

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY



SCHOOL OF APPLIED NATURAL SCIENCE

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MSC THESIS

**PHYTOCHEMICAL, ANTIBACTERIAL AND ANTIOXIDANT STUDIES OF THE
FLOWER EXTRACTS OF *VERNONIA AMYGDALINA***

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Adama, Ethiopia

**PHYTOCHEMICAL, ANTIBACTERIAL AND ANTIOXIDANT STUDIES OF
THE FLOWER EXTRACTS OF *VERNONIA AMYGDALINA***

M.Sc. THESIS

Abere Habtamu Manayia

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ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

**PHYTOCHEMICAL, ANTIBACTERIAL AND ANTIOXIDANT
STUDIES OF THE FLOWER EXTRACTS OF *VERNONIA*
*AMYGDALINA***

**A Thesis Submitted to the school of applied natural science,
Program of applied chemistry, School of Graduate Studies.**

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

**In Partial Fulfillment of the Requirements for the Degree of
MASTER OF SCIENCE IN CHEMISTRY (ORGANIC CHEMISTRY)**

By

Abere Habtamu

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ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

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We hereby certify that we have read and evaluated this Thesis titled “**phytochemical, antibacterial and antioxidant studies of the flower extracts of *Vernonia amygdalina***” proposed under our guidance by **Abere Habtamu Manayia**. We recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

This thesis manuscript is dedicated to all my families and my beloved brother, **Charter Habtamu**, who departed from this world when I was grade four student, on November 21, 2000.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this thesis is my own work and I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis and compilation of this thesis. Any scholarly matter that is included in the thesis has been given recognition through citation.

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ABBREVIATIONS AND ACRONYMS

Br. s	broad singlet	MIC	Minimum Inhibition Concentration
CC	Column Chromatography	NMR	Nuclear Magnetic Resonance
COSY	CORrelation SpectroscopY	PTLC	Preparative Thin Layer Chromatography
DEPT	Distortionless Enhancement by Polarisation Transfer	Q	Quartet
<i>d</i>	Doublet	<i>s</i>	Singlet
DPPH	2,2-Diphenyl-1-Picrydylhydrazil	TLC	Thin Layer Chromatography
EtOAc	Ethyl Acetate	TM	Traditional Medicine
HMBC	Heteronuclear Multiple Bond Correlation	TMP	Traditional Medicinal Plants
HSQC	Heteronuclear Single Quantum Correlation	<i>t</i>	Triplet
IR	Infrared Spectroscopy	UV-Vis	Ultraviolet Visible Spectrophotometer
<i>m</i>	Multiplet	WHO	World Health Organization
MeOH	Methanol		

BIOGRAPHIC SKETCH OF THE AUTHOR

The author was born on October 07, 1989 in Amhara region, East Gojjam zone, Gozamn Woreda, Debre Markos. He first started his education at Mehal-Amba Primary School in 1995 and continued in Yebokila Primary School from 2000 to 2004. He then attended his secondary and preparatory educations at Ginbot-20 Senior Secondary and Preparatory School from 2005 to 2008. He first joined Debre Markos University in 2009 and graduated with ranked first within B.Sc. in Chemistry in 2011. After graduation, the author had worked in Wonji-Shoa sugar factory as of quality control expert and worked as of such position in there, he joined Adama Science and Technology University, school of applied natural science program of applied chemistry in 2015/16 to pursue his postgraduate studies in Organic Chemistry.

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Contents	Pages
STATEMENT OF THE AUTHOR	VI
ABBREVIATIONS AND ACRONMYS	VII
BIOGRAPHIC SKETCH OF THE AUTHOR.....	VIII
ACKNOWLEDGEMENTS	IX
LIST OF TABLES	XIII
LIST OF FIGURES, PICTURE AND SCHEME	XIV
ABSTRACT	XV
1. INTRODUCTION.....	1
1.1 Background	1
1.2 Statement of the Problem.....	3
1.3 Justification of the study	3
1.4 Significance of the study.....	3
1.5. Objectives of the Study	4
1.5.1. General objective.....	4
1.5.2. Specific objectives.....	4
2. LITTERATURE REVIEW.....	5
2.1. The family of Asteraceae	5
2.2. The genus <i>Vernonia</i>	5
2.2.1. Biological activities of the genus <i>Vernonia</i>	6
2.2.2. Phytochemistry of the genus <i>Vernonia</i>	6
2.3. <i>Vernonia amygdalina</i>	8
2.3.1. Botanical Classification.....	8
2.3.2. Reported Biological Activities.....	9
2.3.3. Reported Chemical Constituents.....	11
3. MATERIALS AND METHODS	13
3.1. Experimental sites.....	13

3.2. Instruments and Apparatus.....	13
3.3. Chemicals and Reagents	13
3.4. Experimental Methods	14
3.4.1. Collection of the plant material.....	14
3.4.2. Extraction.....	14
3.5. Phytochemical screening tests of the flower extracts of <i>V. amygdalina</i>	16
3.6. Isolation of compounds from the crude extracts of the flowers of <i>V. amygdalina</i>	17
3.6. 1. Isolation of compounds from the chloroform extract.....	17
3.6.2. Isolation of compounds from the acetone extract	18
3.7 Biological activities of the extracts and isolated compounds of <i>V. amygdalina</i>	20
3.7.1. Antioxidant activity	20
3.7.2. Antibacterial activities.....	21
4. RESULTS AND DISCUSSION	22
4.1. General overview.....	22
4.2. Percentage yield of the crude extracts	22
4.3. Phytochemical Screening of crude extracts of <i>V. amygdalina</i>	22
4.4 Antioxidant activity	23
4.5. Antibacterial activities	25
4.6. Structural elucidation of isolated compounds from the chloroform extract	28
4.6.1. Characterization of compound VAC-F1.....	28
4.6.2. Characterization of compound VAC-F16	29
4.7. Structure elucidation of isolated compound from acetone extract	32
4.7.1. Characterization of compound VAA-F16-17	32
5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS	37
5.1 Summary	37
5.2. Conclusion and Recommendation.....	38
6. REFERENCES	39

7. LIST OF APPENDIX.....	48
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LIST OF TABLES

Tables	Pages
Table 1: Column chromatographic fractionation of the chloroform extracts of the flowers <i>V. amygdalina</i>	18
Table 2: Column chromatographic fractionation of the acetone extracts of the flowers of <i>V. amygdalina</i>	19
Table 3: Fractionation of VAA-F22 of the acetone extract of the flowers of <i>V. amygdalina</i>	20
Table 4: Phytochemical screening results of chloroform and acetone extracts of flowers of <i>V. amygdalina</i>	23
Table 5: Radical scavenging activities of the extracts and constituents of flowers of <i>V. amygdalina</i>	24
Table 6: Inhibition zone diameter of chloroform extract, acetone extract, vernolide, isorhamnetin, antibiotics and distilled water.....	27
Table 7: ¹ H and ¹³ C-NMR spectral data of VAC-F1	29
Table 8: ¹ H and ¹³ C-NMR spectral data of VAC-F16 and literature reported for vernolide.....	31
Table 9: ¹ H and ¹³ C-NMR spectral data of VAA-F16-17 and literature reported for isorhamnetin (δ in ppm, J in Hz).....	34
Table 10: ¹³ C-NMR spectral data of minor peaks in VAA-F16-17 and literature reported for luteolin.....	36

LIST OF FIGURES, PICTURE AND SCHEME

Figures	Pages
Figure 1: Sesquiterpene lactones reported from <i>V. amygdalina</i> (1-4) and <i>V. scorpioides</i> (5-6).....	7
Figure 2: Elemanolide sesquiterpene lactones reported from <i>V. lasiopus</i> (7-9).....	7
Figure 3: Steroid glycosides reported from the leaves of <i>V. amygdalina</i> (10-13).....	11-12
Figure 4: Sesquiterpene lactones reported from the leaves of <i>V. amygdalina</i> (14-16).....	12
Figure 5: Chemical structure of tricosane (VAC-F1).....	28
Figure 6: Chemical structure of vernolide (VAC-F16).....	32
Figure 7: Chemical structure of isorhamnetin (VAA-F16-17).....	33
Figure 8: Chemical structure of luteolin (minor peak of VAA-F16-17).....	35
 Pictures	
Picture 1: Aerial part of <i>Vernonia amygdalina</i> from Wonji on February 12, 2017(by Abere Habtamu).....	9
Picture 2: Antibacterial activities of the flower of <i>V. amygdalina</i>	25-26
 Scheme	
Scheme 1: Successive extractions of the flowers of <i>V. amygdalina</i>	15

ABSTRACT

Medicinal plants are the raw materials for both traditional medicine and conventional medicine and 90% of all traditional medicines are estimated to be plant based. Vernonia amygdalina is a soft woody shrub/tree belonging to the family Asteraceae and genus Vernonia. The flowers of V. amygdalina were extracted successively with CHCl₃ and acetone to furnish 1.78 and 1.91%, respectively. The phytochemical screening test of the chloroform extract of the flower of V. amygdalina revealed the presence of secondary metabolites including alkaloids, quinones, saponins, steroids, tannins and terpenoids where as the acetone extract showed the presence of alkaloids, flavonoids, phenols, quinones, saponins, steroids and terpenoids. The CHCl₃ extract after silica gel column chromatography has led to the isolation of two compounds identified as tricosane (VAC-F1) and vernolide (VAC-F16). On the other hand, the acetone extract furnished isorhamnetin (VAA-F16-17) and luteolin. Structure elucidations of these compounds were achieved using various spectroscopic methods such as NMR, IR and UV-Vis. The extracts and constituents were assessed for their radical scavenging activity. Results showed that the acetone extract and isorhamnetin scavenged the DPPH radical by 91.6 and 94%, respectively. The result is comparable with ascorbic acid (97%) used as positive control. Furthermore the extracts, isorhamnetin and vernolide were also evaluated for their antibacterial activity against three gram negative (Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis) and two gram positive (Staphylococcus aureus and bacillus) bacteria. The CHCl₃ extract and vernolide showed strong activity against S. aureus with an inhibition zone of about 21 and 19 mm, respectively. The acetone extract and isorhamnetin were active against all bacterial pathogens tested in this study. The biological activity displayed by the extracts and constituents of this plant corroborate the traditional uses of this plant by the local people against various diseases.

Key words: Medicinal plant, Asteraceae, Vernonia, V. amygdalina, phytochemical screening, Antibacterial, antioxidant, vernolide, isorhamnetin and luteolin

1. INTRODUCTION

1.1 Background

Nature has provided us a variety of valuable resources for the survival of all living organisms on Earth. Among these plants are vital to mankind, animals and environment all over the world. They are primarily used as foods and medicines beginning from prehistoric times. The term medicinal plants include various types of plants used in herbalism and some of these plants have medicinal activities. Medicinal plants are the richest bioresource of drugs for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. These medicinal plants are rich sources of ingredients which can be used in drug development and synthesis. Besides, these medicinal plants play a critical role in the development of human cultures around the whole world. History and numerous studies have shown that medicinal plants are sources of diverse nutrient and non-nutrient molecules. The leaves, flowers, berries, barks and/or roots of plants are traditionally used as antibacterial, antioxidants, antimalarial, analgesics, and for several other ailments which can protect the human body against both cellular oxidation reactions and pathogens [1].

Most African countries are very poor, with highly under developed health care systems. A larger percentage of the African population is living below the poverty line and cannot afford expensive conventional medicinal drugs [2]. Herbal medicine has therefore provided the best alternative method for their health care to treat themselves from different ailments of the disease. Many people are now turning to herbal medicine as a source of medication [3-4].

In view of this, the majority of Ethiopian's population still depend on traditional medicine mainly due to shortage of pharmaceuticals, inadequate coverage of modern medical system and unaffordable prices of modern drugs [5]. Traditional medicinal practices are common in Ethiopia in which about 80% of the population in the country use plant based TM by indigenous knowledge as their major primary health care system [6]. The ways TM are used as diverse as the different cultures existing in the country. Traditional medicine healing practice is not only concerned with curing of diseases but also with the protection and promotion of human physical, spiritual, social, mental and material well-being [7].

Medicinal plant products are affordable especially for the rural people. Most of the plants are locally available, accessible and inexpensive. The other aspect is its biodegradability and therefore causing minimum environmental pollution as opposed to the conventional drugs where drug spillage and their containers are part of the environmental pollution. The medicinal plants are also socially accepted by many communities especially in the rural areas as they have been in use for a long time, Furthermore, the use of medicinal plant products are part of the livelihoods of majority of the people who are involved in conservation, harvesting, marketing and even the traditional practitioners [8].

The other advantage on the usage of medicinal plants is that they have little side effects as compared to conventional drugs and anybody may not expense more money on how to use them since there are local expertise on the usage of these plants. Medicinal plants are also the raw materials for both traditional medicine and conventional medicine and 90% of all traditional medicines are estimated to be plant based [9]. Herbal medicines are currently a major demand for human necessity and their popularity is increasing from day to day. Herbal drugs referred to as plant materials or herbals, involves the use of whole plants or parts of plants, to treat injuries or illnesses [10].

Medicinal plants serve as a vast pool of many organic compounds produced either as a result of the organism adapting to its surrounding environment or for its own survival and defence against predators. These compounds, called secondary metabolites, are responsible for the biological activities elicited by medicinal plants. Hence, an attention was given to isolate these compounds from medicinal plants which are used for their healing properties in addition to their use for the production of plant derived modern drugs. It was also noted that, there have also been increased waves of interest in the field of research in natural products chemistry. This level of interest can be attributed to several factors, including unmet therapeutic needs, the remarkable diversity of both chemical structure and biological activities of naturally occurring secondary metabolites, the utility of novel bioactive natural compounds as biochemical probes, the development of novel and sensitive techniques to detect biologically active natural products, improved techniques to isolate, purify, and structurally characterize these active constituents, and advances in solving the demand for supply of complex natural products [11].

Ethiopia is considered as home for many species of higher plants; making the country one of the most diverse floristic regions in the world. The country possesses a wide range of indigenous and endemic medicinal plants that are used for the treatment of various ailments. *Vernonia amygdalina* is traditionally used to treat many ailments including diabetes [12-13], anti-helminth, anti-malarial, laxative, digestive tonic, appetizer, febrifuge and for the topical treatment of wounds [14]. Stems are used as chew sticks for oral hygiene [14].

In Ethiopia the leaves of the plant are used to treat skin wound by Zay people who live on the islands of Lake Zway, South of Addis Ababa [15]. Its traditional use as antifertility agent is reported. The present study therefore aims to investigate the phytochemical constituents, antioxidant and antibacterial activities of the flower extracts of *V. amygdalina*.

1.2 Statement of the Problem

In some African countries including Ethiopia, *V. amygdalina* is among medicinally significant plant which is used against malaria, helminth infections, gastrointestinal disorders and fever [16]. The species is also used to promote wound healing [17] and to treat microbial infections [18].

1.3 Justification of the study

Despite numerous report on the secondary metabolite profile of the leaves, to the best of our knowledge there is no prior scientific report on the chemical constituents of the flowers of this species. Furthermore, the antioxidant and antibacterial activities of the flower extracts and constituents of *V. amygdalina* have not been reported in the literature. Hence this thesis also addresses the antioxidant and antibacterial activities of the flower extracts and constituents of *V. amygdalina*.

1.4 Significance of the study

In the past, humans used medicinal plants traditionally for controlling different diseases. It is evident that some disease causing micro-organisms that were previously controlled by conventional medicine have undergone mutations and developed resistance to these drugs. Therefore continuous searching of new drugs that helps to overcome this emerging resistance is necessary.

Since many people in Ethiopia use *V. amygdalina* against various disease, this study may provide scientific evidences to support many of the claimed health benefits of this plant. The study made on chemical constituents may help to arrive at new natural products.

The strong antioxidant activity displayed by the extract may be used as a source of natural antioxidant. The study may also provide information for the researchers who want to continue a related study on other related medicinal plant.

1.5. Objectives of the Study

1.5.1. General objective

The main objective of this study is to conduct phytochemical, antibacterial and antioxidant studies of the flower extracts of *V. amygdalina*

1.5.2. Specific objectives

- ✓ To successively extract the flowers of *V. amygdalina* with chloroform and acetone.
- ✓ To isolate compounds from the crude extracts of the flowers of *V. amygdalina* using column chromatography and PTLC.
- ✓ To evaluate the antibacterial activities of the crude and pure constituents of the flower extracts of *V. amygdalina* by Disk diffusion method
- ✓ To investigate the radical scavenging activities of extracts and pure constituents of the flowers of *V. amygdalina* by DPPH method.
- ✓ To elucidate the structures of the isolated compounds employing spectroscopic methods including UV-Vis, IR and NMR.

2. LITTERATURE REVIEW

2.1. The family of Asteraceae

The flowering plants (Angiospermae) are the most wide spread group of land plants. It comprises of about 445 families, 12,000 genera and 300,000 species [19-20]. Asteraceae, probably the largest family of flowering plants, consists of 1,620 genera and more than 23,600 species, growing from sea-level to the highest mountain peak [21]. It is distributed in all continents including North America, Eastern Brazil, Australia, Southern Africa, the Mediterranean region, Central Asia, and South Western China [22]. But it does not grow in Antarctica. The majority of Asteraceae species are herbaceous, yet an important component of the family is constituted by shrubs or even trees occurring primarily in the tropical regions of North and South America, Africa and Madagascar [23].

2.2. The genus *Vernonia*

Vernonia is one of the largest genera in the Asteraceae family [24], consisting of more than 1,000 species distributed in the tropical and sub-tropical region of Africa, Asia and America. It has two major centres of origin, South America and tropical Africa, with approximately five hundred species found in Africa and Asia, three hundred in Mexico, Central and South America [25]. Most of the *Vernonia* species grow in various habitats, forest margins, acacia wood lands, etc, and some of the species grow well in areas that receive little annual rainfall, as low as about 200 mm [26]. They are found distributed in areas with altitude range from 1,600 m to 3,000 m above sea level [27]. Of the 500 species from Africa, about 49 species are grown in Ethiopia [27].

Some of the species in the genus *Vernonia* used in ethnomedicine include *Vernonia amygdalina*, *Vernonia condensata*, *Vernonia cineria*, *Vernonia guineensis* and *Vernonia conferta*. *Vernonia amygdalina* and *Vernonia colorata* are eaten as leafy vegetables and *Vernonia galamensis* is used industrially for its seed oil contents [28]. Different species of the genus *Vernonia* have wide variety of uses. Some of the species are used as medicinal plants [29], some are used as fire wood and others are potential sources of chemicals of industrial importance [30].

2.2.1. Biological activities of the genus *Vernonia*

In traditional medicine, many *Vernonia* species are employed to treat various diseases. The use of many *Vernonia* species for treatment of illnesses is recorded from Tanzania, Nigeria, China and Ethiopia [31-33]. *Vernonia* species are used in the treatment of infectious and parasitic diseases. The majority of these plants are used as vegetables. The vegetables have a bitter taste, hence the name “the bitter was given to the genus *Vernonia*” [34]. Amongst the different *Vernonia* species, *Vernonia amygdalina*, *Vernonia cinerea*, *Vernonia colorata*, *Vernonia guineensis* and *Vernonia kotschyana* are the top five most frequently used species for the treatment of various ailments [28]. So lot of attention has been paid to *Vernonia* species due to their diverse biological activities, particularly in bactericidal, hepatoprotective, and antitumor application. From the pharmacological point of view, species have been investigated revealing many properties, such as antiplasmodial [35], analgesic [36], anti-inflammatory [37], antimicrobial [38], antidiabetes [39], antioxidant [40] and antitumor [41]. Reference has also been made to the effect that the biological activity of several *Vernonia* species could be attributed to the presence of flavonoids in the plants especially their antioxidant activities [42-43].

2.2.2. Phytochemistry of the genus *Vernonia*

The biological activities displayed by many species in the genus has prompted phytochemists to investigate the phytochemical constituents of the *Vernonia* plants, which has led to the identification of many bioactive compounds including alkaloids, flavonoids and terpenoids. A relatively small number of species of the *Vernonia* genus have been reported to contain alkaloids. Qualitative phytochemical studies have led to the identification of alkaloids in *Vernonia ambigua* [34], *Vernonia amygdalina* [44-46], *Vernonia blumeoides* [34], *Vernonia cinerea* [47], *Vernonia colorata* [48], *Vernonia condensata* [49], *Vernonia kotschyana* [50], *Vernonia oocephala* [34] and *Vernonia patula* [51].

Flavonoids are among the major classes of compounds encountered in the *Vernonia* genus [52]. Most of the flavones and phenolic compounds have been isolated from *Vernonia amygdalina* and *Vernonia cinerascens* and have exhibited potent antioxidant as well as urease inhibitory activity [53-54].

Members of the genus *Vernonia* are an excellent source of sesquiterpene lactones which include vernolide (1), vernolepin (2), vernodalin (3) and hydroxyl vernolide (4) [55-58], glaucolide (5) and hirsutinolide (6) [59], 8-desacylvernolide (7), vernomenin (8) and 11,13-dihydrovernodalin (9) [60] (Figure 1 and 2).

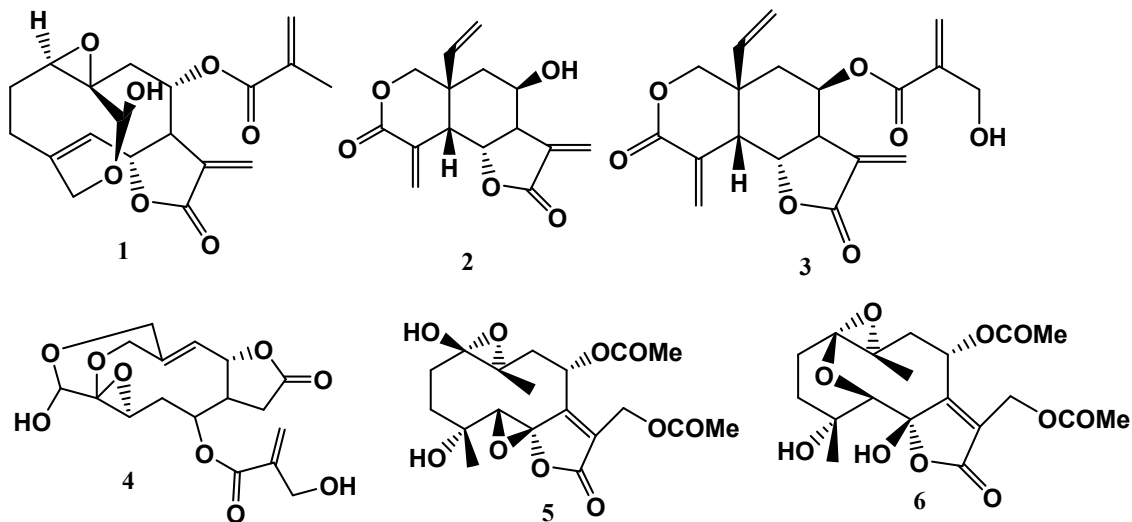


Figure 1: Sesquiterpene lactones reported from *V. amygdalina* (1-4) and *V. scorpioides* (5-6)

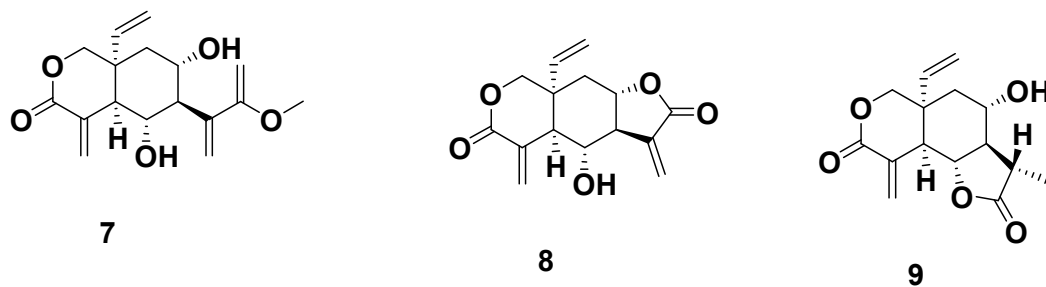


Figure 2: Elemanolide sesquiterpene lactones reported from *V. lasiopus* (7-9)

2.3. *Vernonia amygdalina*

Vernonia amygdalina Del. is a soft woody shrub or tree belonging to the family Asteraceae and genus *Vernonia*. It is a perennial plant characterized by its bitter sap from the leaf which has been widely explored for its medicinal use. This plant grows to 10 m tall with petiole leaf of about 6 mm in diameter (**Picture 1**), and elliptic in shape [61]. It is known by different names by different people across the West and Central African regions as bitter leaf (English), *Buzut*, *Giraw*, *Grawa*, *Ibicha* (Ethiopia), *Ated nkol*, *Suwaaka* (Cameroon), *Etidod*, *Ewuro*, *Ejije*, *Onugbo*, *Shiwaka*, *Olugbo* (Nigeria), and *Musikavakadzi* (Zimbabwe) where it is commonly grown either as vegetable or hedge [61]. In the Ethiopian highland, *V. amygdalina* has been classified by the farmer as a multipurpose fodder tree with high biomass yield, easy propagation, high adaptability and high compatibility with other crops which do not compete with them for soil nutrients or moisture but instead help to improve the soil fertility and growth of perennial crops [62]. *V. amygdalina* is quite commonly used in Ethiopia in the preparation of local beer (TELLA in Amharic) and also as fumigant.

2.3.1. Botanical Classification

According to the international classification code of botanical nomenclature the present taxonomic position of *Vernonia amygdalina* herb scientifically classified as follows:

Kingdom: plantae

Division: Angiosperms

Order: Asterales

Family: Asteraceae

Genus: *Vernonia*

Species: *V. amygdalina*

Botanical Name: *Vernonia amygdalina*

The leaves are succulent and corolla purple or turquoise. Plants annual with the roots not surviving beyond the life span of the aerial stems. Plants perennial with thick root stocks, shrubs or small trees. Outer and middle phyllaries appendaged; appendages usually dark green, narrowly lanceolate and often refluxed; corolla 13-18 mm long.



Picture 1: Aerial part of *Vernonia amygdalina* from Wonji on February 12, 2017(by Abere Habtamu)

2.3.2. Reported Biological Activities

Traditionally the leaves of this plant are used to expel placenta after birth, aid post-partum uterine contraction, induce lactation and control post-partum haemorrhage [63]. Many herbalists and native doctors in Africa recommend its aqueous extracts for their patients as treatment for varieties of ailments ranging from emesis, nausea, diabetes, loss of appetite, dysentery and other gastrointestinal tract problems to sexually transmitted diseases and diabetes mellitus among others [64]. The leaves are used as green leafy vegetable and may be consumed either as a vegetable (leaves are macerated in soups) or aqueous extracts used as tonics for the treatment of various illnesses [65]. In the wild, chimpanzees have been observed to ingest the leaves when suffering from parasitic infections [66-68].

Many experimental studies of *V. amygdalina*, have reported that this plant possesses antibacterial activity. [69] showed that this plant has mild antimicrobial effect on rumen bacteria and protozoa while [70] proved that acetone extract of *V. amygdalina* possesses antibacterial activity towards *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Micrococcus kristinae*, *Staphylococcus aureus*, *Enterobacter cloacae* and *Escherichia coli* growth with minimum inhibition concentration (MIC) of 5 mg/mL.

Although, [71] concluded that *V. amygdalina* was more sensitive towards the gram positive bacteria than gram negative bacteria; some researchers found that the activity of *V. amygdalina* against gram-negative bacteria was comparable to that towards the gram-positive species.

The antibacterial property of *V. amygdalina* was proved to be beneficial in a few applications. For example, it was proposed that this plant is suitable for use in beer industry [72], since it inhibit the growth of *Bacillus circulans*, *Aerococcus viridans*, *Clostridium perfringens* and *Micrococcus spp.* bacteria but promote alcohol production of brewer's yeast. while [73] claimed that the antibacterial activity of its Ethanolic leaf extract against *C. sporogenes* was able to revert the blood, protein and bilirubin urinary to basal level and increase neutrophil and white blood cell count as well as packed cell volume (PCV) in blood, thereby reducing haemolysis caused by infection.

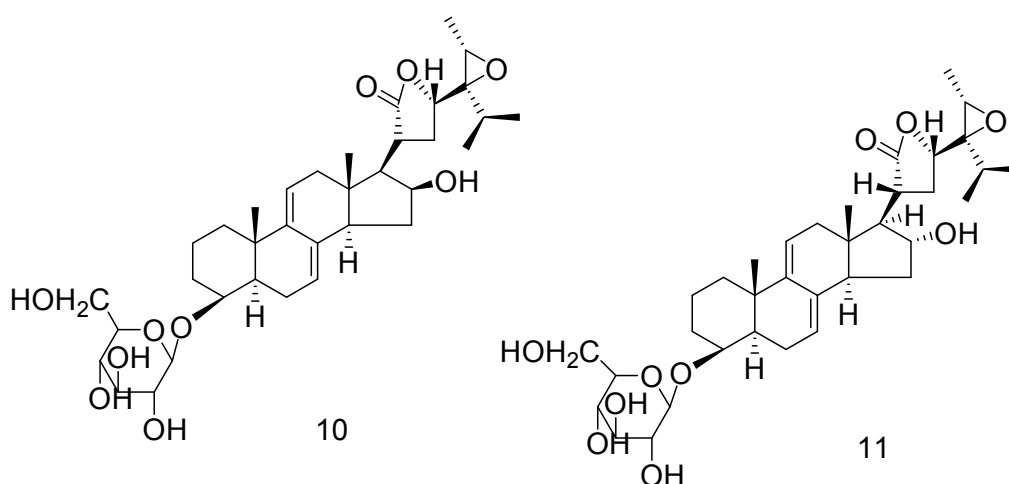
Besides, this plant has also been suggested for use as chewing stick to maintain oral health by dislodging carcinogenic micro-organisms [74], in line with the traditional use of this plant for mouth cleaning. Saliva extracted from *V. amygdalina* chewing stick can maintain oral cleanliness by contributing to gum healing, *agalgesia*, *antisickling*, *haemostasis* and antimicrobial activity and plaque inhibiting effect. This was supported by the finding that cold aqueous extract of *V. amygdalina* whole stem, bark and pulp extract showed bactericidal activity against the oral anaerobic bacteria: *Bacteroides gingivalis*, *B. asaccharolyticus*, *B. melaninogenicus* and *B. oralis* [75].

The water extract of *V. amygdalina* leaves inhibit the growth of *Fusarium moniliforme* on seeds of maize (Zea Mays) as well as mycelial and conidial growths of *Colletotrichum gloeosporioides* in rubber tree [76-78]. Cold water extract of stem bark and root bark (but not leaves) was able to inhibit *Colletotrichum capsici* (Synd) isolated from pepper [79]. On the other hand, the juice of *V. amygdalina* showed stronger effect than its cold water extract where its juice could more effectively inhibited seed borne fungi (*F. moniliforme*, *Botryodiplodia theobromae*, *Aspergillus niger* and *Aspergillus flavus*) in *vitro* and in *vivo* [80].

2.3.3. Reported Chemical Constituents

Several investigators have isolated and characterized a number of chemical constituents of the compounds with potent biological activities from the leaves of *Vernonia amygdalina*. Some of the constituents in which previously isolated from *Vernonia amygdalina* plant include: steroid glucoside and Sesquiterpene lactones. The plant has bitter taste accounted to the presence of anti-nutritional factors such as alkaloids, saponins, tannins, and glycosides [81].

Steroid glucosides are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs [82]. One of the major steroid glucoside compounds that have been identified from *V. amygdalina* is the vernoniosides including: vernonioside A1 (**10**), vernonioside A2 (**11**), vernonioside B1 (**12**) and vernonioside B2 (**13**) (**Figure 3**). These glucoside compounds can be isolated from the leaf, stem and root parts of the plant [83-84].



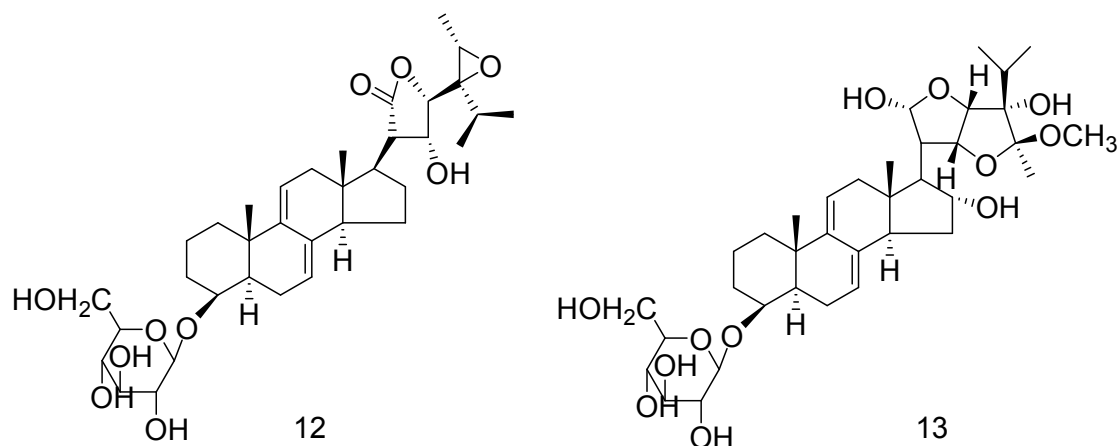


Figure 3: Steroid glucosides reported from the leaves of *V. amygdalina* (10-13)

The sesquiterpene acts as irritants when applied externally and when consumed internally. Their action resembles that of gastrointestinal tract irritant. A number of sesquiterpene lactones phytoconstituents have been isolated from *V. amygdalina* and those have been broad antimicrobial (particularly antiprotozoal) and neurotoxic action. Some of the bioactive sesquiterpene lactone compounds (**Figure 4**) that have been isolated from *V. amygdalina* includes; vernodalin (**3**), vernolepin (**2**), vernomygdin (**14**), vernodalol (**15**) and vernodalinol (**16**) [85-88].

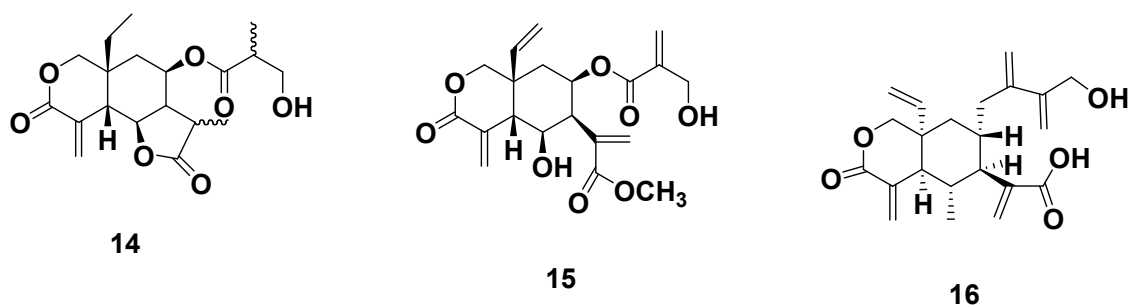


Figure 4: Sesquiterpene lactones reported from the leaves of *V. amygdalina* (14-16)

3. MATERIALS AND METHODS

3.1. Experimental sites

The solvent extraction of the *V. amygdalina* plant was done at Wonji/Shoa sugar factory Research Center. Phytochemical screening test, column chromatographic separation and antioxidant activity assay were done at PG laboratory of the Program of Applied Chemistry of Adama Science and Technology University. Whereas NMR, IR and UV-Vis were generated at Addis Ababa University Department of Chemistry research laboratory. Antibacterial activity tests were done at Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory Centre.

3.2. Instruments and Apparatus

The instruments and other materials that used in this study were: mill grinder (Retsch GmbH, Rheinische straBe 36, 42781 Haan, Germany), Analytical thin layer chromatograms were run on a readymade 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminium plate. Column chromatography was performed using silica gel (230-400 mesh) Merck. ¹H, ¹³C DEPT-135, HH-COSY, HSQC and HMBC spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. Infrared (IR) spectra were obtained on Perkin-Elmer 65FT ((IR $\nu_{\max}$ KBr (4000-400) cm⁻¹) infrared spectrometer using KBr pellets and UV-Vis Spectrophotometer (SAYANO, SP 65, GALANAKAMP, U.K, and PERKIN ELMER, NIR LAMBDA, 950).

3.3. Chemicals and Reagents

The list of chemicals that used in this study were: chloroform (99.4% GC grade Mumbai, India), Acetone (>99% GC grade Mumbai, India), Methanol (99.99% GC grade, United Kingdom), Hexane (99% Ranchem industry and trading) , Ethanol (100% GC grade Mumbai, India), Ethyl acetate (98% UNI-CHEM chemical reagents), Vanillin, Sulphuric acid , Hydrochloric acid, Potassium hydroxide (85% Mumbai, India), acetic anhydrous (British drug house ltd., UK), ferric chloride (British drug house ltd., England), Potassium iodide, Iodine, Distilled water, DPPH and magnesium ribbon.

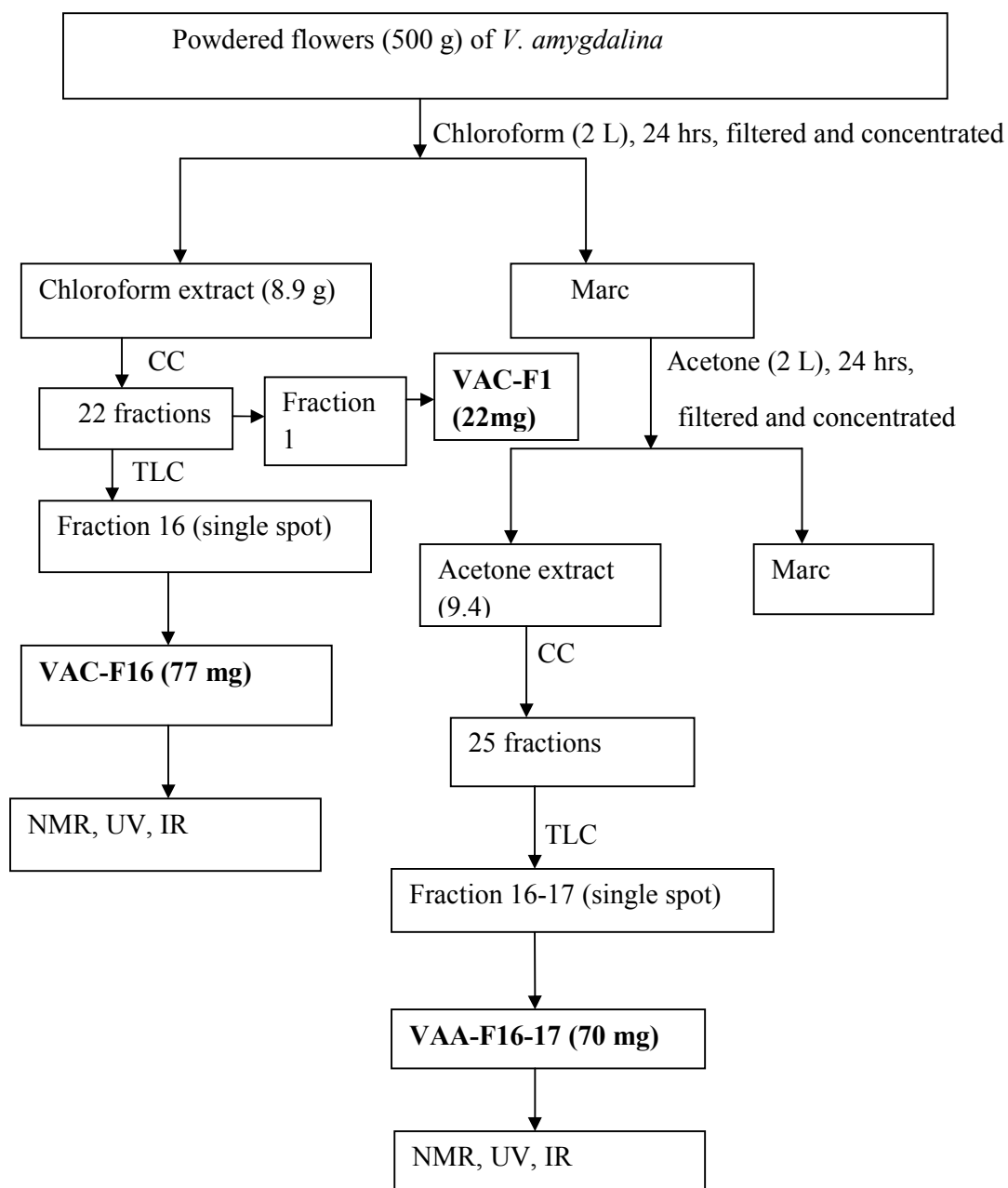
3.4. Experimental Methods

3.4.1. Collection of the plant material

The flowers of *V. amygdalina* were collected on February 12, 2017 from Wonji district, which is located at 107 km Southeast of Addis Ababa, Ethiopia. It was dried in an open air to protect from direct exposure to sun light at the laboratory of Wonji/shoa sugar factory Research Center.

3.4.2. Extraction

The whole air dried powdered flowers of *V. amygdalina* (500 g) were extracted with chloroform (2 L) for 24 hours at room temperature with frequent agitation on the automatic agitator. It was filtered and concentrated to furnish 8.9 g (1.78%). The marc was extracted with acetone (2 L) for 24 hours at room temperature with frequent agitation on the automatic agitator, filtered and concentrated to give 9.4 g (1.91%). The outline of the extraction procedure was shown in Scheme 1.



Scheme 1: Successive extractions of the flowers of *V. amygdalina*

3.5. Phytochemical screening tests of the flower extracts of *V. amygdalina*

The preliminary phytochemical screening for the crude extracts of the flowers of *V. amygdalina* was carried according to standard methods [89-90] to analyze the presence of secondary phytochemical metabolites including alkaloids, cardiac glycosides, flavonoids, phenols, quinones, saponins, steroids, tannins and terpenoids.

3.5.1. Test of alkaloids

Wagner's test: A fraction of extract was treated with 3-5 drops of Wagner's reagent [1.27 g of iodine and 2 g of potassium iodide in 100 mL of water] and observed for the formation of reddish brown precipitate (or coloration) [89].

3.5.2. Test of cardiac glycosides

Kellar-Kiliani test: 2 mL of each extract was treated with 1 mL of glacial acetic acid in a test tube and a drop of ferric chloride solution was added to it. This was carefully underlayered with 1mL concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form [89].

3.5.3. Test of flavonoids

Shinoda test: Four pieces of magnesium fillings (ribbon) are added to the extract in the beaker followed by few drops of concentrated hydrochloric acid. A pink or red color indicates the presence of flavonoids. Colours varying from orange to red indicate flavones, red to crimson indicates flavonoids and crimson to magenta indicates flavonones [90].

3.5.4. Test of phenols

Ferric chloride test: A fraction of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black color [89].

3.5.5. Test of quinones

A small amount of extract was treated with concentrated HCl and observed for the formation of yellow precipitate (or coloration) [89].

3.5.6. Test of saponins

Foam test: To 2 mL of extract was added 6 mL of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins [89].

3.5.7. Test of steroids

Salkowski test: Treat extract in chloroform with few drops of conc. sulfuric acid, shake well and allow standing for some time, red color appears at the lower layer indicates the presence of Steroids.

3.5.8. Test of tannins

Potassium hydroxide test: In to 10 mL of freshly prepared 10% potassium hydroxide (KOH) in a beaker, add 0.5 gm of extract and shake to dissolve. A dirty precipitate is observed which indicates the presence of tannin.

3.5.9. Test of terpenoids

Salkowski test: 1 mL of chloroform was added to 2 mL of each extract followed by a few drops of concentrated sulphuric acid. A reddish brown precipitate produced immediately indicated the presence of terpenoids.

3.6. Isolation of compounds from the crude extracts of the flowers of *V.*

amygdalina

The chloroform and acetone crude extracts of flower of *V. amygdalina* were fractionated using column chromatographic techniques over silica gel.

3.6. 1. Isolation of compounds from the chloroform extract

The chloroform extract of *V. amygdalina* (4.5 g) was first adsorbed in an equal amount of silica gel and eluted with hexane: ethyl acetate: methanol of increasing polarities to furnish 14 combined fractions (**Table 1**). A volume of 100 mL each was collected.

Table 1: Column chromatographic fractionation of the chloroform extracts of the flowers *V. amygdalina*.

Fractions	Eluent	Ratio of solvents	Amount obtained (mg)	Remark
VAC-F1	n-hexane: EtOAc	1:0	22	No spot
VAC-F2-6	„	9:1	709	2 spots
VAC-F7-9	„	4:1 and 7:3	438	Impure
VAC-F10	„	3:2	179	Impure
VAC-F11	„	1:1	120	2 spots
VAC-F12	:	2:3	134	One black spot
VAC-F13	„	3:7	100	2 spots
VAC-F14	„	1:4	41	Impure
VAC-F15	„	1:4	43	Impure
VAC-F16	“	1:9	77	Single spot
VAC-F17	„	0:100	319	Impure
VAC-F18	„	0:100	45	Impure
VAC-F19-21	EtOAc: MeOH	9:1up to 7:3	53	Impure
VAC-F22	“	6:4	52	Single

Where;

Impure= having more than 3 spot on TLC

All fractions after silica gel column chromatography were analyzed with TLC. Two fractions, **VAC-F1** and **VAC-F16**, were found to be pure samples.

3.6.2. Isolation of compounds from the acetone extract

Acetone extract of the flower of *V. amygdalina* (5 g) was adsorbed and fractionated over silica gel (180 g) column chromatography. Gradient elution was done using chloroform: methanol (**Table 2**) to furnish 15 combined fractions. A volume of 100 mL each was collected.

Table 2: Column chromatographic fractionation of the acetone extracts of the flowers of *V. amygdalina*

Fractions	Eluent (Ratio)	Amount in mg	Remark
VAA-F1-2	CHCl ₃	265	Impure
VAA-F3-4	CHCl ₃	82	2 spot
VAA-F5-9	CHCl ₃	87	3 spot
VAA-F10	CHCl ₃	111	Impure
VAA-F11	CHCl ₃ :MeOH (95:5)	156	Impure
VAA-F12	CHCl ₃ :MeOH (9:1)	127	Impure
VAA-F13	CHCl ₃ :MeOH (9:1)	328	Impure
VAA-F14-15	CHCl ₃ :MeOH (9:1)	261	2 spot
VAA-F16-17	CHCl ₃ :MeOH (85:15)	70	pure and single
VAA-F18	CHCl ₃ :MeOH (4:1)	130	Impure
VAA-F19-20	CHCl ₃ :MeOH (7:3)	185	Impure
VAA-F21	CHCl ₃ :MeOH (3:2)	126	Impure
VAA-F22	CHCl ₃ :MeOH (1:1)	100	Seems to pure
VAA-F23-24	CHCl ₃ :MeOH (9:11)	199	Impure
VAA-F25	CHCl ₃ :MeOH (2:3)	191	Impure

After TLC analysis, **VAA-F16-17** displayed a single spot on TLC and hence subjected to spectroscopic methods for determination of its structure.

VAA-F22 obtained from the column chromatographic fractionation of the acetone extract was adsorbed and subjected to silica gel for further purification. The column was eluted with ethyl acetate: methanol to furnish five sub-fractions each 100 mL (**Table 3**).

Table 3: Fractionation of **VAA-F22** of the acetone extract of the flowers of *V. amygdalina*

Fraction	Eluent (Ratio)	Fractions	Remark
1	EtOAc	VAA-F22-1	2 spot
2	„	VAA-F22-2	2 spot
3	EtOAc: MeOH (95:5)	VAA-F22-3	Pure
4	EtOAc: MeOH (9:1)	VAA-F22-4	Pure
5	EtOAc: MeOH (9:1)	VAA-F22-5	Pure

3.7 Biological activities of the extracts and isolated compounds of *V.*

amygdalina

3.7.1. Antioxidant activity

DPPH is the simplest and most widely reported method for screening antioxidant activity in foods and many plant extracts [91-92]. In this work, the DPPH assay was done according to the following procedure [93]: the Chloroform extract was dissolved in methanol to afford 1mg/mL. It was serially diluted in methanol to give concentration of 500, 250, 125 and 62.5 µg/mL. To 1 mL of each concentration, 4 mL DPPH (0.04%DPPH in MeOH) was added which brings the concentration to 100, 50, 25 and 12.5 µg/mL. This was repeated for the acetone extract and pure constituents. Then all the samples prepared were incubated in an oven at 37°C for 30 min. and then absorbance was recorded at 517 nm using UV-Vis spectrometer.

The percentage inhibition was calculated using the formula,

$$\% \text{ Inhibition} = (A_{\text{control}} - A_{\text{extract}}) / A_{\text{control}} \times 100.$$

Where A_{control} is the absorbance of DPPH solution and A_{extract} is the absorbance of the test sample (DPPH solution plus sample).

3.7.2. Antibacterial activities

The antibacterial activities of the extracts and constituents of the flower extracts of *V. amygdalina* were checked against five bacterial pathogens, three gram negative (*Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*) and two gram positive (*Staphylococcus aureus* and *Bacillus*). Bacterial cultures were maintained on nutrient Muller- Hinton agar at 37°C and the cultures were kept in appropriate media slants and stored at 4°C until used.

The antibacterial activities were tested by the disc-diffusion agar method, which is a test of the antibiotic sensitivity of bacteria and it uses the antibiotic discs to test the extent to which bacteria are affected by those antibiotics [94]. 24 hour old culture of 2 to 3 colonies of bacteria was diluted by physiological normal saline (0.85%), to make a 0.1 macfarland standard suspension and then the bacteria inoculated into sterile Petri- dishes of 60 mL of Muller Hinton agar plates. The plates were shaken gently to allow evenly mixing of bacteria cells and agar. Chloroform extract (200 mg) and **VAC-F16** (20 mg) was dissolved in 20 mL and 2 mL of methanol, respectively, to obtain each 10 mg/mL. This was repeated for the acetone extract and **VAA-F16-17** to give each 10 mg/mL. From each sample, 200 µL of each concentration saturated with discs (6.00 mm diameter disc) was placed on plate and incubated at 37°C for 24 hours. The diameters of the inhibition zones were calculated. Clear inhibition zones formed around the discs indicated the presence of antibacterial activity [94].

4. RESULTS AND DISCUSSION

4.1. General overview

In this section, yield of the crude extract, phytochemical screening, antioxidant activities and antimicrobial activities on extracts of the flowers of *V. amygdalina* are discussed. Also incorporated herein is the isolation and structure elucidation compounds isolated (**VAC-F1**, **VAC-F16** and **VAA-F16-17**) from the flower extract of same species.

4.2. Percentage yield of the crude extracts

The crude extraction yield is a measure of the solvent efficiency to extract specific components from the original material of the flowers of *V. amygdalina*. It can be calculated according to the method as follows [95].

$$\% \text{ Yield} = \frac{\text{Weight of crude extract}}{\text{Weight of sample}} \times 100\%$$

The plant material was extracted with chloroform and acetone to furnish 1.78 and 1.91%, respectively.

4.3. Phytochemical Screening of crude extracts of *V. amygdalina*

The CHCl_3 and acetone extracts of the flowers of *V. amygdalina* were analyzed for the presence of secondary metabolites. The results revealed the presence of chemical constituents shown in **Table 4**.

Table 4: Phytochemical screening results of chloroform and acetone extracts of flowers of *V. amygdalina*

Constituents	Tests/Reagents	Chloroform extract	Acetone extract
Alkaloids	Wagner's	+ve	+ve
Cardiac glycosides	Kellar-Kiliani	-ve	-ve
Flavonoids	Shinoda	-ve	+ve
Phenols	Ferric chloride	-ve	+ve
Quinones	Hydrochloric acid	+ve	+ve
Saponins	Foam	+ve	+ve
Steroids	Salkowski	+ve	+ve
Tannins	Potassium hydroxide	+ve	-ve
Terpenoids	Salkowski	+ve	+ve

Key:

-ve = The absence of phytochemical constituents.

+ve = The presence of phytochemical constituents

As we observed in **Table 4**, the acetone extract was found to have flavonoids and phenols. But these metabolites were not detected in the chloroform extract of the flower of *V. amygdalina*. The presence of these secondary metabolites can be taken as good attributes of this plant as they can be used as antioxidant. Many literature reports showed that terpenoids are used against diseases causing bacteria. Hence the presence of terpenoids in the flowers of both extract may justify the use of this plant against pathogens causing microbial diseases.

4.4 Antioxidant activity

DPPH radical scavenging assay is a simple method for finding antioxidants by measuring absorbance at 517 nm due to the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Antioxidant, or free radical scavenging, activities of the crude extracts as well as isolated pure compounds from the respective extracts of the flowers of *V. amygdalina* were determined using DPPH radical scavenging assay. The results are displayed in **Table 5**.

The percent radical scavenging activity of the chloroform and acetone extracts of the flowers of *V. amygdalina* were 54.1 and 91.46% at 100 µg/mL, respectively. At the same concentration standard ascorbic acid scavenged the DPPH radical by 97.7%. It means that the acetone extract of plant at higher concentration have the ability to capture free radicals. The absorbance of 0.04% DPPH in MeOH was 0.597. The radical scavenging activity of ascorbic acid used as positive control was 97% at 100 µg/mL.

Table 5: Radical scavenging activities of the extracts and constituents of flowers of *V. amygdalina*.

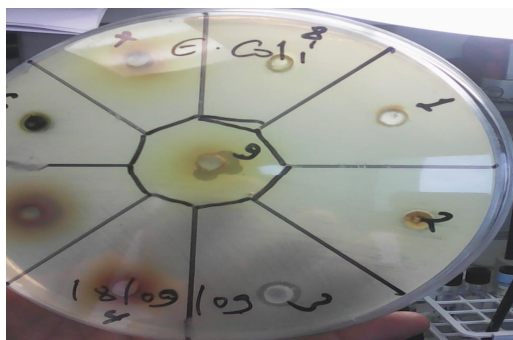
Chloroform extract		Acetone extract	Constituents isolated from the extracts	
			VAA-F16-17	VAC-F16
Concentration µg/mL	%DPPH inhibition	%DPPH inhibition	%DPPH inhibition	%DPPH inhibition
12.5	32.8	69.0	76.5	24
25	37.1	74.5	84	31
50	43.0	83.0	88	37
100	54.1	91.6	94	49

The IC₅₀ value for chloroform extract, acetone extract **VAA-F16-17** and **VAA-F16** were calculated and turned to be 81.55, 71.16, 153.15 and 101.63, respectively.

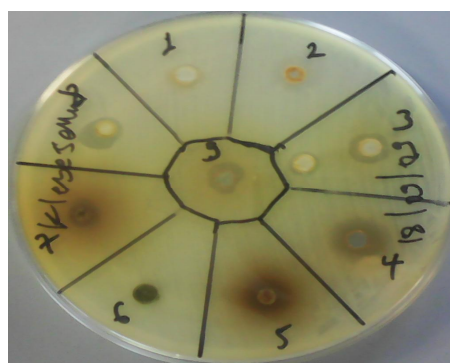
As clearly seen from **Table 5**, the acetone extract of the plant showed a higher radical scavenging activity compared with the chloroform extract. This indicates that the phytochemical constituents responsible for the radical scavenging activities of the flower extract of *V. amygdalina* reside in the acetone extract. The active ingredient responsible for the radical scavenging activity of the acetone extract is likely due to the presence of **VAA-F16-17**. This compound which inhibits the radical by 94% at 100 µg/mL was identified as isorhamnetin (**VAA-F16-17**) which contains phenolics hydroxyl groups. The presence of phenolic hydroxyl groups are of paramount importance in determining the radical scavenging ability of extracts or constituents of plants.

4.5. Antibacterial activities

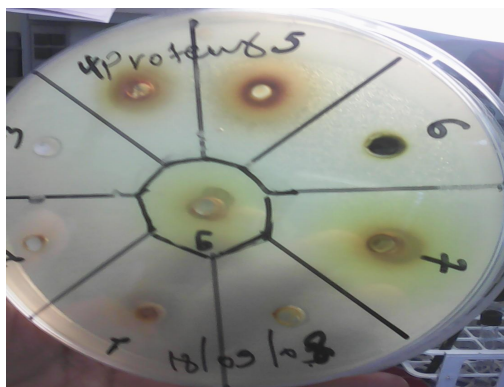
Antibacterial activity was performed with the aim of identifying the zone of inhibition at which the chloroform and acetone extract of *V. amygdalina* flowers as well as the isolated compounds; vernodoline (VAC-F16) and isorhamnetin (VAA-F16-17) were active against the selected bacterial strains. The zone of inhibition was then identified for the bacterial strains that exhibited reasonable activity performed on the prepared concentrations of the extracts and isolated compounds. The inhibitory effects of *V. amygdalina* extracts and isolated compounds were determined against gram negative bacteria's (*E. coli*, *K. pneumoniae* and *P. mirabilis*) and gram positive (*S. aureus* and *Bacillus*) bacteria's. The results of zone of inhibition in diameter against tested pathogens are demonstrated (Picture 2) and presented in Table 6.



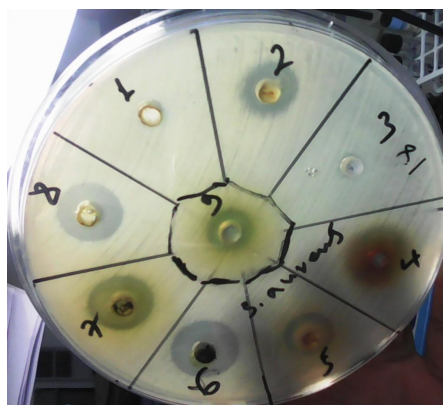
A



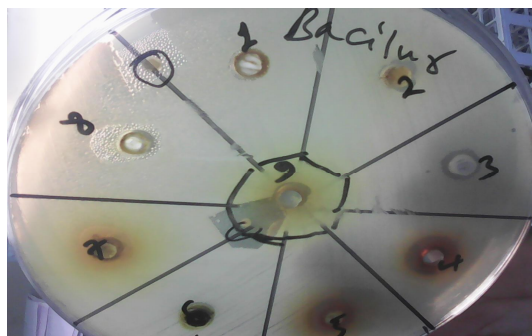
B



C



D



E

Picture 2: Antibacterial activities of the flower of *V. amygdalina*

Where; **A**=*E. coli*, **B**= *K. pneumoniae*, **C**= *P. mirabilis*, **D**= *S. aureus* and **E**= *S. Bacillus*

1, 2, 3, 4 and 5 did not belong to this plant while 6=Chloroform extract, 7=acetone extract, 8=VAC-F16 and 9=VAA-F16-17.

The extracts and isolated compounds demonstrated significant difference in antibacterial activity against all selected bacteria's with zone of inhibition ranging from 6-21 mm. *V. amygdalina* flowers of acetone extract and both isolated compounds demonstrated moderate antibacterial activity (>9 mm) against all bacterial. This value likely to be acceptable compared with Chloramphenicol and Gentamicin standard antibiotics applied against the same antibacterial strain. In contrast, chloroform extract of flowers of *V. amygdalina* did not show any activity against almost all bacterial strains except *S. aureus*, compared with Chloramphenicol and Gentamicin .

Table 6: Inhibition zone diameter of chloroform extract, acetone extract, vernolide and isorhamnetin, antibiotics and distilled water at 0.2 mg/mL

Extract/control	Control for each bacterial strain and standard (mm)	Zone of inhibition (mm)				
		<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>S. aureus</i>	<i>S. bacillus</i>
Chloroform extract	6	6	6	6	21	6
Acetone extract	6	10	12	11	17	12
Vernolide	6	10	12	6	19	12
Isorhamnetin	6	10	14	12	11	9
Chloramphenicol	6	18	18	18	18	18
Gentamicin	6	15	15	15	15	15
Distilled water	6	0	0	0	0	0

The chloroform extract, acetone extract and vernolide displayed strong antibacterial activity against *S. aureus*. The result is significant compared with the positive control corroborating the traditional use of other part of this plant by the community.

4.6. Structural elucidation of isolated compounds from the chloroform

extract

The chloroform extract of the flowers of *V. amygdalina* after silica gel column chromatography has led to the isolation of **VAC-F1** and **VAC-F16**. Herein is the characterization of these compounds.

4.6.1. Characterization of compound VAC-F1

Compound **VAC-F1** (**22 mg**) was obtained as a white power from the CHCl_3 extract of the flower of *V. amygdalina*. Spot was visualized neither after spraying in vanillin nor after dipping in iodine. The ^1H -NMR spectrum (**Appendix 1**) of **VAC-F1** exhibited signal at δ 0.9 (6H, *t*) accounted to the presence of terminal methyl group. A broad singlet observed at δ 1.28 (42H, br. *s*) is characteristic of many overlapping methylene protons. The proton decoupled ^{13}C -NMR spectrum of **VAC-F1** (**Appendix 2**) with the aid of DEPT-135 (**Appendix 3**) suggested the presence of five carbon resonances of which four peaks are due to methylene and one peak is described to methyl group. The carbon signal at δ 14.1 is evident for the presence of terminal methyl group. The other carbon resonances appearing at δ 22.7, 29.7, 29.4 and 31.9 were accounted to the presence of methylene groups in the compound. The NMR spectral data generated showed that compound **VAC-F1** is proposed as tricosane whose structure is shown in **Figure 5**

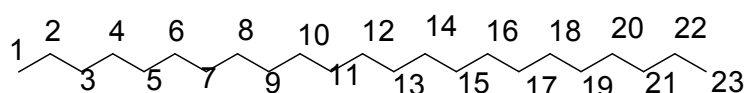


Figure 5: Chemical structure of tricosane (**VAC-F1**)

The ^1H and ^{13}C -NMR spectral data of compound **VAC-F1** was presented in **Table 7**.

Table 7: ^1H and ^{13}C -NMR spectral data of **VAC-F1**

NMR spectral data of compound VAC-F1		
^1H -NMR, δ	^{13}C -NMR, δ	Remark
0.9 (6H, <i>t</i>)	14.1	2-CH ₃
1.28 (42H, <i>bro s</i>)	22.7	2-CH ₂
	29.4	2-CH ₂
	29.7	15-CH ₂
	31.9	2-CH ₂

4.6.2. Characterization of compound **VAC-F16**

Compound **VAC-F16** (77 mg) was obtained as yellow solid from the chloroform extract of the flower of *V. amygdalina*. It was eluted with hexane: ethyl acetate (1:9). The TLC (hexane: EtOAc, 3:2) showed deep yellow spot, which was visualized after dipping in iodine vapor followed by heating in an oven. UV-Vis spectrum (**Appendix 4**) showed absorption band at 320 nm indicating the presence of $\text{n} \rightarrow \pi^*$ transition of the carbonyl group. The FT-IR (**Appendix 5**) spectral data of compound **VAC-F16** displayed absorption band at δ 3383 cm^{-1} indicating the presence of OH stretching. The absorption band at δ 2929 cm^{-1} was suggestive of aliphatic SP^3 C-H stretching frequencies. The strong absorption band at δ 1772 cm^{-1} is indicative of the carbonyl functional group of the exomethylene δ -lactone group. Also the observed band at δ 1723 cm^{-1} is evident for the presence of carbonyl functional group of the acyl group. The weak absorption band around 1625 cm^{-1} is due to presence of C=C double bond stretch.

The ^1H -NMR spectral data of compound **VAC-F16** (**Appendix 6**) showed the presence of exomethylene δ -lactone at δ 6.3 (1H, *d*, Ha-13) and δ 5.9 (1H, *d*, Hb-13). The other diastereotopic methylene protons were observed at δ 6.1 (1H, *d*, Ha-19) and 5.7 (1H, *d*, Hb-19). Another olefinic methine proton is evident at δ 5.5 (1H, *d*, H-5). The signal at δ 5.2 (1H, *t*, H-6) is due to methine proton on oxygenated carbon. An oxygenated methylene proton at δ 4.6 and 3.7 (2H, *d*, H-15) is due to methylene protons. The signal at δ 4.5 (1H, H-14) indicated that the presence of methine proton on carbon bearing two oxygen.

Moreover, the spectral data of this compound showed one methyl group on quaternary carbon at δ 1.9 (3H, s, H-18). The remaining signals are shown in **Table 8**.

The ^1H -NMR spectral data compound **VAC-F16** was compared with literature reported for vernolide and the results was presented in **Table 8**.

The ^{13}C -NMR spectrum of **VAC-F16** (**Appendix 6**) revealed that there are 19 carbon signals, which were sorted by DEPT-135 experiment (**Appendix 7**) as one methyl at δ 18.3 (C-18), six methylene, six methine, six quaternary and two carbonyl carbons (**Table 8**). The signal at δ 126.1 and 127.2 is due to the exomethelene carbons at C-13 and C-19, respectively. The signal at δ 128.7 (C-5) is confirmation of a carbon attached to the carbon of the olefinic and the signal at δ 99.2 (C-14) is assigned to the carbon boded to two oxygen. The signal at δ 58.9 (C-10) is due to the tertiary carbon directly attached to the two methine, one methylene and oxygen and in addition to this the signal at δ 169.5 (C-11) and δ 167.1 (C-16) are indicative as the presence of two ester carbonyl groups. The spectral data generated for **VAC-F16** is in good agreement with that of vernolide and the other NMR spectral data are depicted in **Table 8**.

Table 8: ^1H and ^{13}C -NMR spectral data of **VAC-F16** and literature reported for vernolide

NMR spectral data of compound VAC-F16			Lit. reported for vernolide [96]	
Position	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -NMR
1	66.3	2.7 (1H, <i>dd</i>)		2.7(1H, <i>dd</i>)
2	22.8	1.7 and 2.3 (1H, <i>m</i>)	22.7	1.4 and 2.3(1H, <i>m</i>)
3	33.4	2.4 (2H, <i>m</i>)	33.4	2.4 (2H, <i>m</i>)
4	143.7		143.7	
5	128.7	5.5 (1H, <i>d</i>)	128.6	5.5 (1H, <i>d</i>)
6	77.5	5.2 (1H, <i>t</i>)	77.4	5.2 (1H, <i>t</i>)
7	51.8	3.0 (1H, <i>m</i>)	51.9	3.0 (1H, <i>m</i>)
8	70.7	5.74 (1H, <i>a</i>)	70.7	5.74 (1H, <i>a</i>)
9	41.2	1.4 (1H, <i>dd</i>) and 2.6(1H, <i>d</i>)	41.2	1.4 (1H, <i>dd</i>) and 2.6(1H, <i>d</i>)
10	58.9		58.8	
11	169.5		169.4	
12	134.9		134.8	
13	126.1	5.9 and 6.3 (1H, <i>d</i>)	126.1	5.9 and 6.3 (1H, <i>d</i>)
14	99.2	4.5 (1H, <i>d</i>)	99.2	4.5 (1H, <i>d</i>)
15	64.3	3.6 and 4.6 (1H, <i>d</i>)	64.3	3.6 and 4.6 (1H, <i>d</i>)
16	167.1		167.2	
17	135.7		135.7	
18	18.3	1.9 (3H, <i>s</i>)	18.2	1.9 (3H, <i>s</i>)
19	127.2	5.71 and 6.1(1H, <i>d</i>)	127.2	5.71 and 6.1 (1H, <i>d</i>)

Where;

a is Peak obscured

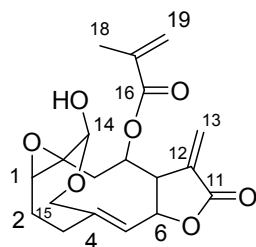


Figure 6: Chemical structure of vernolide (**VAC-F16**)

The structure was confirmed with the COSY(**Appendix 9**) which showed connectivity between H-2 with H-3 and H-1; H-7 with H-6; H-6 with H-7 and H-5 and H-8 with H-7. The HSQC (**Appendix 10**) spectral data showed as H-1, H-2, H-3, H-5, H-6, H-7, H-8, H-9, H-13, H-15 and H-18 with C-1, C-2, C-3, C-5, C-6, C-7, C-8, C-9, C-13, C-15 and C-18, respectively.

Furthermore HMBC (**Appendix 11**) was also generated to analyze the structure of this compound. In view of this the proton on H-9 was correlated with C-7, C-10, C-15, C-8 and C-14; H-15 with C-3, C-14, C-5 and C-4; and H-6 with C-7, C-8 and C-4.

4.7. Structure elucidation of isolated compound from acetone extract

4.7.1. Characterization of compound VAA-F16-17

Compound **VAA-F16-17 (70 mg)** was isolated from the acetone extract of the flower of *V. amygdalina*. It was eluted using CHCl₃: MeOH (85:15) as eluent and visualized as yellow spot after spraying with vanillin in H₂SO₄. The UV-Vis spectral data (**Appendix 12**) showed absorption band at 280 and 310 nm evident for the presence of flavonoids skeleton. The FT-IR (**Appendix 13**) spectral analysis showed band at δ 3410 cm⁻¹ indicating that the OH stretching vibrational frequency and the weak absorption band at δ 3182 cm⁻¹ indicates aromatic C-H multiple bond. The weak absorption band at δ 2920 cm⁻¹ was suggestive as of the aliphatic C-H (SP³ hybridization) asymmetrical stretching vibrational frequencies. The strong absorption band at the δ 1648 cm⁻¹ is indicative of the carbonyl carbon (C=O) functional group and 1610 cm⁻¹ is indicating the presence of C=C double bond stretch.

Analysis of the proton NMR spectrum of compound **VAA-F16-17 (Appendix 14)** showed signal at δ 12.7 (1H, *s*) due to chelated hydroxy group which is evident for the presence of 5-OH. The three aromatic proton signals at δ 7.5 (1H, *d*, *J* = 2.00 Hz, H-2'), 7.6 (1H, *dd*, *J* = 8.4 Hz and 2.00 Hz, H-6') and 6.9 (1H, *d*, *J* = 8.4 Hz, H-5') are typical of flavonoid with 3',4'-disubstituted B-ring.

The other aromatic proton signals at δ 6.4 (1H, *d*, *J* = 2 Hz, H-8) and 6.2 (1H, *d*, *J* = 2 Hz, H-6) are apparently due to *meta* coupled protons on the A-ring of flavonoid. The presence of one methoxy group is evident at δ 3.8 (3H, *s*).

The proton decoupled ^{13}C -NMR spectrum (**Appendix 15**) combined with DEPT-135 (**Appendix 16**) of compound **VAA-F16-17** revealed the presence of only five methine, one methyl and ten quaternary carbons (**Table 10**).

The presence of α , β -unsaturated ketone is evident from the appearance of the carbonyl carbon signal at δ 178.3. Other signals in the ^{13}C -NMR spectrum were observed at δ 164.5 (C-7), 161.7 (C-9), 156.7 (C-5), 156.0 (C-2), 149.1 (C-4'), 145.6 (C-3'), 138.0 (C-3), 121.2 (C-1'), 121.0 (C-6'), 116.1 (C-5'), 115.8 (C-2'), 104.6 (C-10), 98.9 (C-6) and 94.0 (C-8). The carbon resonance at δ 60.0 is due to the presence of methoxy group. Based on the above spectral data, compound **VAA-F16-17** was identified as isorhamnetin whose structure is shown in **Figure 7**.

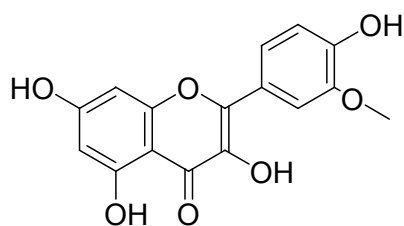


Figure 7: Chemical structure of isorhamnetin (**VAA-F16-17**)

For further confirmation, the NMR spectral data of **VAA-F16-17** was compared with the NMR data reported in the literature for isorhamnetin and the result is presented in **Table 10**.

The upfield chemical shift value of C-6 (98.9 ppm) and C-8 (94.0 ppm) is in support of 5, 7-dioxygenated justification pattern of Ring A.

Table 9: ^1H and ^{13}C -NMR spectral data of **VAAF16-17** and literature reported for isorhamnetin (δ in ppm, J in Hz) [97]

NMR spectral data of compound VAAF16-17			Lit. reported for isorhamnetin [97]	
Position	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -NMR
2	156.0		148.6	
3	138.0		135.6	
4	178.3		175.7	
5	156.7		156.0	
6	98.9	6.2 (1H, <i>d</i> , $J = 2$ Hz)	98.1	6.2 (1H, <i>d</i> , $J = 2$ Hz)
7	164.5		163.7	
8	94.0	6.4 (1H, <i>d</i> , $J = 2$ Hz)	93.5	6.4 (1H, <i>d</i> , $J = 2$ Hz)
9	161.7		160.3	
10	104.6		102.9	
1'	121.2		121.8	
2'	115.8	7.5 (1H, <i>d</i> , $J = 2.00$ Hz)	111.5	7.7 (1H, <i>d</i> , $J = 2.00$ Hz)
3'	145.6		146.4	
4'	149.1		147.2	
5'	116.1	6.9 (1H, <i>d</i> , $J = 8.4$ Hz)	115.3	6.9 (1H, <i>d</i> , $J = 8.4$ Hz)
6'	121.0	7.6 (1H, <i>dd</i> , $J = 8.4$ Hz and 2.00 Hz)	121.6	7.4 (1H, <i>dd</i> , $J = 8.4$ Hz and 2.00 Hz)
1''	60	3.8 (3H, <i>s</i>)		

The spectral data of compound **VAA-F16-17** is in agreement with the data reported in the literature for isorhamnetin [97] whose structure is depicted in **Figure 7**.

The ^{13}C -NMR spectrum of **VAA-F16-17** with the aid of DEPT-135 revealed the presence of minor impurity. Close inspection of the spectrum indicated the presence of fifteen carbon resonances resonating at δ 181.7, 164.4, 161.8, 156.9, 150.1, 146.2, 129.0, 121.5, 119.1, 117.3, 114.2, 103.2, 102.3, 99.1 and 94.2. Among these, the signals observed at δ 129.0, 119.1, 117.3, 114.2, 103.2, 99.1 and 94.2 are due to methine carbon signals. The carbon signal observed at δ 181.7 is ascribed to an α , β -unsaturated carbonyl carbon. Whereas the signal δ 103.2 is attributed to C-3. The presence of five oxygenated aromatic carbon signals is evident at δ 164.4 (C-2), 161.8 (C-9), 156.9 (C-5), 150.1 (C-4') and 146.2 (C-3'). In the ^1H -NMR spectrum the signal at δ 13.0 is apparently due to the presence of hydroxyl attached on C-5. The data likely indicated that the minor peaks observed in the NMR spectra of compound **VAA-F16-17** is ascribed to luteolin whose structure is given in **Figure 8**.

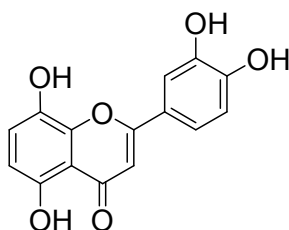


Figure 8: Chemical structure of luteolin (minor peak of **VAA-F16-17**)

The ^{13}C -NMR spectral data of the minor signals in the spectrum of **VAA-F16-17** was compared with the literature reported in the literature for luteolin and the result is presented in **Table 11**.

Table 10: ^{13}C -NMR spectral data of minor peaks in **VAA-F16-17** and literature reported for luteolin

^{13}C -NMR spectral data of minor signals in VAAF16-17 Lit. reported for luteolin [98]		
Position	^{13}C -NMR	^{13}C -NMR
2	164.4	164.0
3	103.2	103.2
4	181.7	181.8
5	156.9	157.6
6	99.1	99.2
7	129.0	164.3
8	94.2	94.7
9	161.8	162.1
10	102.3	103.8
1'	121.5	119.0
2'	114.2	113.2
3'	146.2	146.0
4'	150.1	149.7
5'	117.3	116.8
6'	119.1	120.8

The spectral data generated herein is in agreement with the NMR data reported in the literature for luteolin [98].

5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary

Vernonia amygdalina is among traditionally used medicinal plants in Ethiopia. In view of its traditional uses and absence of scientific literature reports, the flowers of this plant were subjected to systematic phytochemical analysis. The flowers were successively extracted with CHCl_3 and acetone to afford 1.78 and 1.91% yellowish crude extract respectively. The respective crude extracts were subjected to phytochemical screening using standard procedures. The phytochemical screening test of the chloroform extract of the flower of *V. amygdalina* revealed the presence of secondary metabolites including alkaloids, quinines, saponins, steroids, tannins and terpenoids where as the acetone extract showed the presence of alkaloids, flavonoids, phenols, quinones, saponins, steroids and terpenoids.

Moreover, the chloroform extract of the flowers of *V. amygdalina* furnished two compounds namely tricosane and vernolide. While the acetone extract furnished isorhamnetin and luteolin. Chemical structures of these compounds were elucidated using various spectroscopic methods including IR, UV-Vis and NMR. In some cases the structures were confirmed by comparing the NMR data of these compounds with the NMR spectral data reported in the literature for same compound. Further confirmation of the structure of vernolide was achieved using 2D-NMR (COSY, HSQC and HMBC).

The antioxidant activities of the extracts and constituents were assessed for their radical scavenging activity using DPPH. The acetone extract and isorhamnetin displayed radical scavenging activity by 91.6 and 94%, respectively. The result is comparable with ascorbic acid (97%) used as positive control.

Furthermore the extracts, isorhamnetin and vernolide were also evaluated for their antibacterial activity against three gram negative (*Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*) and two gram positive (*Staphylococcus aureus* and *S. bacillus*) bacteria. The CHCl_3 extract and vernolide showed strong activity against *S. aureus* with an inhibition zone of about 21 and 19 mm, respectively. The acetone extract and isorhamnetin were active against all bacterial pathogens tested in this study. The biological activity displayed by the extracts and constituents of this plant corroborate the traditional uses of this plant by the local people against various diseases.

5.2. Conclusion and Recommendation

Currently, the growing interest of consumers in substances of natural origin in association with the increasing concern surrounding potentially harmful infections and disease has directed to a rising interest in the use of plant extracts as functional ingredients in many pharmaceutical products.

The presence of secondary metabolites such as alkaloids, quinones, saponins, steroids, tannins, terpenoids, flavonoids and phenols can be taken as a scientific justification of the use of this plant in the traditional system for the treatment of various ailments. The CHCl_3 and acetone extracts of the flowers after silica gel column chromatography has led to the isolation of tricosane, vernolide, luteolin and isorhamnetin.

The high antioxidant activity displayed by the acetone extract and isorhamnetin is a good attributes of the plant to be used as a natural antioxidant.

The extract and pure compounds isolated from the flowers of *V. amygdalina* exhibited pronounceable antibacterial activity. This justifies the traditional uses of this plant against bacteria.

Further studies should be carried on structure activity relationships which may be a guide to a better understanding of the relationships between the structures and bacterial and antioxidant activities of these compounds. In addition to this the following recommendations were made.

- The antibacterial activity test was conducted on limited bacterial strains. So further testing work should be carried out on large variety of bacterial strains so as to conclude that the plant have good antibacterial activities.
- Further phytochemical and biological activity studies should be conducted on this plant to isolate more constituents of the compound and to identify the specific antibacterial activities of the plant.

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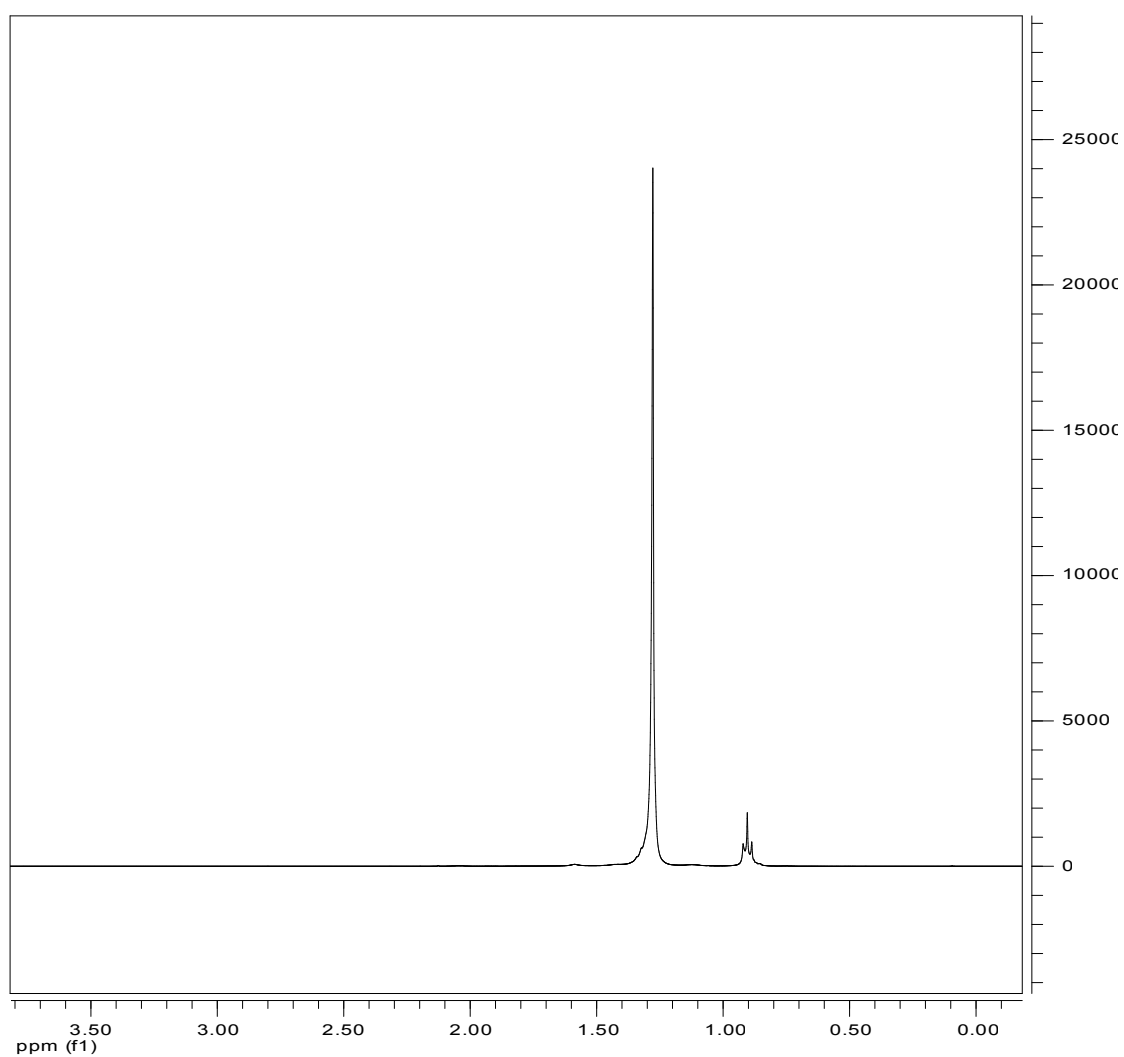
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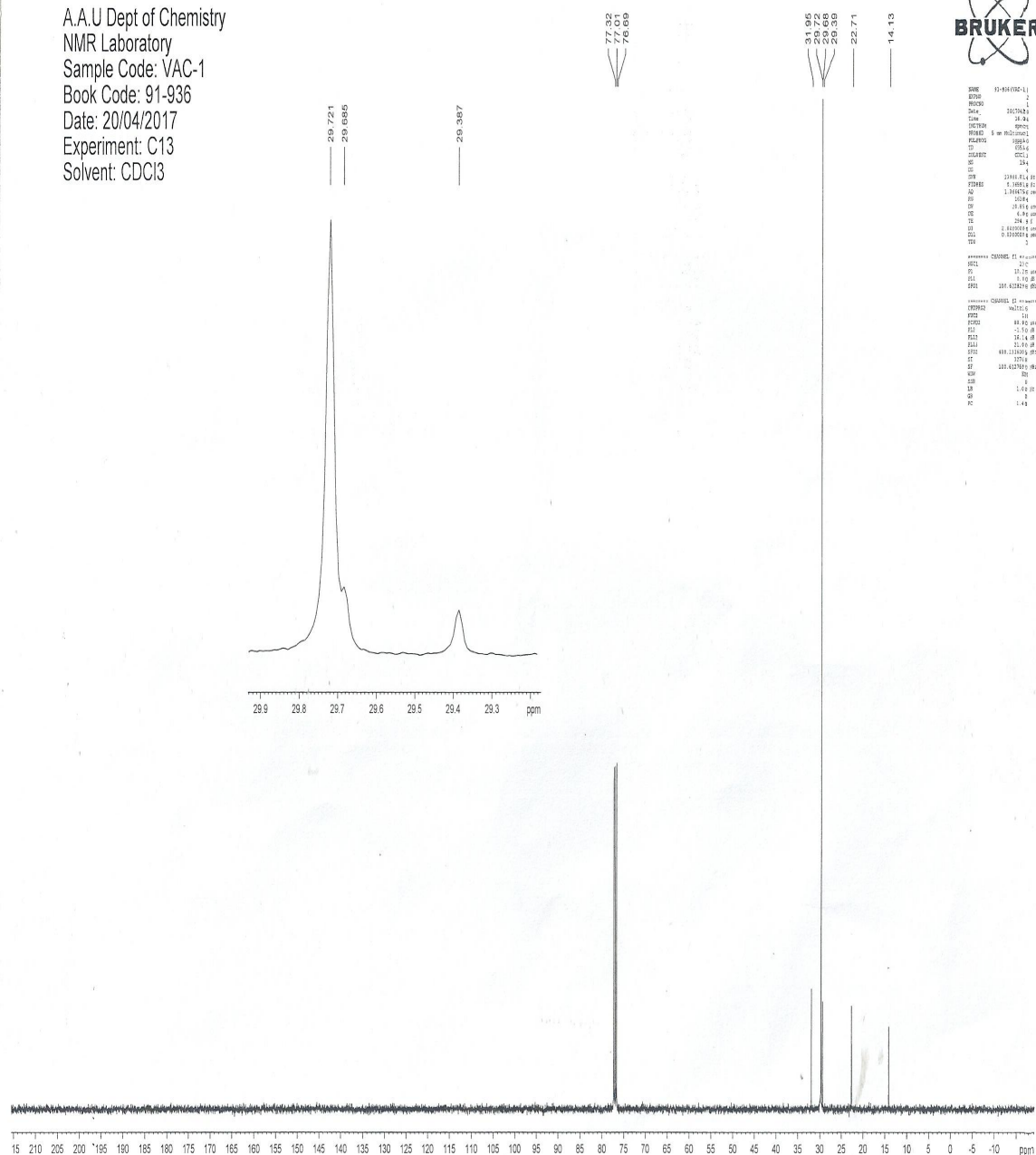
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7. LIST OF APPENDIX



Appendix 1: ^1H -NMR spectrum of VAC-F1

A.A.U Dept of Chemistry
NMR Laboratory
Sample Code: VAC-1
Book Code: 91-936
Date: 20/04/2017
Experiment: C13
Solvent: CDCl3



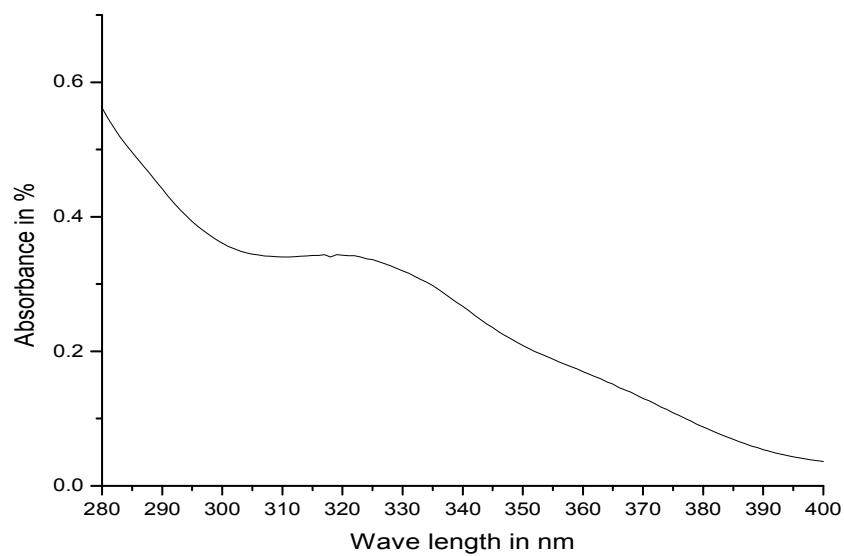
Appendix 2: ^{13}C -NMR spectrum of compound VAC-F1

A.A.U Dept of Chemistry
 NMR Laboratory
 Sample Code: VAC-1
 Book Code: 91-936
 Date: 20/04/2017
 Experiment: DEPT-135
 Solvent: CDCl₃

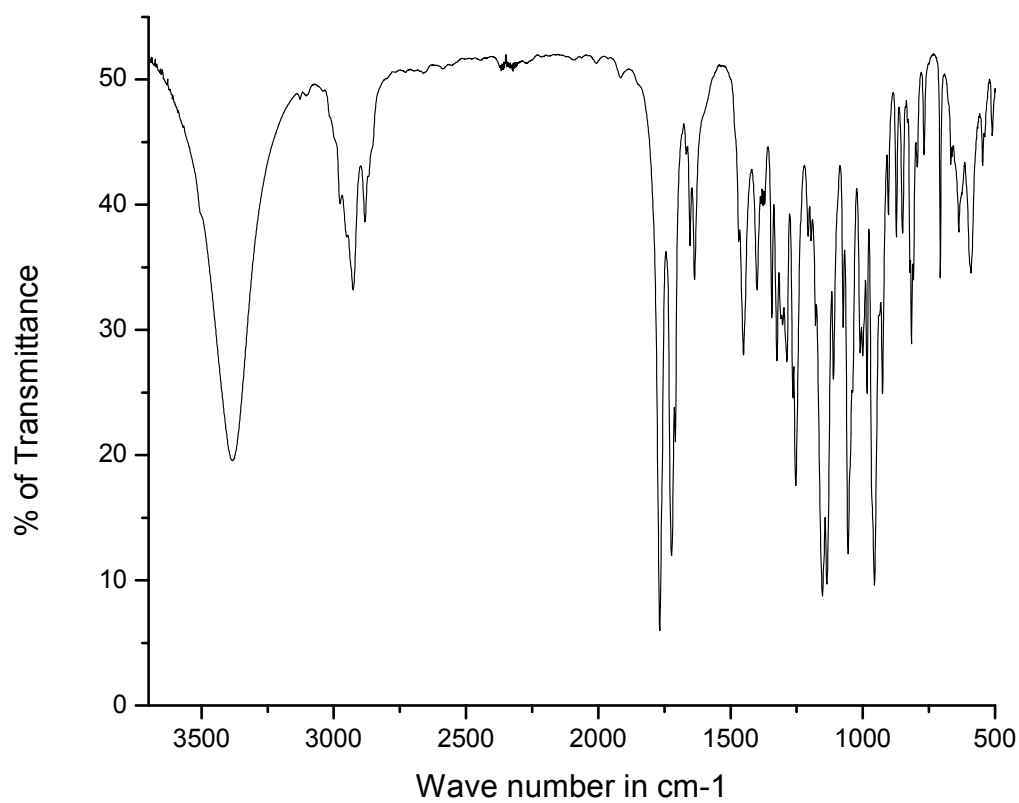


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 PROCNO: 1
 INET: 2DFT400
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 TIME2: 10.00
 DATE_2: 20170420
 PROBHD: 5 mm HX-135C
 PULPROG: zgpg30
 TD: 65536
 SFO: 400.135
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 RG: 327.5
 DWDW: 1.000000
 DE: 1.000000
 FIDRES: 0.000100
 ADF: 0.000000
 QF: 0.000000
 E2: 0.000000
 E3: 0.000000
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Appendix 3: DEPT-135 spectrum of compound VAC-F1



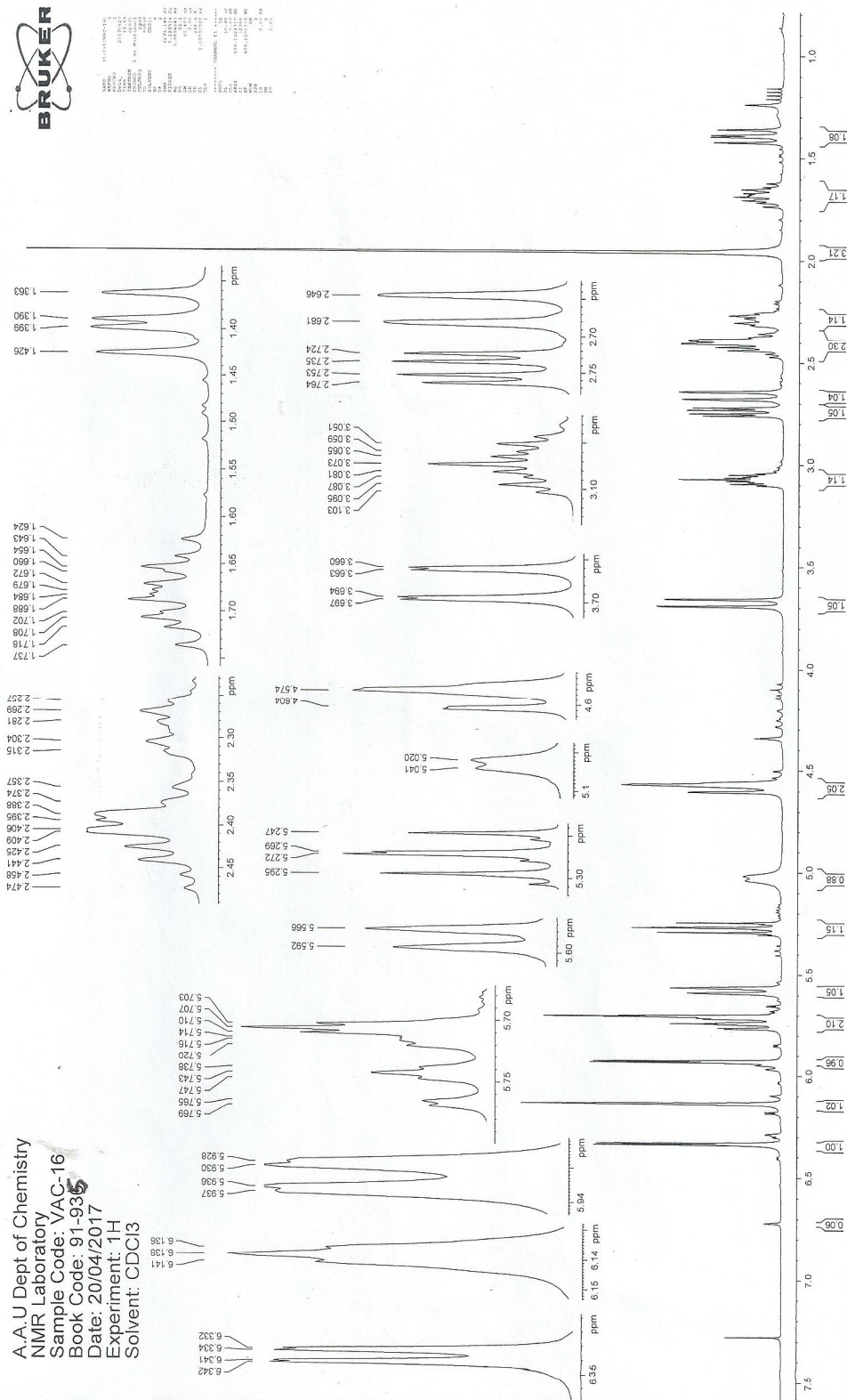
Appendix 4: UV-Vis spectrum of compound **VAC-F16**



Appendix 5: FT-IR spectrum of compound **VAC-F16**

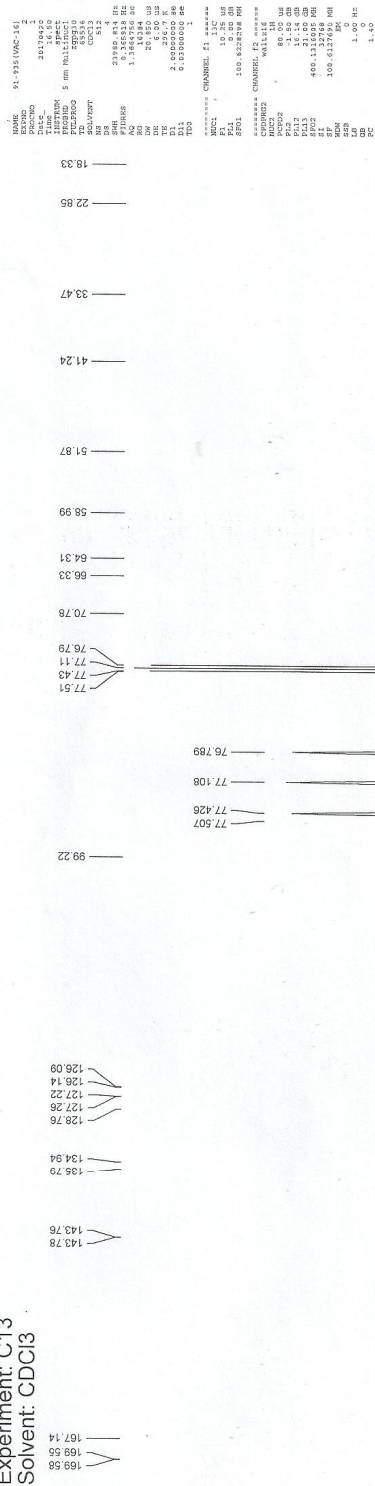


A.A.U Dept of Chemistry
NMR Laboratory
Sample Code: VAC-16
Book Code: 91-935
Date: 20/04/2017
Experiment: 1H
Solvent: CDCl3

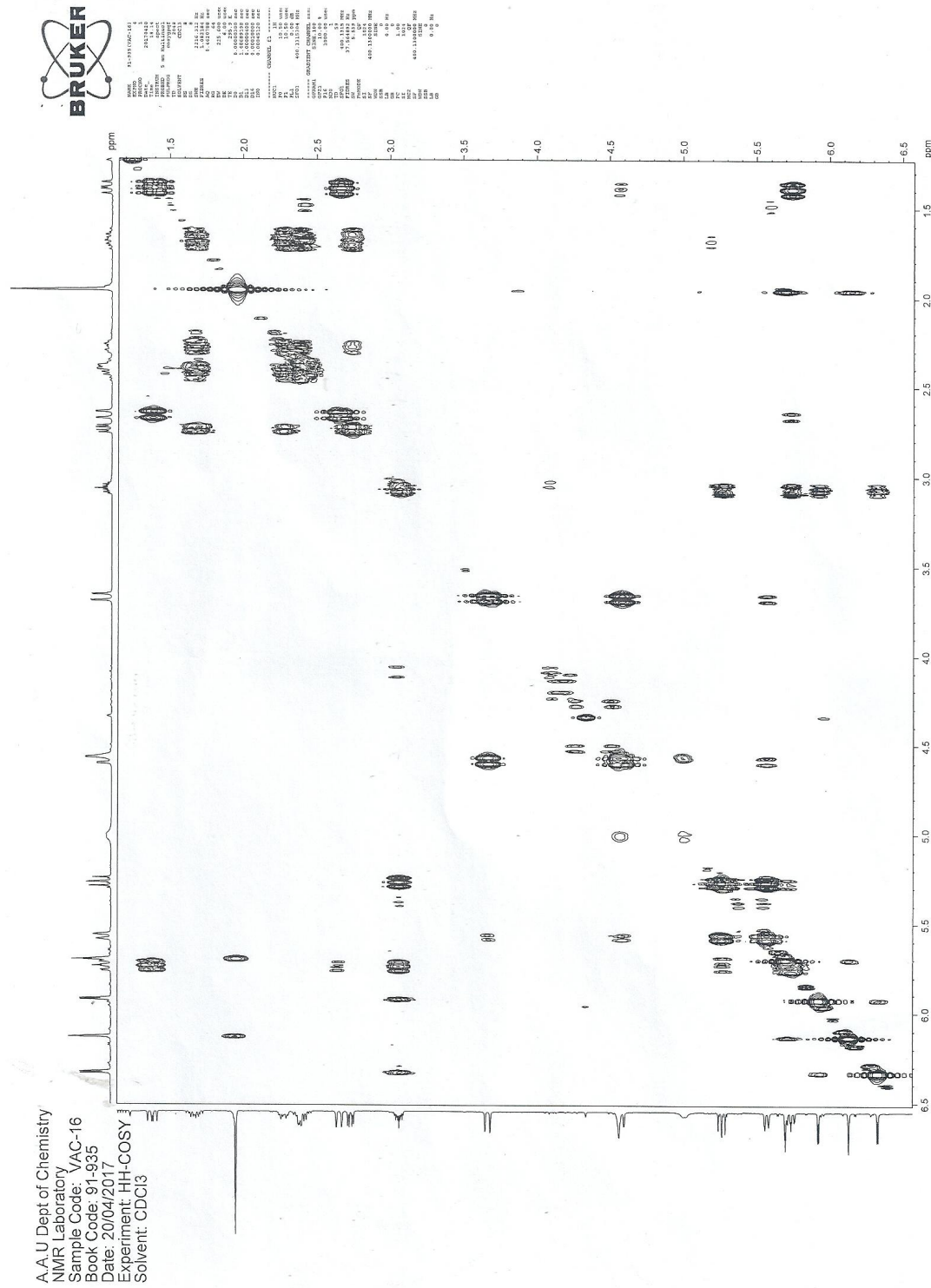


Appendix 6: ^1H -NMR spectrum of compound VAC-F16

A.A.U Dept of Chemistry
 NMR Laboratory
 Sample Code: VAC-16
 Book Code: 91-935
 Date: 20/04/2017
 Experiment: C13
 Solvent: CDCl3

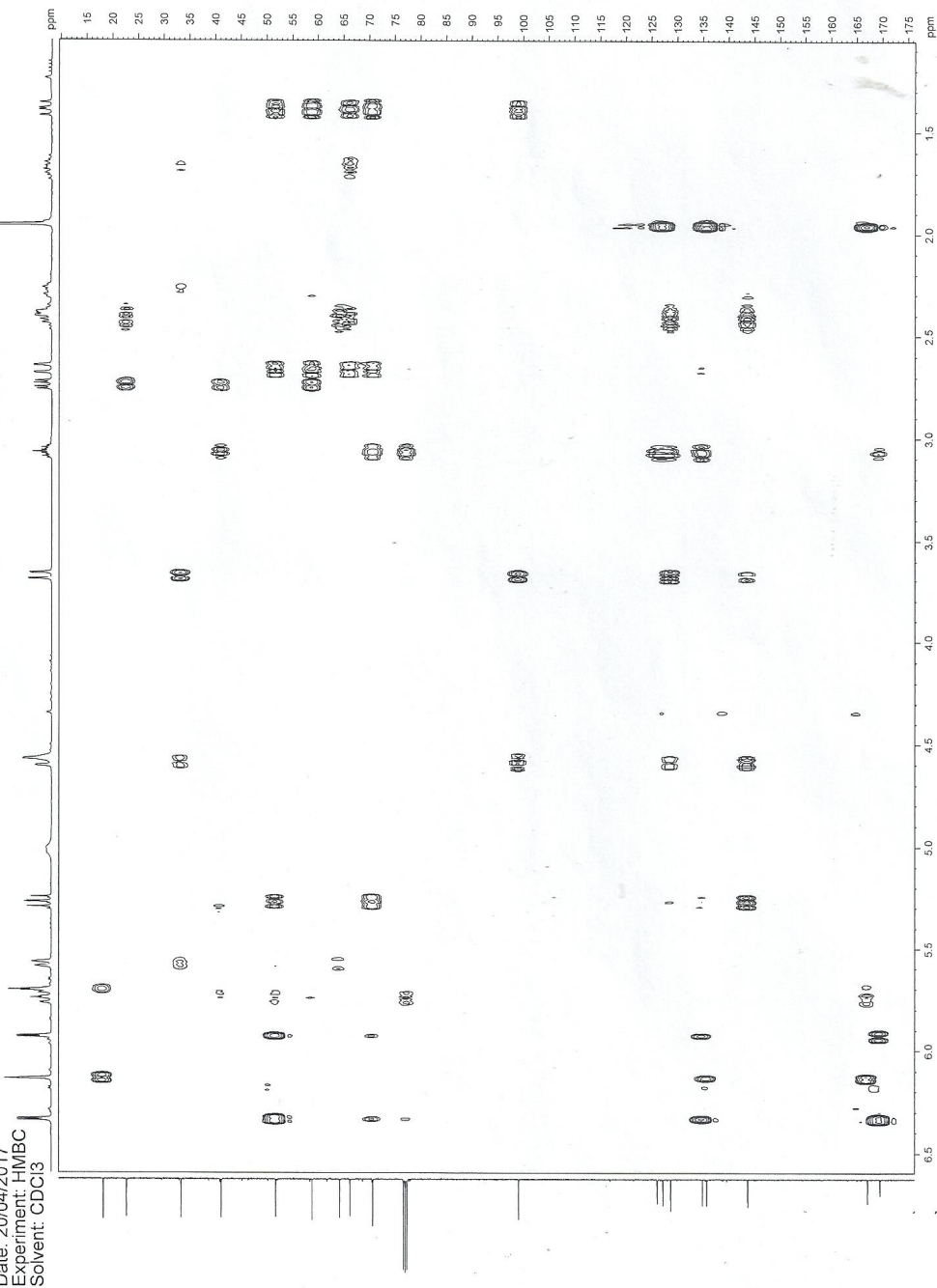


Appendix 7: ^{13}C -NMR spectrum of compound VAC-F16

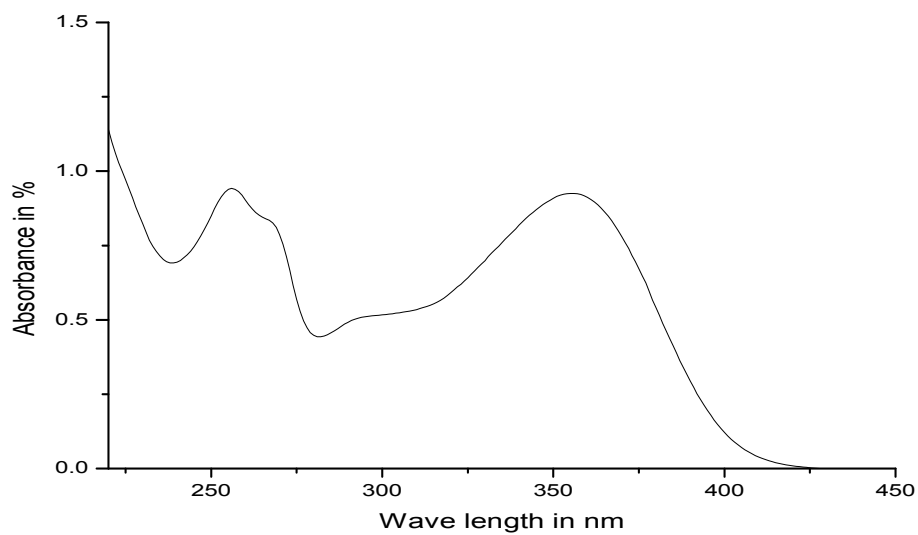


Appendix 9: HH-COSY spectrum of compound VAC-F16

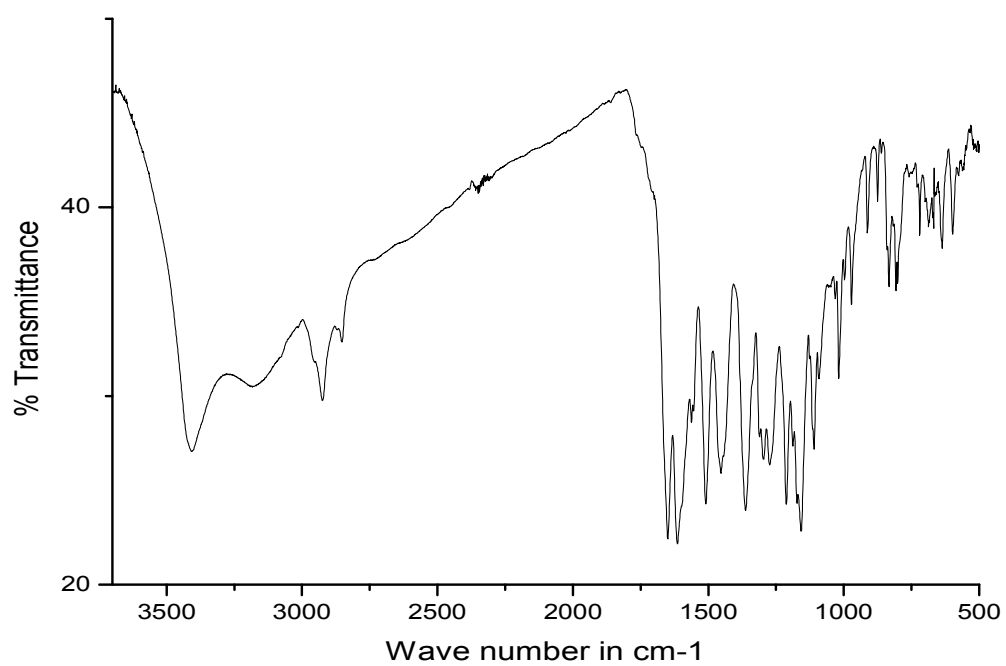
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TIME	18:19
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COLLOC	4036
SOLVENT	CCl2
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NUC2	1H
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CHN2	125.274
CHN3	8.000000
CHN4	0.000000
CHN5	0.000000
CHN6	0.000000
CHN7	0.000000
CHN8	0.000000
CHN9	0.000000
CHN10	0.000000
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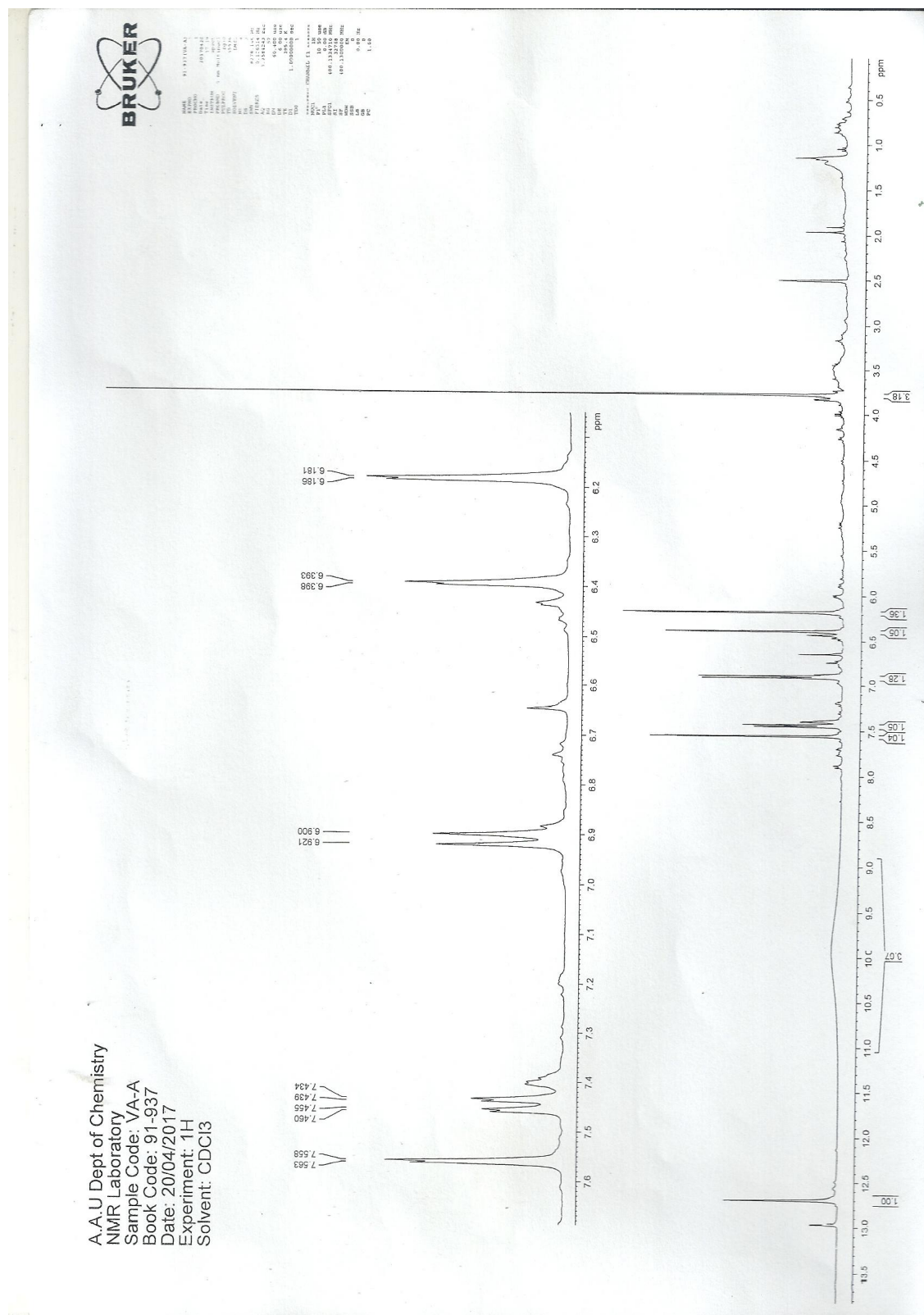
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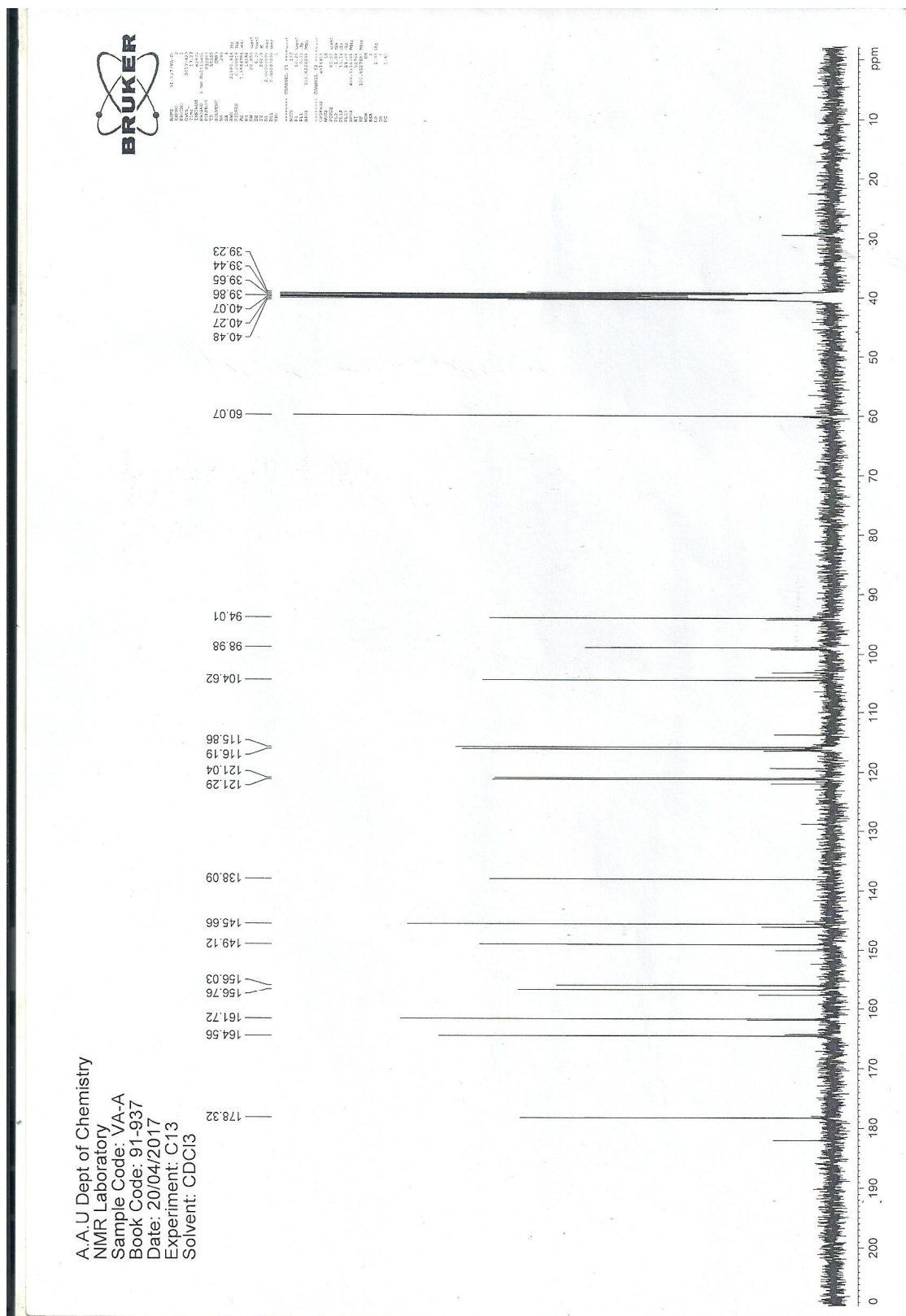
Appendix 12: UV-Vis spectrum of compound **VAA-F16-17**



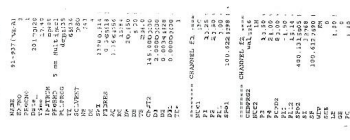
Appendix 13: FT-IR spectrum of compound **VAA-F16-17**



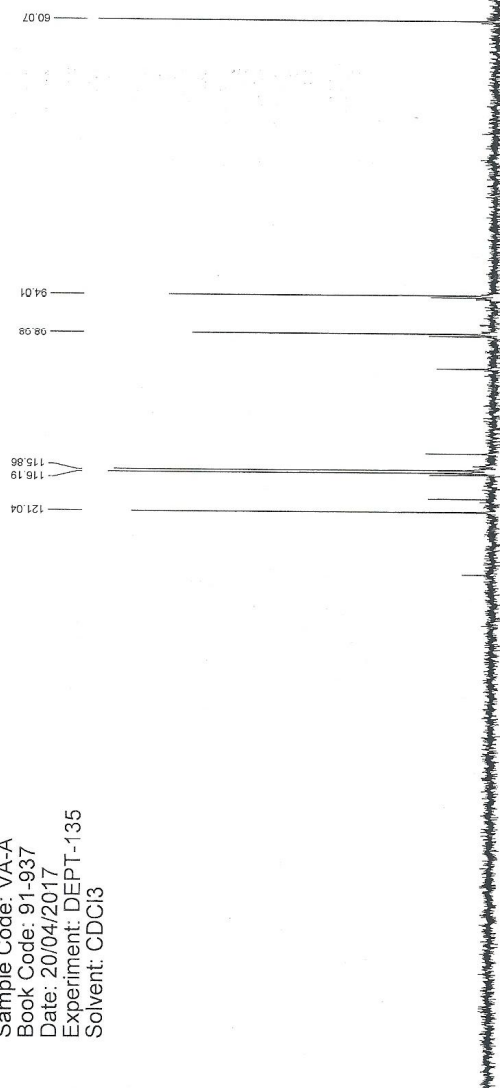
Appendix 14: ¹H-NMR spectrum of compound VAA-F16-17



Appendix 15: ^{13}C -NMR spectrum of compound VAA-F16-17



A.A.U Dept of Chemistry
NMR Laboratory
Sample Code: VA-A
Book Code: 91-937
Date: 20/04/2017
Experiment: DEPT-135
Solvent: CDCl3



Appendix 16: DEPT-135 spectrum of compound **VAA-F16-17**