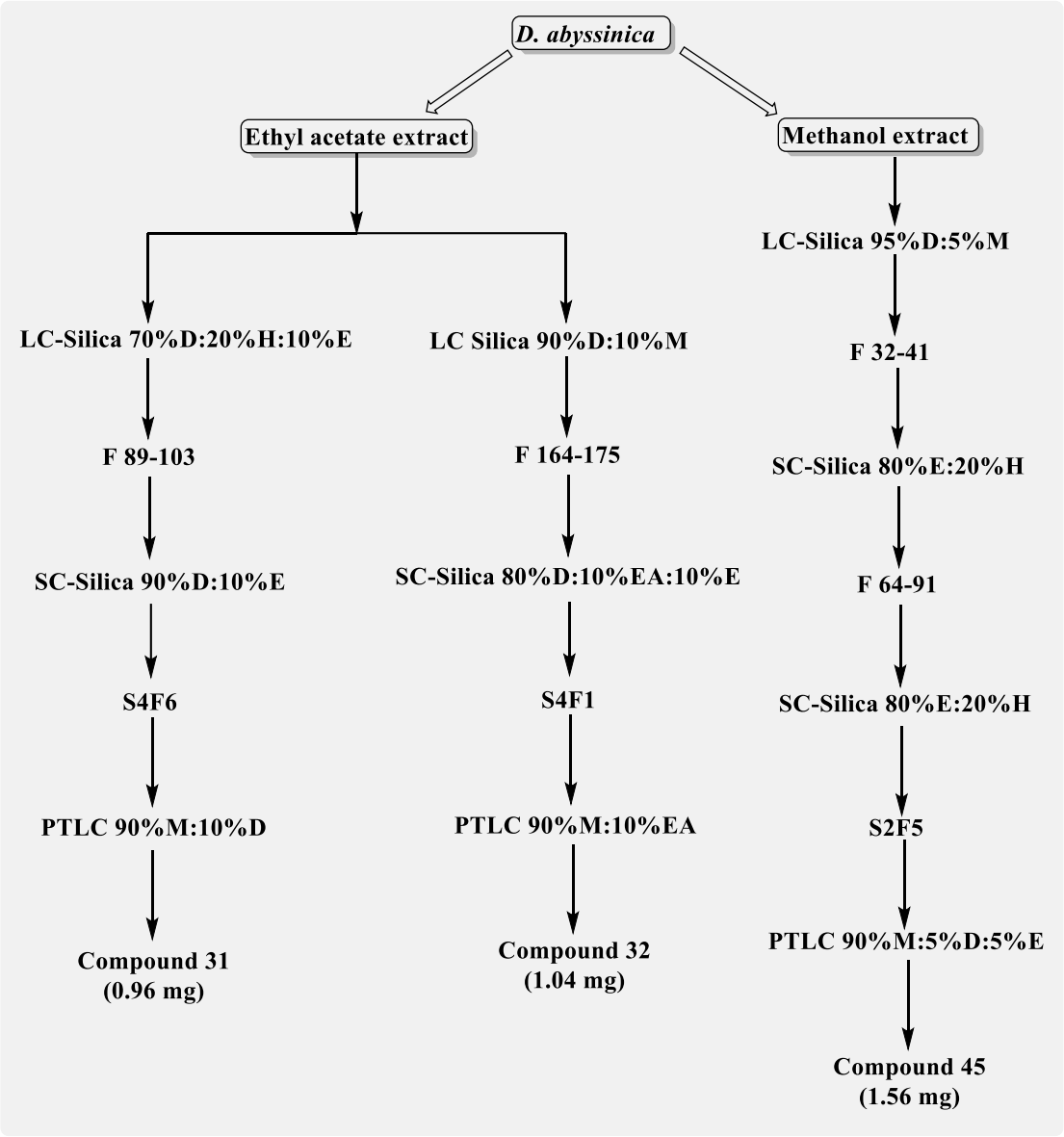


Figure 14: A bar plot of scavenging capacities of the four extracts

The results of IC_{50} showed that the methanol extract had a relatively comparable IC_{50} (4.4037 µg/mL) to the control (1.8492 µg/mL). A value that exceeded the extract with the least IC_{50} in this study (55.8 µg/mL).

4.5 Separation and Purification

The two most polar extracts registered interesting scavenging capacities of the DPPH radical and hence pursued their chemical compounds. The conventional column chromatography was employed to isolate the compounds from the two extracts. The solvent systems used for elution comprised different ratios of solvents: hexane (H), methylene chloride (D), ethyl acetate (EA), diethyl ether (E), and methanol (M). This afforded key fractions, S4F6, S4F1, and S2F5, which were further separated via PTLC to obtain purified compounds (Scheme 7). F, LC, SC, D, H, E, EA, M, and PTLC = fraction, large-column, small-column, methylene chloride, hexane, ethyl acetate, diethyl ether, methanol, and preparative TLC, respectively.



Scheme 7: Schematic illustration of realising compounds **31**, **32**, and **45**

4.6 Data Analyses

After TPC and scavenging capacity experiments, several data parameters were subjected to data analyses, from raw data to visualisation. A one-way analysis of variance (ANOVA) was followed by Tukey's honestly significant difference test (Tukey's HSD), which was performed at a 95% confidence level to determine the difference between means. Barlett's, Levene's, and Fligner-Killeen's tests were carried out where necessary to statistically determine the homogeneity of variances of the means. The normality of populations was also factored in and tested by

visualising using residuals versus fitted and quantile-quantile plots before using the Shapiro-Wilk normality tests.

4.6.1 Total Phenolic Content

The one-way ANOVA results for the quadruplicate TPC experiments of the four extracts revealed a p-value of 2.0×10^{-16}. This is a value of less than 0.05, indicating a significant difference in the means, thus rejecting the null hypothesis that the TPC means are equal and accepting the research or alternative hypothesis that the means are not all equal (Appendix 7.1). The adjusted p-values after the post-hoc test for all comparisons of the four extracts with each other were relatively close to zero (Appendix 7.1), thus removing any doubts about the interactions of these means. Figure 15 shows the homogeneity of variances and normality of the populations of the multiple phenolic contents. The Levene's test and Fligner-Killeen test for homogeneity of variances and Shapiro-Wilk normality test gave p-values of 1.276×10^{-5}, 0.003111, and 0.01516, respectively. Supporting outputs of R Studio for Levene's test, Fligner-Killeen test, and Shapiro's test for TPC experiments can be found in Appendix 7.1.

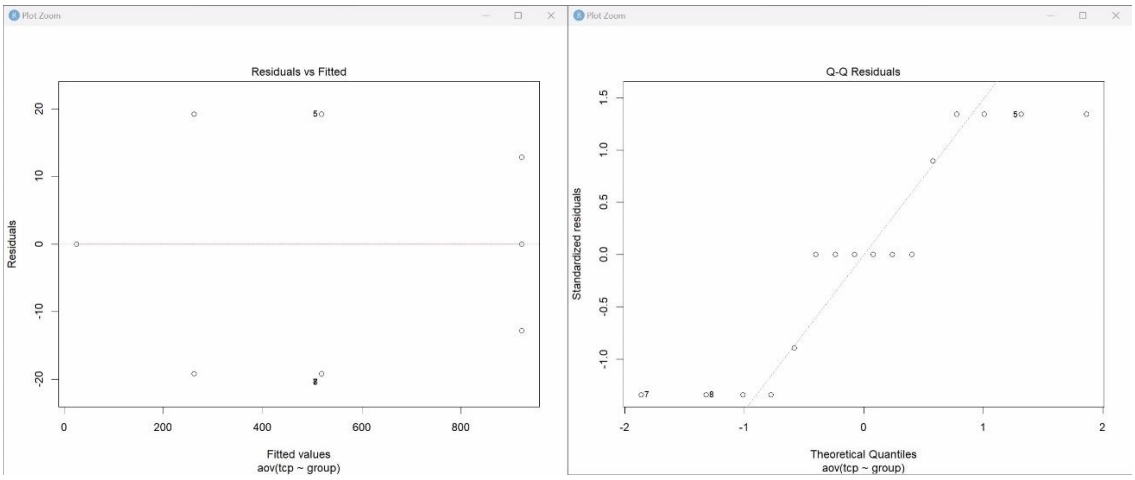


Figure 15: Residuals vs. Fitted and quantile-quantile plots from TCP experiments

4.6.2 DPPH Radical Scavenging Capacity of Extracts

One-way ANOVA was used to determine if there were statistically significant differences among the means of scavenging capacities from the different concentrations, individually across each extract, i.e., each extract's scavenging capacities means were analysed individually before

performing a two-way ANOVA on the combined dataset of concentration of extracts, scavenging capacities, and the type of extracts. The null hypothesis that the one-way ANOVA was testing was that the means of scavenging capacities are equal. Table 7 shows that the one-way ANOVA p-values are way less than α 0.05; this led us to reject the null hypothesis in each of the four extracts. Tukey'sHSD test for all comparisons of each of the four extracts with each other was relatively close to zero (Appendix 7.2), thus removing any doubts of equality of any pair of these means. The p-values of ANOVA, Barlett's tests, Levene's tests, Fligner-Killeen's tests for homogeneity of variances, and Shapiro-Wilk normality tests for scavenging capacities for the four extracts and the control are shown in Table 7. Additional outputs of R Studio for all these test parameters of the four extracts and the control can be found in Appendix 7.2.

Table 7: p-value from analysis of scavenging capacities

Analysis parameter	p-value for means of scavenging capacities (α = 0.05, n = 4)				
	Control (L-ascorbic acid)	MeOH extract	EtOAc extract	DCM extract	Hexane extract
ANOVA	2.0×10^{-16}	2.0×10^{-16}	2.0×10^{-16}	2.0×10^{-16}	2.0×10^{-16}
Bartlett's test	0.2805	N/A	0.6201	0.3947	N/A
Levene's test	N/A	0.6201	N/A	N/A	0.3496
Fligner-Killeen's test	N/A	0.3088	N/A	N/A	0.4762
Shapiro's test	0.3319	0.0004924	0.07174	0.7313	0.0396

Notes: Experiments that showed the non-normal distribution of data proceeded with Levene's and Fligner-Killeen's tests. Experiments that showed a normal distribution of data proceeded with Bartlett's tests only. N/A = not applicable.

Their Levene's p-values of 0.6201 and 0.3496 and Fligner-Killeen's p-values of 0.3088 and 0.4762 for methanol and hexane extracts, respectively, indicated that we had to accept the null hypothesis in this case that all population variances are equal – they were all greater than the α, 0.05. Ethyl acetate extract, methylene chloride extract, and L-ascorbic acid (control) means of scavenging capacities were normally distributed and hence followed Barlett's test of homogeneity

of variances. Their p-values, 0.6201, 0.3947, and 0.2805, respectively, indicated that we had to accept the null hypothesis in this case that all population variances are equal.

Secondly, the assumption was that ANOVA could only be conducted if the dependent variable (scavenging capacities) was normally distributed. This assumption was statistically tested using the Shapiro-Wilk normality tests. From the p-values (Table 7), it was inferred that only the methanol and hexane extract were non-parametric due to their non-normal distribution of populations, while the other three (ethyl acetate, methylene chloride, and the control) were normally distributed. The normality of residuals from the scavenging capacity experiments was visualised by generating residuals vs. fitted and quantile-quantile plots. These have been provided in Figures 16 to 20.

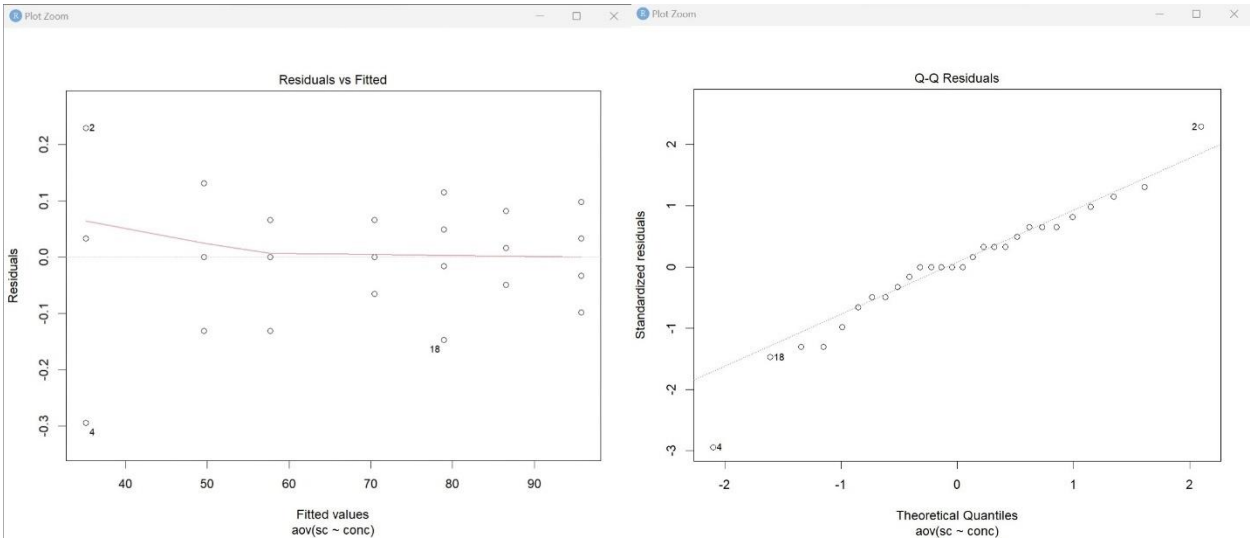


Figure 16: Residuals vs. Fitted and quantile-quantile plots for means of SC of L-ascorbic acid

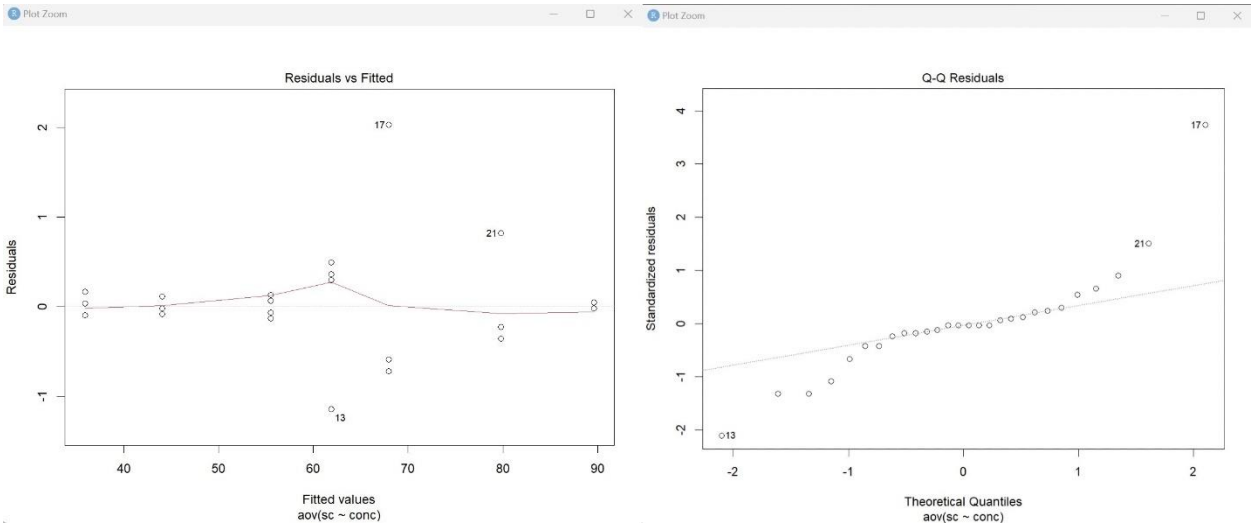


Figure 17: Residuals vs. Fitted and quantile-quantile plots for means of SC of MeOH extract

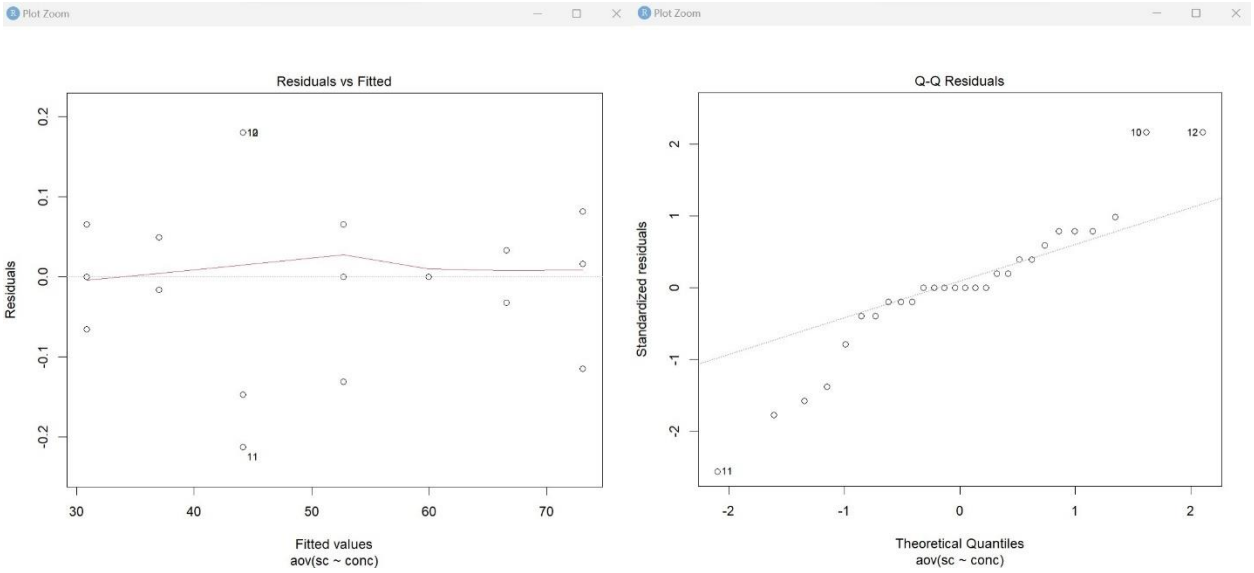


Figure 18: Residuals vs. Fitted and quantile-quantile plots for means of SC of ethyl acetate

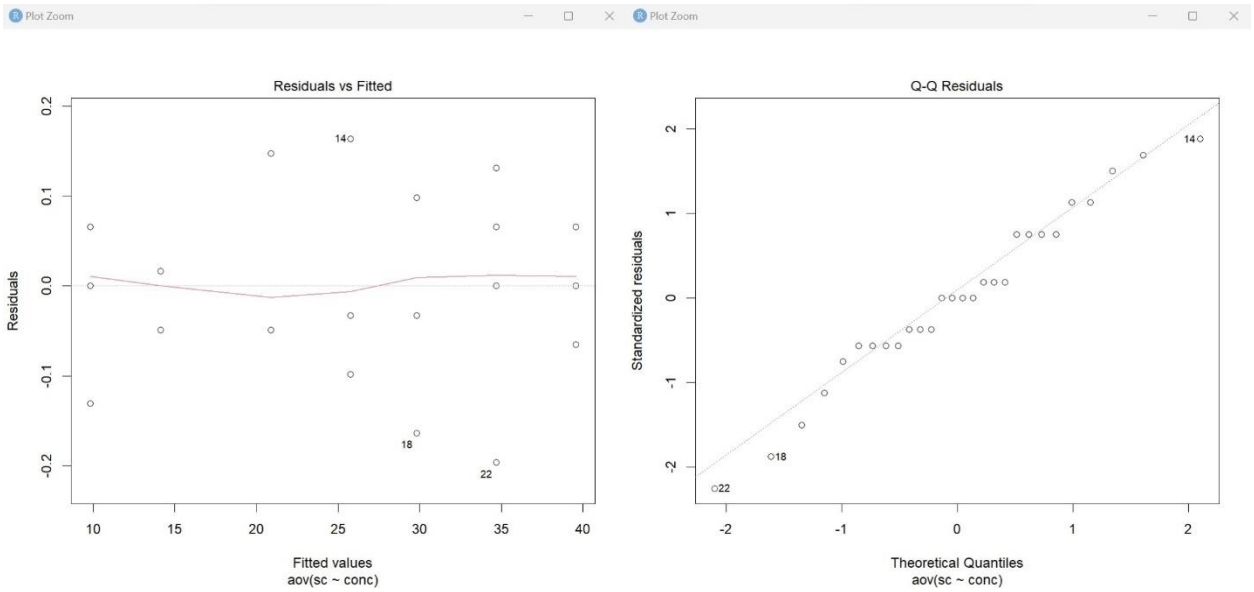


Figure 19: Residuals vs. Fitted and quantile-quantile plots for means of SC of CH_2Cl_2

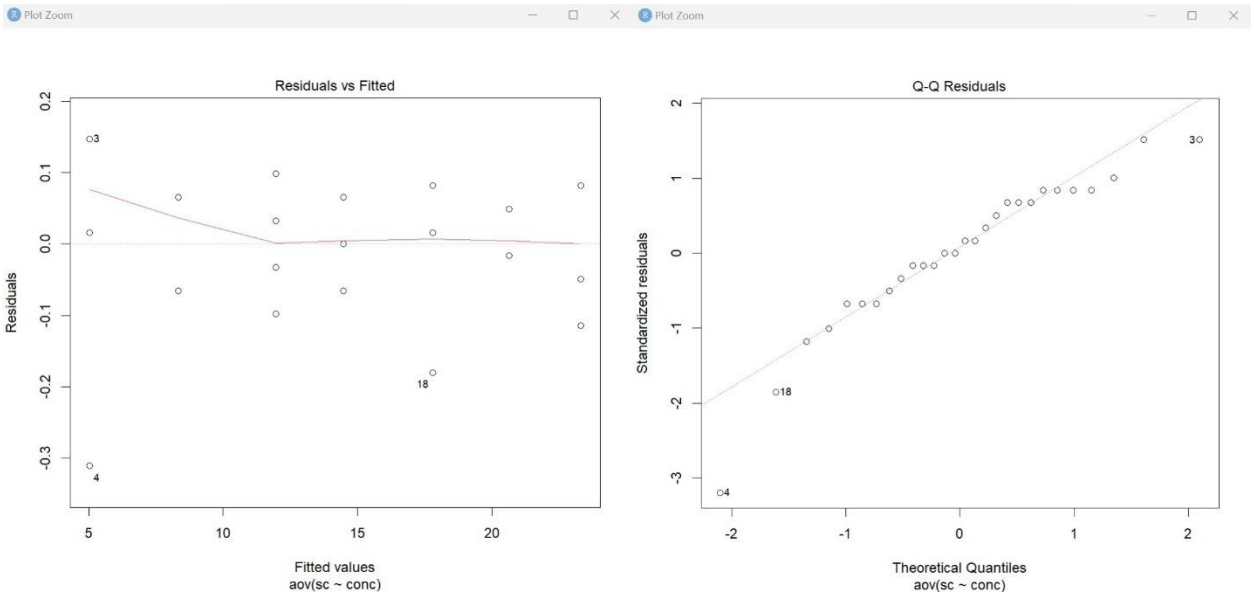


Figure 20: Residuals vs. Fitted and quantile-quantile plots for means of SC of hexane extract

Two-way ANOVA for the means of scavenging capacities of the four extracts and the control gave p-values less than 2.0×10^{-16} for all tested interactions of means. An explicit R Studio output of this two-way ANOVA can be found in Appendix 7.3. From the p-values of this analysis, we concluded that there was a significant difference among all extracts (p-value less than α 0.05), a significant difference among the concentrations (treatment factors) used (p-value less than α 0.05), and a significant difference between the interactions of extracts and their concentrations (p-value

less than α of 0.05). This led us to reject two hypotheses of this two-way ANOVA: the means scavenging capacities of extracts grouped by the seven concentrations are the same, and the means scavenging capacities of extracts grouped by the four extracts are the same. However, we failed to reject the third hypothesis that there is no interaction between the scavenging capacities of the four extracts and their respective seven concentrations. Correlation analysis of TPC and scavenging capacity results are illustrated in Figure 21. A Pearson correlation coefficient of 0.97 was realised. This was a strong positive correlation coefficient, which was relatively close to a positive one.

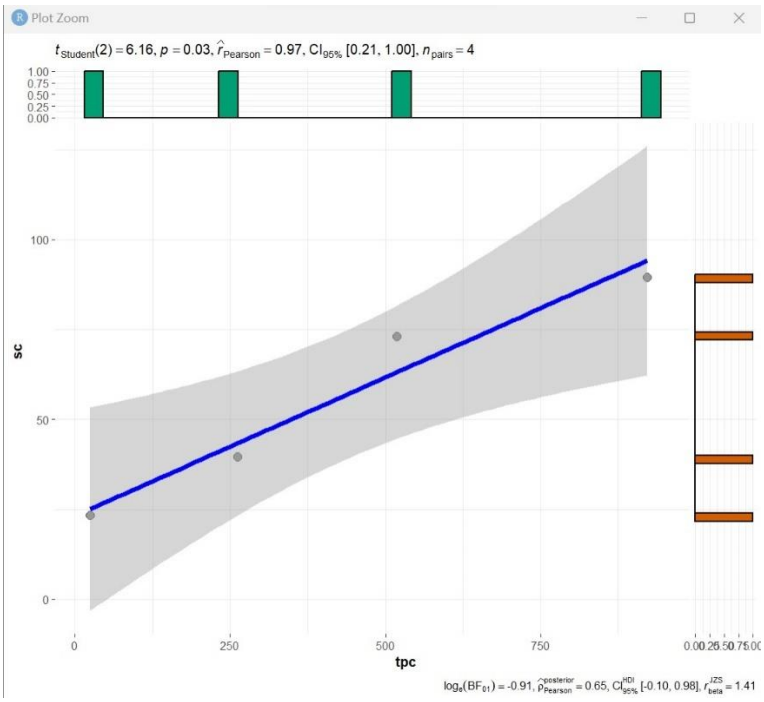


Figure 21: Correlation analysis of TPC and scavenging capacity

4.7 Structure Characterization of the Isolated Compounds from *D. abyssinica*

4.7.1 Betulinic Acid (Compound 31)

Its ^{13}C-NMR spectrum indicated 30 carbon signals: six methyls, eleven methylenes, six methines, and seven quaternary carbon signals. Its ^{1}H NMR spectrum revealed characteristic resonances for an isopropenyl group present in lupane pentacyclic triterpenoids, with the two methylene proton resonances at δ_H 4.74 (d, J = 2.0 Hz) and 4.61 (d, J = 2.0 Hz) ascribable to the two H-29 protons of betulinic acid. Moreover, the ^{1}H NMR spectrum showed an oxymethine proton resonance at δ_H 3.18 (dd, J = 11.2 Hz, 5.0 Hz H-3), which was seen to correspond to the

carbon resonance at δ_C 79.2 (CH, C-3) in the heteronuclear single quantum coherence coupled with distortionless enhancement by polarization transfer (HSQCDEPT) spectrum, Appendix 7.4.6. The ^{13}C NMR spectrum revealed a carbonyl due to a carbon resonance at δ_C 178.1 (C-28), which in heteronuclear multiple bond correlation (HMBC) spectroscopy (Appendix 7.4.7) correlated with δ_C 1.60 m (H-18) as shown in Figure 22. Additionally, Table 8 showed that the obtained data were consistent with the ^{1}H and ^{13}C NMR data from Sharma *et al*. (2010). Therefore, compound **31** (Figure 22) was assigned as betulinic acid.

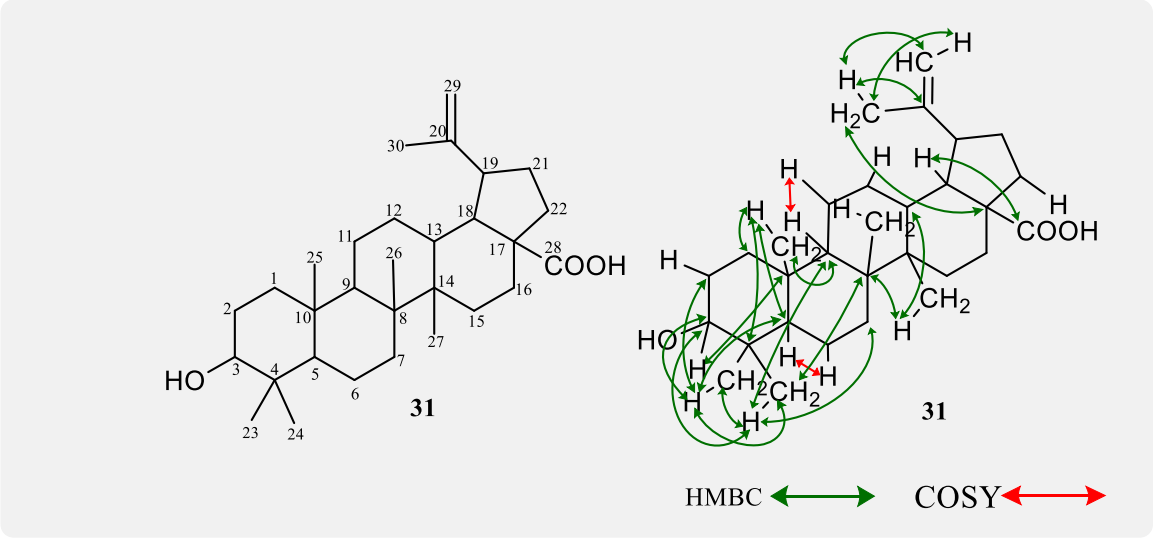


Figure 22: Betulinic acid and its HMBC and COSY correlations (compound **31**)

Table 8: ^{13}C and ^{1}H NMR spectral data of compound **31** and that of betulinic acid

Carbon	δ_C (ppm)	Type	δ_H (ppm) (multiplicity, *J*)	δ_C (ppm) (Sharma *et al.*, 2010)	δ_H (ppm) (multiplicity, *J*) (Sharma *et al.*, 2010)
1	38.9	CH_2		39.0	
2	28.1	CH_2		27.6	
3	79.2	CH	3.18 (dd, 11.2, 5.0)	78.2	3.28
4	39.0	C		39.1	
5	55.5	CH		55.5	
6	19.5	CH_2		18.4	
7	34.5	CH_2		34.5	
8	40.9	C		40.8	
9	50.7	CH		50.7	
10	37.4	C		37.3	
11	21.0	CH_2		21.0	
12	25.7	CH_2		25.7	
13	38.5	CH		38.1	
14	42.6	C		42.5	
15	30.7	CH_2		30.2	
16	32.3	CH_2		32.9	
17	47.0	C		47.1	
18	49.4	CH	1.60 (m)	48.1	
19	56.3	CH	2.25 (m)	49.2	2.29
20	150.5	C		150.1	
21	29.9	CH_2			
22	37.2	CH_2			
23	27.6	CH_3	0.94 (s)	27.9	0.91
24	15.5	CH_3	0.75 (s)	15.5	0.79
25	16.3	CH_3	0.82 (s)	16.4	0.84
26	18.4	CH_3	1.37 (o)	16.7	0.98

Carbon	δ_C (ppm)	Type	δ_H (ppm) (multiplicity, *J*)	δ_C (ppm) (Sharma *et al.*, 2010)	δ_H (ppm) (multiplicity, *J*) (Sharma *et al.*, 2010)
27	14.8	CH_3	0.97 (s)	15.0	0.99
28	178.0	C		180.3	
29	109.8	CH_2	4.74 (d, 2.0) 4.61 (d, 2.0)	108.9	4.78 4.65
30	19.5	CH_3	1.69 (s)	19.6	1.70

Notes: J (coupling constant in Hertz), Hz (Hertz), s (singlet), d (doublet), dd (doublet of doublets), o (overlap), and m (multiplet)

4.7.2 Sitosterol (Compound 32)

Its ^{1}H NMR showed the presence of six methyl proton signals, one olefinic proton, and a methylene proton. Furthermore, the ^{1}H NMR spectra of compound **32** revealed a proton corresponding to the proton associated with the C-3 hydroxy group (Figure 23), which appeared as a triplet of triplets. ^{13}C NMR confirmed twenty-nine distinct carbon signals. Prominently, an oxygenated carbon at δ_C 72.0 (CH, C-3), two olefinic carbon signals at δ_C 140.0 (CH, C-5) and 121.9 (CH, C-6), and six methyls at δ_C 19.2 (CH_3, C-26), 19.0 (CH_3, C-21), 12.2 (CH_3, C-18), and 12.1 (CH_3, C-29). It significantly lacked the olefinic shifts at C-22 characteristic of stigmasterol as shown with its correlations in Figure 23. Additionally, Table 9 showed that the obtained data were consistent with the ^{1}H and ^{13}C NMR data from Ododo *et al*. (2016). Therefore, compound **32** (Figure 23) was confirmed to be sitosterol.

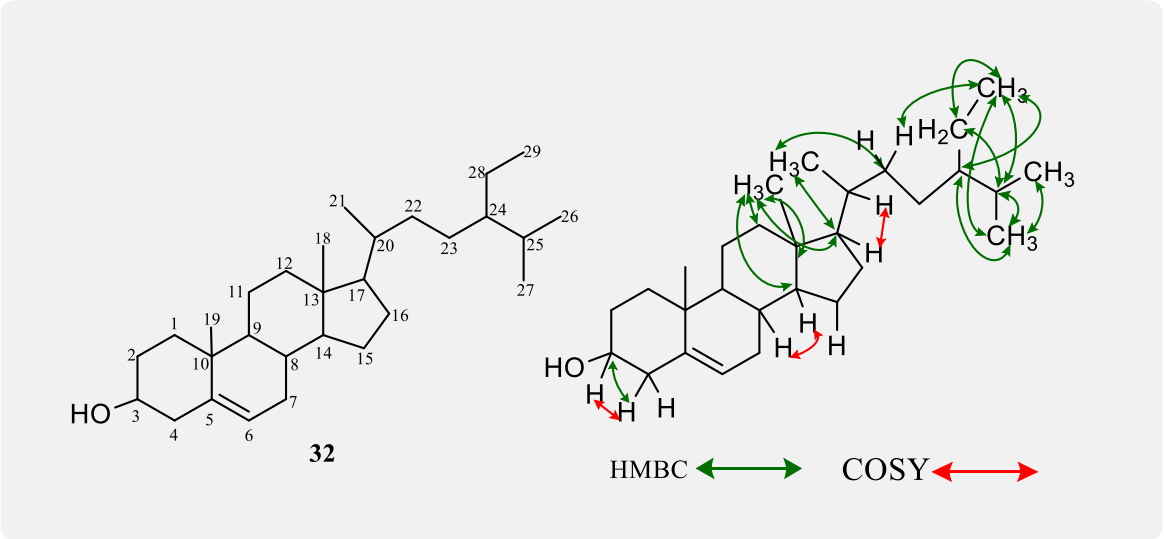


Figure 23: Sitosterol and its HMBC and COSY correlations (compound **32**)

Table 9: ^{13}C and ^{1}H NMR spectral data of compound **32** and that of sitosterol

Carbon	δ_C (ppm)	Type	δ_H (ppm), (multiplicity, *J*)	δ_C (ppm) (Ododo *et al.*, 2016)	δ_H (ppm), (multiplicity, *J*) (Ododo *et al.*, 2016)
1	37.5	CH_2		37.3	
2	31.9	CH_2		31.7	
3	72.0	CH	3.53 (tt, 11.12, 4.52)	71.8	3.54 (m)
4	42.5	CH_2	2.27 (m)	42.3	2.32 (m)
5	140.0	CH		140.8	
6	121.9	CH	5.35 (t, 5.30)	121.7	5.37 (o)
7	32.1	CH_2	2.00 (m)	31.9	2.04 (m)
8	32.1	CH	1.50 (m)	31.9	1.69 (m)
9	50.4	CH		50.2	
10	36.7	C		36.5	
11	21.3	CH_2	1.48 (m)	21.1	1.52 (m)
12	40.0	CH_2		39.8	
13	42.5	CH		42.3	
14	57.0	CH		56.8	
15	24.5	CH_2	1.56	24.3	1.58 (m)
16	28.4	CH_2	1.84 (m)	28.3	1.85 (m)
17	56.3	CH		56.1	
18	12.2	CH_3	0.68 (s)	11.9	0.70 (s)
19	20.0	CH_3	1.01 (s)	19.4	1.03 (s)
20	36.3	CH		36.2	
21	19.0	CH_3	0.92 (d, 6.55)	18.8	0.94 (o)
22	34.2	CH_2	1.01	34.0	0.93 (m)
23	26.3	CH_2	1.14 (m)	26.1	1.15 (m)
24	46.1	CH	1.01 (s)	45.9	1.38 (m)
25	29.4	CH	1.65 (m)	29.2	1.57 (m)

26	19.2	CH_3	0.81 (o)	19.8	0.84 (o)
27	19.6	CH_3	0.82 (d, 1.89)	19.1	0.86 (d)
28	23.3	CH_3	1.25 (m)	23.1	1.10 (m)
29	12.1	CH_3	0.84 (s)	12.0	0.82 (o)

Notes: J (coupling constant in Hertz), Hz (Hertz), s (singlet), d (doublet), t (triplet), tt (triplet of triplets), m (multiplet), and o (overlap)

4.7.3 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol (Compound 45)

The ^{1}H NMR data revealed the presence of eight aromatic protons as two pairs of doublets at δ_H 7.78 (2H, d, J = 8.8 Hz, H-2″), 7.75 (2H, d, J = 8.8 Hz, H-2′), and δ_H 6.72 (2H, d, J = 8.7 Hz, H-3″), 6.72 (2H, d, J = 8.7 Hz, H-3′), which showed correlations in the correlation spectroscopy (COSY) spectrum (Appendix 7.6.4), indicating the presence of two *p*-substituted benzene moieties. The eight proton resonances at δ_H 5.18 (1H, t, J = 9.7 Hz, H-4), 4.08 (1H, dd, J = 11.2 Hz, 5.5 Hz, H-1b), 3.90 (1H, ddd, J = 11.2 Hz, 9.7 Hz, 5.5 Hz, H-2), 3.65 (1H, m, H-6b), 3.63 (1H, m, H-5), 3.55 (1H, dd, J = 11.8 Hz, 5.4 Hz, H-6a), and 3.42 (1H, t, J = 11.2 Hz, H-1a) revealed a sugar moiety which was characterized as a 1,5-anhydro-D-glucitol. The two aryl groups were characterized as 4-hydroxybenzoate due to the ester carbonyl carbons at δ_C 167.9 (C, 3-OCO), 167.2 (C, 4-OCO) respectively, and the ester groups were attached to C-3 and C-4 of 1,5-anhydro-D-glucitol as confirmed by HMBC correlations between δ_H 5.37 (1H, t, J = 9.4 Hz, H-3) and δ_C 167.9 (C, 3-OCO), and between δ_H 5.18 (1H, t, J = 9.7 Hz, H-4) and δ_C 167.2 (C, 4-OCO). Therefore, the structure of compound **45** (Figure 24) was established as the known 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol. The findings from Yalo *et al*. (2022) were consistent with the ^{1}H and ^{13}C NMR data obtained in this study, as shown in Table 10, and used to affirm the structure of compound **45**.

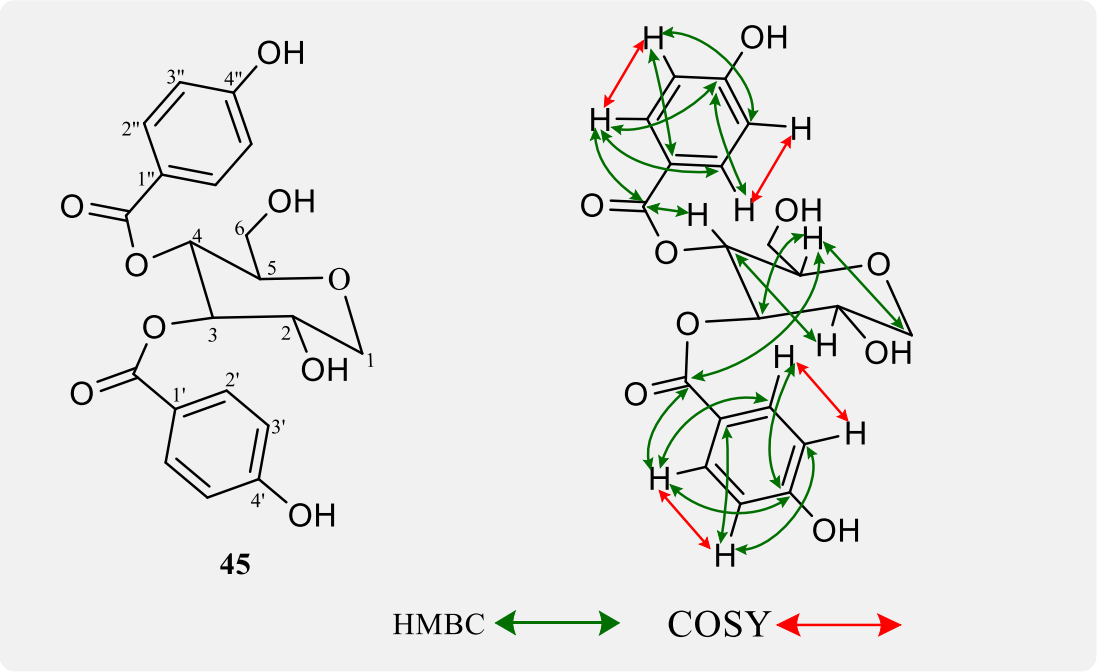


Figure 24: 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol and its HMBC and COSY correlations (compound **45**)

Table 10: ^{13}C and ^{1}H NMR spectral data of compound **45** and that of *3*,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol

Carbon No.	δ_C (ppm)	Type	δ_H (ppm) (multiplicity, *J*)	δ_C (ppm) (Yalo *et al.*, 2022)	δ_H (ppm) (multiplicity, *J*) (Yalo *et al.*, 2022)
1	71.0	CH_2	3.42 (t, 11.2, H-1_a) 4.08 (dd, 11.2, 5.5, H-1_b)	69.9	3.33 (dd, 11.1, 5.5, H-1_a) 3.94 (dd, 11.1, 5.5, H-1_b)
2	69.8	CH	3.90 (ddd, 11.2, 9.7, 5.5)	68.3	3.76 (m)
3	78.6	CH	5.37 (t, 9.4)	77.5	5.27 (t, 9.3)
4	70.7	CH	5.18 (t, 9.7)	69.7	5.05 (t, 9.7)
5	80.7	CH	3.63 (m)	79.5	3.59 (ddd, 4.2, 2.3, 2.2)
6	62.5	CH_2	3.55 (dd, 11.8, 5.4, H-6_a) 3.65 (m, H-6_b)	61.2	3.38 (dd, H-6_a) 3.45 (dd, H-6_b)
1′	121.7	C	-	120.8	-
2′	133.1	CH	7.75 (2H, d, 8.8)	132.0	7.71 (d, 8.8)
3′	116.1	CH	6.72 (2H, d, 8.7)	115.6	6.78 (d, 8.8)
4′	164.0	C		162.3	-
3-OCO	167.9	C		165.6	-
1″	121.0	C	-	120.1	-
2″	133.0	CH	7.78 (2H, d, 8.8)	131.9	7.68 (d, 8.8)
3″	116.3	CH	6.72 (2H, d, 8.7)	115.7	6.78 (d, 8.8)
4″	164.5	C		162.6	-
4-OCO	167.2	C		165.0	-

Notes: J (coupling constant in Hertz), Hz (Hertz), s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), and m (multiplet).

CHAPTER FIVE
DISCUSSION

5.1 Total Phenolic Content

Edibility of *D. abyssinica* fruits in the tropics, as documented by Ruffo *et al*. (2002), especially its widespread consumption (raw or cooked) among various ethnic groups in Kenya and the wider Eastern Africa, led us to pursue its total phenolic content. Methanol extract of the fruit pulp exhibited the highest phenolic concentration, $921.79 \pm 1.63 \times 10^{-3}$ mg GAE/100g. A value that closely agreed with 1128 ± 60 mg GAE/100g for total polyphenols of the dried fruit pulp of *D. abyssinica* (Waweru *et al.*, 2022). However, it is worth appreciating that the other study, Waweru et al. (2022), used a different extraction protocol (decocting in water at 60 °C) to the one used in the current study. In the current study, maceration at room temperature with solvents of increasing polarities was applied, and extractions were done successively. The phenolic contents of the extracts were lowest in the hexane extract and highest in the methanol extract. This was attributed to methanol being more soluble in phenols than hexane.

Four samples were evaluated for their TPC. Regarding numerical values, the TPC contents of all the extracts ranged from 24.36 to 921.79 mg GAE/100 g. This range of TPC – increasing with an increase in extract polarity, mirrors the antioxidant capacities of these extracts, with the most polar extract exhibiting the most AC. These TPC values are significantly different from the figures reported in previous studies for *D. abyssinica* fruit, which were in the range of 1128 and 1203 mg GAE/100 g (Waweru *et al.*, 2022). This discrepancy in extraction methods contributes to the observed differences in reported TPC.

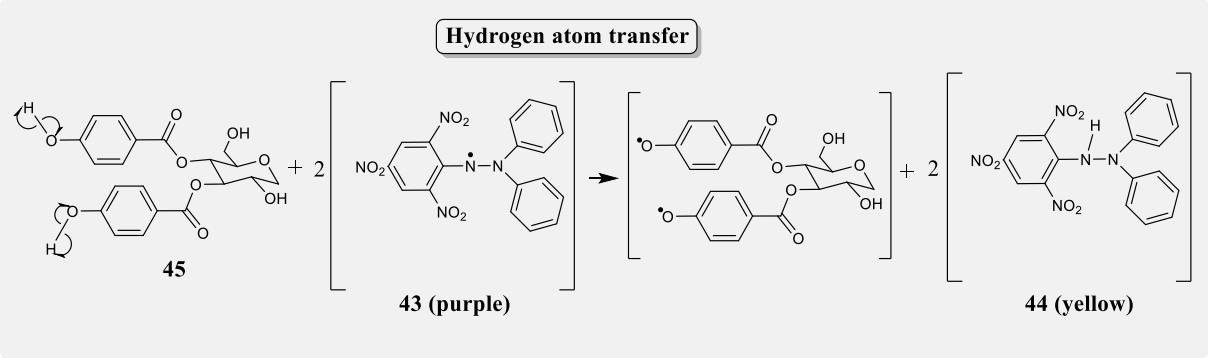
5.2 DPPH Radical Scavenging Capacity of the Crude Extracts

It is safe to say that the extraction medium used when subjecting a plant extract to the DPPH assay can significantly impact the IC_{50} values. Methanol is a common solvent used in the DPPH assay. Alongside its low viscosity, it exhibits polar and non-polar properties. In contrast, solvents like ethyl acetate, methylene chloride, and hexane may not be as effective in dissolving the antioxidant compounds, leading to much lower IC_{50} values.

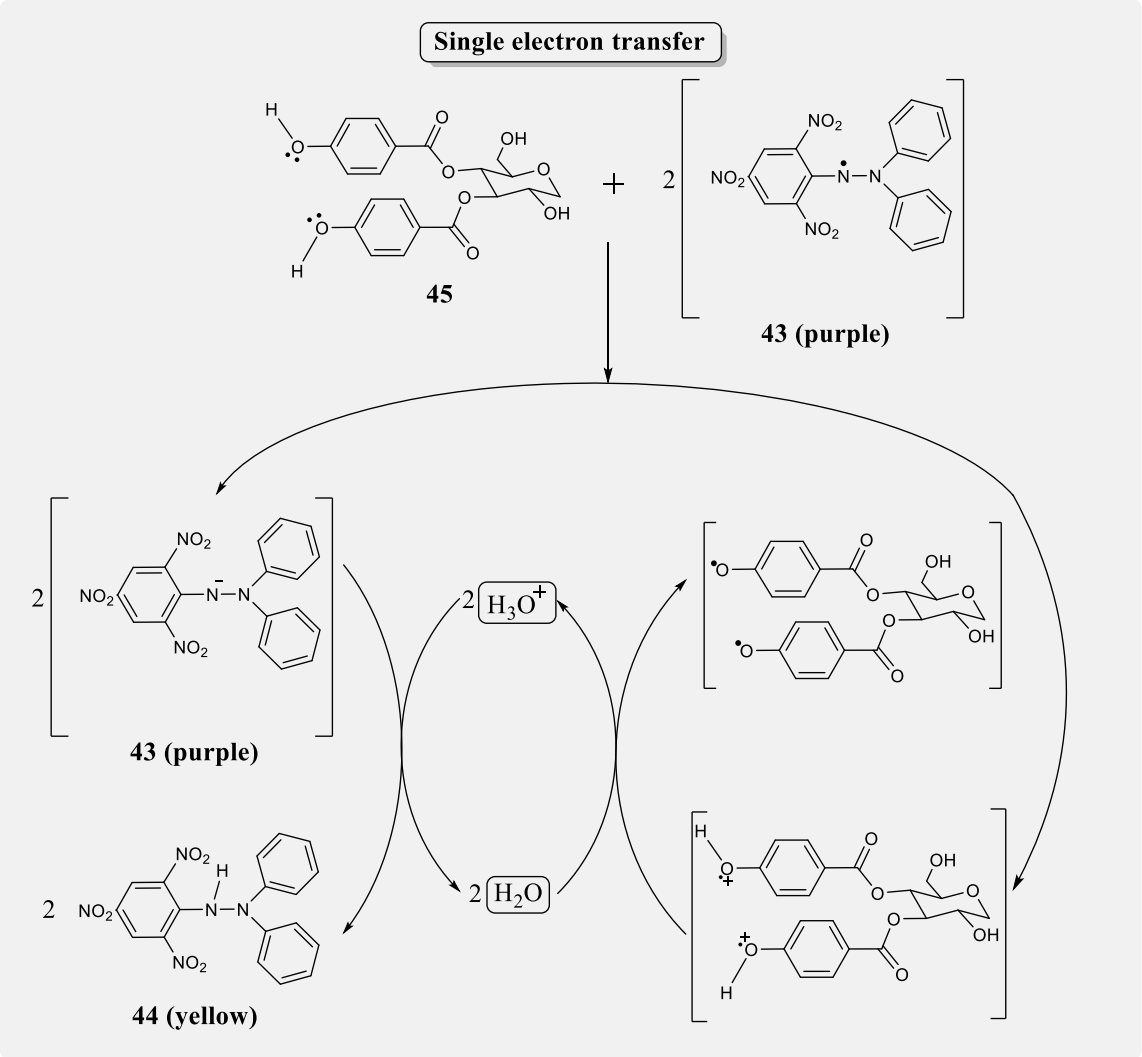
There is a considerable comparison of other studies that report the IC_{50} values of methanol extracts from various edible fruits using the DPPH assay. For instance, a study by Siregar *et al*. (2022) revealed a high antioxidant activity on methanol fruit extracts compared to other extracts.

Another study on biochemical and antioxidant activity of wild edible fruits of the Eastern Himalaya, India reported IC_{50} values higher in methanol extract (Rymbai *et al.*, 2023). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from four varieties of guava fruits showed a higher activity in methanol extract than in methylene chloride extract (Thaipong *et al.*, 2006). These IC_{50} findings, including the ones presented in this study, show that methanol extracts from fruits have the greatest potential in reporting high IC_{50} values.

Since compound **45** was isolated from the methanol extract, and the methanol extract showed significantly higher DPPH radical scavenging capacity compared to the other extracts, it was necessary to try to explain the reaction mechanism of compound **45** scavenging the DPPH radical. The bifurcated mechanisms of action can be used to explain what might be happening here. Perhaps, the samples are following either of the two paths or both simultaneously as shown in Schemes 8 and 9.



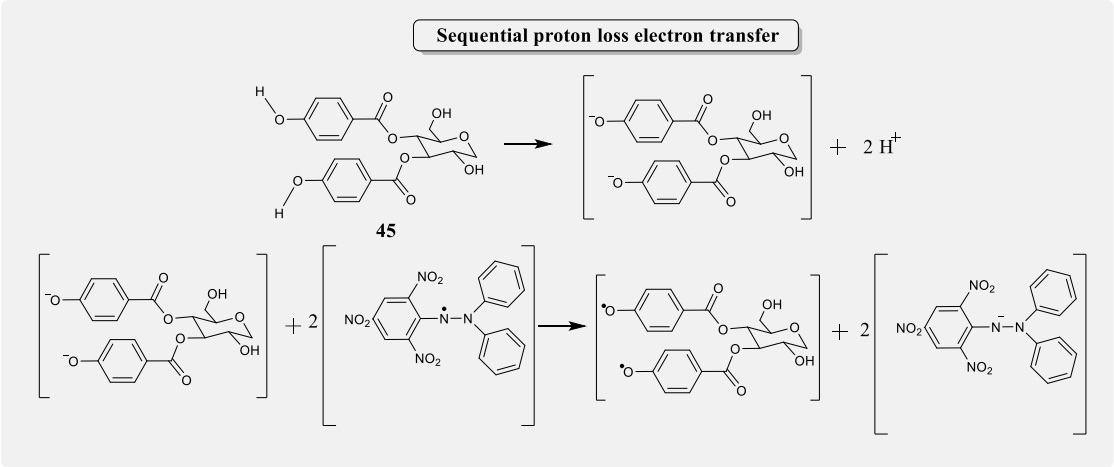
Scheme 8: A proposed HAT mechanism of compound **45** with two molecules of DPPH



Scheme 9: A proposed SET mechanism of compound **45**

Both Schemes 8 and 9 result in the formation of a new radical. They both underscore the achievement of a new radical that is stabilised through resonance. However, it is important to acknowledge that Scheme 9 navigates through the ionization of both the antioxidant and the DPPH molecule (Leopoldini *et al.*, 2011). It is unclear at this moment which mechanism is favoured in this study. For this reason, the formation of an antioxidant-DPPH intermediate complex cannot be ruled out. Additionally, the reaction could be simultaneously taking both routes in a shared or thermodynamically favoured route over the other, as depicted in Scheme 10. Moreover, in a more concerted way, it can be argued that the solvent of extraction is influencing the mechanism to be

followed. As identified by Litwinienko and Ingold (2003), this solvent influence is such that aprotic solvents lean towards Scheme 9 and vice versa.



Scheme 10: Proposed mixed mechanisms of HAT and SET

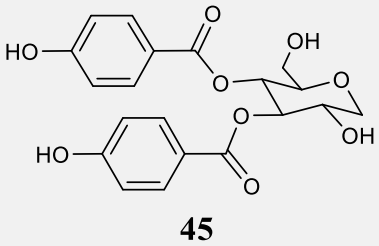
5.3 Separation, Purification, and Chemical Characterisation of Compounds

Several studies have reported the isolation of compounds **31** and **32**. A study on fruit pulp extracts of *Azanza garckeana* yielded compound **31** (Dikko *et al.*, 2016). This species, and widely, the family Malvaceae, has reported compounds **31** and **32** prevalently from plant parts other than the fruits. Isolation and purification studies from fruits of *Solanum mauense* were reported by Chirchir et al. (2018). In this report, fruits of *Solanum mauense*, which were collected from the Southwest Mau Forest in Kenya, yielded compound **31**. In 2023, the isolation and characterisation of triterpenoids from the stem of *Diospyros gracilishe* reported the preparation of three extracts in a similar protocol as the one in the current study. Among the fractions purified, compound **31** was isolated and characterised using NMR spectroscopy – this is the most recent identical procedure of isolation and chemical characterisation of compound **31** to the current study. The family Ebenaceae has reported compound **31** prevalently from other plant parts and not from the fruits. Kumar *et al*. (2016), determined the *in vitro* antimicrobial activity of compound **32** isolated from the extract of fruits of *Helicteres isora*. Later on, Suja *et al*. (2017) isolated and characterized antimycobacterial compounds from fruits of *Aegle marmelos*, among which compound **32** was isolated after repeated silica gel and Sephadex column chromatography, followed by an NMR analysis.

The characterization of compounds from *Protea cynaroides* leaves afforded four new compounds, among them compound **45** (Yalo *et al.*, 2022). Currently, this is the only available literature for compound **45**. The ethyl acetate and butanol extracts were chromatographed repeatedly using silica gel and Sephadex column chromatography to yield compound 41 alongside four other new compounds. The family Proteaceae has not reported compound **45** before.

5.3.1 An Anhydroglucitol from *D. abyssinica*

The current study was able to isolate, purify, and characterise 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol (compound **45**), an anhydroglucitol, from the fruits of *D. abyssinica* (Figure 25). A thorough search of the literature revealed only one study that had isolated this compound from the leaves of *Protea cynaroides* before (Yalo *et al.*, 2022). By then, this compound was a new report from the fruits of *D. abyssinica*.

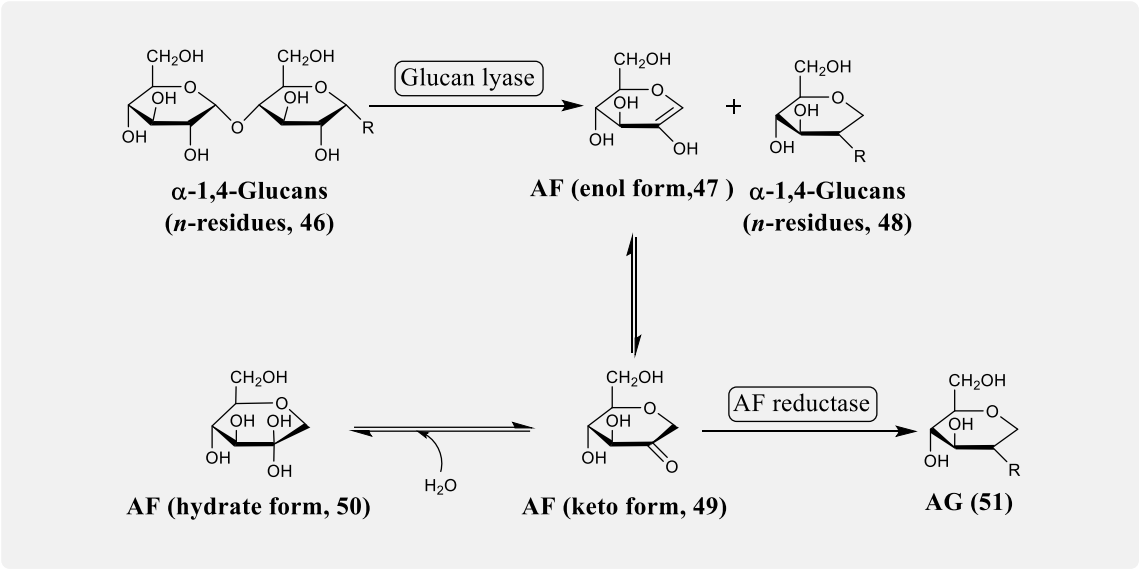


45

Figure 25: An anhydroglucitol isolated and characterised from the current study

5.3.2 Biosynthesis of Anhydroglucitols

The breakdown of α-1,4-glucans, which include glycogen, starch, and maltosaccharides, through the action of α-1,4-glucan lyase, results in the production of anhydrofructose. Scheme 5 shows that the anhydrofructose pathway is responsible for the metabolism of various metabolites, including 1,5-anhydro-D-fructose (AF) keto-enol tautomers, and 1,5-anhydro-D-glucitol (AG) via the AF reductase. Bacteria, fungi, algae, and even some mammalian tissues are known to contain AF or the AF-forming glucan lyase (Fiskesund *et al.*, 2010).



Scheme 11: A proposed biosynthetic pathway for Anhydroglucitol

Source Fiskesund *et al*. (2010)

5.4 Data Analyses

Evidence from one-way ANOVA showed that the four different extracts portrayed significantly different TPC and IC_{50} values. This observation was related to a study whose subject was to determine the best solvent and optimum extraction conditions for the extraction of maximum antioxidant phenolic compounds and antioxidant activity from strawberry fruits *Fragaria ananassa* (Koraqi *et al.*, 2023).

During the two-way ANOVA, it was evident that there was no interaction among the means of scavenging capacities of the four extracts and their respective seven concentrations. This observation had earlier been reported by studying the antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts of *Torilis leptophylla* (Saeed *et al.*, 2012).

Among the four extracts, it was observed that the higher the total phenolic content in an extract, the stronger the scavenging capacity. This observation was drawn from the strong Pearson correlation coefficient of 0.97 and was in line with the first reported studies (Velioglu *et al.*, 1998) that fruits' antioxidant activity positively correlates with their phenolic compounds' content.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study aimed to enrich the literature associated with *D. abyssinica* by investigating the medicinal properties of this plant – specifically, its antioxidant properties. The findings herein provide alternatives for health scientists and institutions like the Kenya Medical Research Institute for a more natural source of antioxidants. These antioxidants freely occur in nature, are environmentally friendly, and support sustainable development in the drug industry in Kenya. All these align with SDGs 2.1 and 3.7 on improved nutrition and well-being promotion. This research study had four key outputs that were in line with its aims.

i. From this study's total phenolic content report, it is evident that extracts from the fruits of *D. abyssinica* have significant polyphenols that contribute to free radical scavenging capacities.
ii. This study's in-vitro antioxidant capacity assay report shows that extracts from the fruits of *D. abyssinica* have significant radical scavenging capacities.
iii. The extracts from the fruits of *D. abyssinica* contain numerous compounds, as evidenced by the chromatography data. Amongst them, three compounds were successfully isolated; compounds betulinic acid, sitosterol, and 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol
iv. Compounds **31** and **32** were identified as triterpenoids (betulinic acid and sitosterol, respectively), and compound **45** was identified as an anhydroglucitol (3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol). This is the first time the three compounds were isolated from fruits of *D. abyssinica*, although they had been isolated previously from other parts of the plant.

6.2 Recommendations

A study has been presented on the secondary metabolites from *D. abyssinica* and the antioxidant effects of its fruits. However, more studies must be conducted to consolidate and cement these findings.

i. The nature of antioxidants is sometimes complicated, as are these antioxidant capacity assays. We should not rely solely on these *in-vitro* results; additional *in-vivo* tests should be done to consolidate their validation.
ii. The anhydroglucitol's antioxidant activity should also be determined, and more compounds should be isolated from *D. abyssinica* and their antioxidant activities should be determined.
iii. To ascertain the identity of compound **45**, structure confirmation through total synthesis, high-resolution mass spectrometry, or single-crystal X-ray diffraction for the three compounds should be done.
iv. The continued use of these fruits as a food source is highly recommended. Therefore, these fruits can be used as a readily available source of natural antioxidants and as promising food supplements to address nutritional deficiencies.

6.3 Suggestions for Further Research

The study presents various aspects of inquiry to guide future research on antioxidants derived from the plant's secondary metabolites. Some of these aspects include suggested further research on antioxidants from secondary metabolites in plants in general. Here are three suggestions for further research:

i. First is addressing the limitations in the methodology approach of sample size and type. This is owed to the fact that plants from different geographical locations might feature different amounts or types of metabolites. Therefore, sampling should be extended to represent the different geographical locations of the plant to enhance the validity and reliability of findings.
ii. Secondly, by building upon the findings of compound **45**, it is necessary to explore its biosynthetic pathway and determine its key precursors. This information can be crucial in finding alternative synthetic routes for the compound to enable more sustainable production.
iii. Thirdly, to understand the balance of ROS and antioxidants in a living organism, and to expand the theoretical framework of oxidative stress, there needs to be empirical studies that give guidelines on the required amount of these species, especially in complex

organisms such as humans. The quantification of these species provides a reliable argument that explains the research problem better.

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APPENDICES

Appendices 1: R Studio Outputs

7.1 Quadruplicate TPC Experiments

```
            Df  Sum Sq Mean Sq F value Pr(>F)
group        3 1770053  590018    2154 <2e-16 ***
Residuals   12    3287     274
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> #Post-hoc test
> TukeyHSD(Results)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = tcp ~ group)

$group
                   diff       lwr       upr p adj
EtOAc-MeOH    -403.8462 -438.5927 -369.0997     0
DCM-MeOH      -660.2564 -695.0029 -625.5100     0
Hexane-MeOH   -897.4359 -932.1824 -862.6894     0
DCM-EtOAc     -256.4102 -291.1567 -221.6638     0
Hexane-EtOAc  -493.5897 -528.3362 -458.8432     0
Hexane-DCM    -237.1795 -271.9259 -202.4330     0
```

```
> leveneTest(tcp ~ group, data = mydata)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value    Pr(>F)
group  3      27 1.276e-05 ***
      12
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> shapiro.test(residuals(Results))

        Shapiro-Wilk normality test

data:  residuals(Results)
W = 0.85315, p-value = 0.01516
```

7.2 R Studio outputs for One-way ANOVA of the DPPH Radical Scavenging Capacities

7.2.1 Barlette's test, and Shapiro-Wilk's test

```
> # ANOVA
> res.aov <- aov(sc ~ conc, data = mydata)
> # Summary of the analysis
> summary(res.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
conc         6  11053    1842  137711 <2e-16 ***
Residuals   21      0       0
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> TukeyHSD(res.aov)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = sc ~ conc, data = mydata)

$conc
                  diff       lwr       upr p adj
0.78-0.39   14.440079 14.174223 14.705934     0
1.56-0.39   22.560576 22.294721 22.826431     0
3.13-0.39   35.330714 35.064859 35.596569     0
6.25-0.39   43.795023 43.529168 44.060878     0
12.25-0.39  51.424361 51.158506 51.690217     0
25-0.39     60.576293 60.310438 60.842148     0
1.56-0.78    8.120498  7.854643  8.386353     0
3.13-0.78   20.890635 20.624780 21.156490     0
6.25-0.78   29.354944 29.089089 29.620799     0
12.25-0.78  36.984283 36.718428 37.250138     0
25-0.78     46.136215 45.870360 46.402070     0
3.13-1.56   12.770138 12.504282 13.035993     0
6.25-1.56   21.234447 20.968592 21.500302     0
12.25-1.56  28.863785 28.597930 29.129640     0
25-1.56     38.015717 37.749862 38.281572     0
6.25-3.13    8.464309  8.198454  8.730164     0
12.25-3.13  16.093648 15.827793 16.359503     0
25-3.13     25.245580 24.979724 25.511435     0
12.25-6.25   7.629339  7.363483  7.895194     0
25-6.25     16.781270 16.515415 17.047126     0
25-12.25     9.151932  8.886077  9.417787     0
```

```
> bartlett.test(sc ~ conc, data = mydata)

        Bartlett test of homogeneity of variances

data:  sc by conc
Bartlett's K-squared = 7.4589, df = 6, p-value =
0.2805
```

```
> shapiro.test(residuals(res.aov))

        Shapiro-Wilk normality test

data:  residuals(res.aov)
W = 0.9591, p-value = 0.3319
```

7.2.2 Levene's Test, Fligner-Killeen's Test, and Shapiro-Wilk's Test

```
> # ANOVA
> res.aov <- aov(sc ~ conc, data = mydata)
> # Summary of the analysis
> summary(res.aov)
             Df Sum Sq Mean Sq F value Pr(>F)
conc          6   8628  1438.1    3640 <2e-16 ***
Residuals    21      8     0.4
---
Signif. codes:
0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
> #Tukey honest significance difference test
> TukeyHSD(res.aov)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = sc ~ conc, data = mydata)

$conc
                  diff       lwr       upr p adj
0.78-0.39    8.169614  6.724810  9.614417     0
1.56-0.39   19.613621 18.168818 21.058425     0
3.13-0.39   25.998690 24.553887 27.443493     0
6.25-0.39   32.056320 30.611516 33.501123     0
12.25-0.39  43.876883 42.432080 45.321686     0
25-0.39     53.683694 52.238890 55.128497     0
1.56-0.78   11.444008  9.999205 12.888811     0
3.13-0.78   17.829077 16.384273 19.273880     0
6.25-0.78   23.886706 22.441903 25.331509     0
12.25-0.78  35.707269 34.262466 37.152072     0
25-0.78     45.514080 44.069277 46.958883     0
3.13-1.56    6.385069  4.940266  7.829872     0
6.25-1.56   12.442698 10.997895 13.887501     0
12.25-1.56  24.263261 22.818458 25.708064     0
25-1.56     34.070072 32.625269 35.514875     0
6.25-3.13    6.057629  4.612826  7.502433     0
12.25-3.13  17.878193 16.433389 19.322996     0
25-3.13     27.685003 26.240200 29.129806     0
12.25-6.25  11.820563 10.375760 13.265366     0
25-6.25     21.627374 20.182571 23.072177     0
25-12.25     9.806811  8.362008 11.251614     0
```

```
> leveneTest(sc ~ conc, data = mydata)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  6  0.7447 0.6201
      21
```

```
> fligner.test(sc ~ conc, data = mydata)

        Fligner-Killeen test of homogeneity of variances

data:  sc by conc
Fligner-Killeen:med chi-squared = 7.1322, df = 6,
p-value = 0.3088
```

```
> #Shapiro test for the normality of residuals
> shapiro.test(residuals(res.aov))

        Shapiro-Wilk normality test

data:  residuals(res.aov)
W = 0.83593, p-value = 0.0004924
```

7.2.3 Levene's test and Shapiro-Wilk's test.

```
> # ANOVA
> res.aov <- aov(sc ~ conc, data = mydata)
> # Summary of the analysis
> summary(res.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
conc         6   8628  1438.1    3640 <2e-16 ***
Residuals   21      8     0.4
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
> #Tukey honest significant difference test
> TukeyHSD(res.aov)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = sc ~ conc, data = mydata)

$conc
                  diff       lwr       upr p adj
0.78-0.39    8.169614  6.724810  9.614417     0
1.56-0.39   19.613621 18.168818 21.058425     0
3.13-0.39   25.998690 24.553887 27.443493     0
6.25-0.39   32.056320 30.611516 33.501123     0
12.25-0.39 43.876883 42.432080 45.321686     0
25-0.39     53.683694 52.238890 55.128497     0
1.56-0.78   11.444008  9.999205 12.888811     0
3.13-0.78   17.829077 16.384273 19.273880     0
6.25-0.78   23.886706 22.441903 25.331509     0
12.25-0.78 35.707269 34.262466 37.152072     0
25-0.78     45.514080 44.069277 46.958883     0
3.13-1.56    6.385069  4.940266  7.829872     0
6.25-1.56   12.442698 10.997895 13.887501     0
12.25-1.56 24.263261 22.818458 25.708064     0
25-1.56     34.070072 32.625269 35.514875     0
6.25-3.13    6.057629  4.612826  7.502433     0
12.25-3.13 17.878193 16.433389 19.322996     0
25-3.13     27.685003 26.240200 29.129806     0
12.25-6.25 11.820563 10.375760 13.265366     0
25-6.25     21.627374 20.182571 23.072177     0
25-12.25     9.806811  8.362008 11.251614     0
```

```
> leveneTest(sc ~ conc, data = mydata)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  6  0.7447 0.6201
      21
```

```
> shapiro.test(residuals(res.aov))

        Shapiro-Wilk normality test

data:  residuals(res.aov)
W = 0.83593, p-value = 0.0004924
```

7.2.4 Barlette's Test and Shapiro-Wilk's Test

```
> # ANOVA
> res.aov <- aov(sc ~ conc, data = mydata)
> # Summary of the analysis
> summary(res.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
conc         6 2782.1   463.7   45869 <2e-16 ***
Residuals   21    0.2     0.0
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
> #Tukey multiple pairwise-comparisons
> TukeyHSD(res.aov)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = sc ~ conc, data = mydata)

$conc
                 diff       lwr       upr p adj
0.78-0.39    4.305828  4.074714  4.536943     0
1.56-0.39   11.051081 10.819966 11.282195     0
3.13-0.39   15.946300 15.715186 16.177414     0
6.25-0.39   20.006549 19.775434 20.237663     0
12.25-0.39  24.885396 24.654282 25.116511     0
25-0.39     29.731500 29.500385 29.962614     0
1.56-0.78    6.745252  6.514138  6.976366     0
3.13-0.78   11.640472 11.409357 11.871586     0
6.25-0.78   15.700720 15.469606 15.931835     0
12.25-0.78  20.579568 20.348453 20.810682     0
25-0.78     25.425671 25.194557 25.656786     0
3.13-1.56    4.895219  4.664105  5.126334     0
6.25-1.56    8.955468  8.724354  9.186583     0
12.25-1.56  13.834316 13.603201 14.065430     0
25-1.56     18.680419 18.449305 18.911533     0
6.25-3.13    4.060249  3.829135  4.291363     0
12.25-3.13   8.939096  8.707982  9.170211     0
25-3.13     13.785200 13.554085 14.016314     0
12.25-6.25   4.878847  4.647733  5.109962     0
25-6.25      9.724951  9.493837  9.956065     0
25-12.25     4.846103  4.614989  5.077218     0
```

```
> bartlett.test(sc ~ conc, data = mydata)

        Bartlett test of homogeneity of variances

data:  sc by conc
Bartlett's K-squared = 6.2596, df = 6, p-value =
0.3947
```

```
> #Shapiro test for the normality of residuals
> shapiro.test(residuals(res.aov))

        Shapiro-Wilk normality test

data:  residuals(res.aov)
W = 0.97546, p-value = 0.7313
```

7.2.5 Levene's Test, Fligner-Killeen's Test, and Shapiro-Wilk's Test

```
> # ANOVA
> res.aov <- aov(sc ~ conc, data = mydata)
> # Summary of the analysis
> summary(res.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
conc         6 1042.0  173.67   13716 <2e-16 ***
Residuals   21    0.3    0.01
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
> TukeyHSD(res.aov)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = sc ~ conc, data = mydata)

$conc
                 diff       lwr       upr p adj
0.78-0.39    3.290766  3.032112  3.549421     0
1.56-0.39    6.925344  6.666689  7.183998     0
3.13-0.39    9.446627  9.187973  9.705282     0
6.25-0.39   12.770138 12.511483 13.028792     0
12.25-0.39  15.618861 15.360206 15.877515     0
25-0.39     18.271120 18.012465 18.529774     0
1.56-0.78    3.634578  3.375923  3.893232     0
3.13-0.78    6.155861  5.897207  6.414516     0
6.25-0.78    9.479371  9.220717  9.738026     0
12.25-0.78  12.328094 12.069440 12.586749     0
25-0.78     14.980354 14.721699 15.239008     0
3.13-1.56    2.521284  2.262629  2.779938     0
6.25-1.56    5.844794  5.586139  6.103448     0
12.25-1.56   8.693517  8.434862  8.952171     0
25-1.56     11.345776 11.087121 11.604431     0
6.25-3.13    3.323510  3.064856  3.582165     0
12.25-3.13   6.172233  5.913579  6.430888     0
25-3.13      8.824492  8.565838  9.083147     0
12.25-6.25   2.848723  2.590068  3.107378     0
25-6.25      5.500982  5.242328  5.759637     0
25-12.25     2.652259  2.393605  2.910914     0
```

```
> leveneTest(sc ~ conc, data = mydata)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  6  1.1897 0.3496
      21
```

```
> fligner.test(sc ~ conc, data = mydata)

        Fligner-Killeen test of homogeneity of variances

data:  sc by conc
Fligner-Killeen:med chi-squared = 5.5439, df = 6,
p-value = 0.4762
```

```
> shapiro.test(residuals(res.aov))

        Shapiro-Wilk normality test

data:  residuals(res.aov)
W = 0.92232, p-value = 0.0396
```

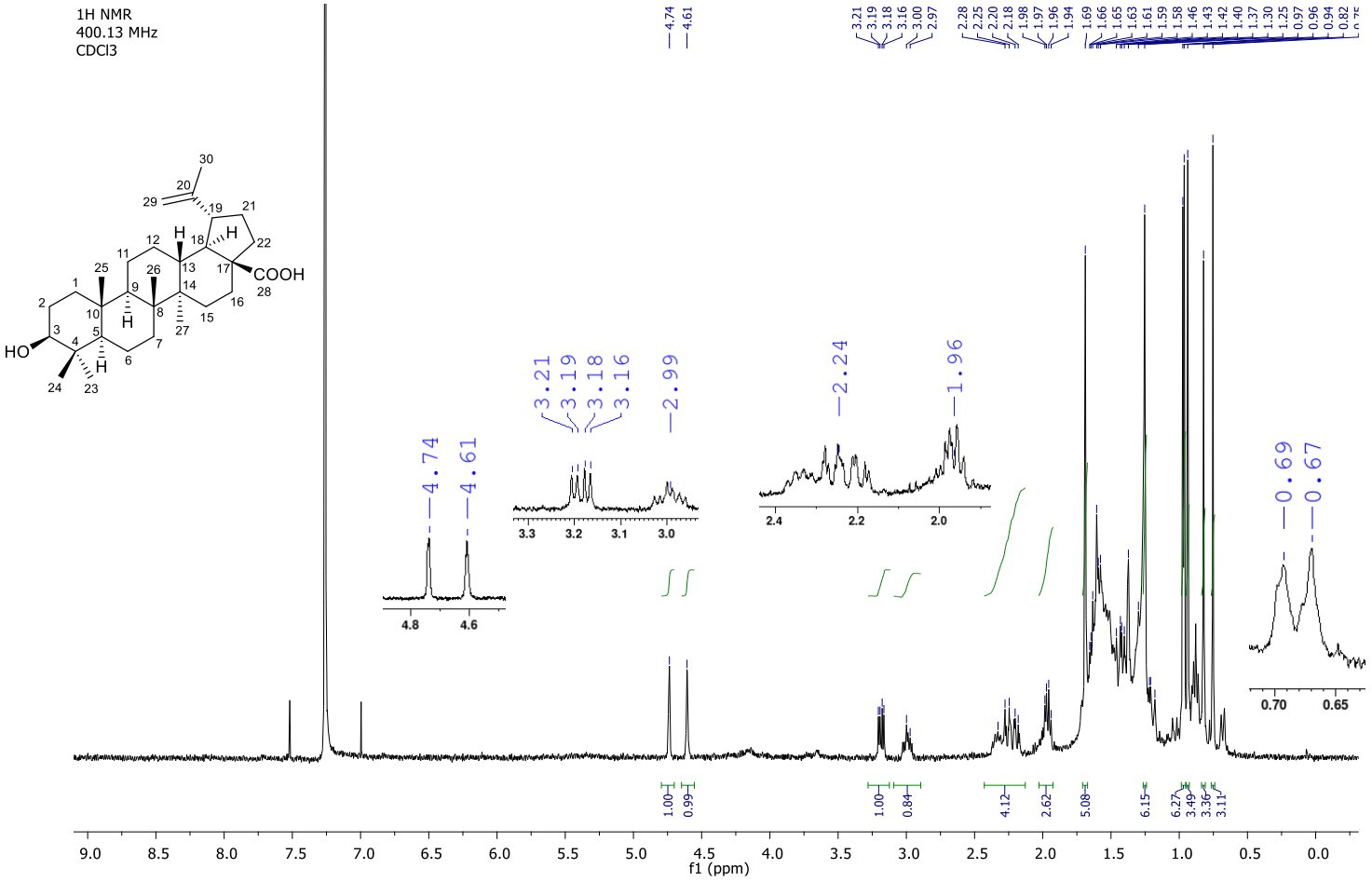
7.3 Two-way ANOVA R Studio Outputs for the DPPH Radical Scavenging Capacities

```
> twoANOVA <- aov(sc ~  extract * factor(conc), data = mydata)
> summary(twoANOVA)
                     Df Sum Sq Mean Sq F value Pr(>F)
extract               4  63085   15771 2808227 <2e-16 ***
factor(conc)          6   3119     520   92558 <2e-16 ***
extract:factor(conc) 24   8112     338   60185 <2e-16 ***
Residuals           105      1       0
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

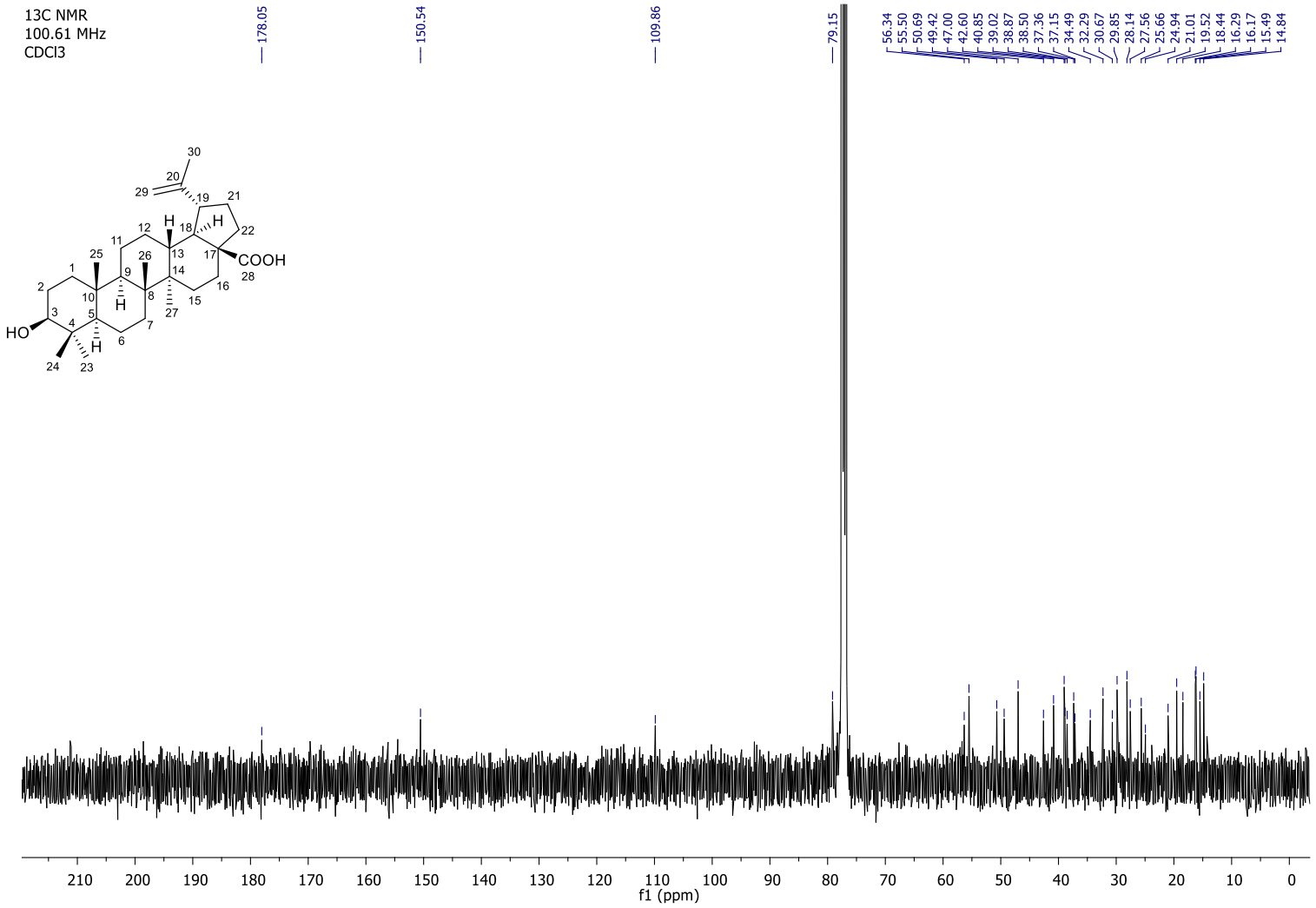
Appendices 2: NMR Spectra of Compounds 31, 32, and 45

7.4 Compound 31

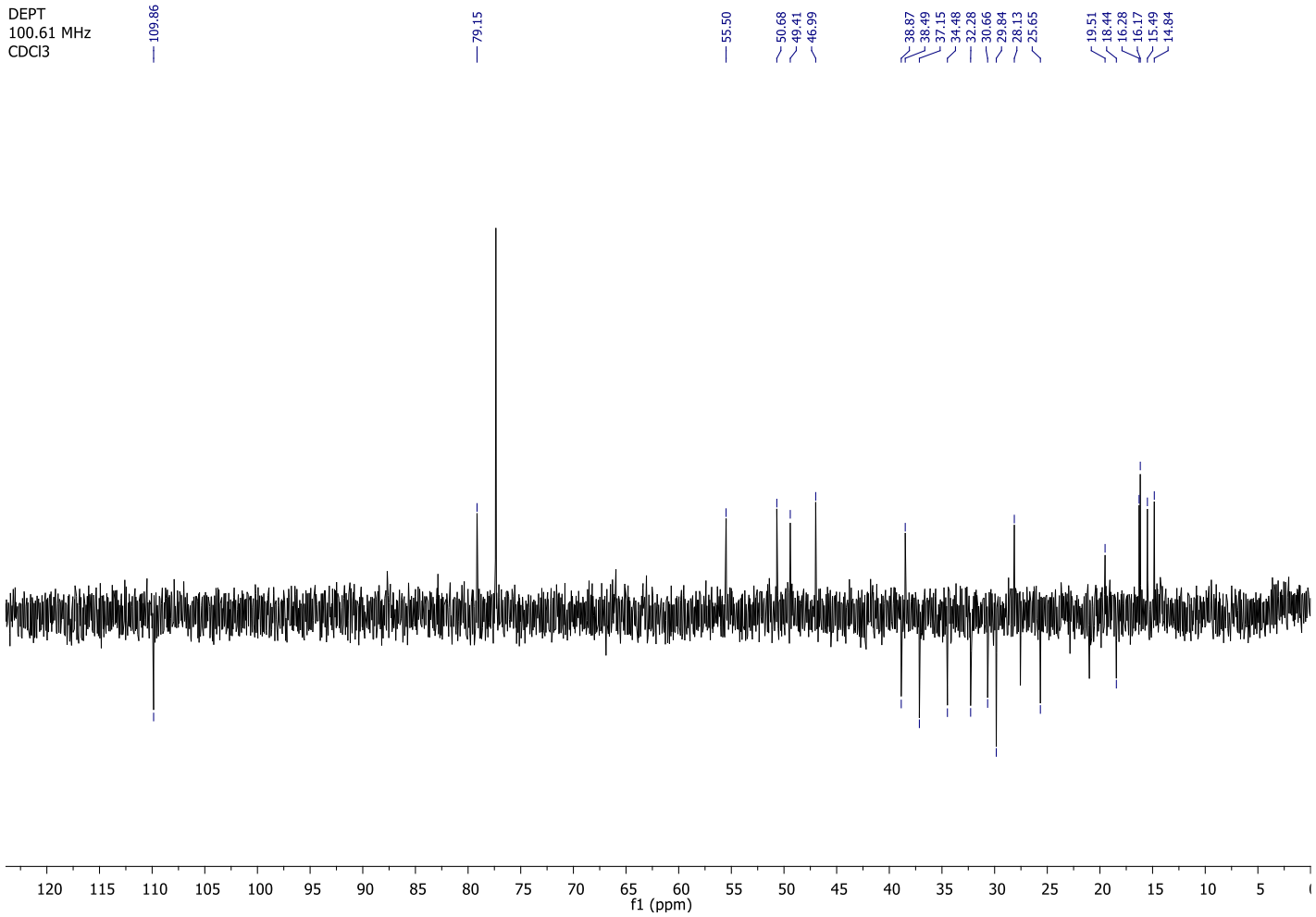
7.4.1 ^{1}H-NMR Spectrum of Compound 31



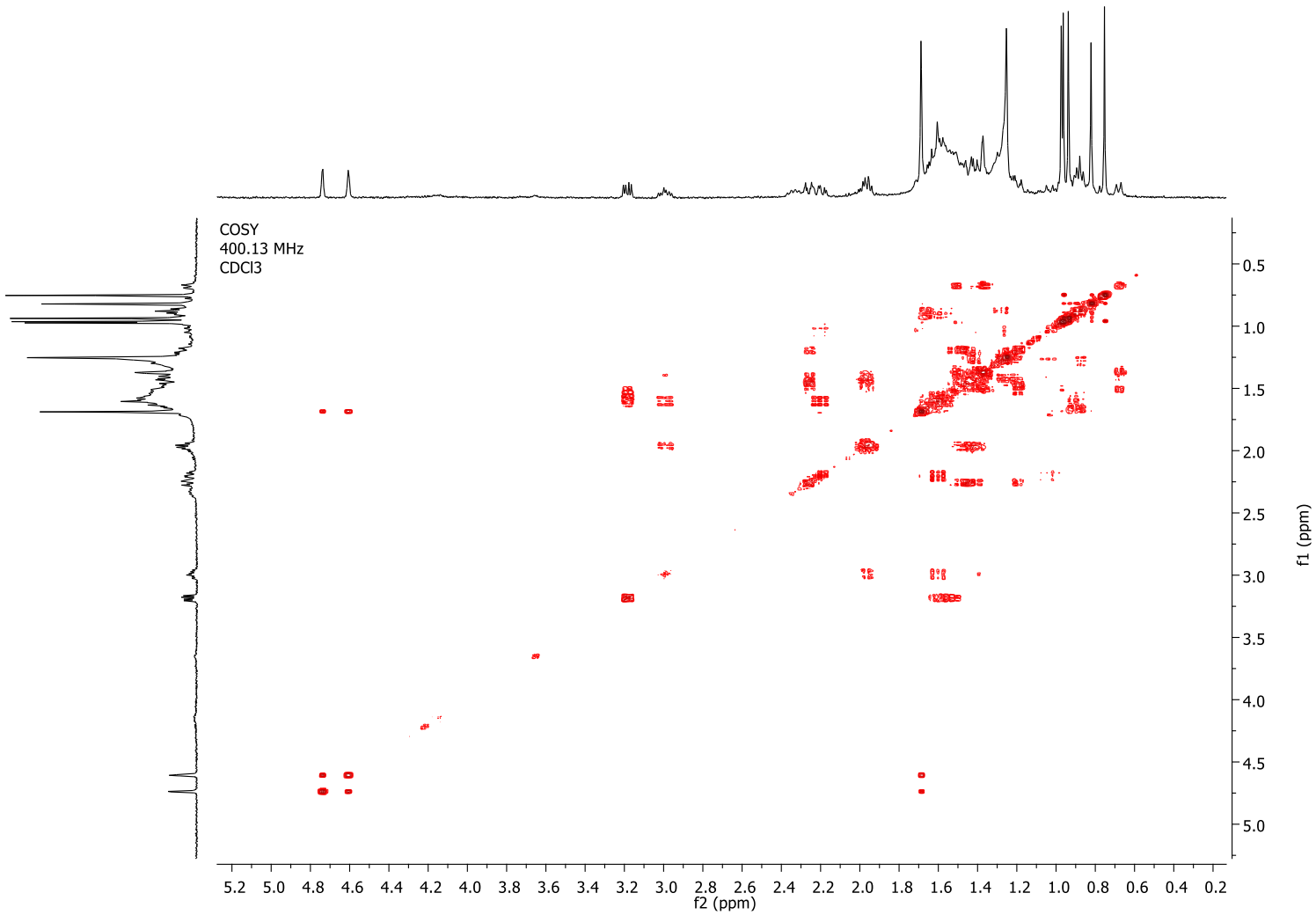
7.4.2 ^{13}C-NMR Spectrum of Compound 31



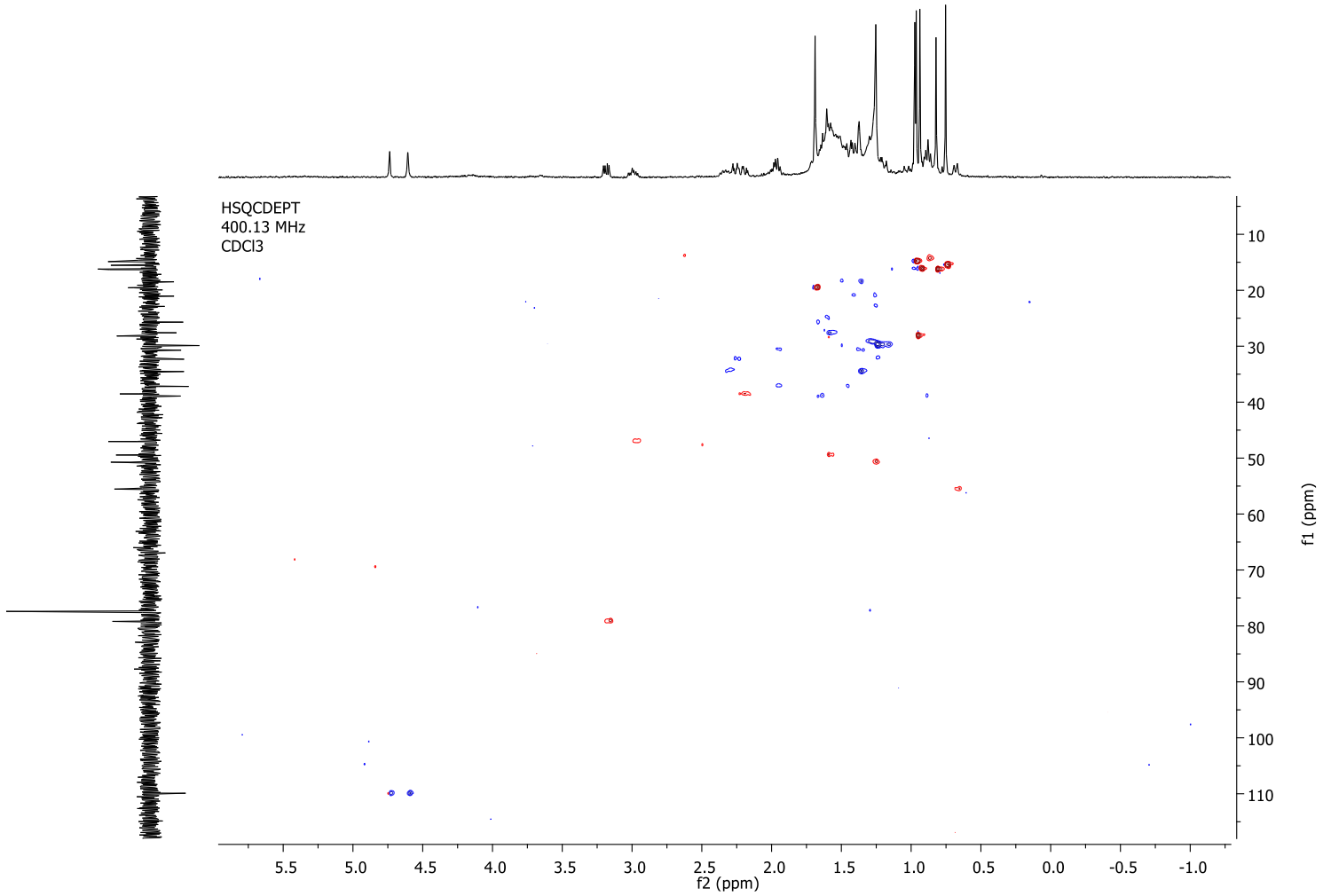
7.4.3 DEPT Spectrum of Compound 31



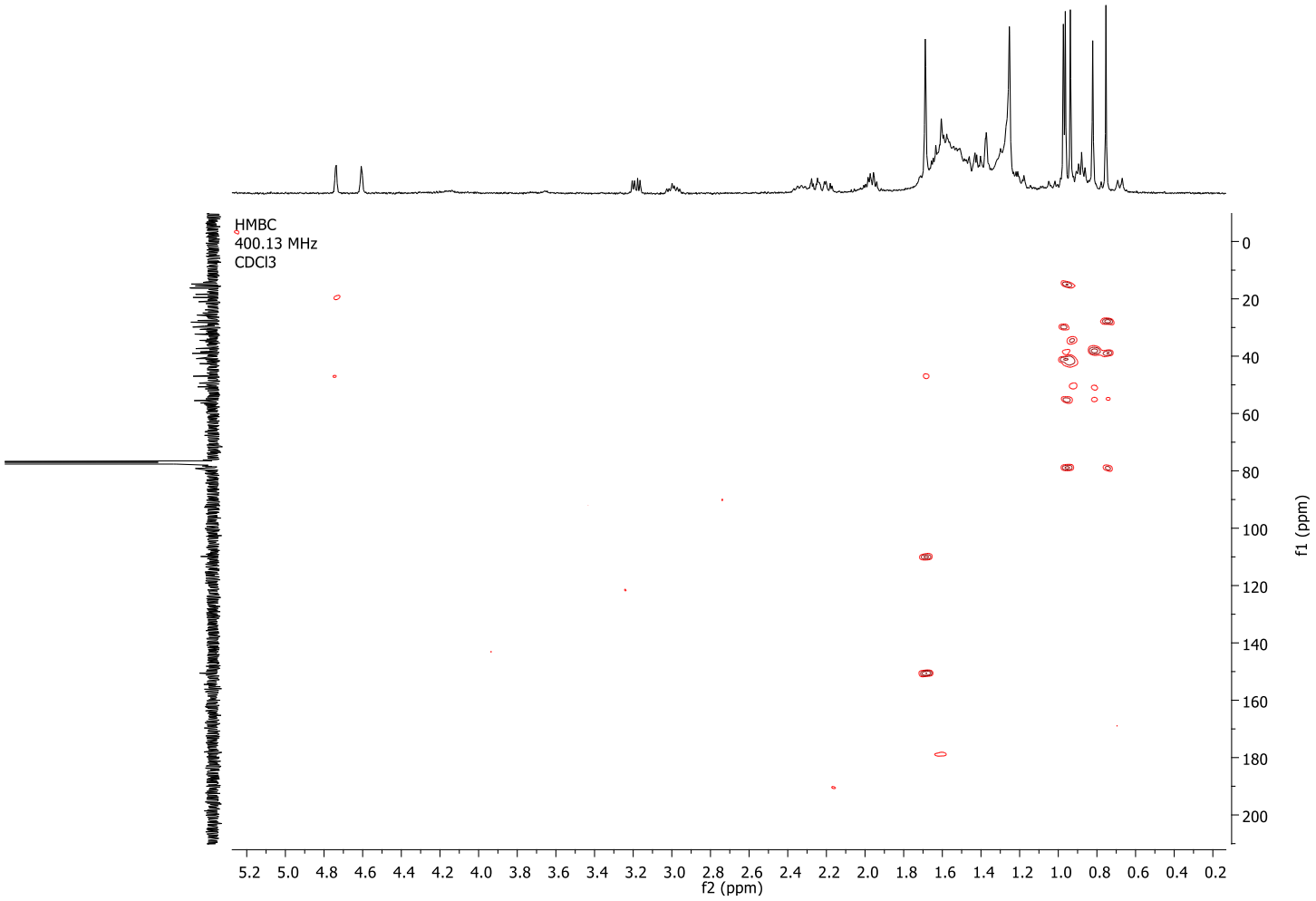
7.4.5 ^{1}H-^{1}H COSY Spectrum of Compound 31



7.4.6 HSQCDEPT Spectrum of Compound 31

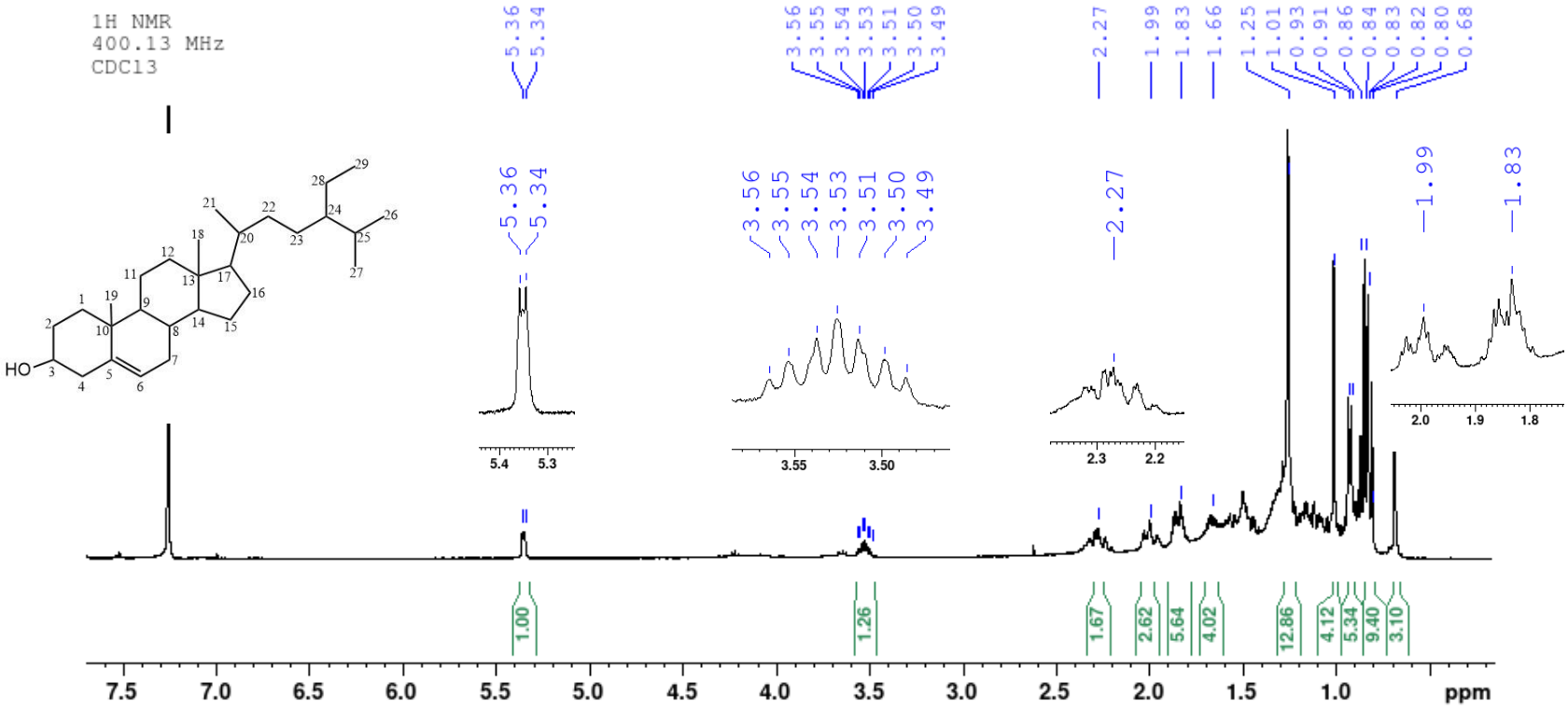


7.4.7 HMBC Spectrum of Compound 31

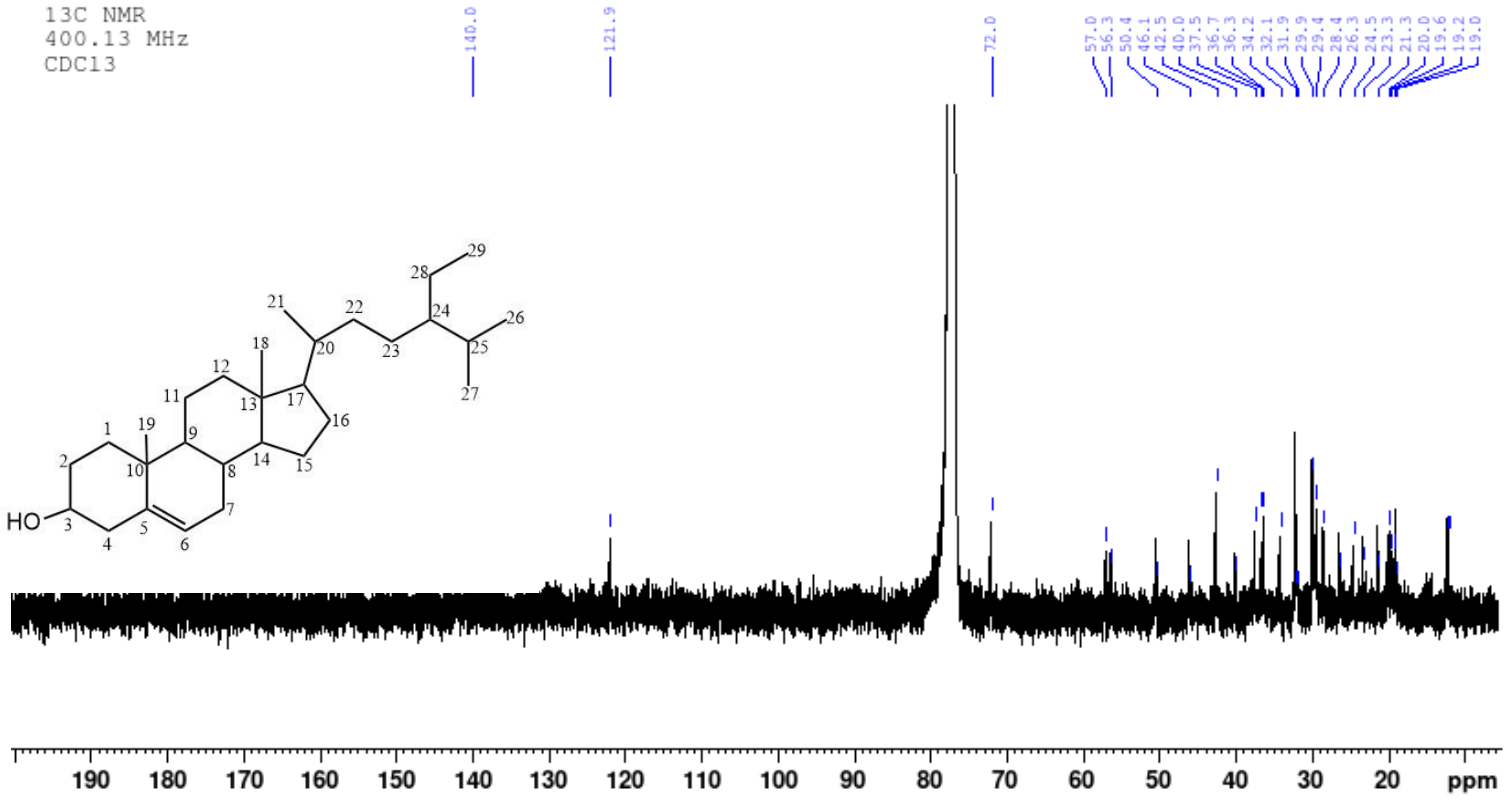


7.5 Compound 32

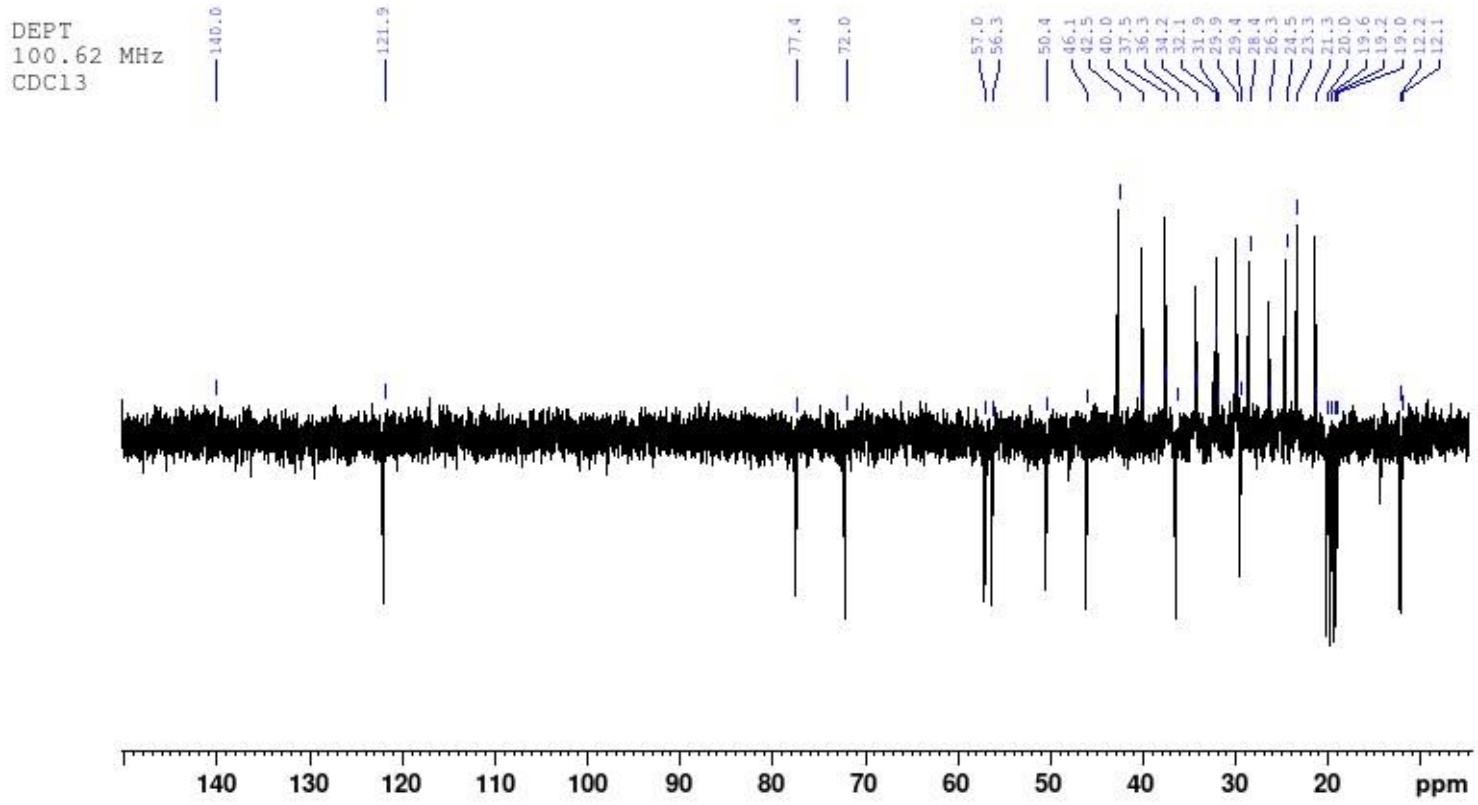
7.5.1 ^{1}H-NMR Spectrum of Compound 32



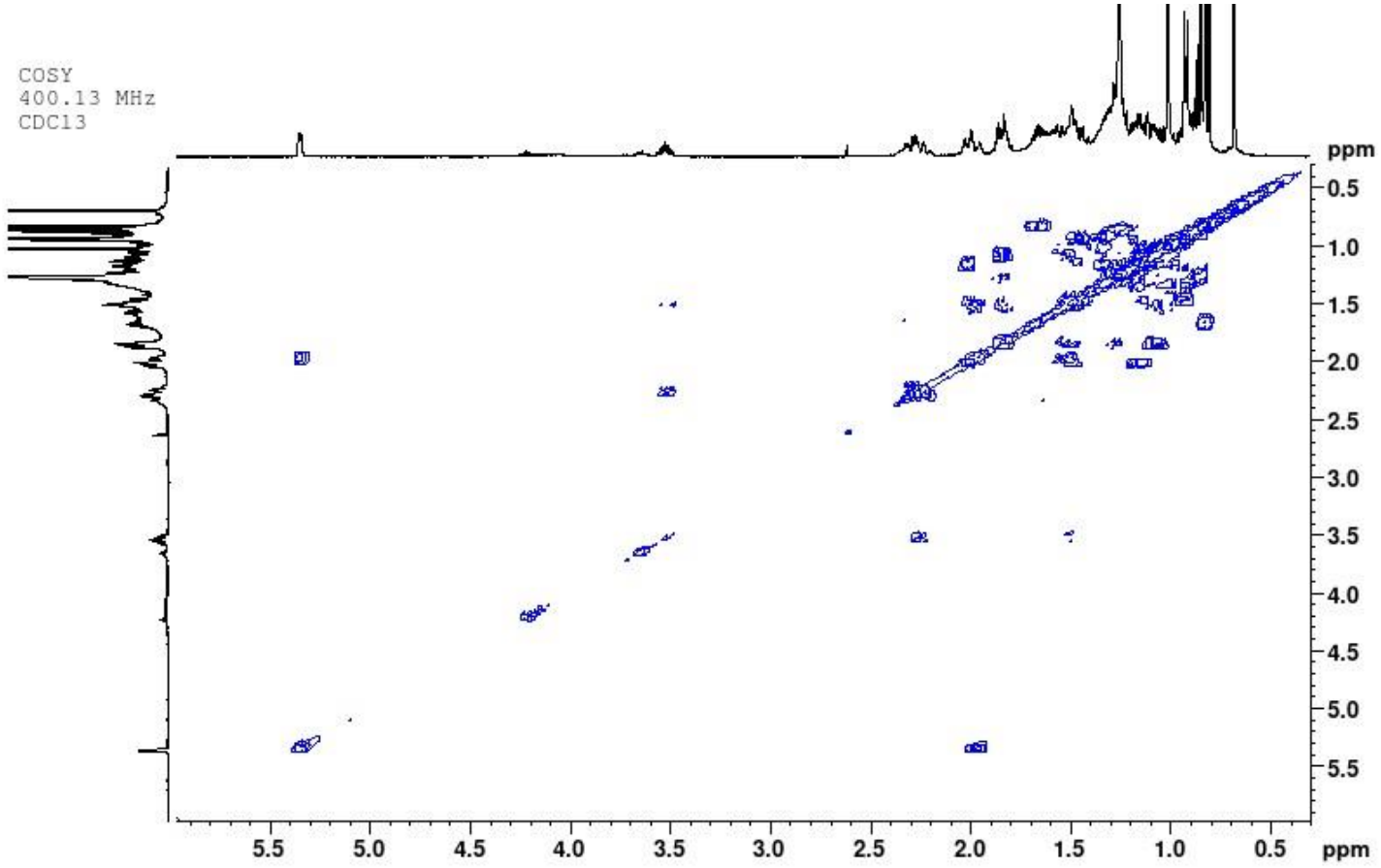
7.5.2 ^{13}C-NMR Spectrum of Compound 32



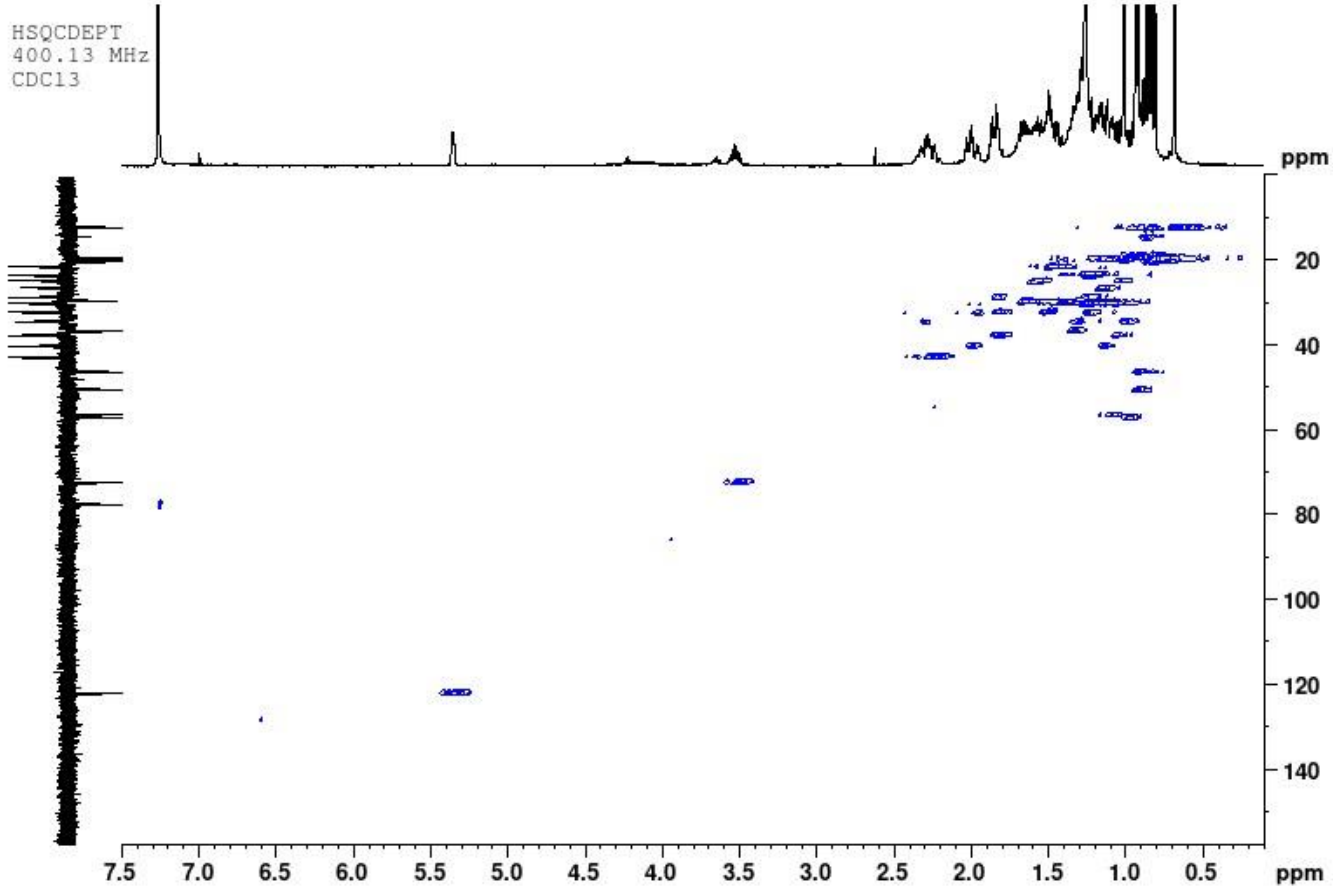
7.5.3 DEPT Spectrum of Compound 32



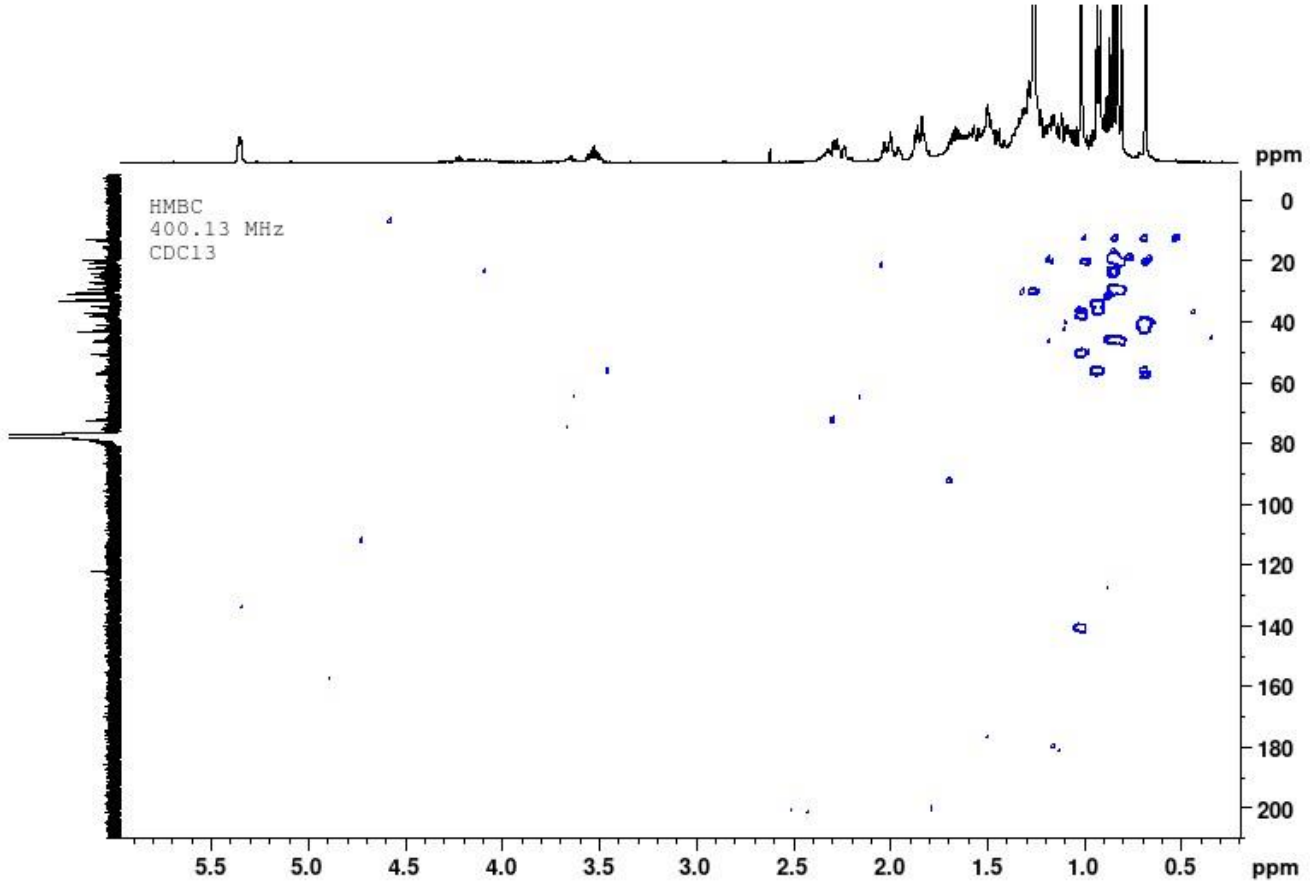
7.5.4 ^{1}H-^{1}H COSY Spectrum of Compound 32



7.5.5 HSQCDEPT Spectrum of Compound 32

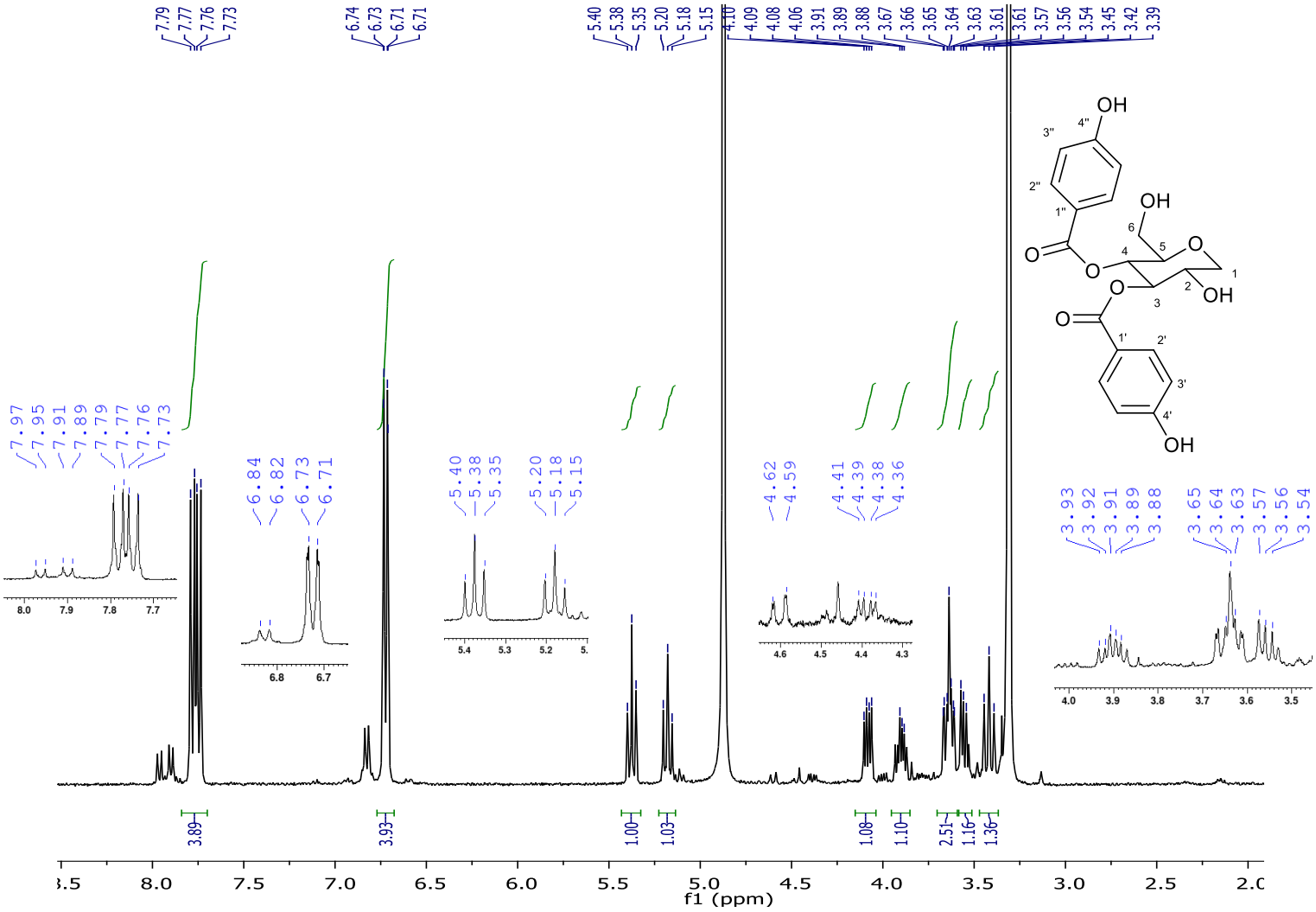


7.5.6 HMBC Spectrum of Compound 32

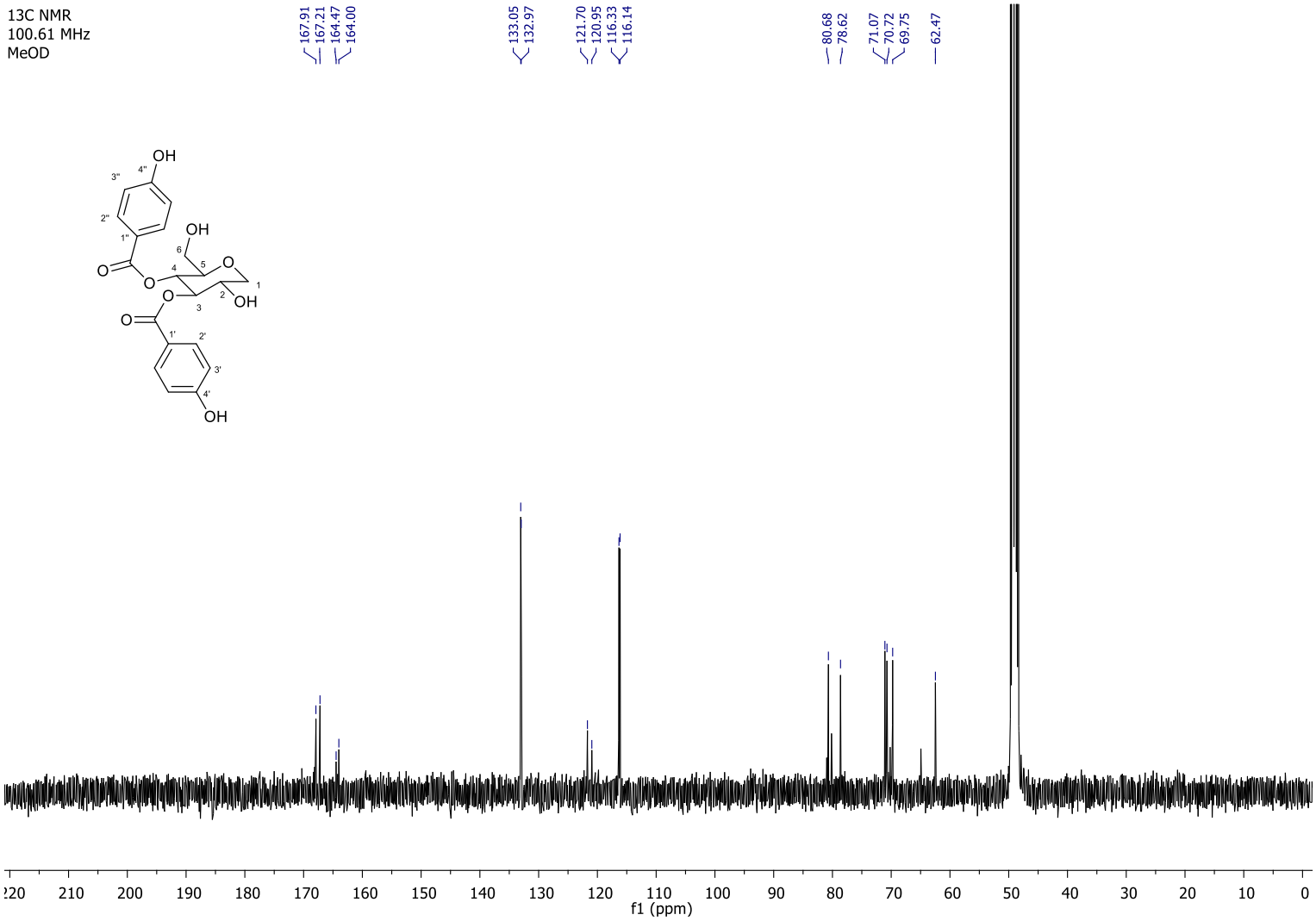


7.6 Compound 45

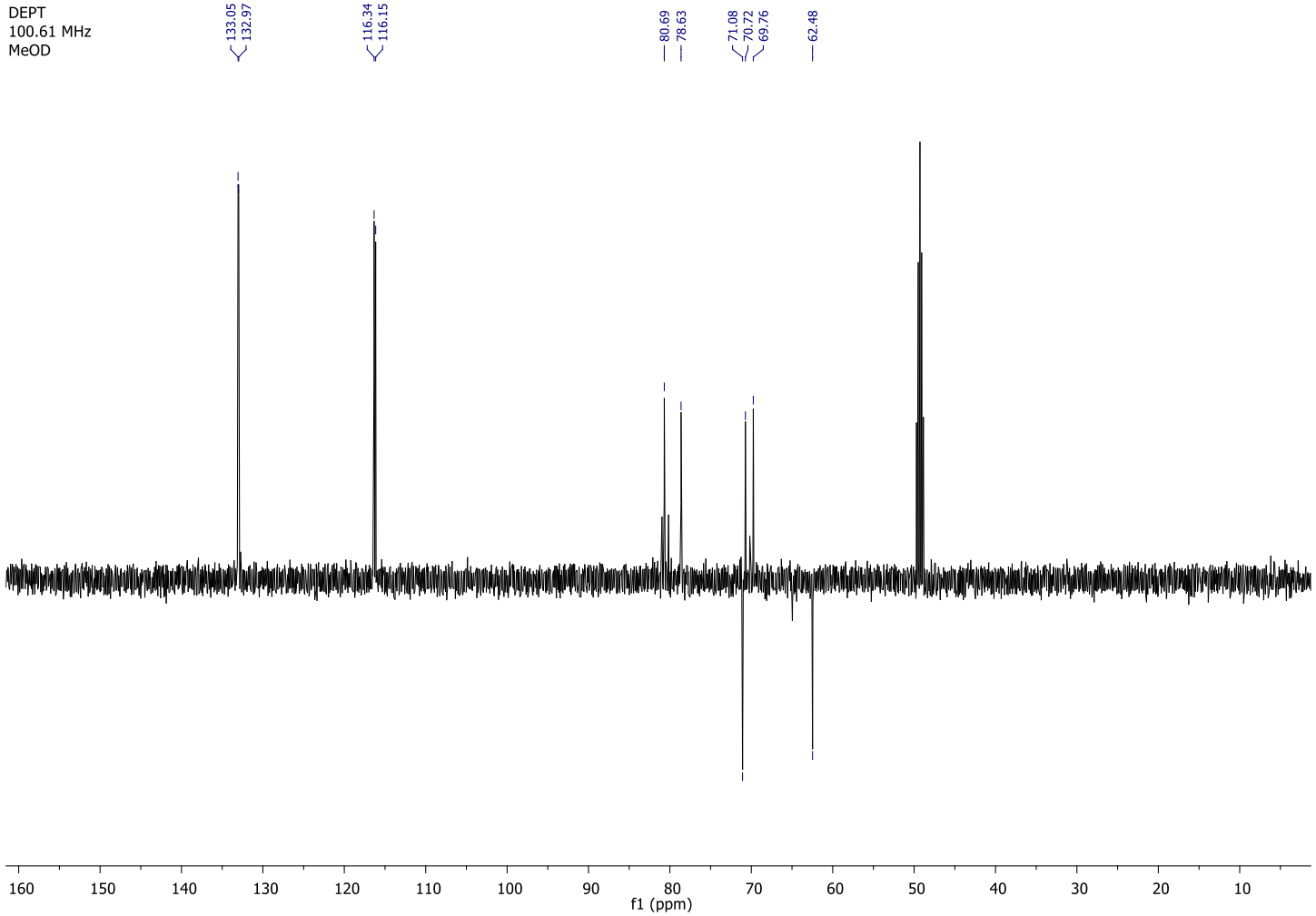
7.6.1 ^{1}H-NMR Spectrum of Compound 45



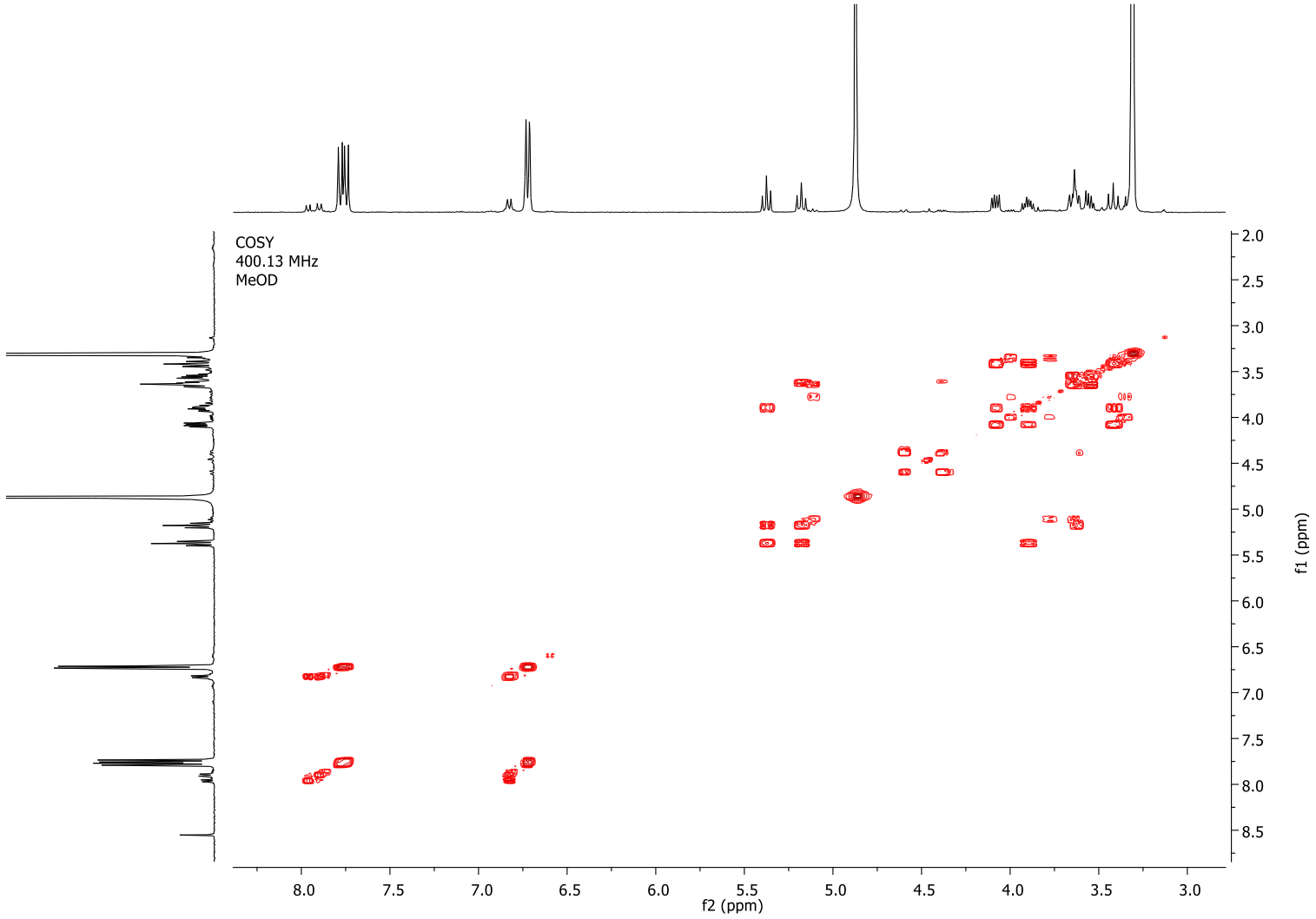
7.6.2 ^{13}C-NMR Spectrum of Compound 45



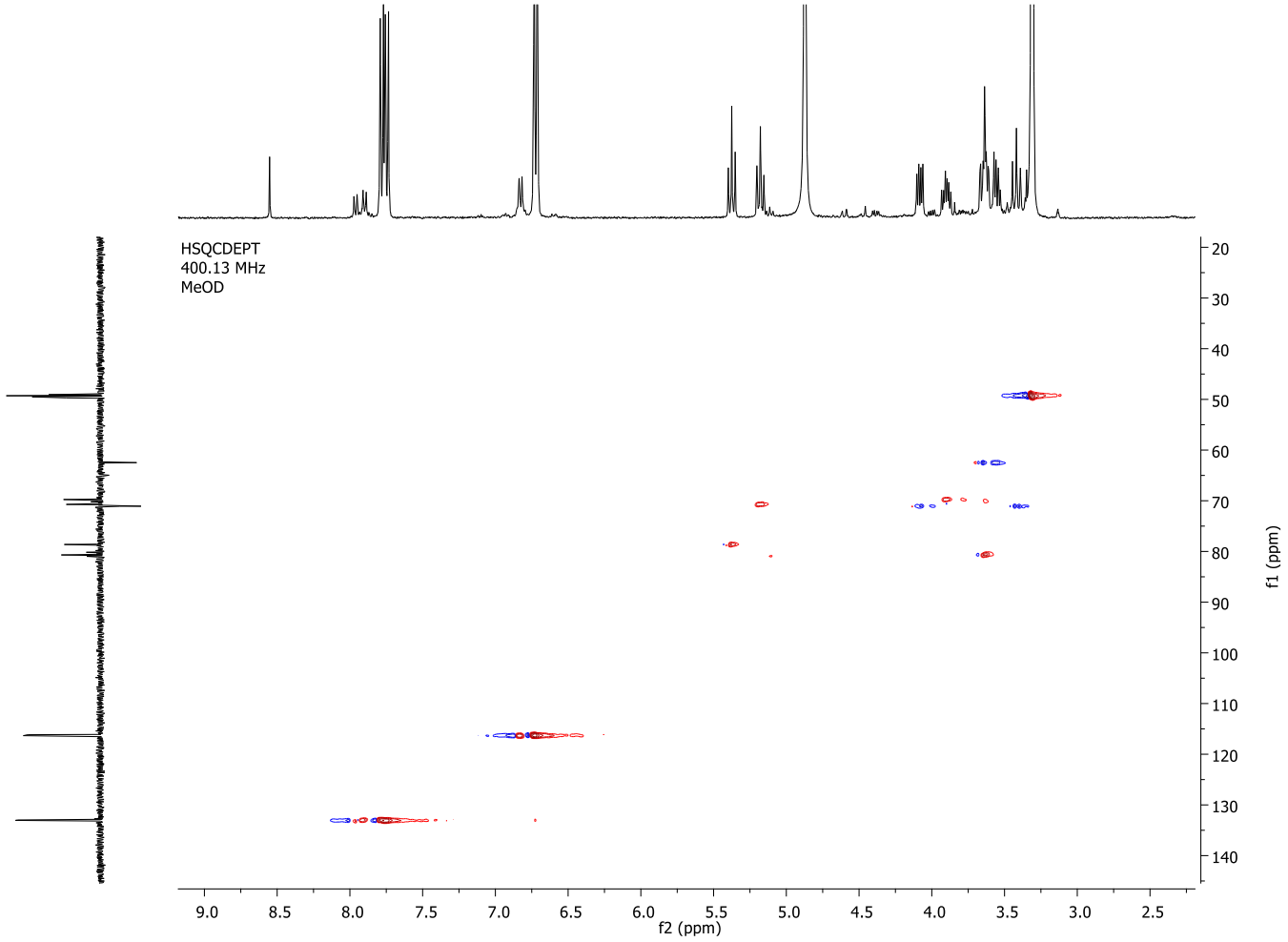
7.6.3 DEPT spectrum of compound 45



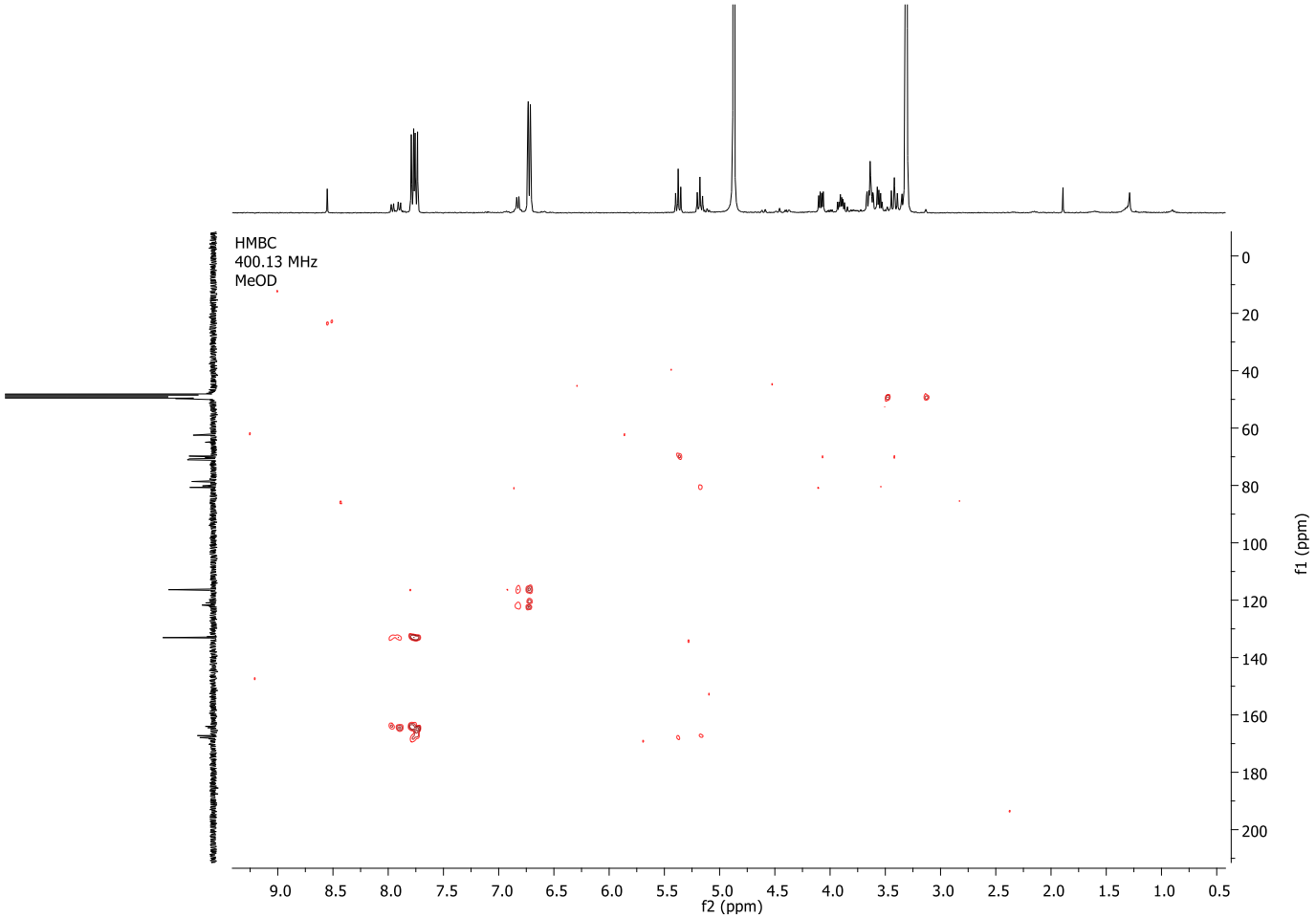
7.6.4 ^{1}H-^{1}H COSY Spectrum of Compound 45



7.6.5 HSQCDEPT Spectrum of Compound 45



7.6.6 HMBC Spectrum of Compound 45



7.7 Research Permit

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Ref No: **907244**

Date of Issue: **25/May/2023**

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7.8 Publication

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SPC

Advanced Journal of Chemistry-Section B
Natural Products and Medical Chemistry

Journal homepage: http://www.ajchem-b.com/

Original Research Article

Chemical Constituents of *Dovyalis abyssinica* (A. Rich.) Warb Fruits and Their Antioxidant Effects

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ABSTRACT

The antioxidant capacity and total phenolic content of extracts from ripe *Dovyalis abyssinica* fruits were examined in this work. The fruits' chemistry was further detailed after isolation and purification studies. Methanol and ethyl acetate extracts yielded 921.79 ± 1.63×10^{-3} mg GAE/100g and 517.95 ± 1.4×10^{-3} mg GAE/100g phenolic content. In contrast, the methylene chloride and hexane extracts gave 261.54 ± 1.0×10^{-3} mg GAE/100g and 24.36 ± 8.2×10^{-3} mg GAE/100g, respectively. From these extracts, data on the IC_{50} of the four extracts showed significantly higher antioxidant capacity in the methanol extract (4.4 µg/mL) than that of the ethyl acetate, methylene chloride, and hexane extracts (8.4 µg/mL, 28.8 µg/mL, and 55.8 µg/mL, respectively). Phytochemical analysis gave three known compounds: betulinic acid (**1**), 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol l (**2**), and sitosterol (**3**) via NMR spectroscopic techniques. The nutritional potential of these fruits was validated by their chemical profile, providing a solid foundation for their future exploitation. The three compounds are new reports, specifically from the fruits of *D. abyssinica*. The anhydroglucitol was particularly interesting, as it is a phenol capable of antioxidant activity. To the best of our knowledge, this is the second time that this compound has been reported through this study.

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