JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF ROOT EXTRACT OF *Vernonia hymenolepis*

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A MASTER THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

BY

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DECLARATION

I, undersigned, declare that this thesis is my original work and has not been presented for a degree or diploma in any other universities and that all sources of materials used for this thesis have been duly acknowledged.

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Jimma University

School of Graduate Studies

Phytochemical Investigation and Evaluation of Antimicrobial Activity of Root Extract of *Vernonia hymenolepis*

By

Alemu Geleta

A Thesis submitted to college of natural science, department of chemistry Jimma University, for the partial fulfillment of the Requirements for the Degree of Masters of Science in Chemistry

Approved by board of Examiners

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List of Abbreviations and Acronyms

^{13}C NMR	Carbon Nuclear Magnetic Resonance
$CDCl_3$	Deuterated Chloroform
d	doublet
1D	One dimension
dd	double of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsuphoxide
^{1}H NMR	Proton Nuclear Magnetic Resonance
m	multiplicity
nm	nanometer
PDA	Potato dextrose agar
R_f	Retention factor
s	singlet
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHO	World Health Organization

ABSTRACT

*Vernonia hymenolepis is a medicinal plant which has been known to be used for the treatments of various diseases including pneumonia, hypertension, toothache, diarrhea, jaundice, amoebiasis, malaria, typhoid, hepatitis, fever, stomach ache and constipation by different communities in Ethiopia. However, its phytochemical and biological information is very limited. Following its medicinal importance, the plant material was collected from Oromia regional state, East Wollega Zone Jimma Arjo woreda. The plant material was air dried and sequentially extracted with chloroform and methanol. The resulting crude extracts were in vitro assayed against four bacterial strains (Bacillus subtilis ATCC11778, Staphylococcus aureus ATCC25923, Escherichia coli ATCC25922, and Pseudomonas aeruginosa ATCC27853) and one fungal strain (Candida albicans) using disc diffusion method. The crude extracts showed promising antibacterial activities with the highest activity observed for chloroform extract against E. coli (18 mm) but lower than that of the reference drug gentamycin (25 mm). The methanol extract showed the highest activity (13 mm) against the fungal strain Candida albicans as compared to the reference drug, clotrimazole, (12 mm). The antimicrobial activity displayed by the root extracts of Vernonia hymenolepis validates the traditional use of this plant against bacterial infections. Finally, silica gel column chromatographic separation of the chloroform resulted in isolation of two compounds (**1** and **2**); betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) and 4-(α-hydroxyacrylic) phenol. The structures of the isolated compounds were established using ^{1}H and ^{13}C-NMR spectroscopic methods and comparison with literature reports.*

Key words; *Vernonia hymenolepis, root extracts, phytochemical, antimicrobial.*

1. INTRODUCTION

1.1 Background of the Study

Plants have been known from prehistoric times to treat a wide array of diseases affecting human beings and livestock. The use of traditional medicine for treating human diseases remains widespread in developing countries. The recent reports of World Health Organization (WHO) indicated that 70 to 90% of world population especially from developing countries uses plant remedies for their health care [1].

Medicinal plants are now more focused than ever because they have the capability of producing many benefits to society indeed to mankind, especially in the line of medicine and pharmaceutical. Plant parts such as leaves, seeds, fruits, roots and barks are used for therapeutic purposes and as well serve as precursors for the synthesis of useful drugs due to their ethno-medical importance in nature. The medicinal potentials of these plants could be due to the bioactive phytochemical constituents such as flavonoids, alkaloids, terpenoids, anthraquinones, lignans, etc that are responsible for the physiological action [1, 2].

Ethiopians have been using medicinal plants and it has become an integral part of traditional practices in Ethiopia. The indigenous peoples of different localities in the country have developed their own specific knowledge of plant resource uses, management and conservation [3].

In view of increasing resistance to existing antimicrobial drugs, herbal drugs are being looked as very importance source for discovery of new agents for treating microbial infections [4]. The loss in efficiency of antimicrobial agents is due to the development of resistant strains and some of the drugs have plugged with unwanted side effects [5]. Efforts are consequently strengthened towards the search and improvements of antimicrobial agents with expectant safety and efficiency [6]. The accumulation of different antibiotic resistance mechanisms within the same strains has led to the appearance of the so called superbugs, or multi-drug resistant bacteria [7].

Due to these aforementioned problems, now more attention is given to isolation of bioactive compounds from plant species, mainly from herbal medicines or medicinal plants, as they may offer a new source of antibacterial, antifungal and antiviral activities [8-10]. *Vernonia hymenolepis* is one of the medicinal plants that have been commonly practiced by traditional healers for the treatments of stomach ache, faint fever and diarrhea in Ethiopia. However, the phytochemical and bioactivity information are not exhaustively studied.

1.2 Statement of the Problem

In Ethiopia, over 70% of the people depend on traditional medicines (TMs) for their healthcare, and more than 95% of the preparations are made from plant origin [11]. Even if they are effective in treating diseases, the phytochemical constituents and bioactivity of most of the plants are unknown. Thus, it needs to be supported by scientific experiment to identify constituents of the plant in searching for new chemically bioactive drugs. *Vernonia hymenolepis* is one of most commonly known medicinal plant used. The plant is used for the treatments of stomach ache, mental illness, faint fever and diarrhea in Jimma Arjo woreda, Oromia regional state. There are little information pertaining to the phytochemical analysis and antimicrobial activity of the leaves part of the plant. That is there are no studies on phytochemical constituents of the roots of the plant. Hence, there is a need to study the phytochemical constitutes and the biologically activity of the root part of the plant.

1.3 Objectives

1.3.1 General Objective

The main objective of this study was to investigate the phytochemical constituents and evaluate the antimicrobial activity of root extract of *Vernonia hymenolepis.*

1.3.2 Specific objectives

- To extract the root of *Vernonia hymenolepis* using organic solvents (chloroform and methanol);
- To evaluate the antimicrobial activities of the extracts against four bacterial strains (*Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli) and fungal strain (candida albicans)* using disk diffusion method;
- To isolate compounds from the root extract using column chromatographic techniques;
- To elucidate structure of the isolated compounds using spectroscopic technique (NMR).

1.4 Significance of the Study

The studies on antimicrobial activities of Ethiopian medicinal plants are still scarce, and there is need for further investigations and evaluate the effectiveness of these plants phytochemical constituents and crude extracts against disease causing agents and their ultimate utilization in drug development. Some herbal remedies may be safe and effective for the treatment of microbial infection. Therefore, this study is expected to give important guide on molecular structures for possible antimicrobial agent development especially in the production of synthetically improved therapeutic agents. The identified/isolated compounds would be used as markers for the standardization of herbal formulation from root extract of *Vernonia hymenolepis.* The bioactivity results of root extract of the plant may provide scientific justification for the traditional medicinal uses of this ethno-remedy and important step towards its acceptance and development as alternative therapeutic agent. Moreover, it is anticipated to provide scientific information for researchers working in similar area.

2. LITERATURE REVIEW

2.1 Botanical Information

Vernonia (Asteraceae) is the largest genus in the tribe *Vernoniae* with close to 1000 species. The genus *Vernonia* is named after William Vernon, an English botanist who collected and identified the genus in Maryland in the late 1600s. The genus is widely distributed in the tropical regions of the world. It grows in a wide range of habitats of broad ecological diversity and climatic conditions including tropical forest, marshes and wet areas, dry plains, tropical savannahs, desert xeric or dry sites [12-14].

Vernonia hymenolepis ('sooyyoma' in Afan Oromo) (Figure 1) is widely grown in different regions of Ethiopia. The plant is an ever green shrub, the stem has spines and it's green in color, the leaves are leathery pubescent and tomentose, beneath the leaves are indigenous vegetable that is commonly cultivated by farmers in Nigeria and Cameroon. *Vernonia hymenolepis* occurs in montane forest, along rivers, old cultivation areas, roadsides, in forest margins and also in bushed grassland [13].

(Photo by Alemu Geleta East Wollega Zone, Jimma Arjo area, 14/8/2019)

Figure 1. The picture of *Vernonia hymenolepis. (*a) Aerial part (b) Root part

2.2 Ethno-medicinal uses of *Vernonia hymenolepis*

Vernonia hymenolepis is one of the known traditionally used medicinal plant with the leaf part of the plant is used to treat tumor [15]. It is used by various communities for treatment of different ailments including oral conditions and toothache, pneumonia, hypertensions and diarrhea and jaundice. It has also been validated by herbalist and communities in Tanzania for the treat

amoebiasis, malaria, typhoid and constipation, although there are lack of sufficient and scientific knowledge about the dose and toxicity of the herbal preparation [13].

It has also been documented that different parts of the plant is used for the treatment of pneumonia, hypertension and also treating diarrhea in babies and jaundice. It is also widely practiced by herbalist in Trans Nzoia County, Kenya for the treatment of oral conditions especially toothache [13]. Different parts of *Vernonia hymenolepis* were also used for the healing of stomach ache, mental illness, faint fever, diarrhoea, hernia, spleen enlargement by diverse community of Tanzania [16]. Whereas, in Ethiopia, it is used for the treatment of cancer and also the crushed leaves of this plant are boiled with water and administered orally for the treatment of hepatitis [16, 17].

2.3 Phytochemistry of the Genus *Vernonia*

Phytochemical studies have indicated that the main constituents of the genus are sesquiterpene lactones, triterpenes, steroids, carotenoids, flavonoids, lignoids, alkaloids and tannins. The most common constituents are flavonoids and sesquiterpene lactones, with the latter being considered for the chemotaxonomic marker in the genus [18].

Vernonia amygdalina is widely cultivated in Yemen, Ethiopia, South Uganda, Kenya, Tanzania, and Brazil for its medicinal uses [19]. Following its wider use, several compounds with promising biological activities such as cytotoxic and antitumor activities have been isolated reported from different parts of this plant. For instance, sesquiterpene lactones such as vernolide (**1**), vernolepin (**2**), vernomenin (**3**), vernodalin (**4**)**,** hydroxyl vernolide (**5**) and vernodalol (**8**) have been isolated from the leaves part of *Vernonia amygdalina* [20, 21]. Two stigmastane-type steroid glycosides namely vernonioside D (**6**) and E (**7**) (**Figure 2**) have also been isolated from the leaves of this plant species [22].

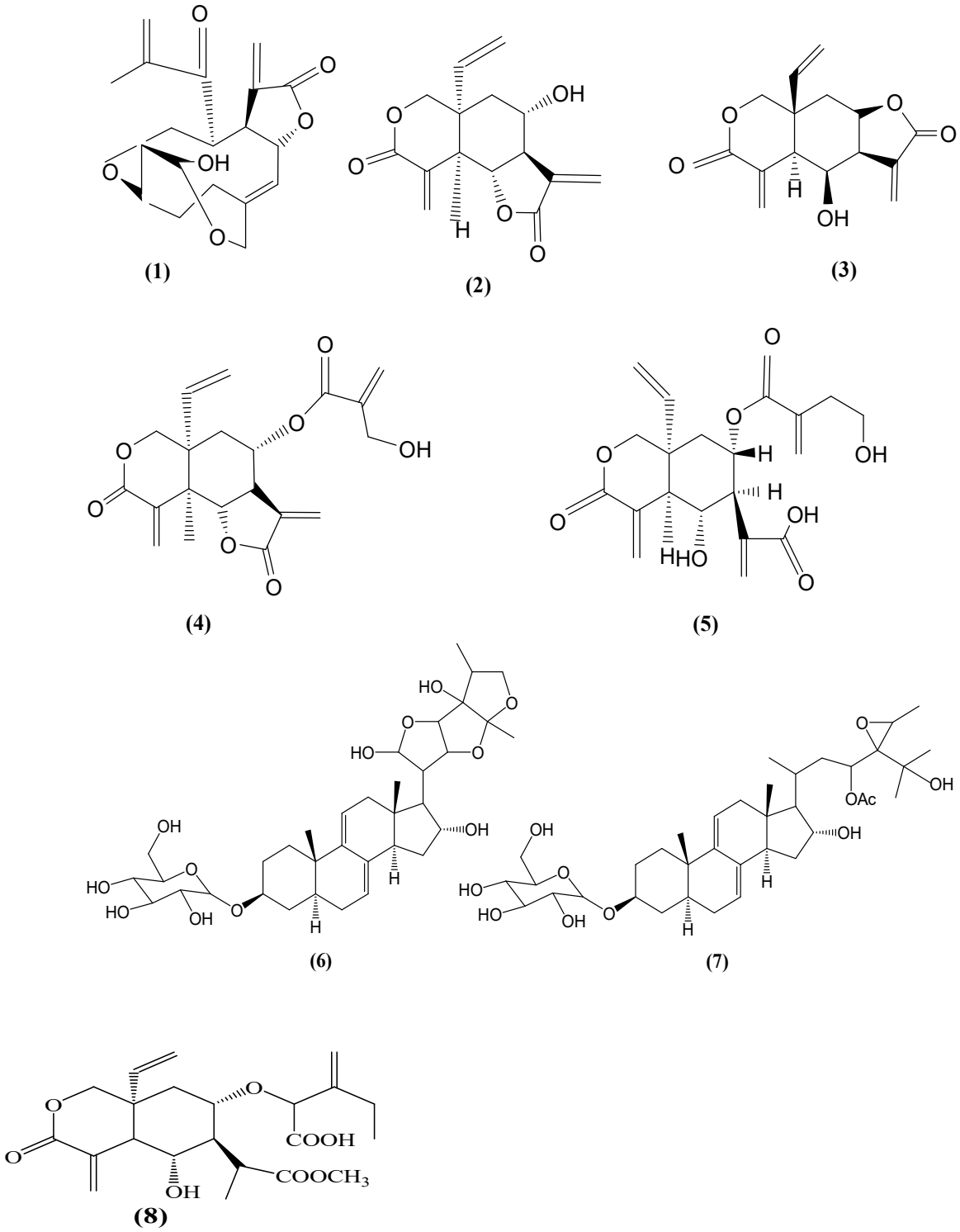
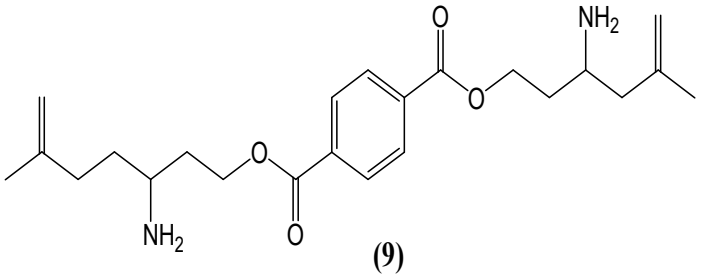


Figure 2. The chemical structures of compounds (1-8) isolated from leaves of *Vernonia amygdalina*

It is worth to mention that several compounds (**9-29**) (**Figure 3**) with good to excellent pharmacological activities have also been reported from *Vernonia amygdalina*, particularly from the polar fraction of the leave extracts [23-27].



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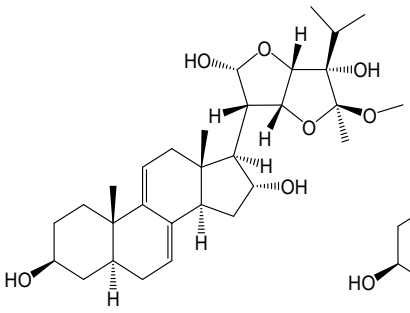

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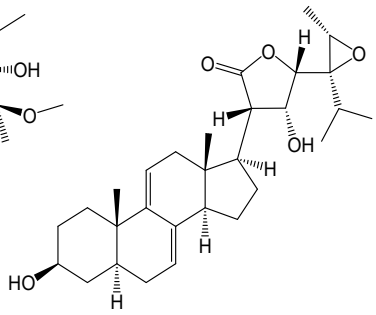

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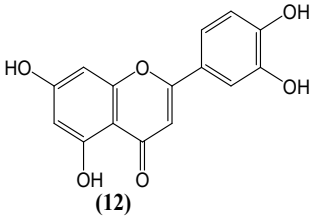

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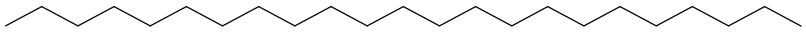

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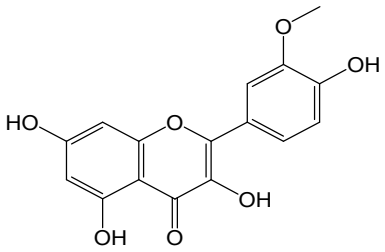

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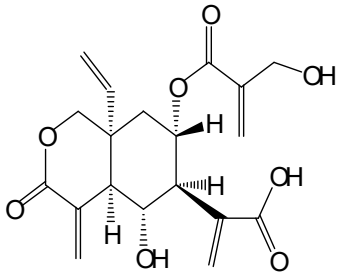

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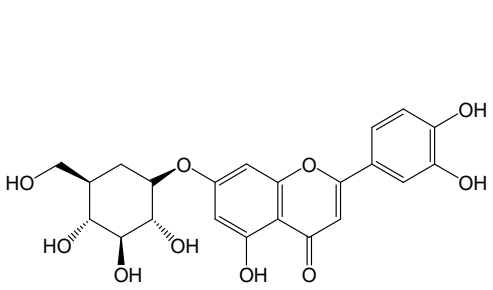

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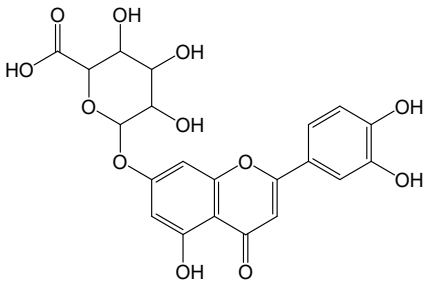

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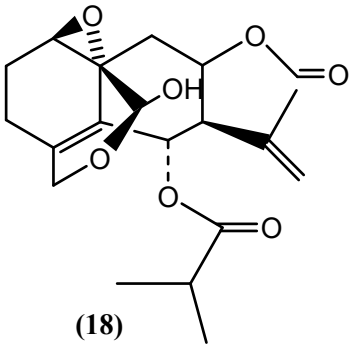

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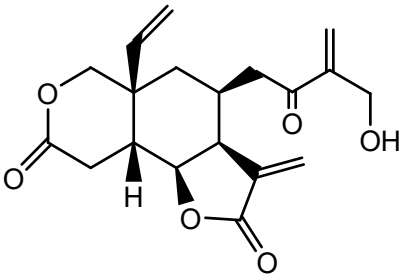

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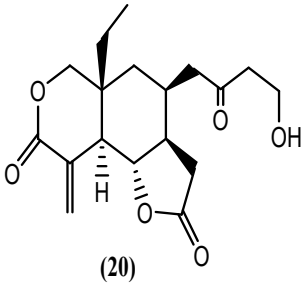

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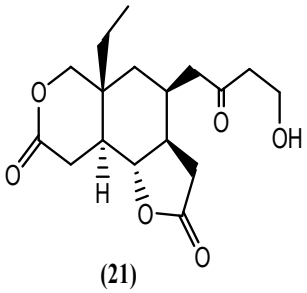

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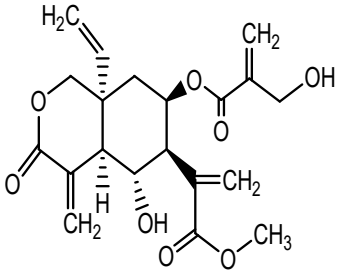

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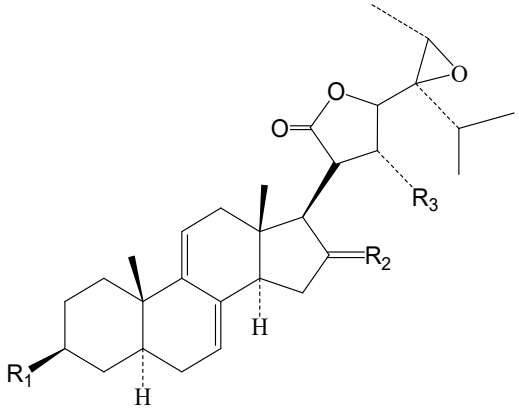

R1	R2	R3
23 *O*-Glc	β- OH, H	H
24 OH	β-OH, H	H
25 *O*-Glc	α-OH, H	H
26 OH	α-OH, H	H
27 *O*-Glc	O	H
28 OH	O	H
29 *O*-Glc	H, HO H	

Figure 3. The chemical structures of compounds (**9-29)** isolated from *Vernonia amygdalina*, particularly from the polar fraction of the leave extracts

Three elemanolide dimers such as vernodalidimer C **(30)**, D **(31)** and E **(32)** were reported from the seeds of *Vernonia anthelmintica* along with known elemanolides such as vernodalinol **(15)**, epivernodalol **(22)**, lasiopulide **(33)**, and elemacarmanin **(34)** [28]. Two new guaianolides, 1R,4R,5S,6R,7R,8S)-8,15-dihydroxyguaia-10(14),11(13)-dien-12,6-olide **(35)** and (1R,4R,5S,6R,7R,8S,11S)-8,15-dihydroxyguaia-10(14)-en-6,12-olide (**36**) , have also been reported along with two known elemanolides, (4S,5R,6R,7R,8S,10R,11S)-11,13-dihydrovernolepin (**37**) and (5R,6R,7R,8S,10R,11S)-melitensin (**38**) (Figure4) from the aerial parts of this species [29].

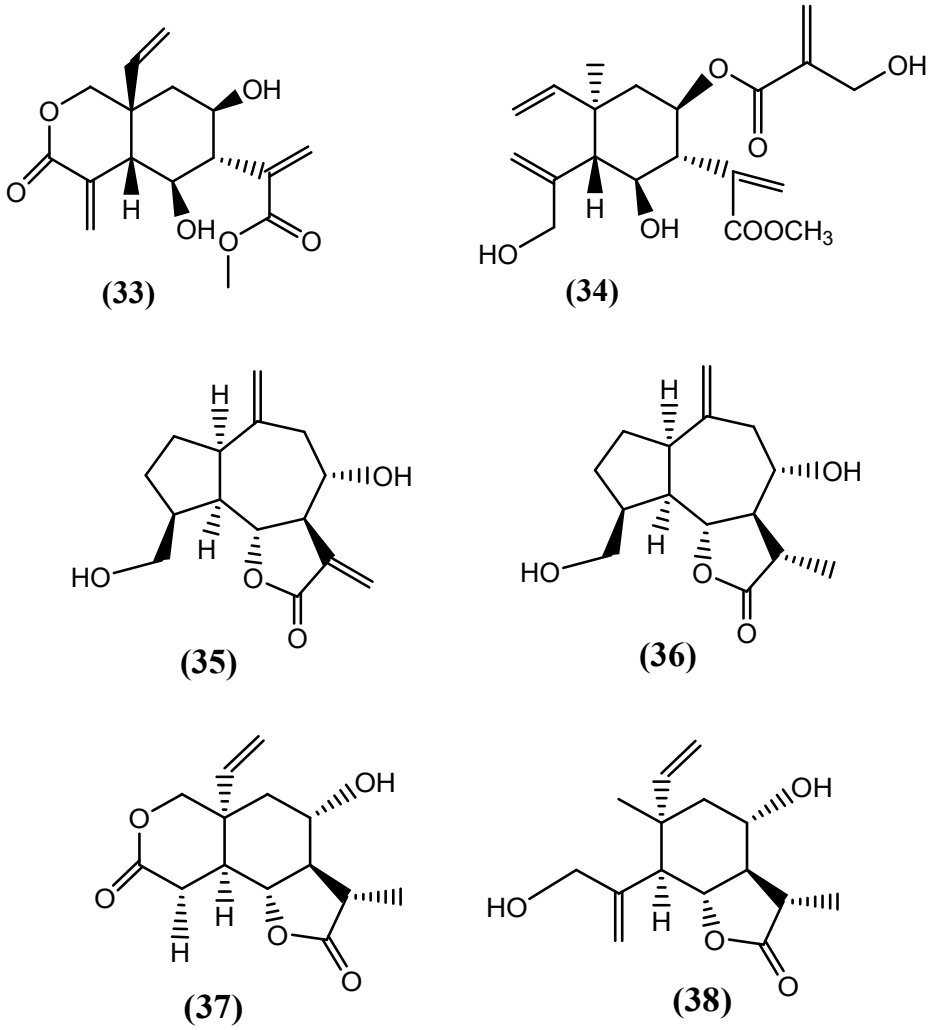

Figure 4. The chemical structure of compounds isolated from the aerial parts of *Vernonia anthelmintica*

A sesquiterpene lactone, zaluzanin D **(39)** (Figure 5) having antifungal activity has been isolated from the aerial parts of *Vernonia arborea* [30].

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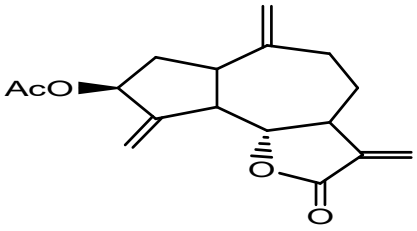
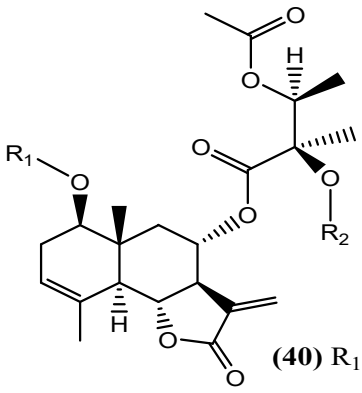


(39)

Figure 5. The chemical structure of compounds isolated from the aerial parts of *Vernonia arborea*

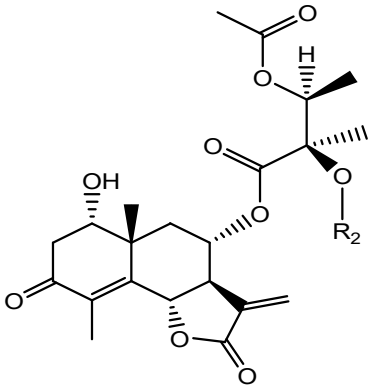
Isolation of eudesmanolide sesquiterpene lactones named blumeoidolide A **(40)**, blumeoidolide B **(41)**, blumeoidol ide C **(42)**, blumeoidolide D **(43**) has been reported from the aerial part of *Vernonia blumeoides* along known compounds; chrysin **(44)** and apigenin **(45)** luteolin **(12)** stigmasterol **(46)** and lupeol **(47)** (Figure 6) [31].



(40) $R_1 = C(O)CH_3$, $R_2 = H$

(41) $R_1 = H$, $R_2 = C(O)CH_3$

(42) $R_1 = H$, $R_2 = H$



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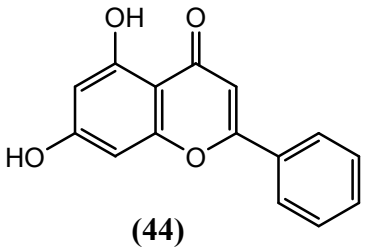

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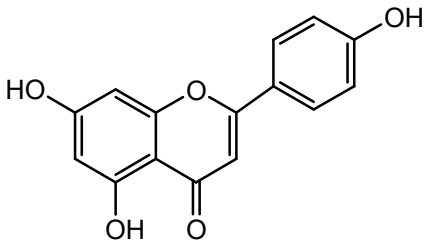
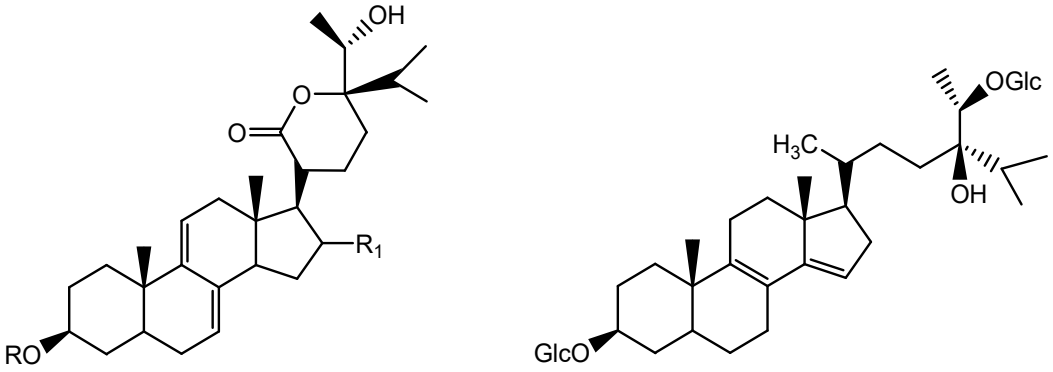


(45)

Figure 6. The chemical structure of compounds isolated from the aerial parts of *Vernonia blumeoides*

Seven stigmastane-type steroidal glucosides namely vernocuminoside **(48)**, vernocuminoside **(49)**, vernocuminoside J **(50)**, vernocuminoside **(51)**, vernocuminoside **(52)**, vernocuminoside **(53)** and vernocuminoside **(54)** (Figure 7) were isolated from ethanolic extract of stem bark of *Vernonia cumingiana* [32].



(52)

(48) R = β-D-Glc; R_1 = H, H

(49) R = β-D-Gal-(1→2)- β-D-Glc; R_1 = H, H

(50) R = β-D-Glc-(1→2)- β-D-Glc; R_1 = H, H

(51) R = β-D-Gal-(1→2)- β-D-Glc; R_1 = β-CH_3COO

Figure 7. The chemical structure of compounds isolated from ethanolic extract of stem bark of *Vernonia cumingiana*

Isolation of four sesquiterpene lactones named (8α,13-Diacetoxy-1α,10α,-5β,6β-diepoxygermacra-7(11)-en-12-olide **[55]**, 10α,4α-Dihydroxy-5β,6β-isoglaucolide B **(56)**, 8α,13-Diacetoxy-1α,10α,-1β,5β-diepoxygermacra-7(11)-en-12-olide **(57)** and 8α,13-Diacetoxy-1α,10α-hydroxy-5,6-epoxy-hirsutinolide **(58)**, together with diacetylpiptocarphol **(59)**, 8-acetyl-13-etoxypiptocarphol **(60)**, and ethyl caffeate **(61)** from fresh leaves and flowers of *Vernonia scorpioides* were reported [33].

2.4 Pharmacological activities of Genus *Vernonia*

The genus *Vernonia* is one of the largest, predominantly in Africa and South America. Its species have wide ethno-pharmacological use for treatment of several diseases. In Ethiopia, a study of nine plants with the ethno-medicinal use indicated that *Vernonia* species are used for the treatment of eye infections, for wound healing and the treatment of bone related problems such as fractures [18].

The sesquiterpene lactone vernolepin was isolated from *Vernonia hymenolepis* collected in Ethiopia. It is used medicinally as a cure for pneumonia, and a hot leaf placed on a wound is said to stop bleeding. Juice from crushed leaves is used to treat diarrhoea in babies and jaundice. In Kenya a root decoction is used as a purgative and to treat abdominal pains. Dry branches and stems serve as fuel [34].

2.4.1 Antibacterial Activity

In *Vernonia amygdalina* the sesquiterpene lactones vernolide **(1)** and vernodalol **(8)** were reported to have antibacterial and antifungal activity. In Tanzania the petroleum ether extract of the leaves of *Vernonia adoensis* was assayed against the bacteria *Pseudomonas aeruginosa Bacillus subtilis*, *Escherichia coli* and *S. aureus* strains using disk diffusion and agar diffusion methods and bioactivity against the *E. coli* strain. The methanol extract of the seeds of *Vernonia anthelmintica* was tested against *P. aeruginosa*, *S. flexneri*, *E. coli* and *S. aureus* strains. The extracts showed antibacterial activity against all strains tested except *Y. aldovae*. The essential oil of leaves of *Vernonia brasiliana*, extracted by hydro-distillation, was tested against *P. aeruginosa*, *Enterobacter aerogenes*, *Salmonella choleraeasuis*, *K. pneumoniae*, *S. aureus* and *B. subtilis*, by the disk diffusion method, finding broad-spectrum antibacterial activity against all tested microorganisms [18].

2.4.2 Antifungal Activity

Evaluation of the antifungal activity of the chloroform extract of the stems of *V. scorpioides* against *P. citrinum* fungus showed the formation of growth inhibition zones up to 80 mm in diameter. The presence of vernolide **(1)** and vernodalol **(8)** which belongs to sesquiterpene lactones in the *V. amygdalina* leaves extracts made them exhibit higher antifungal effect against *Penicillium notatum* and *Aspergillus flavus* with LC_{50} values of 0.4 mg/ml [18]. Zaluzanin D, a sesquiterpene lactone which has been isolated from the aerial parts of *Vernonia arborea* assayed for antifungal activity against six plant-pathogenic fungi, the compound showed 100% inhibition in mycelial growth of *Rhizoctoniasolani*, the effect being ca.75% with *Curvularialunata* and *Botrytiscinereaat* 200 ppm concentration. With *Colletotrichum lindemuthianum*, *Fusariu mequisetii*, *F. oxysporum* only moderate activity (60%) was observed at 200 ppm [30].

2.4.3 Anticancer Activity

Medicinal plants have been a rich source of clinically effective anticancer agents for the past few decades. Over 60% of the currently used anticancer drugs are either directly derived from plants or inspired by their novel phytochemicals and/or unique ligands as secondary metabolites[35]

Breast cancer has been the second leading cause of deaths in the world. *Vernonia amygdalina* leaves extracts had been reported to inhibit the proliferation of MCF-7 and MDA-MB-231 which involved the stimulation of cell-type specific G1/S phase cell cycle arrest in only MCF-7 cells but not in MDAMB-231 cells given an approximate of 70% of diagnosed breast cancer express ER-α. Owoeye et al. 2010 has also reported the presence of epivernodalol in the methanolic extract of *Vernonia amygdalina* leaf which was active against HT-144 (skin melanoma) cell line [18].

2.4.4 Antioxidant Activity

The crude extracts from *Vernonia amygdalina* has been reported to possess an antioxidant property by scavenging the free radicals cells. The aqueous extracts from the leaf showed a significant reduction in the malondialdehyde levels of oxidative stressed streptozotocin-induced diabetic rats. The leaves extracts had been examined to scavenge 75-99.3% DPPH radicals and 96.2-100% of the ABTS radicals. The presence of flavonoids in the *Vernonia amygdalina* extracts has been attributed to have antioxidant property [18].

2.4.5 Anti-diabetic Activity

Studies have shown that aqueous extracts from *Vernonia amygdalina* leaves reduced the blood glucose, increased the serum triglyceride levels and serum MDA, increased the LDL-cholesterol, and normalized cholesterol concentrations in streptozocin-induced diabetic rats. In another study on the effect of *Vernonia amygdalina* leaf extracts on blood glucose of diabetic rats, the results showed that decrease in blood glucose after administration of the extracts may be associated with the presence phytochemicals, vitamins and other nutrients in the extracts [26]. The acetonic extract of the leaves of *Vernonia colorata* induced a significant decrease of blood glucose in normoglycaemic rats. Therefore, it has an antidiabetic activity in hyperglycaemic rat models [36].

2.4.6 Anti-inflammatoryActivity

Vernonia amygdalina leaves extracts had been reported to possess anti-inflammatory activity when applied to the ear of rat suffering from inflammation. It produced a significant reduction when compared with the application of acetylsalicylic acid. The percentage of inhibition of leaves extracts was higher than roots extracts [27]. The anti-inflammatory activity of vernocuminosides isolated from ethanolic extract of stem bark of *vernonia cumingiana* was assayed by measuring the inhibition of the platelet activating factor (PAF)-induced release of β-glucuronidase from the ratpolymorphonuclear leucocytes *in vitro* [32].

2.4.7 Anti-malarial Activity

Leaves and roots of *Vernonia amygdalina* extract possessed antimalarial effect against drug sensitive *Plasmodium berghei* in mice which resulted in 67% and 53.5% suppression of parasitaemia after the four days of administration, respectively. The leaves extracts had exhibited a significant antiplasmodial effect in mice against *Plasmodium berghei* with 73% inhibition. In the same vein, isolated sesquiterpene lactones from *Vernonia amygdalina* had been reported to show antiplasmodial property with IC_{50}< 4 µg/ml against *Plasmodium falciparum* [27]. The antiplasmodial activity of two sesquiterpene lactones which were isolated from acetonic leaves extract of *V. colorata* has been reported. They show excellent antimalarial activity: vernolide (IC_{50}= 1.87µg/ml) and vernodalin (IC_{50}= 0.52µg/ml) with selective indices of 1.02 and 2.79 respectively [37].

3. MATERIALS AND METHODS

3.1 Collection of Plant Material

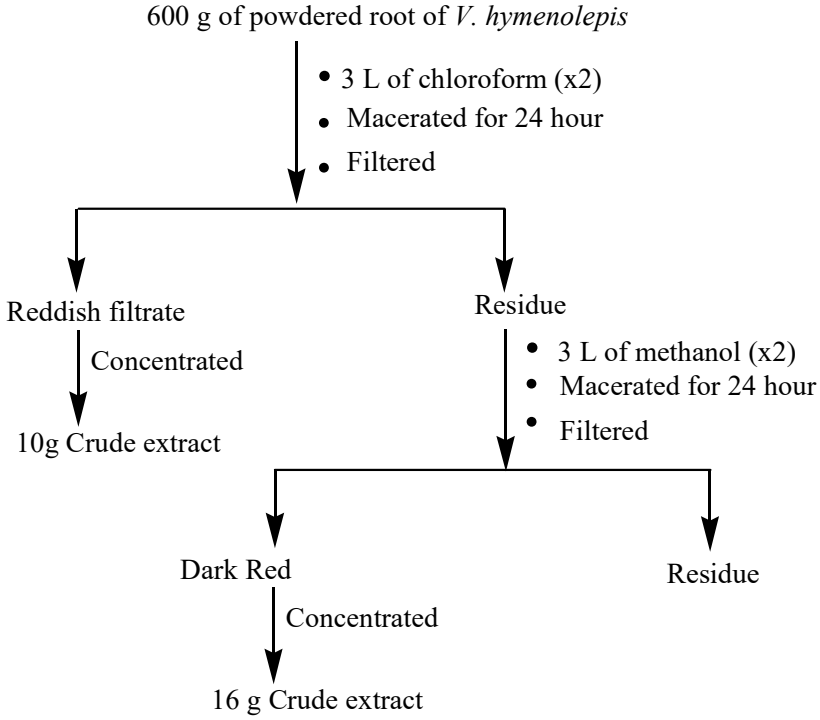
The roots of the *Vernonia hymenolepis* were collected from Oromia regional state, East Wollega Zone, Jimma Arjo woreda around Arjo Dedessa Sugar Factory on August 14, 2019. The plant material was air dried under shade at Chemistry laboratory, Jimma University. The air-dried plant material was pulverized using an electric grinder to increase the surface area.

3.2 chemicals and apparatus

The powdered plant material was extracted by cold maceration method and solvent was freed using rotary evaporator. Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Spots were detected by observation under UV light (254 nm and 365) followed by inserting in iodine chamber. Column chromatography was performed on oxalic acid impregnated silica gel (60-120 mesh) Merck. Analytical grade solvents (petroleum ether, acetone, chloroform, ethyl acetate and methanol) were used for extraction and separation. Standard drug (Gentamicin and Clotrimazole), culture medium (Mueller Hinton agar, nutrient agar, potato dextrose agar) and DMSO were used during antimicrobial test. 1D NMR analysis was recorded on a Bruker 400MHz spectrometer with tetra methyl silane as internal standard; $CDCl_3$ and DMSO-d_6 as solvent.

3.3 Extraction of the crude extracts

A 600 g of powdered plant material was extracted sequentially in 3 L chloroform and methanol using cold maceration technique two times for 24 hour each. The extracts were filtered with Whatmann No.1 filter paper and concentrated using rotary evaporator to at 40 °C (Scheme 1).



Scheme 1: The General procedure of extraction of root of *Vernonia hymenolepis*

3.4. Isolation of Compound

About 7 g of chloroform extract was adsorbed onto an equal amount of silica gel and subjected to silica gel column chromatography separation. The mixture was eluted with increasing gradient of petroleum ether ethyl acetate: methanol to afford 28 fractions each 25 mL (Table 1).

Table 1: Column chromatography fractionation of chloroform extract

Solvent system	Proportion (V:V)	Fractions	Resolved spots
Petroleum ether	50:0	1	Nothing
		2	
Petroleum ether: Ethyl acetate	45:5	3	
		4	
Petroleum ether: Ethyl acetate	40:10	5	
		6	
Petroleum ether: Ethyl acetate	35:15	7	
		8	
Petroleum ether: Ethyl acetate	30:20	9	Two spots
		10	
Petroleum ether: Ethyl acetate	30:20	11	Two spots
		12	
Petroleum ether: Ethyl acetate	25:25	13	
		14	
Petroleum ether: Ethyl acetate	20:30	15	Two spots
		16	
Petroleum ether : Ethyl acetate	20:30	17	
		18	
Petroleum ether : Ethyl acetate	10:40	19	Three spots
		20	
Ethyl acetate	50	21	
		22	
Ethyl acetate: Methanol	45:5	23	
		24	
Ethyl acetate: Methanol	40:10	25	Four spots
		26	
Ethyl acetate: Methanol	30:20	27	
		28	

Among the 28 fractions collected, fractions (F1-F8) were colorless and could probably contain fats. Fractions 9-12 (petroleum ether/ethyl acetate, 3:2) showed white precipitate and washed successively with petroleum ether afforded compound **1** (8 mg). Whereas fractions from 19- 24 were combined based on their TLC profile and subjected to small column chromatography, eluted with petroleum ether increasing gradient of ethyl acetate and resulted compound **2** (13 mg)

3.5 Antimicrobial susceptibility testing

3.5.1 Antibacterial Test

The *in vitro* antibacterial activities of the root extracts of *Vernonia hymenolepis* were evaluated using Agar well diffusion method against four bacterial strains, two gram negative; *Escherichiacoli (*ATCC25922) and *Pseudomonas aeruginosa (*ATCC27853) and two gram positive; *Staphylococcus aureus* (ATCC25923) and *Bacillus subtilis (*ATCC11778). The bacteria stock cultures were maintained on the nutrient agar slants, which were stored at 40°C. The test solutions were prepared by dissolving 200 mg of the test samples to achieve final stock concentrations of 200 mg/mL in DMSO. The same concentration of Gentamycin (positive) control was also used. The freshly grown liquid culture of the test pathogens solution of having similar turbidity with 0.5 McFarland were seeded over the Müeller-Hinton Agar medium plates. The plates were shaken gently to allow evenly mixing of bacterial cells and agar. Sterile paper discs (6 mm diameter, Whatmann No.3) were separately soaked in the above stock solutions (samples and standards) and then applied over the seeded plates at equidistance. Control experiments were carried out under similar conditions by using gentamycin for antibacterial activity as a standard drug. The plates were then inverted and incubated at 37 °C for 24 hour. After the incubation period, the plates were observed for a clearance zone around the disks. Clear inhibition zones formed around the discs indicated the presence of antibacterial activity and measured in millimeter. Each experiment was carried out in triplicates. The mean of the inhibition zone of each test sample was taken for evaluating the antibacterial activity. The inhibition zones produced by the plant extracts were compared with the inhibition zones produced by commercial standard antibiotics [38].

3.5.2 Antifungal Test

Using the same method used for antibacterial testing, a fungal strain *Candida albicans* was also used; potato dextrose agar (PDA) as test media; clotrimazole as standard anti-fungal drug (positive control) and longer incubation period 72 hour were employed [38].

RESULTS AND DISCUSSION

4.1 Yield of Extracts

A 600 g of powdered plant material which was extracted sequentially using 3 L of chloroform and methanol with cold maceration technique two times for 24 hour each yield 10g of crude extract in chloroform and 16g in methanol solvents .The color of chloroform filtrate was reddish and that of methanol was dark red. The yield obtained using methanol was better than that of chloroform and the result for chloroform extract could be due to concentration.

Table 2-Extraction summery

Solvents	Crude Extracts (g)	Yield (%)
Chloroform	10	1.67%
Methanol	16	2.71%

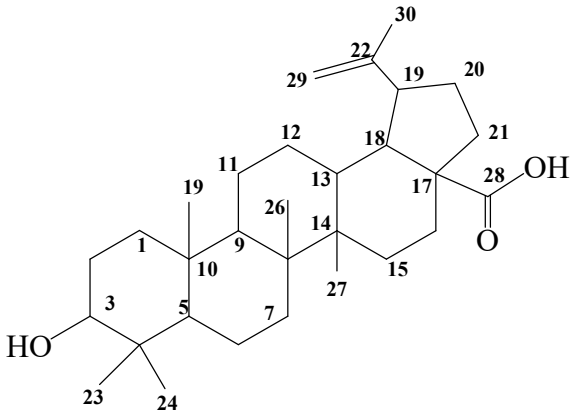
4.2 Structural elucidation of the isolated compounds

Compound **1** was isolated as a white powder with R_f value of 0.62 (30% ethyl acetate in petroleum ether). The ^{1}H NMR spectrum ($CDCl_3$, 400MHz) (Appendix 1) revealed signals at δ_H 0.96 (3H, s, H-27), 0.93 (3H, s, H-25), 1.25 (3H, H-23), 1.27(3H, s, H-24), 1.37 (3H, s, H-26) and 1.70 (3H, s, H-30) for six methyl group; two germinal olefinic proton at δ_H 4.73 (1H, H-29) and 4.60(1H, H-29), and methine proton at δ_H 3.25 (1H, m, H-28) and one oxymethine proton atδ_H 3.15 (1H, m, H-3)T he^{13}C NMR spectrum ($CDCl_3$, 400MHz) (Appendix 2) displayed signals for thirty carbon atoms corresponding to one downfield shifted carbon signals at δ_C 175.5, which indicates the presence of carboxylic acid, assignable to C-28 and other six quaternary carbons at δ_C 37.69 (C-4), 42.15 (C-8), 35.95 (C-10), 42.15 (C-14), 51.54 (C-17), and 145.74 (C-22); five methine carbons at δ_C 51.54 (C-5), 51.54 (C-9), 37.69 (C-13), 44.51 (C-18), 45.77 (C-19), and one oxymethine carbon at δ_C 74.26 (C-3); eleven methylene carbons at δ_C 37.69 (C-1), 25.83 (C-2), 20.76 (C-6), 34.12 (C-7), 23.25 (C-11), 25.83 (C-12), 32.46 (C-15), 33.62 (C-16), 29.58 (C-20), 34.01 (C-21) and 104.91 (C-21); 6 methyl carbons at δ_C 29.58 (C-23), 16.11 (C-24), 16.11 (C-25), 16.11 (C-26), 13.55 (C-27) and 24.9 (C-30). The carbon signals at δ_C104.91 (C-21) and 145.74 (C-22) are attributed to the olefinic carbons, as in lupeol. Therefore, based on the above spectroscopic data and comparison of these data with the related literature [39], the structure of compound **1** was identified to be betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid).

This compound has previously been isolated from stem bark of *Feretia canthioides* [39] and the root of *Vernonia guineensis* [40]. Betulinic acid is a naturally occurring pentacycliclupane type triterpenoid which exhibits a variety of biological and medicinal properties such as inhibition of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antinociceptive, anti-HSV-1, and anticancer activities [41] which also agrees with this plants traditional use.

Table 2: ^{13}CNMR (400MHz) data of compound **1** (in $CDCl_3$), and lupeol-28-oic acid (in DMSO) **[**39**]**

Position	^{13}C NMR Compound **1**	lupeol-28-oic acid ^{13}C NMR data [39]	Carbon Type
1	37.69	39.43	CH_2
2	25.83	27.36	CH_2
3	74.26	77.04	CH
4	37.69	38.71	C
5	51.54	55.12	CH
6	20.76	18.17	CH_2
7	34.12	34.13	CH_2
8	42.15	39.97	C
9	51.54	50.15	CH
10	35.95	36.93	C
11	23.25	20.67	CH_2
12	25.83	25.29	CH_2
13	37.69	37.78	CH
14	42.15	42.19	C
15	32.46	30.33	CH_2
16	33.62	31.93	CH_2
17	51.54	55.63	C
18	44.51	46.84	CH
19	45.77	48.73	CH
20	29.58	29.43	CH_2
21	34.01	36.57	CH_2
22	145.74	150.52	C
23	29.58	28.30	CH_3
24	16.11	15.94	CH_3
25	16.11	15.94	CH_3
26	16.11	16.09	CH_3
27	13.55	14.58	CH_3
28	175.46	177.48	COOH
29	104.91	109.82	CH_2
30	20.76	19.14	CH_3



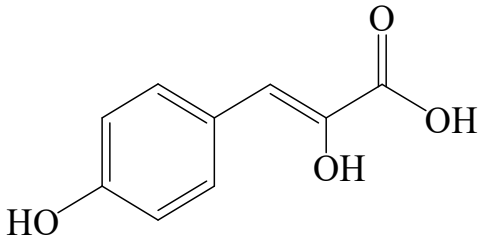
Compound **1**

Compound **2** was obtained as light yellow powder with R_f value of 0.45 in methanol :chloroform (3:7). The ^{1}H NMR spectrum (Acetone-d_6, 400MHz) (Appendix 3) showed only two set of aromatic protons at δ_H 7.04 and 8.22, integrated for two protons each and were assigned to H-3 and H-5 and (H-2 and H-6), respectively. It also showed a down field shifted olefinic proton at δ_H 7.39 (H-7) due to its peri-position to the carbonyl group.

The ^{13}C NMR spectrum (Acetone-d_6, 400 MHz) (Appendix 4) showed the presence of seven signals for nine carbon atoms. The signal at δ_C167.9 corresponds to the carbonyl carbon (C-9), the signals at δ_C 155.5, and 129.9 correspond to C-1, and C-4, respectively, and the intensified carbon signals (due to the symmetric effect) at 131.9 and 113.35 corresponding to two set of meta carbons (C-2 and C-6) and (C-3 and C-5), respectively. Whereas the carbon signals at δ_C 128.2 and 118.1 were assigned to C-8 and C-7, respectively. Thus, based on the above spectral data and comparison with data with literature [42], the structure of compound 2 was found to be 4-(α-hydroxyacrylic) phenol.

Table 3: ^{1}H- and ^{13}C-NMR (in Acetone-d_6) spectral data of compound **2** and 4-(α-hydroxyacrylic acid) phenol **[42]**

Position	Compound 2 δ_H (*m*)	δ_C	4-(α-hydroxyacrylic acid) phenol δ_H (*m*)	δ_C
1		155.5		159.67
2	7.04 (dd)	131.9	7.44 (d)	130.56
3	6.85 (d)	113.35	6.78 (d)	116.45
4		129.9		126.24
5	6.85 (d)	113.5	6.78 (d)	116.45
6	7.04 (dd)	131.9	7.44 (d)	130.56
7	7.39 (d)	118.1	6.27 (d)	117.38
8		128.2		144.02



Compound **2**

4.3 Antimicrobial activities

Preliminary screening of phytochemicals is a valuable step in the detection of bioactive principles present in medicinal plants and may lead to novel environmentally friendly bioherbicides and drug discover [43].

The two crude extracts (chloroform and methanol extracts) were screened for their *in vitro* antimicrobial activity using disk diffusion method and showed varying degrees of responses against the four bacterial strains and one fungal strain (Table 5).

Table 4: Inhibition diameter (mm) of bacterial and fungal growth

Extracts and standards	Growth inhibition zone (mm)				
	Bacterial strains				Fungal strain
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
Chloroform extract	14	17	18	11	NI
Methanol extract	13	12	14	13	13
Gentamycin (Ref)	17	23	25	12	NT
Clotrimazole (Ref)	NT	NT	NT	NT	12
DMSO (Solvent)	NI	NI	NI	NI	NI

Key: NI= No Inhibition, NT = Not Tested

The extracts showed a promising antibacterial activity against all gram positive and gram negative bacteria strains with diameter zones of inhibition 11 to 18 mm. Better antibacterial activities were observed for chloroform extract against *B. subtilis*, *S. aureus* and *E. coli* with inhibition zones of 14 mm, 17 mm and 18 mm, respectively, whereas moderate activities were displayed for methanol extract. Both methanol and chloroform extracts exhibited significant (considerable) antibacterial activities against Gram negative bacterium, *Pseudomonas aeruginosa* with zones of inhibition 13 mm and 11 mm, respectively which is equivalent to that of reference drug, gentamycin (12 mm). The observed activity may be due to the presence of phytochemicals sterols/triterpenes, phenolics and flavonoids found this plant [44, 45]. Methanol extract showed the highest activity (13 mm) against *C. albicans* but no remarkable activity was observed for chloroform extract. These *in vitro* activities are in good agreement with previous report in Kenya [12].

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Silica gel column chromatographic separation of chloroform root extract afforded two compounds (**1** and **2**). The extracts showed a promising antibacterial activity against all gram positive and gram negative bacteria with diameter zones of inhibition 11 to 18 mm. The findings of antimicrobial study concur with the results of previous work. Therefore, the antimicrobial activity displayed by the root extracts of *Vernonia hymenolepis* substantiates the traditional use of this plant against bacterial infections. In agreement with the previous study, the wide traditional use of the plant may be attributed to its rich terpene and phenolic chemical constituents.

5.2 Recommendation

Based on the present study the following recommendations are forwarded:

- In this study only the phytochemical investigation of root extract of plant was carried out. Thus, we recommend conducting phytochemical investigation on other aerial parts like leaves and stems by using different solvent system and separation techniques
- Isolated compounds antimicrobial activity test is recommended so as to validate the traditional medicinal use of the plant.
- Additional studies are needed with this plant to evaluate for *in vivo* bioassay.
- The crude TLC showed still couple of unidentified minor compounds. Thus these minor constituents can be isolated using high-tech separation techniques including HPLC techniques.

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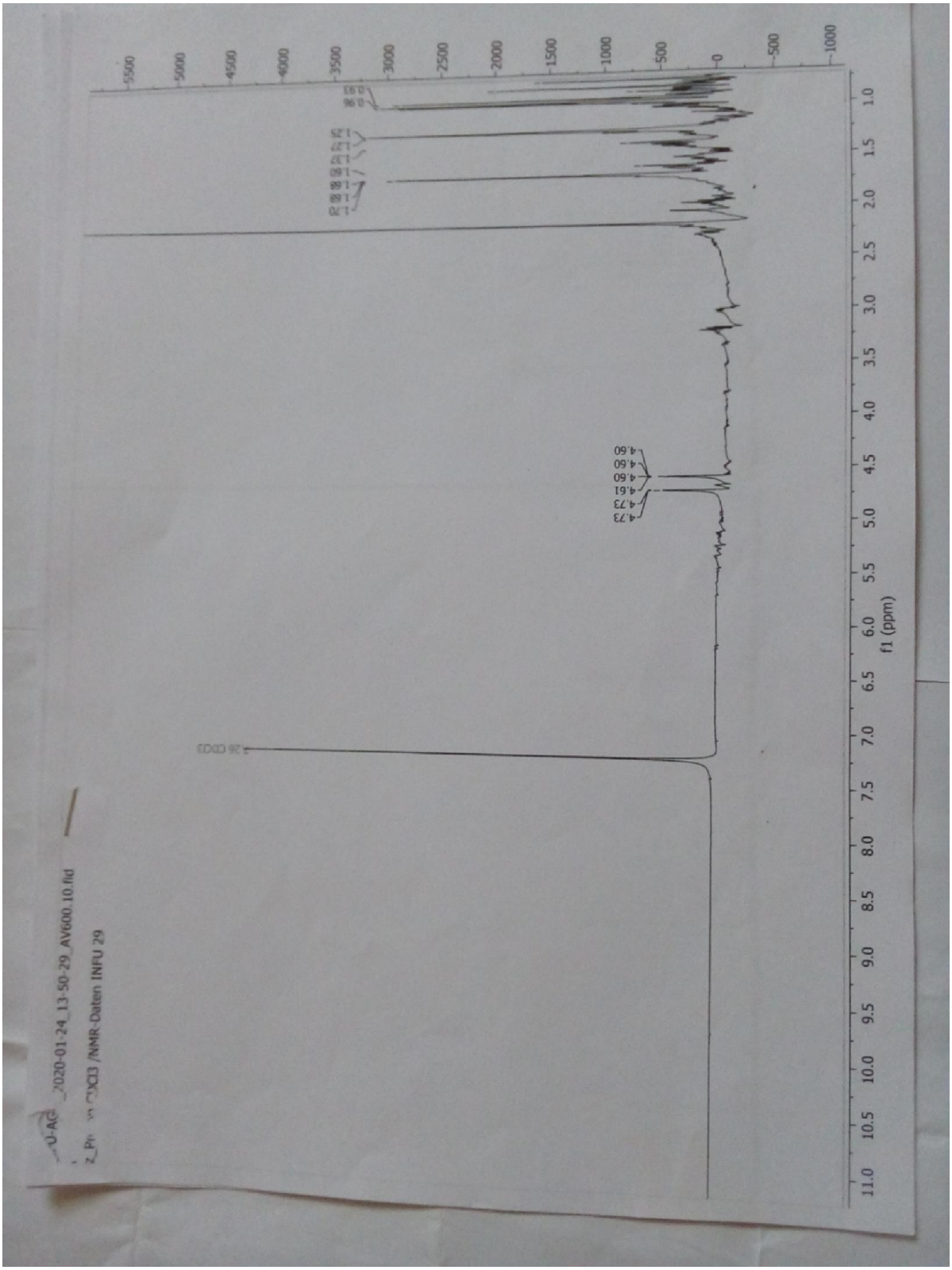
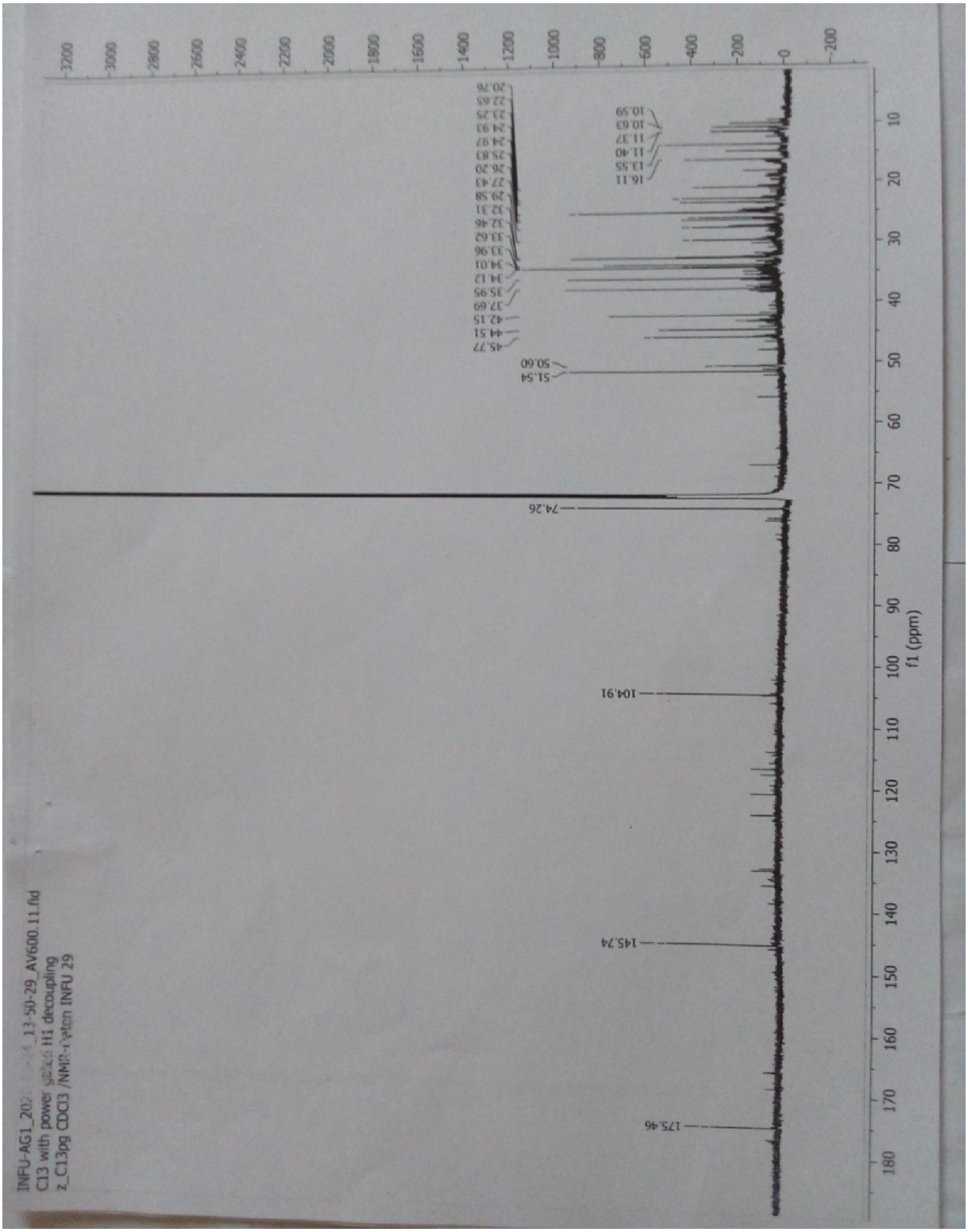
APPENDICES

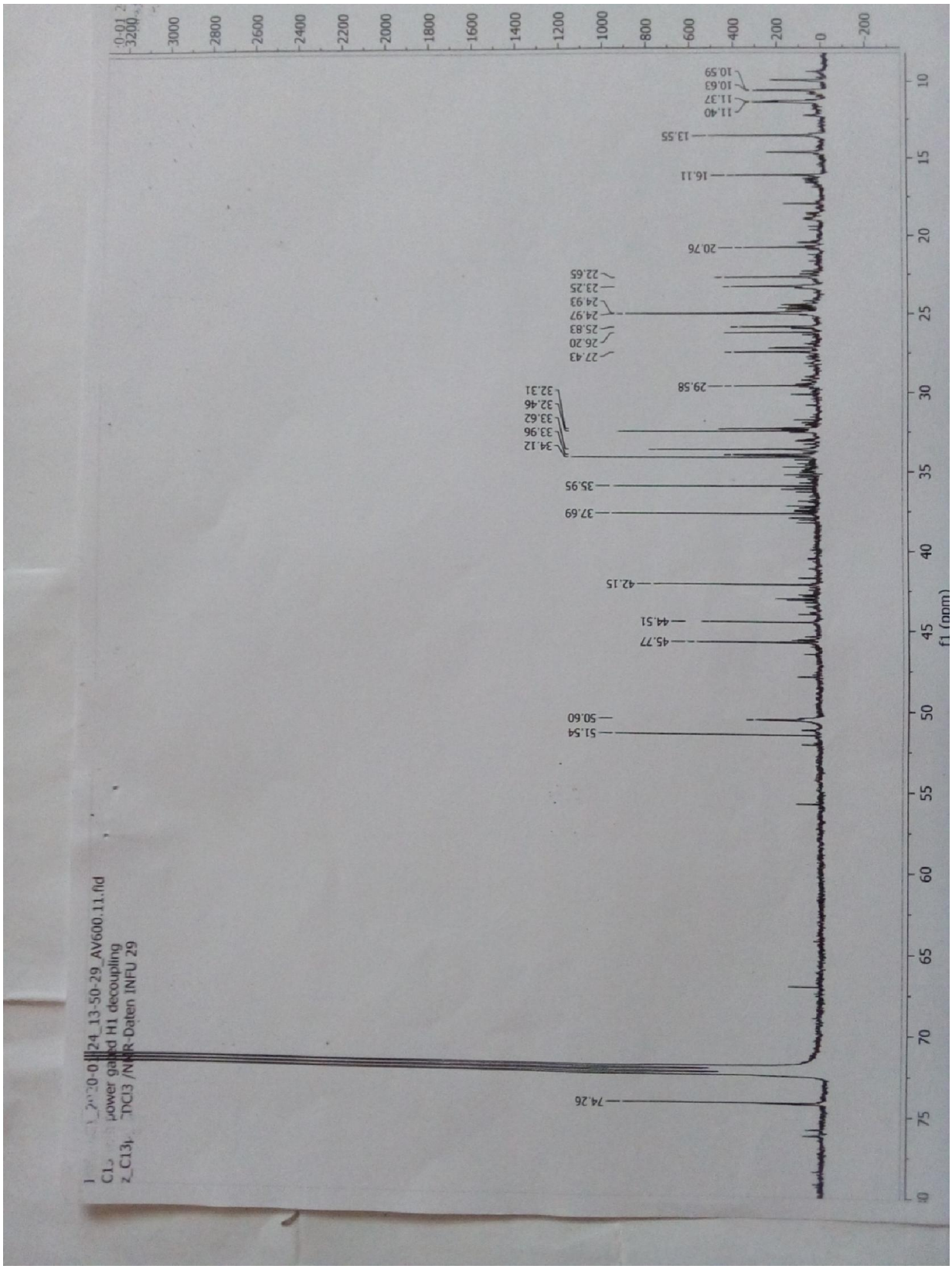
Appendix 1: The ^{1}HNMR spectrum of compound **1** in $CDCl_3$



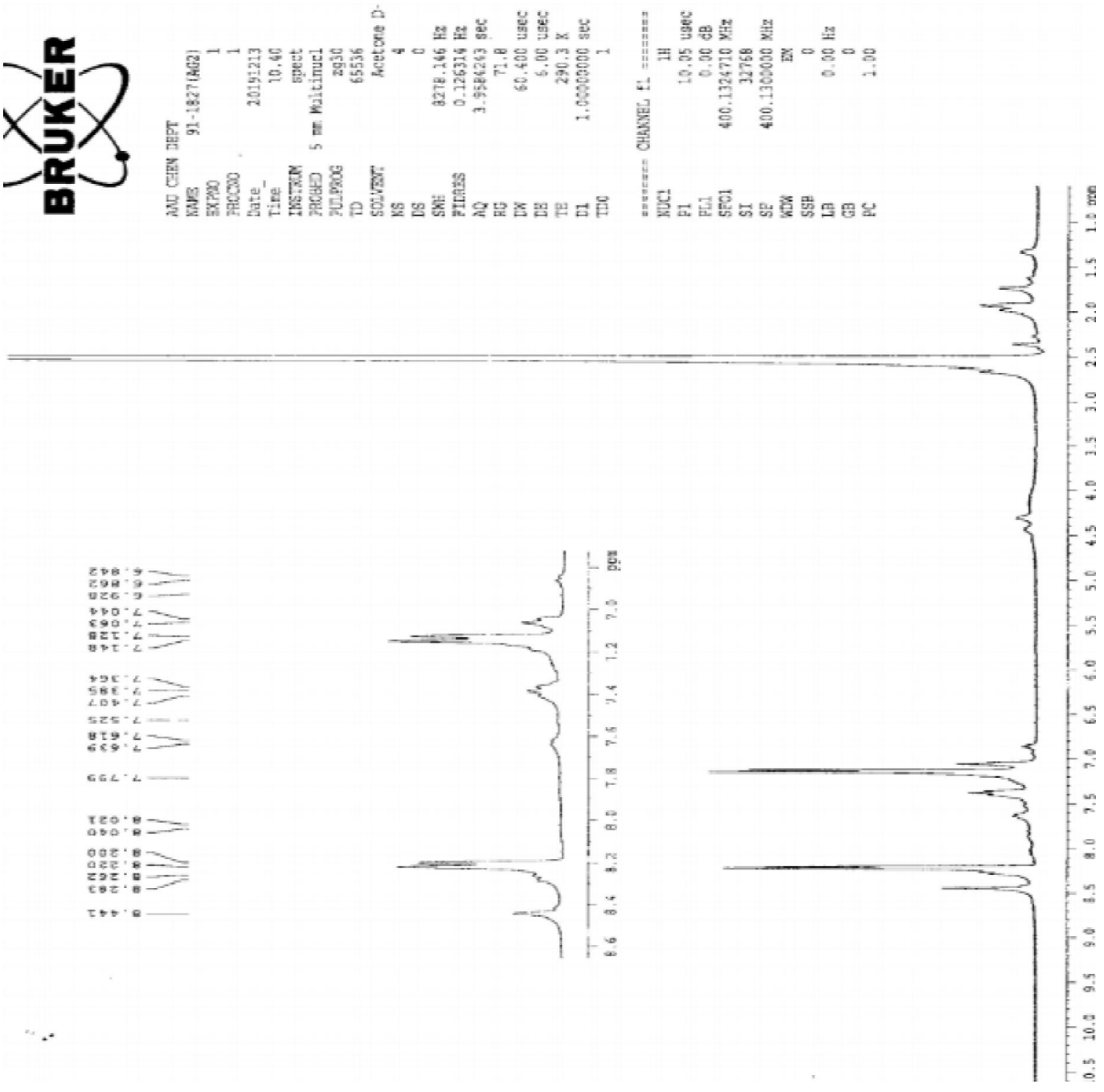
Appendix 2: The ^{13}C NMR spectrum of compound **1** in $CDCl_3$



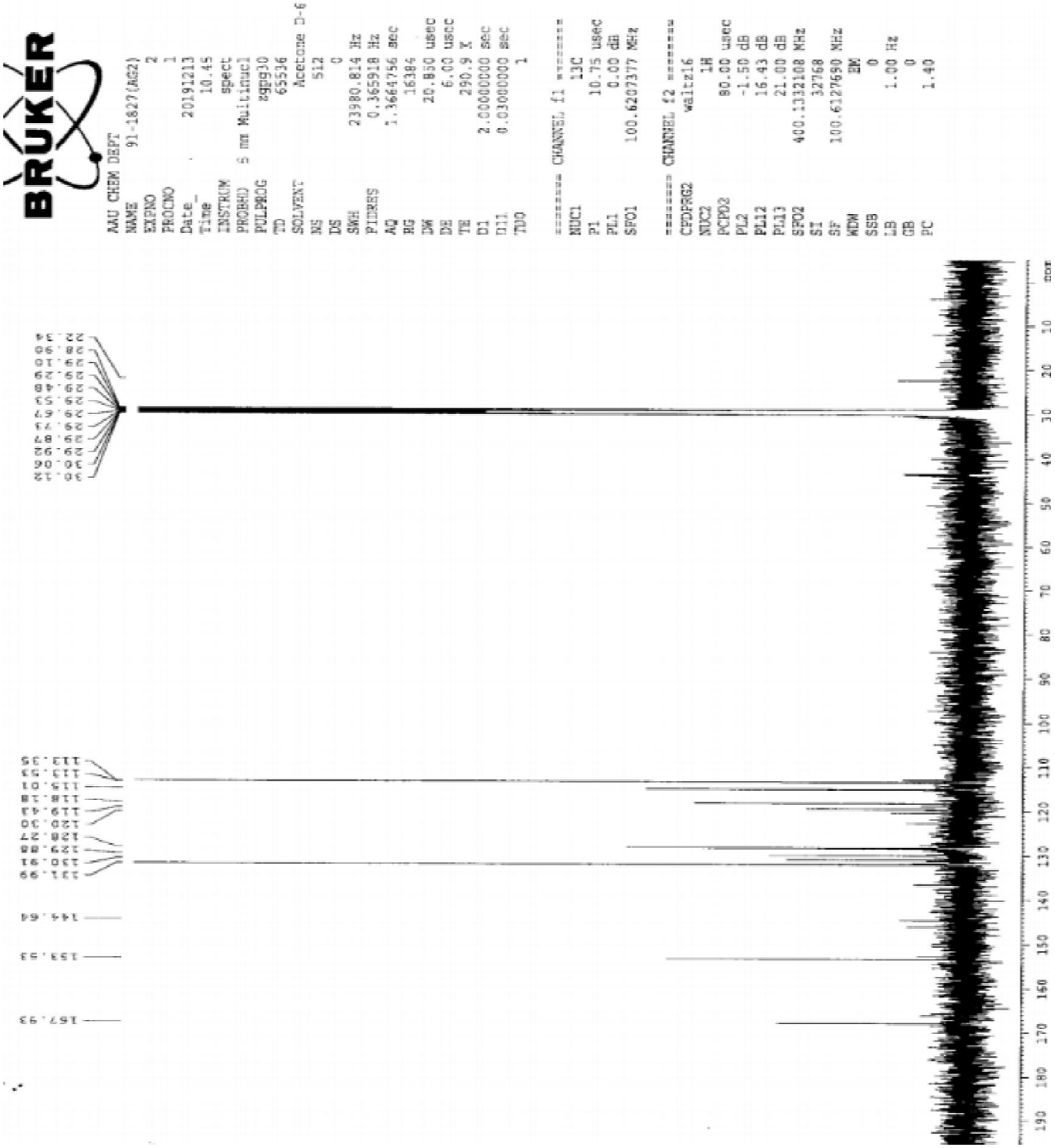
Appendix-2: The ^{13}C NMR spectrum of compound **1** in $CDCl_3$ (Expanded)


74.26
51.54
50.60
45.77
44.51
42.15
37.69
35.95
34.12
33.96
33.62
32.46
32.31
29.58
27.43
26.20
25.83
24.97
24.93
23.25
22.65
20.76
16.11
13.55
11.40
11.37
10.63
10.59
f1 (ppm)

Appendix 3: The ^{1}H NMR spectrum of compound**2** in Acetone-d_6



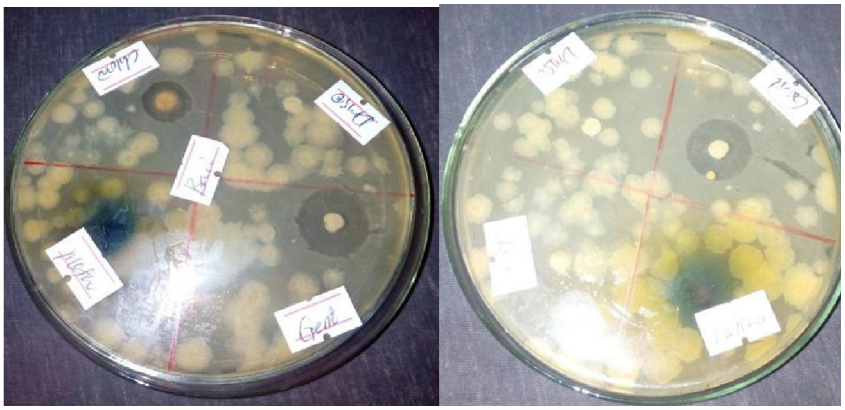
Appendix 4: The ^{13}C NMR spectrum of compound**2** in Acetone-d_6



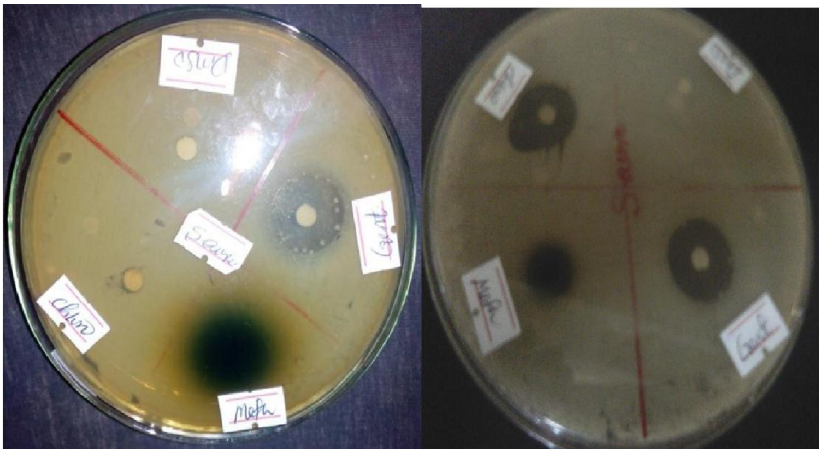
Appendix 5: Picture of Bioassay Activity

Antibacterial Activity Test

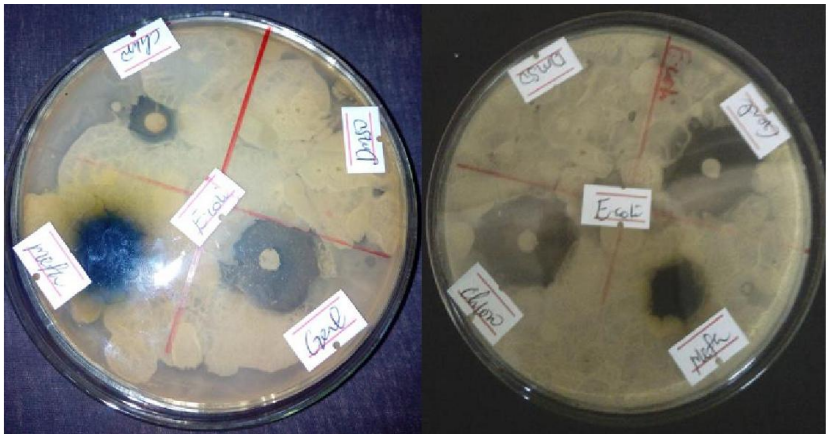
A) Bacillus subtilis



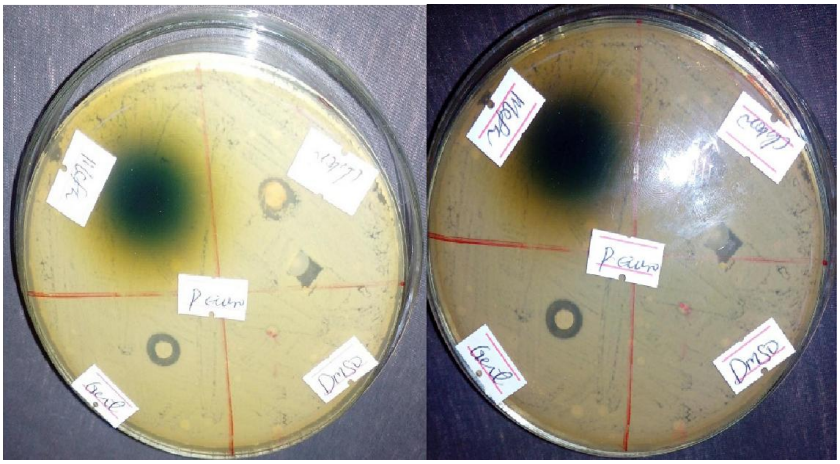
B) Staphylococcus aureus



D) Escherichia coli



D) Pseudomonas aeruginosa



Antifungal Activity Test

Candida albicans