Chemical Constituents and Antidiabetic Activities of The Aerial Parts Of *Tiliacora triandra*

Emmanuel Ayobami Makinde

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Thesis Title	Chemical constituents and antidiabetic activities of the aerial parts of *Tiliacora triandra*
Author	Mr. Emmanuel Ayobami Makinde
Major Program	Thai Traditional Medicine

Major Advisor

……
(Dr. Opeyemi Joshua Olatunji)

Examination Committee:

…… Chairperson
(Assoc. Prof. Dr. Kornkanok Ingkaninan)

……..…... Committee
(Asst. Prof. Dr. Sunee Chansakaow)

…….. Committee
(Dr. Opeyemi Joshua Olatunji)

……...Committee
(Asst. Prof. Dr. Chitchamai Ovatlarnporn)

……Committee
(Asst. Prof. Dr. Nantiya Joycharat)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Master of Thai Traditional Medicine degree in Thai Traditional Medicine.

.....

(Prof. Dr. Damrongsak Faroongsarng)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgment has been made of any assistance received.

……...…...….....Signature

(Dr. Opeyemi Joshua Olatunji)

Major Advisor

……...…….....Signature

(Mr. Emmanuel Ayobami Makinde)

Candidate

I hereby certify that this work has not been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

……..……Signature

(Mr. Emmanuel Ayobami Makinde)

Candidate

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Author	Mr. Emmanuel Ayobami Makinde
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ABSTRACT

Initial investigations revealed that the ethanol extract of the whole aerial part, *n*-hexane (TT-001) and ethyl acetate (TT-002) fractions of the leaves as well as the *n*-hexane (TT-004) and ethyl acetate (TT-005) fractions of the twigs of *Tiliacora triandra* exhibited potent alpha-glucosidase and alpha-amylase inhibitory activities. Further investigations led to the isolation of 5,7-dihydroxyl-6-oxoheptadecanoic acid (**1**), 5,7-dihydroxyl-6-oxooctadecanoate (**2**), ethyl(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate (**3)** and ethyl(9*Z*,12*Z*)-octadeca-9,12-dienoate (**4**) from the leaves and twigs of *T. triandra.* Compounds **1** and **2** are new compounds while **3** and **4** were isolated from *T. triandra* for the first time. Compounds **1**, **2**, **3** and **4** showed potent inhibitory activity against alpha-glucosidase enzyme while only compound **1** showed alpha-amylase inhibitory activity. Furthermore, the antidiabetic effect of the ethanol extract of the aerial part of *T. triandra* and compound **1** on high-fat diet/streptozotocin induced diabetic rats was evaluated. Treatment with 100 mg/kg and 400 mg/kg of *T. triandra* extract and 25 mg/kg of compound **1** markedly suppressed insulin resistance, hyperglycemia, polydipsia, polyphagia, lipid profile and liver function enzymes. Furthermore, the extract of *T. triandra* and compound **1** enhanced glucose tolerance, improved body weight, haematological parameters, and reversed damages to the pancreas and displayed protective effects against diabetes induced liver, kidney and testicular damage. Findings from this study revealed that *T. Triandra* and its' constituents demonstrated good *in vitro and in vivo* antidiabetic properties and are potential sources of alternative or complementary therapy for the treatment of diabetes and its' complications.

DEDICATION

This dissertation is dedicated to my beloved siblings Rebecca and Funmilayo Makinde and late mom, Christianah Abidemi Makinde.

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List of abbreviations

ACN	Acetonitrile
ALP	Alanine phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
BUN	Blood urea nitrogen
DHA	5,7-Dihydroxyl-6-oxoheptadecanoic acid
EtOH	Ethanol
FBG	Fasting blood glucose
HB	Haemoglobin
HbA1C	Glycated haemoglobin
HCT	Haematocrit
HDL	High density lipoprotein
HFD	High fat diet
HOMA – IR	Homeostatic model assessment of insulin resistance
HOMA – β	Homeostatic model assessment of beta cell function
HPLC	High performance liquid chromatography
IPGTT	Intraperitoneal glucose tolerance test
IR	Infrared spectroscopy
LDL-C	Low density lipoprotein – cholesterol
LY	Lymphocytes
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MO	Monocytes
NEU	Neutrophil
NMR	Nuclear magnetic resonance spectroscopy
PLT	Platelets
RBC	Red blood cell
RDW	Red cell distribution width
STZ	Streptozotocin

List of abbreviations (continued)

TCO_2	Total carbon dioxide
TG	Triglycerides
UV	Ultraviolet spectroscopy
WBC	White blood cell

Chapter 1

Introduction

1.1. General introduction

Diabetes mellitus is a chronic metabolic disease characterized by hyperglycemia which is a persistent rise in blood glucose. In addition to hyperglycemia, diabetes is also characterized by abnormalities in carbohydrate, protein and lipid metabolism. Diabetes occurs when the pancreatic beta cells (β-cells) produces little or no insulin, or when the body develops resistance to insulin. Diabetes is associated with symptoms such as polydipsia, polyuria, polyphagia and excessive weight loss (Egan and Dinneen, 2019; Crawford, 2017; Ozougwu et al., 2013; Mohammed et al., 2017). Diabetes also leads to severe complications such as neuropathy, nephropathy, retinopathy and cardiovascular diseases (International Diabetes Federation, 2017; Chatterjee et al., 2012; Fox et al., 2016). The global prevalence of diabetes is rapidly on the rise and according to reports from International Diabetes Federation, about 451 million adults had diabetes in 2017 and by the end of 2045 there will be at least 693 million persons suffering from diabetes. Diabetes was responsible for about 5 million deaths in 2017, killing a person every six seconds and at least 352 million persons are at risk of being diagnosed with diabetes (International Diabetes Federation, 2017; Cho et al., 2018).

There are two main types of diabetes, type 1 diabetes which is also known as insulin dependent diabetes mellitus which occurs when the pancreatic β-cells secretes very little or no insulin and type 2 diabetes known as non-insulin dependent diabetes mellitus which is characterized by insulin resistance caused by pancreatic β-cell dysfunction (Egan and Dinneen, 2019; International Diabetes Federation 2017; Martinez et al., 2019). Type 1 diabetes is an autoimmune disease and the pathogenesis remains unclear. However, factors such as obesity, unhealthy diet, excessive consumption of alcohol and cigarette, environmental toxins and sedentary lifestyle are primarily the cause of type 2 diabetes (Chatterjee et al., 2017; Olokoba et al., 2012; Ozougwu et al., 2013; Rios et al., 2015). Genetics is also believed to play an important

role in the development of type 1 and type 2 diabetes because about 25% of the persons suffering from type 1 and type 2 diabetes have a family history of diabetes (Rother, 2007; Chatterjee et al., 2017).

There is no known cure for diabetes, however, diabetes can be managed successfully through a combination of medication and lifestyle changes like physical exercises and improved diet (Rios et al., 2015). Presently, the therapeutic approach for the management of diabetes is focused on keeping the blood glucose level within the normal limits (fasting blood glucose between 70 and 126 mg/dL) and this can be achieved using conventional drugs of various mechanisms (Katisart and Rattana, 2017; Tang et al., 2017; Rios et al., 2015; Heller and Novodvorsky, 2019). Commercially available glucose lowering drugs includes insulin sensitizers such as biguanides and thiazolidinediones, insulin secretagogues such as meglitinides and sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors as well as alpha-glucosidase and alpha-amylase inhibitors (Prabhakar and Doble, 2011; Mohammed et al., 2017, Olokoba et al., 2017). However, these drugs are expensive and associated with serious adverse effects such as hypoglycemia, neurological deficits, gastrointestinal discomforts, lactic acidosis amongst others (Ibrahim et al., 2016; Ekperikpe et al., 2019; Prabhakar and Doble, 2011). At present the management of diabetes without side effects remains a challenge in pharmaceutical research and these drawbacks have spurred increased research interest in medicinal and edible plants with the aim of developing cheaper and more effective treatment for diabetes with little or no side effects (Rios et al., 2015, Tang et al., 2017).

Nature, before the advent of modern medicine was the only source of therapeutics and for thousands of years man solely depended on nature for healthcare and disease prevention (Cragg and Newman, 2005; Lahlou, 2013; Rey-Ladino et al., 2011). The use of medicinal plants for the treatment of various ailments during the ancient civilization of the Indians, Chinese and North Africans are well documented and the use of medicinal plants have formed the basis of today's medical system (Cragg and Newman, 2013; Phillipson, 2001; Butler, 2004).

Medicinal plants or their extracts have been employed by humans since time immemorial for the treatment of different diseases and they continue to provide new and important natural product leads against various pharmacological targets

including diabetes, cancer, cardiovascular diseases, malaria and neurological disorders (Kumar et al., 2015a; Prahabkar and Doble, 2011). Ethnobotanical reports suggest that at least 800 plants are used globally for the treatment and management of diabetes. However, only about 30% of these plants have been evaluated biologically with the bioactive substances responsible for their antidiabetic potential largely unknown (Arumugam et al., 2013; Chinsembu, 2019). Furthermore, less than 1% of the world's over 250,000 higher plants has been screened for their antidiabetic activity, consequently there are huge prospects of discovering new antidiabetic medications from medicinal plants (Arumugam et al., 2013).

A few natural products derived active principles have been used in the treatment of diabetes and some of them are precursors for a number of antidiabetic drugs which are in use today (Chinsembu, 2019; Rios et al., 2015). These includes classes of compounds like alkaloids, phenolics, terpenoids, steroids, guanidine, glycopeptides, amino acids and polysaccharides (Ríos et al., 2015; Joseph and Jini, 2013; Bedekar et al., 2010; Prahabkar and Doble, 2008; Yin et al., 2014). The discovery of metformin a widely used hypoglycemic drug belonging to the class biguanides came from the traditional approach of using *Galega officinalis* for the treatment of diabetes in Europe and Asia (Bailey and Day, 2004). This plant is rich in galegine, a guanidine, derivative with hypoglycemic activity which is present in the basic structure of metformin. Although guanidine was found to be too toxic for clinical use, it formed the basis for the development of biguanindes and later the development of metformin which is one of the most widely prescribed drug for the treatment of diabetes today (Bailey and Day, 2004; Bailey and Day, 1989; Bailey, 1988; Marles and Farnsworth, 1995). Acarbose a widely used alpha-glucosidase and alpha-amylase inhibitor is of microbial origin and was obtained from *Actinoplanes* sp. (Wehmeier, 2003). Also, miglitol which is an α-glucosidase inhibitor was first isolated from different strains of *Bacillus* and *Streptomyces* (Bedekar et al., 2010; Ríos et al., 2015). Valiolamine which was isolated from *Streptomyces hydroscopicus* was the precursor for the synthesis of voglibose which is an alpha-glucosidase inhibitor (Matsuo et al., 1992).

1.2. Alpha-glucosidase and alpha-amylase enzymes

Alpha-glucosidase and alpha-amylase are enzymes which are intricately involved in the breakdown of carbohydrates into smaller molecules in the digestive system. Large insoluble polysaccharides are hydrolysed into smaller absorbable oligosaccharides by alpha-amylase which is found in the saliva and pancreatic juice while alpha-glucosidase which is found in the mucosal brush border of the small intestine finally hydrolyses the oligosaccharides into smaller absorbable glucose molecules before being absorbed into the blood (Ademiluyi and Oboh, 2013; Bhandari et al., 2008; Kazeem et al., 2013; Rizvi et al., 2019). Inhibition of alpha-glucosidase and alpha-amylase causes a delay in the breakdown of carbohydrates to absorbable glucose molecules, which leads to a reduction in the rate of glucose absorption into the blood and consequently lowers the postprandial blood glucose level. Inhibition of alpha-glucosidase and alpha-amylase has been found to be an effective approach in the management of diabetes (Ademiluyi and Oboh, 2013; Kazeem et al., 2013; Kasipandi et al., 2019; Luo et al., 2019; Rizvi et al., 2019).

Synthetic drugs such as acarbose, voglibose and miglitol are commercially available alpha-glucosidase and alpha-amylase inhibitors. However, they show some undesirable side effects like diarrhea, flatulence and abdominal pains. These drugs also have toxic effects on the liver (Irons and Minze, 2014; Kasipandi et al., 2019; Luo et al., 2019; Rizvi et al., 2019). These challenges have necessitated the search for natural alternatives with enhanced potency and lesser or no side effects.

1.3. High-fat diet/streptozotocin induced diabetes model

Over the years, various animal models have been used for diabetic studies and most of these models make use of very toxic chemicals like alloxan and streptozotocin (STZ). STZ which was first used in 1993 is the most commonly used chemical for the induction of diabetes (Ghasemi et al., 2014; Szkudelski, 2001; Lenzen, 2008). Streptozotocin is a nitrosourea analogue which has selective cytotoxic effect on β-cells. When introduced into the body STZ is transported to the β-cells through the glucose transporters 2 (GLUT 2), causing necrosis of the β-cells through DNA alkylation or breakage. This essentially results in mild or severe impairment of insulin secretion depending on the dose of STZ used (Lenzen, 2008). A major disadvantage of

STZ is that at high doses, STZ severely impairs insulin secretion and may also lead to ketone body formation and spillage into the urine. However, induction of diabetes with low dose of STZ mildly impairs insulin secretion, eliminates the possibility of ketone spillage and ketone body formation but does not result in insulin resistance as seen in diabetes, especially in type 2 diabetes (Gheibi et al., 2017a; Kashfi et al., 1995). It has been reported that feeding animals with high fat diet (HFD) causes insulin resistance and obesity but not necessarily diabetes. Thus, this formed the basis for the combination of HFD and low dose of STZ for the induction diabetes (Kern et al., 1990; Kraegen et al., 1991; Reed et al., 2000).

High fat diet/streptozotocin (HFD/STZ) diabetic model was first reported by Reed et al. (2000). This model was improved in 2005, when diabetes was induced using a lower dose of STZ after dietary manipulation with HFD (Srinivasan et al., 2005). The key advantage of HFD/STZ induced diabetes is that it makes it possible to reproduce the pathogenisis of human type 2 diabetes in animal models (Gheibi et al., 2017a; Reed et al., 2000). This is because insulin resistance is a key feature in diabetes (especially type 2) and initial dietary manipulation of animals with HFD causes insulin resistance in the animals which makes their pancreatic β-cells more susceptible to the diabetogenic effect of STZ at low dose (Asrafuzzaman et al., 2017; Skovso, 2014).

1.4. The genus *Tiliacora*

Tiliacora is a genus of the *Menispermaceae* family consisting of approximately 22 species. Amongst these species, 20 are reportedly distributed in Africa while two are native to Southeast Asia (De Wet et al., 2016). Although the genus *Tiliacora* consists of 22 species, information available in literatures are limited to only four species of this genus (*T. triandra*, *T. acuminata*, *T. funifera* and *T. dinklagei*). Species of this genus are climbing plants characterised by shiny dark green leaves with ovate shape and thin apex. Plants of this genus are used traditionally as antimalarial agents and to treat snake bites, gastrointestinal diseases and menstrual problems (De Wet et al., 2016; Molander et al., 2012; Oliver-Bever, 1983).

The root of *T. funifera* is mixed with other plants to increase women fertility in South Africa while in Congo the fruits and the sap from the fruit are included in herbal remedies for prevention of insanity. In Ghana, *T. funifera* is used to treat liver

diseases, gastric fever, hernia and menstrual problems (De Wet and Van Wyk, 2008, 2016; Tackie, 1973). In Ayurvedic medicine *T. acuminata* is used as antidotes for snake bites, insect bites and to treat filariasis. The root extract and the methanolic extract of the leaves of *T. acuminata* has been reported to possess acetylcholinesterase inhibitory activity, antimicrobial, antitumor, antibacterial, antioxidant and nootropic activities (Hossain et al., 2013; Vivek et al., 2017). The ethanolic extract of the leaves of *T. acuminata* displayed potent antinociceptive and antidiarrheal activities (Ben et al., 2013; Chandrakanthan et al., 2014; Hossain et al., 2013).

Several phytoconstituents have been isolated from the genus *Tiliacora* with the majority being bisbenzylisoquinoline alkaloids. Bisbenzylisoquinoline alkaloids such as tiliacorine, tiliacorinine, tiliamosine, tiliaresine, tiliarine, *N*-methyltiliamosine, tiliacosine, magnoflorine and tiliasine were previously isolated from *T. acuminata* and these alkaloids were reported to possess hypotensive, hepatoprotective, antimicrobial, antimalarial, antitumor, antifungal and antioxidant activities (Ray et al., 1989; Ray et al., 1990; Vivek et al., 2017; Sylvester et al., 2018). Nortiliacorine A and nortiliacorinine A were isolated from *T. funifera* (Tackie et al., 1972). Oblongine, dinklacorine and tiliageine were also isolated from the roots of *T. dinklagei* (Dwuma-Badu et al., 1980). Two novel esters octyl (benzoylamino)acetate and heptadeca-4-ene-acetate were isolated from *T. acuminata* (Joseph et al., 2009).

1.5. *Tiliacora triandra*

T. triandra (Colebr.) Diels known in Thailand as Ya-nang is a flowering plant native to Southeast Asia especially Thailand and Laos. It is a common vegetable in the cuisines of northeast Thailand and Laos (Singthong et al., 2009; Singthong et al., 2010). *T. triandra*, is a climbing plant with deep green ovate shaped leaves and yellow flowers. In Thailand, the extract from the root of *T. triandra* is used for the treatment of fever and malaria while the juice made from the leaves is used traditionally as an anticancer and immunomodulator (Wiriyachitra and Phuriyakorn, 1981; Rattana et al., 2010; Rattana et al., 2016; De wet et al., 2016). According to traditional healers, drinking a decoction of the root or climber of *T. triandra* lowers the blood glucose level in diabetic patients (Neamsuvan et al., 2015a). In Thailand, decoction made from the root of *T. triandra* is used for the treatment of gastrointestinal diseases and the flower

is used for the treatment of various skin diseases (Neamsuvan et al., 2015b; Neamsuvan et al., 2016). Boiled fresh roots, leaves and twigs of *T. triandra* were used in the treatment of hypertension in Thai folklore medicine (Neamsuvan et al., 2018).

Figure 1. *Tiliacora triandra*

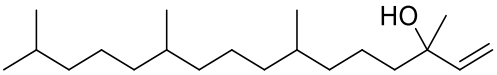
The methanolic extract of the stem bark and root of *T. triandra* demonstrated potent antifungal activity and antibacterial activity. The highest antibacterial activity was observed against gram-negative *Escherichia coli* and the extracts also showed moderate activity against *Shigella sonnei*, *S. dysenteriae*, *Agrobacterium spp* and *Aspergillus niger* (Rahman et al., 2017). The methanolic extract from the root of *T. triandra* showed acetylcholinesterase inhibitory activity, demonstrating its potency as a treatment for neurological disorders such as Alzheimer's disease (Ingkaninan et al., 2003; Mukherjee et al., 2007). The water extract of the aerial part of *T. triandra* is effective in the treatment of neurological disorder induced by alcohol and it also possesses antitumor and anti-cancer activity (Janeklang et al., 2014; Phunchago et al., 2015). The leaf extracts of *T. triandra* has high antioxidant activity due to its high flavonoid and phenolic content (Katisart and Rattana, 2017). Essential oils found in *T. triandra* has been reported to exhibit antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Salmonella specie* (Naibaho and Kerdchoechuen, 2012). *T. triandra* was said to be a potential source of novel chemosensitizer because the hexane extract of the leaves reduced the etoposide resistance of A549RT-eto cell line (Assavalapsakul et al., 2014).

Chemical analysis have shown that the leaves of *T. triandra* contains high level of β-carotene and minerals like calcium and iron (Singthong et al., 2009). Studies have been conducted to identify the chemical composition of the essential oils from the leaves of *T. triandra*, which were identified as isophytol, linoleic acid, hexadecanoic acid, linalool, α-terpineol, *p*-vinylguaiacol, 1-hexanol, β-damascenone, neophytadiene, tetradecanoic acid, 3-hexen-1-ol, spathulenol and benzeneacetaldehyde (Naibaho and Kerdchoechuen, 2012). Also, analysis of the hexane extract of the leaves of *T. triandra* showed that the extract contains a mixture of hexadecanoic acid, octadecanoic acid and (*Z*)-6-octadecenoic acid (Assavalapsakul et al., 2014).

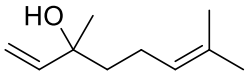
T. triandra like other species of *Tiliacora* is rich in bisbenzylisoquinoline alkaloids. Tiliacorine and tiliacorinine were isolated from the root of *T. triandra*. Tiliacorinine was reported to possess antimalarial activity at an IC_{50} value of 2.09 μg/mL (Nutmakul et al., 2016; Pavanand et al., 1989; Saiin and Markmee, 2003). Tiliacorinine inhibited the proliferation of human cholangiocarcinoma cell lines at an IC_{50} value of 4.5-7 μM and also reduced tumour growth in cholangiocarcinoma xenografted mice at a dose of 10 mg/kg (Janeklang et al., 2014).

Another bisbenzylisoquinoline alkaloid, nortiliacorinine A isolated from the roots of *T. triandra* have been reported to exhibit antimycobacterial activity against clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*, with MIC value of 3.1 μg/mL (Sureram et al., 2012). Yanangcorinine which was also isolated from the root of *T. triandra* was reported to show potent anti-plasmodial activity at an IC_{50} value of < 2 μg/mL (Nutmakul et al., 2016). Also, tiliacorinine 2′-*N*-oxide was isolated from the root of *T. triandra* (Wiriyachitra and Phuriyakorn, 1981). Dinklacorine, tilianangine, noryanangine, norisoyanangine, yanangine, nortiliacorine A, tilitriandrine, 2′-*N*-methyltilitriandrine and tiliageine are other bisbenzylisoquinonine alkaloids isolated from the aerial parts of *T. triandra* but there are no reports on their pharmacological activity (Pachaly and Khosravian, 1988a; Pachaly and Khosravian, 1988b; Pachaly and Tan, 1986). Magnoflorine which was isolated from the aerial part of *T. triandra* displayed potent alpha-glucosidase inhibitory against at an IC_{50} value of 7.8 and 9.8 μg/mL for maltase and sucrase, respectively (Patel and Mishra, 2012). Oxoanolobine was isolated from the leaves of *T. triandra* and have been reported to demonstrate anti-cancer activity. Oxoanolobine showed moderate activity against NCI-

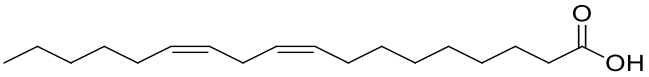
H187 cell lines at an IC_{50} value of 27.60 μg/mL (Rattana et al., 2016). Toxicity studies revealed that *T. triandra* when administered orally does not cause acute or sub-chronic toxicities in either male or female rats (Sireeratawong et al., 2008) and due to its long history of consumption as food and its use as traditional medicine especially amongst the Thais, *T. triandra* is considered safe for human consumption (Chaveerach et al., 2016).



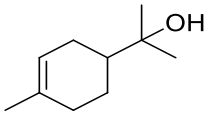
Isophytol



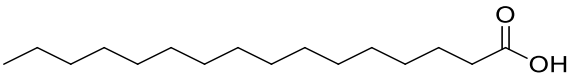
Linalool



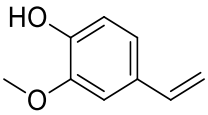
Linoleic acid



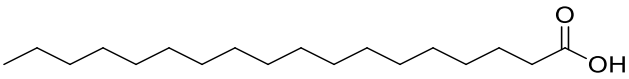
α-Terpineol



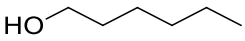
Hexadecanoic acid



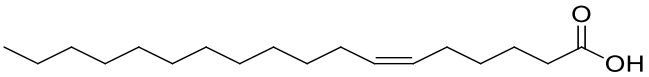
p-Vinylguaiacol



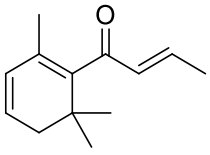
Octadecanoic acid



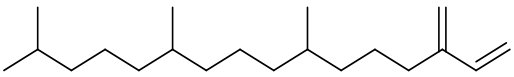
1-Hexanol



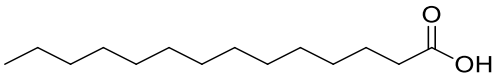
(*Z*)-6-octadecenoic acid



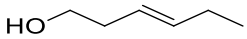
β-Damascenone



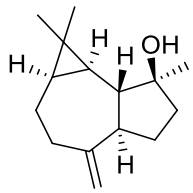
Neophytadiene



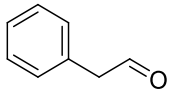
Tetradecanoic acid



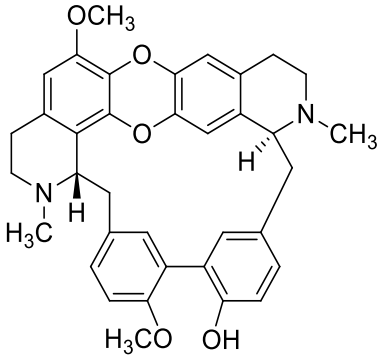
3-Hexen-1-ol



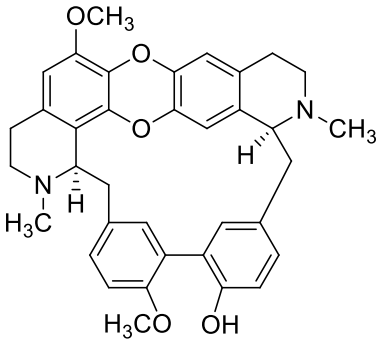
Spathulenol



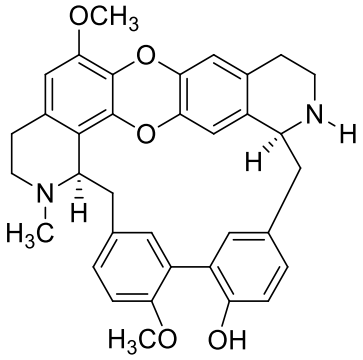
Benzeneacetaldehyde



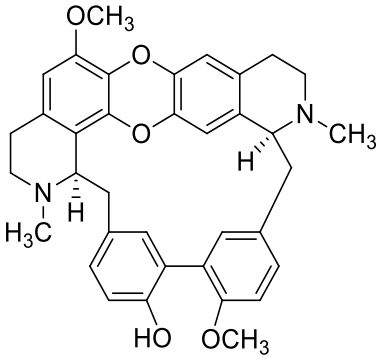
Tiliacorine



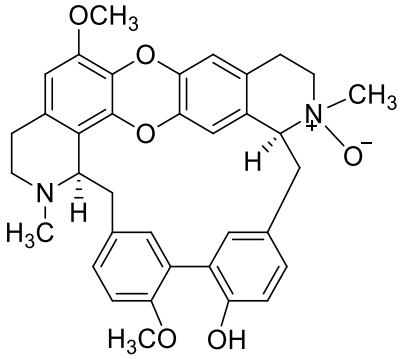
Tiliacorinine



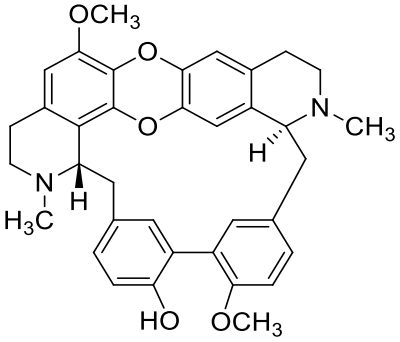
Nortiliacorinine A



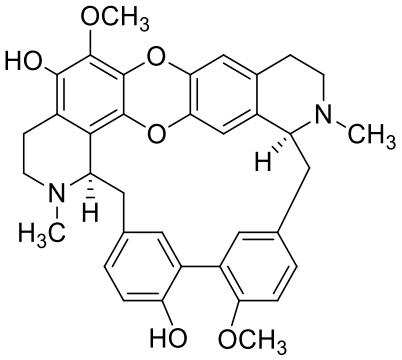
Yanangcorinine



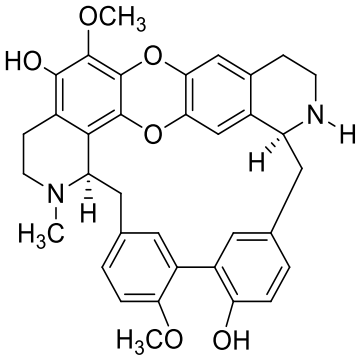
Tiliacorine 2´-*N*-oxide



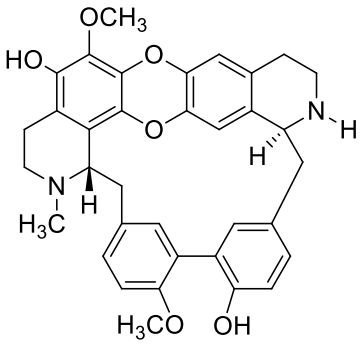
Dinklacorine



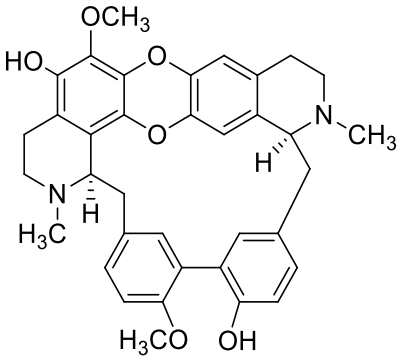
Tilianangine



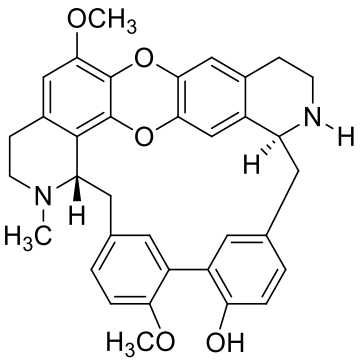
Noryanangine



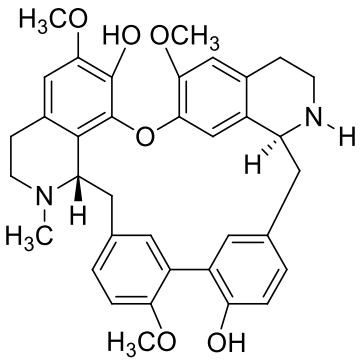
Noryisoyanangine



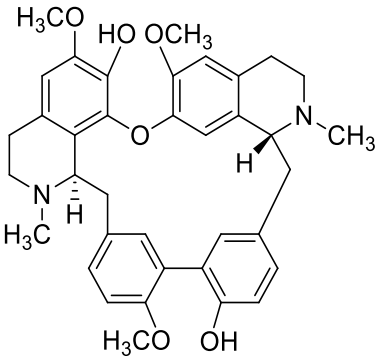
Yanangine



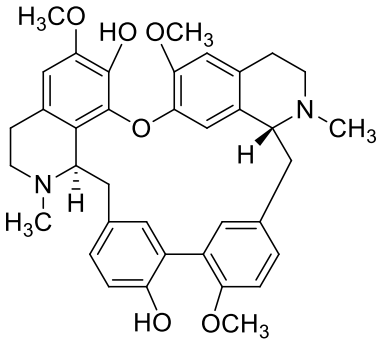
Nortiliacorine A



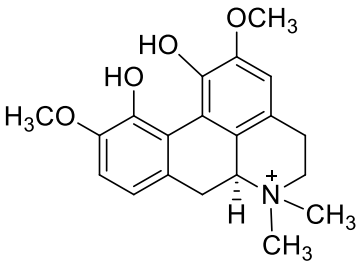
Tilitriandrine



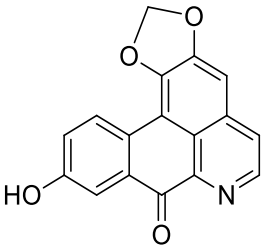
2´-*N*-methyltilitriandrine



Tiliageine



Magnoflorine



Oxoanolobine

1.6. Rationale and objectives

Today, diabetes is not just a global public health epidemic, it is also a huge societal challenge causing sufferings to families by imposing huge financial burden. The prevalence of diabetes has resulted in increased global public health expenditure and governments worldwide are struggling to cope with the cost of diabetic care (Chatterjee et al., 2017; International Diabetes Federation, 2017; Olokoba et al., 2012). Despite the huge progress made towards developing an effective treatment for diabetes,

drugs which are currently used to treat diabetes are expensive and also associated with various side effects such as hypoglycemia, heart failure, diarrhea, nausea and vomiting (Mohammed et al., 2017). These challenges have necessitated the exploration of natural products for new antidiabetic treatments.

Given the rising prevalence of diabetes on a global scale and the limitations associated with the management of the disease and its complications it is evident that there is a need to urgently explore nature particularly medicinal plants for bioactive agents with antidiabetic properties as this could provide a more potent, safer and cheaper alternatives to available treatments. Previous reports have suggested that *T. triandra* is possesses antidiabetic potential, a study reported that the ethanol extract of the leaf of *T. triandra* displayed potent hypoglycemic activity in STZ induced diabetic rats (Katisart and Rattana, 2017). However, the compounds responsible for this activity remains unknown and there are also no reports on the ability of *T. triandra* and its constituents to ameliorate diabetes induced complications. Hence, the research described in this dissertation is focused on unraveling the antidiabetic capabilities of the extracts and constituents from the aerial parts of *T. triandra.*

The objectives of this research are:

- To establish the alpha-amylase and alpha-glucosidase inhibitory activities of the extracts of *T. triandra.*
- To isolate and characterize bioactive chemical constituents from the aerial part of *T. triandra.*
- To determine the alpha-amylase and alpha-glucosidase inhibitory activities of the isolated compounds
- To assess the effect of the crude ethanol extract and the major active constituent of the aerial part of *T. triandra* in high-fat diet/streptozotocin induced diabetic rats.

Chapter 2

Materials and Methods

2.1. General experimental procedures

All chromatographic materials and chemicals were used as purchased except otherwise stated. Solvents used for silica gel column chromatography and other general purposes were of commercial grade and were distilled prior to use. High performance liquid chromatography (HPLC) analysis were performed using HPLC grade acetonitrile (RCI Labscan Ltd., Bangkok Thailand) and ultrapure water. Thin layer chromatography was performed using SiO_2 pre-coated aluminum sheets, 0.2 mm thickness (DC-Fertigfolien ALUGRAM® Xtra SIL G/UV_{254}). Column chromatography was performed using SiO_2 60, purchased from VertiFlash Silica (60 1001-1405 Å, 60-200μm, Vertical Chromatography Co., Ltd., Bangkok, Thailand). Semi-preparative HPLC was performed on Jasco HPLC-4000 preparative (Jasco Inc., Easton, USA) using Vertisep GES C18 HPLC column (10 x 250 mm, 5 μm, Vertical Chromatography Co., Ltd., Bangkok, Thailand).

Nuclear magnetic resonance spectrum was recorded on Fourier Transform NMR spectrometer 500 MHz, Unity Inova (Varian, Germany, 500 MHz ^{1}H; and 125 MHz ^{13}C; $CDCl_3$ as solvent). Mass spectrum was recorded on 1290 Infinity II LC-6545 Quadrupole-TOF spectrometer (Agilent Technologies, USA). Infrared spectroscopy was done using Perkin Elmer FT-IR Spectrum Gx (Massachusetts, USA) and UV spectrum were recorded on Shimadzu UV-Vis Spectrophotometer UV-2600, (Shimadzu, Kyoto, Japan). Optical rotations were recorded on Kruss A. Kruss Optronic polarimeter (Kruss, Germany).

2.2. Plant material

The aerial parts of *T. triandra* was collected from Phattalung province in Thailand and authenticated at the faculty of Traditional Thai Medicine, Prince of Songkla University Hat Yai Thailand. Voucher specimen with voucher number TTM/TT/001 was deposited at the herbarium of the Faculty of Traditional Thai Medicine, Prince of Songkla University Hat Yai Thailand.

2.3. Extraction and isolation

The leaves and twigs of *T. triandra* were dried separately and ground to fine powder. The powdered leaves (800 g) and twigs (500 g) of *T. triandra* were exhaustively extracted by cold maceration in ethanol. The solvent was filtered off and the resulting filtrate was evaporated under reduced pressure to yield crude ethanol extract of leaves (55.87 g) and twigs (28.33 g). The crude ethanol extracts were resuspended in water and partitioned with hexane, ethyl acetate and butanol. This yielded hexane (TT-001, 16.74 g), ethyl acetate (TT-002, 9.83 g) and butanol (TT-003, 2.36 g) extracts of the leaves, as well as the hexane (TT-004, 3.71 g), ethyl acetate (TT-005, 6.91 g) and butanol (TT-006, 1.89 g) extracts of the twigs. In order to check for similarities and to determine the best solvent system for elution during column chromatography all the extracts were monitored using thin layer chromatography (TLC).

TT-002 (1.5 g) was further separated using silica gel column chromatography and eluted with a gradient solvent system of petroleum ether:acetone (4:1 to 1:4). This yielded 58 fractions which were monitored with TLC and combined based on similarities in their TLC fingerprints to obtain 9 pooled fractions: F1 (284.3 mg), F2 (132.5 mg), F3 (151.5 mg), F4 (48.7 mg), F5 (26.7 mg), F6 (15.4 mg), F7 (76.1 mg), F8 (18.0 mg) and F9 (33.7 mg).

Compound **1** (6.7 mg) was obtained from F3 (151.5 mg) after it was purified by reverse phase semi-preparative HPLC using a mobile phase containing 87% acetonitrile (ACN) and 13% H_2O, a flow rate of 3.5 mL/min and detected using a photodiode array detector at a wavelength of 210 nm.

TT-005 (1.8 g) was separated by silica gel column chromatography using a gradient solvent system of petroleum ether:acetone (4:1 to 1:4) to yield 60 fractions. These fractions were monitored using TLC and fractions showing similar TLC fingerprints were pooled to yield 9 fractions: F10 (268.4 mg), F11 (337.8mg), F12 (152.6 mg), F13 (223.0 mg), F14 (128.5 mg), F15 (31.5 mg), F16 (39.4 mg), F17 (145.9 mg) and F18 (240.2 mg). Fraction F11 (337.8 mg) was subjected to reverse phase semi-preparative HPLC using a mobile phase of 90% ACN and 10% H_2O, a flow rate of 4 mL/min and detected using a photodiode array detector at a wavelength of 210 nm. This

afforded compounds **2** (47.7 mg), **3** (9.4 mg) and **4** (27.1 mg). Compound **1** (98.6 mg) was also isolated from fractions F12 (152.6 mg) and F13 (223.0 mg) after they were purified with reverse phase semi-preparative HPLC using 87% ACN and 13% H_2O as the mobile phase, a flow rate of 3.5 mL/min and detected using a photodiode array detector at a wavelength of 210 nm.

2.3.1. 5,7-dihydroxyl-6-oxoheptadecanoic acid (**1**): yellow oil, $[\alpha]_D^{25}$ -15 (*c* 0.015, EtOH); UV (EtOH) λ_{max} (log ε): 410 (1.74), 666 (0.75); FT-IR ν_{max} 3446, 2923, 2852, 1736, 1365, 1217, 1094; HR-ESI-MS *m/z* 317.20748 $[M + H]^+$, (calcd. 317.2322 for $C_{17}H_{33}O_5$). ^{13}C and ^1H NMR data are summarised in Table 2.

2.3.2. 5,7-dihydroxy-6-oxooctadecanoate (**2**): yellow oil, $[\alpha]_D^{25}$ 24 (*c* 0.012, EtOH), UV (EtOH) λ_{max} (log ε): 414 (0.41), 434 (0.48) 657 (0.08), FT-IR ν_{max} 3442, 2925, 2854, 1735, 1647, 1455, 1376, 1260; HR-ESI-MS *m/z* 391.2859 $[M + CH_3OH + H]^+$, (calcd. 391.3054 for $C_{21}H_{43}O_6$). ^{13}C and ^1H NMR data are summarised in Table 3.

2.3.3. Ethyl(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate (**3**) (CAS No: 1191-41-9): yellow oil, $[\alpha]_D^{25}$ 8 (*c* = 0.010, EtOH), UV (EtOH) λ_{max} (log ε): 208 (3.24), 432 (0.76), FT-IR ν_{max} 3011, 2927, 2854, 1737, 1460,1376, 1261; HR-ESI-MS *m/z* 329.2448 $[M + Na]^+$, (calcd. 329.4683 for $C_{20}H_{34}O_2Na$). ^{13}C and ^1H NMR data are summarised in Table 4.

2.3.4. Ethyl(9*Z*,12*Z*)-octadeca-9,12-dienoate (**4**) (CAS No: 544-35-4): yellow oil, UV (EtOH) λ_{max} (log ε): 210 (3.24), 432 (0.76), FT-IR ν_{max} 2970, 1736, 1365, 1217; $C_{20}H_{36}O_2$. ^{13}C and ^1H NMR data are summarised in Table 5.

2.4. *In vitro* antidiabetic activity

2.4.1. Determination of alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity was determined using a previously reported method with some modifications (Kumar et al., 2013). Stock samples of each extract and compound from *T. triandra* were prepared in ethanol at a concentration of 10,000 µg/mL and further diluted to concentrations ranging from 0.1–5,000 µg/mL with phosphate buffer (pH 6.9, 50 mM). A reaction mixture containing 50 µL of extracts or compounds and 50 µL of 0.57 unit/mL alpha-glucosidase enzyme was added to a 96-well plate and incubated for 10 mins at 37°C. After incubation, 50 µL of *p*-nitrophenyl-α-$_D$-glucopyranoside (1.5 mg/mL) was added and the mixture was further incubated for 20 mins. The reaction was stopped with 50 µL Na_2CO_3 (1.0 M)

and absorbance was measured at 405 nm. Acarbose was used as the standard drug and phosphate buffer was used instead of the extracts and compounds as the negative control and instead of α-glucosidase enzyme as the blank. The concentration of extracts or compounds which inhibits 50% of alpha-glucosidase enzyme (IC_{50}) was calculated from linear regression. Percentage inhibition was determined according to the equation:

$$\%\text{inhibition} = [A_n - (A_s - A_{bs})/A_n)] \times 100$$

Where: A_n = absorbance of negative solution (no extract or compound)

A_s = absorbance of sample solution

A_{bs} = absorbance of blank sample solution (no enzyme)

2.4.2. Determination of alpha-amylase inhibitory activity

The alpha-amylase inhibitory activity was determined using previously reported method with slight adjustments (Chakrabarti et al., 2014; Sudha et al., 2011). Each of the extract and compound were dissolved in ethanol and prepared at a stock concentration of 10,000 µg/mL. Afterward, the stock solution of extracts and compounds were diluted with 20 mM phosphate buffer (pH 6.9) to concentrations ranging from 0.5–5,000 µg/mL. A mixture containing 20 µL of the extracts or compounds, 20 µL of phosphate buffer and 20 µL of 1% starch solution was added to a 96-well plate and incubated in a shake-incubator at 37°C for 3 mins. After incubation, 20 µL of porcine pancreatic α-amylase enzyme solution (12.8 units/mL) was added and the resulting mixture was further incubated at 37°C for 15 mins. The reaction was stopped with 1 M hydrochloric acid (20 µL), followed by the addition of 100 µL of 2.5 mM iodine test solution. Acarbose was used as the standard drug and absorbance was measured at 630 nm. The concentration of compound or extract which inhibits 50% of alpha-amylase enzyme (IC_{50}) was calculated from linear regression. The percentage inhibition was determined according to the equation below:

$$\%\ \text{inhibition} = [1 - (A_{bs} - A_s)/(A_{st} - A_n)] \times 100$$

Where: A_s = absorbance of sample solution

A_{bs} = absorbance of mixture without the enzyme

A_n = absorbance of mixture without the sample

A_{st} = absorbance of mixture without sample and enzyme

2.5. Preparation of *T. triandra* extract for animal experiment

Freshly collected aerial part of *T. triandra* was washed and dried at 50 ^{o}C. The dried aerial part was pulverized into fine powder and extracted exhaustively by cold maceration in ethanol. The mixture was filtered daily and the filtrate was concentrated under reduced pressure with a rotary evaporator to yield the crude ethanol extract.

2.6. Animal experiment

2.6.1. Animal model and induction of diabetes

Male Sprague Dawley rats weighing between 160–200 g were purchased from Nomura Siam International Co. Ltd. Bangkok, Thailand. The animals were kept in steel cages and housed at the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Thailand at a temperature of 23 ± 2 ^{o}C and relative humidity of 50 ± 5 %, while a 12-hour light/dark cycle was maintained. The animals were fed with normal pelletized diet and allowed to adjust to their new environment for seven days. After the period of acclimatization, the animals were separated into two groups and given two different feeding regimes. The first group were fed with normal rat pellets, while the second group was fed with high-fat diet (HFD) and 15% (w/v) fructose solution for four weeks. The HFD was formulated based on previous literature as shown in Table 1 (Gheibi et al., 2017b). After 4 weeks of dietary manipulation, animals were fasted for 12 hours and diabetes was induced by intraperitoneal injection of 35 mg/kg STZ, which was freshly prepared in ice cold 0.1M citrate buffer (pH 4.5). Blood was collected from the tail vein of the animals and used to determine the fasting blood glucose (FBG) level 3 – 5 days after diabetes was induced with the aid of a glucometer (Accu-Chek Perfoma, Mannheim, Germany). Animals which had FBG level ≥ 250 mg/dL were considered diabetic and included in the study. This study received ethical clearance approval from the Institutional Animal Care and Use Committee, Prince of Songkla University with approval number MOE0521.11/1231.

Table 1. Composition of High-fat diet

Composition	Amount (g)
Normal pelletized diet	500.00
Butter	300.00
Mineral mix	28.70
Soya	73.20
DL methionine	1.80
Coconut oil (mL)	200.00

2.6.2. Experimental design

The rats were separated into five groups of five animals each. Non-diabetic normal control group (NC) treated with 1% tween-80, diabetic control group (DC) treated with 1% tween-80, *T. triandra* 100 (TT100) group treated with 100 mg/kg of ethanol extract of *T. triandra*, (TT400) group treated with 400 mg/ kg of ethanol extract of *T. triandra* and DHA25 group which was treated with 25 mg/kg of compound **1** (5,7-dihydroxyl-6-oxoheptadecanoic acid, and abbreviated as DHA). DHA was chosen because it demonstrated the best alpha-glucosidase and alpha-amylase inhibitory activities amongst all the isolated compounds.

The extract and DHA were dissolved in 1% tween-80 and doses were orally administered daily for 30 days. Changes in FBG, body weight, food and water intake of all the groups were monitored throughout the experiment. After 30 days of treatment, the rats were fasted overnight, anaesthetized by intraperitoneal injection of sodium thiopental (150 mg/kg) and sacrificed by cervical dislocation. Blood samples were collected into EDTA and heparin tubes through cardiac puncture. The blood collected into heparin tubes was centrifuged for 10 mins at 2500 rpm and 4^{o}C to obtain the serum which was used for biochemical analysis and the blood collected into EDTA tubes was used for haematological analysis. The pancreas, liver, kidney and testes were rapidly excised, washed in ice cold phosphate buffered saline, weighed and stored in 10%

buffered formalin for histological assessment. The liver, kidney and testes index were calculated based on the equation given below:

$$\frac{\text{Organ weight}}{\text{Body weight}}$$

2.6.3. Intraperitoneal glucose tolerance test

On the 30th day of treatment, all the groups were fasted for 16 hours, treated with *T. triandra* extract (100 and 400 mg/kg) and DHA25 group (25 mg/kg). The animals were given 2 g/kg of glucose by intraperitoneal injection 30 mins after treatment. The blood glucose level of the animals was checked at 0, 30, 60 and 120 mins after glucose administration.

2.6.4. Homeostatic model assessment of insulin resistance and beta cell function (HOMA-IR and HOMA-β)

HOMA-IR was calculated according to the equation below:

$$\frac{\text{Insulin (μU/mL) x FBG (mg/dL)}}{405}$$

HOMA-β was calculated according to the equation below:

$$\frac{\text{360 x Insulin (μU/mL) \%}}{\text{FBG (mg/dL)} - 63}$$

2.6.5. Biochemical assay

The serum levels of insulin, glycated hemoglobin (Hb1Ac), total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein-cholesterol (LDL-C), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total protein, globulin, albumin, total bilirubin, direct bilirubin, creatinine and blood urea nitrogen (BUN) were determined using Dirui CS 600B autochemistry analyzer. Serum levels of total carbon dioxide (TCO_2), sodium (Na^+), potassium (K^+) and chloride (Cl^-) were analyzed using Siemens Advia 1800 clinical chemistry system.

2.6.6. Haematological analysis

Haematological parameters such as red blood cell (RBC), haemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell (WBC), neutrophil (NEU), lymphocyte (LY), monocyte (MO) and platelet count (PLT) were determined using Horiba ABX Pentra 60 diagnostics haematological series.

2.6.7. Histology of pancreas, liver, kidney and testes

The fixed pancreas, liver, kidney and testes tissues were processed in a tissue processor and embedded in paraffin. The embedded tissues were cut into sections of 5 μm thickness, mounted onto slides, stained with hematoxylin-eosin and examined under light microscope.

2.7. Data analysis

Data are presented as mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$. Graphpad prism 7.0 (San Diego, California USA.) was used for all statistical test.

Chapter 3

Results and Discussion

3.1. Isolation and structure elucidation

The hexane and ethyl acetate extracts from the leaves and twigs of *T. triandra* were subjected to series of chromatographic techniques which led to the isolation four compounds. This section describes the structure elucidation of these compounds. Their structures were determined by extensive use of various spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, ultraviolet/visible (UV/Vis) spectroscopy and mass spectrometry (MS).

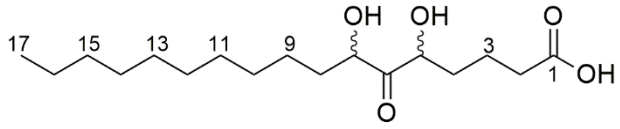
Compound **1** was isolated as a yellow oil, its' UV spectrum displayed maximum absorption at 410 nm and 666 nm, while IR spectrum showed a broad absorption band at 3449 cm^{-1} which corresponds to the stretching band of a hydroxyl group and a sharp band at 1736 cm^{-1} which is characteristic of carbonyls. The HR-ESI-MS spectrum showed a molecular ion peak at *m/z* 317.2090 $[M + H]^+$, corresponding to a molecular formula of $C_{17}H_{32}O_5$ and two degrees of unsaturation.

The ^1H NMR spectrum showed signals of one hydroxyl proton at δ_H 10.00, two hydroxymethine protons at δ_H 5.09 (1H, H-7), 5.05 (1H, H-5); twelve methylenes at δ_H 2.39 (2H, t, *J*= 7.5 Hz, H-2), δ_H 2.04 (2H, dq, *J*= 7.5, 3.0 Hz, H-4), δ_H 1.97 (2H, dq, *J*= 7.5, 3.0 Hz, H-8), δ_H 1.75 (2H, qnt, *J*= 7.5 Hz), δ_H 1.39 (2H, qnt, *J*= 7.5 Hz, H-9), δ_H 1.28 (2H, H-16), δ_H 1.26 (12H, H-10 to H-15) and a methyl at δ_H 0.88 (3H, t, *J*= 7.0, H-17). The examination of the ^{13}C and DEPT NMR spectrum indicated the presence of 17 carbons which consists of a ketone at δ_C 204.0 (C-6), a carbonyl at δ_C 180.2 (C-1), two hydroxymethine carbons at δ_C 91.5 (C-7) and δ_C 89.6 (C-5), 12 methylenes at δ_C 33.3 to 22.6 (C-2 to C-4 and C-8 to C-16) and a methyl carbon at δ_C 14.0 (C-17). ^1H-^1H COSY correlations were observed for H-2/H-3, H-3/H-4, H-4/H-5, H-7/H-8, H-9/H-10, H-14/H-15, H-15/H-16 and H-16/H-17 which suggests the presence of two spin systems, a butanol unit (H-2 to H-5) and an undecanol unit (H-7 to H-17). HMBC correlations from H-4, H-5, H-7 H-8 to C-6 confirmed the ketone was at C-6 and the position of the terminal carboxylic acid group at C-1 was assigned by HMBC correlations from H-2 and H-3 to C-1. HMBC cross peaks from H-5 to C-3, C-4, C-6, and C-7 as well as from H-7 to C-5, C-8 and C-9 established the position of the

hydroxymethine protons while additional HMBC correlations from H-17 to C-15 and C-16 confirmed the position of the methyl group. These data suggested that compound **1** is a keto acid with two hydroxyl substitutions. Based on these spectruml evidences compound **1** was determined to be 5,7-dihydroxyl-6-oxoheptadecanoic acid which is a new compound.

Table 2. NMR data of compound **1** (^{1}H: 500 MHz, ^{13}C: 125 MHz; $CDCl_3$).

Position	δ_C (ppm)	δ_H (ppm, *J* in Hz)	COSY (H→H)	HMBC (H→C)
1	180.2, C	-	-	-
2	33.3, CH_2	2.39 (t, 7.5)	3	1, 3, 4
3	23.9, CH_2	1.75 (qnt, 7.5)	2, 3	1, 2, 4, 5
4	28.1, CH_2	2.04 (dq, 7.5, 3.0)	3, 4	2, 3, 4, 5, 6,
5	89.6, CH	5.05 (overlap)	4	3, 4, 6, 7
6	204.0, C	-	-	-
7	91.5, CH	5.09 (overlap)	8	5, 8, 9
8	28.9, CH_2	1.97 (dq, 7.5, 3.0)	7, 9	6, 7, 9, 10
9	29.1, CH_2	1.39 (qnt, 7.5)	8, 10	7, 8, 10
10-14	29.2-29.6, CH_2	1.26 (overlap)	-	-
15	31.9, CH_2	1.26 (overlap)	14, 16	14, 16, 17
16	22.6, CH_2	1.28 (overlap)	15, 17	14, 15, 17
17	14.0, CH_3	0.88 (t, 7.0)	16	16, 15
COOH	-	10.00 (brs)		



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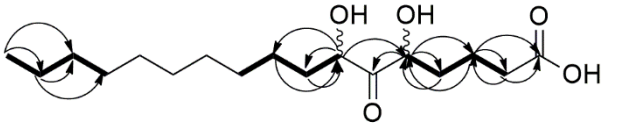


COSY correlations HMBC correlations

Figure 2. Key HMBC and COSY correlations of compound **1.**

Compound **2** was obtained as a yellow oil. The UV spectrum displayed absorption at 414, 434 and 657 nm while the IR spectrum showed a broad absorption band at 3442 cm^{-1} and a sharp absorption peak at 1735 cm^{-1} which are characteristic absorptions for hydroxyl and carbonyl groups respectively. The HR-ESI-MS spectrum showed molecular ion peak at *m/z* 391.2859 $[M + CH_3OH + H]^+$, which corresponds to the molecular formula of $C_{20}H_{38}O_5$ and two degrees of unsaturation.

The ^1H NMR spectrum of compound **2** indicated the presence of two hydroxymethine protons at δ_H 5.08 (1H, H-7), 5.04 (1H, H-5), one oxymethylene at δ_H 4.13 (1H, q, *J*=7.0 Hz, H-1′), 13 methylenes at δ_H 2.33 (2H, t, *J*= 7.5 Hz, H-2), δ_H 2.03 (2H, dq, *J*= 7.5 Hz, 3.0, H-4), δ_H 1.97 (2H, dq, *J*= 7.5, 3.0 Hz, H-8), δ_H 1.74 (2H, qnt, *J*= 7.5 Hz, H-3), δ_H 1.38 (2H, qnt, *J*= 7.5 Hz, H-9), δ_H 1.26 (16H, H-10 to H-17) and two methyl signals at δ_H 1.25 (3H, t, *J*= 7.0 Hz, H-2′) and δ_H 0.88 (3H, t, *J*= 7.0, H-17). The ^{13}C NMR of compound **2** showed 20 carbons signals which consists of a ketone at δ_C 204.0 (C-6), an ester at δ_C 173.7 (C-1), two hydroxymethines at δ_C 91.4 (C-7) and δ_C 89.8 (C-5), an oxymethylene carbon at δ_C 60.2 (C-1′), 13 methylenes at δ_C 33.6 to 22.7 (C-2 to C-4 and C-8 to C-17) and two methyl carbons at δ_C 14.2 (C-2′) and δ_C 14.1 (C-18). The ^1H NMR and ^{13}C NMR spectrum of compound **2** showed obvious and remarkable similarities to that of compound **1** except for the presence of additional signals belonging to the ethoxy group (C-1′ at δ_C 60.1, H-1′ at δ_H 4.13 and C-2′ δ_C at 14.2, H-2′ at δ_H 1.25) and a methylene (C-15 at δ_C 29.2–29.6, H-15 at δ_H 1.26). These differences suggested that compound **2** was a fatty acid ethyl ester. The presence of the fatty acid ethyl ester moiety was confirmed by HMBC correlations from H-1′, H-2′, H-2, H-3 to C-1 and the ketone carbonyl was assigned to C-6 based on HMBC correlations from H-4, H-5, H-7 H-8 to C-6. HMBC correlations from H-4 to C-5 and C-6, H-5 to C-7, H-7 to C-5 and H-8 to C-7 and C-6 confirmed the position of the hydroxymethine protons while the methyl at C-18 was assigned based on HMBC cross peaks from H-18 to C-16 and C-17. These confirmed that compound **2** is a keto ester with two hydroxyl substitutions and the structure was determined to be ethyl 5,7-dihydroxy-6-oxooctadecanoate, a new compound.

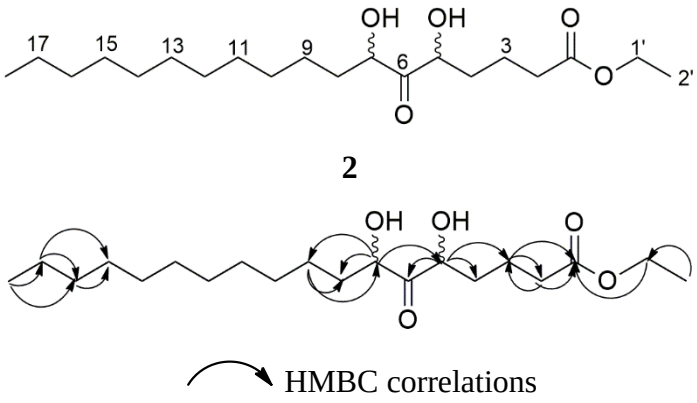


Figure 3. Key HMBC correlations of compound **2**.

Table 3. NMR data of compound **2** (^{1}H: 500 MHz, ^{13}C: 125 MHz; $CDCl_3$).

Position	δ_C (ppm)	δ_H (ppm, *J* in Hz)	HMBC (H→►C)
1	173.7 C	-	-
2	33.6, CH_2	2.33 (t, 7.5)	1, 3, 4
3	24.3, CH_2	1.74 (qnt, 7.5)	1, 2, 4, 5
4	28.3, CH_2	2.03 (dq, 7.5, 3)	3, 4, 5, 6
5	89.8, CH	5.04 (overlap)	7
6	204.0, C	-	-
7	91.4, CH	5.08 (overlap)	5
8	28.9, CH_2	1.97 (dq, 7.5, 3)	6, 7, 9
9	29.1, CH_2	1.38 (qnt, 7.5)	7, 8, 10
10-15	29.2-29.6, CH_2	1.26 (overlap)	-
16	31.9, CH_2	1.26 (overlap)	14, 15, 17, 18
17	22.7, CH_2	1.26 (overlap)	15, 16, 18
18	14.1, CH_3	0.88 (t, 7.0)	16, 17
1′	60.2, CH_2	4.13 (q, 7.0)	1, 2′
2′	14.2, CH_3	1.25 (t, 7.0)	1′

Compound **3** was obtained as a yellow oil with a molecular formula of $C_{20}H_{34}O_2$ based on the molecular ion peak at *m/z* 329.2448 $[M + Na]^+$, indicating four degrees of unsaturation. The IR spectrum showed an absorption at 1736 cm^{-1} which is

characteristics of carbonyl groups and the UV spectrum showed maximum absorption at 432, 413 and 472 nm.

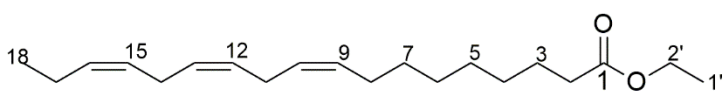
The ^{1}H NMR spectrum of compound **3** indicated the presence of six olefinic protons at δ_H 5.40 (1H, H-9), δ_H 5.39 (1H, H-16), δ_H 5.37 (1H, H-12), δ_H 5.36 (2H, H-10 and H-13) and δ_H 5.34 (1H, H-15) which indicates the presence of three olefinic bonds. It also revealed the presence of an oxygenated methylene at δ_H 4.12 (2H, q, *J*=7.0 Hz, H-1′) and protons attributed to 10 methylenes at δ_H 2.81 (2H, H-14), δ_H 2.80 (2H, m, H-11), δ_H 2.28 (2H, t, *J*=7.5 Hz, H-2), δ_H 2.08 (2H, H-17), δ_H 2.04 (2H, H-8), δ_H 1.63 (2H, H-3) and δ_H 1.31 to 1.37 (8H, H-4 to H-7) as well as two methyl groups at δ_H 1.25 (3H, t, *J*=7.0 Hz, H-2′) and δ_H 0.97 (3H, t, *J*=7.0 Hz, H-18). Examination of the ^{13}C and DEPT NMR spectrum of compound **3** revealed the presence of 20 carbons which includes an ester at δ_C 173.9 (C-1), six olefinic carbons at δ_C 131.9 (C-16), 130.2 (C-9), δ_C 128.3 (C-13), δ_C 128.2 (C-12), δ_C 127.7 (C-10), and δ_C 127.1 (C-15), one oxymethylene carbon at δ_C 60.1 (C-1′), 10 methylene carbons at δ_C C-2 to C-8, C-11, C-14 and C-17 and two methyl carbons at δ_C 14.3 (C-2′) and δ_C 14.2 (C-18). The ^{1}H-^{1}H COSY spectrum showed couplings between H-2/H-3, H-3/H-4, H-4/H-5, H-5/H-6, H-6/H-7, H-7/H-8, H-8/H-9, H-9/H-10, H-10/H-11, H-11/H-12, H-12/H-13, H-13/H-14, H-14/H-15, H-15/H-16, H-16/H-17, H-17/H-18 and H-1′/H-2′ which suggests the presence of a heptadecatriene unit and an ethoxy group. The signals belonging to the ester carbonyl and the ethoxy group suggested the presence of an ethyl ester moiety which was confirmed by HMBC correlations from H-1′, H-2′, H-2 and H-3 to C-1, while the positions of the double bonds were confirmed by HMBC correlations from H-8 to C-9 and C-10, H-10 to C-8, H-11 to C-12 and C-13, H-13 to C-11 and H-14 to C-15 and C-16. The methyl protons H-18 also showed HMBC cross peaks to C-16 and C-17.

The geometry of the double bonds were assigned as *Z* based on previous reports that the carbon chemical shift of a methylene which is attached to an *E* double bond resonates around 33 ppm while the carbon chemical shift of a methylene attached to a *Z* double bond resonates around 27 ppm (Cardellina et al., 1983; Costantino et al., 1993; Dong et al., 2016; Li et al., 2017). According to the ^{13}C NMR data, the chemical shifts of C-8 which is adjacent to C-9 is 27.2 ppm and the chemical shifts of C-11 and C-13 which are attached to C-12 and C-15 respectively is 25.2 ppm. Based on these

data the structure of compound **3** was confirmed as ethyl(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate. Comparison of the NMR data from this experiment with existing NMR data confirmed that ethyl(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate is a known compound (Huh et al., 2010). However, this is the first report on the isolation of ethyl(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate from *T. triandra.*

Table 4. NMR data of compound **3** (^{1}H: 500 MHz, ^{13}C: 125 MHz; $CDCl_3$).

Position	δ_C (ppm)	δ_H (ppm, *J* in Hz)	COSY (H→H)	HMBC (H→C)
1	173.9, C	-	-	-
2	34.3, CH_2	2.28 (t, *J* = 7.5)	3	1, 3, 4
3	24.9, CH_2	1.63 (overlap)	3, 4	1, 2, 4, 5
4-7	29.1-29.5, CH_2	1.31-1.37 (overlap)	-	-
8	27.2, CH_2	2.04 (overlap)	7, 9	6, 7, 9, 10
9	130.2, CH	5.40 (overlap)	8, 10	7, 8, 10, 11
10	127.7, CH	5.36 (overlap)	9, 11	8, 9, 11, 12
11	25.6, CH_2	2.80 (overlap)	10, 12	9, 10, 12, 13
12	128.2, CH	5.37 (overlap)	11, 13	10, 11, 13, 14
13	128.3, CH	5.36 (overlap)	12, 14	11, 12, 14, 15
14	25.5, CH_2	2.81 (overlap)	13, 15	12, 13, 15, 16
15	127.1, CH	5.34 (overlap)	14, 16	13, 14, 16, 17
16	131.9, CH	5.39 (overlap)	15, 17	14, 15, 17, 18
17	20.5, CH_2	2.08 (overlap)	16, 18	15, 16, 18
18	14.2, CH_3	0.97 (t, *J* = 7.0)	17	16, 17
1′	60.1, CH_2	4.12 (q, *J* = 7.0)	19	1, 2′
2′	14.3, CH_3	1.25 (t, *J* = 7.0)	20	1′



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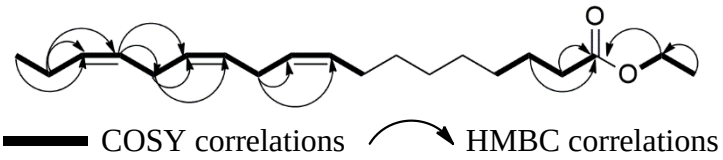


Figure 4. Key HMBC and COSY correlations of compound **3**.

Compound **4** was isolated as a yellow oil with a molecular formula of $C_{20}H_{34}O_2$. The IR spectrum exhibited a sharp absorption peak characteristic of carbonyls at 1736 cm^{-1} and UV absorption maxima at 432, 413 and 473 nm.

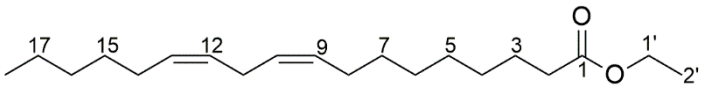
The ^1H NMR spectrum of compound **4** revealed the presence of four olefinic protons at δ_H 5.41 (1H, H-9), δ_H 5.35 (1H, H-12), δ_H 5.33 (1H, H-10) and δ_H 5.30 (1H, H-13) indicating the presence of two olefinic bonds. The ^1H NMR spectrum also showed signals of an oxygenated methylene at δ_H 4.13 (2H, q, *J*=7.0 Hz, H-1´) and signals attributed to 12 methylenes at δ_H 2.78 (2H, t, *J*= 7.0 Hz, H-11), δ_H 2.29 (2H, t, *J*=7.5 Hz, H-2), δ_H 2.05 (4H, H-8 and H-14), δ_H 1.65 (2H, qnt, 7.5 Hz, H-3) and δ_H 1.28 to 1.37 (14H, H-4 to H-7 and H-15 to H-17), two methyl groups at δ_H 1.25 (3H, t, *J*=7.0 Hz, H-2´) and δ_H 0.89 (3H, t, *J*=7.0 Hz, H-18). The ^{13}C NMR spectrum of compound **4** showed 20 carbon signals which includes an ester carbonyl at δ_C 173.9 (C-1), four olefinic methine carbons at δ_C 130.2 (C-9), δ_C 130.1 (C-12), δ_C 128.0 (C-10) and δ_C 127.9 (C-13), one oxygenated methylene at δ_C 60.2 (C-1´), 12 methylenes from δ_C 22.6 to 34.4 (C-2 to C-8, C-11, and C-14 to C-17) and two methyl carbons at δ_C 14.2 (C-18), and δ_C 14.1 (C-2´). The ^1H and ^{13}C NMR data of compound **4** showed obvious and remarkable similarities to that of compound **3**. However, the ^1H and ^{13}C NMR spectrum of compound **4** established that compound **4** contains four olefinic carbons and protons which corresponds to two olefinic bonds, but compound **3** has six olefinic carbons and protons which accounts for its' three olefinic bonds. The positions of the olefinic bonds were confirmed to be C-9 and C-12 based on HMBC correlations from H-8 to C-9 and C-10, H-10 to C-11 and C-12, and H-11 to C-12 and C-13.

Like compound **3**, the geometry of the double bonds was assigned as *Z* based on the carbon chemical shifts of the methylenes next to the double bonds (Cardellina et al., 1983; Costantino et al., 1993; Dong et al., 2016; Li et al., 2017). Based on these spectruml evidences the structure of compound **4** was deduced to be ethyl(9*Z*,12*Z*)-octadeca-9,12-dienoate. Comparisons of NMR data from this

experiment with existing NMR data revealed that compound **4** has been previously isolated (Huh et al., 2010; Park et al., 2014; Uranga et al., 2016a; Uranga et al., 2016b). However, this is the first report on the isolation ethyl(9*Z*,12*Z*)-octadeca-9,12-dienoate from *T. triandra.*

Table 5. NMR data of compound **4** (^{1}H: 500 MHz, ^{13}C: 125 MHz; $CDCl_3$)

Position	δ_C (ppm)	δ_H (ppm, *J* in Hz)	HMBC (H ⟶ C)
1	173.9, C	-	-
2	34.4, CH_2	2.29 (t, 7.5)	1, 3, 4
3	24.9, CH_2	1.65 (qnt, 7.5)	1, 2, 4, 5
4-7	29.1–29.4, CH_2	1.28-1.37 (overlap)	-
8	27.2, CH_2	2.05 (q, 7.5)	6, 7, 9, 10
9	130.2, CH	5.41 (overlap)	7, 8, 10, 11
10	128.0, CH	5.33 (overlap)	8, 9, 11, 12
11	25.6, CH_2	2.78 (t, 7.0)	9, 10, 12, 13
12	130.1, CH	5.35 (overlap)	10, 11, 13, 14
13	127.9, CH	5.30 (overlap)	11, 12, 14, 15
14	27.2, CH_2	2.05 (q, 7.5)	12, 13, 15, 16
15	31.5, CH_2	1.28 1.37 (overlap)	13, 14, 16, 17
16	29.6, CH_2	1.28-1.37 (overlap)	14, 15, 17, 18
17	22.6, CH_2	1.28-1.37 (overlap)	15, 16, 18
18	14.2, CH_3	0.89 (t, 7.0)	16, 17
1′	60.2, CH_2	4.13 (q, 7.0)	1, 2′
2′	14.1, CH_3	1.25 (t, 7.0)	1′



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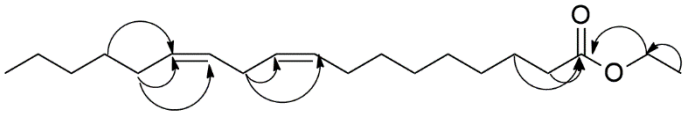

Figure 5. Key HMBC correlations of compound **4**.

3.2. Pharmacological studies

3.2.1. Alpha-glucosidase and alpha-amylase inhibitory activities

In this study, the crude ethanol extract of the whole aerial part of as well as the hexane and ethyl acetate fractions of the leaves and twigs of *T. triandra* were found to display alpha-glucosidase and alpha-amylase inhibitory activities (Table 6). The alpha-glucosidase inhibitory activity of TT-crude was about 5 times better than acarbose. Furthermore, fractions TT-002, TT-004 and TT-005 displayed the best α-glucosidase inhibitory activities amongst all the tested fractios as their IC_{50} values were over 61, 85 and 95 times better than of acarbose, respectively, while fraction TT-001 displayed a poorer activity when compared to acarbose. The alpha-amylase inhibitory activity of TT-crude (97.23 μg/mL) was slightly better than acarbose (114.67 μg/mL). Also, all fractions of *T. triandra* showed alpha-amylase inhibitory activity. TT-001 and TT-004 showed the best activity of 94.41 and 93.74 μg/mL respectively, which was better than the activity of acarbose while the activity of TT-005 at 115.46 μg/mL was comparable to acarbose. These findings confirmed that *T. triandra* possesses antidiabetic activity providing a good basis for the isolation of its antidiabetic constituents.

Compounds **1**, **2**, **3** and **4** also displayed potent alpha-glucosidase inhibitory activity at IC_{50} of 11.58, 105.61, 104.77 and 424.06 μM, respectively. Compound **1** displayed the best activity which was 43 times better than acarbose (500.91 μM). The activities of compounds **2** and **3** were 4.7 times better than acarbose while that of compound **4** was comparable to acarbose. Furthermore, only compound **1** demonstrated α-amylase inhibitory activity at an IC_{50} of 26.27 μM which was 6.8 times better than acarbose which had an IC_{50} of 177.65 μM (Table 6). Consequently, compound **1** being the most potent of all the isolated compounds from the aerial part of *T. triandra* was chosen for the *in vivo* experiment.

Table 6. Alpha-glucosidase and alpha-amylase inhibitory activities (IC_{50}).

	alpha-glucosidase (µg/mL)	alpha-glucosidase (µM)	alpha-amylase (µg/mL)	alpha-amylase (µM)
TT-crude	67.47 ± 0.34 [a]	-	97.23 ± 0.42 [a]	-
TT-001	497.45 ± 0.03[b]	-	94.41 ± 0.08[b]	-
TT-002	5.24 ± 0.08[c]	-	323.03 ± 0.09[c]	-
TT-004	3.40 ± 0.06[d]	-	93.74 ± 0.05[b]	-
TT-005	3.77 ± 0.08[d]	-	155.46 ± 0.06[d]	-
Compound **1**	3.66 ± 0.04[d]	11.58 ± 0.32[a]	8.30 ± 0.45[e]	26.27 ± 1.11[a]
Compound **2**	37.81 ± 0.09[e]	105.61 ± 6.12[b]	NA	NA
Compound **3**	31.85 ± 0.09[f]	104.77 ± 4.56[b]	NA	NA
Compound **4**	130.61 ± 3.67[g]	424.06 ± 15.25[c]	NA	NA
Acarbose	323.39 ± 0.02[h]	500.91 ± 10.26[d]	114.67 ± 0.12[f]	177.65 ± 0.88[b]

TT-crude: ethanol extract of the whole aerial part, TT-001: hexane fraction of leaves, TT-002: ethyl acetate fraction of leaves, TT-004: hexane fraction of twig, TT-005 ethyl acetate fraction of twigs, NA: Not Active. Data were expressed as mean ± SD (n=5). Values having different lowercase letters as superscript across the same column indicates significant differences ($p < 0.05$).

The results also revealed that the fractions and constituents of *T. triandra* are better inhibitors of alpha-glucosidase rather than alpha-amylase. This gives an edge to *T. triandra* and its constituents over existing synthetic alpha-glucosidase and α-amylase inhibitors. This is because the excessive inhibition of alpha-amylase enzyme which occurs with the use of synthetic drugs has been associated with undesirable and sometimes severe side effects such as abdominal cramps, flatulence, diarrhea and bloating (Fujisawa et al., 2005). Keeping postprandial blood glucose level within normal limits (FBG between 70 and 126 mg/dL) is very pivotal in the management of diabetes and the inhibition of alpha-glucosidase and alpha-amylase enzymes offers a very effective means of lowering the postprandial blood glucose. This underscores the relevance of alpha-glucosidase and alpha-amylase inhibitors in the management of diabetes (Ademiluyi and Oboh, 2013; Kazeem et al., 2013; Kasipandi et al., 2019).

3.2.2. Effect of *T. triandra* extract and DHA on fasting blood glucose, HbA1c and insulin level

HFD/STZ induced diabetes caused a marked increase in the fasting blood glucose (FBG) of the rats in the DC groups. However, treatment with 100 and 400 mg/kg of extract and 25 mg/kg DHA reduced the FBG level of the treated groups by 70%, 66% and 74%, respectively (Fig 6A). A key characteristic of diabetes is the sustained and exponential increase in blood glucose also known as hyperglycemia. Hyperglycemia is chiefly responsible for most of the complications associated with diabetes and findings in this study shows that *T. triandra* extract and DHA are able to reverse hyperglycemia.

The HbA1c level of the rats in the DC group increased significantly as a result of diabetes (5.68%) when compared to the NC group (2.42%). However, this was reversed by treatment with 100 and 400 mg/kg of *T. triandra* extract and 25 mg/kg of DHA. There was a reduction in the HbA1c level observed in the treated groups, the HbA1c level in TT100, TT400 and DHA25 was found to be 3.2%, 3.04% and 3.1%, respectively (Fig 6B). As observed in the DC group, diabetes is characterized by an increased level of HbA1c which is a product formed from the non-enzymatic reaction of glucose and free amino acid of haemoglobin. Hb1Ac is a biomarker used to confirm long-term glycemic control. It is also used to predict the chances of development or

progression of diabetes related complications (Sherwani et al., 2016). Past studies showed that a 10% reduction in Hb1Ac levels in diabetic patients reduced the risk of diabetic nephropathy, neuropathy and retinopathy by 25-44%, 30% and 35%, respectively (Ullah et al., 2017). This further shows that *T. triandra* and DHA have the potential to restore glycemic control and reduce the risk of diabetes associated complications.

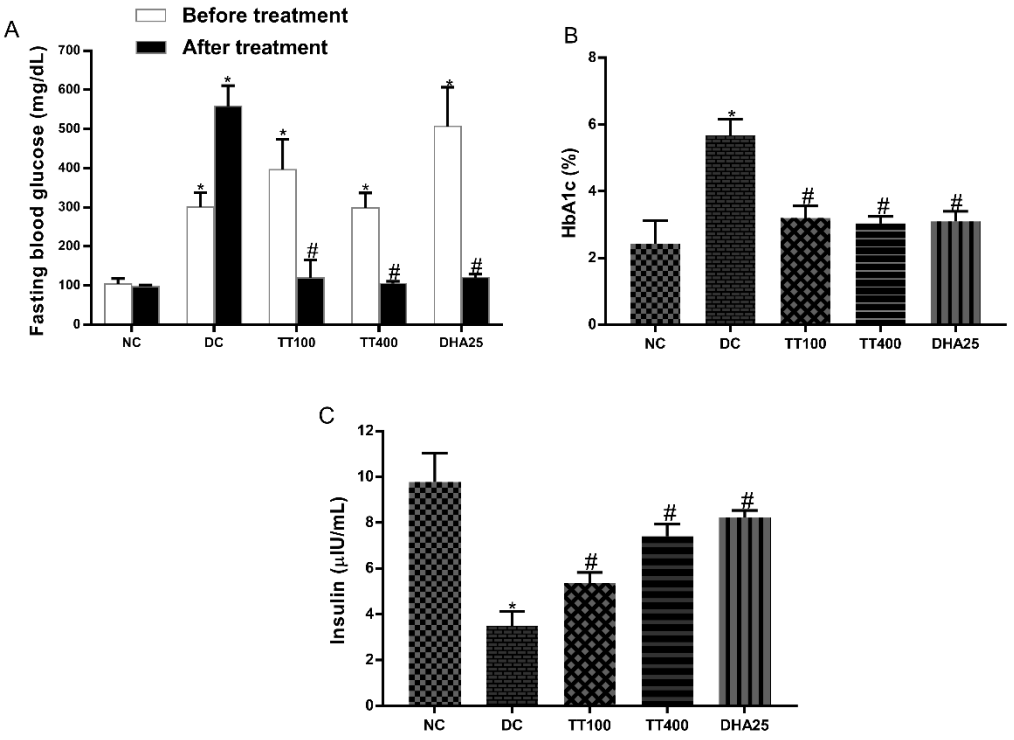

Figure 6A-C. Effect of *T. triandra* extract and DHA on FBG, HbA1c and insulin level. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

In contrast to the NC group which had an insulin level of 9.78 µU/mL, diabetes caused a marked reduction of the insulin level of rats in the DC group (3.48 µU/mL). Secretion of insulin was boosted by treatment with 100 and 400 mg/kg of *T. triandra* extract and 25 mg/kg of DHA. DHA showed the best insulinotropic activity with insulin level of 8.24 µU/mL followed by 400 mg/kg and then 100 mg/kg of *T. triandra* extract with their insulin level at 7.40 and 5.36 µU/mL respectively (Fig. 6C).

Insulin is mainly responsible for glucose absorption from the blood stream, hence an impairment of insulin function results in hyperglycemia (Egan and Dinneen, 2019; Martinez et al., 2019). Findings from this study revealed that DHA and the ethanol extract of *T. triandra* ameliorated hyperglycemia by stimulating insulin secretion from the pancreatic β-cells. This agrees with previous report that the ethanol extract of leaves of *T. triandra* reduced hyperglycemia through the stimulation of insulin secretion (Katisart and Rattana, 2017).

3.2.3. Effect of *T. triandra* extract and DHA on intraperitoneal glucose tolerance test

A marked increase in the blood glucose level of the animals in DC group was observed after glucose administration. The DC group showed a steady increase in blood glucose level from 506.8 mg/dL at 0 min to over 600 mg/dL after 120 mins. However, the blood glucose level of the TT100, TT400 and DHA25 groups were found to be significantly lower than DC group and comparable to NC group. After 120 mins, the blood glucose levels of NC, TT100, TT400 and DHA25 groups were 106, 120, 114 and 118 mg/dL, respectively (Fig.7).

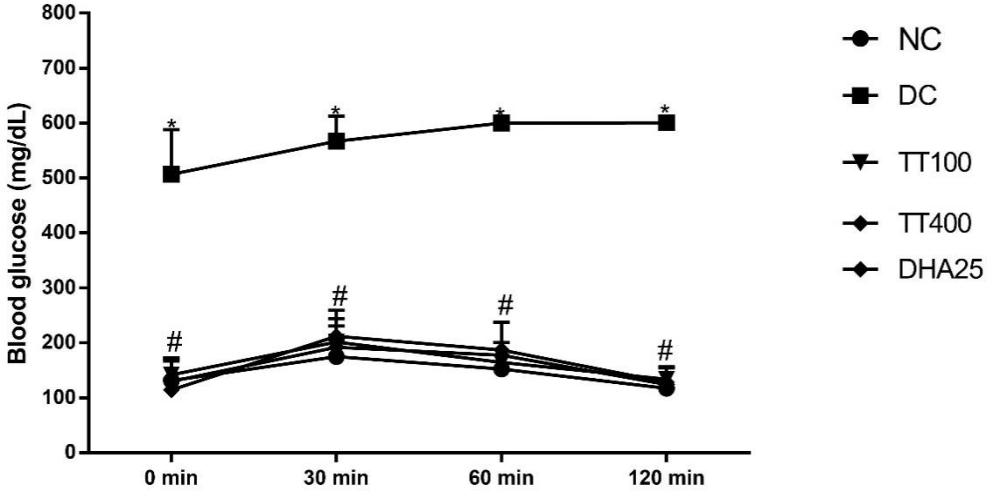

Figure 7. Effect of *T. triandra* extract and DHA on intraperitoneal glucose tolerance test. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

These results suggest that administration of the *T. triandra* extract and DHA improved glycemic control of the diabetic rat by enhancing glucose homeostasis. Glucose tolerance test is used to determine the extent of body glucose homeostasis, to establish the extent of glycemic control, insulin resistance and impaired β-cell function. Glucose tolerance test is also used to diagnose diabetes (Bartoli et al., 2011; Kim et al., 2017; Chanprasertpinyo et al., 2017). Persons with impaired glucose tolerance have a higher risk of developing cardiovascular diseases and other diabetes associated complications (DeFronzo and Abdul-Ghani, 2011; Nathan et al., 2007).

3.2.4. Effect of *T. triandra* extract and DHA on food intake, water intake and body weight

Diabetes caused an upsurge in the rate of food and water consumption of the rats in the DC group when compared to the NC group. Each rat in DC group consumed an average of 45.33 g of food and 280.19 ml of water daily while each rat in NC group consumed an average of 33.37 g of food and 43.77 mL of water daily. Treatment with *T. triandra* extract and DHA caused a marked reduction in the food and water intake of the treated groups. TT100 group consumed 37.17 g of food and 106.44 ml of water daily, TT400 group consumed 28.93 g of food and 106.44 mL of water daily, while the daily food and water intake rate of DHA25 group was 35.54 g and 106.88 mL, respectively (Fig. 8A-B). Increased food and water consumption are common symptoms observed in diabetes and this is because impaired insulin function causes glucose which is needed in the body for cellular energy to accumulate in the blood rather than being taken up by the cells. This essentially increases the demand for glucose by body cells and consequently results in increased appetite called polyphagia which is usually accompanied by increased water intake called polydipsia (Oyedemi et al., 2011b).

After 30 days the body weight of the rats in NC group increased by 27.7% but there was a 3.95% reduction in the weight of the rats in DC group. Treatment however caused 27.4%, 26.0% and 14.5% increase in the body weights of TT100, TT400 and DHA25 groups respectively (Fig. 8C). Weight loss is a common occurrence in diabetic patients and this usually arises from the degradation of structural proteins or muscle wasting. In hyperglycemic conditions, glucose is not available for the body cells

to use as energy therefore the body burns structural fats and muscles to supply the needed energy. This essentially results in a marked reduction in body weight of diabetic patients (Oyedemi et al., 2011b; Chandran et al., 2014; Adefegha et al., 2016). Results obtained from this study showed that *T. triandra* extract and DHA ameliorated body weight loss, polydipsia and polyphagia which is attributable to improved glucose metabolism.

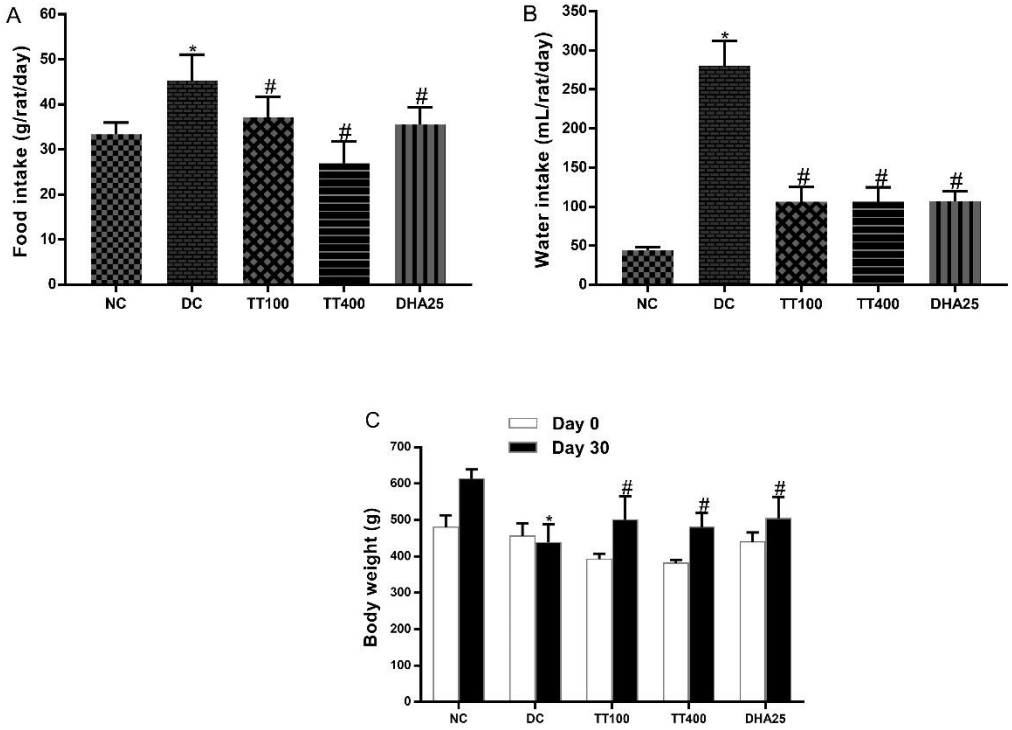

Figure 8A-C. Effect of *T. triandra* extract and DHA on body weight, food and water intake. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

3.2.5. Effect of *T. triandra* extract and DHA on HOMA-IR and HOMA-β

There was a significant increase in the HOMA-IR of rats in DC group when compared to the NC group, but treatment with 100 and 400 mg/kg of the extract and 25 mg/kg of DHA caused a significant reduction of the HOMA-IR values for all the treated groups. HOMA-IR is a model used to estimate the degree of insulin resistance and it is considered an effective biomarker for the prediction of insulin sensitivity (Xia et al.,

2019). The observed reduction in HOMA-IR value of the treated diabetic rats indicates the alleviation of insulin resistance and enhanced insulin sensitivity (Fig. 9A).

In contrast to the NC group, the results from this study revealed that there was a decline in the pancreatic β-cell function of the DC group as indicated by their very low HOMA-β value, but there was a marked improvement in pancreatic β-cell function of TT100, TT400 and DHA25 groups as indicated by their higher HOMA-β values. (Fig. 9B). HOMA-β is a mathematical homeostatic model used to estimate pancreatic β-cell function (Khamchan et al., 2018). Hence, these results suggest that treatment with *T. triandra* extract and DHA enhanced the β-cell function of the treated diabetic groups.

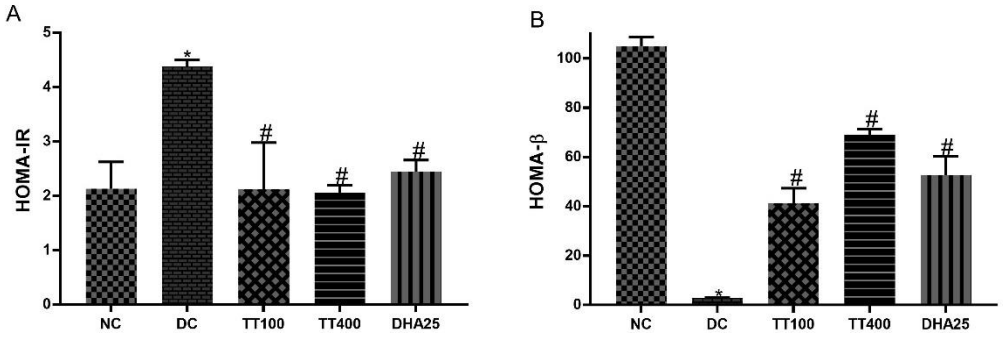

Figure 9A-B. Effect of *T. triandra* extract and DHA on HOMA-IR, HOMA-β and HbA1c. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

3.2.6. Effect of *T. triandra* extract and DHA on lipid profile

The concentration of serum TC, TG, LDL-C and HDL was significantly higher in the DC group (97.78, 100, 69.67 and 15.02 mg/dL, respectively) when compared to NC group (71.60, 34.70, 31.23 and 12.22 mg/dL, respectively). The concentration of TC, TG, LDL-C and HDL was lower in the treated diabetic groups in comparison to the DC group except in TT100 group which had a higher concentration of TG of 12.30 mg/dL Fig. 10A-D).

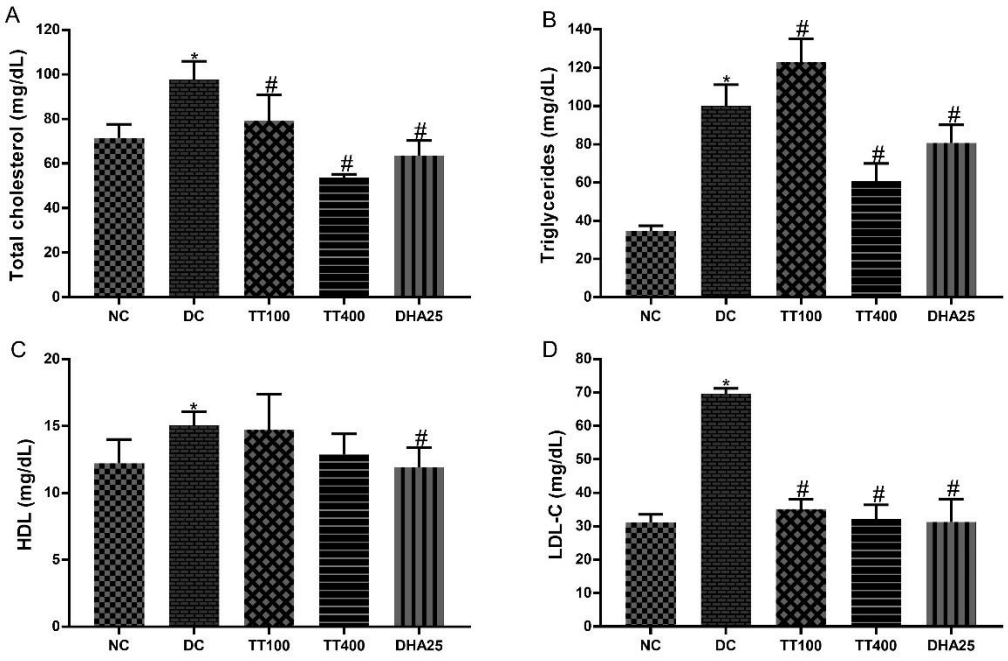

Figure 10A-D. Effect of *T. triandra* extract and DHA on lipid profile. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of T. triandra, TT400: treated with 400 mg/kg of T. triandra, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *$p<0.05$ when DC is compared to NC; #$p<0.05$ when TT100, TT400 and DHA25 are compared to DC.

Previous research evidences have confirmed that diabetes is accompanied by imbalances in lipid profile and that diabetes patients have a higher risk of developing hyperlipidemia. This is because insulin deficiency causes lipids to accumulate in the blood which blocks the blood vessels and increases the risk of cardiovascular diseases, a common complication in diabetes (Mooradian, 2009; Chandran et al., 2014; Adefegha et al., 2014; Giribabu et al., 2014). The enzyme lipoprotein lipase is responsible for the hydrolysis of triglycerides in the body and this enzyme is normally activated by insulin but not in sufficient quantities when insulin function is impaired. This results in elevated level of triglycerides, a condition called hypertriglyceridemia (Kumar et al., 2015b). Furthermore, hormone sensitive lipase which liberates free fatty acids from the adipose tissues can be activated due to lack of insulin thereby causing an increase in the level of serum lipids (Mollica et al., 2017). Results obtained from this experiment shows that the extract of *T. triandra* and DHA restored the lipid profile of the treated rats.

3.2.7. Effect of *T. triandra* extract and DHA on liver function enzymes

The DC group had very high serum concentrations of ALT (836.0 U/L), AST (330.0 U/L) and ALP (235 U/L) when compared to NC group which had a much lower AST, ALT and ALP concentrations of 102.2, 55.0 and 83.4 U/L, respectively. Treatment with *T. triandra* extract and DHA suppressed the serum concentration of AST, ALT and ALP in the TT100, TT400 and DHA25 groups (Fig. 11A-C). The concentrations of AST, ALT and ALP was 356.26, 211.25 and 139.5 U/L, respectively in TT100 group; 176.50 U/L, 137.25 U/L and 89.60 U/L respectively in TT400 group and 215.00 U/L, 125.00 U/L and 125.33 U/L, respectively in DHA group.

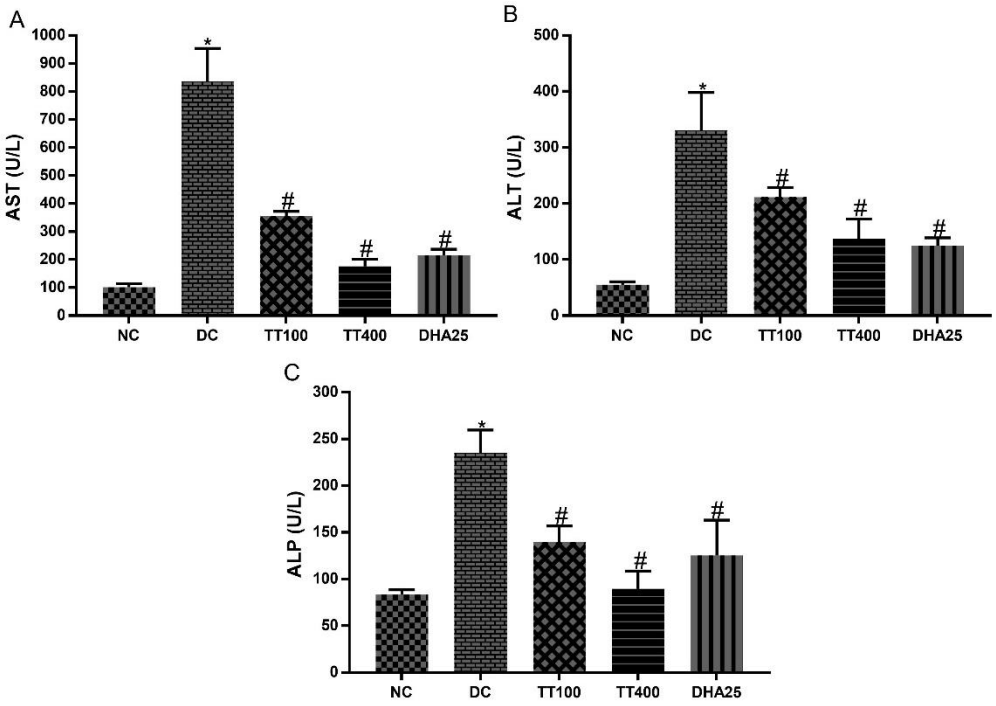

Figure 11A-C. Effect of *T. triandra* extract and DHA on liver enzymes. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of T. triandra, TT400: treated with 400 mg/kg of T. triandra, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

The liver is an important organ in the body because it performs pivotal metabolic functions. The liver is responsible for carbohydrate and lipid metabolism as well as insulin clearance and glucose regulation. However, the ability of the liver to perform these functions effectively is impeded in diabetic conditions (Chaudhary et al.,

2016; Johnson et al., 2019). Elevated levels of ALT, AST and ALP are well-known markers of hepatocellular damage. These liver enzymes are normally present in the liver in small quantities. However, the level of these enzymes increases and leaks into the blood as a result of hyperglycemia induced hepatic injury (Balakrishnan et al., 2019; Goboza et al., 2019). Observations made in this study revealed that treatment with *T. triandra* extract and DHA ameliorated diabetes induced liver injury in the treated diabetic rats.

3.2.8. Effect of *T. triandra* extract and DHA on serum protein levels

When compared to the NC group which had total protein concentration of 6.02 g/dL, albumin concentration of 2.66 g/dL and globulin concentration of 2.44 g/dL, the DC group had lower concentrations of the total protein, albumin and globulin which were 4.97, 2.44 and 2.43 g/dL respectively. This trend was reversed by treatment with 100 and 400 mg/kg of *T. triandra* extract and 25 mg/kg of DHA. TT100, TT400 and DHA25 groups had serum total protein concentration of 5.63, 5.42 and 5.35 g/dL respectively, albumin concentration of 2.70, 2.78, 2.70 g/dL respectively and globulin concentration of 2.93, 2.56, 2.47 g/dL, respectively (Fig. 12A-C). Low level of serum albumin, globulin and total protein as seen in the DC group is also an indictive of liver injury. Hyperglycemia is known to cause the breakdown and degradation of structural proteins due to increased conversion of amino acids into glucose to meet the energy demands of the body cells which imparts the synthesis of proteins such as albumin and globulin negatively (Ekperikpe et al., 2019; Goboza et al., 2019).

3.2.9. Effect of *T. triandra* extract and DHA on serum bilirubin levels

The DC group had a significantly higher level of total and direct bilirubin (0.46 mg/dL and 0.25 mg/dL, respectively) when compared to NC group which had a total bilirubin content of 0.31 mg/dL and direct bilirubin content of 0.20 mg/dL. Elevated bilirubin level as observed in the DC group is an indicator of hepatic injury and this can be attributed to decreased uptake of bilirubin by the liver, decreased bilirubin conjugation and increased bilirubin formation (Ekperikpe et al., 2019). When compared to the DC group, it was found that treatment with *T. triandra* extract and DHA caused a marked decrease in the total bilirubin level of TT100, TT400 and DHA25 groups (0.17 mg/dL, 0.13 mg/dL and 0.18 mg/dL, respectively) and there was

also a significant reduction of the direct bilirubin concentrations in TT400 and DHA25 groups (0.14 and 0.05 mg/dL respectively). However, treatment with 100 mg/kg of *T. triandra* extract did not cause any reduction in the direct bilirubin level (Fig. 13A-B).

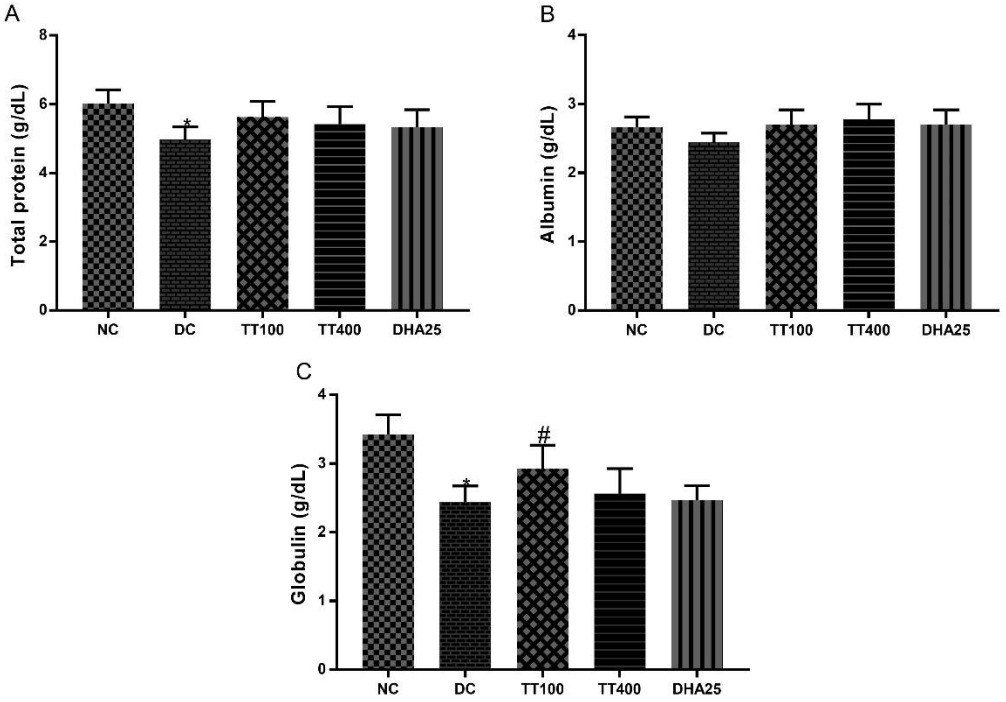

Figure 12A-C. Effect of *T. triandra* extract and DHA on serum protein levels. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

3.2.10. Effect of *T. triandra* extract and DHA on blood urea nitrogen and creatinine

There was a significant increase in the serum BUN and creatinine levels of the untreated DC group (BUN: 41.25 mg/dL, creatinine: 0.43 mg/dL) when compared to the NC group (BUN: 23.85 mg/dL, creatinine: 0.34 mg/dL). However, there was an obvious reduction of BUN in all the treated groups (TT100: 23.97 mg/dL, TT400: 20.03 mg/dL and DHA25: 16.57 mg/dL) while there was also an insignificant decrease in the level of creatinine of rats in TT400 (0.38 mg/dL) and DHA25 (0.41 mg/dL) groups. Whereas, TT100 group exhibited lower creatinine level than TT400 and DHA25 at 0.33 mg/dL (Fig. 14). Elevated levels of BUN and creatinine is a sign of renal dysfunction

which is one of the many abnormalities caused by diabetes (Kotha et al., 2019). Creatinine is generated as a waste product during muscle contraction while BUN is generated as a byproduct during the breakdown of protein. The kidney is responsible for the excretion of both BUN and creatinine and assaults on the kidney causes BUN and creatinine to accumulate in the blood (Al-Attar and Alsalmi, 2019). Based on these discoveries it is evident that *T. triandra* and DHA has a protective effect on the kidney.

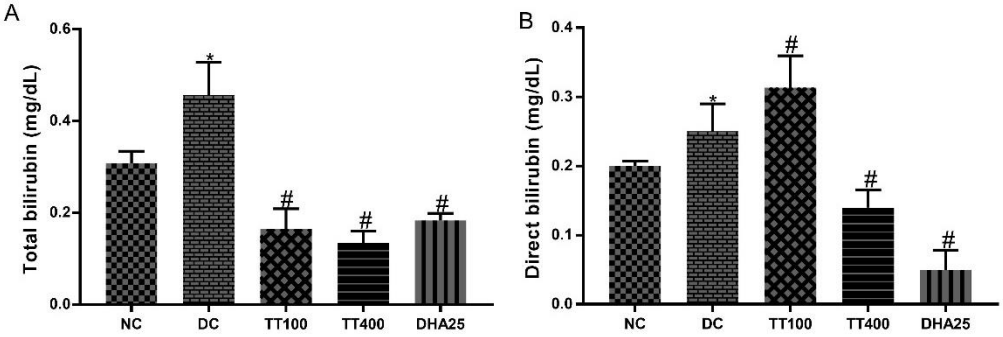

Figure 13A-B. Effect of *T. triandra* extract and DHA on bilirubin levels. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

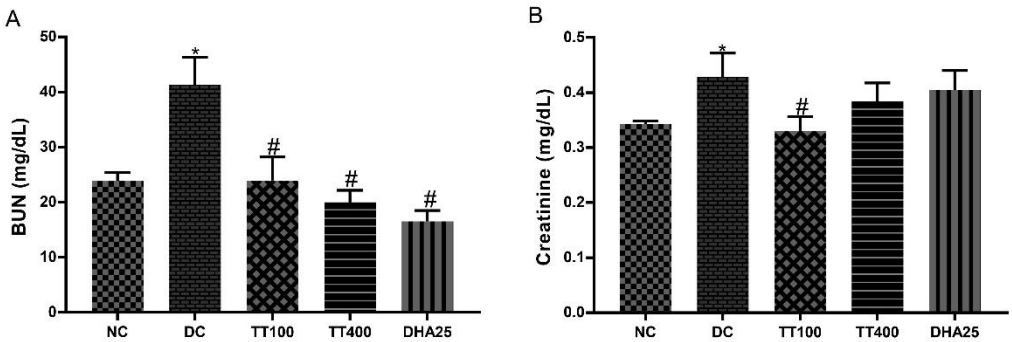

Figure 14A-B. Effect of *T. triandra* extract and DHA on BUN and creatinine. NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of *T. triandra*, TT400: treated with 400 mg/kg of *T. triandra*, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *p<0.05 when DC is compared to NC; #p<0.05 when TT100, TT400 and DHA25 are compared to DC.

3.2.11. Effect of *T. triandra* extract and DHA on serum electrolytes

In this study, there was no significant difference in the level of Na^+ across all the groups. However, there was a marked decrease in the level of K^+ (hypokalemia) in the rats in DC group (4.73 mEq/L) compared to the NC group (9.34 mEq/L). This was reversed by treatment with 400 mg/kg of extract and 25 mg/kg of DHA. The serum concentration of K^+ in the TT400 group was 7.67 mEq/L and that of DHA25 was 4.8 mEq/L. Diabetes is characterized by distortions in electrolyte homeostasis caused by polyuria which accelerates electrolyte loss in the body due to increased osmotic diuresis. Loss of electrolytes such as sodium (Na^+) and potassium (K^+) are indicators of diabetic nephropathy and it also predisposes diabetic patients to hypovolumic shock which can lead to death in uncontrolled diabetes mellitus (Atangwho et al., 2007; Chen et al., 2016; Ogar et al., 2019).

It was observed that diabetes caused an obvious increase in TCO_2 level of the DC group (26.3 mEq/L) which was significantly suppressed by treatment with 400 mg/kg of extract (20.2 mEq/L). An insignificant reduction of TCO_2 was also observed in the DHA25 group (25.5 mEq/L) but there was no noticeable change in TT100 group. The concentration of CO_2 in the blood is a useful index for the monitoring of blood acidity (Ogar et al., 2019). Furthermore, hypochloremia which is a decrease in the concentration of blood Cl^- was observed in the DC group. This was reversed by treatment with 25 mg/kg of DHA but treatment with 100 mg/kg and 400 mg/kg of the extract showed no effect on hypochloremia.

Except for DHA25 group which had a serum Cl^- concentration of 10.25 mEq/L, the Cl^- level of the all the diabetic groups were slightly lower when compared to the NC group (101.2 mEq/L). The Cl^- concentration in DC, TT100 and TT400 groups were 97.2, 96.3 and 95.2 mEq/L respectively. The increased CO_2 level and decreased level of chloride (Cl^-) of the DC group as observed in this study is indicative of blood alkalinity, a condition called ketoalkalosis (Ogar et al., 2019). Although there are fewer reports on diabetic ketoalkalosis when compared to ketoacidosis which is more frequently encountered in diabetes mellitus, the observations made in this study agrees with previous reports that dehydration due to osmotic diuresis in combination with hyperglycemia can cause ketoalkalosis (Kumar et al., 2018, Yasuda et al., 2016).

Table 7. Effect of *T. triandra* extract and DHA on serum electrolytes.

	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^-(mEq/L)	TCO_2 (mEq/L)
NC	141.80 ± 1.64	9.34 ± 0.96	101.20 ± 1.92	17.00 ± 1.58
DC	142.80 ± 3.03	4.73 ± 0.44*	97.20 ± 3.56	26.33 ± 2.31*
TT100	144.25 ± 1.50	3.95 ± 0.65#	96.25 ± 0.50	26.75 ± 1.50
TT400	141.60 ± 1.82	7.07 ± 0.38#	95.20 ± 1.64	20.20 ± 1.30#
DHA25	146.25 ± 1.71	4.80 ± 0.17#	102.50 ± 1.29#	25.50 ± 1.29#

NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of T. triandra, TT400: treated with 400 mg/kg of T. triandra, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *$p<0.05$ when DC is compared to NC; #$p<0.05$ when TT100, TT400 and DHA25 are compared to DC.

3.2.12. Effect of *T. triandra* extract and DHA on haematological parameters

Haematological parameters provides valuable insights into the overall health status of patients and it is very instrumental in the diagnosis of various diseases. Disturbances in haematological parameters are frequent occurrences in diabetes (Oyedemi et al., 2011a; Dalar et al., 2018). The decreased level of red blood cell parameters such as red blood cell count (RBC), haemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW) as witnessed in the diabetic control group is indicative of anaemia (Pradeep and Srinivasan, 2018; Erukainure et al., 2013). In this study, treatment with 25 mg/kg DHA ameliorated anaemia through enhancement the RBC parameters of animals in DHA group, while 100 and 400 mg/kg of extract showed no effect on anaemia (Table 8).

Hyperglycemia causes an increase in the generation of free radicals and reactive oxygen species (ROS) which oxidizes proteins, consequently resulting to lipid peroxidation. Increased level of lipid peroxides causes the haemolysis of RBCs and by

extension anaemia (Ekperipe et al., 2019; Chaudhary et al., 2016; Kolanjiappan et al., 2002). Another factor responsible for anaemia is the deficiency in erythropoietin secretion caused by diabetic nephropathy. Erythropoietin is a hormone secreted by the kidney to stimulate the production of RBCs from the bone marrow and insults to the kidney can affect the secretion of this hormone (Pradeep and Srinivasan, 2018; Chaudhary et al., 2016; Ekperipe et al., 2019). Anaemia is a risk factor for cardiovascular diseases in people suffering from diabetes (Medhi and Toto, 2009). The ameliorative effect of DHA on anaemia might be due to its ability to reduce the glycation of RBC membrane proteins or its ability to attenuate diabetic nephropathy or both.

There was a decrease in the level of white blood cell (WBC) and lymphocyte (LYM) as well an increased level of monocytes (MON) and neutrophils (NEU) in the diabetic control rats. However, treatment with 25 mg/kg of DHA increased the WBC and LYM while there was no improvement in TT100 and TT400 groups. Furthermore, 100 and 400 mg/kg of *T. triandra* extract as well as 25 mg/kg of DHA caused a marked decreased in the level of MON and NEU. The diminished level of WBC count and LYM as well as the increased level of MON and NEU in the untreated diabetic rats indicates immunosuppression, because these cells are vital components of the immune system. Suppression of the immune system can contribute to the progression of diabetes associated complications (Oyedemi et al., 2011a; Rashid et al., 2019; Abdellatief et al., 2017). High platelets count as observed in the DC group can be attributed to poor glycemic control, inflammation and presence of infections (Ekperipe et al., 2019; Osigwe et al., 2017). However, this was reversed by treatment with *T. triandra* extract in TT100 and TT400 as indicated by their low platelet counts.

Table 8. Effect of *T. triandra* extract and DHA25 on RBC parameters.

	NC	DC	TT100	TT400	DHA25
RBC (10^{12}/L)	9.308 ± 0.24	6.32 ± 1.26*	4.76 ± 0.93$^{\#}$	4.87 ± 0.66$^{\#}$	9.40 ± 0.21
Hb (g/dL)	16.62 ± 0.50	11.86 ± 2.10*	9.00 ± 1.47$^{\#}$	9.23 ± 0.68$^{\#}$	15.63 ± 3.05
HCT (%)	49.3 ± 2.24	33.56 ± 6.99*	30.4 ± 5.65$^{\#}$	25.45 ± 3.14$^{\#}$	50.40 ± 0.61$^{\#}$
MCV (fL)	53.2 ± 1.48	52.8 ± 1.48	51.5 ± 0.57	52.6 ± 1.81	53.5 ± 1.00
MCH (pg)	18.88 ± 0.56	17.82 ± 0.43	18.8 ± 0.67	18.94 ± 1.15	19.68 ± 2.83
MCHC (g/dL)	35.58 ± 1.18	33.68 ± 0.64*	36.3 ± 1.22	35.98 ± 2.38	36.85 ± 5.67$^{\#}$
RDW (%)	11.96 ± 0.50	11.56 ± 0.74	11.75 ± 0.34	11.6 ± 0.99	11.68 ± 1.30

NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of T. triandra, TT400: treated with 400 mg/kg of T. triandra, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *$p<0.05$ when DC is compared to NC; #$p<0.05$ when TT100, TT400 and DHA25 are compared to DC.

Table 9. Effect of *T. triandra* extract and DHA of WBC parameters.

	NC	DC	TT100	TT400	DHA25
WBC x 10^3 ($cells/m^3$)	4.83 ± 0.70	4.35 ± 0.64	3.55 ±0.49	2.30 ± 0.14$^{\#}$	6.20 ± 1.91$^{\#}$
Neu (%)	26.00 ± 2.00	30.00 ± 4.00*	14.50 ± 0.71$^{\#}$	13.00 ± 1.00$^{\#}$	13.75 ± 4.03$^{\#}$
Lym (%)	78.00 ± 6.24	59.67 ± 3.01*	71.33 ± 8.02$^{\#}$	73.5 ± 5.92$^{\#}$	75.25 ± 6.95$^{\#}$
Mo (%)	4.75 ± 0.5	11.75 ± 1.5*	4.67 ± 0.58$^{\#}$	7.67 ± 1.53$^{\#}$	8.50 ± 0.71$^{\#}$
PLT x 10^3 ($cells/m^3$)	119.00 ± 1.00	611.67 ± 34.67*	163.00 ± 32.57$^{\#}$	191.67 ± 41.28$^{\#}$	722.37 ± 151.45$^{\#}$

NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of T. triandra, TT400: treated with 400 mg/kg of T. triandra, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *$p<0.05$ when DC is compared to NC; #$p<0.05$ when TT100, TT400 and DHA25 are compared to DC.

3.2.13. Effect of *T. triandra* extract and DHA on liver, kidney and testes index

No significant change was noticed in the liver index of the animals across all groups the diabetic groups when compared to the NC. There was also no obvious difference in the kidney index of the across all groups except in the DHA25 group reduced significantly when compared. There was a marked reduction of the testes index of the DC group when compared to the NC group which indicates necrosis of the testes. However, this was reversed significantly by treatment with 100 and 400 mg/kg of *T. triandra* extract, while the testes index of the DHA25 group was comparable to DC group (Table 10).

Organ index is used as a parameter for the prediction of injury, physiological disturbance or toxicity of target organs. An increase in organ weights suggests hypertrophy while a decrease suggests necrosis of the organ in question. Although organ weights provide very vital information, data from organ index must be interpreted alongside clinical parameters and histopathological findings on the organ in question (Sellers et al., 2007; Teo et al., 2002; Ugwah-Oguejiofor et al., 2019). Except for data obtained from the testicular index which suggests testicular necrosis, results obtained for the liver and kidney index in this study were largely comparable. However, interpreting these data alongside other results like lipid profile, liver function enzymes, serum protein, bilirubin, BUN and creatinine levels as well as the histological observations indicates that extract of *T. triandra* and DHA displayed protective effects on the liver, kidney and testes.

Table 10. Effect of *T. triandra* extract and DHA on liver, kidney and testicular index.

	Liver		Kidney		Testes	
	Weight (g)	Liver index	Weight (g)	Kidney index	Weight (g)	Testes index
NC	21.50 ± 3.11	0.034 ± 0.004	6.20 ± 1.64	0.010 ± 0.002	6.60 ± 0.89	0.011 ± 0.001
DC	14.28 ± 1.29*	0.035 ± 0.007	4.50 ± 0.44*	0.010 ± 0.006	3.53 ± 0.23*	0.008 ± 0.003*
TT100	17.50 ± 0.71$^{\#}$	0.032 ± 0.002	5.75 ± 0.50$^{\#}$	0.010 ± 0.004	5.50 ± 0.58$^{\#}$	0.010 ± 0.001$^{\#}$
TT400	16.80 ± 1.64$^{\#}$	0.033 ± 0.004	5.60 ± 0.89$^{\#}$	0.010 ± 0.001	4.20 ± 1.30$^{\#}$	0.010 ± 0.001$^{\#}$
SS025	17.12 ± 0.99$^{\#}$	0.033 ± 0.004	3.86 ± 0.32$^{\#}$	0.008 ± 0.001$^{\#}$	3.62 ± 0.17	0.007 ± 0.08

NC: Normal control, DC: Diabetic control, TT100: treated with 100 mg/kg of T. triandra, TT400: treated with 400 mg/kg of T. triandra, DHA25: treated with 25 mg/kg of compound **1**. Data were expressed as mean ± SD (n=5). *$p<0.05$ when DC is compared to NC; #$p<0.05$ when TT100, TT400 and DHA25 are compared to DC.

3.2.14. Effect of *T. triandra* extract and DHA on the pancreatic histology

Histological examination revealed that the pancreas of the NC group had normal morphology and architecture with normal islets and no signs of injuries. Their islets of Langerhans (encircled) were also larger than the islets of DC group (Fig. 15A). However, there were abnormalities in the pancreatic tissue architecture of the DC rats and obvious destruction of the islets of Langerhans of DC group as indicated by their smaller sizes and reduced β-cell mass (Fig. 15B). These observations are consistent with previous reports (Chaudhary et al., 2016.; Ekperikpe et al., 2019; Katisart and Rattana, 2017; Kotha et al., 2019) and it explains why there was a significant drop in the insulin level of the diabetic rats, since the β-cells of the islets of Langerhans are critical to insulin function (Matveyenko and butler, 2008). The pancreas of the animals in TT100, TT400 and DHA25 groups showed varying signs of regeneration with larger islets and increased β-cell mass compared to the DC group (Fig. 15C-D).

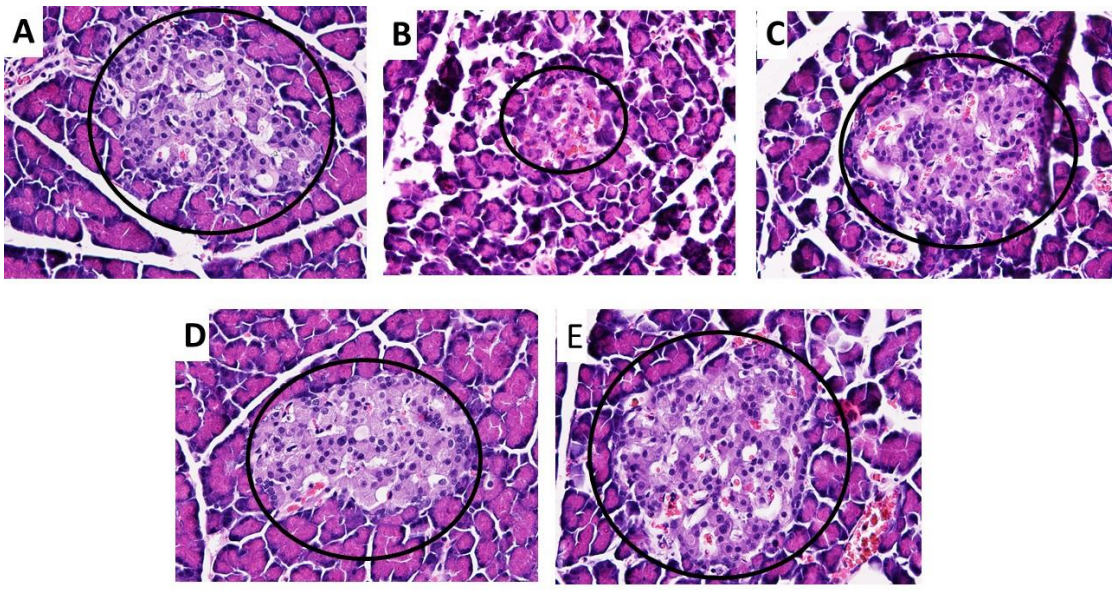

Figure 15A-E. Picture showing the effect of *T. triandra* extract and DHA the pancreas (Magnification: 40x). Circle: Islet of Langerhans. A: Normal control, B: Diabetic control, C: TT100, D: TT400, E: DHA25.

The apoptosis and dysfunction of the pancreatic β-cells is a key factor in the etiology of diabetes. Diabetes occurs due to the failure of the β-cells to secret insulin or due to a decline in insulin function which is usually a consequence of impaired β-cell function and reduced β-cell mass (Khamchan et al., 2018; Matveyenko and Butler, 2008; Meier and Bonadonna, 2013). These anomalies where reversed in all the treated

groups which agrees with the findings in this research that *T. triandra* extract and DHA reduced hyperglycemia by enhancing insulin secretion.

3.2.15. Effect of *T. triandra* extract and DHA on hepatic histology

Histological examinations revealed that the liver of the NC rats showed normal morphology which includes normal central vein (encircled) and well arranged hepatic cells with distinct nucleus (Fig. 16A). The rats in the DC group had abnormal architecture with irregular and swollen liver cells (short arrow), irregular cavities (arrowhead), excessively prominent central vein (encircled) and necrotic hepatocytes (long arrow) (Fig. 16B). These changes agrees with previous reports on the effect of diabetes on the liver (Ekperipe et al., 2019; Li et al., 2019). It was found that treatment with 100 and 400 mg/kg of *T. triandra* extract and 25 mg/kg of DHA reversed hepatic damage as their liver showed similar architecture to the NC group (Fig. 16C-E).

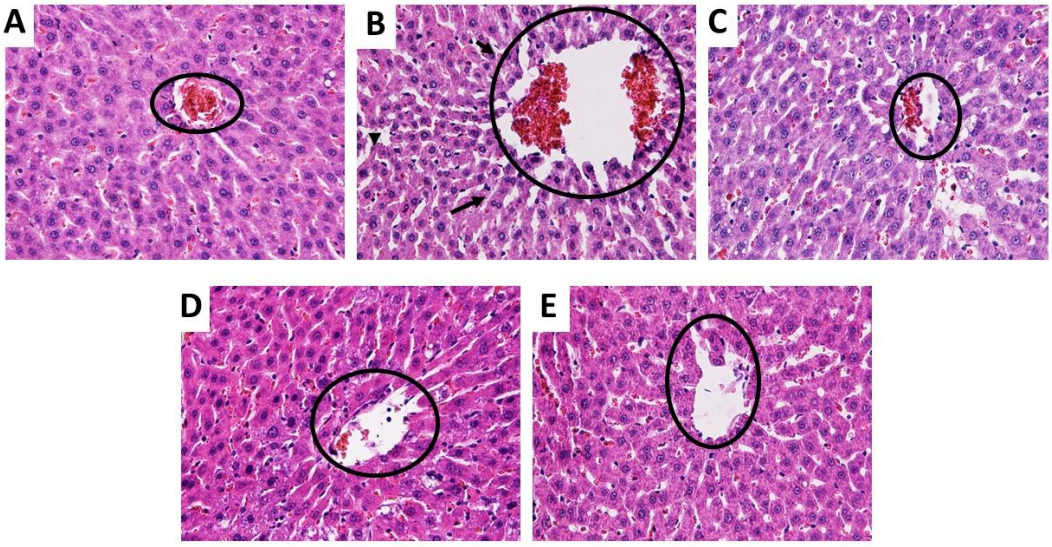

Figure 16A-E. Picture showing the ffect of *T. triandra* extract and DHA on the hepatic histology (Magnification: 40x). Circle: Central vein, Arrowhead: Irregular cavities, Short arrow: Liver cells, Long arrow: Hepatocytes. A: Normal control, B: Diabetic control, C: TT100, D: TT400, E: DHA25.

3.2.16. Effect of *T. triandra* extract and DHA on renal histology

The kidney of the normal rats showed normal histological feature with detailed renal parenchyma, intact glomeruli and normal tubules while the renal corpuscles (encircled) appeared as dense, round shaped structures (Fig. 18A). The

untreated diabetic rats showed deformed renal architecture with atrophied renal corpuscles and degenerated glomeruli (Fig. 17B). These observations are consistent with previous reports on the effect of diabetes on renal histopathological changes (Rashid et al., 2019; Han et al., 2019; Chandran et al., 2016). The kidney of the TT100, TT400 and DHA25 groups showed obvious signs of regeneration as indicated by their improved overall renal architecture and dense round shaped corpuscles which appears bigger compared to the DC group (Fig. 17C-E). These observations agree with previous reports and confirms that *T. triandra* and DHA can protect against kidney damage, which is one of the most severe complications that is associated with diabetes (Ekperipe et al., 2019).

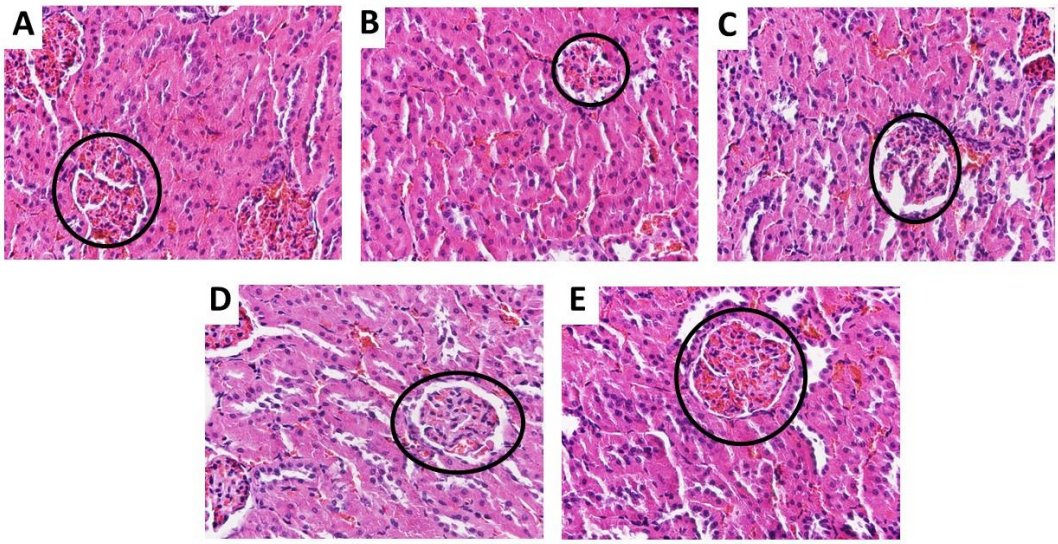

Figure 17A-E. Picture showing the effect of *T. triandra* extract and DHA on renal histology (Magnification: 40x). Circle: Renal corposcules. A: Normal control, B: Diabetic control, C: TT100, D: TT400, E: DHA25.

3.2.17. Effect of *T. triandra* extract and DHA on testicular histology

The testes of rats in NC group showed normal histological morphology with normal seminiferous tubules and normal spermatogonial cells (Fig. 18A). However, there was severe damages to the testes of the rats in the DC group due to apoptosis which was enhanced by diabetes. The testicular degeneration observed in the DC group was characterized by atrophic seminiferous tubules (long arrow) and significant loss of spermatogonial cells (short arrow) (Fig. 18B). These observations are consistent with previous reports with respect to the effect of diabetes on the testes (Soliman et al., 2019;

Khosravi et al., 2019; Koroglu et al., 2015). Treatment with 100 and 400 mg/kg of *T. triandra* extract and 25 mg/kg of DHA reversed these alterations in the TT100, TT400 and DHA25 groups as their testes had close resemblance to that of the NC group (Fig. 18C-E).

Numerous evidences have linked diabetes to sexual dysfunctions and infertility in both male and female. Almost 90% of individual living with diabetes suffer from sexual dysfunction which includes impotence, infertility and decreased libido. Testicular dysfunctions causes impairment of spermatogenic functions which leads to erectile dysfunction in men (Soliman et al., 2019; Koroglu et al., 2015). These observations also agrees with the data obtained from testes index which suggested that there was a necrosis of the testes. Hence, *T. triandra* and DHA demonstrated the potential to prevent or reverse diabetes induced sexual dysfunction.

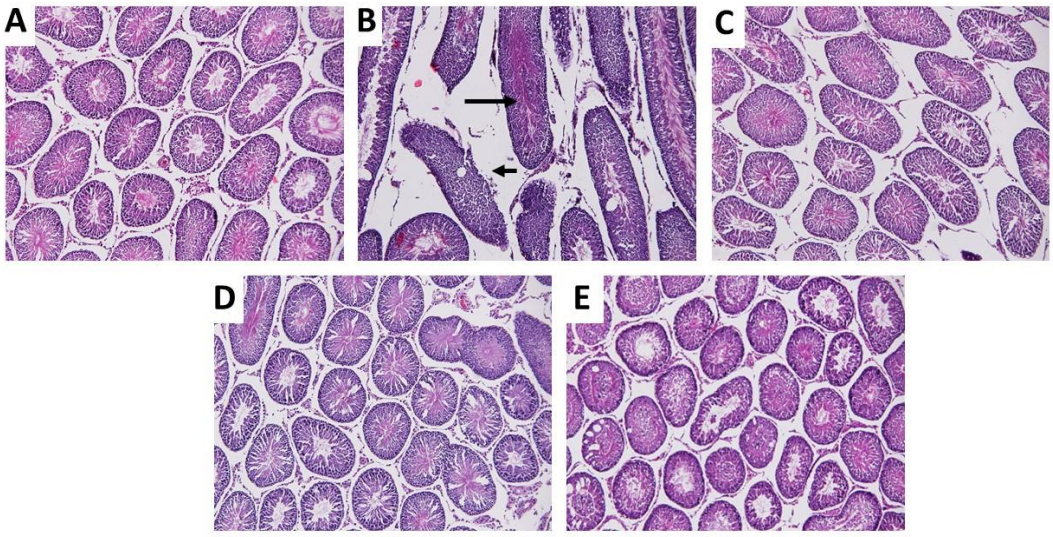

Figure 18A-E. Picture showing the effect of *T. triandra* extract and DHA on the testicular histology. (Magnification: 40x). Long arrow: Seminiferous tubule, Short arrow: Spermatogonial cells. A: Normal control, B: Diabetic control, C: TT100, D: TT400, E: DHA25.

Chapter 4

Conclusion

This research was focused on establishing the antidiabetic activity of *T. triandra* as well as identifying the antidiabetic constituents. This investigation led to the isolation of four compounds; 5,7-dihydroxyl-6-oxoheptadecanoic acid (**1**), ethyl, 5,7-dihydroxyl-6-oxooctadecanoate (**2**), (9Z,12Z,15Z)-ethyl octadeca-9,12,15-trienoate (**3**) (9Z,12Z)-ethyl octadeca-9,12-dienoate (**4**).

The crude ethanol extract of the aerial part of *T. triandra* and fractions TT-001, T-002, TT-004 and TT-005 showed inhibitory activity against alpha-glucosidase enzyme at IC_{50} values of 67.47, 497.45, 5.24, 3.40, and 3.77 μg/mL, respectively, as well as alpha-amylase inhibitory activity at IC_{50} values of 97.23, 94.41, 323.03, 93.74, and 155.46 μg/mL, respectively. All the isolated compounds were also assessed for their alpha-glucosidase and alpha-amylase inhibitory activity. Compound **1** showed α-glucosidase inhibitory activity at IC_{50} value of 11.58 μM and α-amylase inhibitory activity at 22.27 μM, while compounds **2**, **3**, and **4** all showed inhibitory activity against α-glucosidase enzyme at IC_{50} values of 104.77, 424.06 and 105.61 μM, respectively.

Compound **1** (25 mg/kg) and the ethanol extract (100 and 400 mg/kg) of the aerial parts of *T. triandra* also displayed good antidiabetic potentials in high fat diet-streptozotocin induced diabetic rats. Treatment for 30 days caused a suppression of hyperglycemia, increased insulin secretion, improved body weight, and regenerated pancreatic β-cells of the animals. Furthermore, treatment with *T.triandra* extract and compound **1** also alleviated insulin resistance and restored β-cell function as indicated by the HOMA-IR and HOMA-β values. Reduction in the level of glycated hemoglobin of the treated animals proved that treatment with the extract and compound **1** improved glycemic control of the diabetic animals and treatment with *T. triandra* extract and compound **1** also caused improvements in the lipid profile of the treated groups. Furthermore, the extract of *T. triandra* and compound **1** protected against diabetes induced liver damage as indicated by the reduction in the level of liver function enzymes (AST, ALT and ALP), increased total protein, globulin and albumin levels and a decreased level of total and direct bilirubin. Treatment with *T. triandra* extract

and compound **1** also caused a decrease in the level of creatinine and BUN. This implies that *T. triandra* and compound **1** protected against renal dysfunction in diabetic rats. Furthermore, treatment with 400 mg/kg of *T. trinadra* extract and 25 mg/kg of compound **1** reversed hypokalemia and metabolic alkalosis. Compound **1** ameliorated anaemia by improving the red blood cell parameters of the diabetic rats and it also enhanced the immune system of the rats through the improvement of white blood cell parameters. Treatment with *T. triandra* extract lowered the platelet counts of TT100 and TT400 groups. Histopathological assessment revealed that *T. triandra* and compound **1** reversed the damages induced on the pancreas, liver, kidney and testes by diabetes.

Findings in this dissertation revealed that the aerial part of *T. triandra* possess potent antidiabetic activity and it is a prospective source for safer and cheaper natural remedy for diabetes and its associated complications such as diabetic nephropathy, cardiovascular diseases and diabetes induced liver damage. These findings also underline the fact that nature is an ancient pharmacy and a vast deposit of therapeutic solutions.

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Appendix

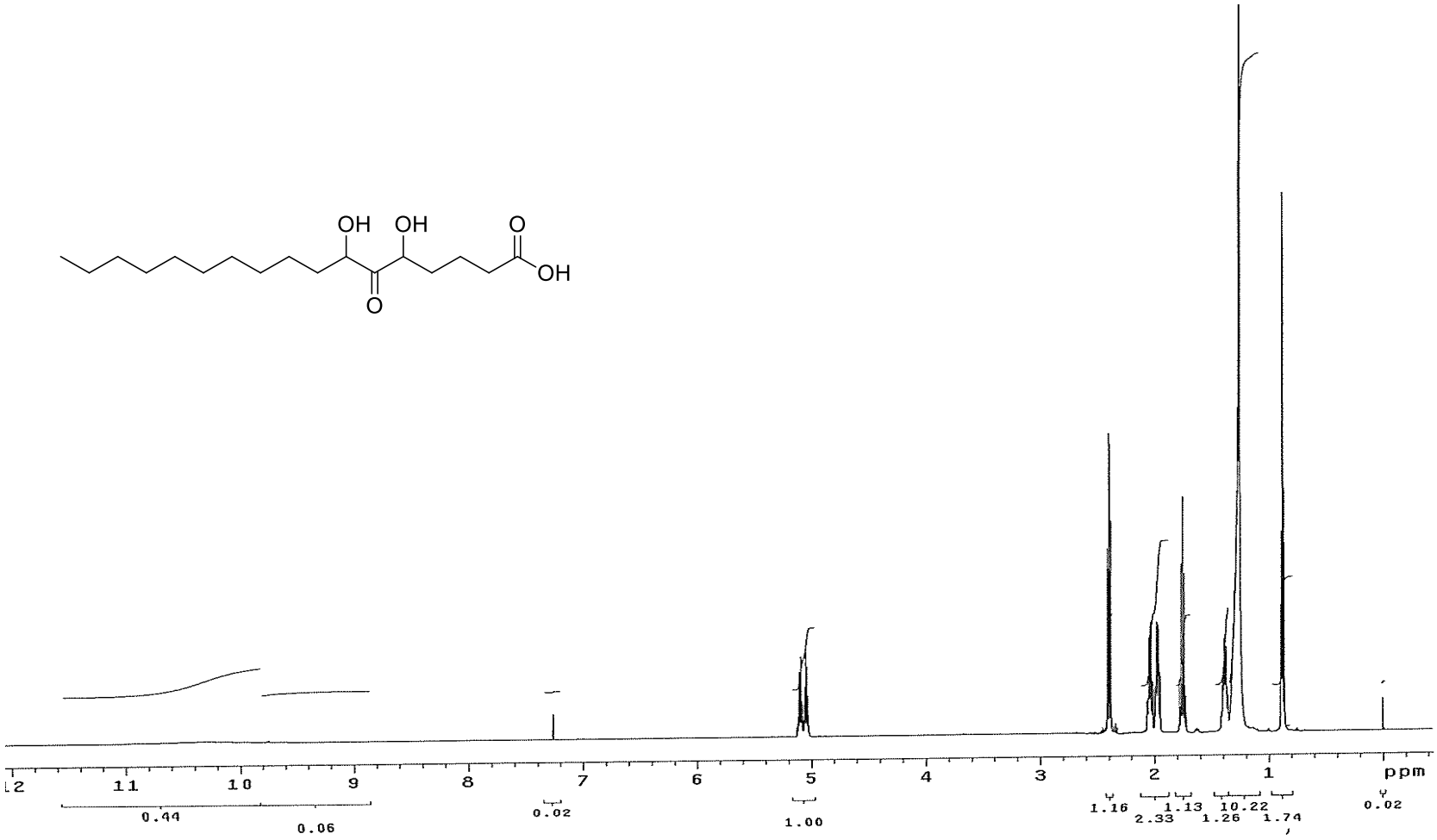

Figure 19. ^{1}H NMR spectrum of compound **1** (500 MHz, $CDCl_3$).

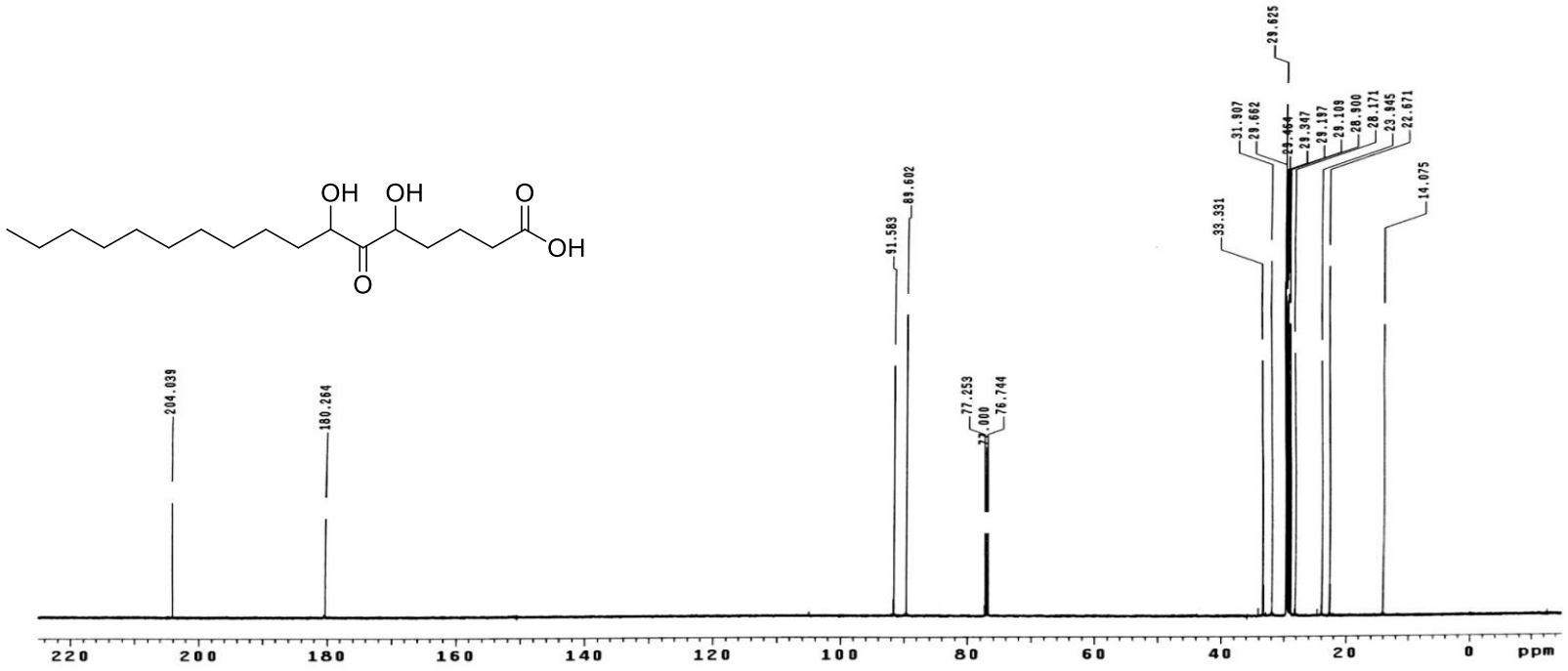

Figure 20. ^{13}C NMR spectrum of compound **1** (125 MHz, $CDCl_3$).

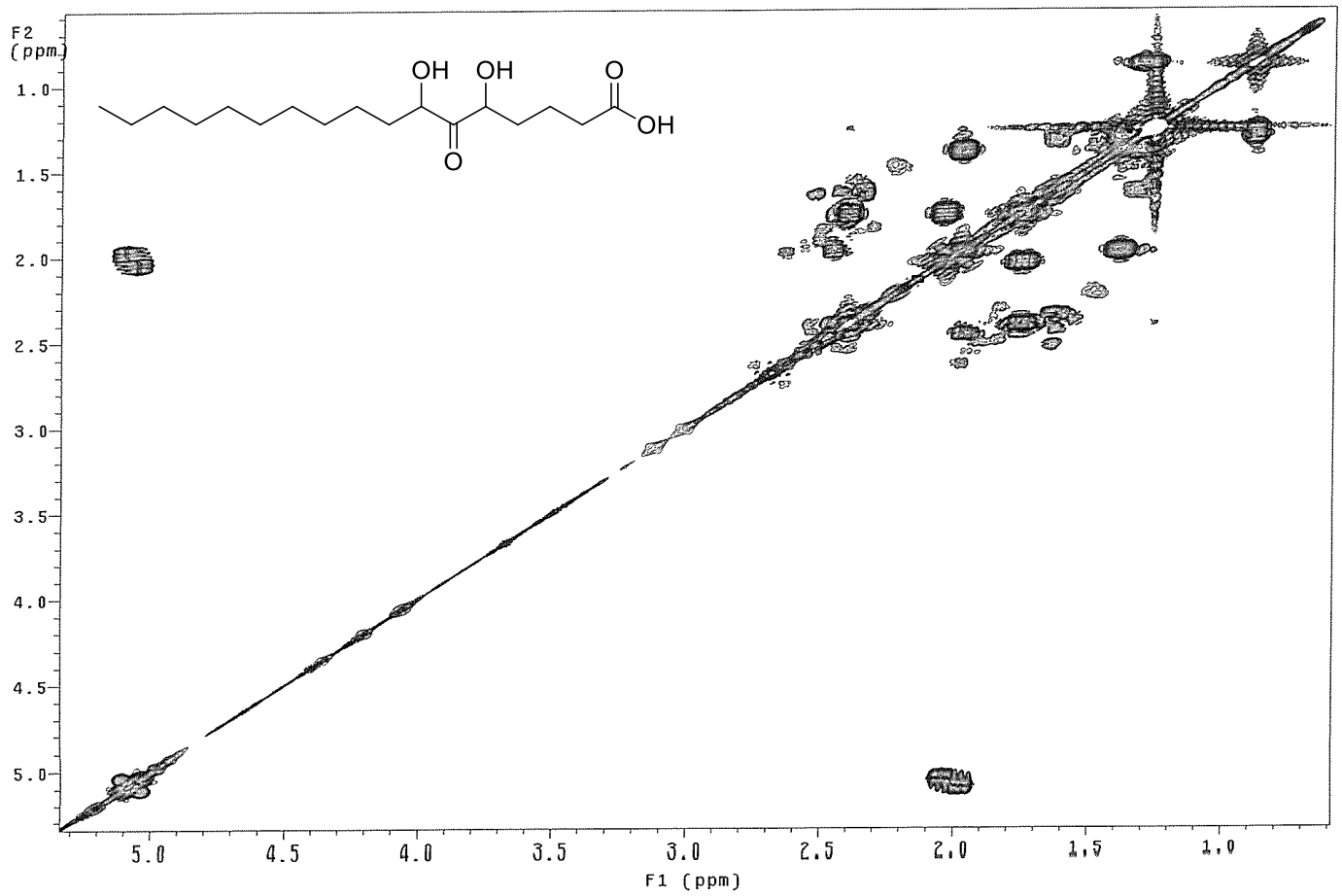

Figure 21. ^{1}H-^{1}H COSY spectrum of compound **1** ($CDCl_3$).

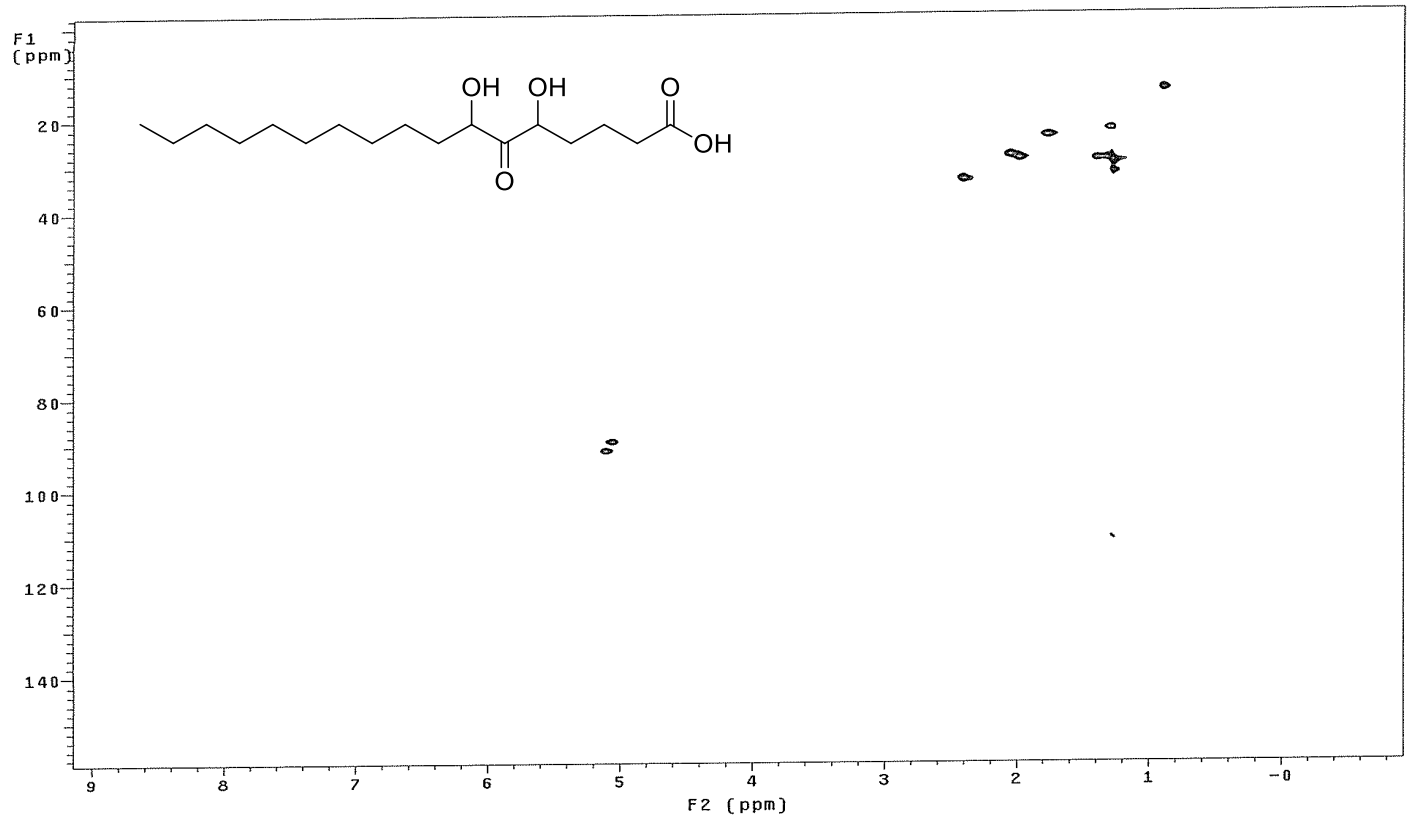

Figure 22. HMQC spectrum of compound **1** ($CDCl_3$).

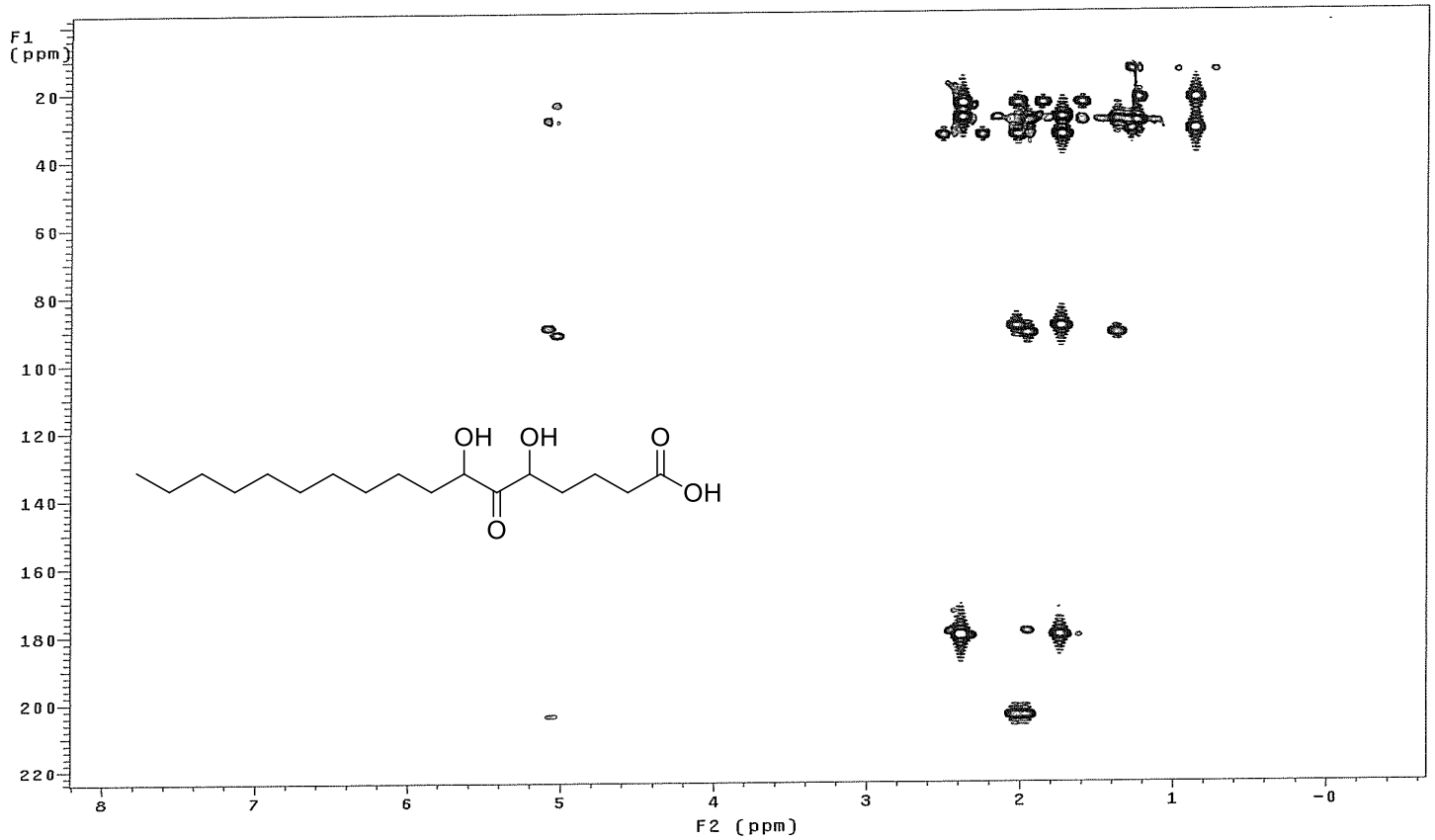

Figure 23. HMBC spectrum of compound **1** ($CDCl_3$).

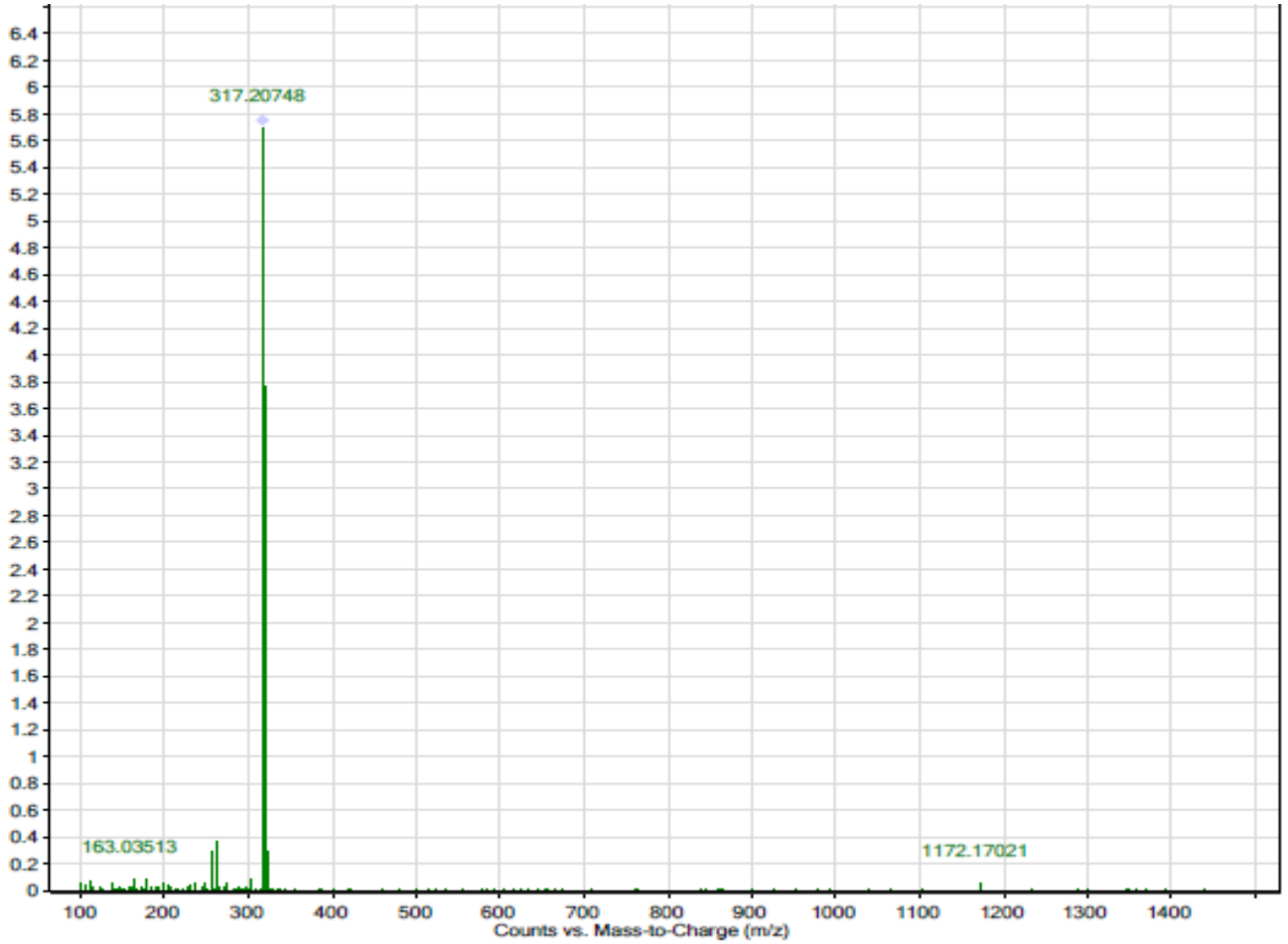

Figure 24. HRESIMS spectrum of compound **1.**

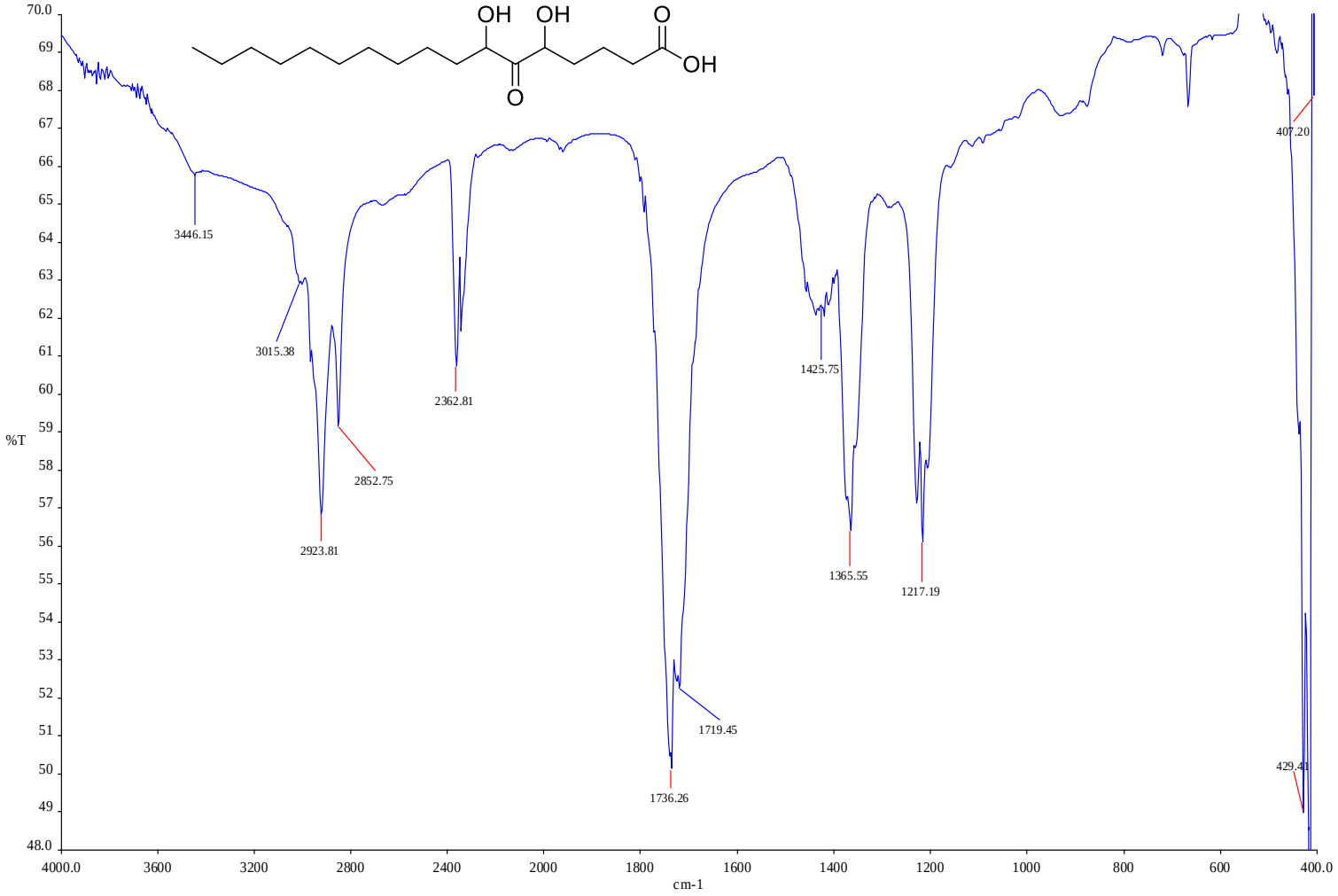

Figure 25. IR spectrum of compound **1** (Neat).

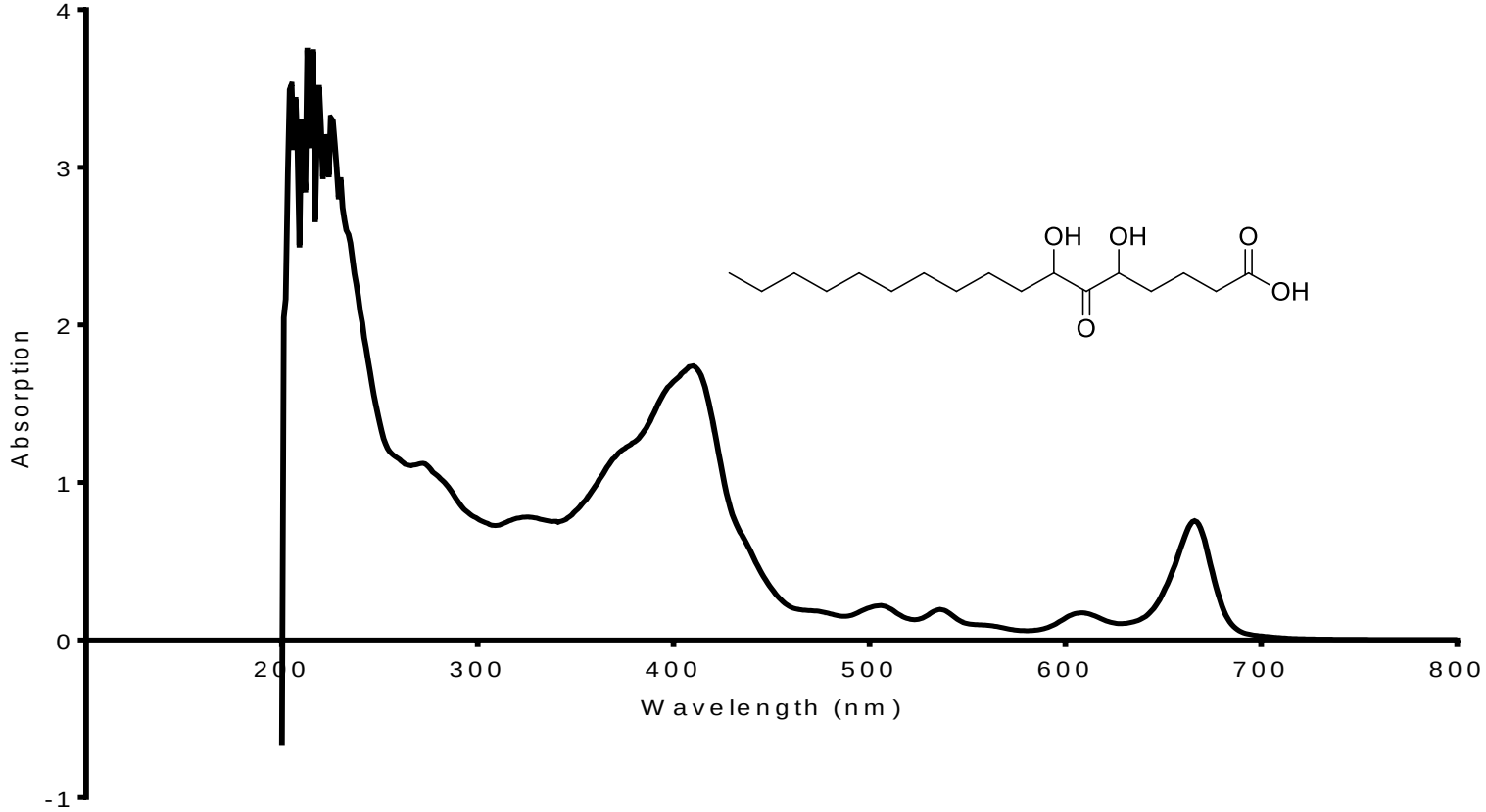

Figure 26. UV spectrum of compound **1** (EtOH).

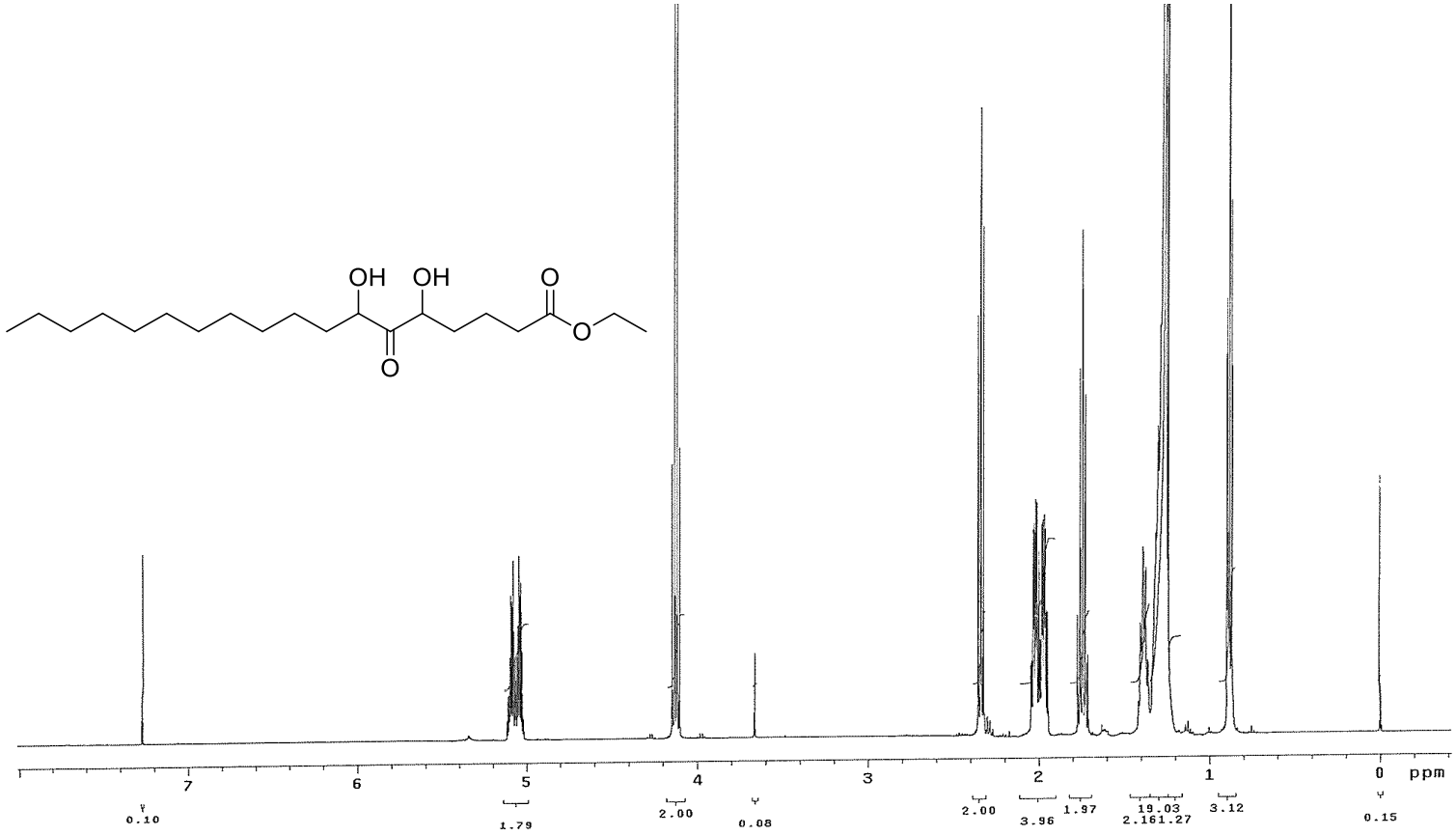

Figure 27. ^{1}H NMR spectrum of compound **2** (500 MHz, $CDCl_3$).

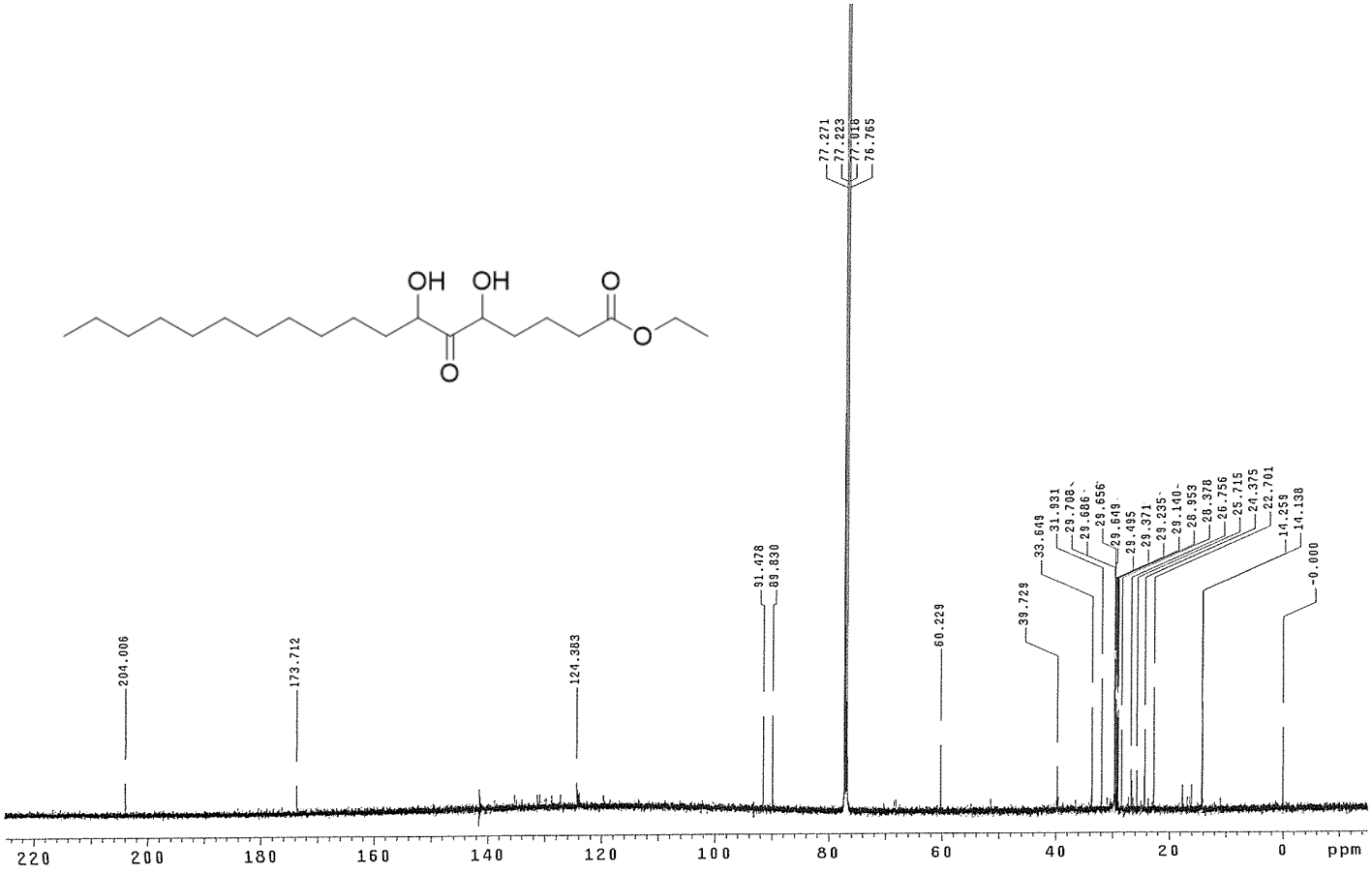

Figure 28. ^{13}C NMR spectrum of compound **2** (125 MHz, $CDCl_3$).

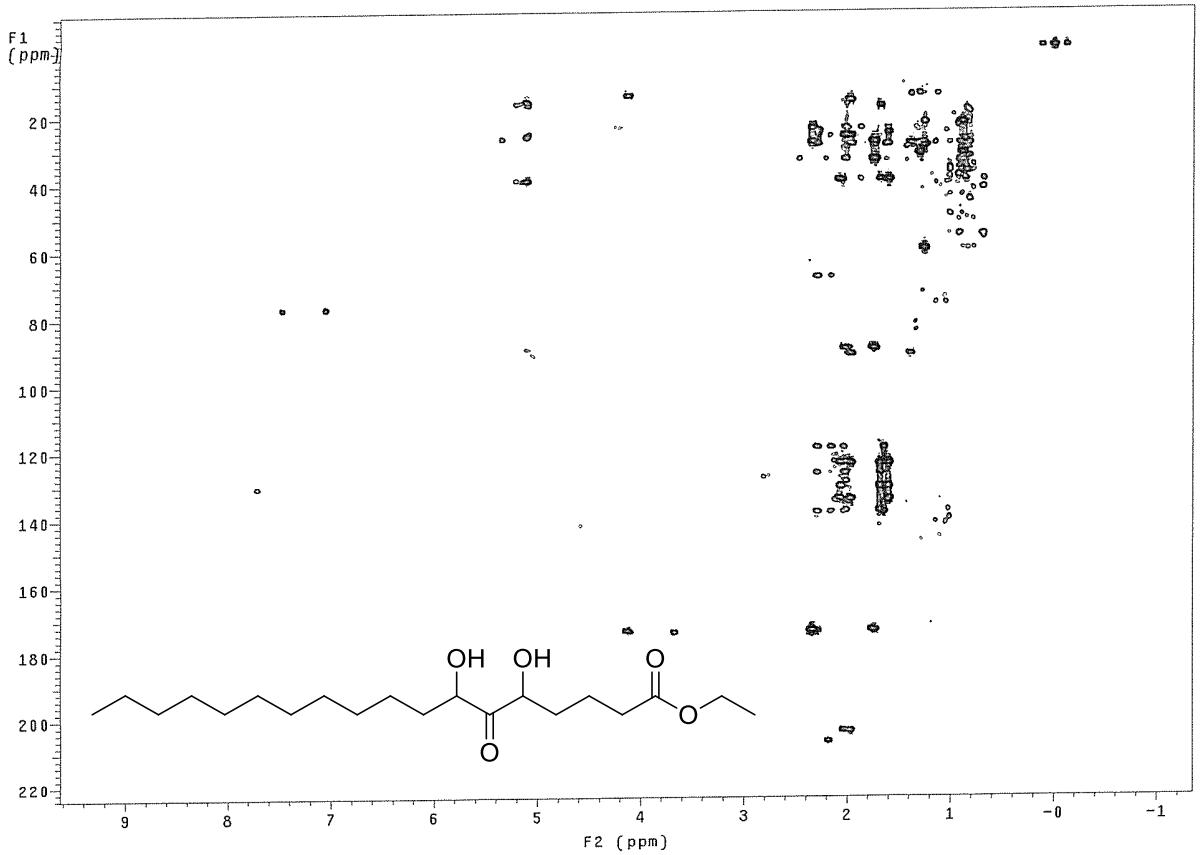

Figure 29. HMBC spectrum of compound **2** ($CDCl_3$).

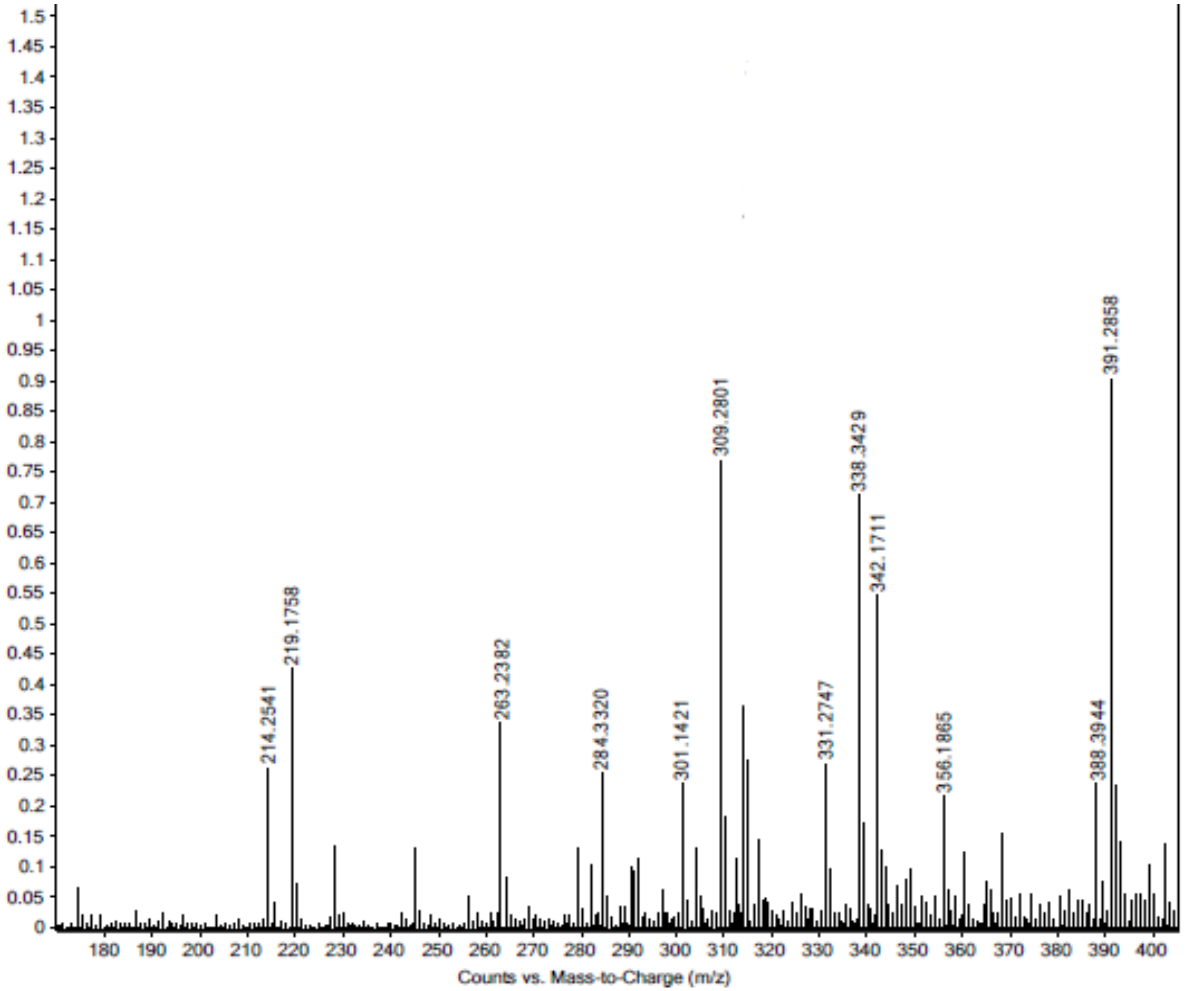

Figure 30. HRESIMS spectrum of compound **2**.

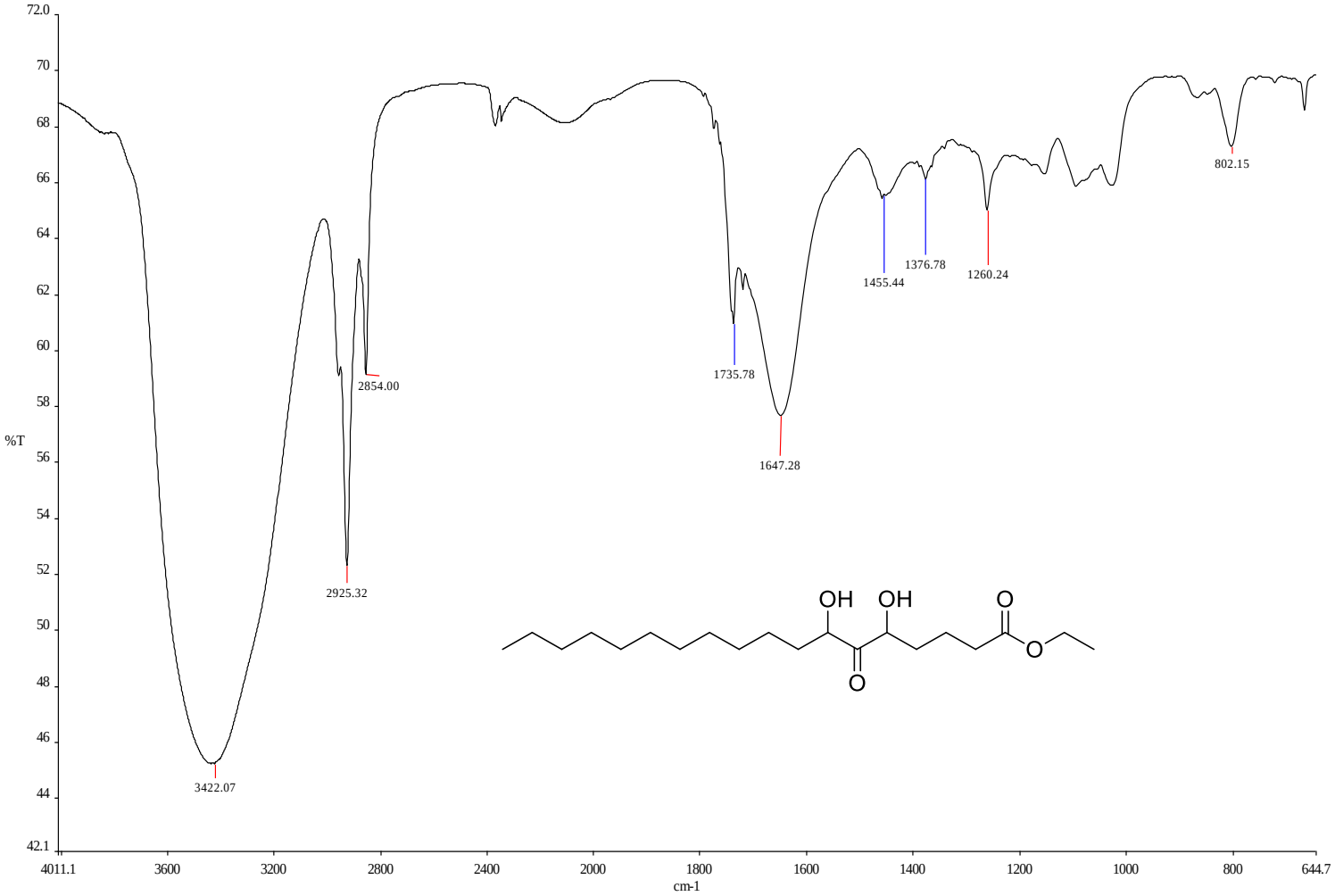

Figure 31. IR spectrum of compound **2** (Neat).

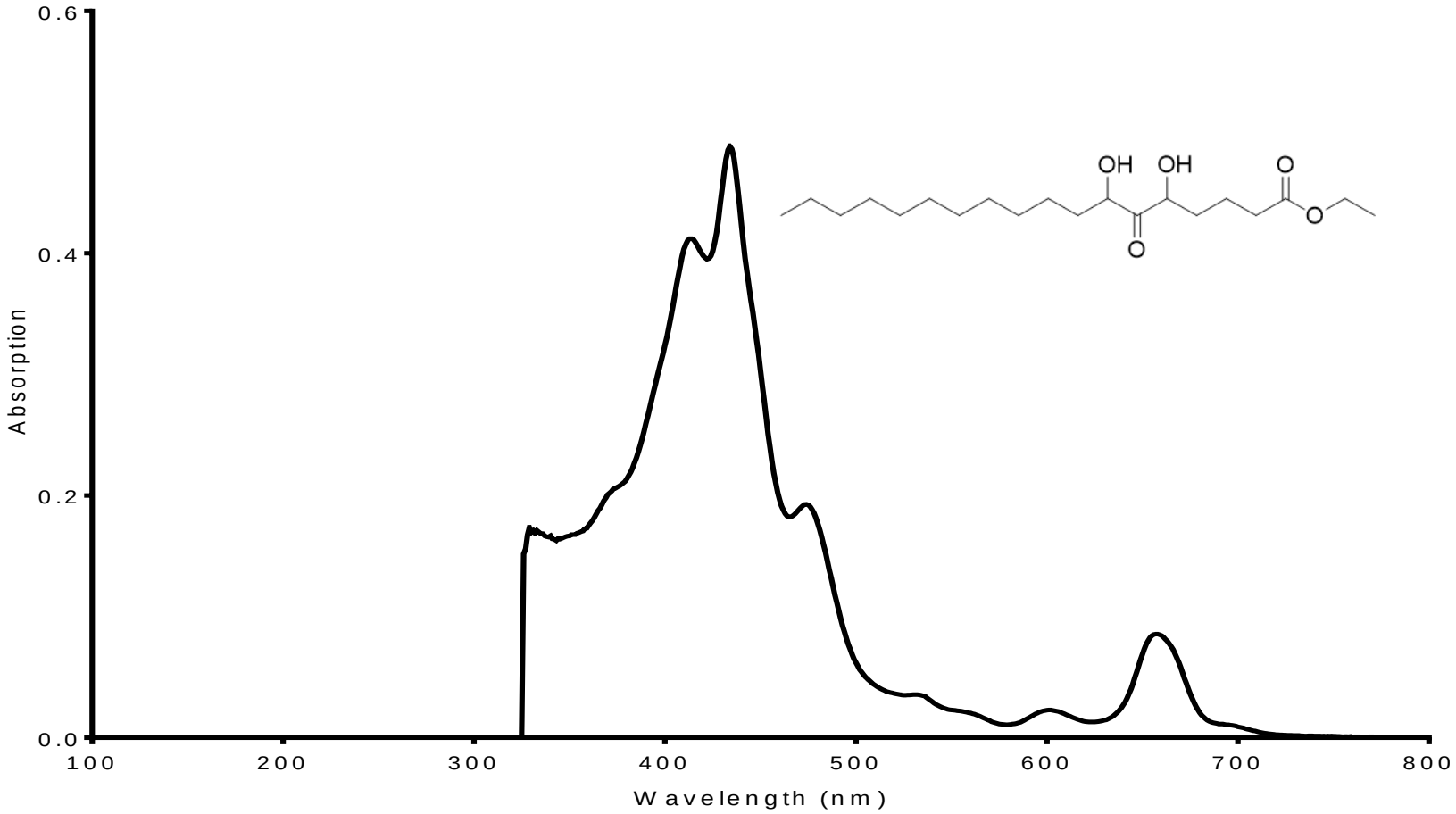

Figure 32. UV spectrum of compound **2** (EtOH).

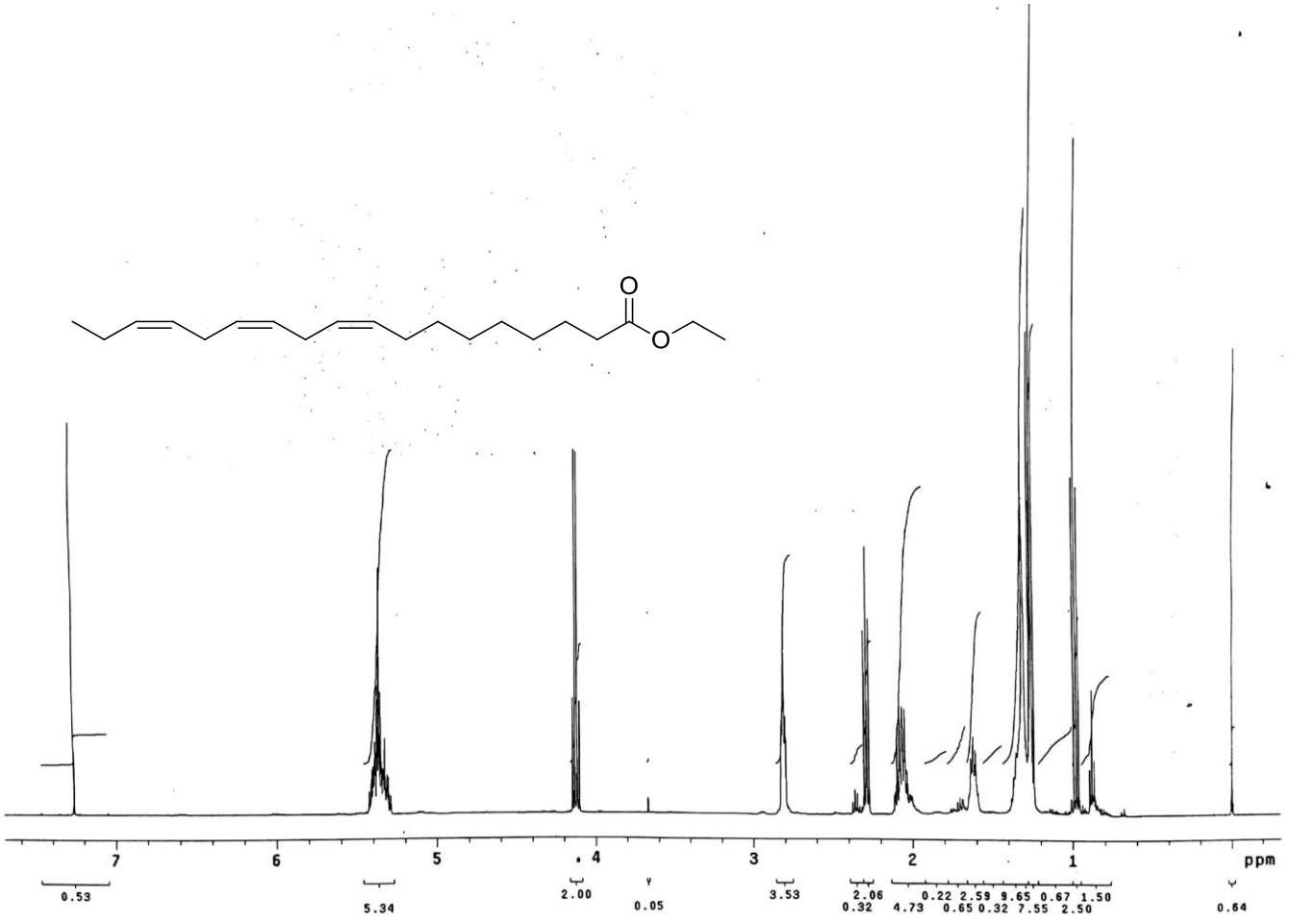

Figure 33. ^{1}H NMR spectrum of compound **3** (500 MHz, $CDCl_3$).

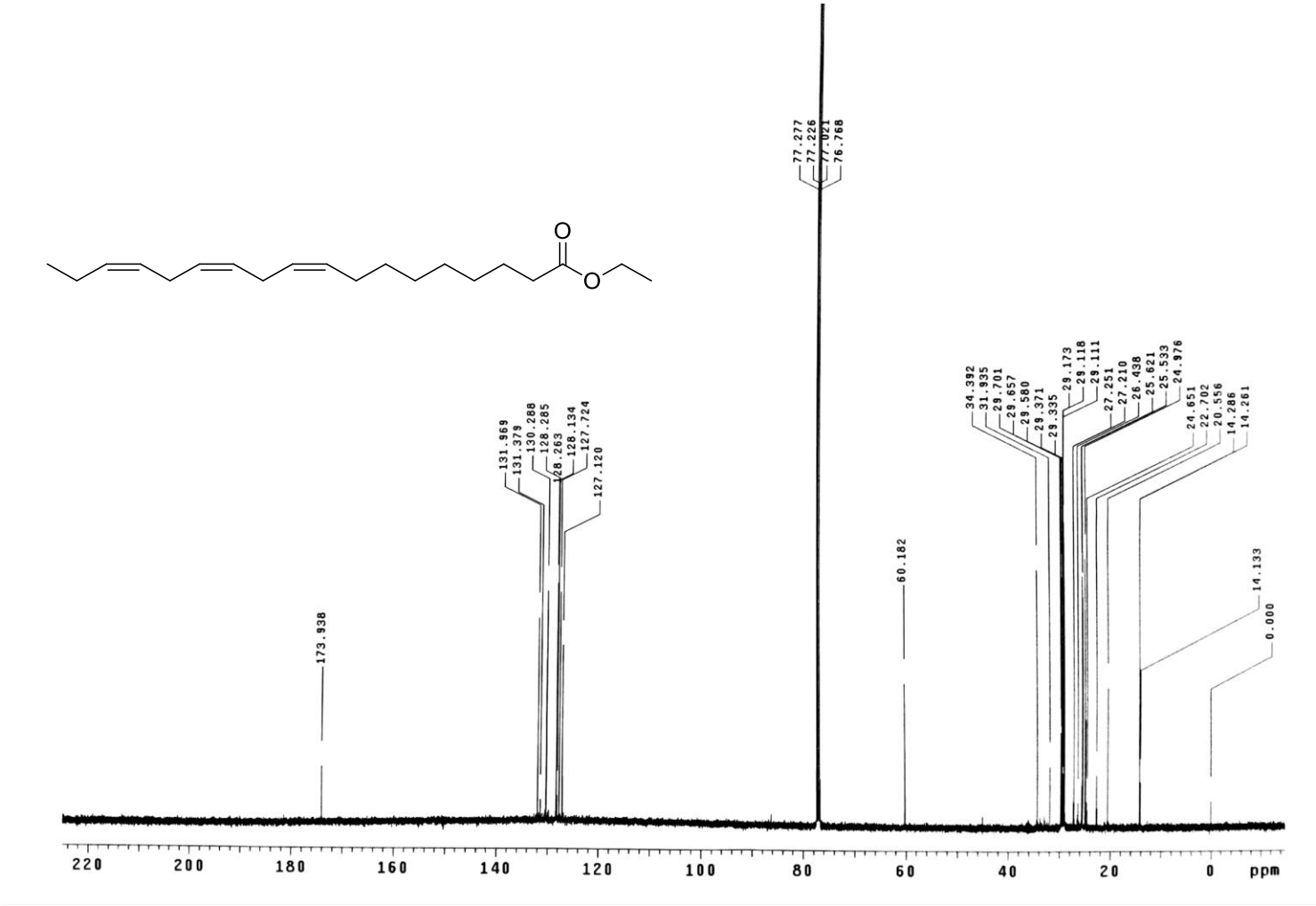

Figure 34. ^{13}C NMR spectrum of compound **3** (125 MHz, $CDCl_3$).

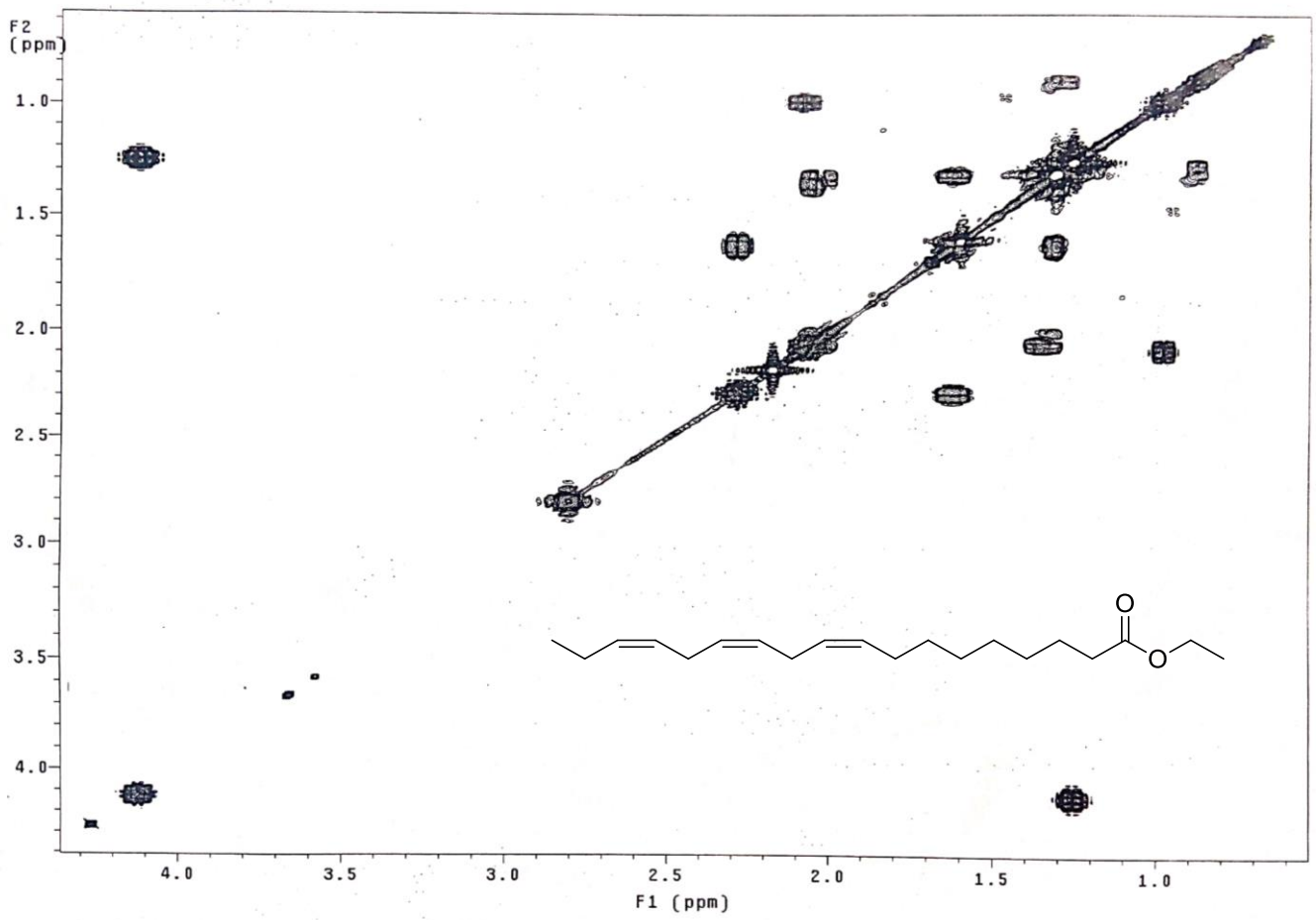

Figure 35. ^{1}H-^{1}H COSY spectrum of compound **3** ($CDCl_3$).

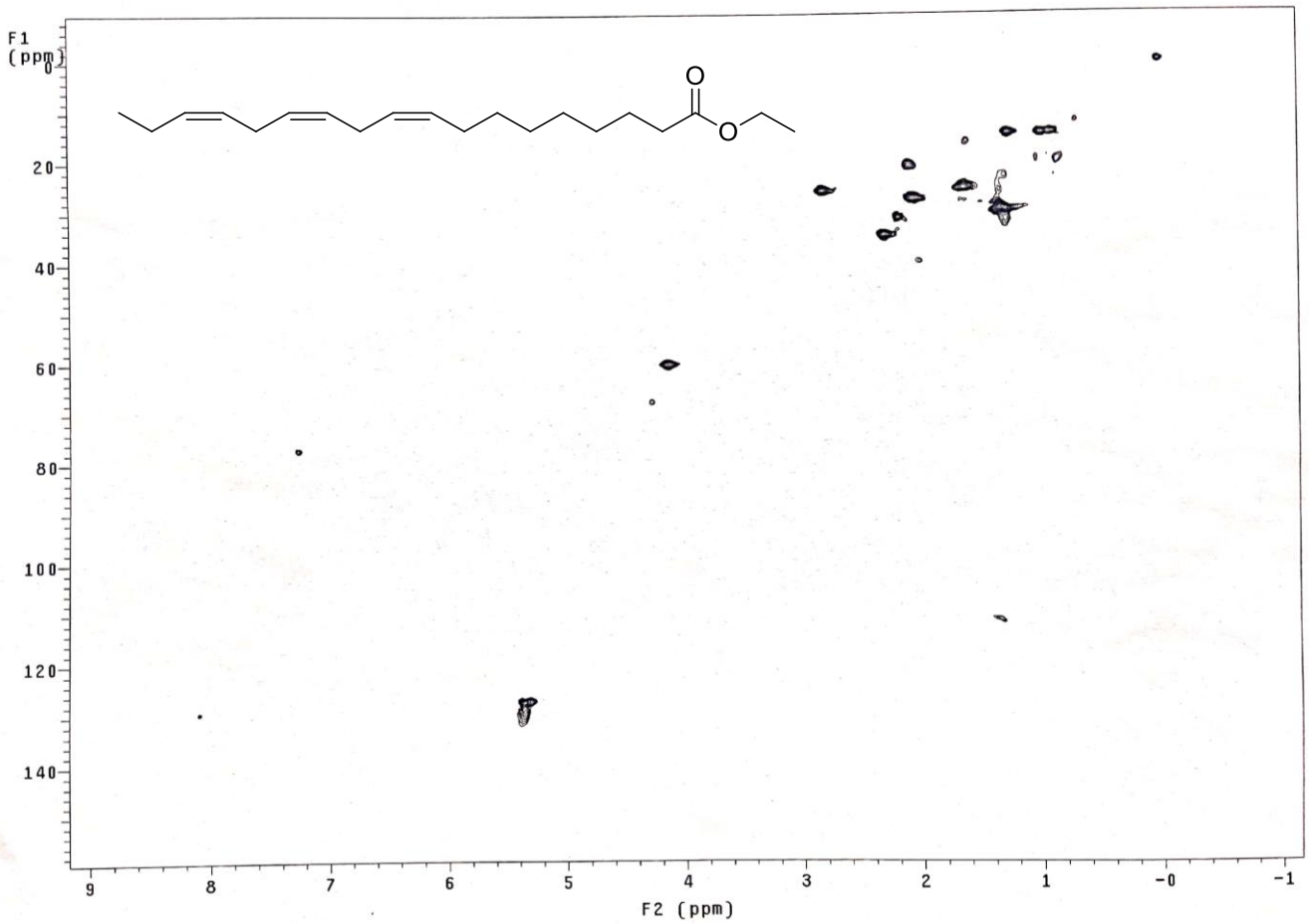

Figure 36. HMQC spectrum of compound **3** ($CDCl_3$).

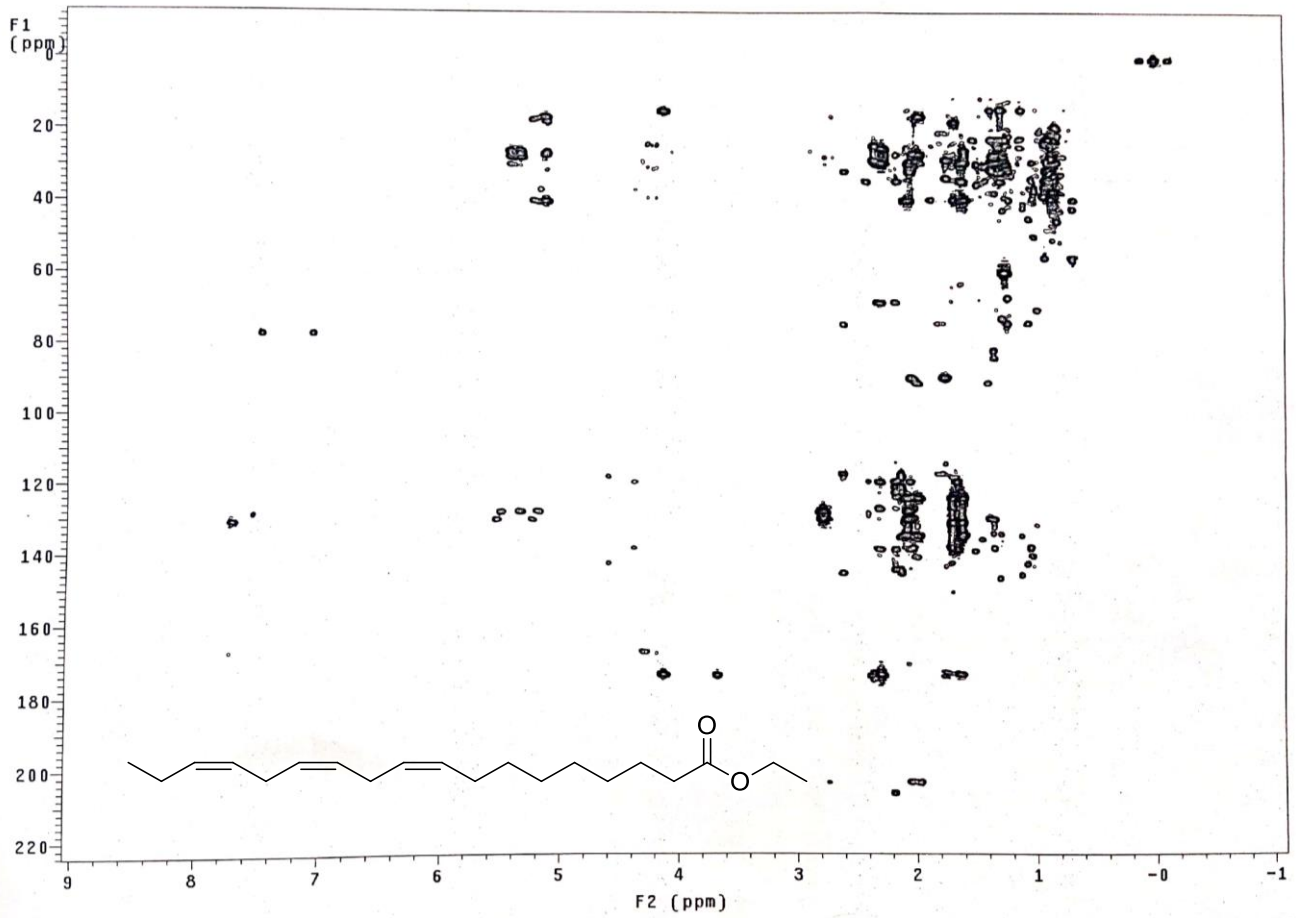

Figure 37. HMBC spectrum of compound **3** ($CDCl_3$).

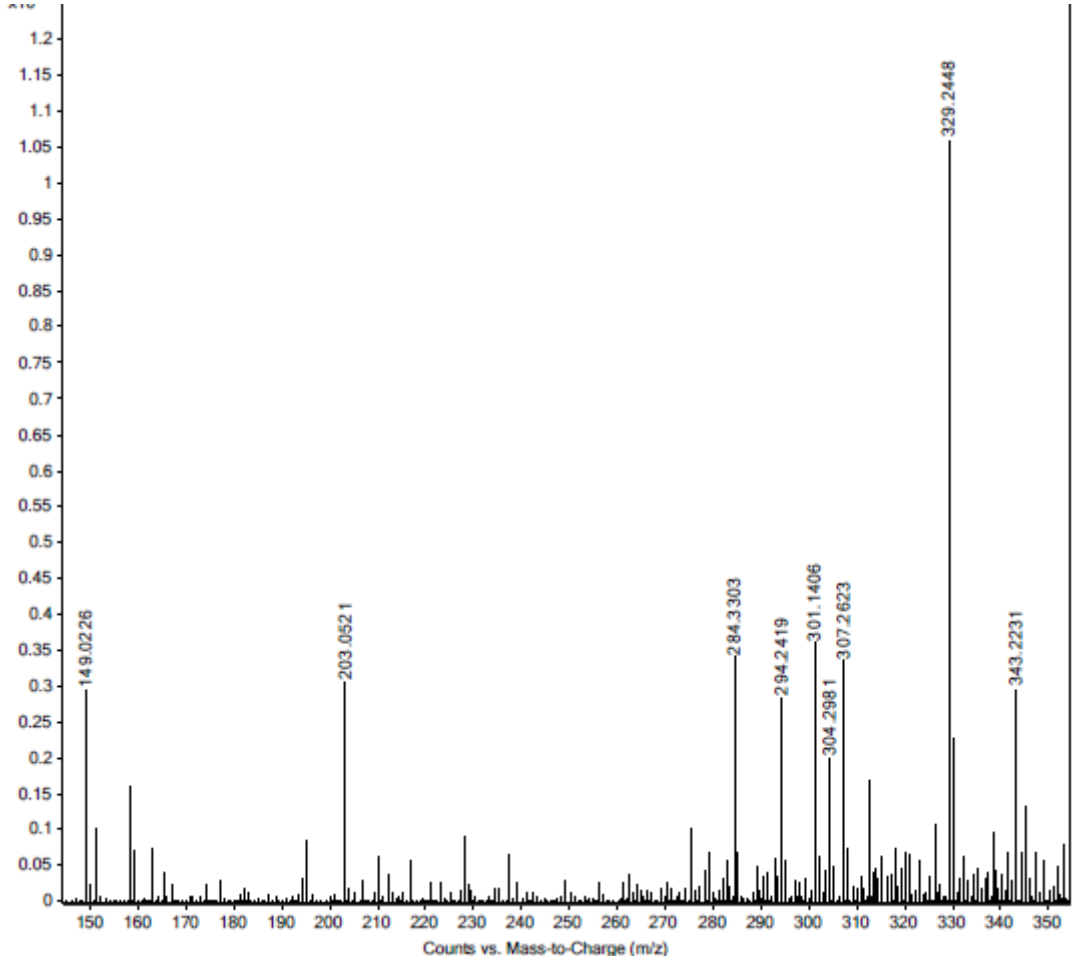

Figure 38. HRESIMS spectrum of compound **3**.

Figure 39. IR spectrum of compound **3** (Neat).

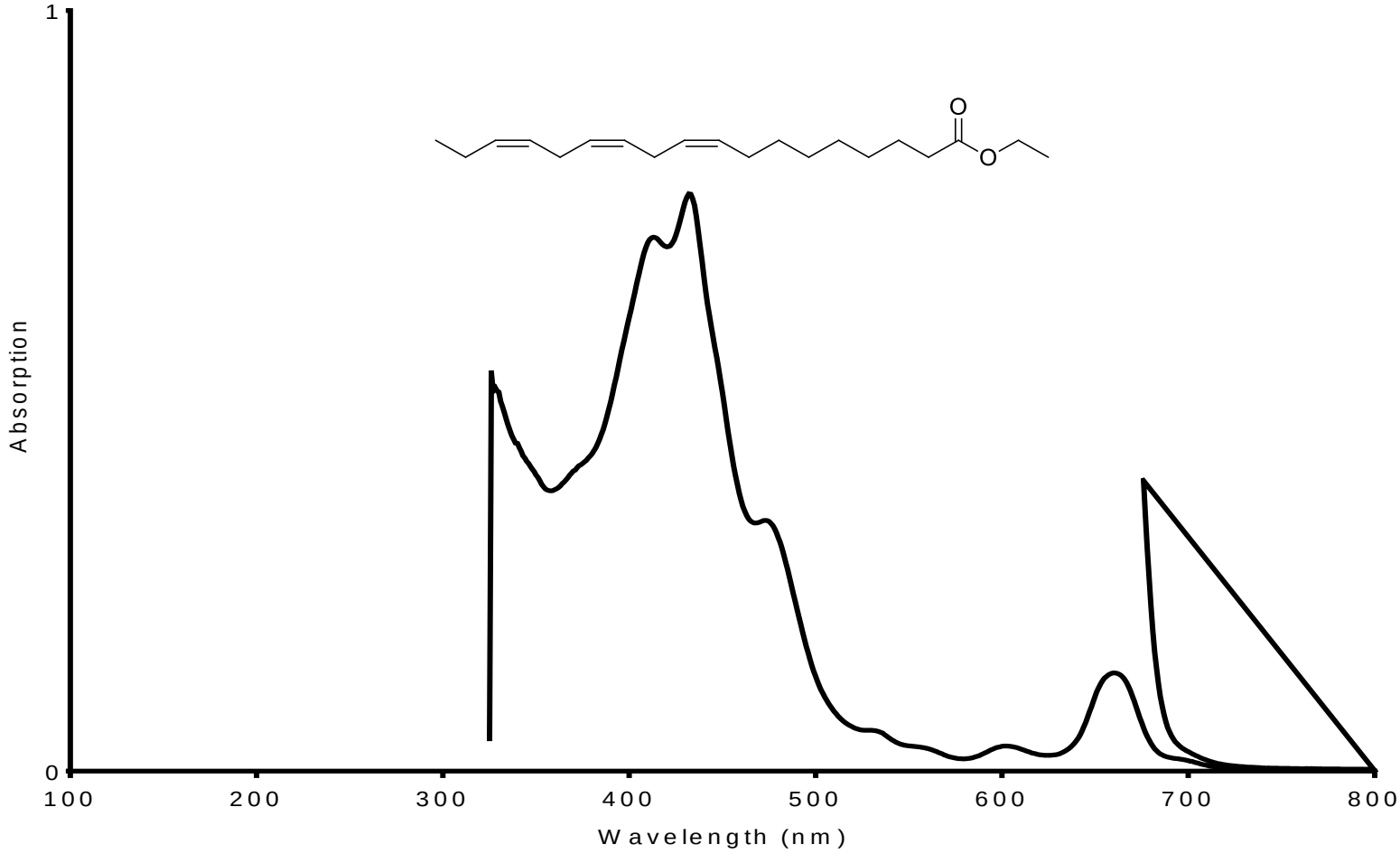

Figure 40. UV spectrum of compound **3** (EtOH).

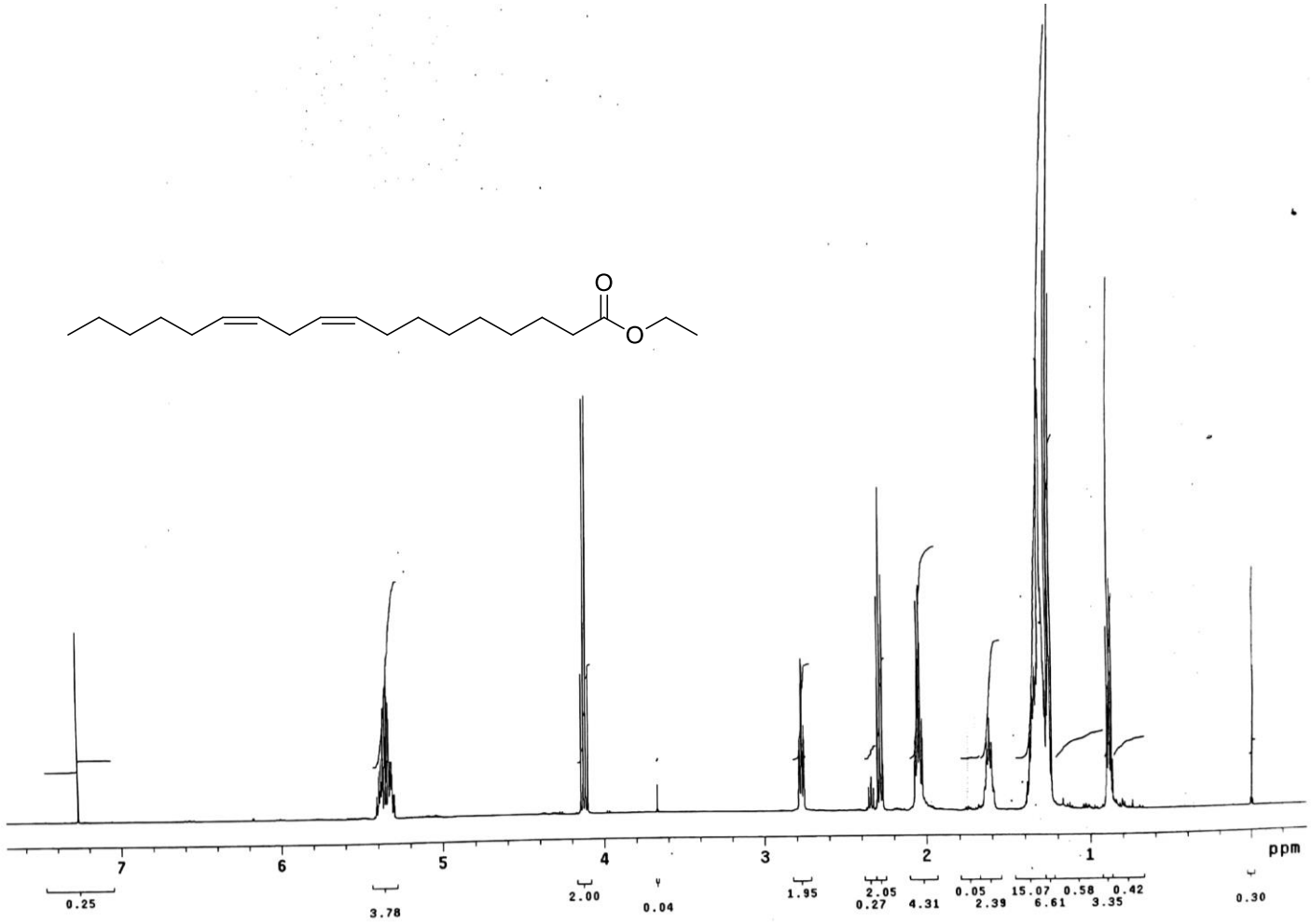

Figure 41. ^{1}H NMR spectrum of compound **4** (500 MHz, $CDCl_3$).

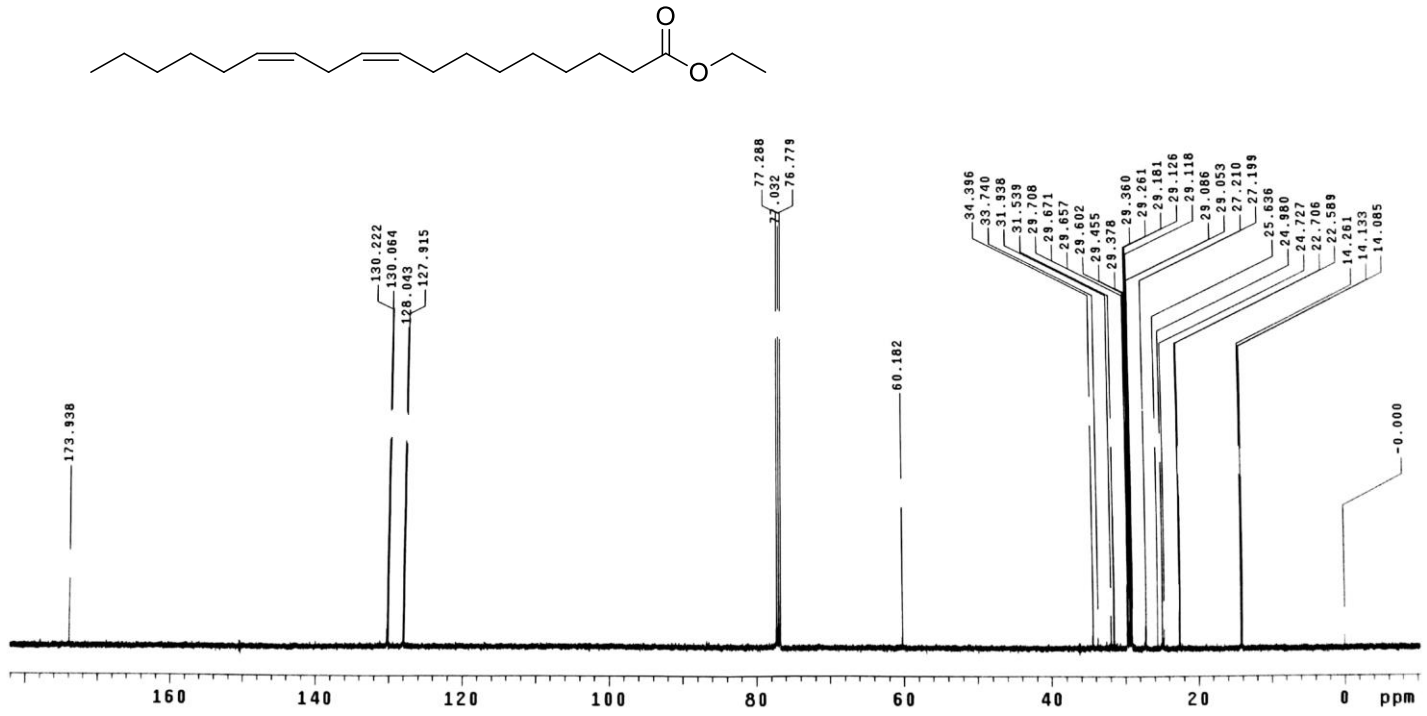

Figure 42. ^{13}C NMR spectrum of compound **4** (125 MHz, $CDCl_3$).

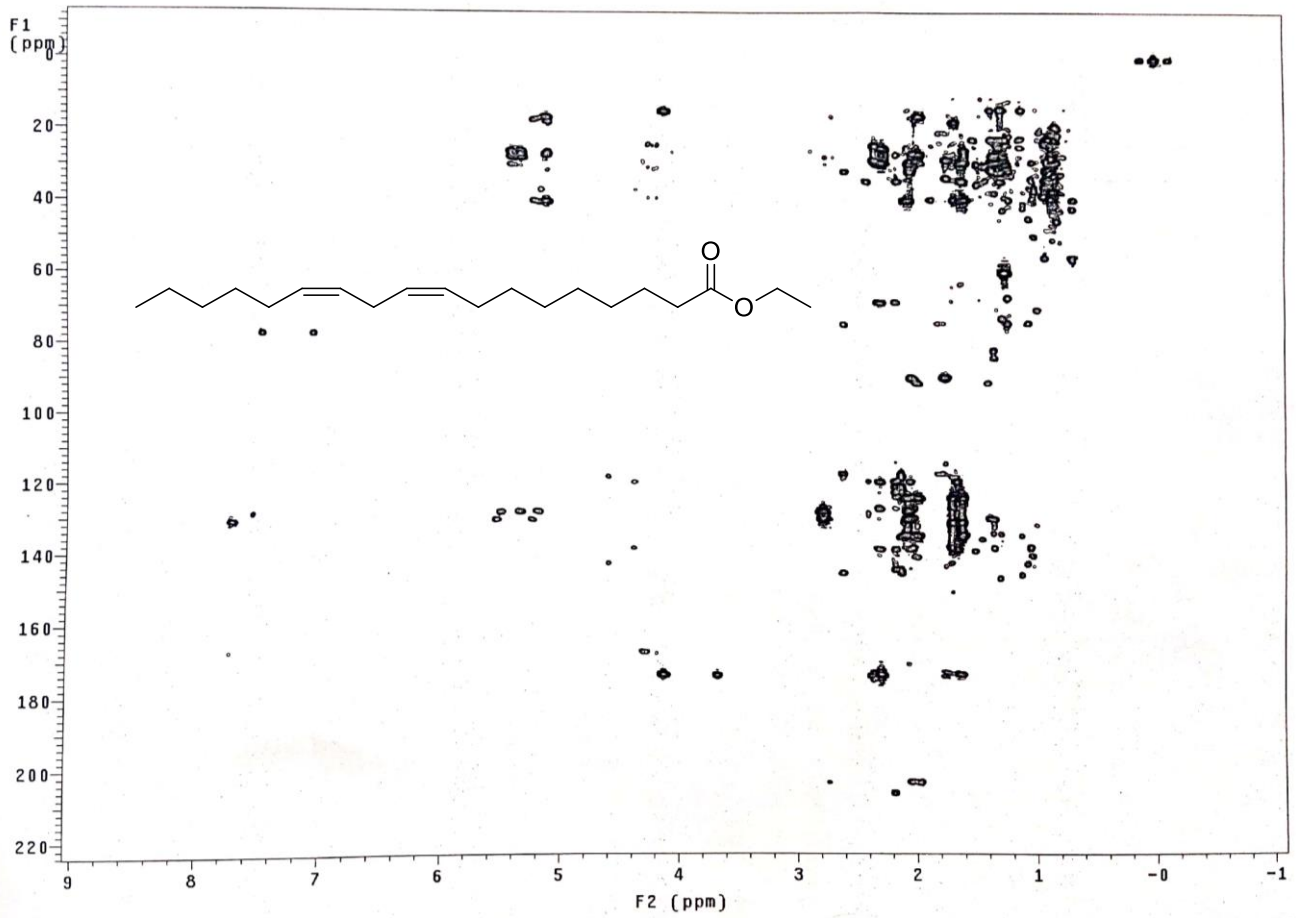

Figure 43. HMBC spectrum of compound **4** ($CDCl_3$).

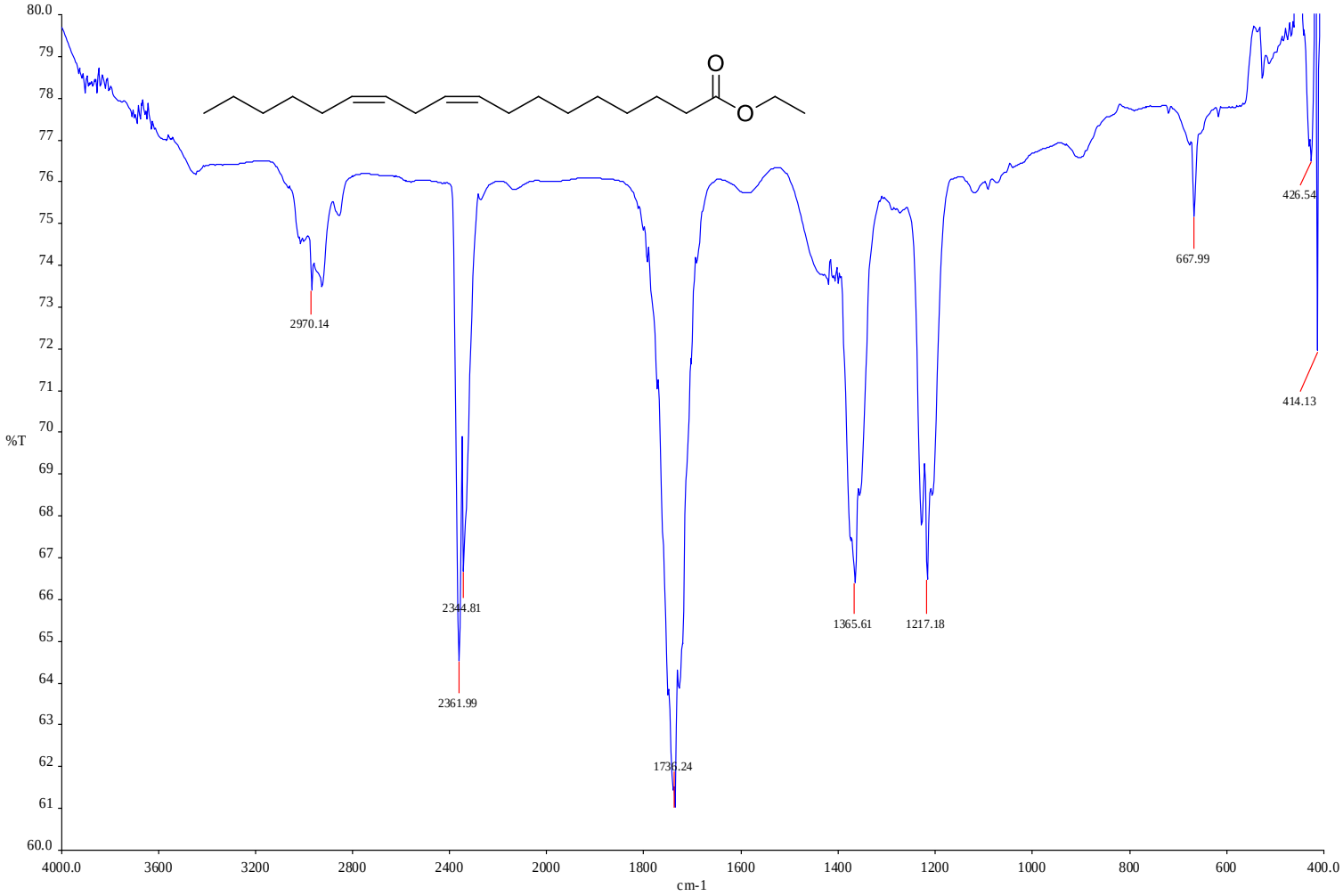

Figure 44. IR spectrum of compound **4** (Neat).

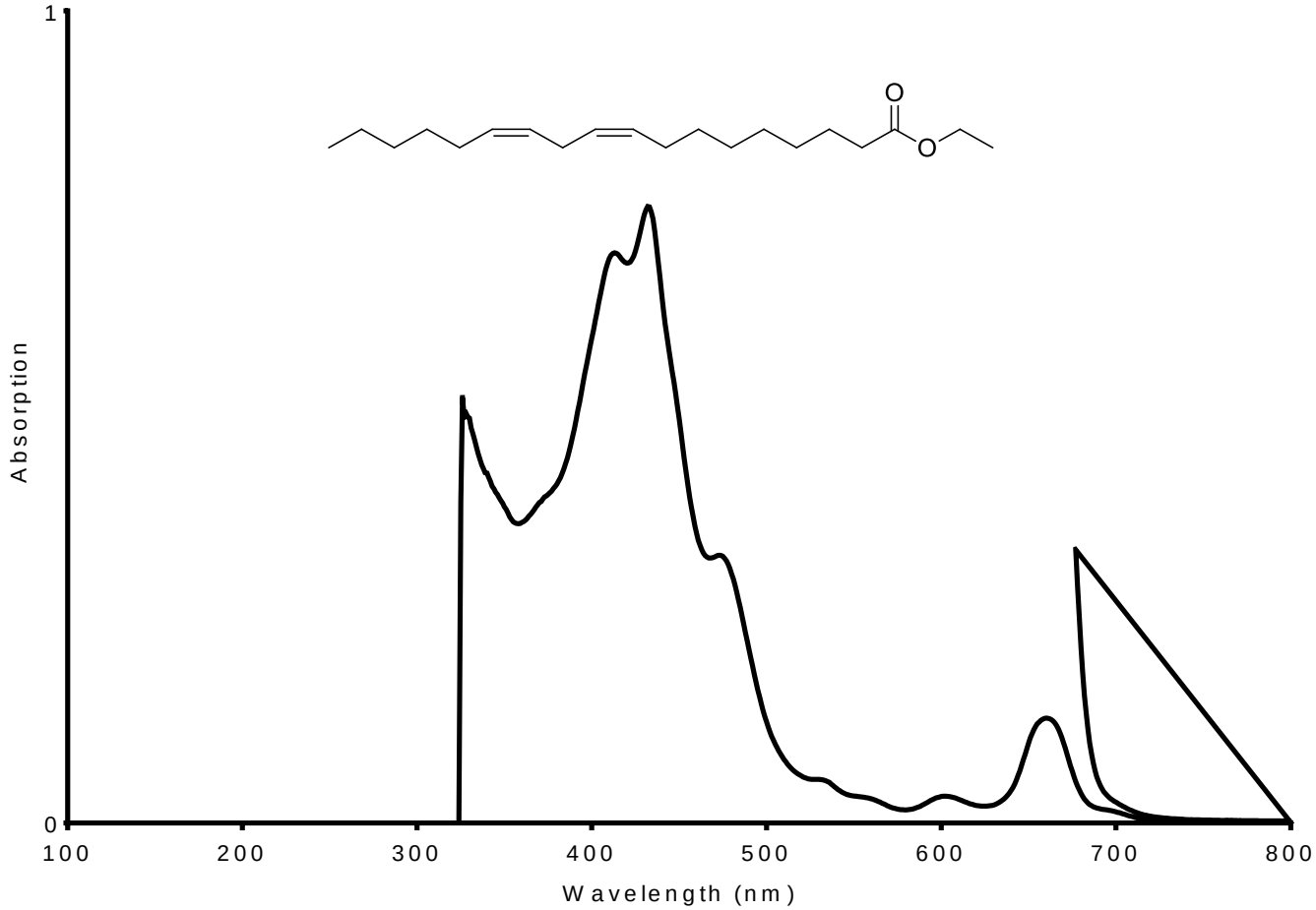

Figure 45. UV spectrum of compound **4** (EtOH).

VITAE

Name Emmanuel Ayobami Makinde

Student ID 6011420001

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Industrial Chemistry)	University of Ilorin, Nigeria	2014

Scholarship Awards during Enrolment

- Thailand Education Hub for ASEAN Countries (TEH-AC) award for Masters study year 2017-2019 (Grant No. TEH-AC 029/2017).
- Graduate School Dissertation Funding for Thesis (2019).

List of Publication

- Makinde, E. A., Ovatlarnporn, C., Adekoya, A. E., Nwabor, O. F. and Olatunji, O. J. Antidiabetic, antioxidant and antimicrobial activity of the aerial part of *Tiliacora triandra*. *S. Afr. J. Bot.* **2019**, 125, 337–343.
- Zhao, L. L., Makinde, E. A., Shah, M. A., Olatunji, O. J., and Panichayupakaranant, P. Rhinacanthins-rich extract and rhinacanthin C ameliorate oxidative stress and inflammation in streptozotocin-nicotinamide induced diabetic nephropathy. *J. Food Biochem.* **2019**, 43, e12812.