INVESTIGATION OF SECONDARY METABOLITES FROM *Withania somnifera* AND *Hagenia abyssinica* FOR THE CONTROL OF MAIZE AND BEAN PATHOGENS

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EGERTON UNIVERSITY

OCTOBER 2018

DECLARATION AND RECOMMENDATION

DECLARATION

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

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DEDICATION

I dedicate this work to my children, Josephine, Rael and Hezron who through their perseverance this work was accomplished.

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ABSTRACT

Maize (*Zea mays* L.) and the common bean (*Phaseolus vulgaris* L.) are the two most important staple foods not only in Kenya but also in Sub-Saharan Africa. Despite the importance of these two crops, there have been production constraints due to diseases caused by bacterial and fungal pathogens. The major diseases affecting maize production are Gray Leaf Spot (GLS) and maize ear rot which are caused by fungal pathogens, *Cercospora zeae-maydis, Fusarium moniliforme* and *Fusarium graminearum*. Fungal pathogens, *Colletotrichum lindemuthianum* and *Pythium* spp. are the major causes of anthracnose and root rot diseases in beans respectively. Common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *phaseoli* and halo blight (HB) caused by *Pseudomonas savastanoi* pv. *phaseolicola* are among the most important bean bacterial diseases in Africa. This research investigated the activity of methanolic extracts from *Withania somnifera, Hagenia abyssinica* and essential oil (EO) from the leaves of *H. abyssinica* against these pathogens. The essential oil was extracted by hydro-distillation in a modified Clevenger apparatus. Disk diffusion method was used to determine the antimicrobial activities. The methanolic extracts of both plants (100 mg/ml) inhibited the growth of both bacterial and fungal pathogens. Extracts from *W. somnifera* had an inhibition zone of 12 mm and *H. abyssinica* 15 mm against *C. lindemuthianum,* which was much higher than the reference standard (Nystatin) inhibition zone of 10.6 mm. The activity of the essential oil was equal to that of standard Chloramphenicol (30 mm) against the two bacterial pathogens. Further isolation of the methanolic extracts through column chromatography yielded three compounds which were identified through 1D and 2D nuclear magnetic resonance. Two steroidal lactones were isolated from *W. somnifera*; 5α, 17β-dihydroxy-6α, 7β-epoxy-1-oxo-witha-2, 24-dienolide (**19**) and 4, 5, 6, 15 tetrahydroxy-1-oxo-witha-7-enolide (**20**). A pentacyclic triterpene, 2, 3, 19-trihydroxyurs-12-en-28-oic acid (**21**) was isolated from *H. abyssinica*. The isolated compounds, **19** and **20** from *W. somnifera* did not show any antimicrobial activity. This implies that the activity observed in the extract was due to synergistic effect, or other active compounds in the extracts. The GC-MS analysis of EO from the leaves of *H. abyssinica* revealed the oil was majorly constituted of sesquiterpenes (53.4%) and monoterpenes (33.2% of the total oil concentrations). The major sesquiterpenes were; ɗ-gurjunene (13.6%), aromadendrene (11.3%) and palustrol (6.2%) while major monoterpenes were endobornyl acetate (19.7%), camphor (3.6%) and azulene (3.9%). These findings show that these plants can be used as lead source of compounds in the development of new, biodegradable, environmentally friendly and cheaper antimicrobials.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ii

COPYRIGHT iii

DEDICATION iv

ACKNOWLEDGEMENT v

ABSTRACT vi

TABLE OF CONTENTS vii

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF APPENDICES xi

LIST OF ABBREVIATIONS xii

CHAPTER ONE 1

INTRODUCTION 1

1.1 Background information 1

1.2 Statement of the problem 3

1.3 General objective 4

1.3.1 Specific objectives 4

1.4 Hypotheses 4

1.5 Justification 4

CHAPTER TWO 6

LITERATURE REVIEW 6

2.1 Use of botanicals for the control of crop pathogens 6

2.2 *Withania somnifera* 7

2.3 *Hagenia abyssinica* 9

2.4 Bean pathogens 10

2.4.1*Colletotrichum lindemuthianum* 11

2.4.2 *Pythium* spp. 11

2.4.3 *Pseudomonas savastanoi* pv. *phaseolicola* 12

2.4.4 *Xanthonomas axonopodis* pv. *phaseoli* 13

2.5 Maize pathogens 14

2.5.1 *Fusarium moniliforme* 14

2.5.2 *Fusarium graminearum* 14

2.5.3 *Cercospora zea-maydis* 14

CHAPTER THREE **16**
MATERIALS AND METHODS **16**
3.1 Collection and identification of the plants 16
3.2 Distillation of essential oil 16
3.2.1 Analysis of the essential oil by GC-MS 16
3.3 Extraction of the non volatiles 17
3.4 Preparation of culture media 18
3.5 Screening for anti-fungal activity 18
3.6 Screening for antibacterial activity 19
3.6.1 Inoculation of Bacteria 19
3.6.2 Determination of the Minimum Inhibitory Concentration (MIC) 20
3.7 Thin layer chromatography (TLC) 20
3.8 Isolation and purification by Column Chromatography 20
3.9 Nuclear magnetic resonance (NMR) spectroscopy 21
CHAPTER FOUR **22**
RESULTS AND DISCUSSION **22**
4.1 Bioassays of extracts from *W. somnifera* and *H. abyssinica* 22
4.2 Structure elucidation of isolated compounds 26
4.2.1 Structure elucidation of compound 19 26
4.2.2 Structure elucidation of compound 20 31
4.2.3 Structure elucidation of compound 21 32
4.3 Determination of the chemical composition of the essential oil 36
CHAPTER FIVE **42**
CONCLUSIONS AND RECOMMENDATIONS **42**
5.1 Conclusions 42
5.2 Recommendations 42
REFERENCES **44**
APPENDICES **53**

LIST OF TABLES

Table 1: Antifungal activity of extracts from *Withania somnifera* and *Hagenia abyssinica* (100 mg/ml) 25

Table 2: Antibacterial activity of methanol extract and essential oil of *Hagenia abyssinica*.. 25

Table 3: Antibacterial activity of methanol leaves extract of *Withania somnifera* (mm) 26

Table 4: NMR data of compound 19 in $CDCl_3$.. 30

Table 5: NMR data of compound 20 in $CDCl_3$.. 32

Table 6: NMR data of compound 21 in $CDCl_3$.. 35

Table 7: Chemical constituents of essential oil from *Hagenia abyssinica* 37

LIST OF FIGURES

Figure 1: Bioactive compounds from natural sources 7

Figure 2: *Withania somnifera* plant 8

Figure 3: Compounds isolated from the roots and leaves of *W. somnifera* 9

Figure 4: Hagenia abyssinica tree 10

Figure 5: Compounds isolated from Rosaceae family 10

Figure 6: Anthracnose symptoms on different parts of beans 11

Figure 7: Root rot in beans due to *Pythium* spp. 12

Figure 8: Symptoms of halo blight on bean 13

Figure 9: Bean leaf showing common bacterial blight 13

Figure 10: Maize affected by ear rot...14

Figure 11: Leaf of maize showing gray leaf spot 15

Figure 12: A flow chart showing the summary of isolation and analysis of essential oil 17

Figure 13: Flow chart summary of methodology for extraction of non-volatiles..................18

Figure 14: Inhibition of the extracts and essential oil against bacterial pathogens 24

Figure 15: Structure of compound 19 28

Figure 16: Structure of compound 19 showing COSY and HMBC correlations 29

Figure 17: Structure of compound 20 31

Figure 18: Structure of compound 21 33

Figure 19: Structure of compound 21showing COSY and HMBC correlations 36

Figure 20:Structures of major compounds of the essential oil from *Hagenia abyssinica* 39

Figure 21: Mass Spectrum of compound 29 40

Figure 22: Mass spectrum for compound 39 40

Figure 23: Mass spectrum for compound 49 41

LIST OF APPENDICES

Appendix 1: ^{13}C NMR of compound **19** 53
Appendix 2: HSQC spectrum of compound **19** 54
Appendix 3: COSY Spectrum of compound **19** 55
Appendix 4: HMBC Spectrum of compound **19** 56
Appendix 5: ^{13}C NMR of compound **20** 57
Appendix 6: HSQC Spectrum of compound **20** 58
Appendix 7: HMBC Spectrum of compound **20** 59
Appendix 8: COSY Spectrum of compound **20** 60
Appendix 9: DEPT Spectrum of compound **20** 61
Appendix 10: ^{13}C NMR of compound **21** 62
Appendix 11: HSQC Spectrum of compound **21** 63
Appendix 12: COSY Spectrum of compound **21** 64
Appendix 13: HMBC Spectrum of compound **21** 65

LIST OF ABBREVIATIONS

CIMMYT	International Maize and Wheat Improvement Centre
EO	Essential oil
DMSO	DimethylSulfoxide
FAO	Food and Agricultural Organization
GC-MS	Gas chromatography-Mass Spectrometry
GLS	Gray Leaf Spot
MIC	Minimum Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
PDA	Potato Dextrose Agar
TLC	Thin Layer Chromatography

CHAPTER ONE
INTRODUCTION
1.1 Background information

Maize (*Zea mays* L.) is Africa's most important food crop and is grown in a wide range of environments. Demand for maize grain is projected to increase by 50% globally by 2020, including 93% in Sub-Saharan Africa (Pingali and Pandey, 2001; Walters, 2009). Maize is processed to offer various product ranges, which include whole maize meal flour, sifted maize meal, vegetable oil, flour for confectionery, dough, corn flakes, snacks and crackers, starch converted to processed sugars like glucose syrup and dextrose. Maize is a very important staple food in Kenya, grown in almost all agro-ecological zones and on two out of every three farms. It accounts for about 40% of daily calories and has per capita consumption of 90 kilograms; this translates to between 30 and 34 million bags (2.7 to 3.1 million metric tonnes) of annual maize consumption in Kenya (Alakonye *et al.*, 2008). Over 85% of the rural population derives its livelihood from agriculture, most of who engage in maize production. Maize is also important in Kenya's crop production patterns, accounting for roughly 20% of gross farm output from the small-scale farming sector (Jayne and Argwing, 1997).

With the projected increase in maize demand, and the existing shortfall in maize production, diseases still rank highly as maize production constraint. It is estimated that at least 10% of food production is lost to diseases (Strange and Scott, 2005). Any shortage of maize production due to diseases has a major implication on the economy because the government has to use large amounts of the country's foreign exchange to bridge the gap (The East Africa Magazine, July 2012). Maize ear rot and gray leaf spot (GLS) are among the most important maize diseases in Kenya (Alakonye *et al.*, 2008; Macdonald and Chapman, 1997). The major ear rot pathogens belong to the fungi of the genera *Fusarium, Stenocarpella, Aspergillus, Penicillium*, *Nigrospora* and *Macrospora.* Gray leaf spot (GLS) of maize is associated with the fungus *Cercospora zeae-maydis* (Latteral and Rossi, 1983). It was first detected in Kenya in 1995 (Siameto and Okoth, 2011), although it had been recorded in other countries previously (Ward *et al.*, 1999).

Common bean (*Phaseolus vulgaris* L.) is the most important legume in Kenya and is second only to maize in importance as a food crop (Gethi *et al.*, 1997). In Kenya beans are grown mostly as an intercrop with maize (Siameto and Okoth, 2011). It is a major staple in the diet of people of all income categories, especially as a major source of proteins for the poor who

cannot afford meat products, hence the reference as ‘meat of the poor’. The country consumes approximately 450,000 tonnes of beans against a local production level of between 150,000 and 200,000 tonnes harvested from about 800,000 hectares (Nekesa *et al*., 1998) Apart from providing the cheapest source of protein, bean is also the fastest and most reliable means to generate income. Fungal and bacterial infection of beans severely constraints the production in Kenya especially where soil fertility is low and bean farming is intensive (Mwang’ombe *et al*., 2008). Root rot pathogens attack beans at all growth stages and cause damping-off at the seedling stage, yellowing of leaves, stunted growth, and death if severe (Otsyula *et al*., 1998). Crop rotation which contributes to minimization of the fungal infestation in soil is not feasible due to small size of land for many farmers, while fungicides are out of reach to the small scale farmers due to high prices (Siameto and Okoth, 2011). Moreover the pathogens have developed resistance against fungicides and in addition these fungicides pose great challenge to safety of environment for both humans and animals (Nzungize *et al*., 2011). Infestation of these pathogens on bean crop cause direct damage, discoloration, off-odours, taints, off-flavours, reduced yields, and loss of nutritive value (Allen *et al*., 1996).

The major diseases that affect bean production in Kenya are due to the bacterial species *Pseudomonas savastanoi* pv. *phaseolicola* (halo blight), *Xanthomonas axonopodis* pv *phaseoli* (common bacterial blight), *P. syringae* pv. *syringae* (bacterial brown spot), and the fungal *Fusarium* spp. (root rots), *C. lindemuthianum* (anthracnose), *Phaeoisariopsis griseola* (angular leaf spot) and oomycete *Pythium* spp. (root rots). *Xanthomonas axonopodis* pv. *phaseoli* is one of the major constraints to common bean production in the world (Coyne *et al.*, 2003). This pathogen is known to exist epiphytically on maize and therefore has a bearing on intercropping and rotation of maize and beans. The maize plants can act as reservoirs of the pathogen which will affect the bean (Gent *et al*., 2005). The most effective way of controlling these diseases is by use of conventional fungicides and bactericides. But their high cost is not achieved by the poor resource farmers. In addition environmental pollution and residues that lead to carcinogenic risks makes this means of control ineffective (Tasleem *et al*., 2011). It is therefore essential that new bioactive secondary metabolites from natural sources are developed to ensure sustained food production for an increasing world population. The increasing demand for better and cheaper anti-pathogens and at the same time safeguarding of the environment for both humans and animals put emphasis on the importance of more environmentally-conscious research (Tasleem *et al*., 2009).

Natural products have been the most productive source of leads for the discovery and development of drugs over the years. Medicinal plants serves as one of important sources of drugs worldwide since they possess interesting biological properties (Jachak, 2017) Medicinal plants are the “backbone” of traditional medicine, which means more than 3.3 billion people in the less developed countries utilize medicinal plants on a regular basis (Roy *et al.*, 2018). Plants have secondary metabolites called phytochemicals. These compounds protect plants against microbial infections or infestations by pests. Phytochemicals are active ingredients which possess therapeutic properties that are considered as a medicine or drug (Shakya, 2016). Natural products are widely exploited in pharmaceutical industries as a valuable source for lead drugs that have assurance of safety, quality, and efficacy. Medicinal plants and herbal products has now become a key issue in industrialized and in developing countries (Singh, 2015). This research investigated the antimicrobial activities of secondary metabolites isolated from two plants; *W. somnifera* and *H. abyssinica.*

1.2 Statement of the problem

Maize ear rot is an important disease in Kenya and causes yield loses and also contaminates infected grain with mycotoxins. Gray leaf spot is a serious threat to the food security situation in Kenya where maize is a leading staple food for about 90% of the population. *Pythium* root rot and *Colletotrichum lindemuthianum* constitutes a highly damaging constraint on the common bean grown in several areas of Eastern and Central Africa. Common bacterial blight (CBB) caused by *X. axonopodis* pv. *phaseoli* and halo blight (HB) caused by *P. savastanoi* pv. *phaseolicola* are among the most important bean diseases in Africa. These diseases mainly cause significant losses in quality, nutritive value and yield on maize and bean. There are many specific fungicides, which have already proven to be efficient in controlling diseases such as Pythium root rot, but given the economic situation prevailing in Sub-Saharan Africa, poor farmers cannot easily afford these fungicides. Crop rotation which contributes to minimization of the fungal infestation in soil is not feasible due to small size of land for many farmers. Moreover, the large scale use of synthetic fungicides constitute a source of soil and water contamination, while at the same time exposing poorly educated farmers to health risks. Consequently, most of the effective pesticides have been banned leaving the less effective ones in the market. Though farmers are constantly encouraged to use conventional methods of applying fungicides and bactericides, their inappropriate use have been found to possess adverse effects on ecosystems and a possible carcinogenic risk than insecticides and

herbicides. Moreover, resistance by pathogens to fungicides and bactericides has rendered them ineffective. Any threat to maize and bean production is a threat to food security in Kenya, and continuous use of conventional means of controlling these pathogens constitutes a risk in public health.

1.3 General objective

To control maize and bean pathogens using secondary metabolites from *W. somnifera* and *H. abyssinica.*

1.3.1 Specific objectives

1. To screen the crude extracts of *W. somnifera* and *H. abyssinica* for activity against selected maize and bean pathogens.
2. To isolate the bio-active secondary metabolites from *W. somnifera* and *H. abyssinica*
3. To screen the isolated secondary metabolites against selected maize and bean pathogens
4. To determine the structures of the secondary metabolites from *W. somnifera* and *H. abyssinica.*

1.4 Hypotheses

1. The crude extracts from *W. somnifera* and *H. abyssinica* have no activity against maize and bean pathogens.
2. The bio-active secondary metabolites cannot be isolated from *W. somnifera* and *H. abyssinica.*
3. The isolated secondary metabolites have no activity against the selected maize and bean pathogens

1.5 Justification

The control of maize and beans pathogens using secondary metabolites from *W. somnifera* and *H. abyssinica* becomes the best alternative due to the fact that, botanicals are biodegradable and pose no dangers on the environment. Continuously increasing poverty levels and drop in food crop production in Kenya has been attributed mainly to pest and diseases caused by phytopathogens. There are many effective fungicides and bactericides in the market, but given the economic situation of many small-scale farmers, controlling these diseases is beyond their reach because of the high cost of these fungicides and bactericides. Also the possible carcinogenic risks when handling these fungicides and bactericides is of more concern due to their level of education. More so these diseases have developed resistance to the conventional means. Therefore using plant based compounds in crop

management and protection remains the only safe alternative to replace the conventional methods being used. The most prevalent way of achieving this is by screening and evaluating alternative antifungal and antibacterial activities of secondary metabolites from plant-based sources to which development of phytopathogens' resistance has not been reported. In addition, there are millions of plants with different secondary metabolites for which not much research has been done. Moreover the ethno botanical information available validates the local use of these plants. Plant- based compounds do not pose any danger to both humans and animal's environments. This new means of controlling these phytopathogens will offer the best alternative since they will be easy to access; less toxic and their cost will be greatly reduced.

CHAPTER TWO

LITERATURE REVIEW

2.1 Use of botanicals for the control of crop pathogens

Botanicals are extracts from plant origin which are specific agents for the control of fungal infestation and diseases. They may act by inhibiting, repelling or killing the fungus or bacteria causing the diseases (Tasleem *et al*., 2009). Plants contain a variety of secondary metabolites which constitute an important source of pesticides, herbicides, fungicides, bactericides and many pharmaceutical drugs. Plants have an almost limitless ability to synthesize aromatic substances of different functional groups that have been found to have antifungal and antibacterial activities (Tasleem *et al*., 2011). Most of the functional groups are phenols or their oxygen substituted derivatives of which at least 13,000 have been isolated, that is less than 10% of the total. In many cases, these substances serve as plant defence mechanisms against predation by microorganisms, insects, and herbivores (Saidulu *et al.*, 2014).

Secondary metabolites from plant origin for instance flavonoids have broad applicability in management of crop diseases caused by fungal or bacteria pathogens. Flavonoids inhibit a number of root pathogens, especially fungal ones, and in general, isoflavones, flavones and flavanones are acknowledged as efficient anti-microbial agents. Studies on barley mutants showed that proanthocyanidins or even small amounts of dihydroquercetin are involved in the protection against *Fusarium* sp. (Mierziak *et al.*, 2014). Also flavonoids namely 3, 7, 5'-trihydroxy anthocynidines and 3, 5-dihydroxy-7-methoxy anthocynidines isolated from *Monanthotaxis littoralis* (Annonaceae) inhibited the growth of mycotoxigenic fungi from three genera; *Aspergillus*, *Fusarium* and *Penicillium* isolated from maize samples (Clara *et al.*, 2014).

The essential oils of *Cinnamomum cassia, Cinnamomum zeylanicum, Syzygium aromaticum* and *Thymus vulgaris* have been found to inhibit the growth of fungus *Colletotrichum gloeosporioides* a major plant pathogen worldwide known to cause anthracnose in many crop plants (Rabari *et al.*, 2018). The essential oil which was isolated from *Monanthotaxis littoralis* (Annonaceae) inhibited the growth of mycotoxigenic fungi from three genera; *Aspergillus*, *Fusarium* and *Penicillium* isolated from maize sample (Clara *et al.*, 2013).

In recent years, many studies have reported the antifungal and anti bacterial activity of phenolic compounds from natural sources (Tasleem *et al*., 2011; Roser *et al*., 2013). Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Generally the many major antifungal and antibacterial compounds from plants comprise of phenolic compounds which include saponins (**1**), quinones (**2**), flavonoids (**3**) coumarins (**4**), xanthones (**5**), as shown in Figure 1.

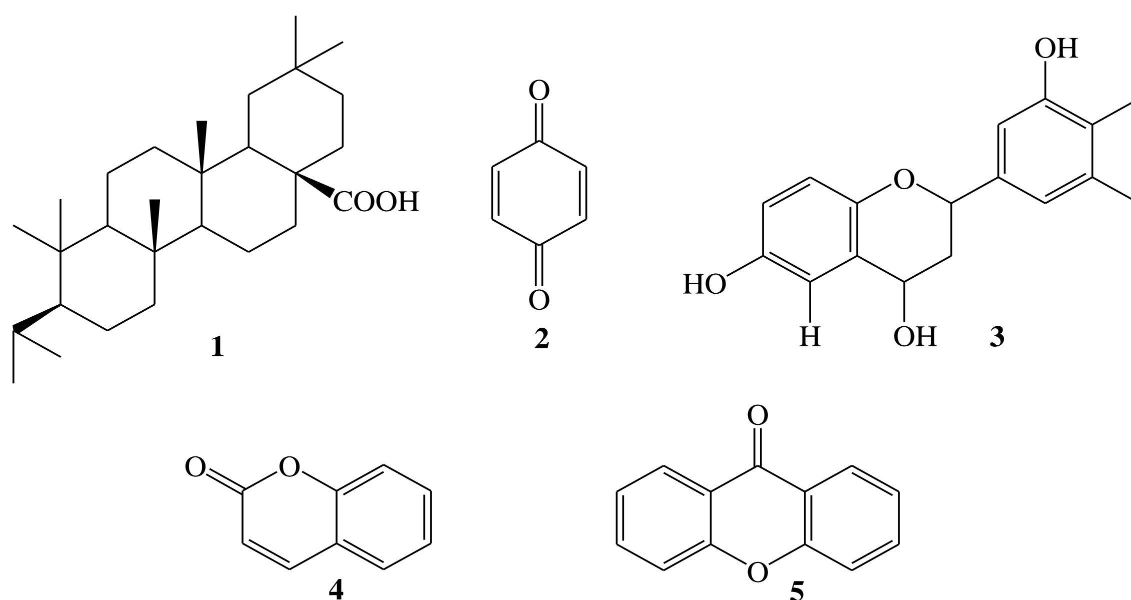

Figure 1: Bioactive compounds from natural sources

2.2 *Withania somnifera*

Withania somnifera (Figure 3), known commonly as ashwagandha in India, Indian ginseng, poison gooseberry, or winter cherry (English), is a plant in the genus Solanaceae or night shade family. This species is a short annual shrub growing 1 to 2 meters tall. Its tomentose branches extend radially from a central stem. The flowers are small and green (Baskaran and Velu, 2012). The ripe fruit is orange-red (Figure 2). *Withania somnifera* is the most common and widespread species in the genus and occurs naturally, mainly in the drier regions, from the Mediterranean through tropical Africa to South Africa and from the Canary and Cape Verde Islands to the Middle East and Arabia, India, Sri Lanka and southern China. It is cultivated in gardens in the warmer parts of Europe and has become a naturalized weed in South Australia and New South Wales. Ashwagandha is grown in India and elsewhere as a medicinal crop plant, mainly for its fleshy roots and is the main herb used in Ayurvedic

medicine (Asha *et al.*, 2012). In Kenya *W. somnifera* occurs naturally in Rift valley especially in Baringo and Marakwet regions and parts of Western Kenya (Kipkorir *et al*., 2014).

Withania somnifera contains more than 80 chemical compounds, mainly alkaloids and steroids (withanolides), (Laxminarain *et al*., 2007. Alkaloids isolated include anaferine **(6)** and somniferine **(7)** which are mainly found in the roots. The leaves contain the steroidal lactones (withanolides), notably withaferine A (**8**) which was the first to be isolated from the leaves (Mirjalili *et al*., 2009). Numerous studies have been published on the activities of these compounds, mostly obtained from the leaves and roots. These studies have demonstrated antibiotic, anti-inflammatory, cytotoxic, anti-tumour and cholesterol-lowering activities (Singh *et al.*, 1986; Kamaljit *et al.*, 2007; Oza *et al.*, 2010; Singh *et al.*, 2010). *Withania somnifera* is an important plant in the traditional medicine of Africa and Asia. Ashwagandha is perhaps the best known of all the plants used in Ayurvedic medicine.

Figure 2: *Withania somnifera* plant

Research has demonstrated narcotic, sedative and diuretic properties and counteracts the symptoms of stress (Singh *et al*, 1982; Bhattacharya and Muruganandam, 2003; Prasanna *et al*., 2009). Ashwagandha (Indian ginseng') like the real ginseng from Chinese medicine (Panax ginseng), is used as a general tonic and considered to be effective against a large number of ailments (Singh *et al.*, 2010). In traditional medicine in southern Africa the leaves are used to heal open as well as septic, inflamed wounds, abscesses, inflammation, haemorrhoids, rheumatism and syphilis; a paste of leaves is applied or ointments are made with fat or oil (Sharma L, 2014). For internal use the dried roots are taken in the form of a decoction, infusion or tincture. Other isolated compounds from the leaves include; withanolide A (**9**), withanolide D (**10**), 27-deoxywithaferineA (**11**) and withanone (**12**)

(Kamaljit *et al.*, 2007). Isolated glycoprotein from W. *somnifera* demonstrated activity against phytopathogenic fungi, *Aspergillus flavus, Fusarium oxysporum, Fusarium verticilloides,* and *Aspergillus niger* (Girish *et al.,* 2006). The most recent report demonstrated that leaf extracts showed activity against *F. oxysporum*, *Colletotrichum capsici,* and *A. niger,* (Premlata *et al.*, 2012; Saidulu *et al.*, 2014).

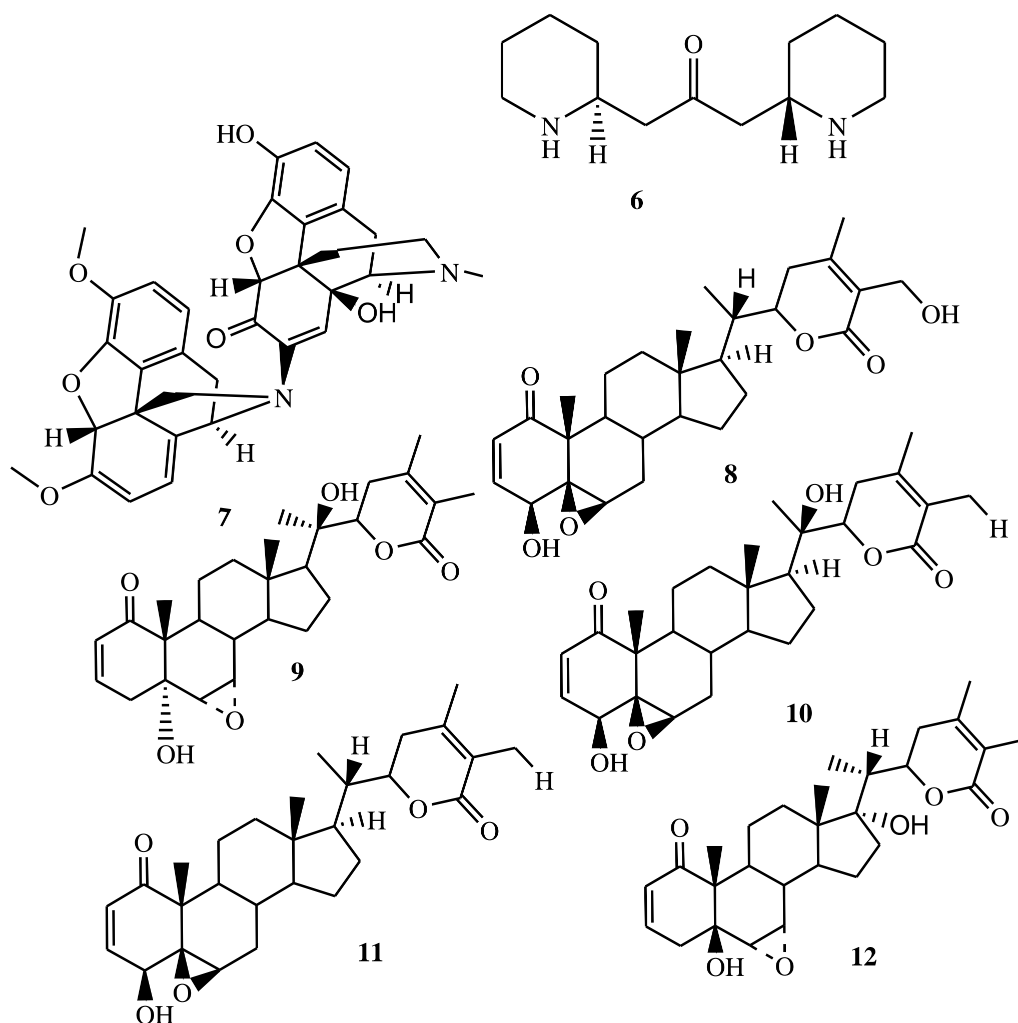

Figure 3: Compounds isolated from the roots and leaves of *W. somnifera*

2.3 *Hagenia abyssinica*

Hagenia abyssinica (Figure 4) belongs to the family Rosaceae. It is indigenous to montane regions of eastern, central and southern Africa, mostly above 2000 m altitude. The species is found in the Democratic Republic of Congo, Sudan and Ethiopia, and south to Malawi, Zambia and Zimbabwe. It is found spread all over East Africa in high altitude areas. It is an important medicinal plant with flowers reported as antihypertensive, taenicidal, anthelminthic, trypanocidal (Belachew, 1995; Nibret and Wink, 2010). The bark has been

reported to be used for treatment of stomach-ache and diarrhea while the roots for the treatment of malaria and general illness (Kokwaro, 1976).

Figure 4: Hagenia abyssinica tree

Phytochemical tests of the leave extracts have shown presence of phenolic compounds, terpenoids and anthraquinones (Figure 3). Studies have also reported presence of phloroquinols (13, 14), (α-kosin, kokotoxin and protokosin) which exhibit antitumor activities (Woldemariam *et al.*, 1992). It has also been reported that leave extracts (hexane and dichloromethane) have antimicrobial activities (Ng'eny *et al.,* 2013). A number of phenolic compounds have been isolated from the Rosaceae family (figure 4). Stigmasterol (15) and β-sitosterol (16) were isolated from *Rubus sauvissmus* (Chaturvedula and Prakash, 2012). Compoud 17 was isolated from *Prunus cerasoides* (Ali and Shaheen, 2013) and compound 18 isolated from *Prunus grayana,* both of rosaceae family.

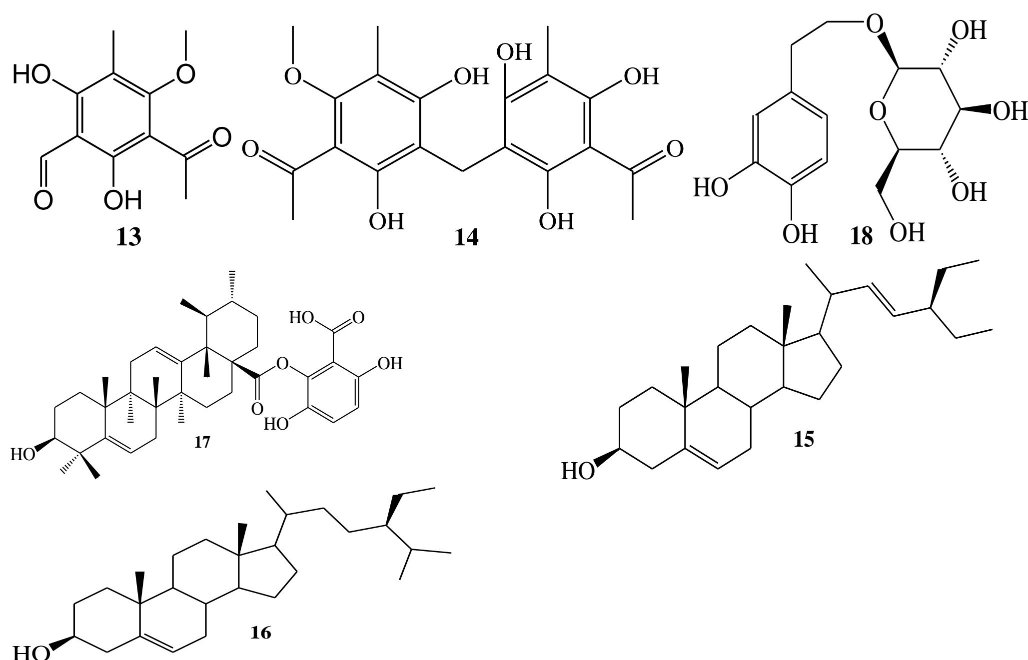

Figure 5: Compounds isolated from Rosaceae family

2.4 Bean pathogens

2.4.1*Colletotrichum lindemuthianum*

Bean anthracnose is caused by the fungal pathogen *Colletotrichum lindemuthianum*. The pathogen causes disease on all classes of dry edible beans and can cause infection on all above-ground parts of dry bean plants (Souza *et al*., 2015). Anthracnose symptoms are most recognizable. Seedlings grown from infected seeds often have dark brown to black sunken lesions on the cotyledons and stems. Severely infected cotyledons senesce prematurely, and growth of the plants is stunted diseased areas may girdle the stem and kill the seedling (Tavernier *et al*., 2007). Under moist conditions, small, pink masses of spores are produced in the lesions. Spores produced on cotyledon and stem lesions may spread to the leaves (Geffroy *et al*., 2000). Symptoms generally occur on the underside of the leaves as linear, dark brick-red to black lesions on the leaf veins. As the disease progresses, the discoloration appears on the upper leaf surface. Leaf symptoms often are not obvious and may be overlooked when examining bean fields (Sicard *et al*., 1997). The most striking symptoms develop on the pods. Small, reddish brown to black blemishes and distinct circular, reddish brown lesions are typical symptoms. Mature lesions are surrounded by a circular, reddish brown to black border with a greyish black interior. During moist periods, the interior of the lesion may exude pink masses of spores. Severely infected pods may shrivel, and the seeds they carry are usually infected. Infected seeds have brown to black blemishes and sunken lesions (Dillard, 1988; Souza et al., 2015) (Figure **6**).

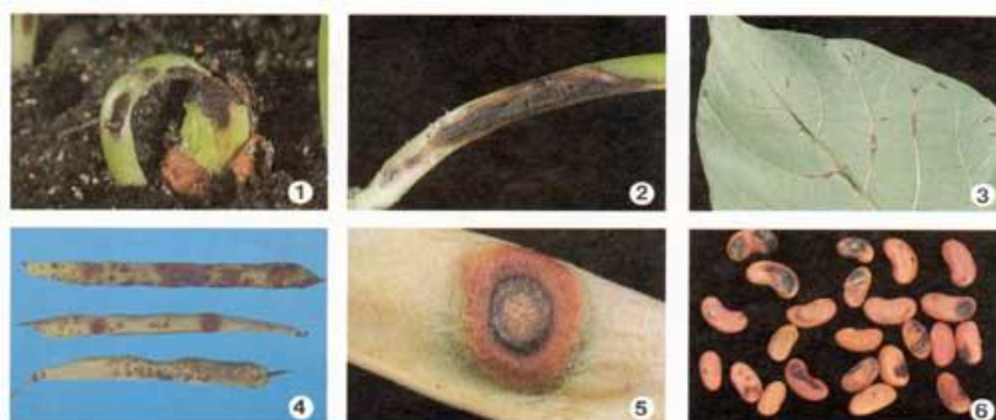

Figure 6: Anthracnose symptoms on different parts of beans

2.4.2 *Pythium* spp.

Pythium spp. usually cause pre-emergence rot and in some cases damping-off of the young seedlings. Symptoms include water-soaked lesions with eventual collapse of the hypocotyls

at or below ground (Lodhi *et al.,* 2013) (Figure **7**). Occasionally, older plants are infected and develop water-soaked lesions that extend some distance up the stem, causing a linear band of dead cortical tissue (Zirillo *et al*., 2013). *Pythium* root rot constitutes a highly damaging constraint on the common bean grown in several areas of Eastern and Central Africa (Nzungize *et al*., 2012). Yield losses of up to 70% in traditional local bean cultivars have been reported in Kenya (Otsyula *et al*., 1998). *Pythium* is a complex genus containing over 200 described species with a broad host range and occupying a variety of terrestrial and aquatic ecological habitats (Nzungize *et al*., 2011).

Figure 7: Root rot in beans due to *Pythium* spp.

2.4.3 *Pseudomonas savastanoi* pv. *phaseolicola*

Halo blight, caused by *Pseudomonas savastanoi* pv. *Phaseolicola* is an important bacterial disease on common bean. It is a pathogen that is present in all countries where bean is cultivated, (Audy *et al*., 1996). Typical symptoms of the disease can be detected over all the aerial parts of the bean plant. On cotyledons, the lesions are water soaked and round to irregular in shape; on leaves, they are yellowish/reddish brown, equally water soaked, with a large light green halo, and there may be curling and deformation of leaflets; on stems, lesions sometimes crack under the weight of pods and may cause bacterial ooze; and, on pods, the symptoms remain longer than on leaves, and seeds may be infected through their vascular system (Marques and Regine, 2016; Yan *et al*., 2016), (Figure 8).

Figure 8: Symptoms of halo blight on bean

2.4.4 *Xanthonomas axonopodis* pv. *phaseoli*

Common bacterial blight of beans is caused by *Xanthomonas axonopodis* pv. *phaseoli (Xanthomonas campestris* pv.*phaseoli), (*Zannata et al., 2007*).* Commom blight is a serious disease in many important snap and dry beans-producing regions of the world (Mahuku *et al*., 2006). Estimate losses due to this disease range from 10% to 45% (Darrase *et al*., 2007) Leaves first develop water soaked spots that then enlarge into initially flaccid but then brown and necrotic lesions with lemon-yellow margins (Figure 9). It then leads to extensive tissue damage and finally to defoliation. *Xanthomonas oxonopodis* pv *phaseoli* is seedborne, and can survive in the seed for over 15 years. As seed germinate the bacterial contaminate the surface of the expanding cotyledon and spread to the leaves via natural openings and wounds and eventually even to the vascular system. The bacteria can be spread to the other parts of the plant by wind-driven rains or hail, insects, farm implements or humans. Localised lesions on pods and systemic invasion of pods lead to external and internal seed contamination (Hagedorn and Inglis, 1986).

Figure 9: Bean leaf showing common bacterial blight

2.5 Maize pathogens

The major fungal species which affect maize crops in the field are*; F. graminearum, Cercospora zea-maydis* and *F. moniliforme*. Most of the pathogenic species are either soil-borne or seed-borne (Loren *et al.*, 2012). Each of these causes different diseases on the leaves, stalk and roots. Fungal infection in maize results in mycotoxins contamination, affects the quality, nutritive value, and the production yields (Ahmad *et al.*, 2014).

2.5.1 *Fusarium moniliforme*

Fusarium moniliforme is the causal agent of ear rot and stalk rot diseases of the maize crop. *Fusarium* ear rot is a common disease in hail-damaged corn. It can infect any part of the ear and penetrates into wounds created by insects or hail. It mainly causes significant losses in quality, nutritive value and yield (Ahmad *et al.*, 2014). It also contaminates grain with mycotoxins (Agustine *et al*., 2007). Environmental factors such as temperature, humidity and pH have great influence on the fungal development (Ramesh *et al.*, 2014).

2.5.2 *Fusarium graminearum*

Fusarium graminearum (also known as *Gibberella zeae*) is the causal agent of Gibberella ear rot, seedling blight, root and stalk rot of maize and *Fusarium* head blight of small grain crops (Figure 10). A large number of cereals are affected by this pathogen, including wheat, barley, rye, sorghum and maize. Other factors contributing to infection are optimum climatic conditions and susceptible cultivars. *Fusarium graminearum* affect growth, quality and the development of the infected maize crops (CIMMYT, 2004) and is a global threat to maize production.

Figure 10: Maize affected by ear rot

2.5.3 *Cercospora zea-maydis*

Cercospora zea-maydis causes Gray leaf spot (GLS) (Figure 11), which is a foliar fungal disease that affects maize. Symptoms seen on corn include leaf lesions, discoloration

(chlorosis), and foliar blight. The fungus survives in debris of topsoil and infects healthy crop via asexual spores called conidia. Environmental conditions that best suit infection and growth include moist, humid, and warm climates; poor airflow, low sunlight; overcrowding, improper soil nutrient and irrigation management, and poor soil drainage can contribute to the propagation of the disease. Major outbreaks of gray leaf spot occur whenever favourable weather conditions are present. The initial symptoms of GLS emerge as small, dark, moist spots that are encircled by a thin, yellow radiance (lesions forming) (CIMMYT, 2004).

Figure 11: Leaf of maize showing gray leaf spot

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection and identification of the plants

The leaves of *W. somnifera* and *H. abyssinica* were collected at the Egerton University botanical garden alongside with a taxonomist from Biological Sciences Department, who identified the plants. A voucher specimen was taken and deposited in the herbarium at the Biological department. The *W. somnifera* and *H. abyssinica* leaves were divided into two, one batch of the leaves was taken to chemistry laboratories where they were dried indoors at room temperatures (25°C) for three weeks. The other batch of the fresh leaves was taken to Biotechnology laboratory for extraction of essential oil.

3.2 Distillation of essential oil

The fresh leaves of *H. abyssinica* were subjected to hydro distillation in a modified Clevenger apparatus to extract essential oils. The leaves were cut into small pieces. Approximately 1000g was put in a 3litre distillation flask and boiled with 1000 ml of water in a modified Clevenger apparatus until no more oils formed (after 4-6 hrs). This process was repeated severally. The essential oil collected was dried using anhydrous Na_2SO_4 and refrigerated at 4°C. The leaves of *W. somnifera* did not produce any oil after subjecting them to the hydro distillation for more than six hours several times. A summary flow chart for the oil analysis is shown in Figure 12.

3.2.1 Analysis of the essential oil by GC-MS

The essential oil was analysed by use of an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS ('Restek') (30 m x 0.25 mm, 0.25 µm film thickness) fused-silica capillary column. The carrier gas used was Helium (at 0.8 ml/min).The column was maintained at 70°C for 2 min and then programmed to 240°C at 4°C/min. The pressure was maintained at 11.05 Psi. The essential oil was diluted in methyl-t-butyl ether (MTBE) (1:100). Sample was injected in the split mode at a ratio 50:1; the syringe size was 10µl and the injection volume was 1µl. The total flow rate was 64.2 ml/min.

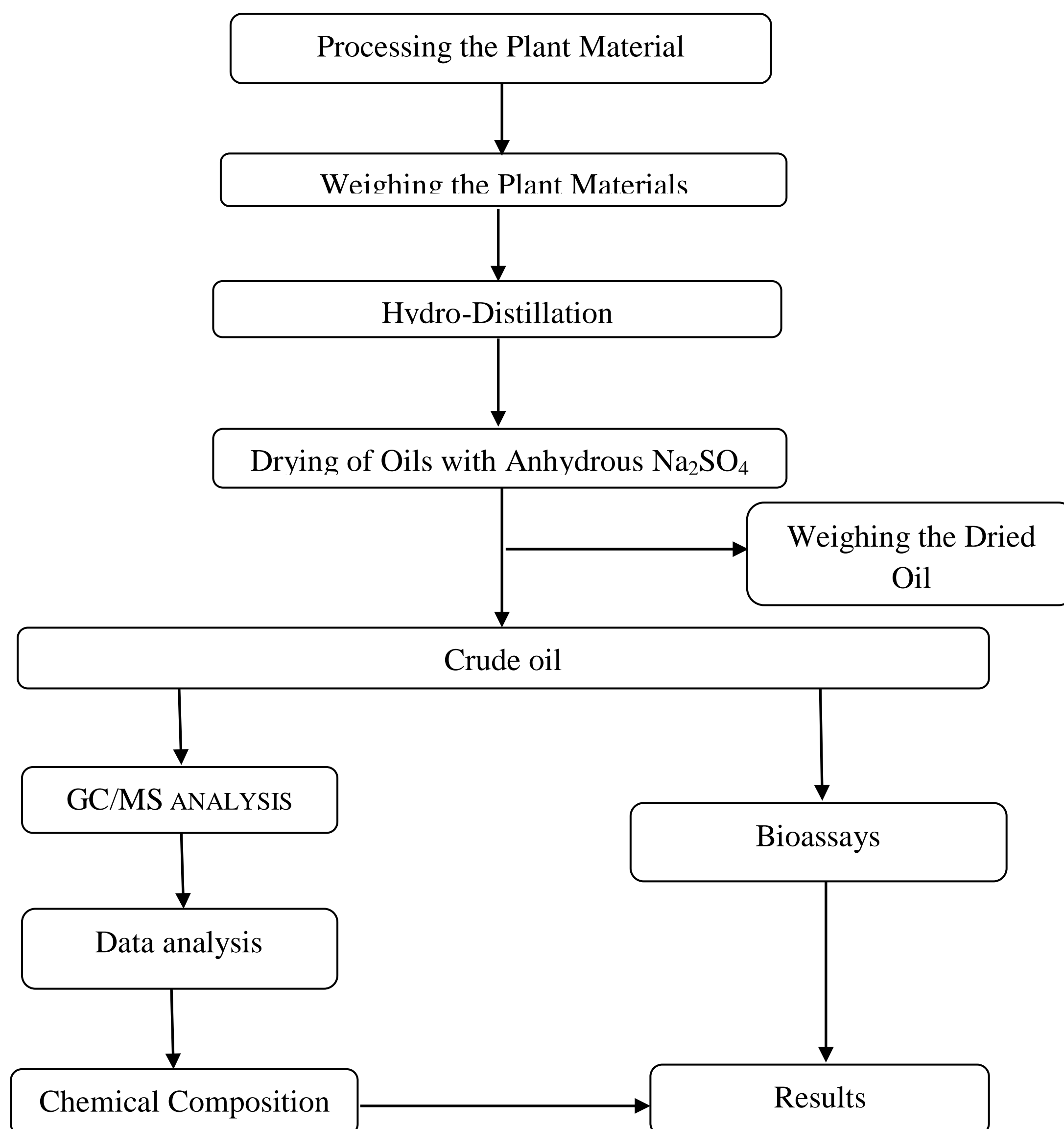

Figure 12: A flow chart showing the summary of isolation and analysis of essential oil

3.3 Extraction of the non volatiles

The dried leaves were ground into fine powder using a grinding mill (Thomas-Wiley mill model 4). Then about 500g of each sample was weighed and put in separate two litre Winchester bottles. The ground samples were soaked with about 700mls of methanol for 72 h after which they were decanted and chlorophyll was first removed with five spoonfuls of activated charcoal. Filtration was done using Whatman no. 1 filter paper. The filtrates were then evaporated under vacuum at 40°C. The concentrates were put in sample bottles and left open in the fume chamber to evaporate any traces of solvent. A summary of the procedure is indicated in the flow chart in figure 13

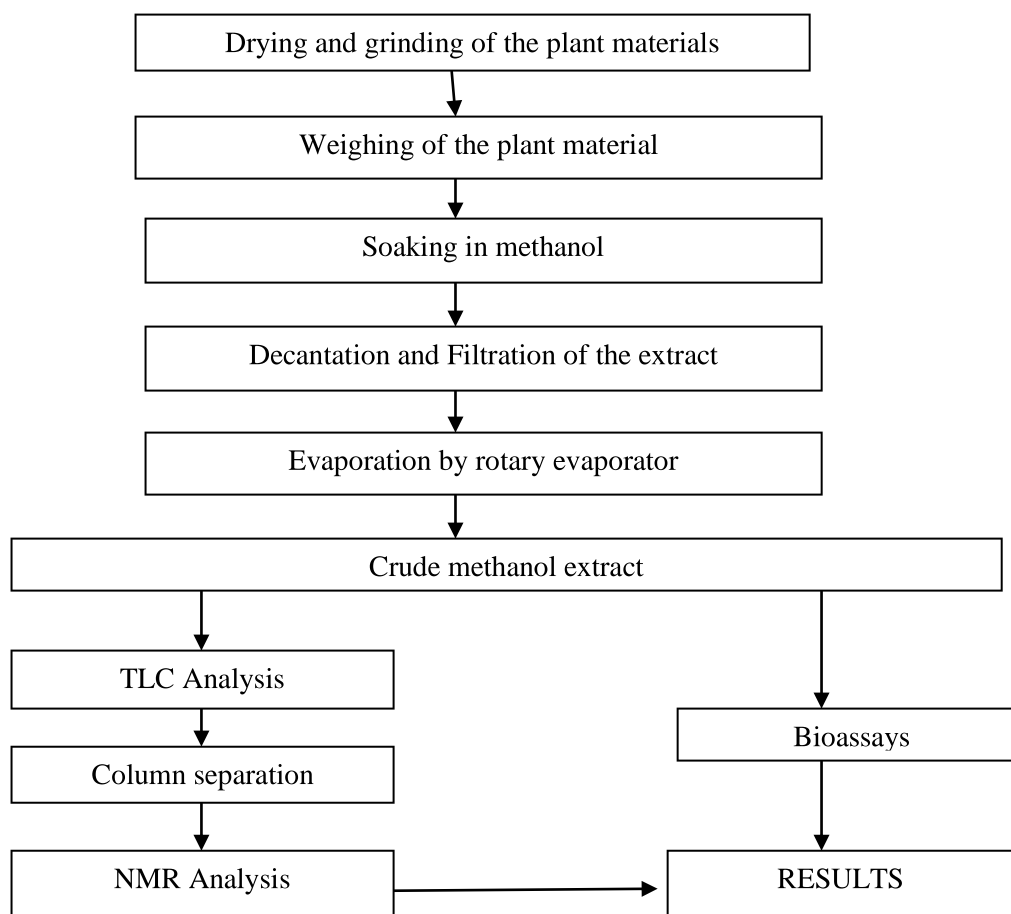

Figure 13: Flow chart summary of methodology for extraction of non-volatiles

3.4 Preparation of culture media

To prepare the Mueller Hinton plates, 21g of Mueller Hinton Agar powder was weighed and suspended in one litre of distilled water. It was then heated till boiling to completely dissolve the powder and finally autoclaved at 121°C for 15 minutes. After cooling, about 20ml was poured into plastic petridishes and left to cool.

To prepare the Potato Dextrose Agar (PDA), 39g of the PDA powder was weighed and suspended in one litre of distilled water. It was heated to boiling till the media was completely dissolved. It was then sterilized by autoclaving at 121°C for 15 minutes, then left to cool. The sterilized and cooled media was then mixed well and dispensed in plastic petridishes.

3.5 Screening for anti-fungal activity

Both the crude extracts and the purified compounds were screened for antifungal activities using the paper disc diffusion method as described by Premlata *et al*, (2012). Fungal

pathogens were obtained from Biotechnology Laboratory of Egerton University and then sub cultured. The subculture was done by cutting a small block of agar containing mycelium using a sterilized needle. The block was then transferred into a container containing 5 ml of distilled sterilized water. It was then crushed using a sterilized glass rod to make a fungal spore suspension. One millilitre of fungal suspension (approximately 10^6 spores) was uniformly spread on sterile PDA media in Petri dishes using a cotton swab. From the dried methanol extracts 100mg was weighed and dissolved in 1ml of DMSO to make a concentration of 100mg/ml. Sterilized 6mm paper discs (obtained by punching whatman no.1 filter paper) were soaked in the prepared concentration and placed at the centre of the inoculated culture plates. The plates were incubated at 25°C. The inhibition zones were clearly visible after 72 h of incubation and measurement of the diameters of inhibition zones was done. Nystatin was used as a reference standard (positive control) and DMSO as the negative control. The most bioactive extracts were determined by the size of the inhibition zones. The bigger the inhibition zone the more active the compound and vice versa (Yao and Livermore, 1994). The fungal pathogens that were used included; *F. moniliforme, F. graminearum, P. ultimum,* and *C. lindemuthianum.* The experiments were done in triplicate for each pathogen. The Means and the standard deviations were calculated. The comparison for the standard deviations of the extracts and that of reference standard (Nystatin) was done using F-test.

3.6 Screening for antibacterial activity

Disc diffusion method was used to test antibacterial activity of the methanolic crude extracts and essential oils. Two bacterial pathogens, *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola* were used. The pathogens were obtained from Biological Sciences Laboratories and then sub cultured. The subculture was done by touching the fully developed morphologically similar colonies with a sterile loop and transferring to nutrient broth. Then the nutrient broth was incubated at 37°C for 24 h to obtain the bacterial suspension.

3.6.1 Inoculation of Bacteria

To the prepared Mueller Hinton agar plates, 10 µl of the bacterial suspension was spread using sterilized cotton swabs. Sterilized paper discs (6 mm) obtained by punching whatman no. 1 filter paper were pre-soaked with 10µl of the extracts and placed at the centre of the inoculated plates. The plates were incubated at 37°C for 24 h. For positive control, standard Chloramphenicol discs (30µg) were used. For the negative control, 6mm paper discs soaked

in DMSO were used. All the experiments were done in triplicates. The zones of inhibition (clearance of bacterial culture around the discs) were measured in millimetres using a ruler (Wahi *et al*., 2011).

3.6.2 Determination of the Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the minimum concentration of a product that can inhibit the visible growth of a microorganism. The MIC for the extract was determined by dilution of the extract using a double fold serial dilution following the method used by Agbabiaka and Sule, (2008). The initial concentration of 200mg/ml (stock solution) was prepared by measuring 400mg of the methanol extract and dissolving in 2ml of DMSO. By diluting 1ml of 200mg/ml (stock solution) to 2ml using DMSO, 100mg/ml concentration was obtained. From 100mg/ml (stock solution), 1ml was diluted to 2ml to make a concentration of 50mg/ml. The above process was repeated several times to obtain the other concentrations of 25, 12.5, 6.25, and 3.15 mg/ml. Then 6 mm diameter sterile discs were impregnated with 10 μl of the different concentrations and placed at the centre of the inoculated culture plates. After allowing the sample to diffuse for five minutes, the plates were incubated at 37°C for 24 h. The MIC experiments were done in triplicates and the average measurements taken.

3.7 Thin layer chromatography (TLC)

The crude extracts were first subjected to analytical TLC, which was performed on silica gel GF 254nm (Merck, Germany) 0.25mm thickness. The dry extracts were reconstituted in the solvents to make up a final concentration of about 10mg/cm^3. To ensure homogeneity all extracts were thoroughly mixed. Preliminary analysis was performed to identify optimum solvent systems for use as mobile phases. Ethyl acetate, hexane and diethyl ether solvent mixtures were tried and modified accordingly to give optimum separation for all extracts. The solvent mixtures that were giving optimum separations were 7:3 ethyl acetate-hexane mixtures for *W. Somnifera*. For *H. abyssinica* the optimum solvent mixtures were found to be 7:3 ethyl acetate: hexane, and 7:3 diethyl ether hexane mixtures. Visualization of spots was done by illumination under UV lamp (Uvitec-LF-204.LS) at 254 nm and 365nm.

3.8 Isolation and purification by Column Chromatography

The column was packed by slurry method using silica gel 60 0.6-0.2mm (70-230 mesh ASTM) supplied by Indo-lab Suppliers. Approximately 2g of the dry crude extracts was weighed and mixed with 2g of silica gel. Using a pestle and motor the extracts together with silica gel were ground into fine powder. Meanwhile silica gel was soaked in hexane and

thoroughly stirred until no air bubbles were seen. The slurry was then carefully introduced into the column as tapping done regularly to ensure uniform packing. Hexane was passed through the column for a while till the packing was accomplished. The finely ground sample was then introduced into the column and eluted with the solvent system established through TLC analysis (7:3 ethyl acetate, hexane for *W. somnifera*). The eluates were collected in approximately 20cm^3 test-tubes. Fractions of equal volumes were collected and TLC analysis of each fraction was done. Fractions with the same TLC patterns were pooled together and concentrated. The fractions that contained more than two compounds were pooled together, concentrated and smaller columns set until separation was achieved. For *H. abyssinica* the column was first run with 7:3 ethyl acetate: hexane and further purification was done using 7:3 diethylether: hexane. After extensive column purification, two compounds were isolated from *W. somnifera*, and were coded; **WS1** and **WSD.** One compound coded **Ha1** was isolated from *H. abyssinica***.** NMR analyses were done on the compounds and their structures elucidated.

3.9 Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}HNMR, ^{13}C NMR, HSQC, COSY and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrometer. All the readings were done in Deuterated chloroform and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) was used as an internal standard. Chemical shifts were given as δ (ppm). The structures were analysed using ACD NMR manager program to obtain the chemical shifts of proton.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Bioassays of extracts from *W. somnifera* and *H. abyssinica*

Tables 1, 2 and 3 below show antifungal and antibacterial activities of methanol extracts of *W. somnifera* and *H. abyssica* determined after incubating for 72h (antifungal) and 24h for antibacterial (Mahesh and Satish, 2008; Premlata *et al*., 2012). The highest inhibition was observed for both extracts against *C. lindemuthianum*. The negative control (DMSO) did not show any inhibition. Figure 14 shows, the inhibition zones of the extracts from *W. somnifera and H. abyssinica* (A) against the fungal pathogens**.** Figure 14 (B) is the inhibition of methanolic extracts of *H. abyssinica* (1), *W. somnifera* (2) and the essential oils (3) against the bacterial pathogens. The results in Table 1 were observed after 72 h of incubation, and there after the inhibition zones were diminishing. *Withania somnifera* was more active on *C. lindemuthianum* (12 mm) followed by *F. graminearum* (11.5 mm) in contrast with the reference standard with 10.6 mm and 29.6 mm respectively. *Hagenia abyssinica* registered inhibition zones of 10 mm and 15 mm against *F. graminearum* and C. *lindemuthianum* respectively. Methanolic extract from *H. abyssinica* did not show any activity against *F. moniliforme*.

The methanolic extract of *W. somnifera* registered an inhibition zone of 8.0 mm and 7.3 mm against *F. moniliforme* and *Pythium* spp. respectively, while the reference compound registered an inhibition zone of 21 mm and 13 mm, respectively. *Hagenia abyssinica* had an inhibition of 9.0 mm against the *Pythium* spp. The extracts from the two plants showed a greater inhibition zone than the standard, Nystatin (10.6 mm) on *C. lindemuthianum*. The results of the antifungal effects of the methanolic extracts from *W. somnifera* and *H. abyssinica* (Table 1) show that the extracts are effective against most of the tested fungal pathogens. These results confirm reports by Tasleem *et al*, (2011) and Roser *et al* (2013) that plants are potent source for antifungal compounds. The methanolic extract of the two plants were more active against the fungal pathogen *C. lindemuthianum* than the reference standard Nystatin. This implies that further research on these plants could lead to development of new and better antifungal agents. The observed results also support the reports by Mahesh and Satish, (2008), Ng'eny *et al*, (2013) and Saidulu *et al*, (2014) that the extracts from *W. somnifera* and *H. abyssinica* can inhibit the growth of fungal and bacterial pathogens. *Withania somnifera* extract has been reported to be active against human fungal pathogens, (Hardeep *et al*., 2013; Javaid and Akhtar, 2015; Nefzi *et al*., 2016). Compound **19** and **20**

isolated from *W. somnifera* did not show any activity against the pathogens. This implies that, either the concentration used was too low or the activity of the extract was due to synergistic effect or there are other active compounds in the extract which were not isolated. Compound **21** could not be tested because of the small amount that was isolated.

In Table 2 and 3, it is evident that methanolic extracts of *W. somnifera* and *H. abyssinica* could be potent bioactive compounds for the control of the bacterial pathogens. The activity of the extracts increased with increasing concentration. The inhibition zones were visible even after 72 h of incubation and this lead to the determination of the Minimum Inhibitory Concentrations (MIC). The results show that methanol extract from the leaves of *H. abyssinica* (Table 2) is more active against the two pathogens as compared to the extracts from the leaves of *W. somnifera* (Table 3). At 200mg/ml *H. abyssinica* methanol extract registered inhibition zones of 15mm and 14.3mm against *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli* respectively**.** The MIC of extract from *H. abyssinica* was 3.125 and 12.25mg/ml against *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli* respectively while the MIC for *W. somnifera* was found to be 12.5mg/ml and 25mg/ml, respectively . The knowledge of MIC in drug development helps in quantification of the sensitivity of a certain bacteria strain to an antibacterial agent, or is a standard method of determining the susceptibility of organisms to antimicrobials (Andrews 2001) A lower MIC value indicates that less drug is required for inhibiting growth of the organism; therefore, drugs with lower MIC scores are more effective antimicrobial agents and are capable of preventing evolution of drug-resistant microbial strains (Piotrowki *et al*., 2015).

These results show that further research on methanol extracts of *H. abyssinica* and *W. somnifera* could lead to potent bioactive compounds for the control of these pathogens. These results also confirm report by Kulshrestha *et al*, (2015) that *W. somnifera* methanol extract can inhibit *X. axanopodis* pv. *phaseoli*. Reports by Saraj *et al*, (2013) and Bashir *et al*, (2013) shows that *W. somnifera* roots and leaves extracts (methanolic), can inhibit the growth of human bacterial pathogens. *Hagenia abyssinica* is said to contain phenolic compounds, phloroglucinols or kosins which have been reported to posses' activity on microorganisms (Woldemarian, 1992). The triterpenoids isolated from Rosaceae family have also been reported to posses' activity on microorganisms (Chu-Hung *et al.,* 2013). In this study a pentacyclic triterpenoid was isolated from the methanolic extract of *H. abyssinica,* and thus the observed activity could be attributed to presence of such compounds in the extract.

Hagenia abyssinica has been reported to posses' antihypertensive, antibacterial as well as antihelmithic property. It has been tested for acute toxicity and was found to be safe at 5000 mg/kg body weight per day (Belachew, 1995; Ng'eny *et al*., 2013; Karumi *et al*., 2013). *Withania somnifera* is also safe for use by humans (Mizra et *al*., 2000)**.**

The essential oil from *H. abyssinica* exerted inhibition against the bacterial pathogens to the same extent with Chloramphenicol. The GC-MS analysis showed that the oil was majorly constituted of monoterpenes (33.22%) and sesquiterpenes (53.43%). The major compounds were endobornyl acetate, ɗ-gurjunene, aromadendrene and palustrol which had concentrations of 19.67%, 13.55%, 11.27% and 6.22% respectively. The antibacterial property could be attributed to these main constituents, although the possibility of other compounds present in low concentrations contributing to the antibacterial properties cannot be ruled out.

Therefore from the obtained results, secondary metabolites from of *W. somnifera,* and *H. abyssinica* can be potent candidates for the search of cheap, readily available, biodegradable, less toxic and environmentaly friendly bioactive compouds from natural sources (Tasleem *et al.*, 2011). These results also justify the traditional use of the plants for treatment of various diseases.

Figure 14: Inhibition of the extracts and essential oil against bacterial pathogens

A- Methanolic crude extract, **B**- essential oil (3) compared to the crude extracts of *H. abyssinica (1)* and *W. somnifera* (2) zones of inhibition

Table 1: Antifungal activity of extracts from *Withania somnifera* and *Hagenia abyssinica* (100mg/ml)

	Fungal Pathogens			
Sample	***F.G***	***FM***	***P.U***	***C.L***
W. somnifera	11.3±0.6	8±0.0	7.3 ±0.6	12±1.0
H. abyssinica	10±1.0	0±0.0	9±0.1	15±1.0
Compound 19	0±0.0	0±0.0	0±0.0	0±0.0
Compound 20	0±0.0	00±0.0	0±0.0	0±0.0
Nystatin	29.7±0.6	21±0.0	12.5±0.5	10.6±0.6
DMSO	0 ± 0.0	0±0.0	0± 0.0	0± 0.0

Key: FG-*Fusarium graminearum*, **FM**- *Fusarium moniliforme*, **PU**- *Pythium ultimum*, **CL**- C*olletotrichum lindemuthianum*.

The F- test was used to analyse the results at α=0.05 significant level. ($S^2_{larger}/S^2_{smaller}=F$), where S= standard deviation. The F- table for α=0.05, was used. The standard deviation of the standard (Nystatin) was compared with that of the extract. The results showed that there was no significant difference between the activity of the extracts and that of the Standard, since all the calculated values were less than the values on the F- table.

Table 2: Antibacterial activity of methanol extract and essential oil of *Hagenia abyssinica.*

Concentrations mg/ml	*P. savastanoi* pv. *phaseolicola*	*X. axonopodis* pv. *phaseoli*
	Mean ±SD	**Mean ±SD**
200	15±1.0	14.3±0.6
100	14±1.0	11.7±0.6
50	12.7±0.6	11.3±0.6
25	11.3±0.6	9.7±0.6
12.5	9.3 ±0.6	8±1.0
6.25	8±0.0	0±0.0
3.125	7±0.0	0±0.0
H. abyssica oil	30±0	30±0.0
Chloramphenicol	30±0.0	30±0.0

The Pearson's rank correlation was applied; H_0: There was no significant relationship between concentration and activity. H_1: There was a significant relationship between concentration and activity. The test was carried out at α = 0.05 significant level. If p (value) ≥ 0.05, H_0 was accepted; if p≤ 0.05; H_1 was accepted.

Table 3: Antibacterial activity of methanol leaves extract of *Withania somnifera* (mm)

Concentrations (mg/ml)	***P. savastanoi pv. phaseolicola***	***X. axonopodis pv.Phaseoli***
	Mean±SD	Mean±SD
200	13±1.0	13±1.0
100	8± 1.0	12±1.0
50	7±0.0	7±0.0
25	7±0.0	7±0.0
12.5	7±0.0	0±0.0
6.25	0±0.0	0±0.0
3.125	*0±0.0*	*0±0.0*
Chloramphenicol	30±0.0	30±0.0
DMSO	0±0.0	0±0.0

According to the statistics done, there was a significant difference between concentration and activity for *H. abyssinica* methanolic extract against bacteria pathogens *P. savastanoi* pv. *phaseolicola* but there was no significant difference against *X. axonopodis* pv. *phaseoli* with correlation coefficient (P) of 0.015 and 0.055 respectively. For *W. somnifera* (Table 3), there was a significant difference between concentration and activity against the test pathogens, *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli* with coefficient correlation of 0.024 and 0.005 respectively.

4.2 Structure elucidation of isolated compounds

Three compounds were isolated, two from *W. somnifera (***19** and **20***)* whose structures have steroidal lactones skeleton and one pentacyclic triterpenoid from *H. abyssinica* (**21**)**.** Their structures are explained in details below.

4.2.1 Structure elucidation of compound 19

Compound **19** (Figure 15) was obtained as a crystalline solid after running the column on crude methanol extract using ethyl acetate and hexane in the ratio 7:3 respectively. The molecular mass was found to be 470.6028 which corresponded to the molecular formula $C_{28}H_{38}O_6$. The ^{1}HNMR spectrum revealed presence of five methyl signals of withanolides at δ= 0.89 (s), 1.21 (s), 1.07(d, J=7.0 Hz), 1.91 (s), 1.97 (s) for H-18, H-19, H-21, H-27 and H-28, respectively (table 4).

The ^{13}C NMR spectra (Appendix 1) of compound **19** supported the presence of 28 carbon resonances. Which include five methyl, six methylene, nine methyne and eight quaternary carbon atoms. Downfield signals at δ=203.1(s) for C-1; 129.0, d for C-2; 139.7, d for C-3

were indicative of α, β-unsaturated ketone. The signals for E ring (α, β-unsaturated lactone at δ=167.2 for C-26; δ=78.5 for C-22; δ=150.5 for C-24 and δ=121.3 for C-25) imply that C-26 and C-22 have oxidized to form the usual E ring of a withasteroid. The singlet at δ=73.1 for C-5 along with the doublets at δ=56.3 for C-6 and δ=57.2 for C-7 corresponded to the hydroxyl and epoxide groups, respectively, matching with the withanone, (Payere *et al*., 2006). ^{13}C NMR was also indicative of the presence of another hydroxyl at the quaternary carbon C-17 since the 14α- as well as14β-hydroxyls influence the chemical shift of H-18 downfield. Further correlations through HSQC spectrum (Appendix 2) were observed between carbon atoms with the protons directly attached to them. From the spectrum there were HSQC correlations between C-2 and a proton absorbing at δ5.88 while C-3 was attached to a proton resonating at δ 6.62. Carbon-4 was observed to have HSQC correlation with proton resonating at δ 2.55 while C-6 and C-7 had HSQC relation with protons absorbing at δ 3.08 and δ 3.35 respectively. The protons resonating at δ1.81, δ 1.58, δ 2.86 and δ 2.50 correlated with C-8, C-9, C-11, and C-12 respectively in their HSQC. There was a HSQC correlation between protons absorbing at δ 2.05 δ 1.95, and 2.58 with C-14, C-15 and C-16 respectively. Also, C-20 and C-21 correlated with proton at δ2.36 and δ 1.07 while C-22 correlated with a proton at δ4.64 in their HSQC. Also C-23 correlated with proton at δ1.67 while C-27 and C-28 correlated with protons resonating at δ1.91 and δ 1.07 respectively (HSQC).

The proton-proton COSY correlation for compound **19** was determined. COSY spectrum gave information on protons which are attached to adjacent carbons. From the COSY spectrum (Appendix 3), the proton H-2 absorbing at δ 5.88 showed correlations with proton at carbon 3 (δ 6.62), also H-3 absorbing at δ 6.62 showed correlation with proton at carbon 4 (δ 2.55). Proton 6 (δ 3.08) had only one cosy correlation with proton 7 (δ3.35) but 6 and 7 showed cosy correlation with each other. Proton 7 showed two cosy correlations, with 6 (δ 3.08) and 8 (δ 1.81). Furthermore proton 20 (δ 2.36) showed two cosy correlations with proton 21 (δ 1.07) and proton 22 (δ 4.64).

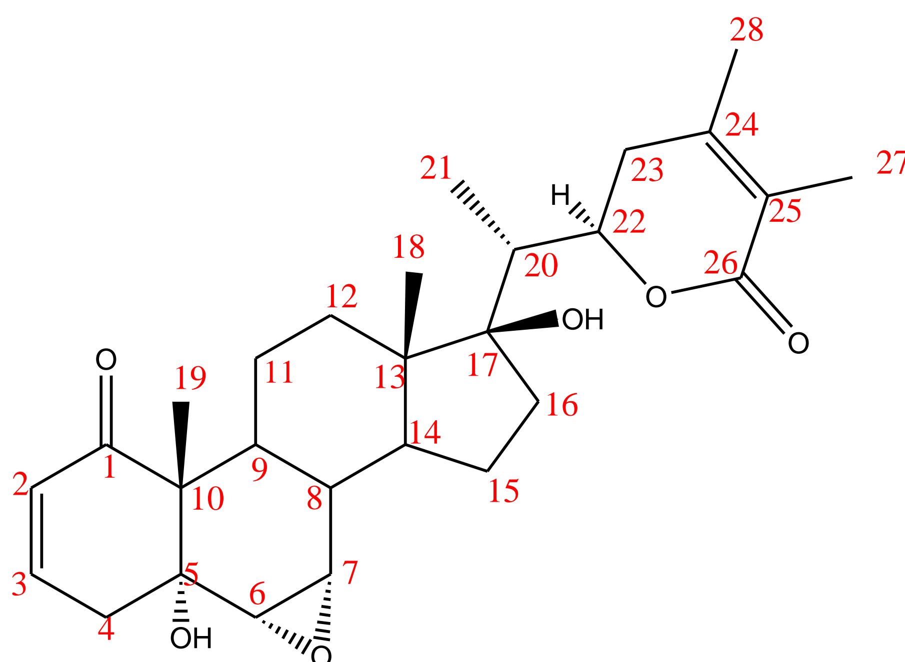

Figure 15: Structure of compound **19**

The proton-carbon HMBC experiments showed correlations between carbons and protons. HMBC spectrum gives information on correlations between carbons and protons which are two to three bonds away. This helps in identification of carbon atoms which are next to each other and those which are two or three bonds away from each other. From the HMBC spectrum (Appendix 5) of compound 19, C-3 and C-4 with protons absorbing at δ 5.88 and δ 6.62 showed correlations. Proton absorbing at δ 2.55(H-4) showed correlations with C-2, C-3, C-5 and C-10 while a Proton absorbing at δ 3.08 (H-6) showed correlations with C-8, C-5 and C-10. C-8 and C-9 correlated with a proton resonating at δ 3.35 (H-7) while proton absorbing at (δ 1.58) showed correlations with C-8 and C-10. H-11 at δ 2.86 correlated with C-9 and C-12 and correlations were also observed between proton at δ 2.50 (H-12) with C-11 and C-13. H-14 absorbing at δ 2.05 showed correlation with C-8, C-9, C-13 and C-15. While a proton absorbing at δ 1.95 (H-15) showed correlations with C-14 and C-16. H-16 absorbing at δ 2.58 correlated with C-14, C-15, C-17 and C-20. H-20 at δ2.58 showed corrections with C-17, C-21 and C-22 while another correlation was observed at H-22 (δ4.64) with C-23. A proton at H-23 absorbing at δ 2.50 correlated with C-20, C22 and C-24. The methyl proton absorbing at δ 1.91 correlated with C-24, C-25 and C-26 while proton absorbing at δ 1.97 (H-28) correlated with C-24, C-25 and C-26. Compound 19 was identified as 5α, 17β-dihydroxy-6α, 7α -epoxy-1-oxo-witha-2, 24-dienolide. The NMR data correlations are shown on Table 4 below. The COSY /HMBC correlations are illustrated on

Figure 16.

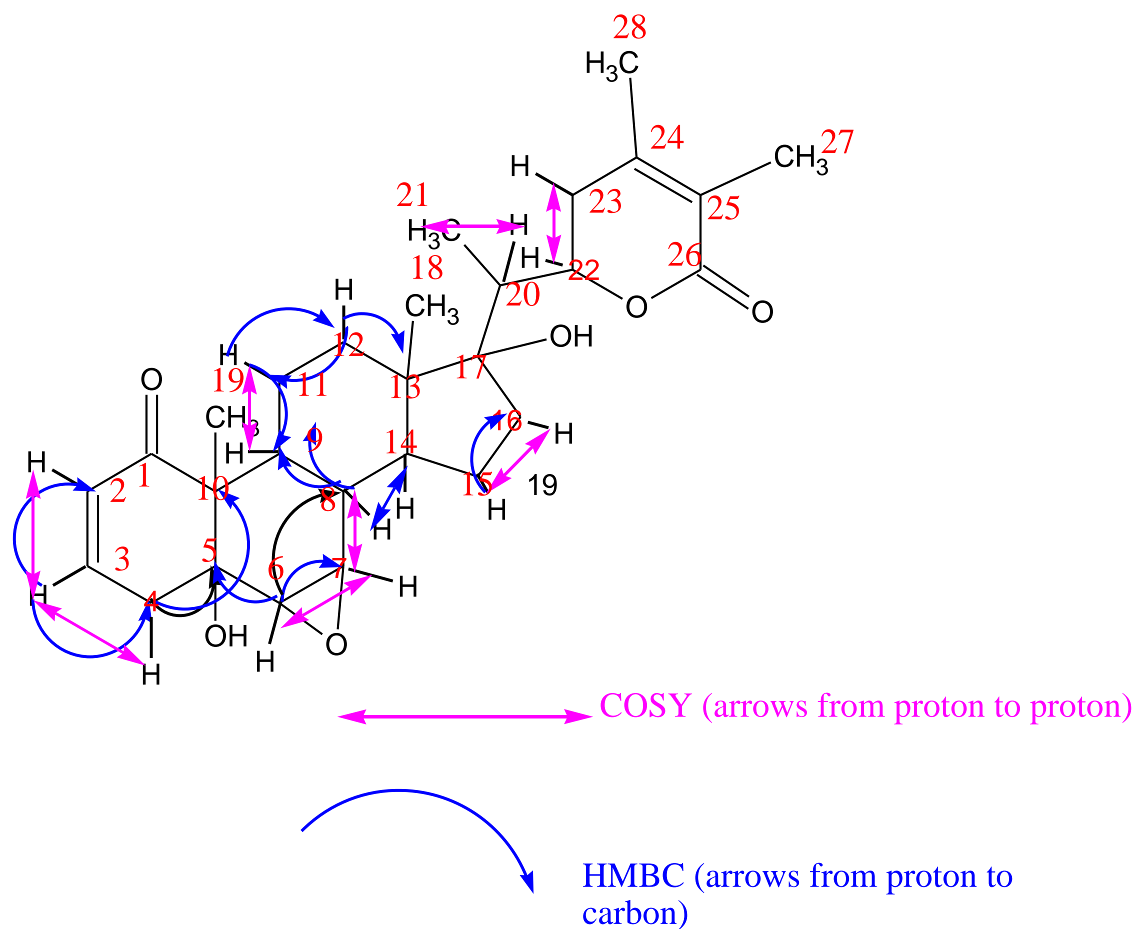

Figure 16: Structure of compound **19** showing COSY and HMBC correlations

Table 4: NMR data of compound 19 in $CDCl_3$

Carbon	$^{13}C(\delta)$	HSQC(δ)	APT	COSY	HMBC	$^{13}C(\delta)$*
1	203.1 s	-	C	-	-	203.1
2	129. d	5.88	CH	3	3,4	129.1
3	139.7 d	6.62	CH	2	2,4	139.2
4	36.8 t	2.55	CH_2	3	2,3,5,10,	36.7
5	73.3 s	-	C	-	-	73.1
6	56.3 d	3.08	CH	7	5, 8,10,	56.3
7	57.2 d	3.35	CH	6,8	8,9	56.7
8	36.0 d	1.81	CH	7,9,14	9,14	37.7
9	35.2 d	1.58	CH	8,11	8,10,11	35.2
10	51.0 s	-	C	-	-	50.9
11	21.6 t	2.86	CH_2	-	9,12	21.6
12	32.8 t	2.50	CH_2	-	11,13	32.6
13	48.7 s	-	C	-	-	48.6
14	45.9 d	2.05	CH	-	8,9,13,15	46.2
15	22.9 t	1.94,1.40	CH_2	16	14,16	22.9
16	36.6 t	2.58	CH_2	15	14,15,17,20	36.7
17	84.7 s	-	C	-	-	85.2
18	15.1 q	0.89	CH_3	-	12,13,1417	15.0
19	14.7 q	1.21	CH_3	-	1,5,9,10	14.7
20	42.9 d	2.36	CH	21,22	17,21,22	40.9
21	9.5 q	1.07	CH_3	20	17,20,22,	12.3
22	78.7 d	4.64	CH	20,23	23	78.5
23	32.5 t	1.67	CH_2	22	20,22,24	32.6
24	150.5 s	-	C	-	-	150.5
25	121.4 s	-	C	-	-	121.3
26	167.2 s	-	C	-	-	167.2
27	12.4 q	1.91	CH_3	-	24,25,26	12.3
28	20.5 q	1.97	CH_3	-	23,24,25	20.3

$^{13}C(\delta)$*- Littérature (Payere *et al*, 2006)

4.2.2 Structure elucidation of compound 20

Compound **20** (Figure 17) was obtained as a crystalline solid after running the column on crude methanol extract using Ethyl acetate and hexane in the ratio 7:3 respectively. The 1H NMR spectrum revealed presence of five methyl signals of withanolides at δ = 0.77, 1.13, 0.72, 1.36, 0.94 for H-18, H-19, H-21, H-27 and H-28, respectively (table 5).

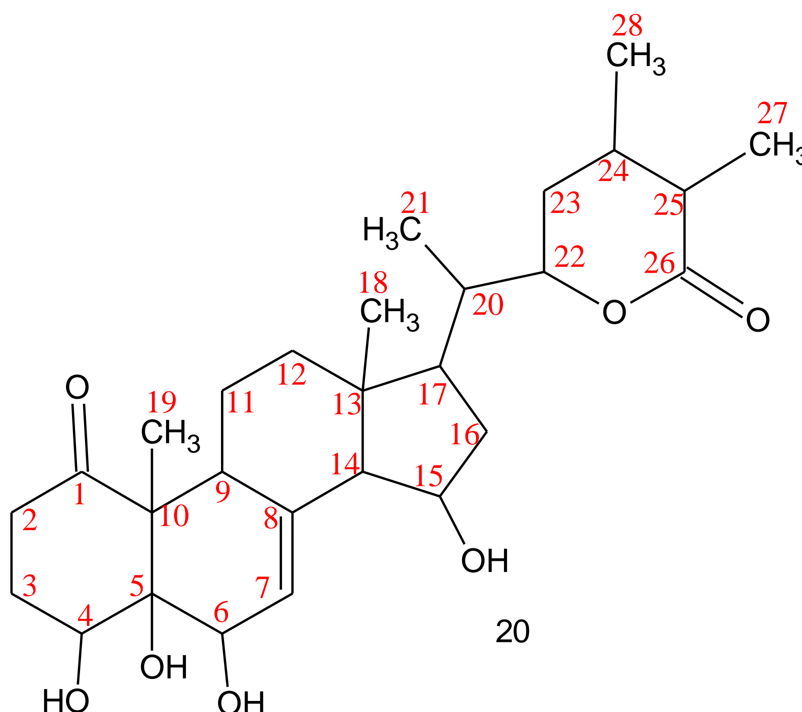

Figure 17: Structure of compound **20**

The ^{13}C NMR spectra of compound **20** (Appendix 5) supported the presence of 28 carbon resonances with molecular mass of 490.629 and molecular formula $C_{28}H_{42}O_7$. Which include five methyl, six methylene, nine methyne and eight quaternary carbons. Compound **20** was similar to compound **19** with the withanolides skeleton structure. The difference occurred in the orientation of the functional groups. At C-4, C-6 and C-15 are hydroxyl groups and a double bond between C-7 and C-8. Between C-24 and C-25, there was no usual double bond leading to C-26 absorbing at δ 182. The HSQC, COSY, HMBC and DEPT were determined and their spectra shown in appendix 6, 7, 8, and 9 respectively. Their correlations are outlined in Table **5.**

Table 5: NMR data of compound **20** in $CDCl_3$

Carbon	$^{13}C(\delta)$	$^{1}H(\delta)$	APT	COSY	HMBC
1	211.4 s	-	C	-	-
2	37.4 t	1.8,1.67	CH_2	3	3,4
3	25.9 t	1.67	CH_2	2	2,4
4	70.9 d	3.8	CH	-	5,10
5	73.3 s	-	C	-	-
6	77.4 d	3.21	CH	7	5,7
7	130 d	3.57	CH	-	5,6
8	136 s	C	C	-	-
9	52.7 d	2.53	CH	-	5,7,8,10,11
10	47.7 s	-	C	-	-
11	25.4 t	1.67	CH_2	-	9,12
12	32.4 d	1.37	CH_2	-	11,13
13	49.6 s	-	C	-	-
14	51.0 d	3.53	CH	15	13,15
15	84.3 d	4.05	CH	14	13,14,16,17
16	26.4 t	2.45, 2.16	CH_2	17	14,17,20
17	48.4 d	2.11	CH	16	13,16,18,21
18	11.9 q	0.77	CH_3	-	12,13,14,17
19	16.2 q	0.65	CH_3	-	1,5,9,10
20	41.1d	1.4	CH	-	17,20,21
21	16.7 q	1.36	CH_3	-	17,20,22,
22	81.2 d	3.97	CH	-	17,21.23,
23	28.3 t	1.7,1.1	CH_2	-	20,22,24,25
24	29.3 d	1.2	CH	-	23,25,26
25	40.8 d	-	CH	-	-
26	182.2 s	-	C	-	-
27	16.3 q	0.94	CH_3	-	24,25,26
28	17.0 q	1.36	CH_3	-	23,24,25

4.2.3 Structure elucidation of compound 21

Compound **21** (Figure 18) was obtained from *H. abyssinica* as white powder after running the column with the methanol extract with 7:3 hexane ethyl acetate; and purifying further with

7:3 diethyl ether**;** hexane. NMR analysis was done on the compound (Table 6) and the correlations are explained in details below.

The ^{13}C NMR (Appendix 10) showed presence of 30 carbons which include seven methyl, eight methylene, seven methyne and eight quaternary carbons. Molecular formula for compound **21** was determined to be $C_{30}H_{48}O_5$ which corresponded to molecular weight 488.699. The ^{13}C NMR, HNMR spectrum (Table 6) of compound **21** signals were consistent with a pentacyclic triterpenoid structure which have been reported to have been isolated from rosaceae families (Hyun *et al*., 2004; Masateru *et al*, 2015).

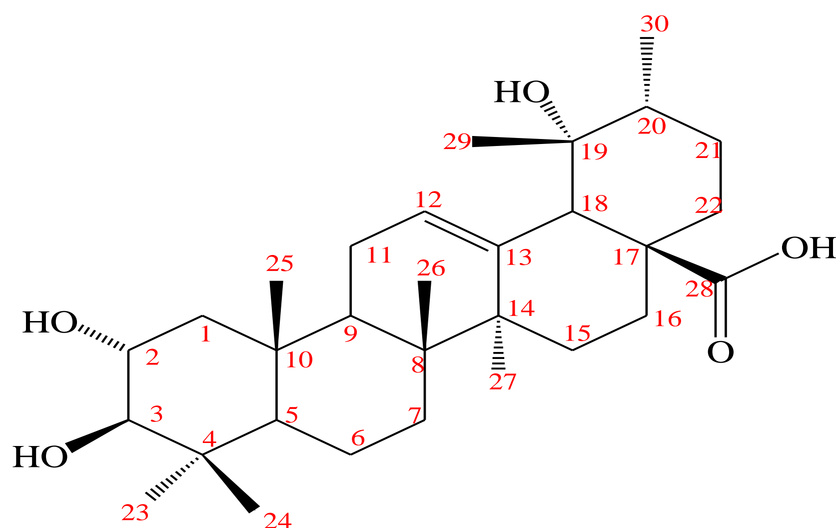

Figure 18: Structure of compound **21**

The first carbon was found to resonate at δ=48.3 (C-1), C-2 (δ 69.5), C-3 (δ 84.6). Quaternary carbons at C-4, C-8, C-10, C-13, C-14, C-17, C-19 and C-28 were found to absorb at δ 39.2, 40.8, 40.5, 139.8, 43.3, 48.5, 73.0 and 181.6 respectively. Carbon 5 absorbed at δ 56.6 while C-6, C-7, C-11, C-14 and C-16 were found to resonate at δ 19.5, 34.2, 24.4, 29.2 and 25.3 respectively. Carbons 21 and 22 absorbed at δ 31.8 and δ 38.1. Carbon 9 and C-23 were determined to absorb at δ 48.9 and δ 29.3. The analysis through HSQC (Appendix 11) showed correlations of six quaternary methyl absorbing at δ= 1.33, 0.85, 0.94, 1.16, 1.01, and 1.05 with C-24, C-25, C-26, C-27, C-29 and C-30 respectively. The methyne C-5, C-9, C-12, C-18 and C-20 were determined to correlate with protons resonating at δ = 0.9, 1.65, 5.28, 2.26 and 1.01 respectively in the HSQC spectrum. The carbon bearing the hydroxyl group at C-2 and C-3 showed HSQC correlation with protons resonating at δ 3.66 and δ 2.96 respectively. The protons resonating at δ 1.99, 0.94 were determined to correlate with C-1. C-6 while C-7 showed HSQC correlations with protons resonating at (δ1.48, 1.59) and (δ1.56,

1.54). C-11 correlated with proton absorbing at δ 1.79, 2.00 while C-15 correlated with protons resonating at δ 1.15, 1.12 in the HSQC spectrum. C-16 correlated with proton absorbing at δ 1.69, 2.11. C-21 correlated with protons absorbing at δ 1.37, 1.54 while a proton resonating at δ 1.67, 1.73 was determined to correlate with C-22 in the HSQC spectrum.

Further analysis through HMBC (Appendix 12) which gave the correlation of carbon to carbon 2-3 bonds away showed that H-1 (δ 1.99) correlated with C-2 and C-3 while H-2 (δ 3.66) showed correlation with C-1, C-3 and C-4. It was also observed that H-3 (δ 2.96) correlated with carbons C-2, C-4, C-5 while H-6 (δ1.59) correlated with C-7 and C-8. H-9 (δ 1.65) showed strong correlation with carbons at positions C-1, C-8, C-10, C-11, C-14 and C-25. Another major correlation was observed at H-11 (δ 2.00) with C-9, C-10 and C-12. C-12 correlated with C-13 and C-14 while H-15(δ1.15) had correlation with C-13, C-14, C-16 and C-17, H-16 (δ 2.11) was determined to correlate with C-14, C-17, C-20 while H-18 (δ 2.26) had correlation with C-12, C-13, C-14 , C-16 and C-17. There was correlation at H-21 (δ 1.54) with C-20 and C-22 while H-22 (δ 1.67) was determined to correlate with C-17, C-20, C-21, C-23 and C-28. H-23 (δ 1.06) correlated with C-3, C-4, C-5 and C-24 while H-24(δ1.33) had correlation with C-3, C-5 and C-23. Correlation was observed at H-25 (δ 0.85) with C-1, C-5, C-9 and C-10. H-26 (δ 0.94) correlated with C-7, C-8 and C-9 while H-27 (0.941.17) showed correlation with C-8, C-13, C-14, and C-15. Both H-29 (δ 1.01) and H-30 (δ 1.05) showed correlation with C-20. The HMBC analysis confirmed the proposed structure for compound 21.

Further confirmation with COSY (Appendix 13) correlations showed that there was a strong correlation between protons at H-1 (δ 1.99) with a proton at H-2 resonating at (δ 3.66). Also H-3 (δ 2.96) showed correlation with a Proton absorbing at δ 3.66 (H-2). H-6 with a proton resonating at δ 1.45 correlated with a proton resonating at δ 1.59 (H-7). H-9 (δ 1.65) correlated with H-11 (δ 2.00) while proton absorbing at δ1.96 (H-15) correlated with a proton resonating at δ 2.11 (H-16). H-21 (δ1.54) correlated with H-22 (δ1.67). The COSY correlation gave information of correlations on protons on adjacent carbon atoms. The COSY /HMBC correlations are illustrated on Figure 19. This analysis helped to confirm the positions of the carbons on the structure. This compound through the NMR analysis and Literature confirmation was named as 2, 3β, 19α -trihydroxyurs-12-en-28-oic acid.

Table 6: NMR data of compound 21 in $CDCl_3$

Carbon	$^{13}C(\delta)$	$^{1}H(\delta)$	APT	COSY	HMBC
1	48.3 t	1.99,0.94,	CH_2	2	2,3
2	69.5 d	3.66	CH	1,3	1,3,4
3	84.5 t	2.96	CH	2	2,4,5
4	39.2 s	-	C	-	-
5	56.7 d	0.90	CH	-	-
6	19.5 t	1.59,1.48	CH_2	7	7,8
7	34.2 t	1.56,1.54	CH_2	6	8
8	40.8 s	-	C		-
9	48.9 d	1.65	CH	11	1,8,10,11,14, 25
10	40.5 s	-	C		-
11	24.4 t	2.0,1.79	CH_2	9	9,10,12
12	126.7 d	5.28	CH		11,13
13	139.8 s	-	C		-
14	43.3 s	-	C		-
15	29.2 t	1.96,1.15	CH_2	16	13,14,16,17
16	25.3 t	1.69, 2.11	CH_2	15	14,17,20
17	48.5 s	-	C		-
18	54.3 d	2.26	CH		12,13,14,16,17,28
19	73 s	-	C		-
20	40.4 d	1.01	CH		-
21	31.8 t	1.37,1.54	CH_2	22	20,22,
22	38.1 t	1.67,1.73	CH_2	21	17,20,21.23,28
23	29.3 q	1.21	CH_3		3,4,5,24
24	18.4 q	1.33	CH_3		3,5,23
25	17.5 q	0.85	CH_3		1,5,9,10,
26	17.6 q	0.94	CH_3		7,8,9
27	24.1 q	1.16	CH_3		8,13,14,15
28	181.6 s	-	C		-
29	21.6 q	1.01	CH_3		20
30	17.2 q	1.05	CH_3		20

The isolated compound was found to be a derivative of Ursonic acid which has been mostly isolated from rosaceae family, (Hyun *et al*., 2004; Nguelefack *et al*., 2011; Chu-Hung *et al*., 2013; Ali and Farzana, 2013; Ibrahim and Babalola, 2013; Shiping *et al*., 2014 and Masateru *et al*., 2015).

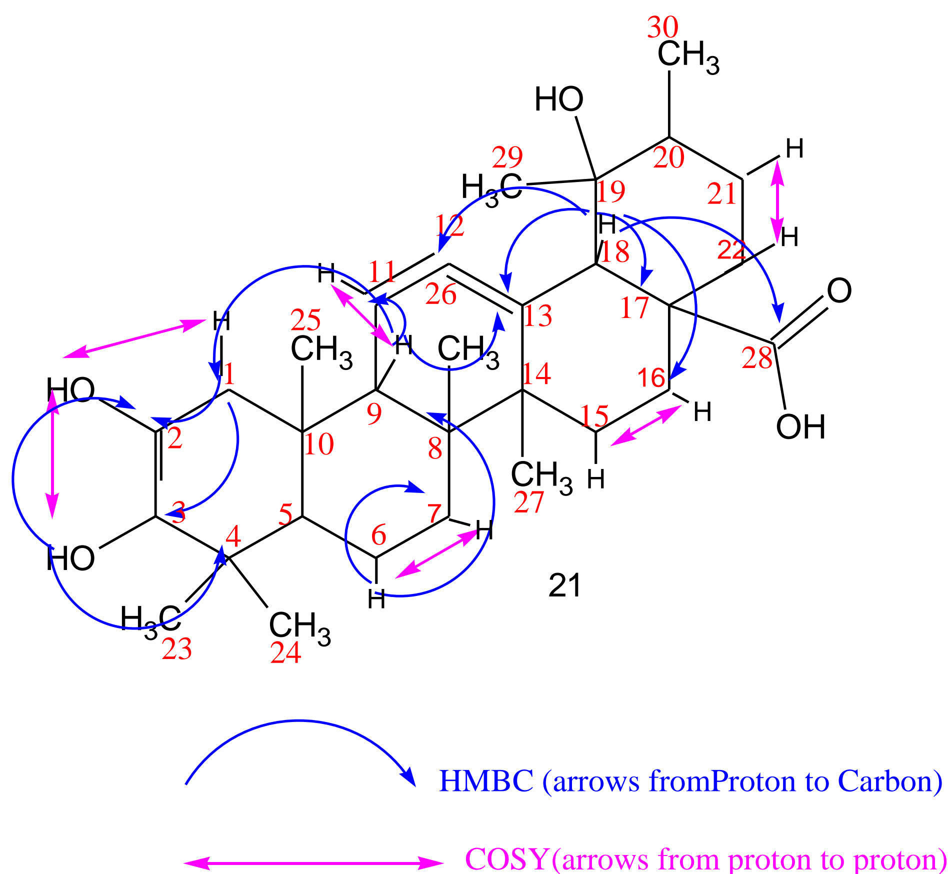

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Figure 19: Structure of compound 21showing COSY and HMBC correlations

4.3 Determination of the chemical composition of the essential oil

The chemical composition of the essential oil extracted from the fresh leaves of *H. abyssinica* was determined by the GC-MS. The identification of the compounds in the oil was determined by comparing the electron impact mass spectrum of the compounds in the oil and those in the Wiley7N.l, FLAVORS.L, HPCH1607.L computer library databases. From the data obtained, thirty one compounds were identified by GC/MS representing 86.65% of the total oil (Table 7). The mass spectrum revealed that the essential oil was dominated by sesquiterpenes and monoterpenes. The sesquiterpene hydrocarbons had the highest concentration of 53.43% and monoterpenes hydrocarbons 33.22%. The major sesquiterpenes hydrocarbons identified were; α-copaene (2.02%), α-gurgunene (2.66%), calarene (4.07%),

Table 7: Chemical constituents of essential oil from *Hagenia abyssinica*

Compound no.	RT (min)	% concentration	Compound name
22	5.27	0.25	Camphene
23	6.28	0.17	Benzene
24	8.95	0.18	Nonanal
25	10.38	1.07	α-campholene aldehyde
26	11.62	3.58	Camphor
27	13.12	0.58	Tran-Pinocarveol
28	13.27	0.28	(-)Myrtenal
29	14.14	19.67	Endobornyl acetate
30	15.32	1.20	Borneol
31	15.27	0.27	Trans-(+)-Carveol
32	16.12	2.73	α-copaene
33	16.51	0.05	(+)-Aromadendrene
34	16.72	0.35	Tetradecane
35	17.48	2.66	Alpha-gurjunene
36	17.87	4.07	Calarene
37	18.90	1.07	Cadina
38	18.96	3.35	1H-cycloprop[e]-azulene
39	19.39	11.27	Aromadendrene
40	20.24	1.30	Ledene
41	20.31	0.37	β-Bisabolene
42	20.45	0.48	Isolongifolene
43	20.74	0.29	2-Tridecanone
44	20.84	0.44	δ-Cadinene
45	21.01	3.93	Azulene
46	21.16	0.40	Tetradecanal
47	23.86	6.22	Palustrol
48	24.82	0.32	Eudesma
49	24.95	13.55	δ –gurjunene
50	25.41	1.01	Naphthalene
51	25.28	3.75	(+)-Spathulenol
52	27.03	0.34	γ-Gurjunene
53	29.87	0.45	2-Pentadecanone

cadina (1.07%), 1H-cycloprop[e]-azulene (3.35%), aromadendrene (11.27%), ledene (1.30%), palustrol (6.22%), δ-gurjunene (13.55%), and spathulenol (3.75%). The major monoterpene hydrocarbons were found to be; endobornyl acetate (19.67%), α-campholene (1.06%), camphor (3.58%), borneol (1.20%) and azulene (3.93%). Their structures are shown on Figure 20.

There were three major compounds with concentrations more than 10% of the total oil which

are discussed here. Compound (**29**) was observed to occur at retention time 14.14. It was identified as endobornyl acetate and had the highest concentration of 19.67% of the total oil and with mass 196 amu. The MS spectrum showed presence of a small peak at m/z 196 corresponding to $[C_{12}H_{20}O_2]^+$. An intense peak was visible at m/z 95 which corresponded to $[C_7H_{11}]^+$ and another fragment at m/z 43 corresponding to $[C_3H_7]^+$. The fragmentations peaks are shown in Figure 21**.** Compound (**39**) was observed to occur at the retention time 19.39 with concentration of 11.27% of the total oil with mass 204 amu. It was identified as aromadendrene and corresponded to peak at m/z 204 amu. The spectrum showed fragments peaks at m/z 161, 133, 105, 91, 79, 69 and 55 (Figure 22). The compound (**49**) occurred at the retention time 24.95 and was identified as ɗ-gurjunene with molecular weight of 204 and was 13.55% of the total oil. The GC-MS spectrum of the oil showed a peak at *m/z* 204 $[C_{15}H_{24}]^+$, m/z 161 corresponding to the fragment $[C_{12}H_{17}]^+$ and the peaks at *m/z*105 corresponding to fragment$[C_8H_9]^+$. The illustrations are shown on Figure 23.

The results of the bioassays showed that the essential oil of *H. abyssinica* exhibited very high activities against the bacterial pathogens tested. The oil exerted an inhibition zone to the same extent with the reference standard (Chloramphenicol) which registered an inhibition zone of 30mm. The antibacterial properties of the oil could be attributed to the high percentage of endobornyl acetate, camphor, palustrol, aromadendrene, α-gurjunene, spathulenol, calarene and azulene which are known to posses very strong antibacterial activities, (Couladis *et al.*, 2003; Jianu *et al.,* 2013; Zamri and Mohd, 2015; Fahmidabinti *et al.*, 2016; Bahri *et al.*, 2016). However, we cannot rule out the possibility of the compounds present in low concentrations contributing to the high antimicrobial activity. Essential oil from the female flowers of *H. abyssinica* has also been reported to exhibit trypanocidal activities (Nibret and Wink 2010).

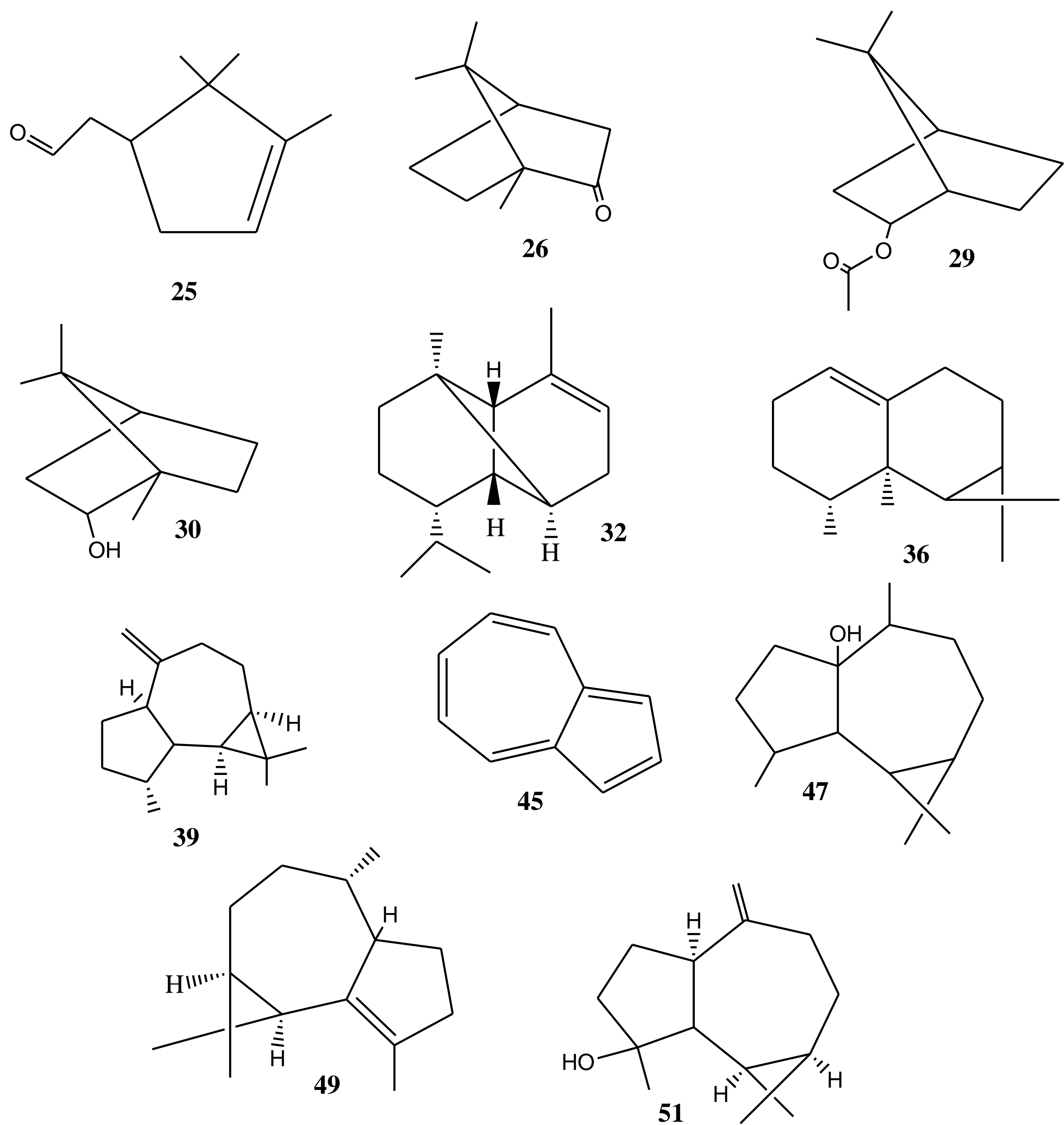

Figure 20:Structures of major compounds of the essential oil from *Hagenia abyssinica*

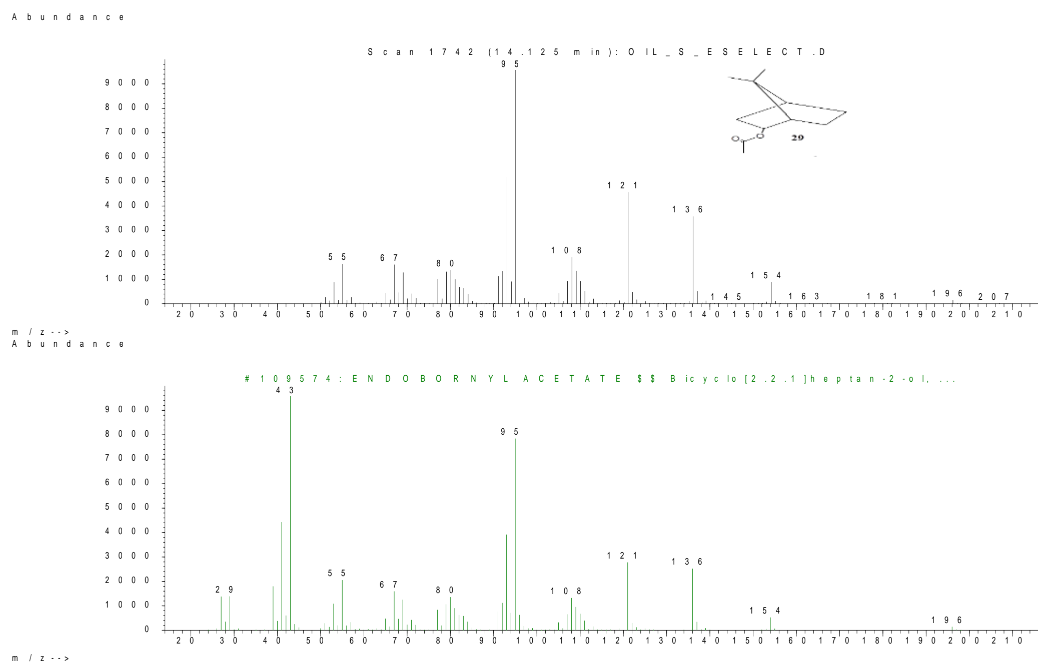

Figure 21: Mass Spectrum of compound **29**

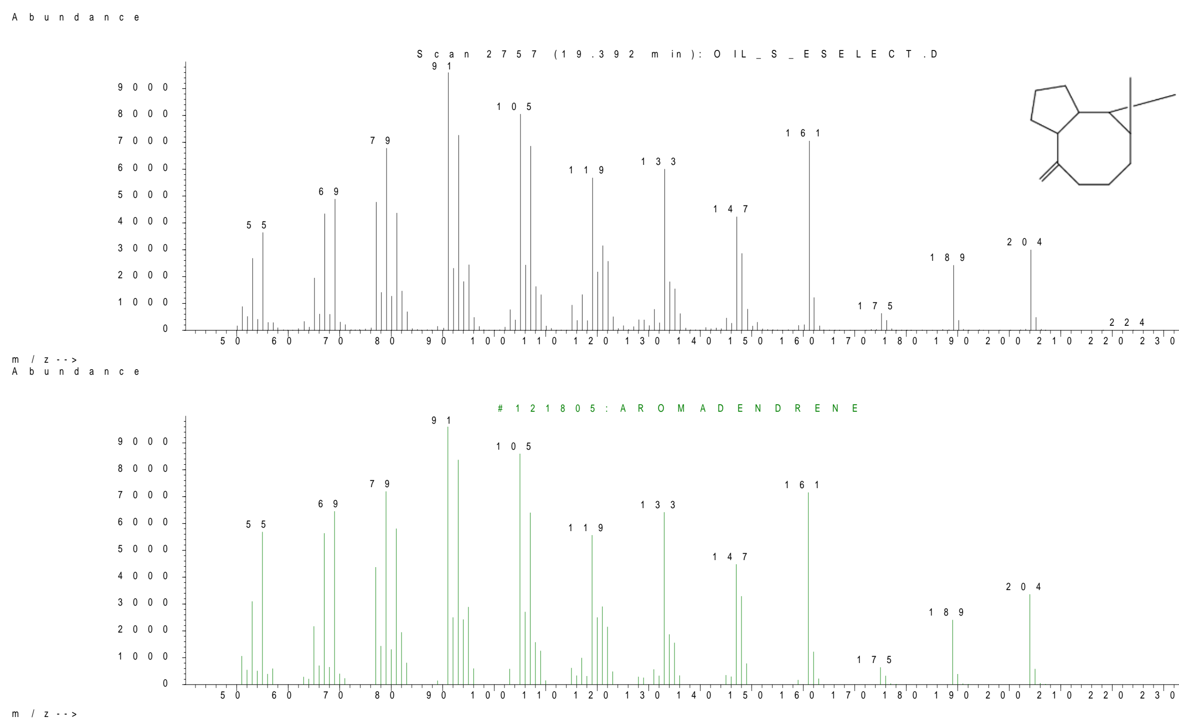

Figure 22: Mass spectrum for compound **39**

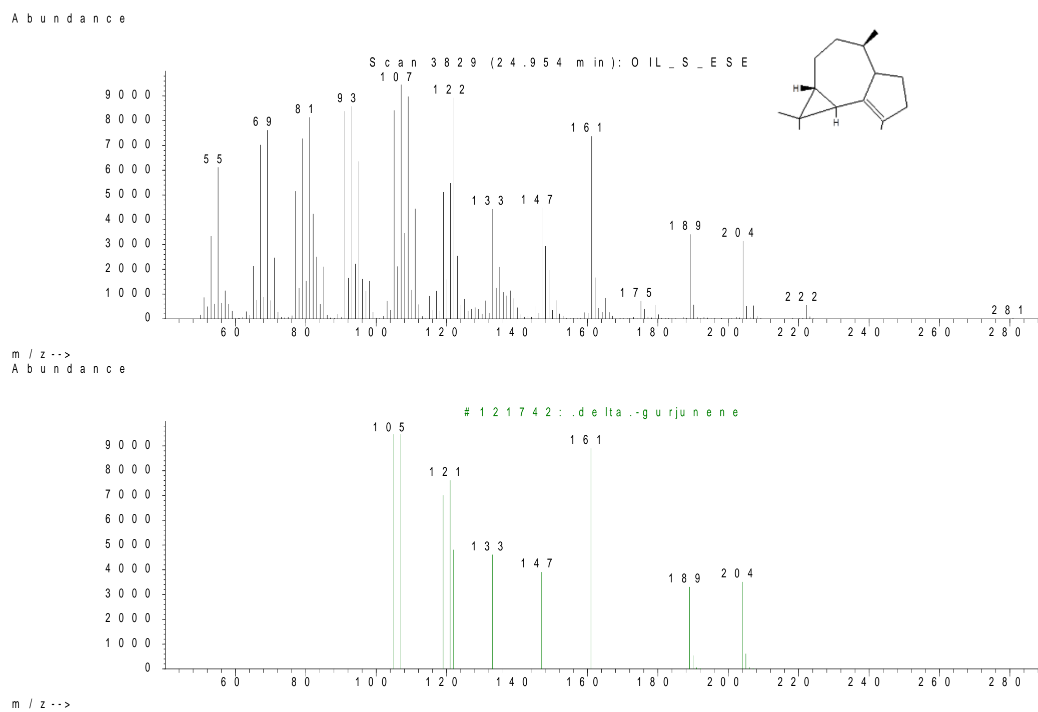

Figure 23: Mass spectrum for compound **49**

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The methanolic crude extracts of *W. somnifera* and *H. abyssinica* showed significant activities against most of the tested microorganisms. *Fusarium moniliforme* is the only fungal pathogen that was not inhibited by the methanolic extract from *H. abyssinica*. The extracts were more active on *C. lindemuthianum* than the reference standard Nystatin.

The antibacterial activities of the extracts increased with increasing concentrations. The extracts from *H. abyssinica* showed more activity on the pathogens than the extracts from *W. somnifera*. The low MICs observed means that the pathogens resistance can be averted by this new means of control.

Compound **21** isolated from *H. abyssinica* was a derivative of ursonic acid and was named 2, 3, 19-trihydroxyurs-12-en-28-oic acid. The compounds isolated from *W. somnifera,* 5α, 17β-dihydroxy-6α, 7β-epoxy-1-oxo-witha-2, 24-dienolide (**19**) and 4,5,6, 15 tetrahydroxy-1-oxo-witha-7-enolide (**20**) have been isolated from the leaves for the first time. The isolated compounds did not show any activity, this could mean that the compounds were synergistically active or there are other active compounds in the extracts that were not isolated.

Results from this study showed that secondary metabolites from the leaves of *W. somnifera* and *H. abyssinica* and the essential oil from *H. abyssinica* have potential applicability in the control of maize and bean diseases caused by the fungal and bacterial pathogens tested. These findings also showed that these plants can be a potent source of lead compounds in the development of new, biodegradable, environmentally friendly, cheaper and readily available means of controlling these diseases.

5.2 Recommendations

In relation to this research, the following recommendations were made;

i. That further isolations and purifications of compounds from these plants should be done and bioassays carried out

ii. Use of crude extracts is recommended instead of the pure inactive compounds.

iii. The essential oil from *H. abyssinica* should be tested against the bacterial pathogens under green house and field conditions.

iv. More of compound 21 should be isolated and screened for bioactivity against the pathogens.

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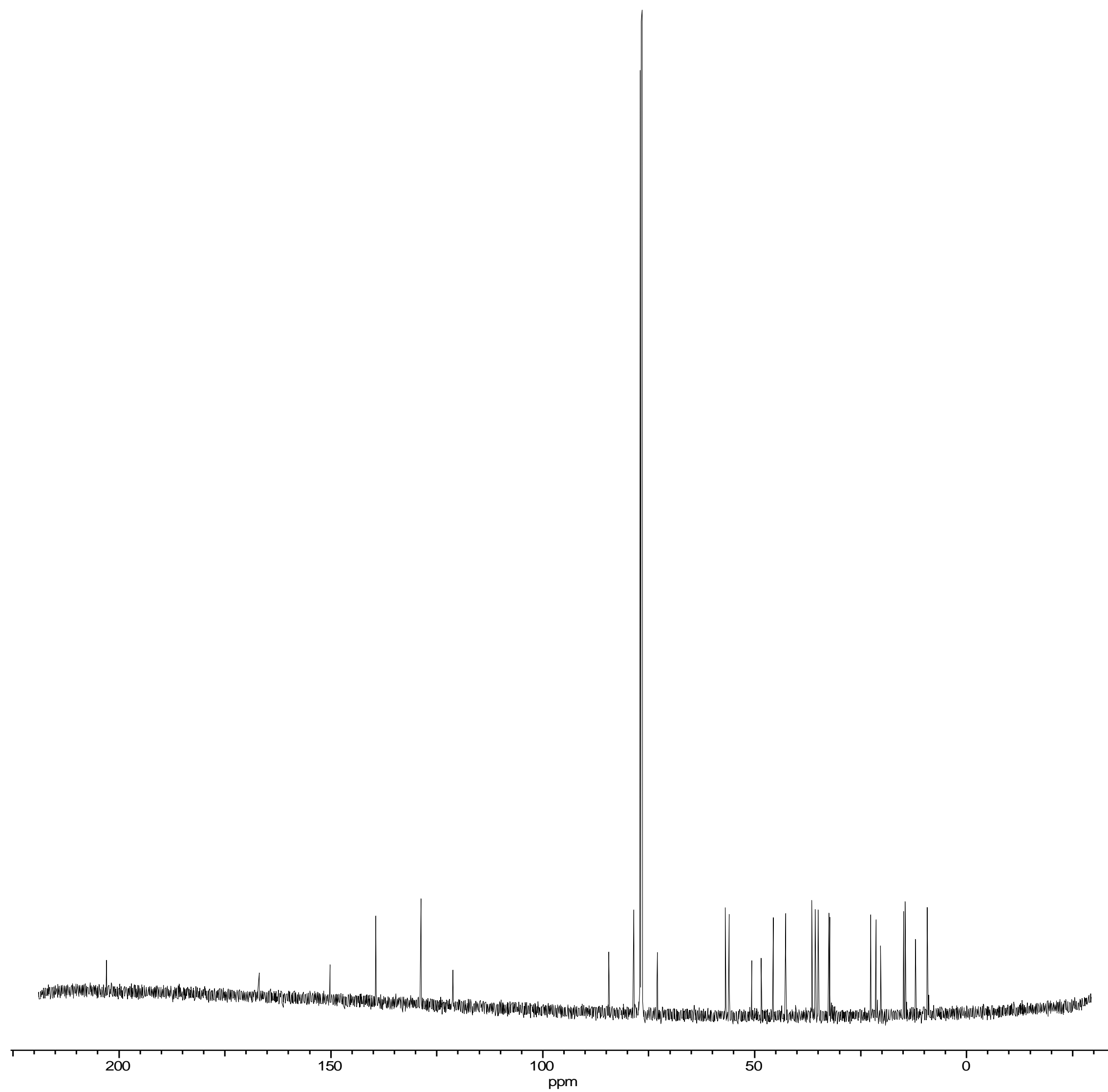
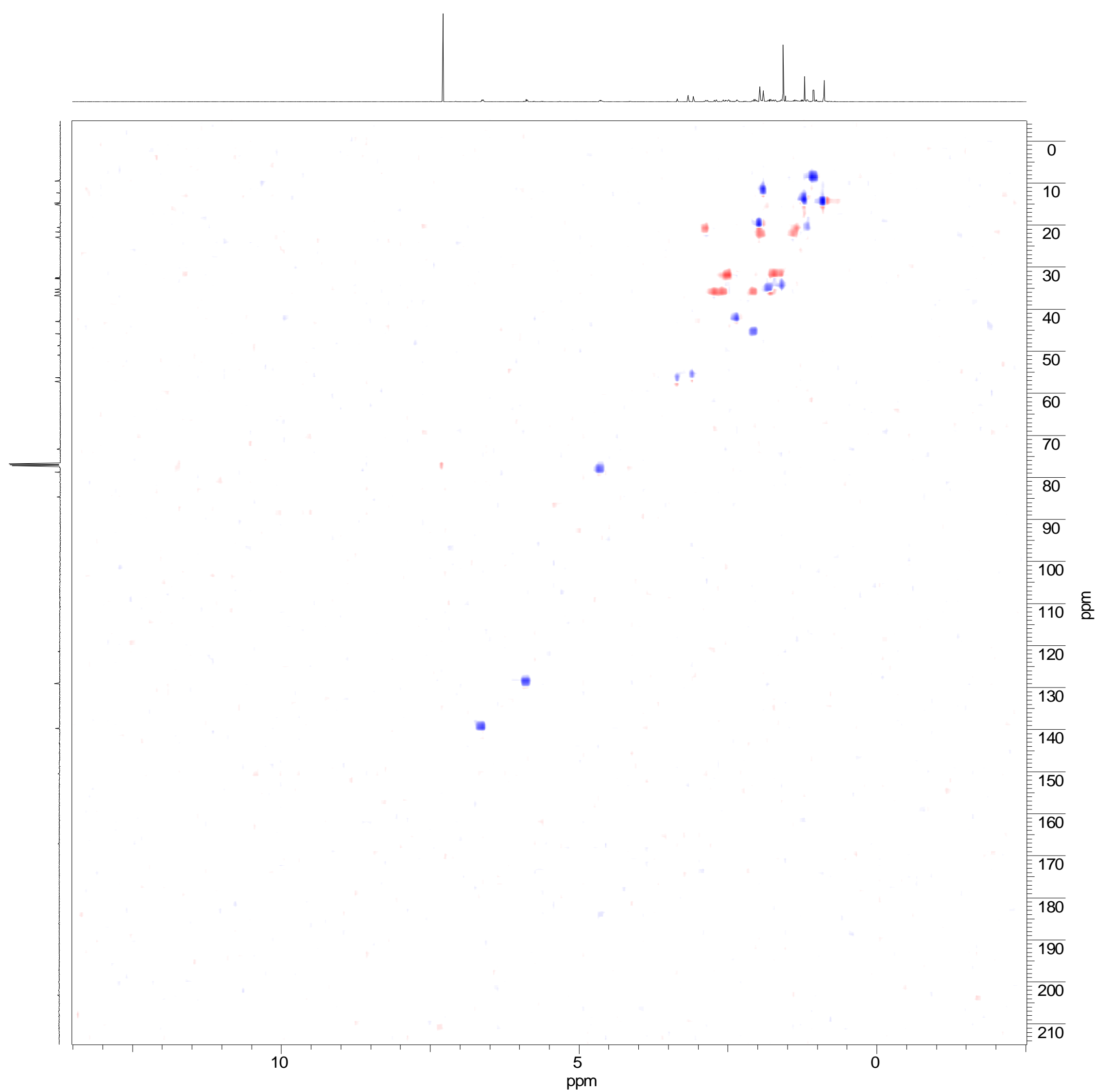
APPENDICES

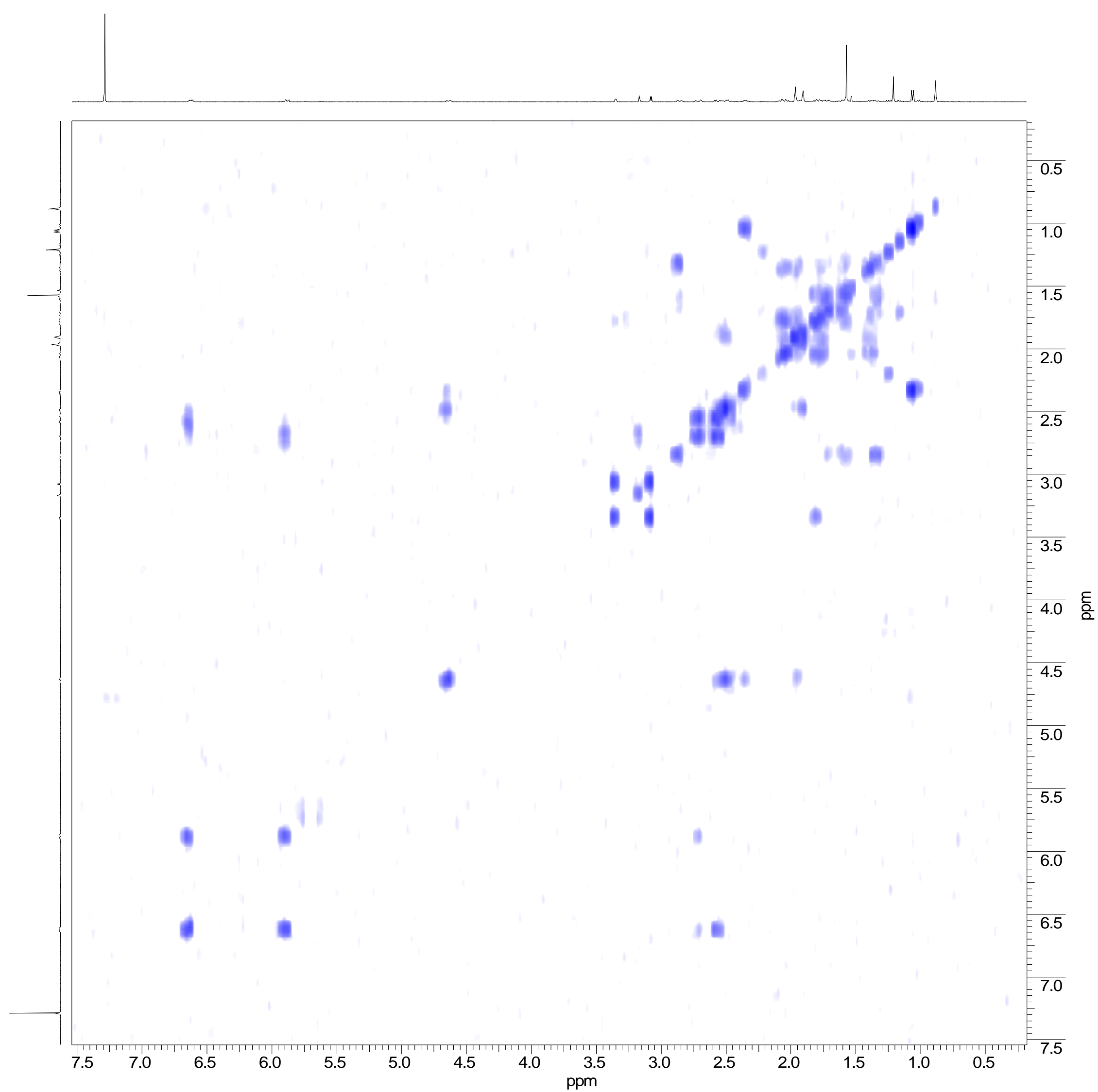
Appendix 1: ^{13}C NMR of compound **19**



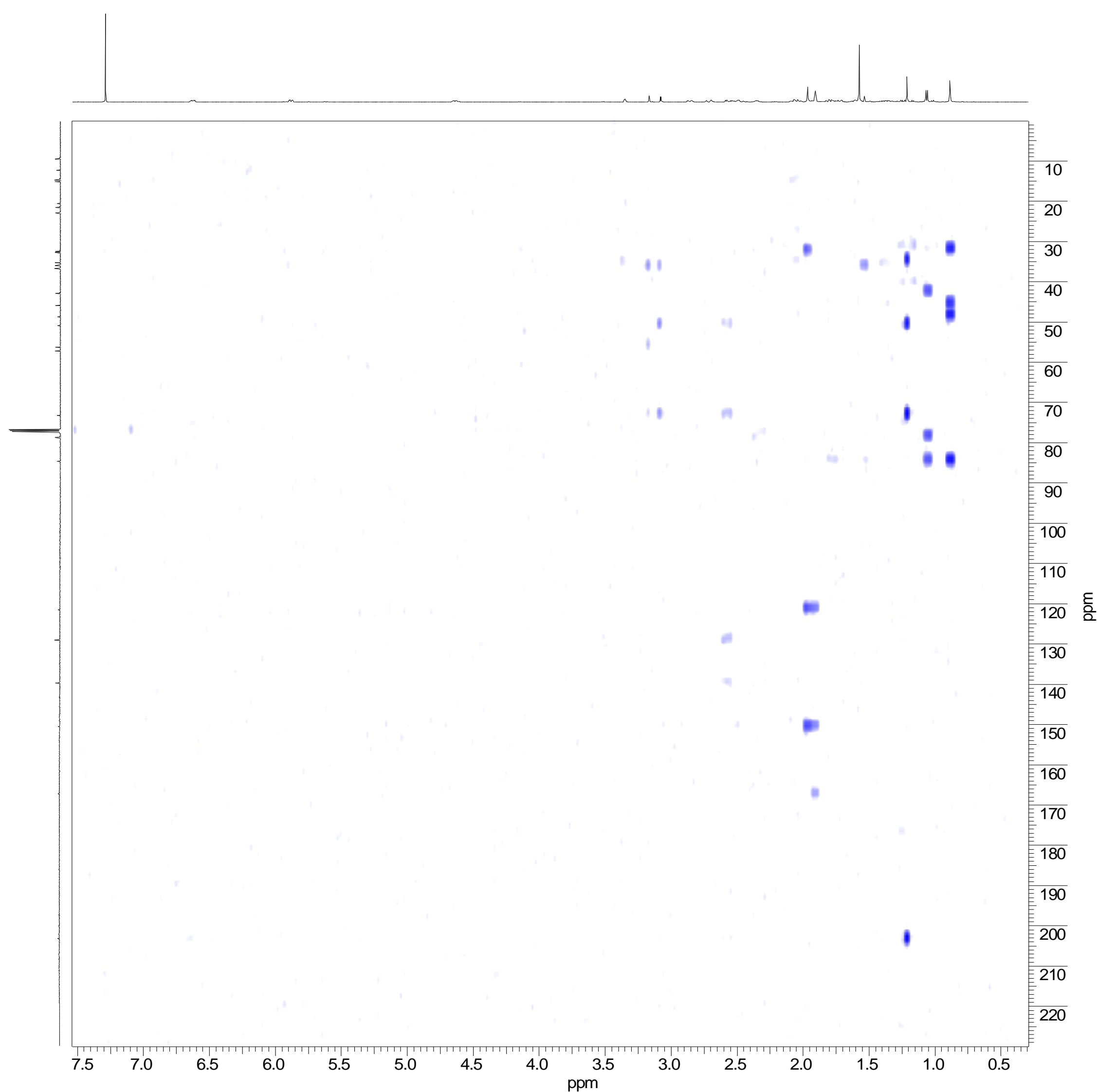
Appendix 2: HSQC spectrum of compound **19**



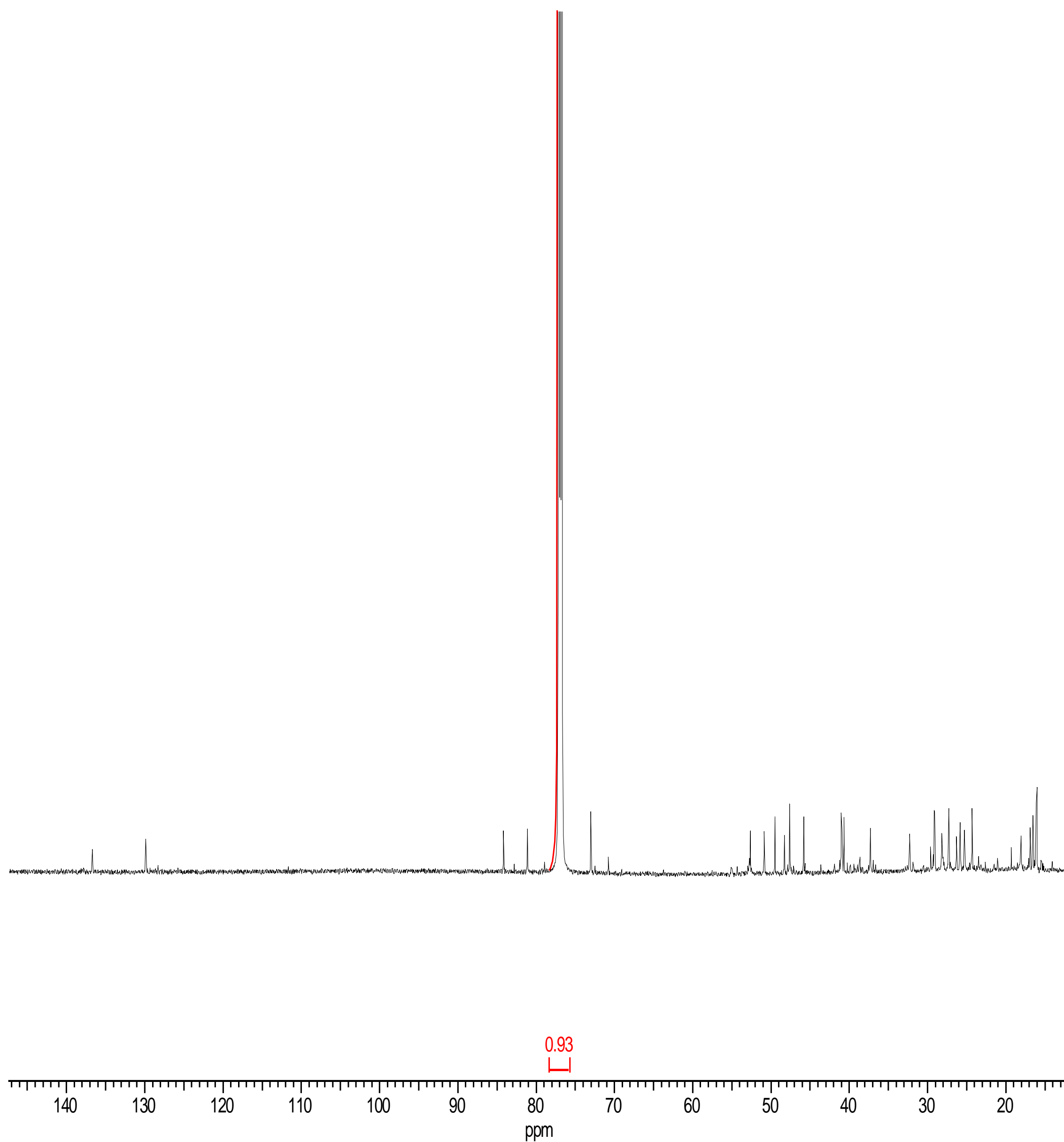
Appendix 3: COSY Spectrum of compound **19**



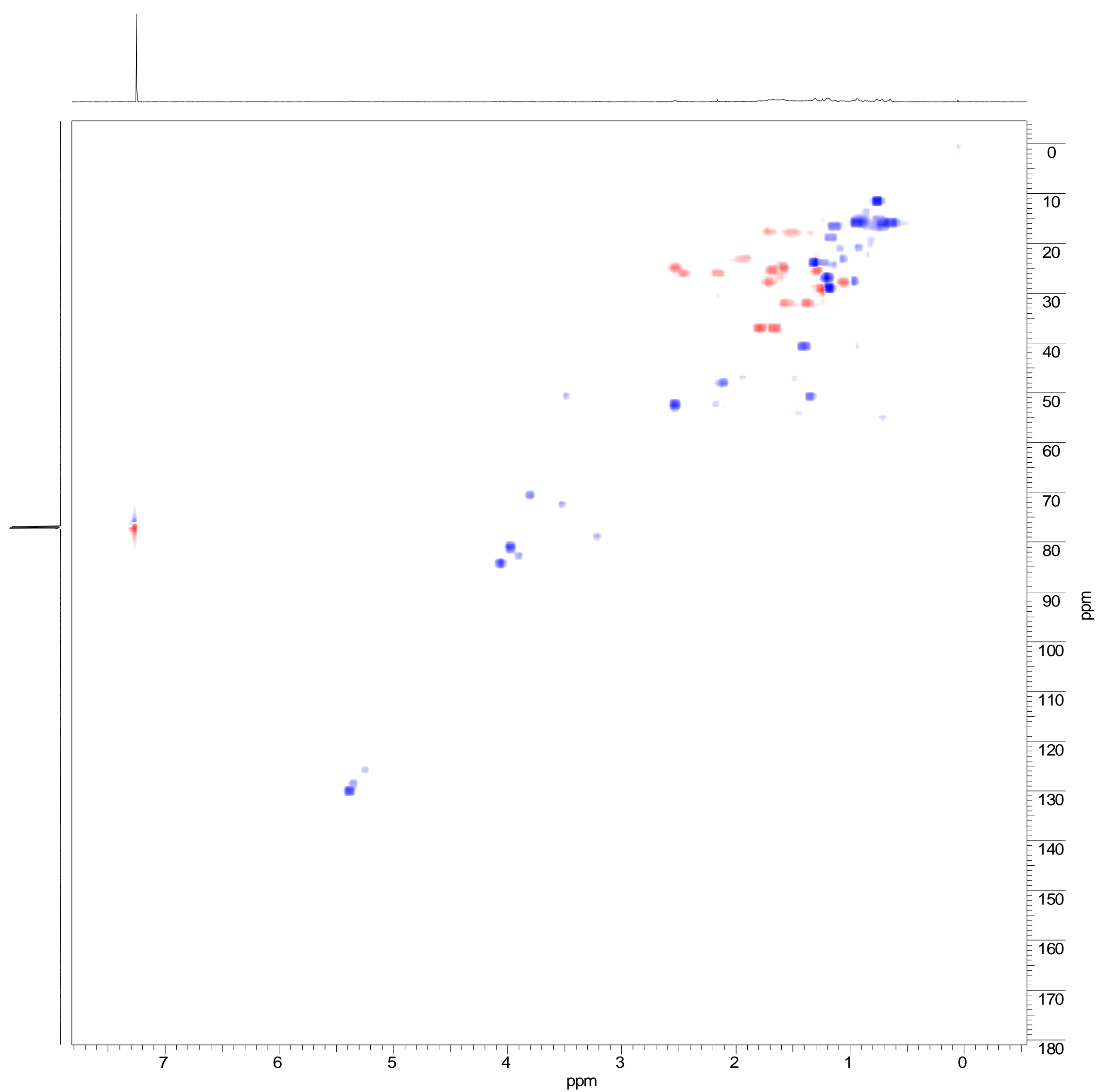
Appendix 4: HMBC Spectrum of compound **19**



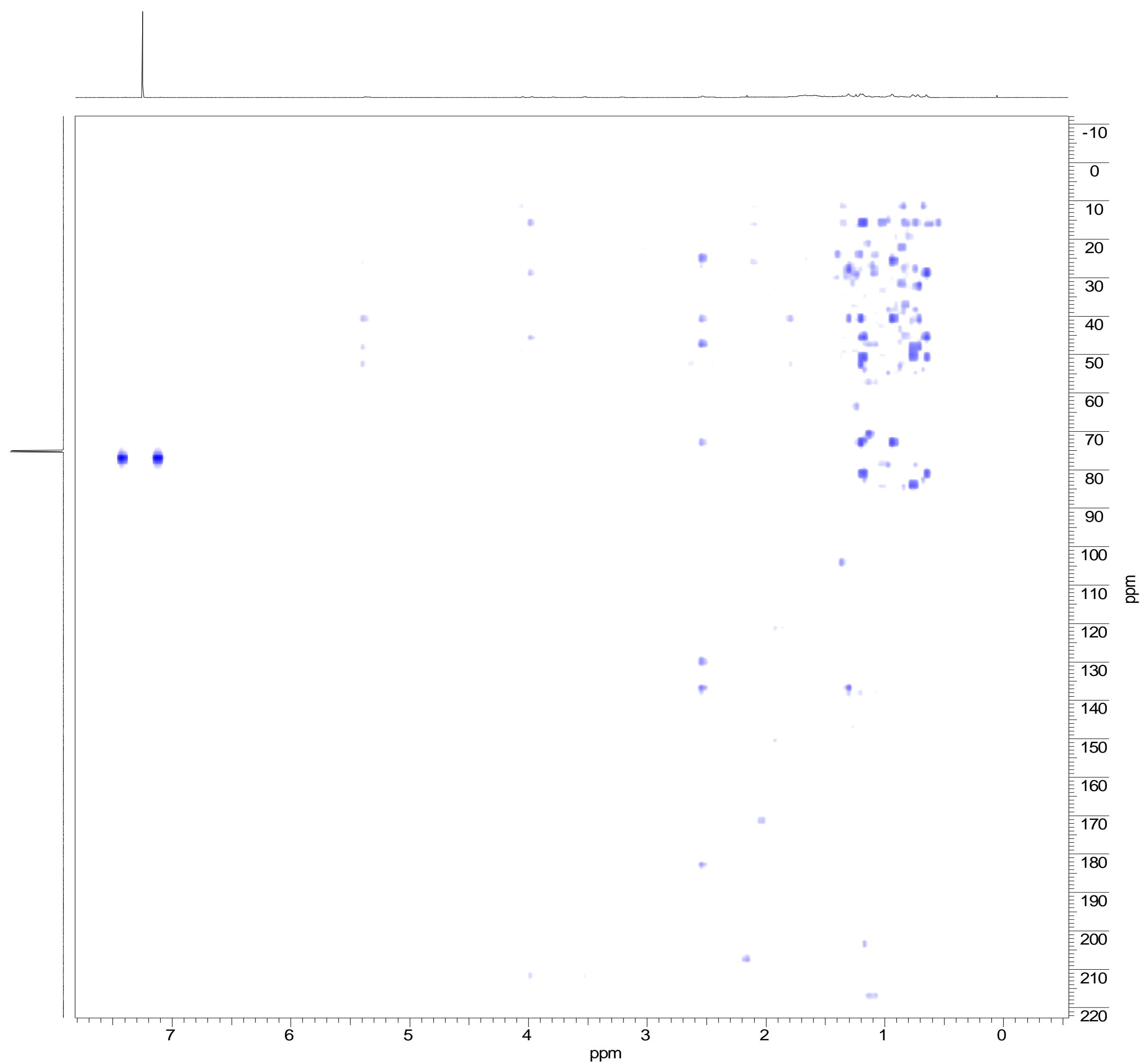
Appendix 5: ^{13}C NMR of compound **20**



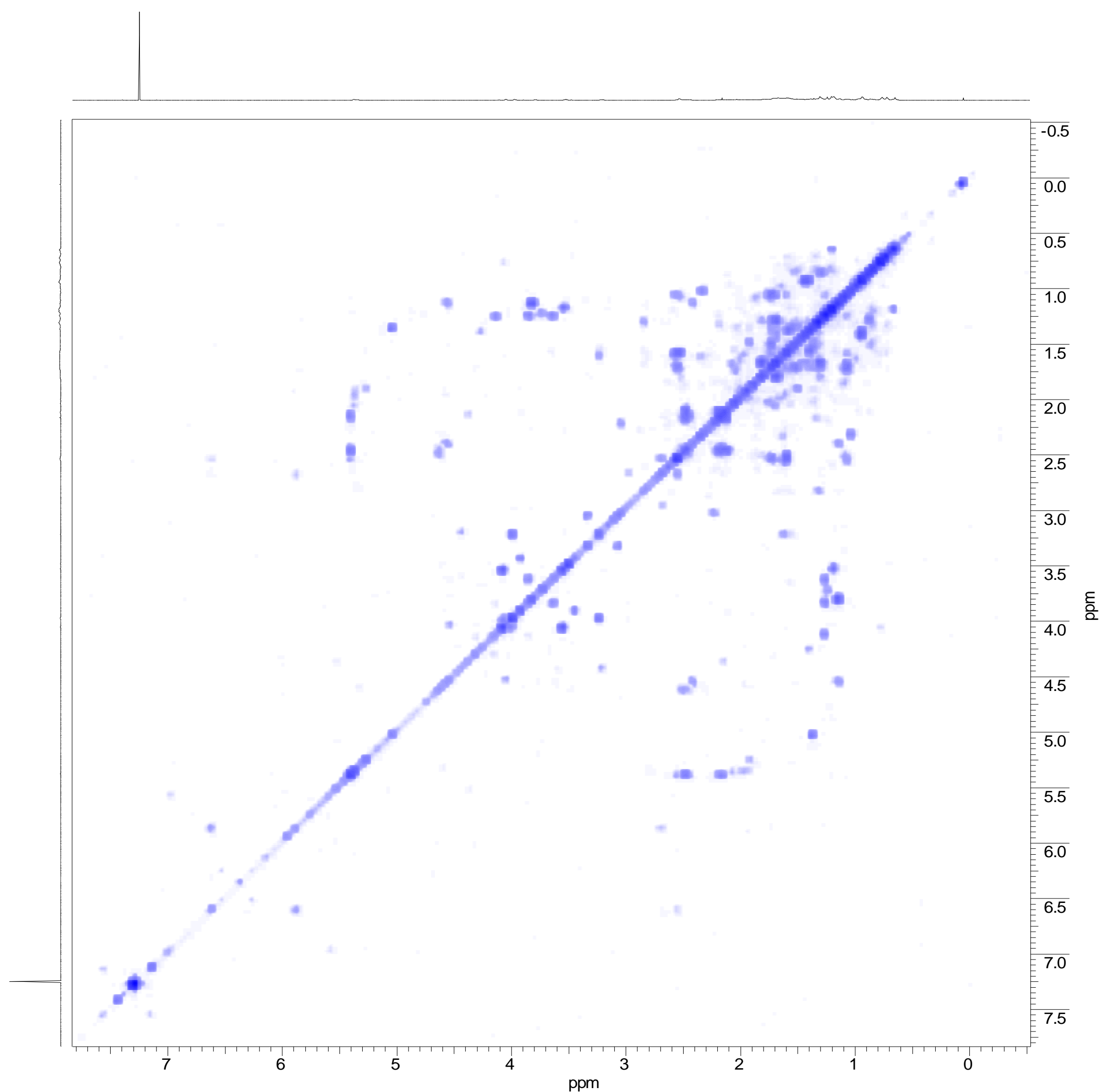
Appendix 6: HSQC Spectrum of compound **20**



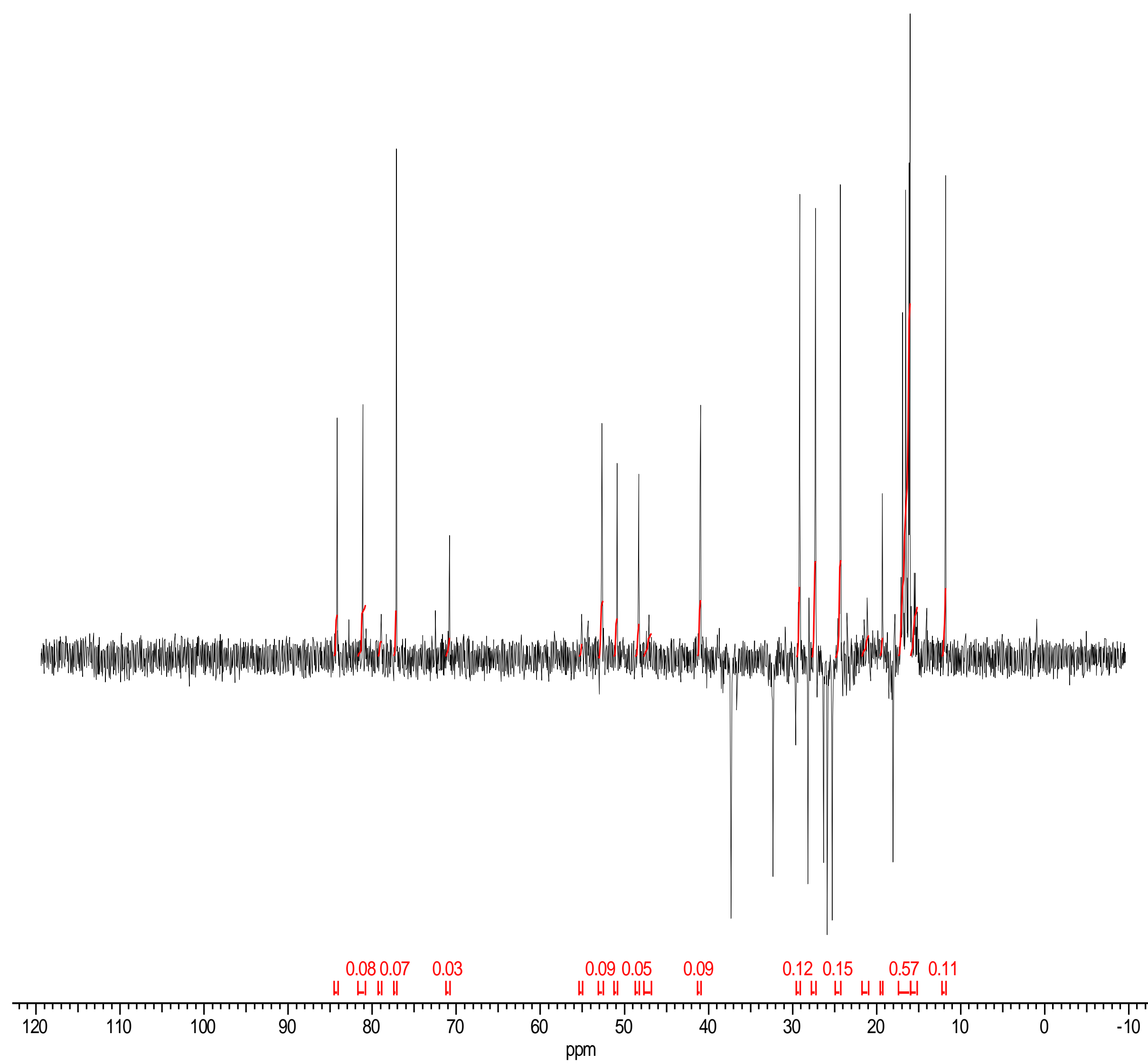
Appendix 7: HMBC Spectrum of compound **20**



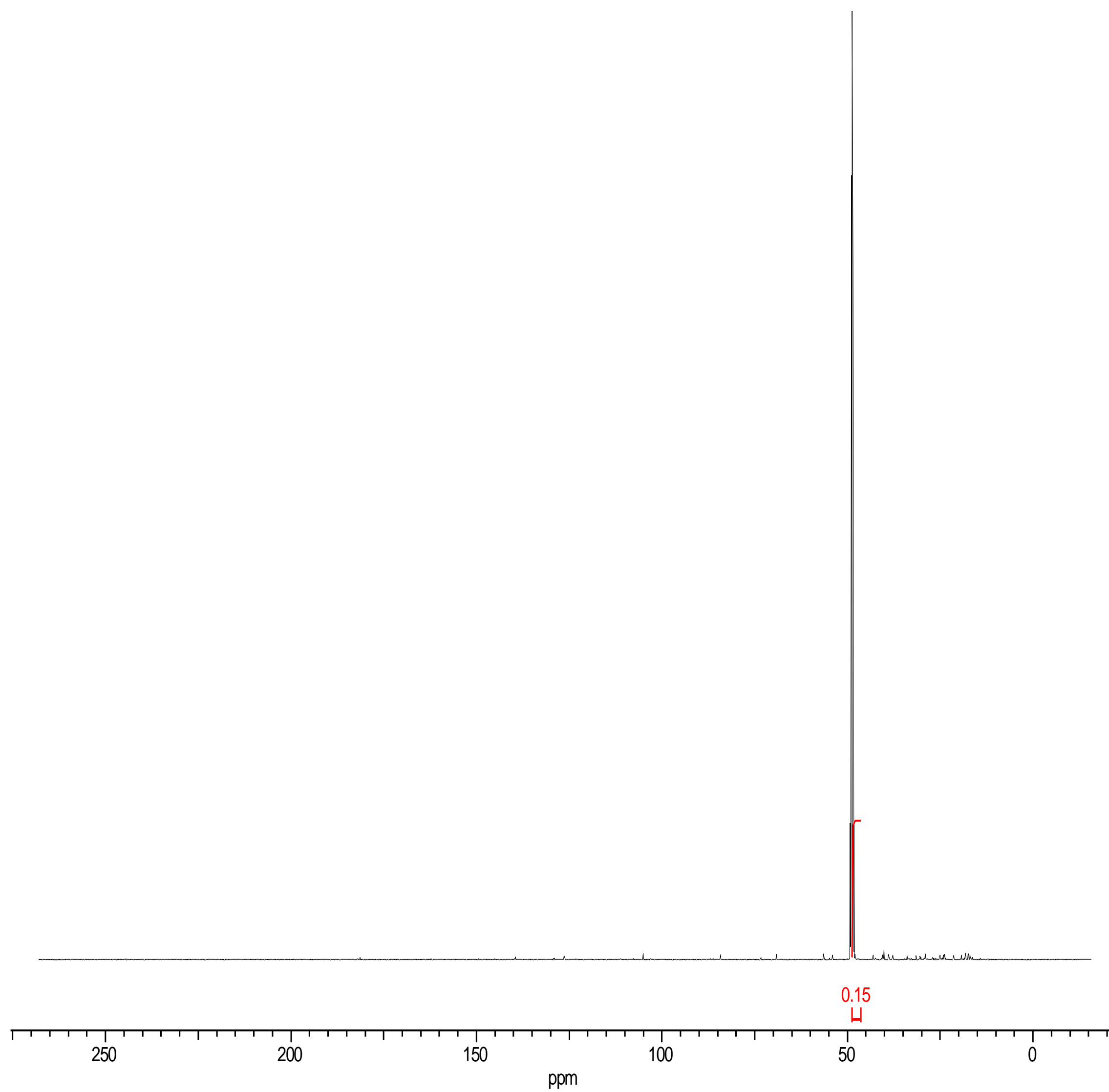
Appendix 8: COSY Spectrum of compound **20**



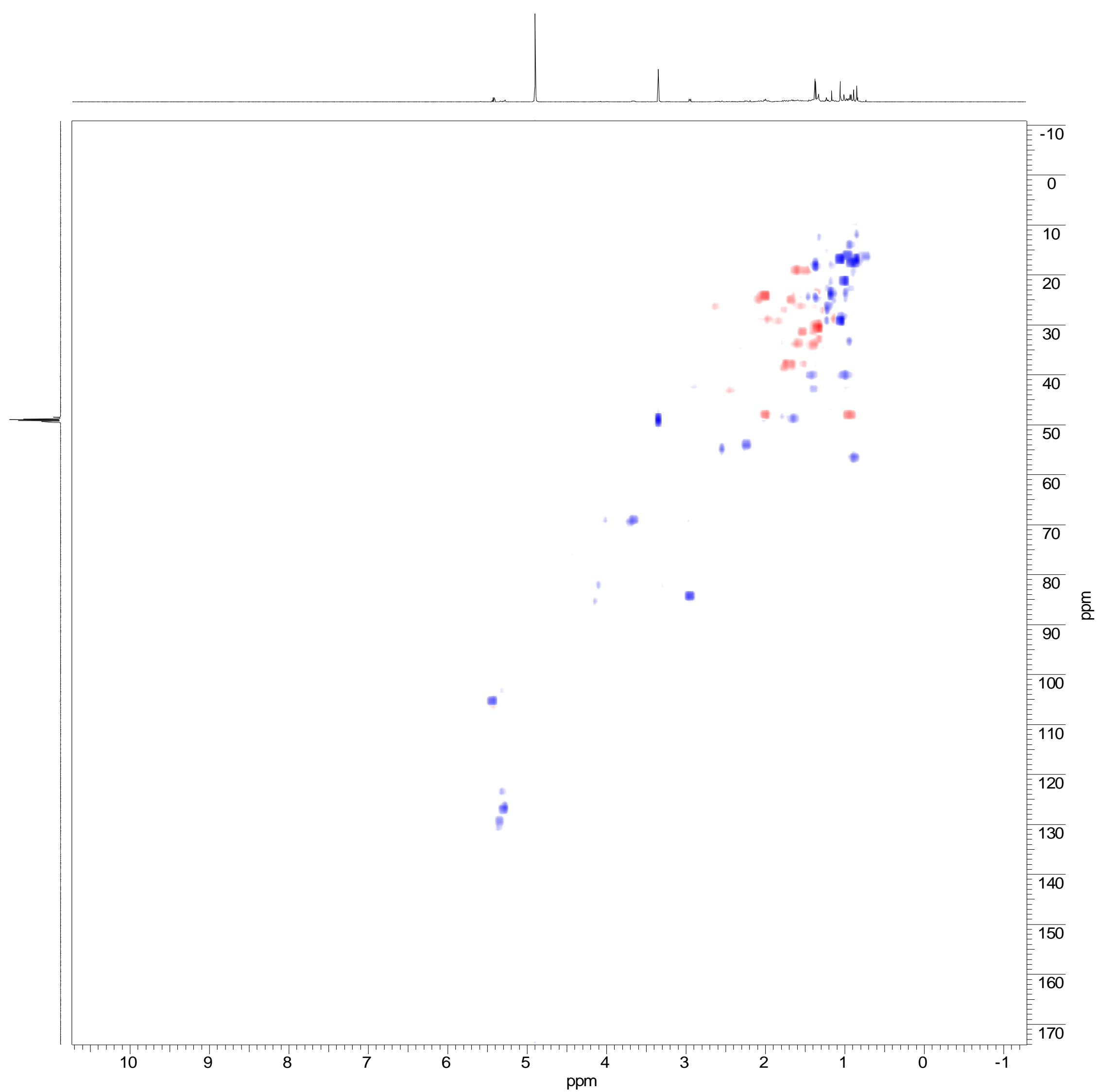
Appendix 9: DEPT Spectrum of compound **20**



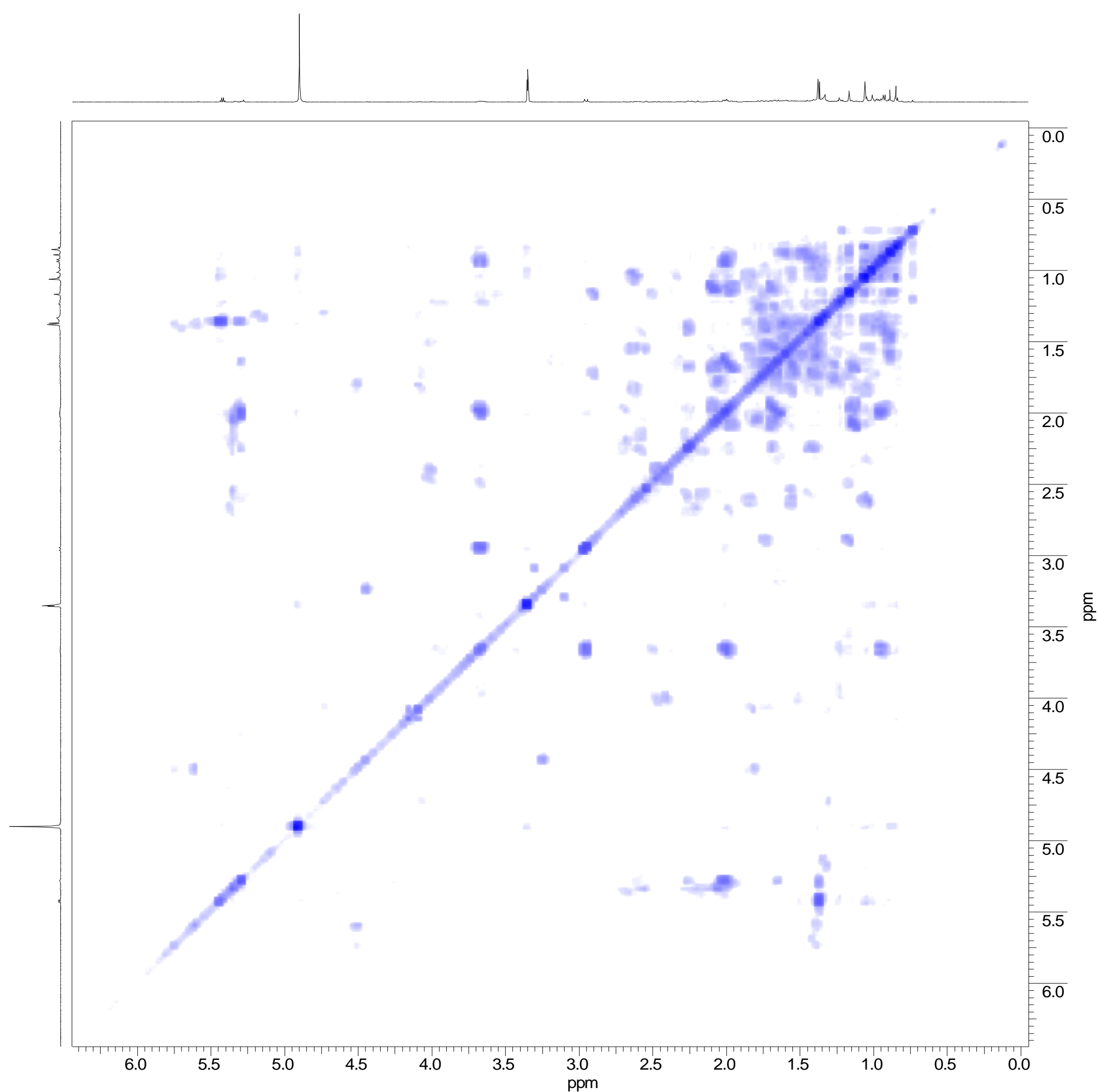
Appendix 10: ^{13}C NMR of compound **21**



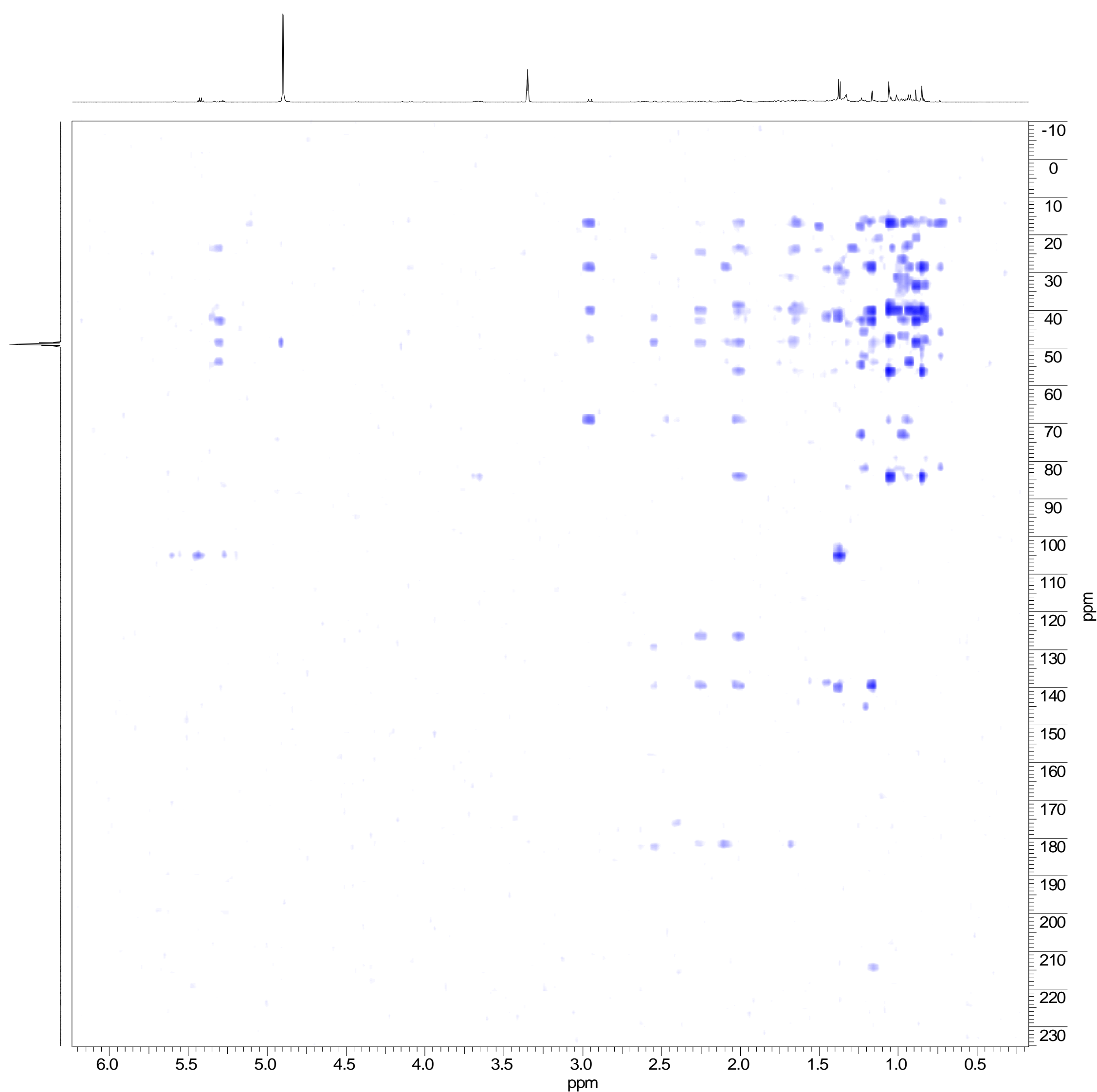
Appendix 11: HSQC Spectrum of compound **21**



Appendix 12: COSY Spectrum of compound **21**



Appendix 13: HMBC Spectrum of compound **21**



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