ANALYSIS OF ANTIOXIDATIVE CONSTITUENTS AND TOTAL PHENOLIC CONTENT OF *MORINGA OLEIFERA*

BY

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Declaration

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Ahmad Ali Yakasai and has not been presented anywhere for the award of a degree or certificate. All sources have been duly acknowledged

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Certification

This is to certify that the research work for this dissertation and subsequent write up (Ayuba Suleiman SPS/11/MCH/00017) were carried out under supervision.

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Approval

This dissertation has been examined and approved for the award of

Masters in Organic Chemistry.

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Acknowledgement

Praise is to Almighty Allah (S. W. T) the Most Gracious, and the Most Merciful for giving me the strength, guidance, courage, good health and the opportunity to undergo this program successfully. Peace and blessing be upon His prophet Muhammad (S.A.W)

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Dedication

I dedicated this work to my lovely parents Alh Suleiman Yakub and Hajiya Asiya Abubakar.

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LIST OF ABBREVIATIONS

%(AA)	Percentage Antioxidant Activity
$[M+H]^+$	Molecular ion
$[M+Na]^+$	Adduct ion (Molecular ion + sodium)
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
ABTS	2, 2- azinobis(3-ethylbenzothiazoline-6-sulfonic acid
BHA	Butylated hydroxyl anisole
BHT	Butylated Hydroxy Toulene
COSY	Correlation Spectroscopy
CUPRAC	Cupric reducing antioxidant power
DAD	Diode Array Detector
DNA	Deoxyribose nucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ESI	Electro spray Ionization
ESI-MS	Electro Spray Ionization Mass Spectrometry
FA	Folin-Ciocalteau
FRAP	Ferric reducing antioxidant power
GAE	Gallic Acid Equivalent
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
$L^{\cdot}$	Allyl radical

LC_{50}	Lethal Concentration at 50%
LC-MS	Liquid Chromatography Mass Spectrometry
L-H	Substrates molecule
$LOO^{\cdot}$	peroxyl radical
M/Z	Mass per Charge ratio
mAU	milIi Absorbance Unit
Mg/GAE	Milligram per Gallic Acid Equivalent
MS	Micro Soft
NMR	Nuclear Magnetic Resonance
ODS	Octa Docane Silane
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffer Solution
ppm	Part Per Million
Prep TLC	Preparative Thin Layer Chromatography
$R^{\cdot}$	Initiation oxidizing radical
Redox	Reduction and oxidation
R_F	Retention Factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen Species
SPSS	Statistical Software
TLC	Thin Layer Chromatography
TPTZ	2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine

UV	Ultra Violet
W.H.O	World Health Organization
λ_{max}	Wavelength of maximum absorption

Abstract

Moringa oleifera is traditionally consumed as food and in the treatment of many various diseases. Air dried leaves and stems of the plant was extracted with ethanol and the crude extract was partitioned using solvents of different polarities to yield crude ethanol extract, methanol fraction, ethyl acetate fraction and n-hexane fraction labelled F1-F4 respectively. The extract and fractions were tested for anti-oxidant (radical scavenging) activity using 1, 1-diphenyl-2-picrylhydraxyl (DPPH) free radical. The result showed that methanol fraction possessed the highest scavenging activity. It was therefore subjected to activity guided column chromatography which yielded 15 pooled fractions. DPPH antioxidant assay on these fractions showed that fractions AS-F4-1-99, AS-F4-1-87 and AS-F4-109 possessed the highest activity with IC_{50} of 23.704, 24.704 and 46.07µg/ml respectively. Further activity guided chromatographic fractions coupled with HPLC and LCMS analysis led to the identification of the chemical constituents responsible for the observed activity. Two compounds were isolated and analyzed by NMR but complete structural elucidation was inconclusive due to small sample size. The combined spectroscopic and mass spectrometric analysis revealed phenolic compounds to be partly responsible for the antioxidant properties of the leaves and stem of Moringa oleifera. This result was further supported from the result of Folin-ciocalteau analysis of the extract and fractions.

CHAPTER ONE

1.0 Introduction

Natural products are organic compounds that are produced by living system (James, 2010). The uses of these compounds as medicines and other forms have been well documented throughout history; although most of the active ingredients contained in these products were chemically unknown (Dias *et al.,* 2012). Natural product compounds are broadly divided into primary and secondary metabolites. Primary metabolites occur in all organisms and play a central role in metabolism and reproduction of the cells. They include carbohydrates, protein, nucleic acid and lipids. While secondary metabolites are those compounds that are characterized for a limited range of species. These classes of natural products attract more interest due to their biological effects on other organisms. They are largely responsible for the biologically active effects of medicinal, commercial and poisonous plants. Studies have indicated that natural product organic compounds constitute more than 40% of all known medicines. They include alkaloids, flavonoids, phenolics, and saponins (Stankovic, 2011). Secondary metabolites produced by plants have important biological and pharmacological applications as antioxidants, antiallergenic, antibiotics, ant malarial, hyperglycemic and anti-cancer (Stankovic, 2011; Sahakitpichan *et al*., 2011).

1.1. Importance of Natural Products

Throughout history, human beings rely on nature to gather most of their basic needs which includes medicines used for the treatment of a wide range of diseases. Plants in particular, have formed the basis of traditional medical systems. The earliest records of human application of natural products as medicines date back to 2600 BCE. Mesopotamian record documented the uses of approximately1000 plant-derived substances. These include oils of *Cedrus species* (cedar) and *Cupressussempevirens* (cypress), *Glycyrrhizaglabra* (licorice), *Commiphora species* (myrrh), and *Papaversomniferum* (poppy juice), all of which are still in use for the treatment of ailments ranging from coughs, colds to parasitic infections and inflammation. The best-known record is the "Ebers Papyrus" dating from 1500 BCE, documenting over 700 drugs, mostly of plant origin. The Chinese *Materia Medica* has been extensively documented over the centuries with the first record dating from about 1100 B. C. (Wu Shi Er Bing Fang, containing 52 prescriptions), followed by works such as the Shennong Herbal (~100 B. C.; 365 drugs) and the Tang Herbal (659 A. D.; 850 drugs). Likewise, documentation of the Indian Ayurvedic system dates from before 1000 B. C. (Charaka; Sushruta and Samhitas with 341 and 516 drugs respectively) (Dias *et al.,* 2012; Cragg & Newman, 2013).

The Greeks and Romans contributed a lot to the development of herbal drugs in the ancient Western World. Dioscorides, a Greek physician (100 CE) has recorded the collection, storage, and use of medicinal herbs during his travels with Roman armies",

whilst Galen (130–200 CE.), a practitioner and teacher of pharmacy and medicine in Rome, is well known for his complex prescriptions and formula used in making drugs. The Arabs, however, preserved much of the Greco-Roman expertise during the Dark and Middle Ages (5th to 12th centuries), and expanded it to include the use of their own resources, together with foreign herbs unknown to the Greco-Roman. The World Health Organization (WHO) estimated in 1985 that approximately 65% of the population of the world predominately relied on plant-derived traditional medicines for their primary health care (Dias *et al.,* 2012; Cragg & Newman, 2013).

The synthesis of complex natural products at the turn of the 21st Century has long posed a challenge worldwide, and has led to dramatic advances in the field of organic chemistry where the active natural medicinal compounds began to be replaced by synthetic analogues even though natural products are still in use (Cragg & Newman, 2013). An anti-inflammatory agent, acetylsalicylic acid (**1)** derived from *Salix herbacea* plant, Codeine (**2)** from *Papavesomniferum* and Digitoxin (**3)** from *Digitalis lanta*are still in clinical use for the treatment of inflammation, painkilling and congestive heart failure respectively. The antimalarial drug quinine (**4)**, isolated from the bark of *Cinchona succirubra* had been used for centuries in the treatment of malaria fever. Pilocarpine (**5)** (Fig. 1.1) an alkaloid produced by *Pilocarpusjaborandi* has been in use as drug in the clinical *treatment* of chronic open-angle glaucoma and acute angle-closure glaucoma for over 100 years (Dias *et al.,* 2012)

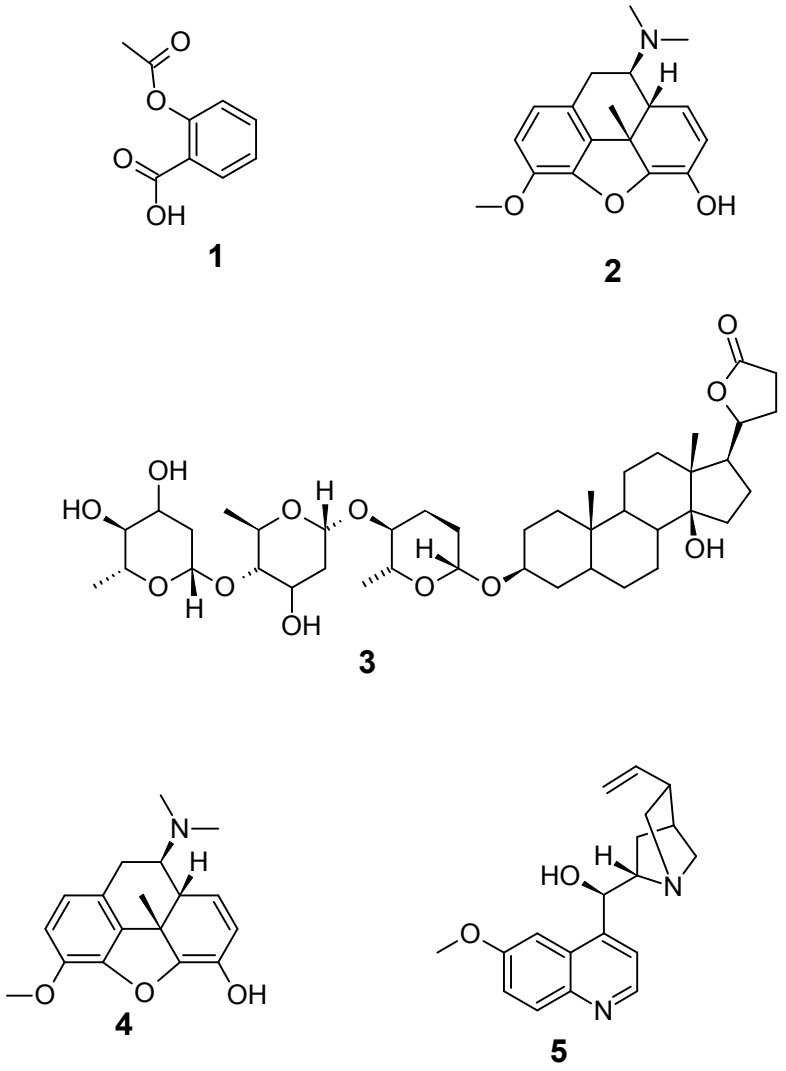


Figure 1.1 Structures of some natural products isolated from plant which are in clinical use

1.2 Oxidative Stress and Chronic Diseases

Oxidative stress is a phenomenon that reflects an imbalance between the production of reactive oxygen species and so-called oxidants, and their elimination by protective mechanisms. These are referred to as antioxidative systems which can detoxify the reactive intermediates, or repair the resulting damage causing toxic effects through the production of peroxides and free radicals that damage all cell components. Oxidative stress results in the production of free radicals as a result of metabolic activities in the living systems. Such free radicals include reactive oxygen (ROS) and reactive nitrogen species (RNS) which are highly reactive and attack important biomolecules resulting in oxidation and the onset of disease condition. Aerobic organisms have integrated antioxidant systems, which include enzymatic and non-enzymatic antioxidants that are usually effective in blocking harmful effects of ROS. Free radicals can also damage the cell and even tissues. They also cause disruptions in normal cellular reaction mechanisms. In humans, oxidative stress is believed to be involved in neurodegenerative diseases, such as cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases and aging processes (Rahman *et al*., 2012; Li *et al.*, 2013; Sulaiman *et a*l., 2013). The free radical theory of aging" has gained universal acceptance, as it is supported by the fact that the production of free radicals and the free radical damage increases with age. This theory postulates that free radicals in the body cause oxidative damage to cellular components, a process that results in altered cellular function, compromised tissue and organ function, ultimately leading to death. Toxicity of free

radicals contributes to the damage or injury to proteins and DNA, inflammation, tissue damage and subsequent cellular apoptosis, Literature reports have indicated plants as a good source of radical scavenging metabolites capable of providing antioxidant protection thereby preventing the occurrence or extent of oxidative stress-linked medical conditions. There is thus increasing awareness on the need for increased consumption of vegetable products. Among the vegetables that receive such attention is *Moringa oleifera* (Kahkonen *et al*., 1999; Mensor *et al*., 2001).

1.3 Antioxidant Compounds

An antioxidant is any chemical substances that acts against the effects of free radical by either lowering the oxidation of substance in body or prevent it. Natural antioxidants are gaining more attentions due to their therapeutic value with few side effects and can also be utilized for the prevention of the oxidative damage related disease (Kin *et al*., 2012). It has been long reported that higher plants possess naturally occurring substances which have antioxidant activity (Mensor *et al*., 2001). Potential sources of antioxidant compounds have been searched in several types of plant materials such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs, and crude plant drugs. A well-known natural antioxidant compounds includes hydroxycinnamic acids such as Caffeic acid (**6)** and Ferulic acid (**7)** as well as Hydroxybenzoic acid such as Gallic acid (**8)** Others include Quercetin (**9)** β-carotene (**10)** etc. which have been reported to contain high amount of antioxidant. The antioxidant activity of compounds such as phenols is mainly attributable to the redox (oxidation and reduction) properties, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Kahkonen *et*

al., 1999; Samuel *et al*., 2014). Antioxidant system of body plays its decisive role in prevention of any loss due to free radicals. A free radical could be an atom or group of atoms possessing at least an unpaired electron in their outermost orbital. These occur in living system when oxygen or nitrogen is bonded to other molecules. They are highly reactive specie capable of initiation other chain reactions. Those radicals produced by living system through either respiration, from normal metabolisms or are induce by UV radiation as pollutants can affects our health system by affecting cell mediated immunity (Stankovic, 2011). However, imbalanced defense mechanism of antioxidants, overproduction or incorporation of free radicals from environment to living system leads to serious condition leading to neuro-degeneration, imbalanced metabolism and production of excess reactive oxygen species (ROS) which lead to a range of disorders such as Alzheimer's disease, Parkinson's disease, aging and many other neural disorders. The excess free radicals circulating in the body oxidize the low-density lipoproteins making them potentially lethal which can also accelerate aging processes and have been linked to other very serious pathologies, such as brain stroke, diabetes mellitus ,rheumatoid arthritis ,and cancer. Natural and synthetic antioxidants, such as butylated hydroxyl anisole BHA **(11),** butylated hydroxyl toluene BHT **(12),** ascorbic acid **(13)** (Fig 1.2) and Isopropyl gallates can effectively scavenge free radicals, absorb light in the ultraviolet (UV) region (100 to 400 nm), and chelate transition metals, thus stopping progressive auto oxidative damage of the tongue and nose. Antioxidants are now being looked upon as persuasive therapeutics against solemn neuronal loss, as they have the capability to neutralizing free radicals. Antioxidants from natural sources increase the

anti oxidative capability of the plasma and decrease the risk of many diseases. At present, diet is major source of antioxidants, while medicinal herbs are attracting more attention to be commercial source of antioxidants. Recognition is given all over towards antioxidant therapy to the oxidative stress which has been proved an effective tool in alteration of any neuronal damage as well as free radical scavenging. Antioxidants have a wide scope to sequester metal ions involved in neuronal plaque formation to prevent oxidative stress. In addition, antioxidant therapy is vital in scavenging free radicals and ROS preventing neuronal degeneration (Uttra *et al*., 2009).

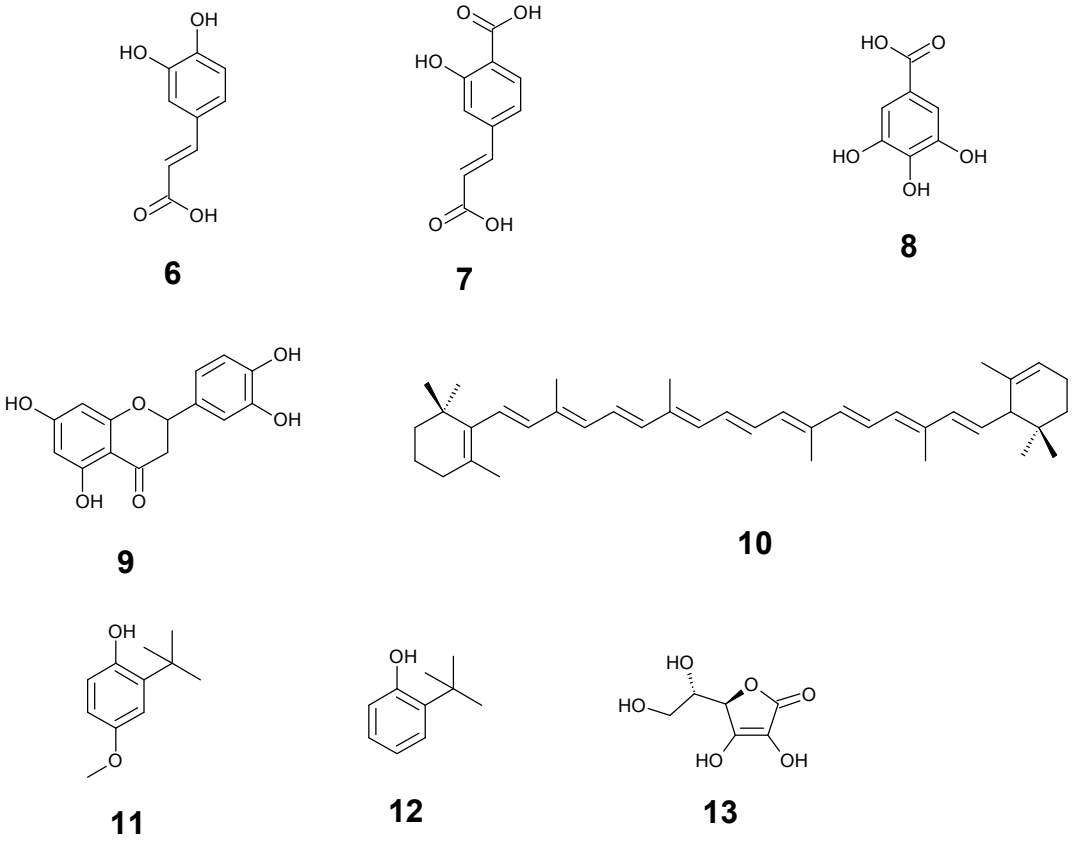


***Figure*1.2** Chemical Structure of Some Antioxidant Compounds

1.4 Aim and Objective of Research

The study is therefore intent to investigate the existence of biologically active antioxidant secondary metabolites compounds present in *Moringa oleifera*

The objectives is

(a) To extracts and fractionates the extract of *Moringa oleifera* using solvents of different polarities.

(b) To screen the fractions for DPPH Antioxidants assay

(c) The DPPH Antioxidants assay was used to identify the fraction posse's high activity and correlates it with total phenolic content using Folin cio calteau method

(d) Then using Chromatographic techniques (I e ^{1}H NMR, ^{13}C NMR, 2 dimensional NMR) and activity guides an antioxidant compound was isolated

(e) The electron spray ionization mass spectrometric (EIMS) and ultra violet (UV) spectroscopic were employed to detects some Secondary Metabolites present in the methanolic extract of the *Moringa oleifera*

CHAPTER TWO

2.0 Literature Review.

Moringa oleifera belongs to the family *Moringaceae,* popularly known as horse radish (Guevara *et al*., 1999). It is native to north India which spreads through the tropics. The plant grows fast reaching up to 12 m long. It possesses a thick grey bark which looks like cork. It is a flowering plant which is made of up to 13 species, out of which *Moringa oleifera* is the most economically important (Murrafo *et al*., 2013).

Figure 2.1: Pictures showing the whole plant of the *Moringa oleifera, and* the leaves including stem.

2.1 Ethno medical Uses of Moringa *oleifera*.

Traditionally, all parts of the plants are used in a variety of ways depending on the geographical location. Moringa plant is known to be rich in nutrients such as proteins and vitamins and largely consumed as food by humans and livestock.

Eating *Moringa* food is good for those suffering from malnutrition due to high proteins and fiber contents (Mahmood *et al*., 2010; Cheenpracha *et al*., 2010)

Different parts of the plant are reported to be applicable in the treatment of various ailments such as diarrhea, gastric ulcer, insect bites, skin infection, anemia, asthma, catarrh, etc across the globe (Cheenpracha *et al*., 2010).

The powdered seed kernels are used in water purification. This powder is found to impart good sedimentation ability in water treatment process similar to that of *Alum* (AluminumSulphate $\mathbf{Al_2\ (SO_4)_3}$). It is promoted to serve as an in expensive and safer replacement to Alum in water treatment (Nath *et al*., 1992; Mahmood *et al*., 2010).

Ancient kings and queens used leaves and fruits extracts of *Moringa oleifera* in their diet to maintain mental alertness and healthy skin. Also Ancient Warriors were fed with extracts of *Moringa oleifera* prior to war which believe to give them extra energy and relieves them from stress and pain during war (Mahmood *et al*., 2010).

2.2 Biological Activities of *Moringa oleifera*.

Several investigations had been carried out leading to isolation of bioactive compounds from various part of the plant. The antihypertensive property of the leaves is as a result of the presence of muster oil glycosides, thiocarbamate glycoside and nitriles like Niazirin **(14)**, β-Sitosterol (**15)** and Niazimicin **(16)**. A glycoside 4-(α-l-rhamnosyloxy) benzyl isothiocyanate **(17)** (Fig. 2.2) is responsible for the antimicrobial activity of the root and stem (Guevara *et al*., 1999).

The leaves of the plant are used to cure headaches, blood loss from shallow cut, anti-

inflammatory effect when applied to wounds or insect bites, gastric ulcer and diarrhea (Sahakitpichan *et al*., 2011).

Their seed are used as anti-biotic, anti-inflammatory to treat arthritis rheumatism, gout cramp, sexually transmitted disease and boils. Roasted seeds help in urination and used as relaxant for epilepsy (Murrafo *et al*., 2013).

Leaves and seed extract of *Moringa oleifera* is used as biopesticide activity which has strong effect against larva and adults of *Trigoderma granarium* that can reduce the incidence of fungi on ground nut seeds. A well known flavanoids of *Moringa oleifera* I. e Zeatin (**18)** is a plant hormone belongs to the cytokinin that can be used to stimulate plant growth and increases crop yield (Mahmood *et al*., 2010; Leone *et al*., 2015).

Moringa oleifera seed is also applicable in the utilization of Biomass for the biodiesel production (Leone *et al*., 2015).

2.3 Secondary Metabolites of *Moringa oleifera*

Several compounds were isolated from this plant and their structure was determined by spectroscopic analysis.

Sarot Cheepracha and his coworkers isolated an Ant Inflammatory compounds including 4-[(2-o-acetyl-α-l-rhamnosyloxy) benzyl] isothiocyanate (**19)** their isomer 4-[(3^1-o--acetyl-α-l-rhamnosyloxy)benzyl]isothiocyanate (**20)** and S-methyl-N-[4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (**21)** (Cheenpracha *et al*., 2010). Fig 2.2

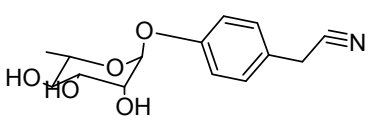
Other compounds isolated from the leaves of *Moringa Oleifera* include glycoside of pyrrole

alkaloid (pyrrolemarumine 4"-O-α-L-rhamnopyranoside) (**22)**. Marumoside A. (4'-hydroxy phenylethanamide-α-L-rhanmopyranoside (**23)** and Marumoside B (3" –O-β-D-glucopyranosyl) (**24)**, which is derivatives of Marumoside A. but there is no any recent development of biological activity of the compounds. (Sahakitpichan *et al*., 2011; Cheenpracha *et al*., 2010).

Moringa oleifera leaves are rich in flavonoids, such as Quercetin (**9**) which contains phenolic hydroxyl group that are the strong class of antioxidant which can protects the cells against the injury damage caused by X- rays radiation and acts in preventing carcinogenesis, they also inhibit the production of nitric oxides when stimulated by injury (Murrafo *et al*., 2013).

Naturally occurring bioactive flavanoid Astragalin (Kaempferol 3-glucoside (**25**) has a diversified pharmacological application such as anti inflammatory, antioxidant, neuroprotective, cardio protective, ant obesity, antiosteoprotic, anticancer, antiulcer, and anti diabetic properties (Riaz *et al*., 2018).

Moringa oleifera is the one of the richest plant that serves as a source of Vitamin A, B (1, 2, 3, 6, 7), C, D, E, and K., vital minerals such as Calcium, Copper, Iron, Potassium, Magnesium, Manganese and Zinc, and they also contains more than 40 natural antioxidants among them are Kaempferol (**26)**, β-Sitosterol (**15)** and Caffeoylquinic (**27)** which are the strong class of antioxidants (Hsu *et al.,* 2006; Mahmood *et al*., 2010; Leone *et al*., 2015).



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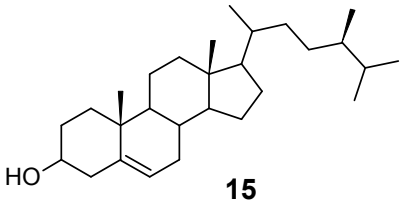

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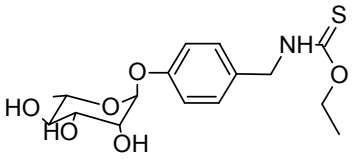

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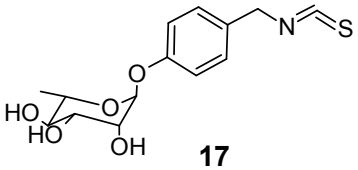

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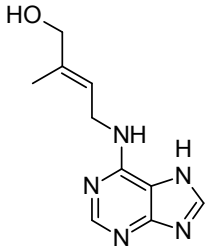

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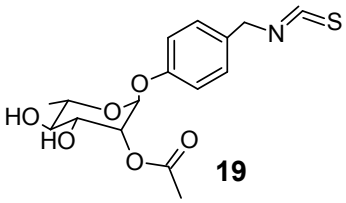

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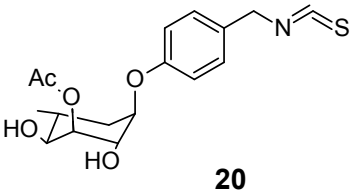

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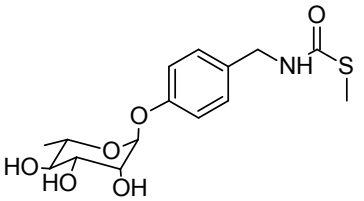

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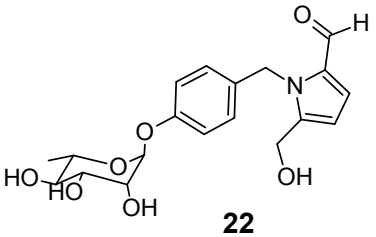

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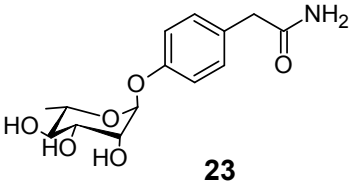

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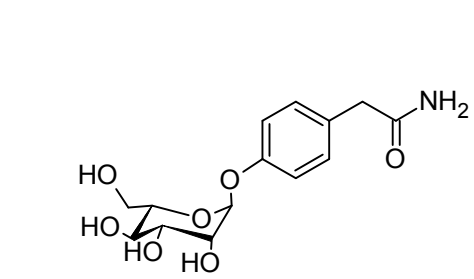

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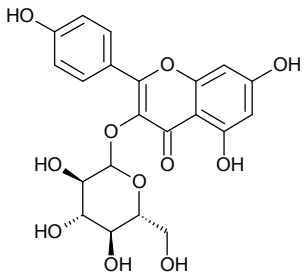

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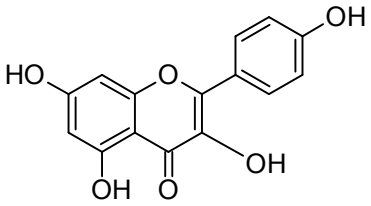

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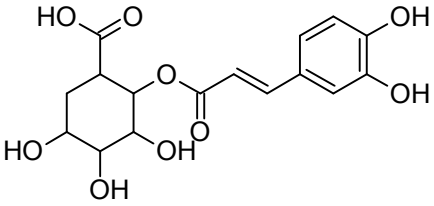

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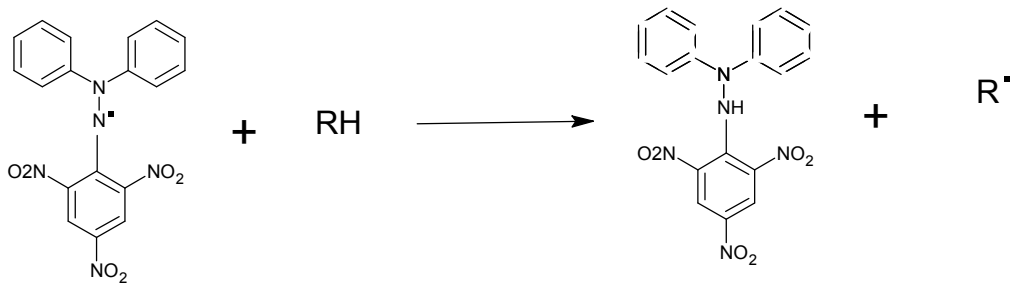
Figure 2.2: Structure of some Secondary Metabolites Isolated from *Moringa oleifera.*

2.4 BIOASSAYS METHODS FOR DETERMINING THE RADICAL SCAVENGING PROPERTIES OF THE COMPOUNDS

2.4.1 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Method

This method is used to measure antioxidant capacity of food substance it is simple and rapid which involves the use of the free radical 2, 2- Diphenyl-1-picrylhydrazyl (DPPH). DPPH radical is stable due to the delocalization of the spare electron on the molecule. The delocalization on the DPPH· molecule determines the occurrence of a purple colour, with an absorption band with a maximum wavelength around 520 nm. When DPPH· reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet colour. Synthetic antioxidants like Butylated hydroxyl Anisole (BHA), Butylated Hydroxyl Toluene (BHT) and Ascorbic acid were used as positive controls. This method is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Figure 2.3). It will also use to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods (Molan *et al*., 2012; Shalaby & Shanab, 2013; Paixao *et al*., 2007).



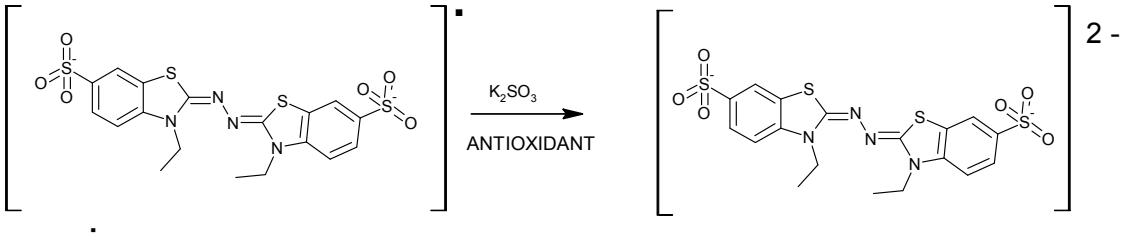
Diphenylpicrylhydrazyl radical (Purple) Diphenylpicrylhydrazine (Yellow)

Figure 2.3: Abstraction of proton by antioxidant from DPPH radical, the oxidized and reduced form of DPPH

2.4.2 ABTS [2, 2- Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Method:

This has been used to screen the relative radical-scavenging abilities of flavonoids and phenolics. ABTS is a better choice than DPPH and more sensitive than DPPH. The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds. It is also useful for measuring antioxidant activity of samples extracted in acidic solvents. The ABTS cation radical (ABTS·+) which absorbs at 743 nm (giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS (2, 2-azino-bis-(3ethylbenzthiazoline-6-sulphonic acid)).

Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing antioxidant activity of samples in different media and is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer solution containing 150 mM NaCl) (PBS). Another advantage of ABTS+ method was that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS), reaching a steady state within 30 min. The DPPH reacted very slowly with the samples, approaching but not reaching steady state after 8 h. This slow reaction was also observed when ABTS was reacted with samples (Figure 2.4). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization. ABTS can be oxidized by potassium per sulphate or manganese dioxide, giving rise to the ABTS cation radical (ABTS·+) whose absorbance diminution at 743 nm was monitored in the presence of Trolox, as standard. (Shalaby & Shanab, 2013; Pisoschi & Negullescu, 2011; Paixao *et al*., 2007).



ABTS (bluish green) $ABTS^{2-}$ (colourless)

λ_{max}= 734 nm

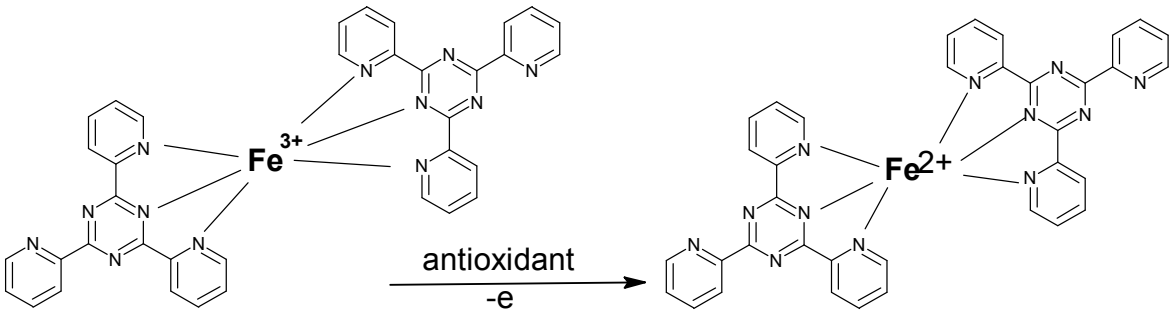
Figure 2.4: Reaction between ABTS radical and antioxidant compound.

2.4.3 The Oxygen Radical Absorbance Capacity (ORAC) Method

This method is used to determine antioxidant capacities of fruits and vegetables. In the ORAC method, a sample is added to the peroxyl radical generator, 2, 2-azobis (2-amidinopropane) hydrochloride (AAPH) and inhibition of the free radical action is measured using the fluorescent compound, B-phycoerthrin or R-phycoerythrin. Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, foods, and beverages and are usually quantified employing Folin's reagent have measured phenolics in fruits and vegetables calorimetrically using the *Folin-Ciocalteu* reagent and determined the fruit and vegetables antioxidant capacity by inhibition of low-density lipoprotein oxidation mediated by cupric ions (Pisoschi & Negullescu, 2011).

2.4.4 FRAP (ferric reducing antioxidant power) Method

The FRAP (Ferric Reducing Antioxidant Power) method takes advantage of electron-transfer reactions. They rely on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2, 4, 6-tri(2-pyridyl)-1, 3, 5-triazine). The binding of $Fe^{2+}$to the ligand creates a very intense navy-blue color. The absorbance can be measured to test the amount of iron reduced and can be correlated with the number of antioxidants Trolox or ascorbic acid are normally used as positive standards (Figure 1.4). (Pisoschi & Negullescu, 2011; Shalaby & Shanab, 2013; Molan *et al*., 2012).



Fe (III)(TPTZ)l_2 Fe(II) (TPTZ) I_2,

Figure2.5: Antioxidant reaction with ferric salt, Fe (III)(TPTZ)l2 to Fe(II) (TPTZ)2,4,6- tripyridyls-triazine.

2.4.5 The CUPRAC (Cupric Reducing Antioxidant Power) Assay:

The standard antioxidants or extracts are mixed with $CuSO_4$ and neocuproine. After 30 min, the absorbance was measured at 450 nm. In the assay, Cu (II) is reduced to Cu (I) through the action of electron donating antioxidants.(Scheme 1).The L-H represents the substrates molecule, $R^{\cdot}$, $L^{\cdot}$ represents initiation oxidizing radical and highly reactive allyl radical that can rapidly react with oxygen to form a peroxyl radical (LOO·) respectively. The peroxyl radicals are the chain carriers of the reaction; they can further oxidize to producing hydro peroxides (Shalaby & Shanab, 2013; Pisoschi & Negullescu, 2011).

$Cu^{2+} + AOH \longrightarrow Cu^{+} + AO^{\cdot} + H^{+}$

$AO^{\cdot} + L\text{-}H \longrightarrow AOH + L^{\cdot}$

$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$

$LOO^{\cdot} + L\text{-}H \longrightarrow LOOH + L^{\cdot}$

$Cu^{+} + LOOH \longrightarrow Cu^{2+} + LO^{\cdot} + OH^{-}$

Scheme1: Reaction of Cupric with Antioxidant Compound AOH

CHAPTER THREE

3.0 Materials and Methods.

3.1 General Equipment and Apparatus

Buchi Rota vapour R200 were used for extracts concentration.

Weighing balance (Mentle Telado AB 54) was used for weighing purposes.

U V Lamp (Allen 425) was used for viewing TLC spots.

96 well Micro plate Reader were used for taking absorbance.

Columns (5.5cm x 80cm) and (2.5cm x35cm) were used for chromatographic purification.

Agilent 1260 quad pump infinity compartment were used for HPLC Analysis.

Agilent Technologies 6120 quadrupole HPLC system were used for ESI-Mass analysis.

Pyrex glass wares were used for the research.

Varian S400 NMR was used for NMR analysis.

SPSS (6.0) and MS Excel software were used for the statistical analysis and computation of IC_{50}

3.2 CHEMICALS AND REAGENTS

All chemicals, reagents and solvents were of analytical grade. Solvents used for HPLC and LCMS analysis are of standard HPLC grade which includes

Diphenylpicrylhydrazyl (DPPH) radical,

butylated hydroxyl toluene (BHT)

Silica gel (Pore size 60Å, 230-270 mesh),

Ascorbic acid.

Precoated TLC plates with silica gel 60 $F_{254,}$ (Merck).

And etc.

3.3 Plant Material

Moringa oleifera were collected on 14/11/2013 from the Faculty of Agriculture, Department of Agronomy, Bayero University Kano and they were identified by Prof Mansur Auwal Bindawa.

The leaves including the stem were dried at room temperature under shed and made into powder using mortar and pestle.

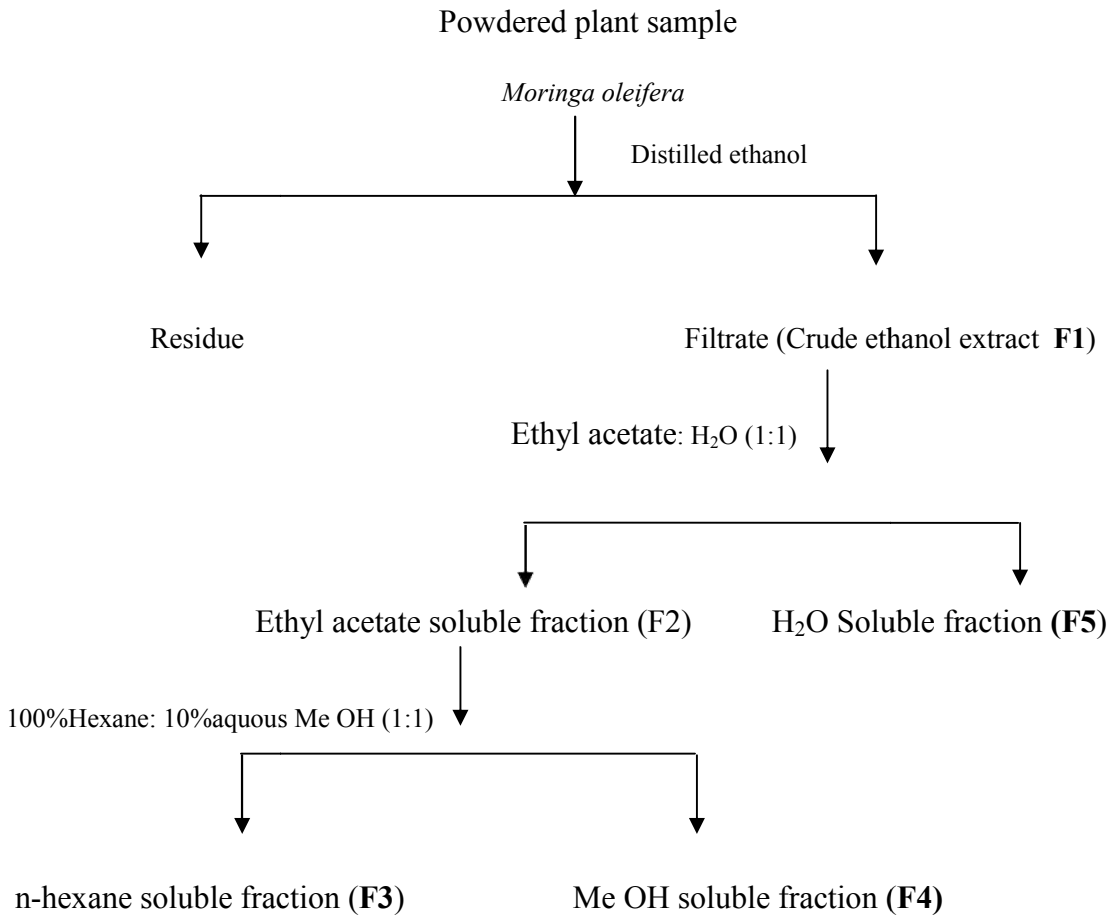
3.4 Extraction of Plant Material

The powdered plants 3.5 Kg were used; each 120 g were soaked in 1.5 L of the distilled ethanol for a period of 2 weeks with occasional shaking and then filtered. The filtrate were concentrated using rotary evaporator at 40^{o}C.

The weights of crude ethanol Extract were recorded as F1.

3.5 Fractionation of Crude Extract

The dark greenish crude ethanol extract were further partitioned with solvent of varying polarities Using separating funnel to obtained Ethyl acetate soluble fraction, n -hexane soluble fraction, methanol fraction and water soluble fraction respectively labelled as F2, F3, F4 and F5 (Scheme 2)



Scheme2: Extraction and Fractionation Protocol

3.6 General Protocols for Thin Layer Chromatographic Analysis

. A TLC analysis was conducted by dissolving 1 mg of the sample in chloroform. The resulting solution was introduced onto aluminum-backed silica gel plate by means of 25µL Hirsman capillary tube. Plates were developed in an appropriate solvent of hexane/chloroform gradient and chloroform/methanol gradient system to achieve reasonable

separation. Plate were viewed using UV light at 254/365 nm, alternatively iodine vapour was used. Distinct spots or bands were marked by their corresponding R^f values

3.7 Column Chromatography

Methanol soluble fraction F4 (21.5 g) was mixed with celite and silica gel and made into a fine powder. 600g of silica gel was made into slurry with n-hexane and loaded into a glass column (5.5cm ×80cm).The powdered mixture of the extract was then loaded onto silica gel bed in the column. Small quantity of silica gel was introduced on top. The chromatography was eluted with hexane/chloroform gradient (100:0, 75:25, 50:50, 25:75 and 0:100%). This was followed by chloroform/methanol gradient (99.5:0.5, 99:1, 98:2, 97:3, 96:4 and 95:5 %). About 2 L was eluted per solvent system and 100 ml portions were collected all through. A total of 15 pooled fractions were obtained after combining 150 fractions with similar TLC profiles. In the case of chromatographic purification of the most active column fractions a smaller column (column dimension: 35 cm ×2.5cm) was used which contains 60 g silica gel. Chloroform/methanol gradient (100:0, 99:1, 98:2, 97:3, 96:4, 95:5 and 94:6%) was used. 500 ml was eluted per solvent system and 50 ml portions were collected. 13 pooled fractions were obtained after combining 81 fractions with similar TLC and HPLC profiles.

3.8 Scavenging Activity on DPPH Radical Assay

The antioxidants activity of the crude extract and column fractions was measured using method described by Seephonkai *et al.*, (2011) and Mensor *et al.*,(2001) with some modification. The test samples were prepared at following concentrations; 1000, 500, 250, 100, 50, 25 and 10 µg/ml, in methanol. 100 µl of each test solutions are placed separately

into 96 wells plate in triplicate after which the absorbance was measured at 517 nm wavelength to serve as blank. To the 96 wells 200 µL of 0.2 mM DPPH solution (78.9mg L^{-1}) in methanol was added. This was incubated for a period of 30 min at 26°C in micro plate reader after which the absorbance of samples was measured at 517nm. Absorbance of 0.2 mM DPPH solution in methanol was taken to serve as a negative control. Standard antioxidants: BHT and ascorbic acid were used as positive controls. (the same concentration and volume with taste samples)

The percentage antioxidant activity % (AA) for each sample were obtained using the following relation:

$$\% AA = 100 - \left(\frac{\text{Ab sample} - \text{Ab blank}}{\text{Ab control}}\right) * 100$$

Where

Ab sample Absorbance of sample (i.e. after DPPH was added).

Ab blank Absorbance of blank (I e sample without DPPH).

Ab control Absorbance of DPPH (I.e. negative control).

The plot of percentage antioxidant activity % (AA) against concentration (µgmL^{-1}) were used to determine the 50 % Inhibitory concentration (IC_{50}) values of each fractions and standards were evaluated by means of SPSS software (Mensor *et al.*,2001)

3.9 Liquid Chromatography Mass Spectrometry (LCMS) and High Performance Liquid Chromatography (HPLC) Analysis

LCMS analysis was conducted using Waters 2795HT Agilent Technologies 6120

quadrupole HPLC system. HPLC analysis and purification were achieved using Agilent 1260 infinity. Extract and fractions were analyzed on various UV wavelengths between 200 and 400 nm. Chromatography (flow rate 1 mL min^{-1}) was done using a Zorbax ODS column (5 µ, C_{18}, 4.6 x 250 mm) following 15 min gradient of HPLC grade H_2O containing 0.05% formic acid; and HPLC grade CH_3CN containing 0.045% formic acid. In case of HPLC purification of the most active column fraction Solution concentration ranging from (10-20) mg/ml of the each sample were prepared. Auto sampler injects 75 µl of each sample. Fractions AS-F4-2-76 and AS-F4-2-81 (most active 2nd column) were further purified by HPLC using acetonitrile/methanol/water gradient system buffed with formic acid. The measurement was done at temperature of 30 ^{0}C.

3.10 Determination of Total Phenolic Content (Folin ciocalteau Method).

3.10.1 Preparation of Solution for Folin-ciocalteau Method.

Folin-ciocalteau phenol reagent was prepared by dilution the reagent at ratio of 1:3 with 96 % methanol prior to use.

Lowry A solution was prepared by dissolving 2 % (w/v) of Na_2CO_3 in 0.1 M NaOH.

Lowry B solution was prepared by dissolving 0.5% (w/v) $CuSO_4$ in 1% Sodium potassium tartrate (Na K $C_4H_4O_6$).

Lowry C is prepared by mixing 50 ml of Lowry A with 1ml of Lowry B.

For a test sample of 1 mg/ml an aliquot of 0.5 mL of the sample were used. Then 1.5 mL of water was added. 2.5 mL of Lowry C was also added. Then the solutions were allowed to stand for 10 minutes. After which 0.25mL Folin- Ciocalteau reagent were finally added.

Allowed 30 more minutes for the stabilization of the blue colour formed. Then the absorbance against a reagent blank was taken at 750 nm (Genwali *et al*., 2013).

3.11 Nuclear Magnetic Resonance (N M R) Spectroscopy Analysis

^{1}HNMR was measured at 500MHz and ^{13}C measured at 125MHz NMR Analysis was conducted using Varian S400 NMR. The most active Second column fractions I e Fractions AS-F4-2-76 and AS-F4-2-81 are further purified by HPLC using acetonitrile/methanol/water gradient system buffed with formic acid to yield the four isolated compounds which were submitted for NMR spectroscopic analysis but due to the smaller sample size only the data for ^{1}H NMR, ^{13}C NMR, COSY and HSQC were acquired for sample AS-F4-2-76-1 and AS-F4-2-76-4, (Fig4.20-Fig.4.26) but complete structural elucidation was inconclusive.

3.12 Mass Spectrometric Analysis

mass spectrometric analysis were performed using Agilent Technologies 6120 quadrupole Liquid Chromatography Mass Spectrometry which is equipped with Electrospray Ionization (ESI) Mass Spectrometer operating in positive and negative ion mode and diode array UV detector operating at 200 – 400 nm. Natural product identification was achieved by studying the combined mass and UV spectral profiles.

CHAPTER FOUR

4.0 Results and Discussion

4.1 Extraction and Fractionation of Plant Material

A 3.5 kg of the plant material was extracted with ethanol and further partitioned with solvents of varying polarities to yield crude ethanol extract, ethyl acetate, n-hexane, and methanol soluble fractions. These fractions are respectively labelled as F1, F2, F3 and F4 the result of the weight of the extractable and physical characteristic of the concentrates are presented in (Table **4.1**)

Table 4.1: Weight and the Physical Appearance of the crude extract and Fractions Recovered

S/No	Sample	Weight (g)	Appearance
1	F1	286.7	Dark-green
2	F2	151.8	Dark-green gum
3	F3	35.8	Bright-greenish gel
4	F4	23.3	Greenish-brown solid

F1, F2, F3, F4, were ethanol, ethyl acetate, n-hexane, and methanol respectively

4.2 DPPH Radical Scavenging Assay of the Crude Extract and Fractions

All the samples (FI, F2, F3, and F4) obtained from the extraction and partition were subjected to DPPH radical scavenging activity assay. This was conducted with view to determining the presence of antioxidant and free radical scavenging natural compounds in the crude samples. The free radical scavenging activity was used as a means of evaluating the antioxidant activity of the plant extracts, expressed in IC_{50} values in µg/ml. The antioxidant potency of the various fractions was referenced to vitamin C (Ascorbic Acid), as standard natural antioxidant and Butylated Hydroxytoluene (BHT) as a reference synthetic antioxidant. The results are presented in (Figure 4.1, 4.2 & Table 4.2).

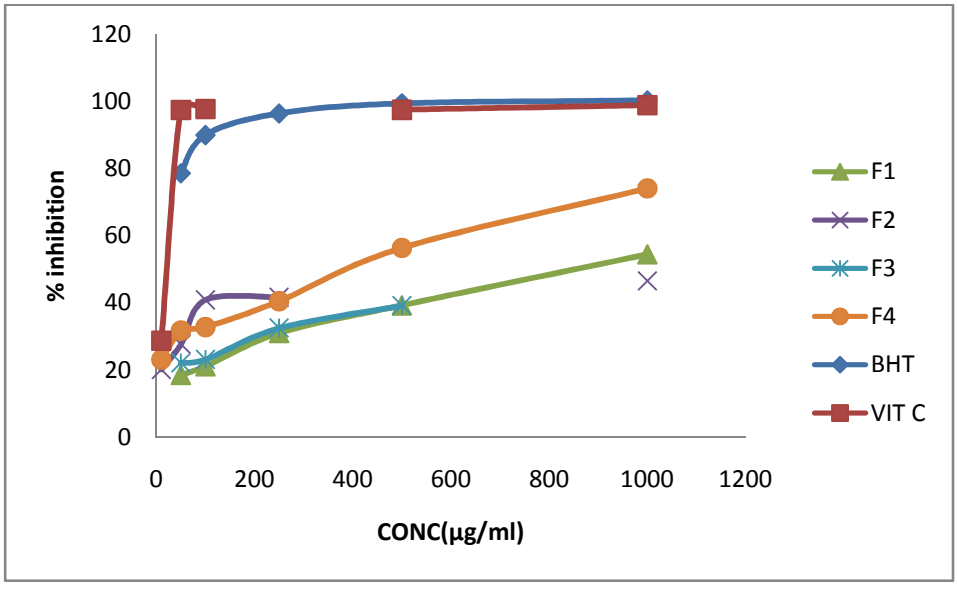


Figure 4.1: DPPH Radical Scavenging Activity as % inhibition of the Extract, fractions and Standards at various concentration

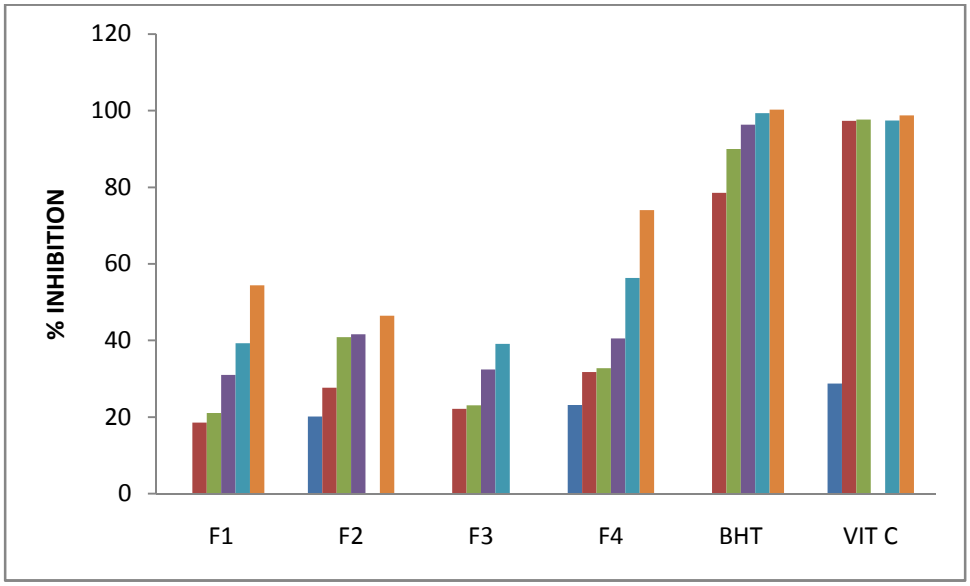


Figure 4.2: Relative % Inhibition of DPPH Radicals by Extract, fractions and Standard

Table 4.2 Comparative Antioxidant Activity of Fractions and Standards

S/no	Fractions	IC 50(µg/ml)
1	F 1	924.6797
2	F 2	663.0336
3	F 3	1820.847
4	F 4	268.5717
5	BHT	10.5995
6	Vitamin C	15.2717

The intensity of the color depends upon the oxidative power of the compound which can be spectrophotometrically measured. The more the intense colour the higher the activity (Fig.4.3/4.4). Due to these chemical properties DPPH radical provided the easiest and most rapid way of evaluating radical scavenging activity

Figure4.3: DPPH Reactions at Varying Concentrations of Extracts Showing Colour Change from Purple to Yellow Due to Radical Abstraction from DPPH

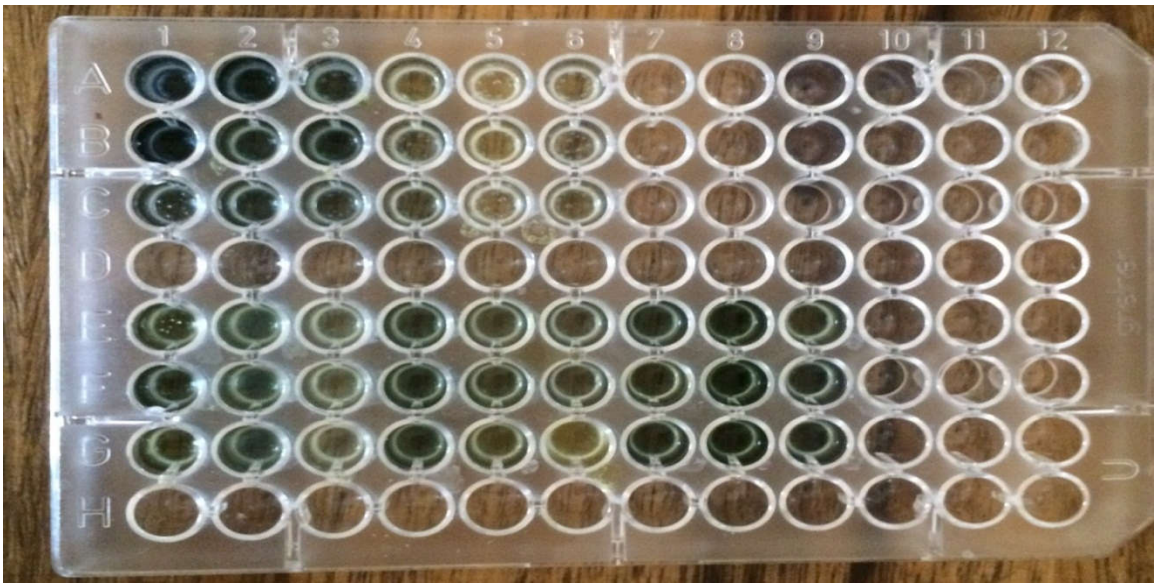


Figure4.4: Antioxidant Activity Reactions on 96 well plates showing strong anti-oxidants by means of colour change at certain doses of the test fractions per well.

The activity of the extract fraction against DPPH radical was evaluated and the IC_{50} of the crudes extract and fractions range from (268.5717-1820.847) µg/ml. Methanol soluble fraction F4 having the highest scavenging activity while n-hexane soluble fraction F3 possessed the least activity, the crude ethanol extract F1 and ethyl acetate soluble fraction F2 possessed the 924.6797 µg/ml and 663.0336 µg/ml respectively, Relative to the standard Vitamin C and BHT (15.2717 and 10.5995) µg/ml. The lower the IC_{50} value the higher the activity.

The methanol soluble fraction F4 showed the highest scavenging activity with least IC_{50} value of 268.5717µg/ml. This is why it was subjected to column chromatography.

4.3 TLC, HPLC and LCMS Analysis of Methanol Extract

The methanol soluble fraction F4 with the lowest IC_{50} as the most active was subjected to TLC, HPLC and LCMS analysis in order to determine its composition of natural product compounds. Both TLC spots, LCMS chromatograms and UV diode array at different retention times contains wave lengths of the maximum absorption which is due to the continuous conjugation and choromaphore (benzene ring and lone pair of electrons from antioxidants) which indicated that the methanol soluble extracts contains natural antioxidant compounds (Figure 4.5- 4.7)

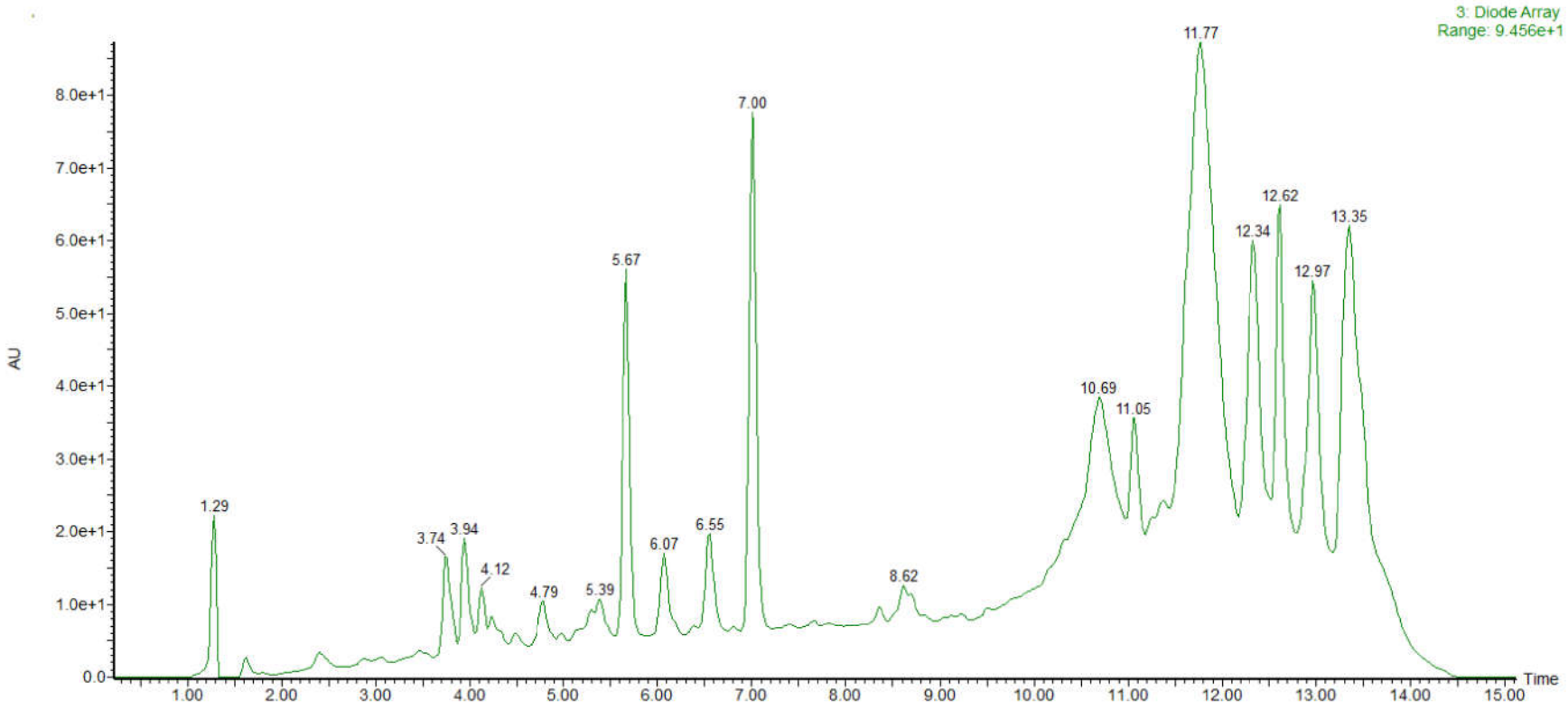


Figure4.5: HPLC diode array chromatogram of the crude methanol extract F4 of *Moringa oleifera* showing signals of compounds at different retention times

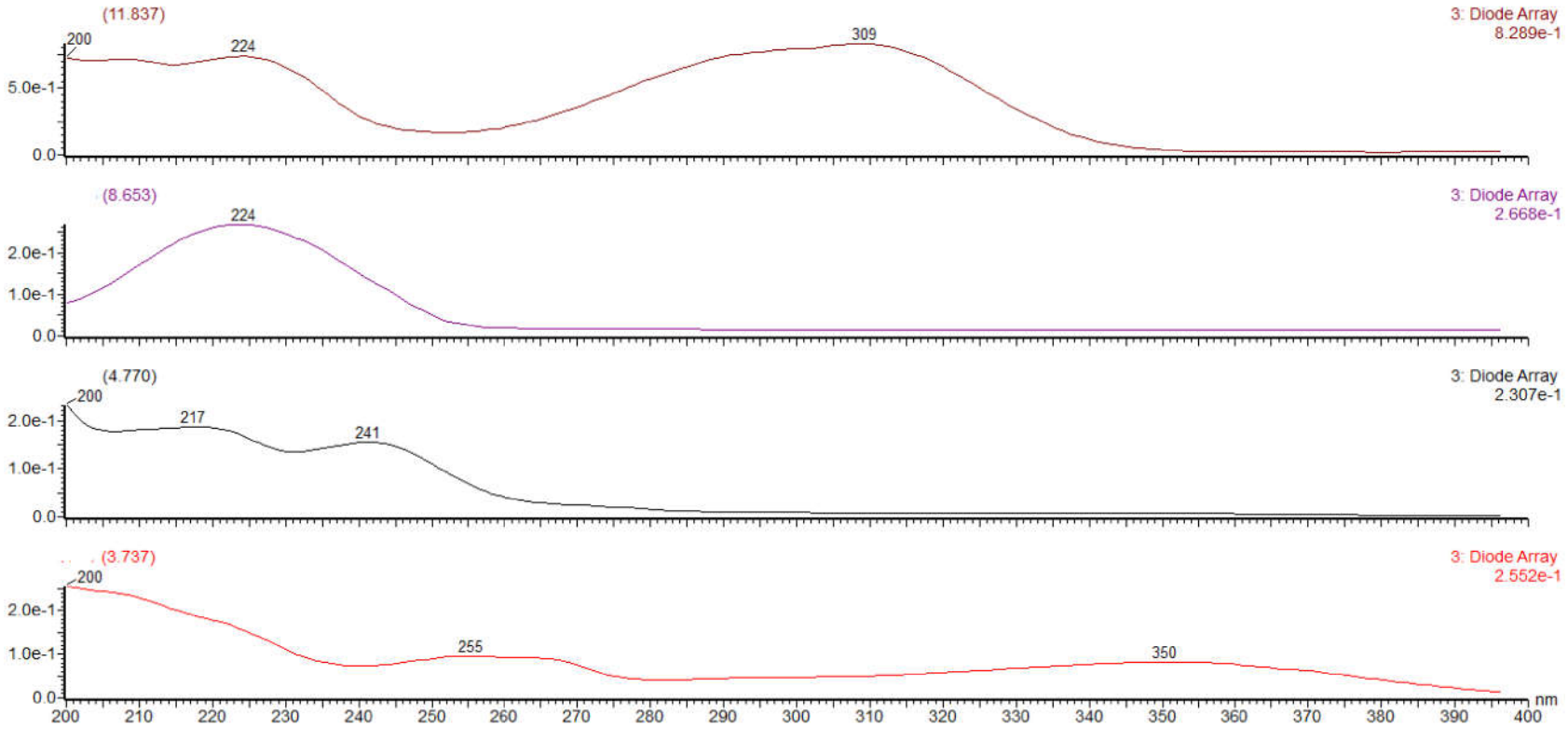


Figure4.6: Diode array UV Spectra showing various pattern of some compound signals at different retention times.

Figure4.7: TLC Analysis of the Crude Methanol fraction F4 of *Moringa oleifera* Viewed inside Iodine Chamber showing Spots of Different Compounds

4.4 Column Chromatographic Purification of Methanol Fraction

The methanol extract was subjected to silica gel column chromatographic purification using gradient of n-hexane, chloroform and methanol solvent system. A total of 130 individual fractions were obtained when collecting 100 ml (Fig 4.8). Eluent fractions with similar TLC profiles were pooled to 15 fractions.

Figure 4.8: Silica-gel Column Chromatography and Eluted Fractions from Chromatographic Purification of Methanol fraction..

4.5 Radical Scavenging Assay on Eluted Fractions of methanol fraction

All the eluted 15 fractions obtained from column chromatography were subjected to DPPH radical scavenging activity assay. This was conducted with view to determining the presence of antioxidant/radical scavenging compounds. The experiment was conducted in triplicate using 96 wells plate on micro plate reader. Reference standards; ascorbic acid and butylated hydroxyl toluene (BHT) were used as positive controls the results are presented in (Fig 4.9 - 4.12 & Table 4.3-4.4)

Table4.3 Yield, Appearance and TLC Analysis Result of Eluted Fractions

Fractions	Weight (g)	Appearances	R_F Value/Remarks
AS-F4-1-15	0.056	Colourless	No spot shown
AS-F4-1- 27	1.558	Yellow oil	Spot shows pink on UV Light at 365nm with $R_F = 0.65$
AS-F4-1- 32	0.524	Yellow oil	Spot shows pink on UV Light at 365nm with R_F Value 0.68
AS-F4-1- 35	0.097	Yellow oil	Spot shows pink on UV Light at 365nm with R_F Value 0.38
AS-F4-1- 39	0.284	Orange color oil	Spot shows pink on UV Light at 365nm with R_F Value 0.3
AS-F4-1- 49	1.044	Orange color solid	Spot shows pink on UV Light at 365nm with R_F Value 0.3
AS-F4-1- 56	0.537	Green solid	Shows 2 spots of pink color on UV Light at 365nm with R_F Values of≈ 0.25, 0.45
AS-F4-1- 58	1.419	Green solid	Shows 2 spots of dark green and red color on UV Light at 254nm with R_f: 0.15, 0.3.
AS-F4-1- 65	2.939	Green solid	Shows 2 spots of green and light brown on iodine chamber with R_F: 0.25, 0.575
AS-F4-1- 75	0.683	Green solid	Shows light brown spot on iodine with R_F Value 0.15.
AS-F4-1- 87	0.986	Green solid	Shows light brown on iodine with R_F Value of 0.25
AS-F4-1- 99	0.475	Green solid	Shows 2 spots of green and light brown on UV light at 254nm with R_F values 0.225, 0.70
AS-F4-1- 109	0.348	Green solid	Shows 2 spots of green and light brown on UV light at 254nm with R_F values 0.225, 0.70
AS-F4-1- 116	0.187	Green solid	Shows 2 spots of green and light brown on UV light at 254nm with R_F :0.1625,0.725
AS-F4-1- 130	0.740	Green solid	2 spots of green and light brown on UV light at 254nm with R_F values of 0.225,0.725

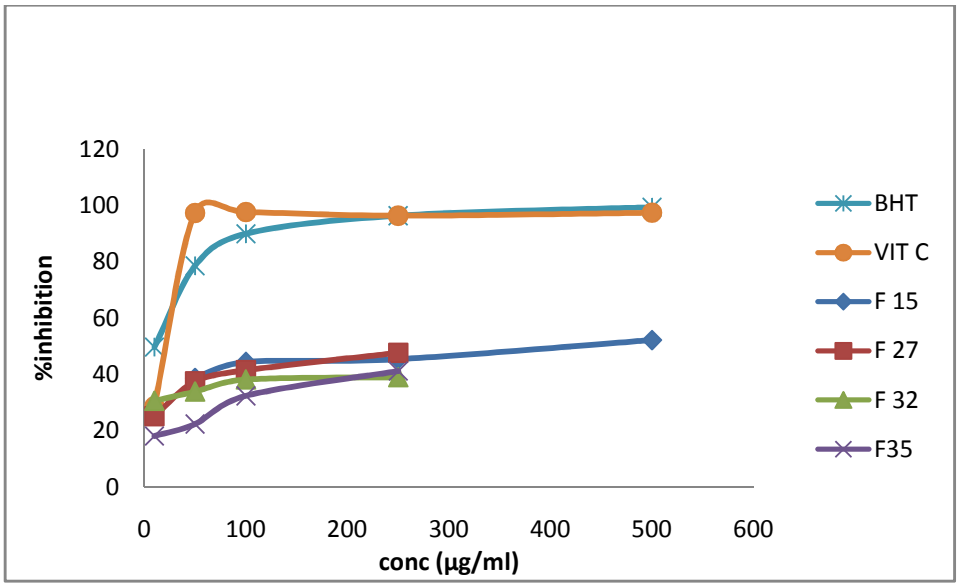


Figure4.9: Result of DPPH Radical Scavenging Activity of AS-F4-1-15 - AS-F4-1-35

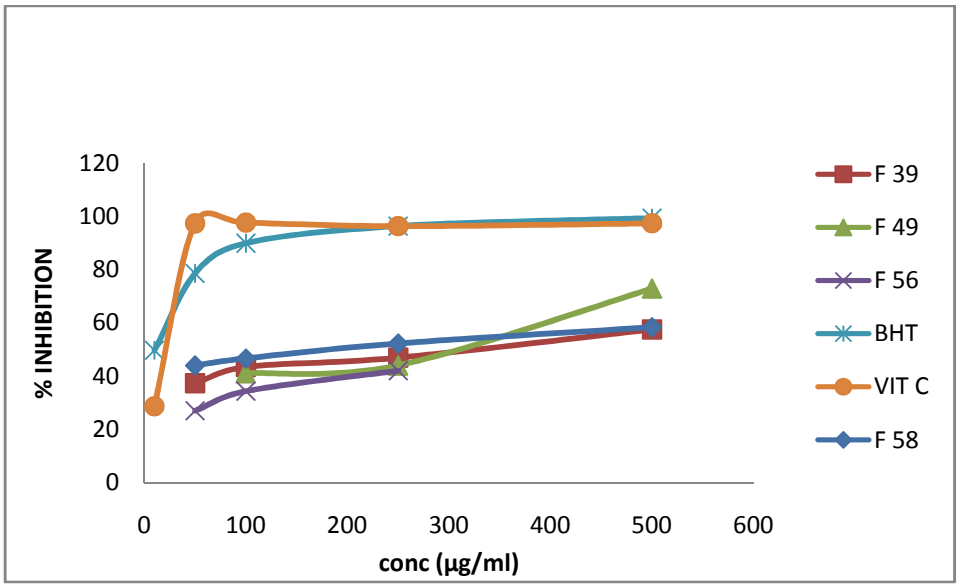


Figure4.10: Result of DPPH Radical Scavenging Activity of AS-F4-1-39 - AS-F4-1-58

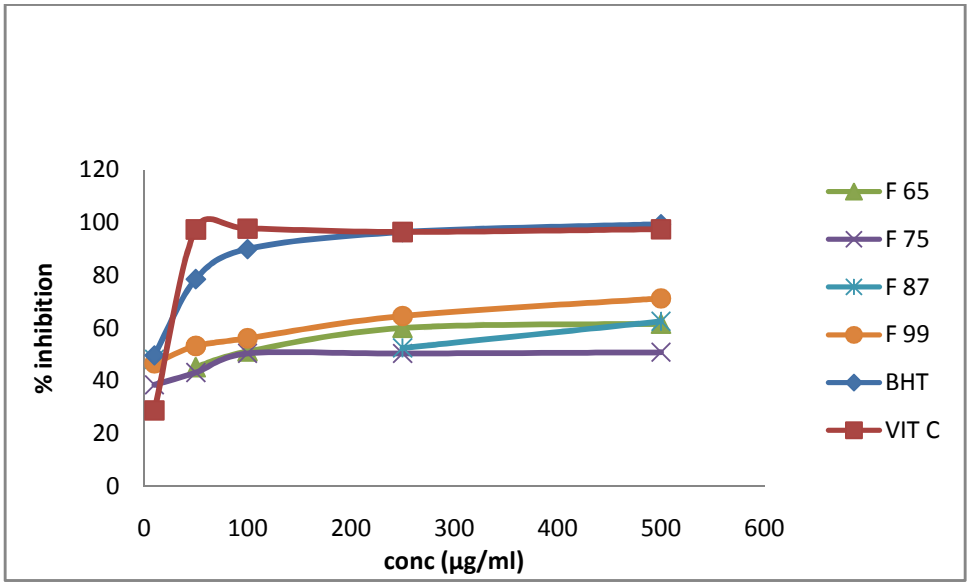


Figure 4.11: Result of DPPH Radical Scavenging Activity of AS-F4-1-65 - AS-F4-1-99

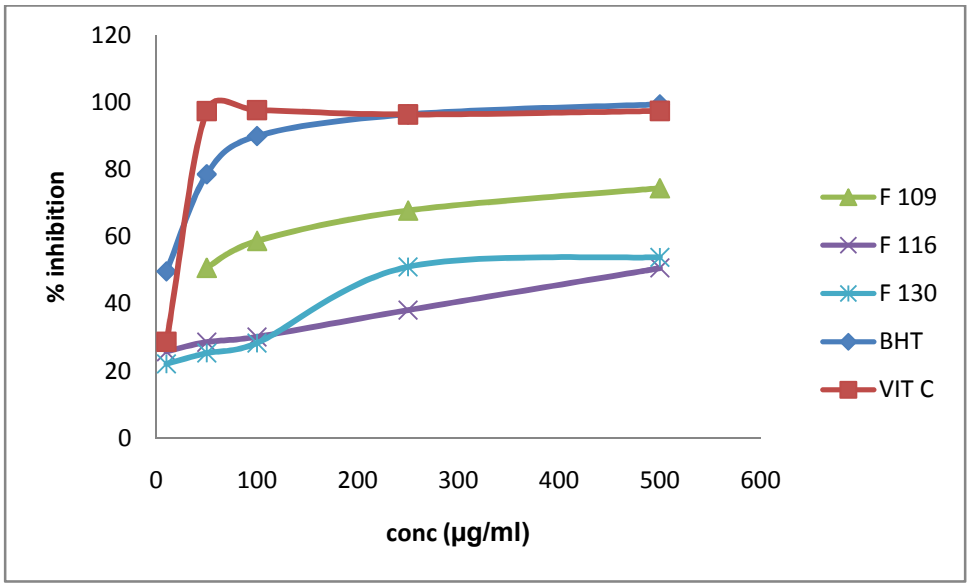


Figure 4.12: Result of DPPH Radical Scavenging Activity of AS-F4-1-109 - AS-F4-1-130

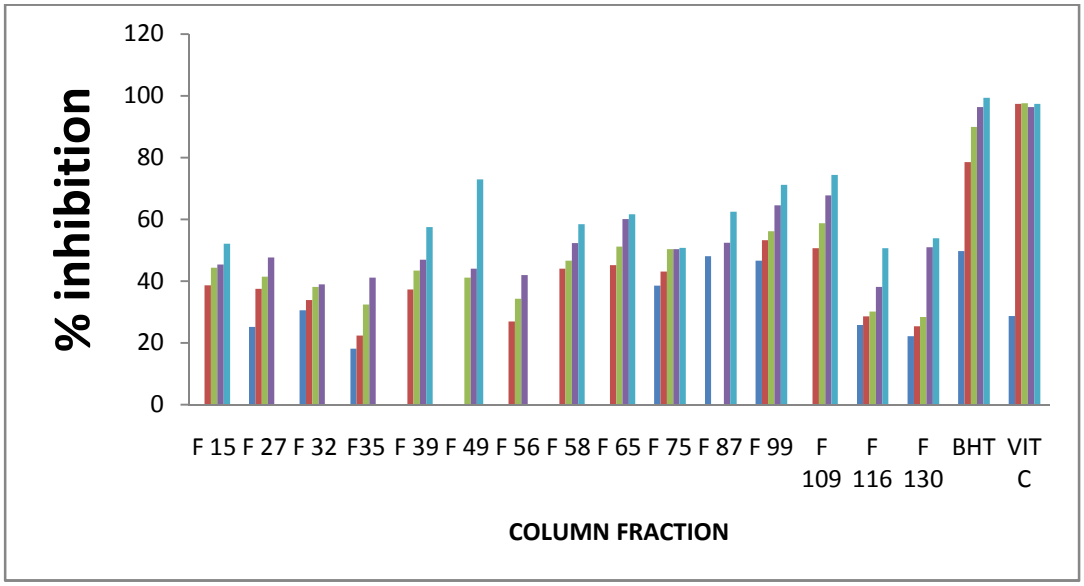


Figure4.13: Comparative DPPH Radical Scavenging Activity of the pooled Column Fractions

Table 4.4: Yield and Result of DPPH Radical Scavenging Assay of Chromatographic Fractions

S/N	Fraction	Yield (mg)	IC_{50}(µg/ml)
1	AS-F4-1-15	56	400.9169
2	AS-F4-1-27	1558	314.761
3	AS-F4-1-32	524	6353.799
4	AS-F1-1-35	97	3235.876
5	AS-F4-1-39	284	249.7848
6	AS-F4-1-49	1044	167.9368
7	AS-F4-1-56	537	536.6557
8	AS-F4-1-58	1419	149.2422
9	AS-F4-1-65	2939	86.4852
10	AS-F4-1-75	683	242.8322

11	AS-F4-1-87	986	24.7043
12	AS-F4-1-99	475	23.7043
13	AS-F4-1-109	348	46.096
14	AS-F4-1-116	187	1097.789
15	AS-F4-1-130	74	406.6241
16	B H T		10.5995
17	VIT C		15.271

The results of the column chromatography indicated that almost all the fractions were found to show radical scavenging activity by displaying IC_{50} values ranging from 23.70 to 6353.79μgml^{-1}. This is relative to IC_{50} values of positive controls ascorbic acid and BHT (15.27μgml^{-1} and10.60) μgml^{-1}. Fractions AS-F4-1-99, AS-F4-1-87 and AS-F4-1-109 showed strong antioxidant activity with IC_{50} values of 23.7043, 24.7043 and 46.096µg/ml respectively. The 3 most active fractions i.e. AS-F4-1-99, AS-F4-1-87 and AS-F4-1-109 were further combined together. As the results obtained from both radical scavenging assay and HPLC analysis shows that in the chromatograms the same compound appeared at a retention time 12.5 minutes in fractions AS-F4-1-99 and AS-F4-1-109 but higher in concentration in AS-F4-1-99 as it reached up to 2000mAU therefore it may be the causes of these high antioxidant activity of the fractions.

Also the chromatograms of fractions AS-F4-1-109 and AS-F4-1-87 the same compound appeared at retention time 16.9 minutes but it higher in concentration at fraction AS-F4-1-87 reached to 2000mAU therefore it may be the causes of the high antioxidant activity

of the fractions. This is why it prep arable to combine the entire three fractions and performed the second column chromatography.

4.6 Total Phenolic Content: Folin-Ciocalteau Method

The Folin-Ciocalteau reaction relies on the transfer of electrons in alkali medium from phenolic compounds to phosphomolybdic /phosphotungstic acid complex which are determined spectrophotometrically at 765 nm (Elizabeth & Kelly 2007).

The total phenolic content of the fractions were determined using Folin-Ciocalteau (FA) method and expressed as Gallic Acid Equivalent (GAE) by employing A calibration was developed by reacting range of concentration of Gallic acid (0.01 – 0.2 mg/ml) under the FA reaction conditions. Corresponding absorbance were measured **(**Table 4.5**).** This was used to develop a linear calibration curve of Gallic acid with R^2 of 0.9854**.** Total phenolic content of the most active crude fractions and the column fractions were evaluated from the calibration equation (*y=5.0742x+0.0392*) and expressed as GAE; where y=absorbance at 765nm and X=concentration of total phenolic compounds in mg/ml of the extracts (Genwali *et al*., 2013).

Table 4.5: Result of Total Phenolic Content of Extracts and Active Eluted Fractions Expressed as Gallic Acid Equivalent

Fractions	Absorbance (nm)	Total Phenolic Content (mg/GAE)
Ethanol Extract (F1)	0.4663	466.30
Ethyl Acetate Extract (F2)	0.6146	614.61
N-hexane Extract (F3)	0.2806	281.14
Methanol Extract (F4)	0.4556	455.74
AS-F1-1-65	0.3184	318.38
AS-F1-1-75	0.2738	274..11
AS-F1-1-87	0.405	405.85
AS-F1-1-99	0.4739	474.48
AS-F1-1-109	0.33258	332.61

4.7 Relationship between Antioxidant Activity (DPPH free radical scavenging activity) and Total Phenolic content (Folin-ciocalteau method) of the Crude extract and the Most Active Column Fractions

Table 4.6 indicated the relationship between total phenolic content (Folin-ciocalteau) and anti-oxidant activity (DPPH free radical scavenging activity).The total phenolic content of the crude extract and fractions i.e. F1 –F4 ranged from (281.414 - 614.614) mg equivalent to Gallic acid (EGA). Ethyl acetate soluble fraction F2 possessed the highest

number of phenolic content of 614.614 mg/GAE with moderates' activity against DPPH radical scavenging activity as they have IC_{50} value of 663.03µg/ml (Table 4.2), even though methanol soluble fraction F4 possesses 455.747 mg/GAE of phenolic content with the highest anti-oxidant activity of IC_{50} 268.5717µg/ml. The reason behind the highest phenolic content of Ethyl acetate soluble fraction F2 is that both n-hexane soluble fraction F3 and methanol soluble fraction F4 is the fraction of ethyl acetate soluble fraction F2 (I.e. F2=F3+F4).

The next in term of phenolic content is ethanol soluble fraction F1 which has 466.3 mg/ GAE with DPPH radical scavenging activity of the IC_{50} 924.7µg/ml.

n-hexane soluble fraction F3 possess the lowest number of phenolic content among all the crudes fractions, as they contained 281.1414 mg/GAE, they also showed the lowest antioxidant activity with highest IC_{50} value of 1820.85 µg/ml.

In term of column fractions the results shows that fraction contained higher number of phenolic content possesses strong antioxidant activity against DPPH radical I.e. lowest IC_{50} and vice-versa.

Fractions AS-F4-1-99, AS-F4-1-87, AS-F4-1-109, AS-F4-1-65 and AS-F4-1-75 having the following radical scavenging activities of IC_{50} Values of 23.704µg/ml, 24.70 µg/ml, 46.0 µg/ml, 86.48 µg/ml and 242.832 µg/ml and the of phenolic content of 474.48 mg/ GAE, 405.847 mg/GAE, 332.614 mg/GAE, 318.38 mg/GAE and 274.114 mg/GAE respectively. The result obtained shows the correlation between antioxidant activity and total phenolic content indicated that the phenolic content is responsible for the DPPH free

radical scavenging activity of the column fraction; the phenolics compounds of *Moringa oleifera* are responsible for this high anti oxidant activities (Table 4.6).

Table 4.6 Relationship between Total Phenolic Content and Total Anti-oxidant Activity of the both Crudes Fraction and most Active Column Fraction.

S/NO	Fraction	Total Phenolic Content (Mg/GAE)	Total Antioxidant Activity IC_{50} (µg/ml)
1	F1	466.3	924.7
2	F2	614.614	663.03
3	F3	281.141	1820.85
4	F4	455.747	268.5717
5	AS-F1-1-65	318.38	86.485
6	AS-F1-1-75	274..114	242.832
7	AS-F1-1-87	405.847	24.704
8	AS-F1-1-99	474.48	23.704
9	AS-F1-1-109	332.614	46.096

4.8 Preparative TLC of Fraction AS- F4-1-58

Fraction AS-F4-1-58 was purified further by means of Prep TLC on glass backed silica gel plate. This was in consideration of its yield (1419 mg) and antioxidant activity (IC_{50} = 149.242µgml^{-1}). 7 different bands at different R_F positions as well as appearance under the $UV_{254/365\ nm}$ were obtained (Table 4.8 /Fig 4.14).These bands were separately recovered and their yield evaluated (Table 4.8**).**

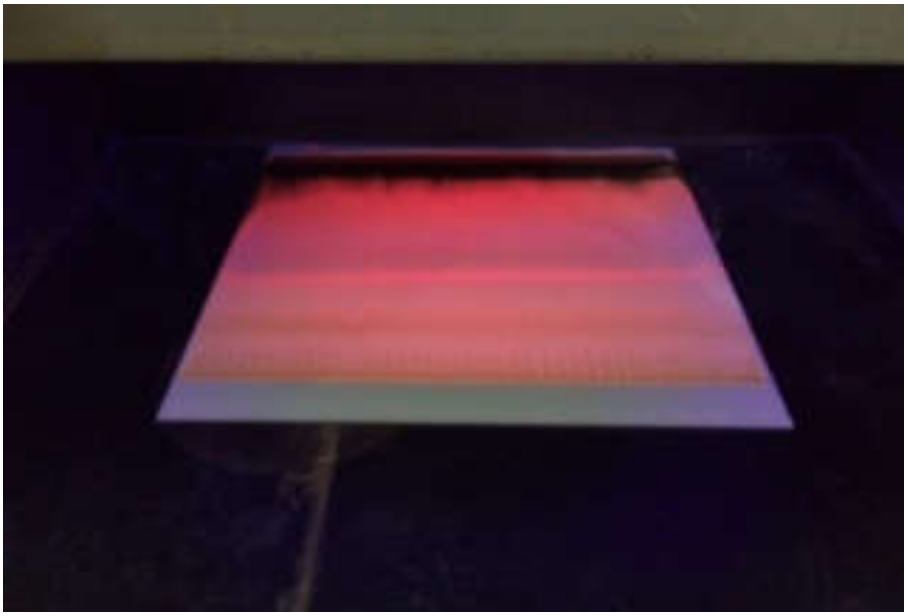


Figure4.14: Eluted Prep TLC Plate of Fraction AS- F4-1-58 Viewed under UV light at 254nm showing Bands at Different R_F Positions

Table 4.7: R_f Value and Yield of Recovered Prep TLC Fraction

Fractions	R_F Value	Weight (mg)
1st band	0.056	9
2nd band	0.167	14
3rd band	0.333	13
4th band	0.722	142
5th band	0.806	152
6th band	0.889	75
7th band	0.917	32

The result of the preparative HPLC analysis indicated that the chromatogram contained a cluster compound which is not separable

4.9 Column Chromatographic Purification of Fraction AS-F4-1-87 To 109

Fractions AS-F4-1-87, AS-F4-1-99 and AS-F4-1-109 were combined based on the similarity in their antioxidant and HPLC profiles. A total weight of 1.57g was loaded using gradient of $CHCl_3$/Methanol as eluting solvents. A total of 81 fractions were obtained when collecting 50 ml which were subsequently pooled into13 fractions (Table 4.8).

4.10 Result of Antioxidant Assays of the Second Column Fractions

The fractions obtained from chromatographic purifications of AS-F4-2-10 to AS-F4-2-81 (Table 4.8) were subjected to radical scavenging activity assay against DPPH radical (Seephonkai *et al*., 2011). The activity was expressed in IC_{50} values (µg/ml), while Vitamin C and BHT were used as positive controls (Fig. 4.15 –4.19).

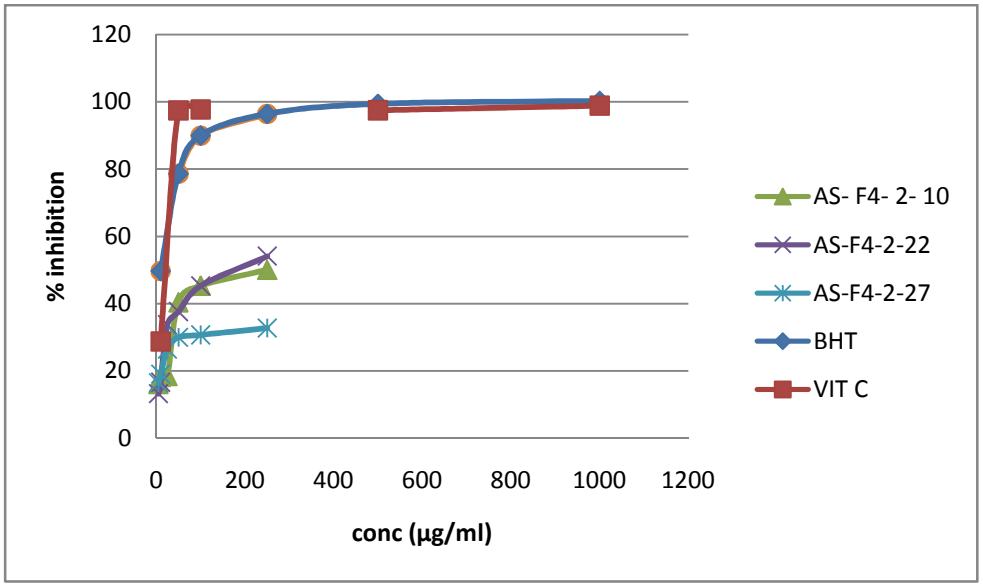


Figure 4.15: Result of DPPH Radical Scavenging Activity of AS-F4-2--10 - AS-F2-2-27

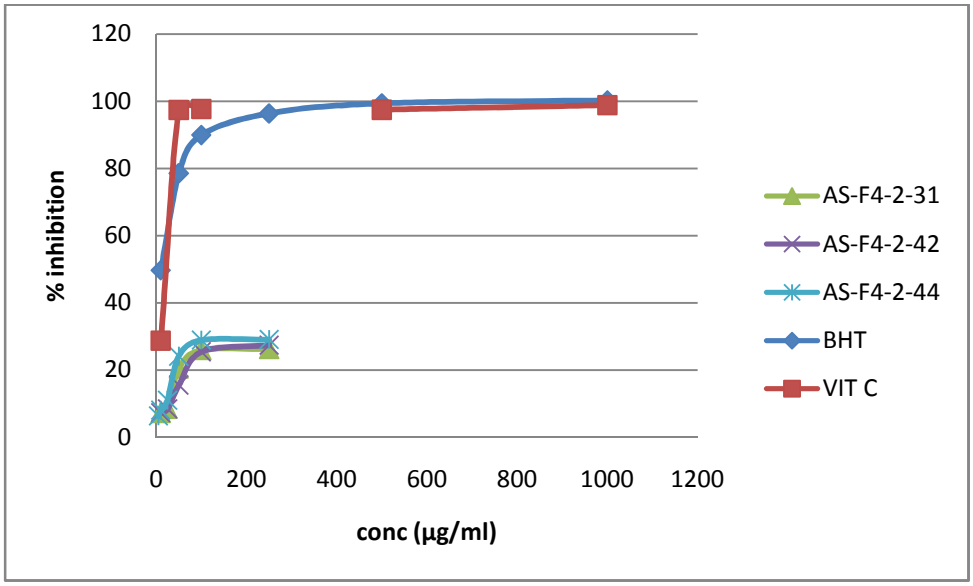


Figure 4.16: Result of DPPH Radical Scavenging Activity of AS-F4-2-31 - AS-F4-2-44

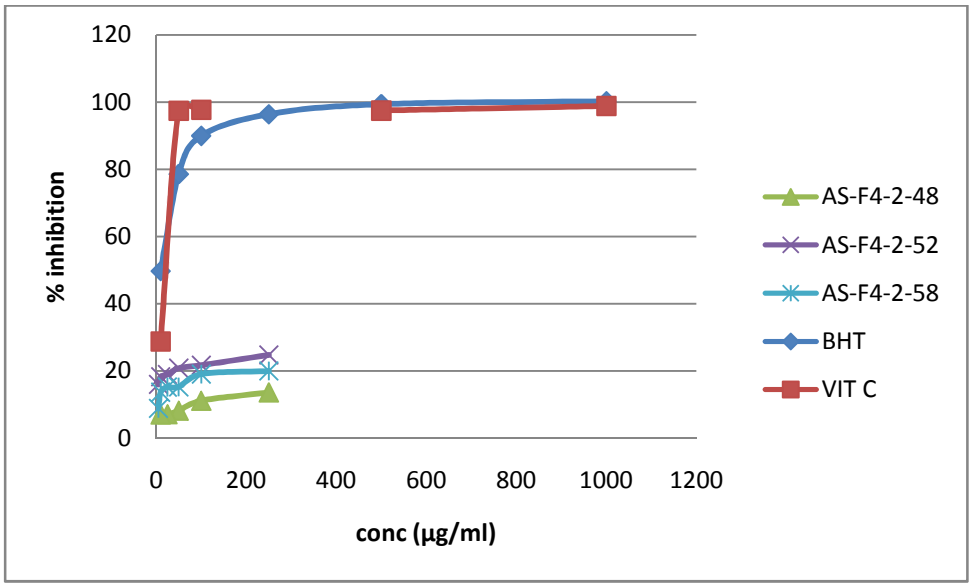


Figure 4.17: Result of DPPH Radical Scavenging Activity of AS-F4-2-48 - AS-F4-2-58

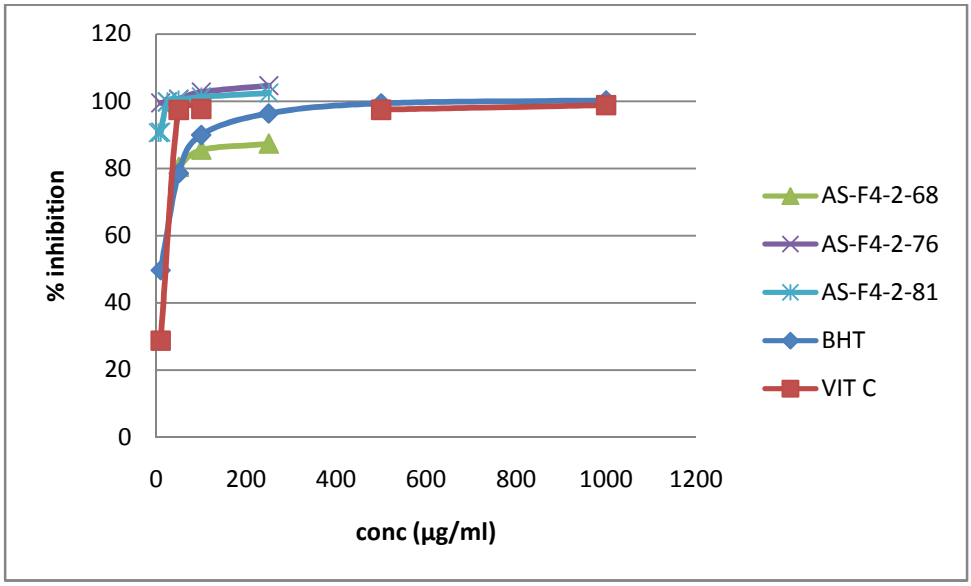


Figure 4.18: Result of DPPH Radical Scavenging Activity of AS-F4-2-68 - AS-F4-2-81

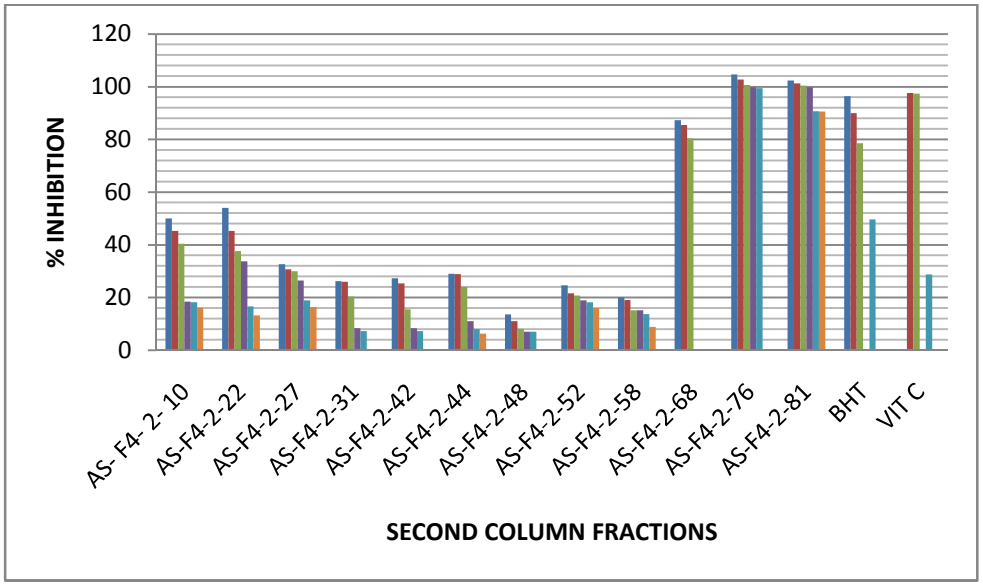


Figure 4.19: Comparative DPPH Radical Scavenging Activity of the Pooled Column Fractions Fraction with BHT and Vitamin C

Table 4.8: Yield and Result of DPPH Radical Scavenging Assay of Eluted Fractions

SN	Fraction	Yield (Mg)	IC_{50} (µg/ml)
1	AS-F4-2-10	44	201.4
2	AS-F4-2-22	1017	148.5
3	AS-F4-2-27	168	>1000
4	AS-F4-2-31	41	>1000
5	AS-F4-2-42	58	>1000
6	AS-F4-2-44	14	>1000
7	AS-F4-2-46	12	>1000
8	AS-F4-2-48	11	>1000
9	AS-F4-2-52	22	>1000
10	AS-F4-2-58	42	>1000
11	AS-F4-2-68	36	655.43
12	AS-F4-2-76	118	0.0080
13	AS-F4-2-81	44	0.019
14	VIT C	-	15.27

4.11 HPLC Purification of Fraction AS-F4-2-76 and AS-F4-2-81

Based on the results of the second column fraction, fraction AS-F4-2-76 and fraction AS-F4-2-81 possesses the high radical scavenging activity with the IC_{50} of 0.008 µg/ml and 0.09µg/ml respectively. They also show similar chemical composition on testing using both analytical HPLC and TLC. Based on this the fractions were combined together and purified by the preparative HPLC. The chromatograms of semi pure compounds are shown in (Fig5.2) when they combined together.

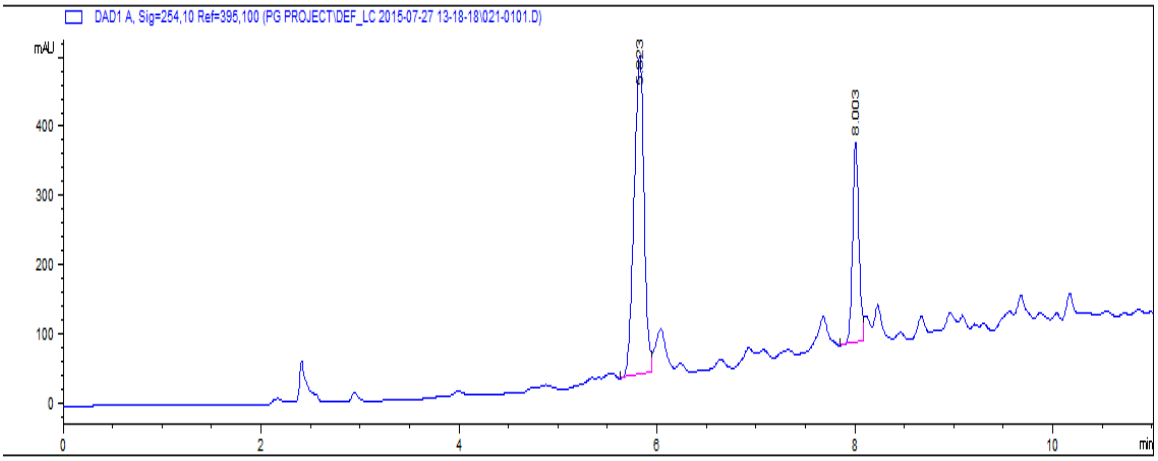


Figure 4.20; HPLC Chromatogram of Semi Pure Compound of the 2 most Active Fractions after they Combined

A total of four compounds were isolated at the different retentions times-5.8, 6.05, 8.0 and 8.2 minutes (Table 4.9)

Table4.9: **Weights of the Pure Compounds Isolated**

SN	Sample	Yield (mg)
1	AS-F4-2-76-1	2.4
2	AS-F4-2-76-2	1.4
3	AS-F4-2-76-3	1.6
4	AS-F4-2-76-4	1.1

The four isolated compounds Table (4.9) were submitted for the Nuclear Magnetic Resonance Spectroscopic analysis. Data for ^{1}H NMR, ^{13}C NMR, COSY and HSQC were acquired for samples AS-F4-2-76-1 and AS-F4-2-76-4, (Fig4.21-Fig.4.27)

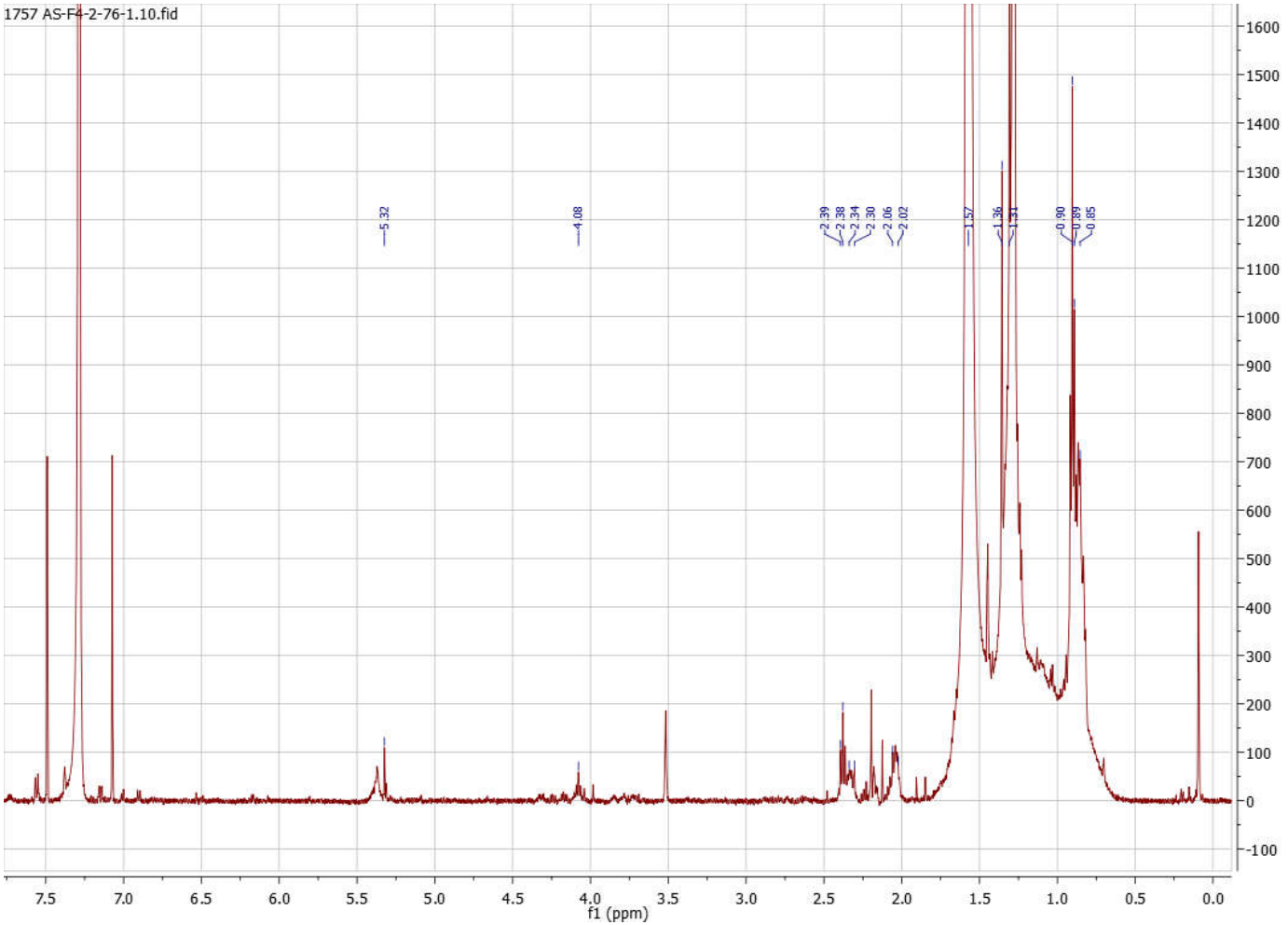


Figure 4.21: ^{1}H NMR Spectrum of Sample AS-F4-2-76-1

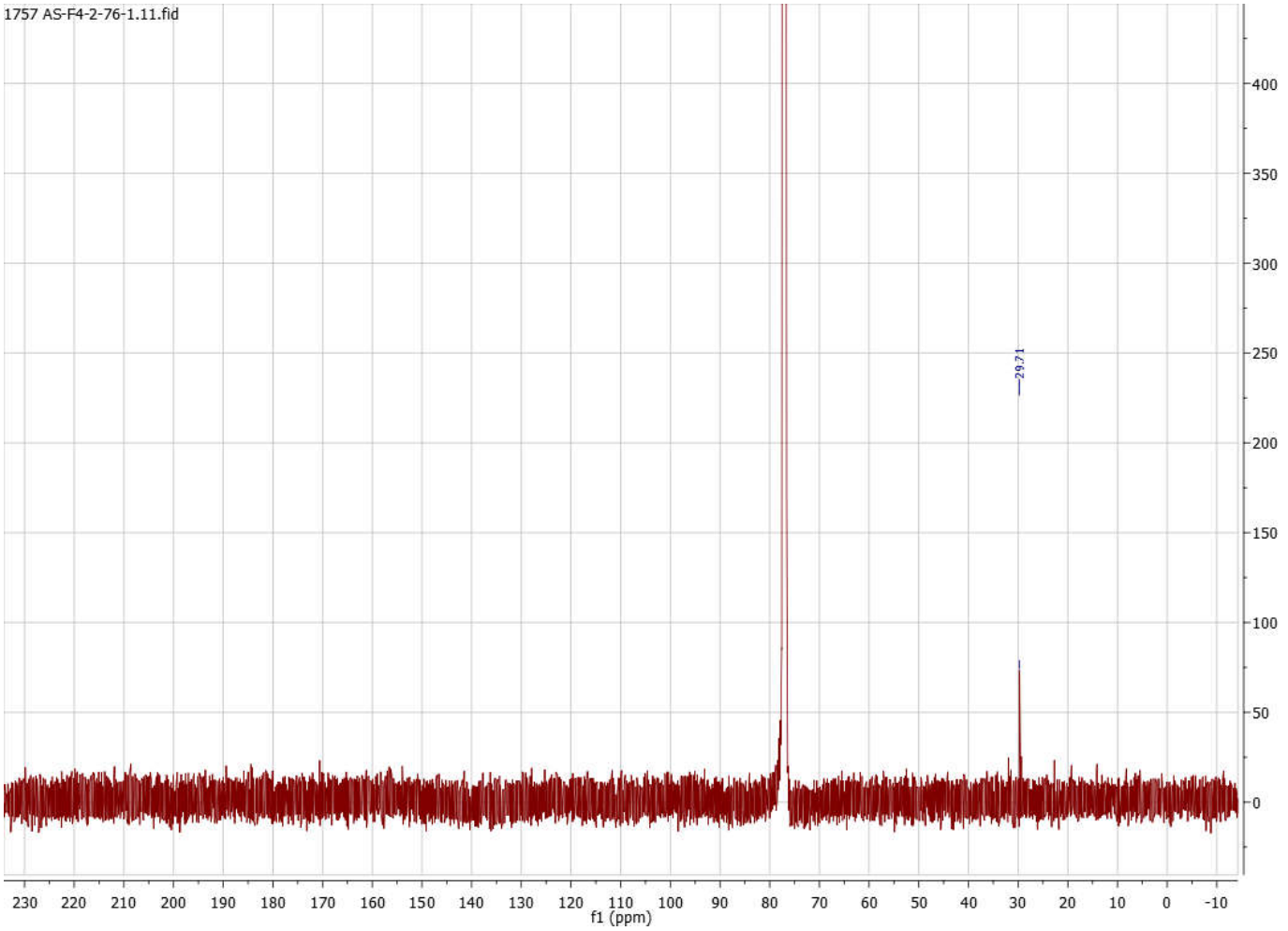


Figure4.22:^{13}C NMR Spectrum of Sample AS-F4-2-76-1

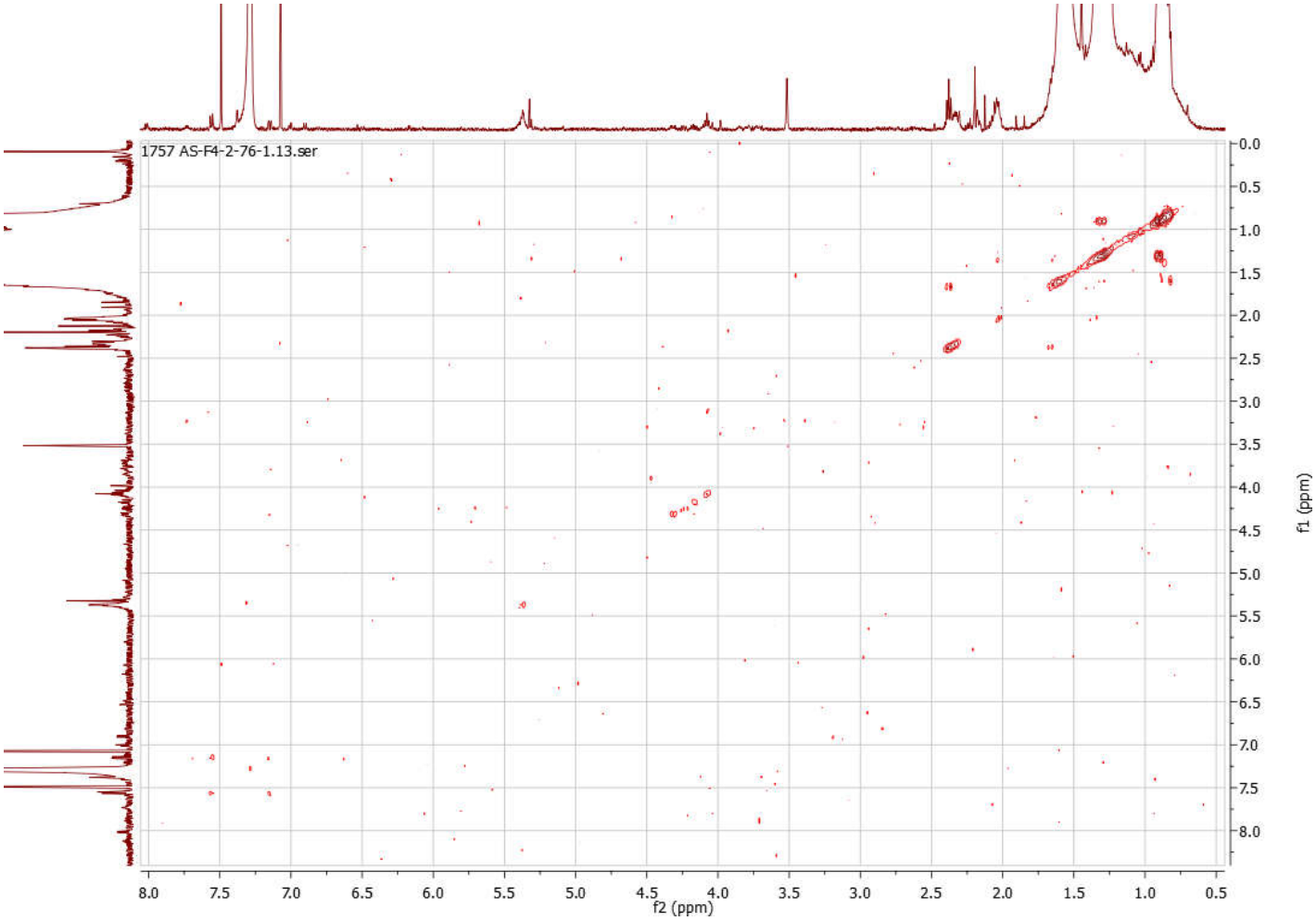


Figure 4.23: COSY Spectrum of Sample AS-F4-2-76-1

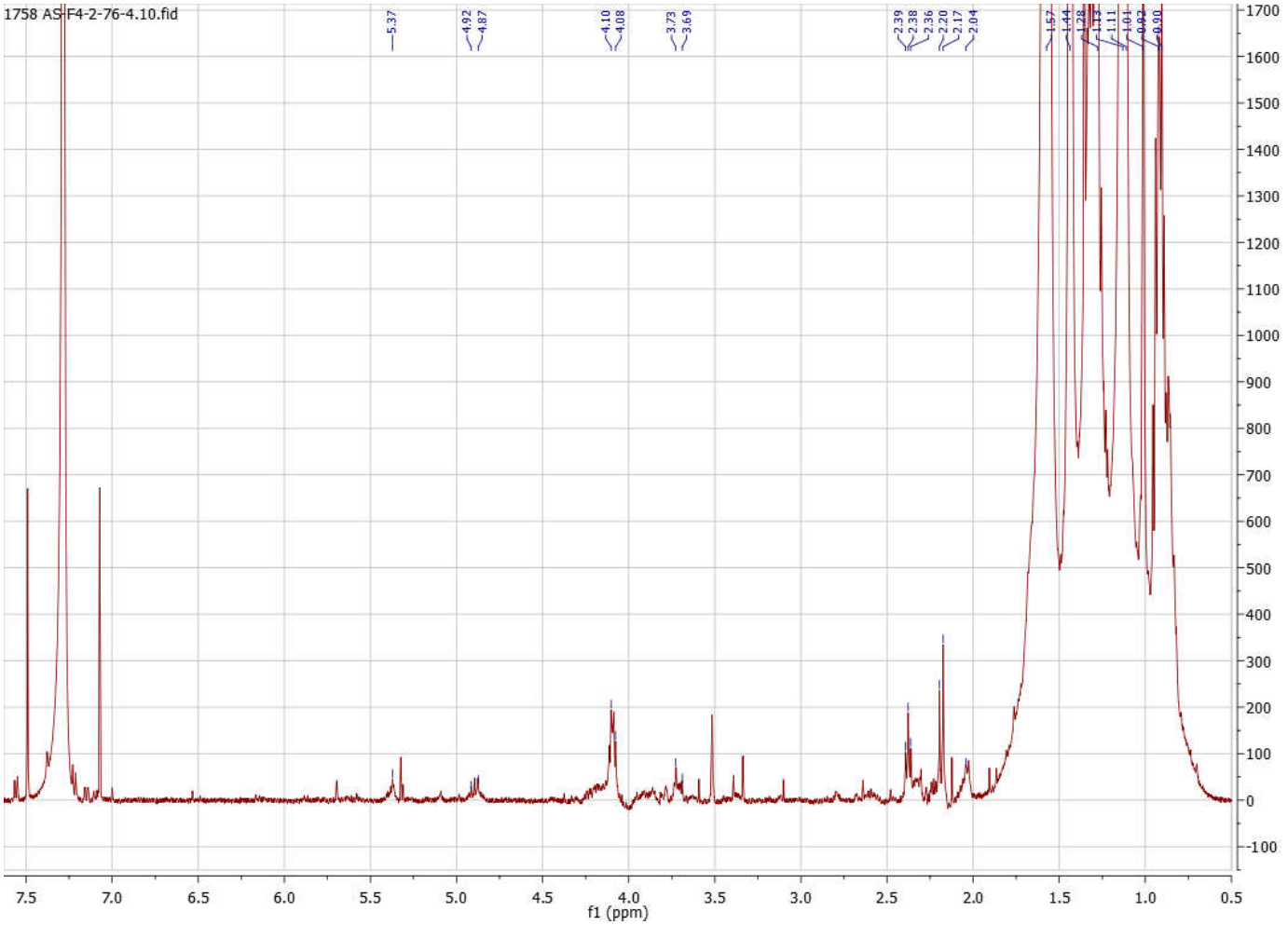


Figure 4.24: ^{1}H NMR Spectrum of Sample AS-F4-2-76-4

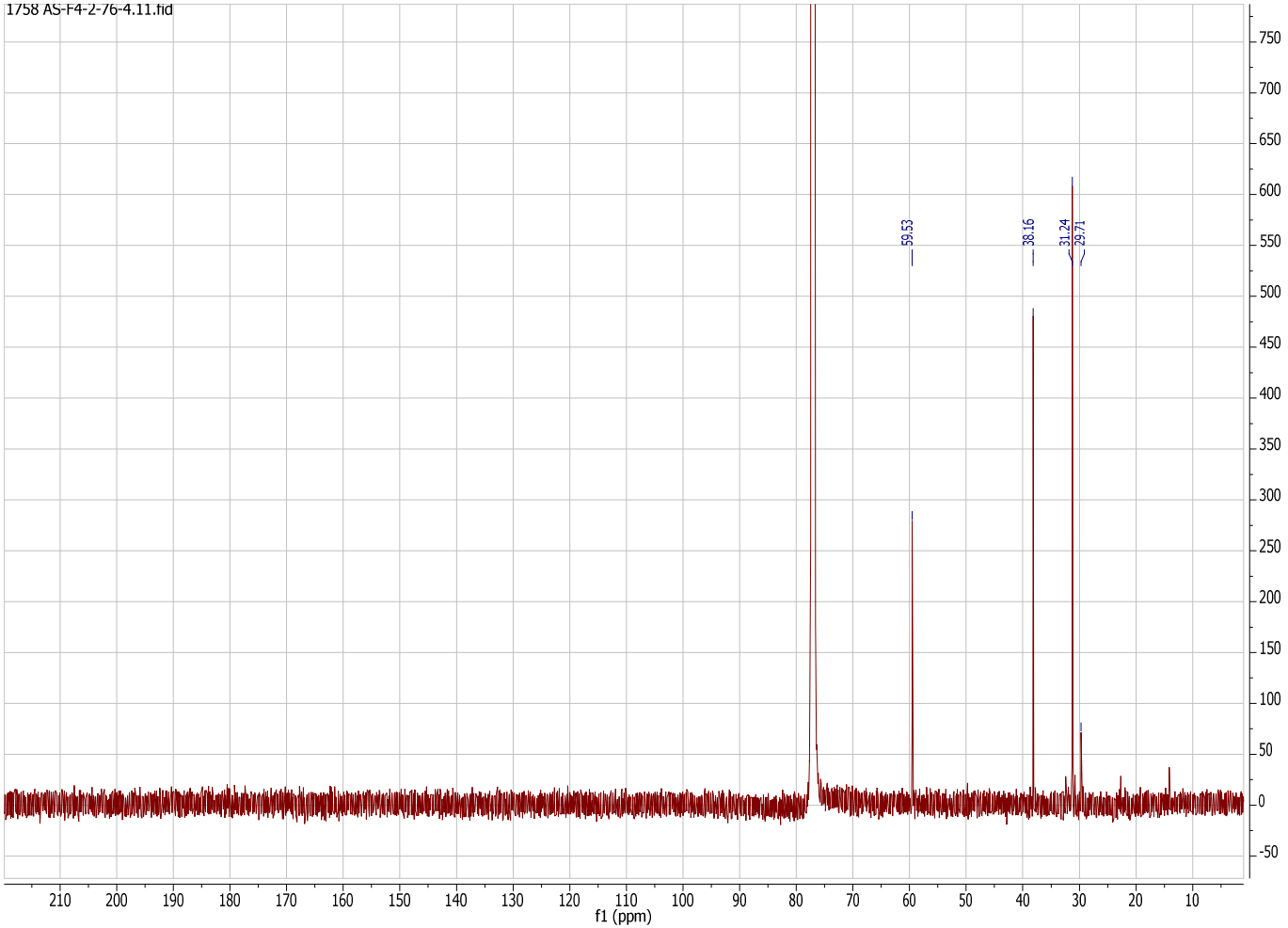


Figure4.25: ^{13}C NMR Spectrum of Sample AS-F4-2-76-4

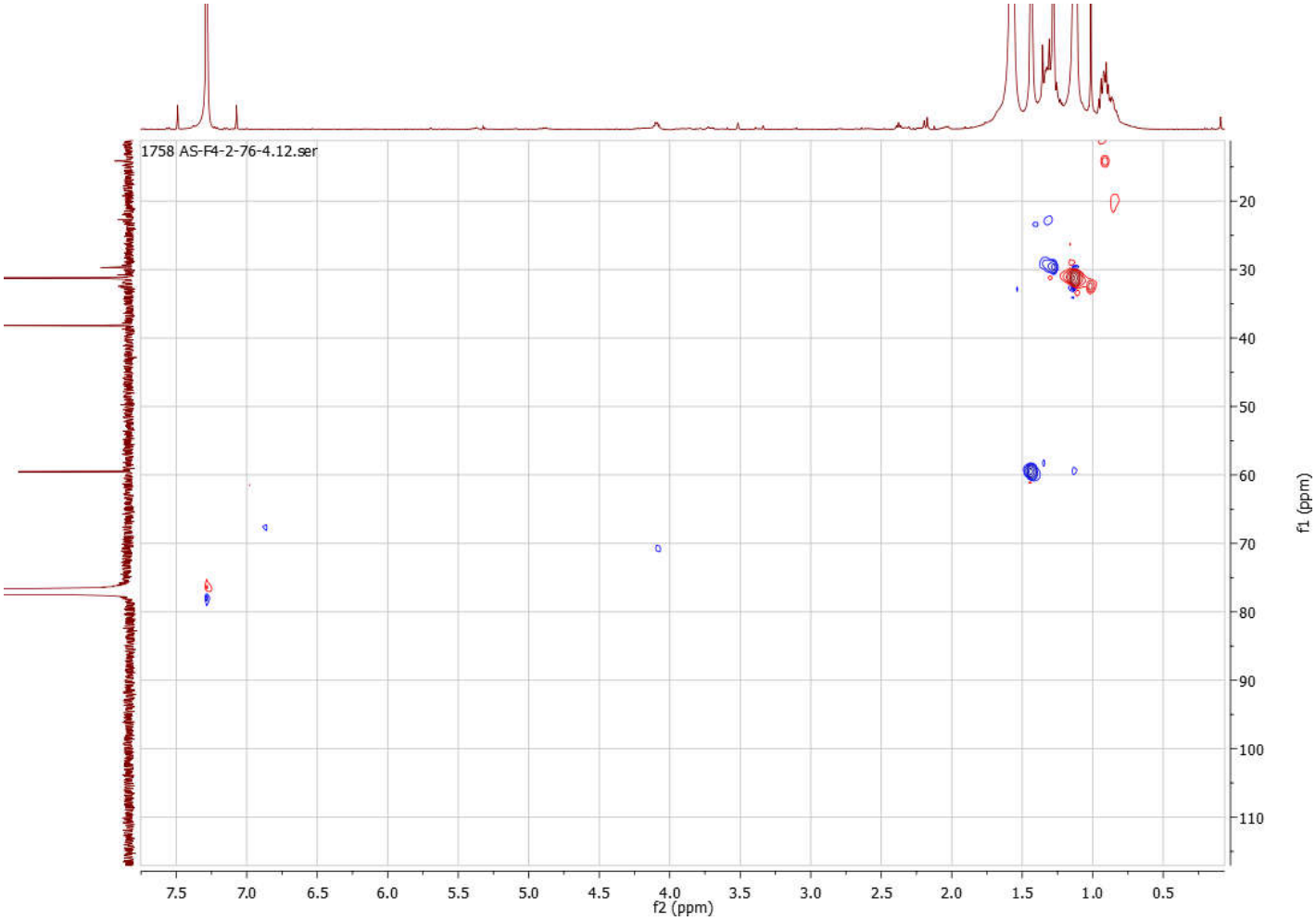


Figure4.26: HSQC Spectrum of Sample AS-F4-2-76-4

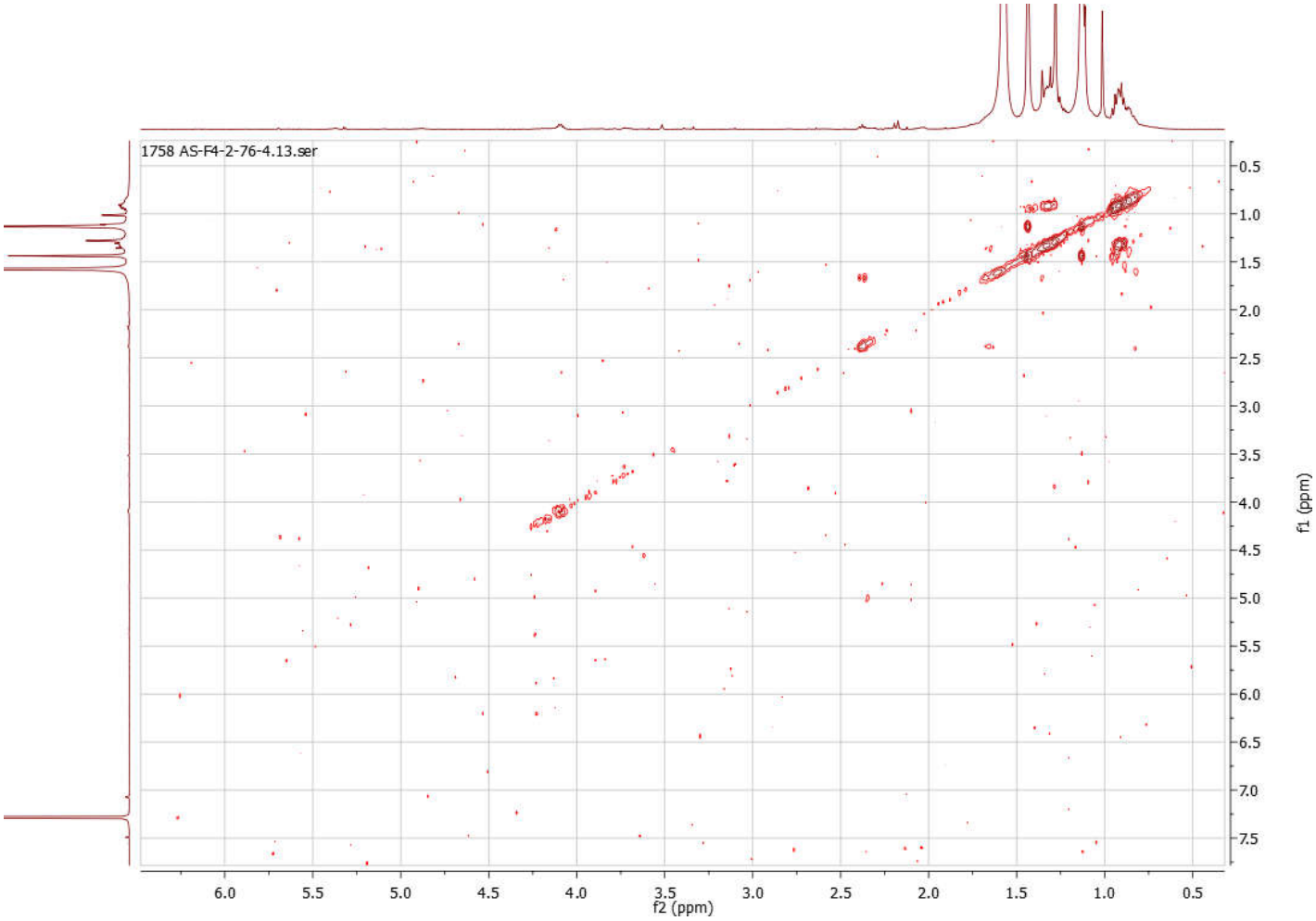


Figure4.27: COSY Spectrum of Sample AS-F4-2-76-4

4.12 Mass Spectrometric Analysis and Detection of Secondary Metabolites from Extract of *Moringa oleifera*

Ethanol extract of *M. Oleifera* were subjected to mass spectrometric analysis by means of Liquid Chromatography Mass Spectrometry. Natural product identification was achieved by studying the combined mass and UV spectral profiles.

Astraglin, Niazidine and Isoquercetin (Tatiano *et al*., 2013; Dayal *et al*., 2013; Sharma *et a*l., 2013; Leone *et al*., 2015) were detected and identified using collision induced electrospray ionization mass spectrometry. The compounds were characterized in both + ve and - ve ion mode.

Astraglin exhibit the following m\z values of the molecular ion, fragments ion and the adduct ion which indicates the characteristics of the molecular compound. Astraglin ion $[M+H]^+$ with molecular ion peak at M\Z 449 (100 %), Adduct ion $[M+Na]^+$ 471 (50%). while the other fragment ions peak at m\z 287 (18 %) and m\z 163 (15 %) are kaempferol ion by losing glycosides and 5-hydroxy-4-oxo-4*H*-chromenium by further losing phenol. (Fig 4.27) The comparism between the +ve and -ve ion mode of the Astraglin are demonstrated in (Fig 4.28) which confirmed the isolation of the compound.

The result obtained from The collision induced electrospray ionization mass spectrometry of Niazidine in +ve ion mode yield the following m\z values of the molecular ion, fragments ion and the adduct ion which indicates the characteristics fragmentation pattern of these molecular compound. Niazidine ion $[M+H]^+$ Indicates molecular ion peak at M\Z 355 (30 %) , adduct ion $[M+Na]^+$ 377 (65%) .and the other fragment ion peaks are at m\z 313 (20%) and 163 (100%) which are the 3,4,5-trihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}benzylidene)amino]methylidene}sulfonium and the most stable

glycosides ion by losing cyanic acid and benzylcarbamothioiccyanate respectively.(Fig 4.29)

The UV absorption spectra of Niazidine shown two peaks of different wavelengths which they correspond to the absorption due to aromatic ring and that of the chromophore groups (Fig 4.30)

ESI-MS (- ve ion and + ve ion) of isoquercetin as shown in (Fig4.31) tally with the structural compound isolated. Both parent and daughter ion illustrated the characteristics properties of the molecular ion. Isoquercetin ion $[M-H]^-$ Indicates molecular ion peak at M\Z 463 (100%) - ve mode and $[M+H]^+$465 (60 %) + ve mode. Adduct ion $[M+Na]^+$ 487 (20%) are shown in the +ve mode. The other doughier fragment peak at.301 (10 %)- ve ion mode and 303 (100 %) +ve ion mode indicates Quercetin ion while 165 (1%) - ve mode indicates 2, 3-didehydro-4*H*-chromen-4-one by losing glycoside and benzene-1, 2-diol respectively (Fig 4.32).

Isoquercetin UV absorption spectra showed two peaks of the maximum absorption at 255 nm and 354nm of which they correspond to the absorption due to continuous conjugation of the aromatic ring and that of the chromophore groups (Fig 4.33)

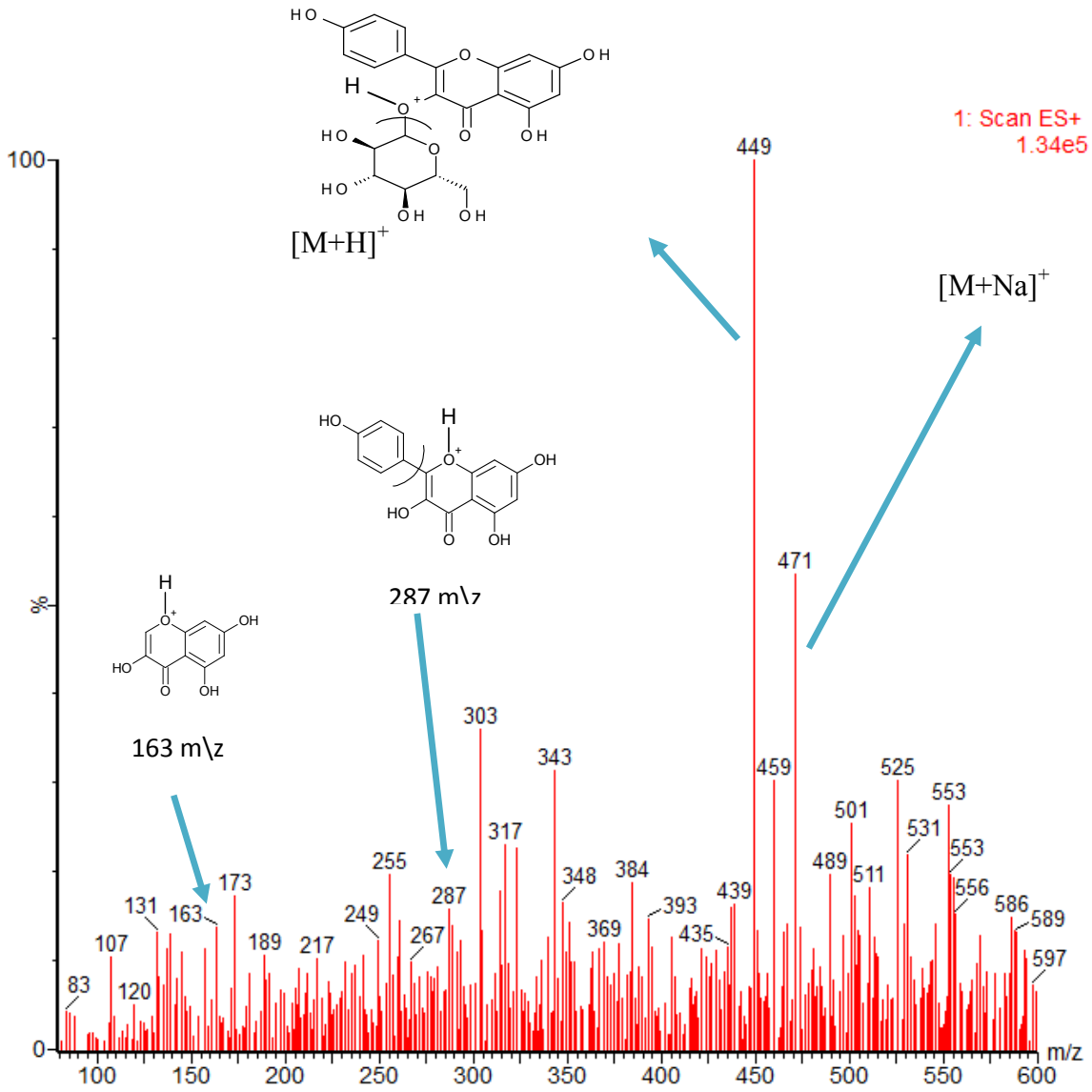


Figure4.28: ESI Mass Spectrum (Positive Ion Mode) of Astragalin from Extract of *Moringa oleifera*

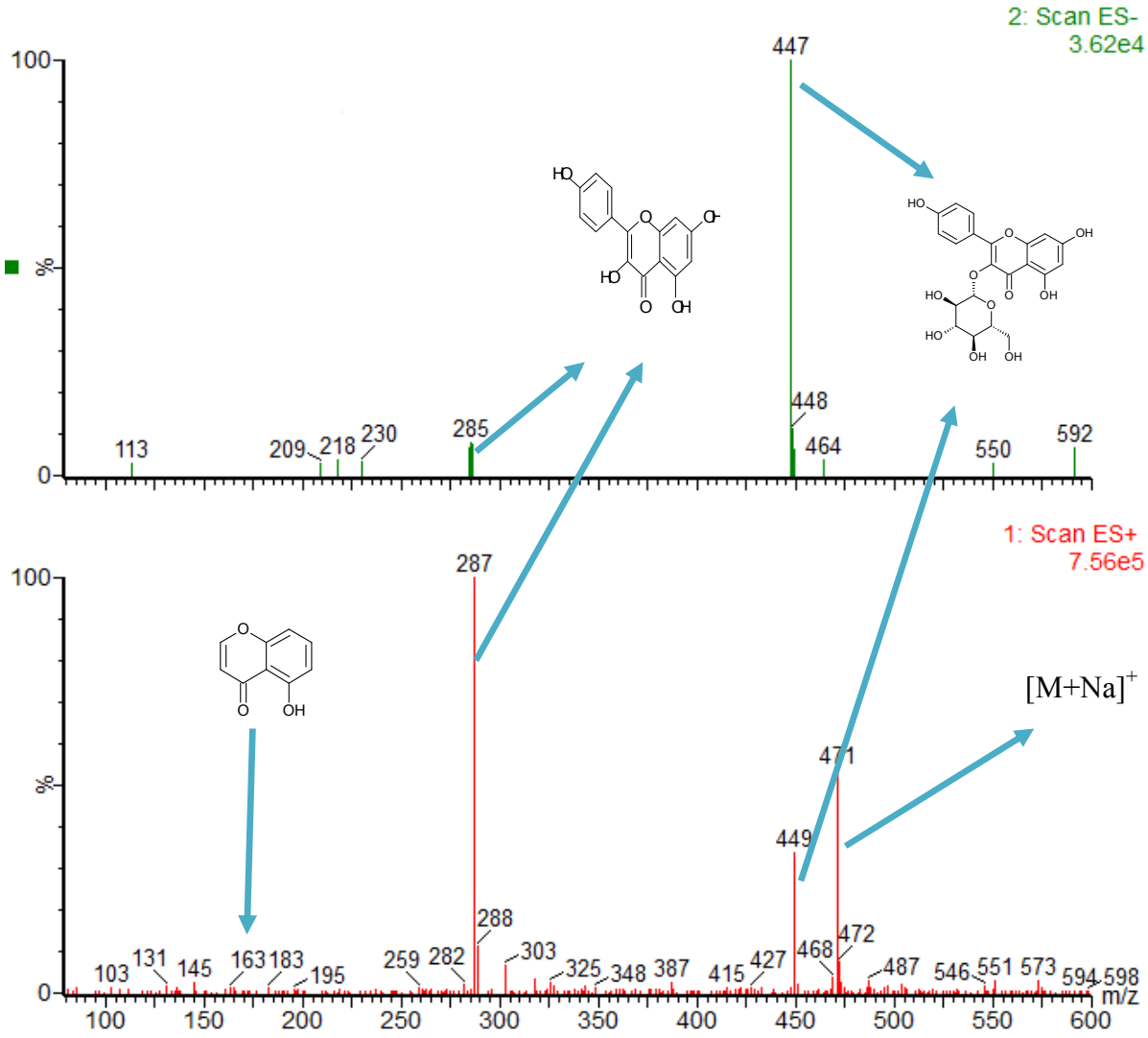


Figure 4.29: Overlay of ESI Mass Spectrum (Positive/Negative Ion Mode) of Astragalin from Extract of *Moringa oleifera*

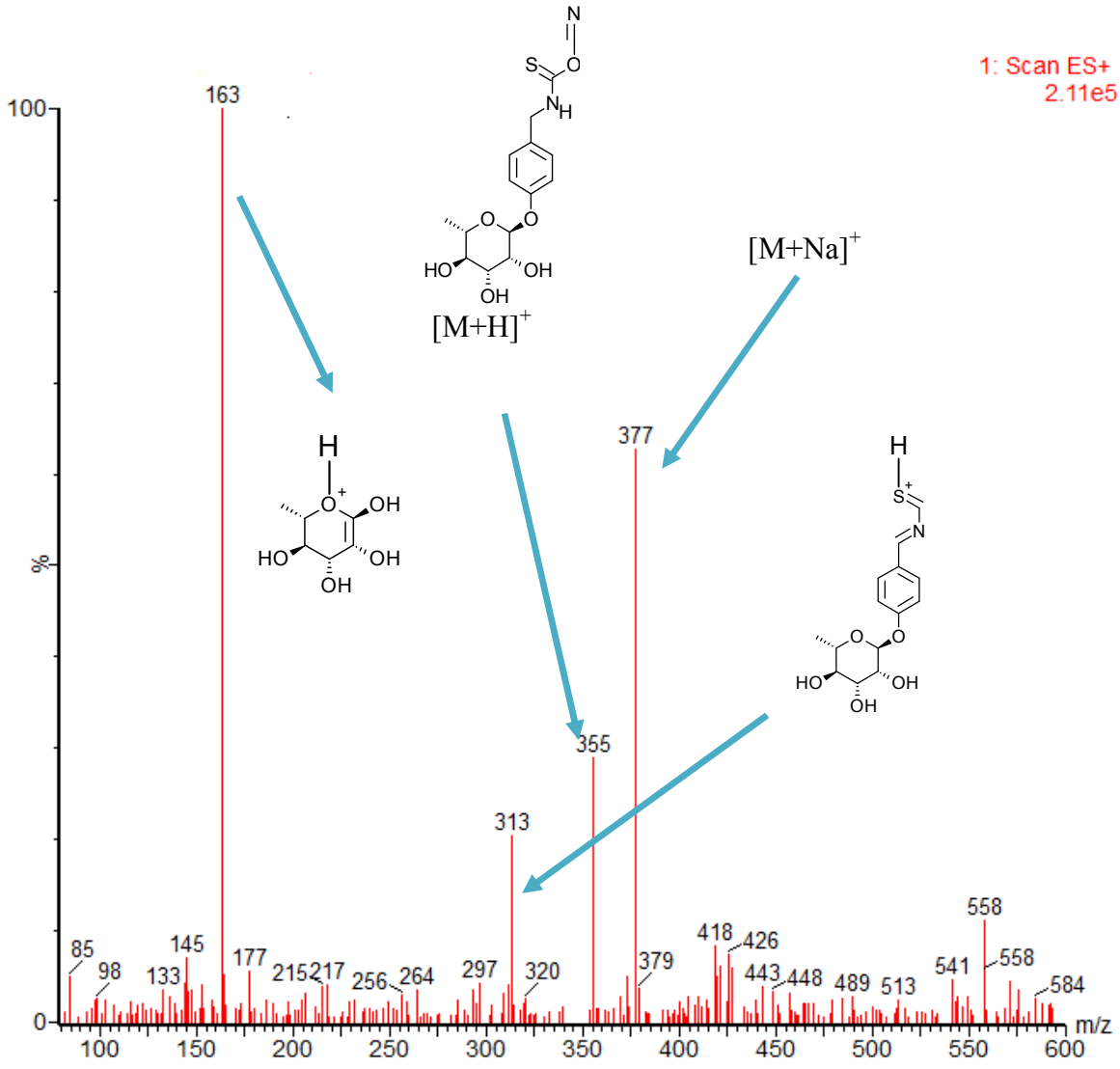


Figure 4.30: ESI Mass Spectrum (Positive Ion Mode) of Niazidine from Extract of *Moringa oleifera*

Figure 4.31: UV Absorption Spectrum of Niazidine showing λ_{max} at 213 and 325nm

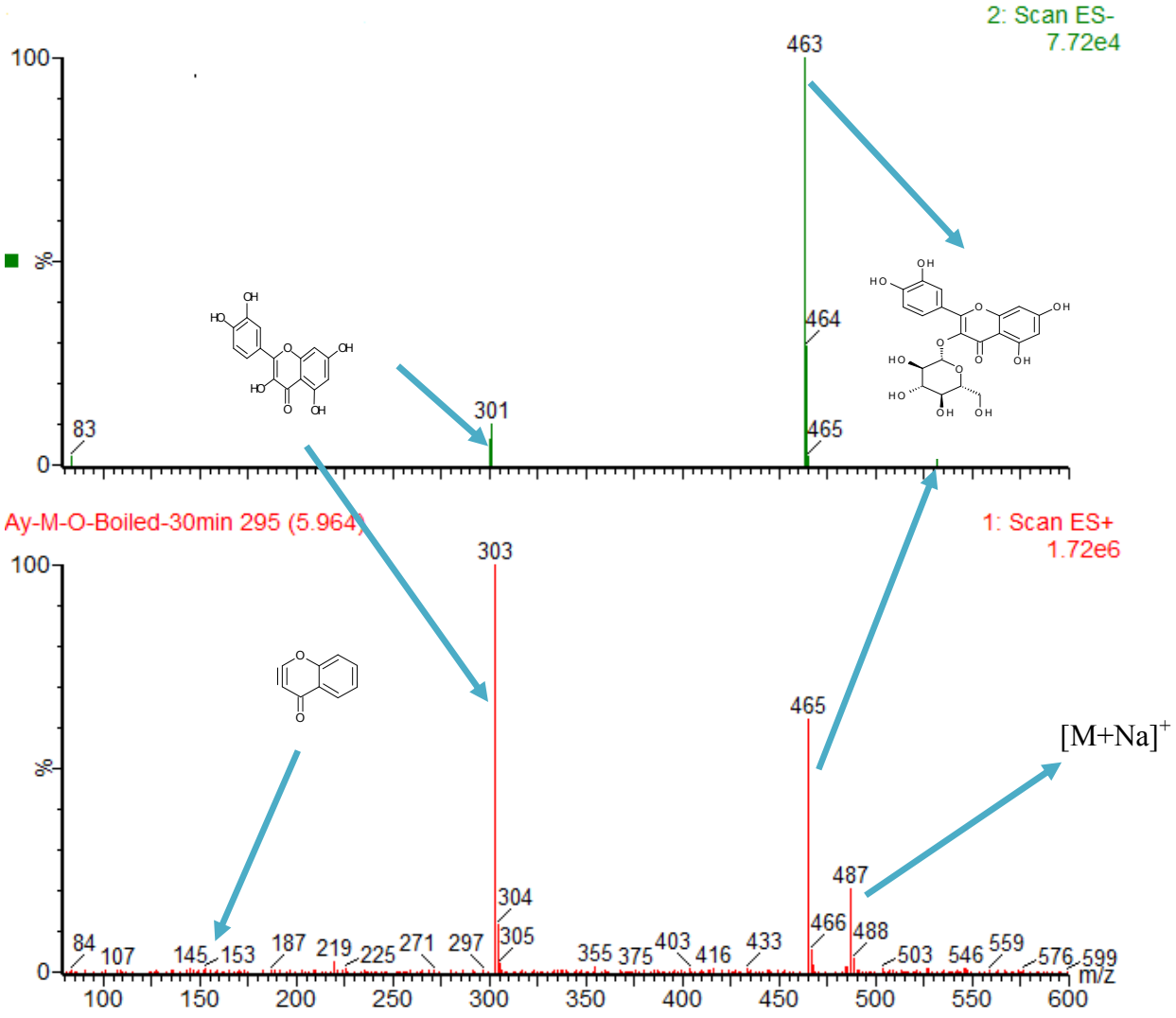


Figure 4.32: Overlay of ESI Mass Spectrum (Positive/Negative Ion Mode) of Isoquercetin from Extract of *Moringa oleifera*

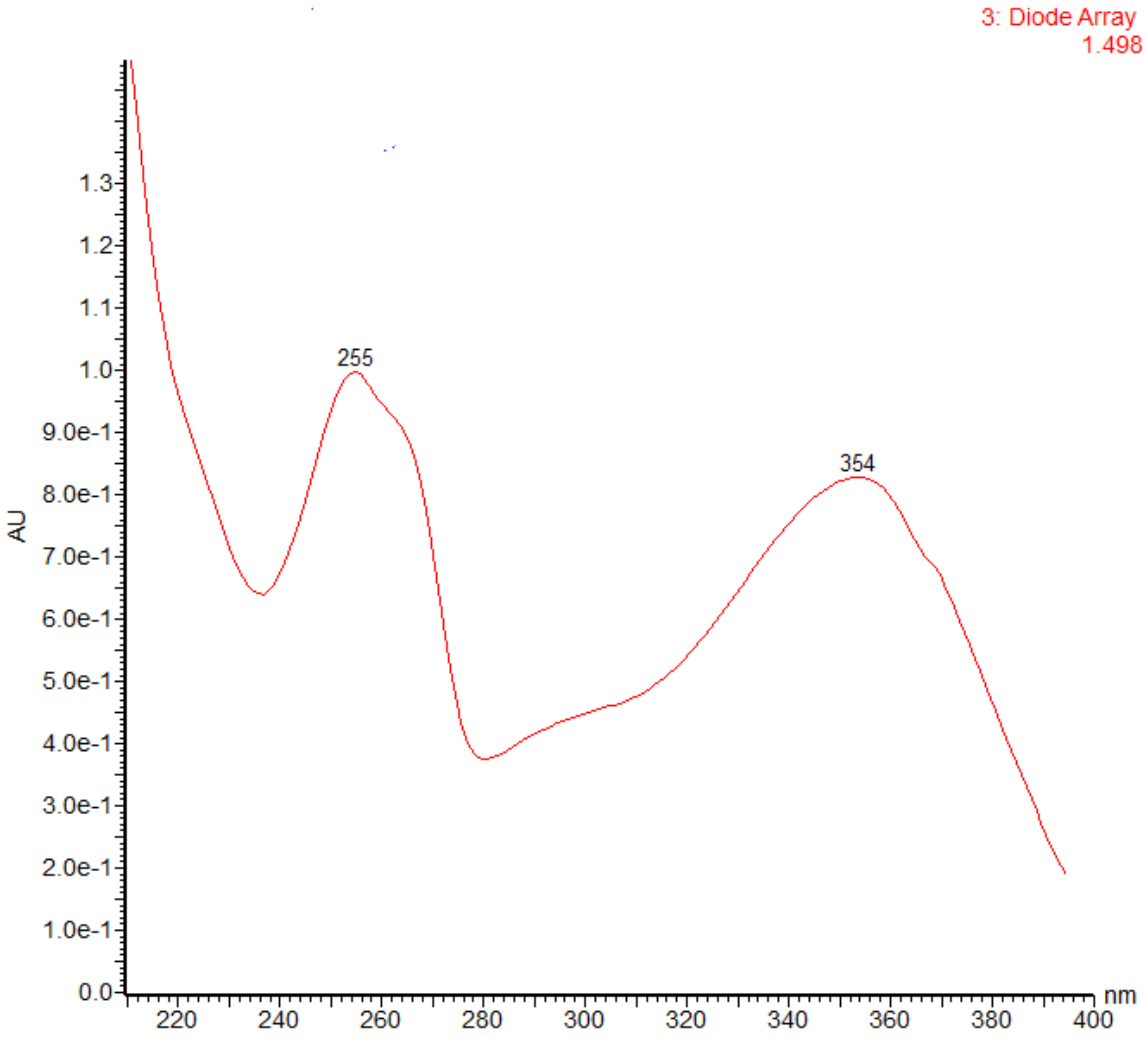


Figure4.33: UV Absorption Spectrum of Isoquercetin showing λ_{max} at 255 and 354nm

CHAPTER FIVE

5.0 Conclusion and Recommendation

Human being relies on nature to gather most of their basic needs. Natural products produced by plant posses' variety of uses especially in medicine even though most of the active secondary metabolites were chemically unknown.

The link between oxidative stress and chronic diseases such as Cancer, diabetes, inflammation etc result in production of free radical which is as a result of metabolic activities in the living system Such free radicals resulting in oxidation and disease condition. Body system is known to have a means of controlling of excess free radical but a system is not enough, hence supplementary sources of natural antioxidant compounds capable of quenching the excess free radicals are required.

Natural antioxidants are gaining more attention due to their therapeutic value with few side effects. Several bioassays are used to determine the antioxidant properties of the compounds.

Moringa oleifera is the one of the most economical importance species traditionally applicable in the treatment of the various ailments in the world.

The plant has been long investigated for its phytochemicals and pharmacological activities supporting ethno medical uses. Traditionally the plant has been validated by several pharmacological investigations. This reported extensively as ant cancerous, antimicrobial, antitumor, anti-inflammatory antihypertensive, and anti-oxidant. However, many of the diseases treated indigenously using the plant have not been confirmed in the

laboratory which leaves an opportunity to explore the species both phytochemically and pharmacologically.

Fractionation and purification of the crude extracts of *Moringa oleifera* using solvents of varying polarities led to the isolation of antioxidant compounds. It is noticed that the highest concentration of phenolic compounds in the extracts were obtained using solvents of high polarity; the methanol fracion manifested greater power of extraction for phenolic compounds from *Moringa oleifera.* LCMS, HPLC analysis as well as bioassay results indicated that the plants are the good sources of the bioactive secondary metabolites.

The results of the DPPH radical scavenging assay of the Crude extracts and column fractions indicated that the plant can be used as a good source of antioxidant compounds.

DPPH radical scavenging assay and total phenolic contents (Folin-ciocalteau assay) of the crude and the most active column fraction suggested that this extracts contain phenolic compounds which are part of antioxidant ingredients.

Based on the Chemspider database search, the compound isolated by LCMS was identified using collision induced electrospray ionization mass spectrometry represents a well known natural antioxidant compounds Astraglin, Niazidine, Quercetin and Isoquercetin as reported before.

Complete spectroscopic structure elucidation of the isolated compounds is recommended. Further effort should be directed towards isolation and identification as well as bioactivity testing of all the compounds identified from analysis of extracts fractions from this plant. Further studies of this plant species should be directed to carry out in vivo

studies of its medicinal active components in order to prepare natural pharmaceutical products of high value

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APPENDICES

Appendix 1: Calibration Concentration of Gallic Acid against Absorbance

Conc. (mg/ml)	Absorbance (nm)
0.200	1.0052
0.150	0.8646
0.100	0.5728
0.050	0.2407
0.010	0.1004

Appendix 2: Calibration graph of the Gallic Acid Equivalent