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M.Sc THESIS

ON

PHYTOCHEMICAL INVESTIGATION AND ANTI-MICROBIAL ACTIVITIES OF *Vernonia biafrae STEM BARK*

BY

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Phytochemical Investigation and Anti-microbial Activities of *Vernonia biafrae Stem bark*

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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

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Abbreviations

DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl Sulfoxide
IR	Infrared
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet visible
WHO	World Health Organization

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Abstract

Bacterial diseases are several causes for mortality and morbidity in all regions of the world due to the resistance of antibiotics. Plants have been used as a primary source of medicine in the traditional healthcare systems around the world due to the presence of bioactive secondary metabolites. The aim of this research work was to investigate phytochemical constituents and evaluate antimicrobial activity on the stem bark extracts of *Vernonia biafrae***.** The dried and powdered steam bark of the plant material was extracted with four types of solvents (petroleum, chloroform, acetone and methanol) respectively. The chloroform extract was subjected to column chromatography for further purification and isolation of the bioactive compounds. The first fraction 2% ethyl acetate in petroleum ether yields compound **1(**1-tricosanol**)**, the second fraction 7% ethyl acetate in petroleum ether yields compound **2** (stigmasterol). TLC was used for monitoring the isolation techniques. The structural elucidation of isolated compounds was done based on data gathered from ^{1}H-NMR and 13C-NMR spectroscopic techniques. Antimicrobial activities of the crude extracts and isolated compounds were carried out on four bacterial strains (*B. subtilus, S. aureus, E. coli* and *S.typhi)* and two of fungal strains (*Candida* and *Fuzarium*). The antimicrobial analysis showed that the acetone extract was the most active followed by petroleum ether, chloroform and methanol extracts. Acetone extract showed the greatest activity in both Gram positive and Gram negative bacteria. It has high potential against *s. typhi, E. coli, and S. aureus* by showing inhibition zone of 7.1, 7.3, and 7.9 mm respectively. Petroleum ether extract showed greater inhibition zone against *E. coli, s.typhe* and *S. Aureus* by showing inhibition zone of 7, 7.2 and 7 mm respectively. Compound **2** showed active antimicrobial activity than compound **1**.

1. Introduction

1.1 Background of the study

Medicinal plants are known to contain substances which could be used for the treatment purposes or used to produce drugs. They are exploited as medicinal source since ancient age. The traditional and folk medicinal system uses plant products for the treatment of various infectious diseases. In recent times, they are being extensively explored for harboring medicinal properties. As studies of various researchers indicate, they are one of the major sources for drug discovery and development of drugs. They are reported by different researchers as antimicrobial, anticancer, anti-inflammatory, antidiabetic, hemolytic, antioxidant, Larvicidal properties etc. Many of such plants known to be used primitively to alleviate symptoms of illnesses have been screened to have medicinal importance.[1]

The World Health Organization (WHO) has indeed recognized medicinal plants as the best source to get a variety of synthetic drugs and defined medicinal plants; as plants that contain properties or compounds that can be used for therapeutic purposes or those that synthesize metabolites to produce useful drugs . Also, it has been generally described that medicinal plant is a plant that has similar properties as conventional pharmaceutical drugs which humans have used throughout history to either cure or lessen symptoms from an illness. Therefore, a various of scientists started to search medicinal plants over the world for treatment of various disease [3].

In current days, as medical and pharmaceutical advancement shows, microbial involve in the change of their metabolism and genetic structure to acquire resistant against the drugs used in the treatment of common infectious disease [2]. Due to drug resistant of these microbial, high mortality rate is observed especially in developing countries and become a great challenge in the pharmaceutical and health care industry. To overcome such challenges, scientists look forward for the development of alternative and novel drugs. Failurity of chemotherapeutics and antibiotics of some drugs exhibited by pathogenic microbial agents has led to other alternatives of several medicinal plants for their potential antimicrobial activity [2].

Plants produce a vast and diverse assortment of organic primary and secondary metabolites, the great majority of which do not appear or participate directly in growth and development. These substances ,traditionally referred to as secondary metabolites, often they are distributed among limited taxonomic groups within the plant kingdom. Their functions are not well known. The

primary metabolites, such phytosterols, acyllipids, nucleotides, aminoacids, and organic acids, are found in large amount. Compounds and extracts derived from the natural product have found to uses in medicine, agriculture , cosmetic, and food in ancient and modern societies around the world [4] . Therefore, the ability to access natural products, understand their usefulness, and drive applications has been a major driving force in the field of natural product research. Natural products isolated from plants and microorganism have been providing noble, clinically active drugs. Individual secondary metabolites may be common to a number of species or may be produced by only one organism [14].

Natural product chemistry covers the chemistry of naturally occurring organic compounds ,their biosynthesis, functioning their environment, metabolism and structure elucidation and synthesis. Natural products are generally classified based on their structure, physiological activity, taxonomy, and biochemical origin. The World Health Organization (WHO) estimated about 80% of the people in the developing countries relies on traditional medicine for primary healthcare needs, they used major proportion corresponds to plant extracts. Herbal remedies have been used for centuries' but more recently, the compounds that are active have been identified, extracted and purified. Synthetic organic chemists have then been able to produce the molecules in vitro and so produce them on large scales.[14]

Phytochemicals are bioactive chemicals which are obtained either from plants or microorganisms. They are regarded as secondary metabolites, because; plants that manufacture them may need little for themselves and naturally synthesized in all parts of the plant body; bark, leaves, stem, root, flower, fruits, seeds, etc. i.e. any part of the plant body may contain active components [3]. They are also available in supplementary forms, but evidence is lacking that they provide the same health benefits as other phytochemicals having biological properties such as antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property [4].

Ethiopian plants have shown very effective medicinal value for some ailments of human and domestic animals. Thus medicinal plants and knowledge of their use provide a vital contribution to human and livestock health care needs throughout the country. The major reasons why medicinal plants are demanded in Ethiopia are due to culturally linked traditions, the trust the

communities have in the medicinal values of traditional medicine and relatively low cost in using them. From those existing medicinal herbs and spice plants only small percent are traded. In Ethiopia, people practice to use traditional medicinal plants for their primary health care or to save themselves from a number of ailments where, *Vernonia biafrae* species is common medicinal plant used by the people for the treatment of various disease. *Vernonia biafrae* species is classified under *Asteraceae* family, which includes about 1000 species in which *Vernonia biafrae* is the most known medicinal species. It is used as medicine, in which its root decoction is drunk as a purgative against abdominal pains. The stem is also chewed to treat mouth and throat diseases and as well as to toughen the skin from microbial infections which are one of the most common public health problems.

1.2 Statement of the Problem

A large number of the plants are claimed to possess the antibiotic properties in the traditional system and are also used extensively by the local people throughout the world specially in Africa. It is now mostly thought that nature has given the cure of every disease in one form or another. Plants have been known to cure people from various diseases. Therefore Studies on species have relevant advantages that are important in medicine and the chemical studies have done worldwide. Culturally *Vernonia biafrae* is used as medicinal plant. But the dose, bioactive components and its medicinal application do not identified well. Therefore this research is mainly focused on isolation, identification and characterization of bioactive compounds in the plant of *Vernonia biafrae and* its antimicrobial effect.

1.3 Objective of the study

1.3.1 General Objective

To investigate bioactive compounds from the stem barks of *Vernonia biafrae* and evaluation of its antimicrobial activity.

1.3.2 Specific objectives

1. To isolate secondary metabolites from the steam barks of *Vernonia biafrae* using column chromatographic techniques.
2. To evaluate the antibacterial activity of crude extracts and isolated compounds using disk diffusion method on selected two gram-positive and two gram negative and two fungus strains
3. To Elucidate the structures of isolated compounds using ^{1}H-NMR and ^{13}C-NMR, .

. 1.4 Significance of the study

All plants produce compounds that are used for growth and defense mechanism. Those substances are known as phytochemicals and accumulated in leaves, fruits, flowers, bark and stem of plants. They also have ability to protect human beings from disease and used as medicine. The finding of this study would be:-

- ✓ Knowing bioactive compounds in steam bark of *Vernonia biafrae* plant.
- ✓ Forecasting medicinal application of *Vernonia biafrae* and initiate chemists for further study on the plant
- ✓ Provide the chemical profile of steam bark of *Vernonia biafra*

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2. Review of Related Literature

2.1 Botanical information

The genus *vernonia* is named for the English botanist William Vernon. There are numerous distinct subgenera and subsections in this genus. This has led some botanists to divide this large genus into several distinct genera. For instance, the *Flora of North America* only recognizes about 20 species in *Vernonia sensustricto*, of which are in North America north of Mexico, with the others being found in South America. Several species of *Vernonia*, including *V. calvoana*, *V. amygdalina*, and *V. colorata*, are eaten as leaf vegetables. Common names for these species include bitterleaf. They are common in most West African and Central African countries. *Vernonia amygdalina* is used in traditional herbal medicine. These leaves are exported from several African countries and can be purchased in grocerystores aiming to serve African clients. One of vernonia genus *Vernonia biafrae* is used in local traditional medicine [6].

Vernonia biafrae(family) also called little iron weed is a perennial grass with erect stem seen in most African countries such as Nigeria, Kameroon, Gabon, Equatorial Guinae, Sudan, Ethiopia, Kenya, Uganda, Tanzania, Zambia, and Malawi. *Vernonia biafrae* have properties such as antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. The traditional uses of Asteraceae plants include wound healing, asthma, dysentery, cold and diarrhoea. It is used for treating cancer gastro intestinal disorders and abortions [9,14]. It also cures urinary incontinence in children [8].Certain species have leguminous property and hence enrich soil fertility [9]. It is used to control fever, inflammation, bleeding, swelling and detoxification [5].

It is also seen to have therapeutic effects against certain gastrointestinal and skin disorders and also some immune modulatory effects [14]. It is seen to have supplementary effects on reducing smoking rate, beta endorphin release and oxidative stress biomarkers [15]. It has antibacterial activity against certain bacterial species like *E.coli*. It has larvicidal activity against mosquito and other filarial species due to flavonoids and thiophene derivatives which are toxic to those species[16]. The roots and leaves of this plant is also known to cure fever, hiccups, kidney disease and stomach discomfort [10]. The plant is used to treat nerve disorders and is also a potent analgesic [13].

The methanolic extract of the aerial parts of the plant showed the presence of steroids, glycosides, triterpinoids and esters during phytochemical screening. The phytochemical screening of the whole plant extract revealed the presence of alkaloid, anthraquinone, courmarin, tannin, glycoside, xanthoprotein and sugar which have potent antibacterial activity and also Antimicrobial activity against a wide range of pathogens.

2.1.1 The family Asteraceae

Asteraceae is large family of flowering plants, with more than 1,620 genera and 23, 600 species. Members of the family have flower heads composed of many small flowers, called florets, that are surrounded by bracts (leaf like structures). Bell-shaped disk florets form the centre of each head. Strap-shaped ray florets extend out like petals from the centre and are sometimes reflexed (bent back). Some species have flowers with only disk or only ray florets. The sepals have been reduced to a ring of hairs, scales, or bristles that is called the pappus on the mature fruit. The one-seeded fruit (an achene) has a hard outer covering. The leaves of *Asteraceae* are simple or occasionally compound, and their arrangement along the stem may be opposite, alternate, or, less commonly, whorled; not infrequently they are opposite toward the base of the stem and alternate above [20].

2.1.2 The Genus *Vernonia*

Commonly called ironweed, is a tall, coarse, upright perennial which typically occurs in the wild in moist thickets, low areas. The genus vernonia plants easily grown in average, medium to wet soils in full sun. Tolerates wide range of soils, but prefers rich, moist, slightly acidic soils. Remove flower heads before seed develops to avoid unwanted self-seeding. Overall plant height may be reduced by cutting back stems nearly to the ground in late spring [21]

2.1.3 Antimicrobial potential of the genus *Vernonia*

Vernonia is one of the largest genus of plants belonging to the tribe *Vernonieae* (Cichorioideae), Asteraceae family, with about 1,500 described species, being the largest genus of the tribe. In traditional medicine, many *Vernonia* species are employed to treat various diseases. From the pharmacological point of view, species have been investigated revealing many properties, such as antiplasmodial, analgesic, anti-inflammatory, antimicrobial,antidiabetes and antioxidant

There have been numerous studies involving extracts, fixed oils and essential oils of *Vernonia* species (Table 1) with potential antimicrobial activity against strains of bacteria, protozoa and fungi that are pathogens to animals and plants, using extracts, fixed oils, and essential oils. In particular, phytochemical screening is promising to identify bioactive compounds with antimicrobial activity. In Ethiopia, a study of nine plants with the ethnomedicinal 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 [22].

2.1.4 The plant *Vernonia biafrae*

Vernonia biafrae has been described as shrub or small tree, occurring in West and Central tropical Africa from the areas of Nigeria, Kameroon, Gabon, Equatorial Guinae, Sudan, Ethiopia, Kenya, Uganda, Tanzania, Zambia, and Malawi. In the most general way, its habit resembles plants of the *Vernonia zanzibarensis.* Less.relationship, but details, such as the acute-tipped sweeping hairs on the styles and structure of the achene wall, shows close relation. *Vernonia biafrae* is categorized under Asteraceae family and vernonia genus [23].

It is a perennial climbing herb, with stem up to 3 m long. It is a strongly branched and succulent plant. The leaves are alternate, simple or deeply pinnately lobed, more or less succulent, stipule absent, with petiole 1-10 cm long. The flower is a headed structure with many flowers, homogamous, peduncle 5-10 cm long, involucral bracts 4-6 with bisexual flowers. It occurs naturally in the forest zone most especially in Cocoa plantation. *Vernonia biafrae* leaf extract is used to stop bleeding from cuts or injury [22].

Figure1**.** *Vernonia biafrae* plant with its flower

2.1.5 Medicinal Use of *Vernonia biafrae.*

Natural product is a major source of drugs, with more than 25% of the pharmaceuticals in use to derived from natural product, therefore interest in natural product research remain strong .Several species of *Vernonia biafrae* have important medicinal properties for teeth ache and stomach ache and they have been the subject of different pharmalogical investigation. The most important medicinal compounds in this species are steroids. Plant extracts containing steroids are being increasingly used for cosmetics, food, dye and pharmaceuticals due to their wide therapeutic and pharmacological properties [24] .

2.2 Secondary Metabolite from *vernonia* Genus

Phytochemical study on the genus *Vernonia* will led to the isolation and characterization of different classes of secondary metabolites including saponins and important class of compounds with important biological properties. These compounds elaborated both by higher and lower plant, are also one of the most well- known naturaly occurring pigments. Saponins, flovonoids, phenols and related compounds in higher plants are located in all parts of the plant, including root, bark, leaves, seeds, and flowers [25]

Table 1 . Some species of the genus *Vernonia* with their secondary metabolities

Scientific name	part used	Biological Preparation	Bioactive substances	References
Vernonia adeonsis	L	PE	Terpenoids	Kisangau (2007)
Vernonia ambigua .	Ap	CE	Alkaloids ,Saponins ,tannins cardiac glycosides, steroids and terpens	Aliyu (2011)
	L	ME	saponins, tannins, flavonoids	Jisaka (1993)
	L	ME	suqiterpens, lactones and steroids	Akinpelu (1999)
	L	EE	phenolic compounds	Otshudi (1999)
	L	ME	saponins, alkaloids& phenols	Kambizi and Afolayan (2001)
	L	EE	susquiterpene lactones	
Vernonia auriculifera	L/st/R	HE/DE	Triterpenes and susquiterpenes	Kiplimo (2011)
	AP	CE	Alkaloids ,flavonoids, tannins ,	Aliyu (2011)

Vernonia bluomides	L	CE	Laconesn	
Vernonia brasiliana	L	EO	susquiterpenes	Maia (2010)
	AP	ME	-	Latha (2010)
Vernonia cinera	L	ME	-	Latha (2010)
Vernonia colorata	L/St/R	ME/EAE	-	Kelmanson (2000)
	L	HE/EAE	Susquiterpene lactones	Rabe (2002)
Vernonia condensate	AP	ME	-	Brasileiro (2006)
Vernonia glamensis	S	FO	Amino compounds	Mbugua (2007)
Vernonia glabra	AP	DE/ME	-	Kitonde (2013)
*Vernonia guineense*s	R	DE/ME	-	Toyang et (2012)
Vernonia hymenolopis	L	ME	Alkaloids, flavonoids and saponins	Noumedem (2013)
Vernonia lasiopus	L/St	aqE	-	Kareru et (2008)
Vernonia oocepala	AP	CE/EE	Alkaloids, saponins, flavonoids tannins ,glycosides, terpenes, steroids and cardial glycosides	Aliyu (2011)
Vernonia polyanthes	L	ME	Alkaloids, terpinoids , saponins & flavonoids	Braga (2007)
Vernonia remotiflora	L	EO	susquiterpenes	Maia (2010)
	St	HE/CE	Susquiterpene lactones	
	L	HE/CE	Susquiterpene lactones	Freire (1996a)
Vernonia scorpiodes	AP	EO	Terpenoids	
	L	EAE	-	
Vernonia smithiana	AP	EO	Terpenoids	Toigo (2004)
Vernonia species	L	ME	-	Kalayou (2012)
Vernonia tenoriana	L/St	ME/EAE/ AE/HE	Alkaloids, tannins, cardiac glycosides & anthraquinones	Ogundar (2006) Ogundare (2006)

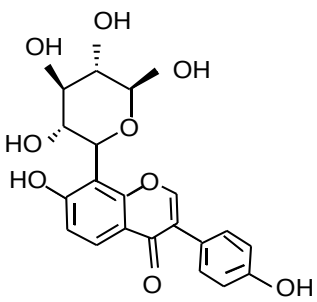
Key:- AP - Aereal part L – Leaves R – Root S – Seed St – Stems

CE - Chloroform extract DE - Dichloromethane extract DEE - Diethyl ether extract EAE - Ethyl acetate extract EE - ethanol extract

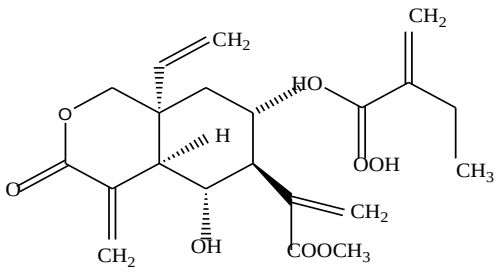
EO - Essential oil FO - Fixed oil; HE - Hexane extract

ME - Methanol extract PE - Petroleum ether extract.

AE - Acetone extract



Flavonoids



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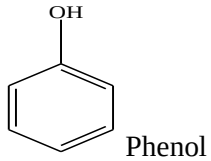
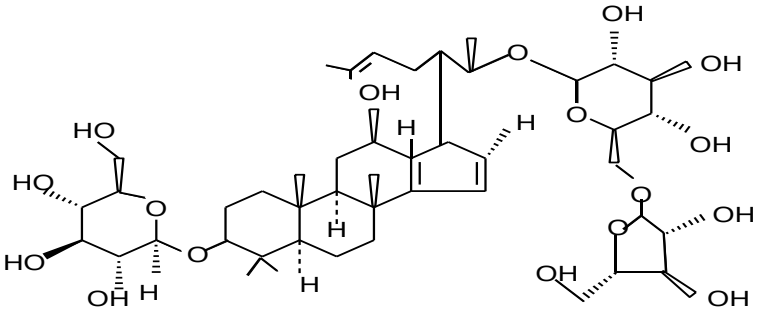


Phenol



Saponins

Figure 2: some compounds isolated from genus of *Vernonia*

3. Materials and Methods

3.1. Chemicals

Chemicals that were used in this research includes petroleum ether , chloroform, acetone, methanol and Ethyl acetate for gradient extraction and column elution; Silica gel 60-120 mm

size, deionized water, iodine for detection of spots on Thin layer Chromatography(TLC). Dimethyl sulfoxide/DMSO, Mueller Hinton agar and nutrient broth as culture was used for antibacterial control in this study. Standard antibiotic drug phenicolcloram and Ketoconazol was also used as standard for antimicrobial test. All the chemicals and reagents were in high analytical grade.

3.2. Apparatus and Instruments

Apparatuses that was used includes:- rotary vapor(Heidolph Laborata 4000) to concentrate the crude, TLC papers for detection of spots under Uv-techc, round bottom flask (50 , 100 and 250)mL for filtration purpose , measuring cylinder for measuring of solvent, pestle and mortar for grinding of the sample, filter papers for filtration of extract, weighing balances for weighing mass of sample, crude extract and isolated compounds, glass columns for column chromatography , Sephadex for further separation and purification of compounds, chamber (UV-techic) for detection of spots on TLC, Petri dishes was also used for anti microbial tests. The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 were recorded on Bruker Advance NMR machine in a chloroform ($CDCl_3$) solvent.

3.3 Sample collection and preparation

The steam bark of plant materials *Vernonia biafrae* were collected from Oromia region Jimma zone, Jiren kebele in July 5, 2010 E.C. Identification of the plant species was done by a plant botanist Dr.Dereje Denu and voucher specimens was deposited at, botanical science laboratory Herbarium with the specification code CH2, in Biology department of Jimma University. The bark, of the plant was air-dried in organic chemistry research laboratory of Jimma University for 3 weeks , finally powdered to suitable size by using mechanical grinder.

3.4 Extraction and isolation

About 1 Kg of the powdered plant bark was socked and sequentially extracted with petroleum ether, chloroform, acetone and methanol respectively using maceration method for 24 hr in each solvent. The crude extracts of each solvent were filtered first through a fresh cotton plug and then through Whatman filter paper. The filtrates were concentrated by evaporation under reduced pressure using a rotary evaporator (Heidolph Laborata 4000) at 40 °C and about 5 , 7.5 , 12 and 25 g of crude extract of petroleum ether, chloroform, acetone, and methanol, respectively were obtained. All the extracts were stored in the desiccators until used for isolation and microbial assay.

3.5 Chromatographic separation technique

The 500 mL column was packed with deactivated 104.5 g of silica gel. Then 7.3 g of chloroform crude extract was subjected into the column. Column elution was carried using binary solvents system (Petroleum ether and Acetone). It was first eluted with 100% petroleum ether and elution process was continued by increasing gradient of Ethyl acetate (1, 2 , 3 , 4 , 5 , 6 , 7 , 8 , 9 , 10 , 12 , 14 , 16 , 18 , 20 , 25 , 30 , 35 , 40 , 45 , 50 mL). A total of 310 fractions each with 30 mL were collected. The fractions were concentrated under reduced pressure using rotary vaporator (Heidolph Laborata 4000) at 40 ^{O}C. The level of separation was checked by using thin layer chromatographic analyses under Ultra violet light at 254 and 365 nm and then by exposure to iodine vapour. Fractions that showed the same TLC profiles (Rf value) were combined together and labeled as pure compounds. After the isolated compounds are dried enough their TLC profile were re-checked and sent for characterization to Addis Ababa University. Characterization of isolated compounds was carried out using spectral techniques ^{1}H-NMR, ^{13}C-NMR and DEPT-135.

3.6 Structural elucidation

Structural elucidation of the compounds was done based on the data which is obtained from ^{1}H-NMR and ^{13}C-NMR analysis. Depending on the obtained result the chemical formula and structure of the desired compounds was identified including their functional group.

3.7 Bioassay Activity

3.7.1 Preparation of test organism and bioassay test

These standard bacterial strains and fungal strains were obtained from the Department of Biology Research lab., Jimma University . The antibacterial activity of crude extract of *venonia biafrae* was tested against four bacteria strains (*S. typhe, E. coli, S. aureus and B. subtilus)* and two fungus strains(*Candida* and *Fuzarium*) by using disc diffusion method. The disc measuring 6mm diameter was prepared from whatman No.1 filter paper sterilized by dry heat at 180^{o}C for 1 hr. The stock solution was prepared by dissolving the crude extract and isolated compounds in1mL DMSO .

The antimicrobial activity test was done using disc diffusion method standard procedures .Stocked bacterial and a fungus strain was cultured on Muller Hinton agar. Bacterial strains and fungus strain were grown and maintained on an agar slant at controlled temperature (4°C). Then strain activation were done for experiment at 37°C for 24 hr for bacteria strains and 48 hours for

fungus strains on nutrient agar, before any experimental screening. The culture media was boiled in distilled water to dissolve the media and autoclaved at 121^{o}C for 2 hr. and poured into sterile Petri dishes refrigerator until use. After the culture media had solidified, organisms were uniformly seeded with it. Four well-isolated colonies of bacteria were selected from an agar plate culture and the top of each colony was touched with a loop, and the growth was transferred into a tube containing 4.5mL of a suitable nutrient broth medium. After the culture media had solidified, organisms were uniformly seeded with it. Four well-isolated colonies of bacteria and two fungus strains were selected from an agar plate culture and the top of each colony was touched with a loop, and the growth was transferred into a tube containing 4.5mL of a suitable nutrient broth medium. Then bacteria and fungus strains were spread on the solid plates with a sterile swab moistened with the bacterial suspension. 200 mg/mL and 5 mg/mL concentration of 20 µL of the working suspension of the same concentration of the sample plant extracts, isolated compound and 1 mL DMSO for negative control was impregnated using Whatman No.1 filter paper disc (diameter 6mm) with the help of micropipette respectively. Positive control using chloramphenicol for bacteria strains and Ketoconazol for fungus strains was applied simultaneously. Plates were left for 10 minutes till the extract diffuse in the medium and incubated at 37°C for 24 hr for bacteria and 48 hr for fungal strains. After incubation, the plates were observed for the zone of inhibition and the diameter of the inhibition zone was measured using transparent ruler.

4. Result and Discussion

4.1 Percent Recovery

Percentage yield is the ratio of mass of crude extract to mass of drained sample multiplied by 100. As the polarity of extracting solvent increase the mass (percentage yield) of the extract also increases. As the polarity of the extract solvent increase gradually the selectivity of the solvent toward the solute increase. This result showed that most secondary metabolites in a *Vernonia biafrae* bark are polar. Percentages yield of crude extract can calculated by;

$$\%\ yield = \frac{weight\ of\ the\ crude\ extract\ (g)}{weight\ of\ the\ draied\ sample\ used\ (g)}\ x\ 100$$

Table2: Percentage yield of extract of each solvents

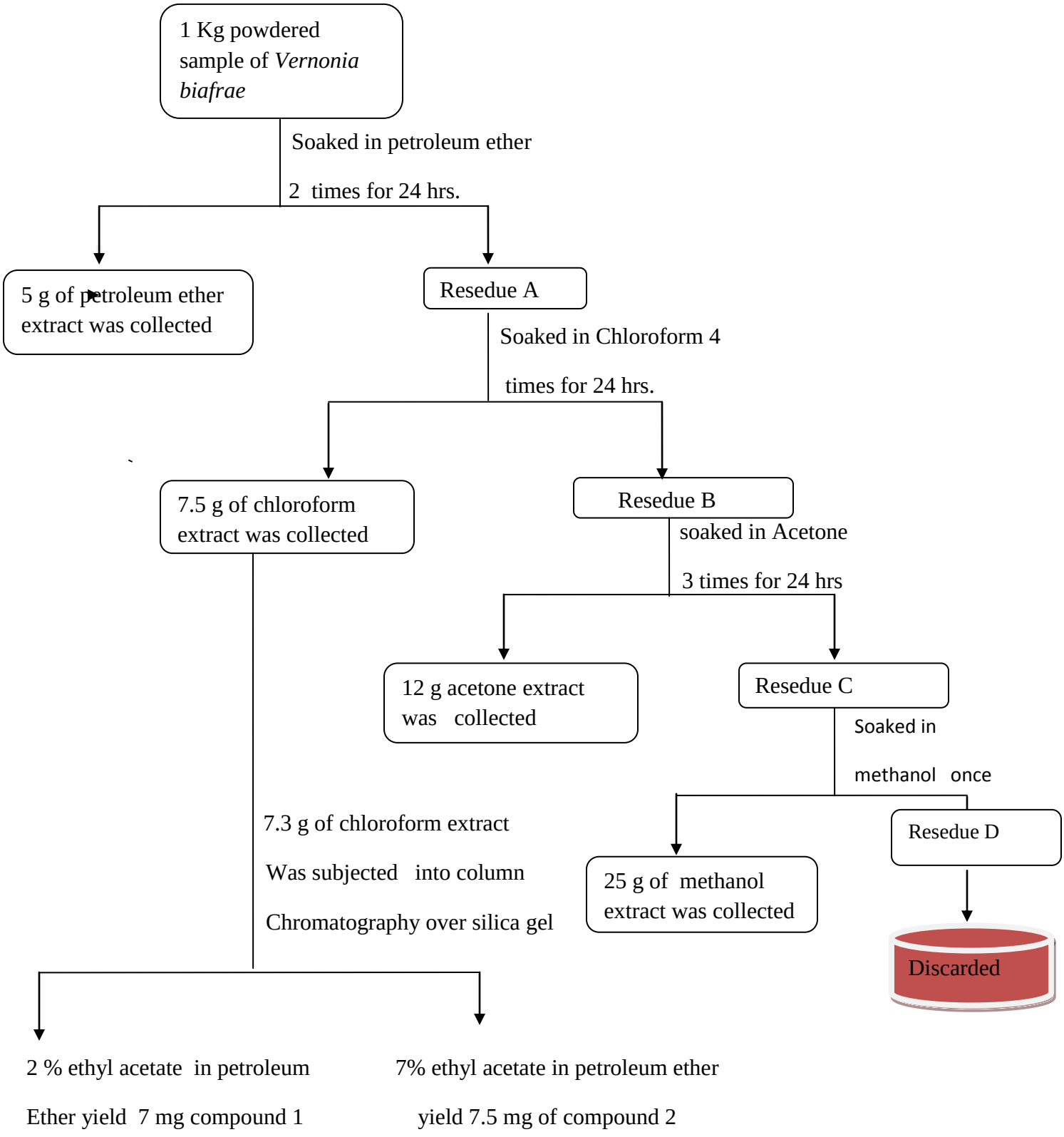
Solvents	mass (g)	% recovery
Petroleum ether	**5**	**0.5**
Chloroform	**7.5**	**0.75**
Acetone	**12**	**1.2**
Methanol	**25**	**2.5**

4.2 Results of extraction and Isolated compounds

Petroleum ether, chloroform, acetone and methanol extract produce 5, 7.5, 12 and 25 g of crudes respectively. Then 7.3 g of chloroform crude extract that shows high number of spots and good separation between spots was subjected to column chromatography for isolation of pure compounds using different combinations of petroleum ether and ethyl acetate as mobile phase with increasing polarity. During the isolation of compounds by using gradient elution of ethyl acetate in petroleum ether a total of 310 fractions each with 30 mL were collected. Among those fractions, 50-65 (2% ethyl acetate in petroleum ether) have shown the same TLC profiles when checked under UV- techc. and iodine vapour. These fractions were combined and the solvent were removed under reduced pressure at 40^0C and **7** mg white amorphous solid were collected and labeled as compound **1**.

The second pure compound (white powder **7.5** mg) was isolated from 93 % petroleum ether with ethyl acetate (fractions 79 – 110) . The compound showed a single spot on TLC after washed with 50 mL of n-hexane and assigned as compound 2. Oily fractions were also collected from fraction 10 to 20 and their TLC profiles were assassed to test the purities of the compounds.

However TLC showed more than one spot with broad tailing. The polarity of elution was further increased to 50:50 petroleum ether :ethyl acetate combination. But most of the fractions become mixed with multiple spots on TLC. The general extraction process is summarized by the following scheme.



Scheme 1: Flow chart of Extraction and isolation from stem bark of *Vernonia biafrae*

4.3 Structural elucidation of the Isolated Compounds

4.3.1Characterization of compound 1

Compound 1 was isolated as white amorphous solid. The 1H-NMR spectrum of compound 1 showed a two proton triplets at δ_H 3.37 (*J*=6.0 Hz) assigned to anomeric H2-1 proton. It also showed the three two proton multiplet at δ 2.30, 2.02 and 1.72 assigned to H2- 2 and one four proton multiplet at δ 1.58 protons. The other protons appeared between δ1.41 and 1.25. A three-proton triplet at δ 0.87 (*J*=6.0 Hz) was accounted to the methyl protons.

Table3. ^{1}H-NMR chemical shifts of compound 1 in $CDCl_3$ (δ400MHZ

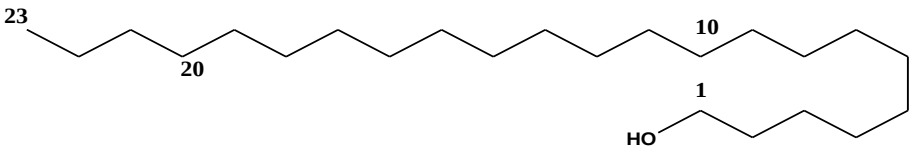
Proton	Observed data(400 In CDCl3) δ	MHz DEPT-135 Multiplicity	Reported data(400MHz Reference (28,29)
H-1	3.66(1H,t)	CH_2	3.34(1H,t)
H-2	3.53(2H,t)	CH_2	3.34(2H,t)
H-3	1.48(2H,d)	CH_2	1.29(2H,t)
Hydroxyl proton	2.34	OH	
H-23	0.9(3H,t)	CH_3	0.87(3H,t)

The ^{13}C-NMR spectrum displayed the presence of 23 carbons. The signal at δ 62.95 indicates the carbon that holds the functional group (C-1),the methyl group is indicated at δ 14.13(C-23). Two methylene groups are also indicated at δ 22.7 , there are other two methylene carbons at δ 31.93 and the other six methylene groups are indicated at δ (24.71,25.74,27.29,28.48,32.77 and 33.96) . The rest eleven methylene carbons are showed between δ29.25-29.71.Totaly 23 carbons are found on compound-1 . On the basis of these spectral data analysis, structure of compound1 was characterized as 1-Tricosanol.

Table 4:-DEPT-135 (400 MHZ) of compound 1in $CDCl_3$

Carbon	Observed data for compound -1	DEPT-135 (multiplicity)	Reported for compound-1	Nature of carbon Reference[28,29]
1	63.10	CH_2	62.89	CH_2
2	33.96	CH_2	33.04	CH_2
3	32.77	CH_2	31.84	CH_2
4	31.93	CH_2	29.90	CH_2
5-18	29.26-29.71	$14CH_2$	29.9	$14CH_2$
19	28.48	CH_2	27.57	CH_2
20	27.29	CH_2	25.37	CH_2
21	25.74	CH_2	22.90	CH_2
22	24.71	CH_2	22.90	CH_2
23	14.13	CH_3	14.32	CH_3

^{1}HNMR ($CDCl_3$): δ 3.34 (2H, t, J = 6.3 Hz, H2-1), 2.04 – 1.34 (8H, m, 4 x CH_2), 1.29 (8H, 4×CH_2), 1.23 (24 12 × CH_2), 0.87 (3H, t, J = 6.6 Hz, Me-23); ^{13}CNMR (CDCl3): δ 62.8 (C-1), 33.0 (CH_2), 31.8(CH_2), 29.9 (16×CH_2), 27.5 (CH_2), 25.3 (CH_2), 22.9 (CH_2), 14.3 (C-23) Based on the ^{1}H-NMR and ^{13}C- NMR values, compound **1** is 1-Tricosanol.



1

Figure 3. The proposed structure of compound **1** (1-Tricosanol)

4.3.1 Characterization of compound 2

Compound 2 was isolated as white powdered solid. The 1H NMR spectrum of compound (2) varied between 0.736 to 5.378 ppm, This spectrum showed the presence of 6 high intensity peaks indicating the presence of six methyl groups at δ 0.736 , 0.843, 0.967, 1.037, 1.200 and 1.534

ppm which were assigned to the H-18,H-27,H-29,H-26,H-21 and H-19 protons respectively. It also shows the presence of olefinic proton at δ 5.364, 5.020 and 5.158. One proton signal appeared at δ 5.364 as the doublet represents the endocyclic double bond proton H-6 of compound 2.The other two olefinic protons appeared as two doublet of doublets at δ 5.020 and δ 5.158 which were assigned for the other olefinic protons at H-23 and H-22respectively.

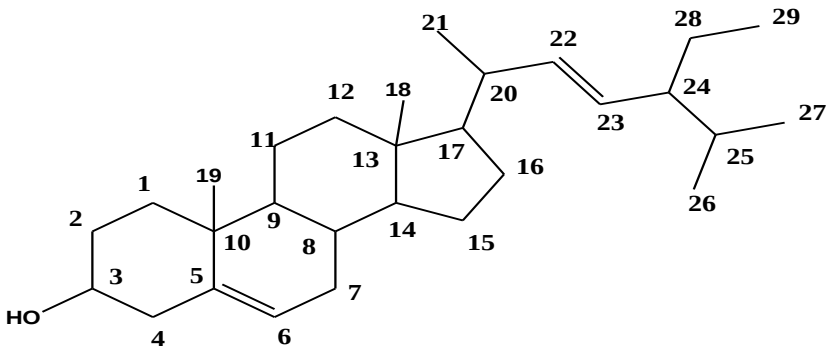
The proton corresponding to the H-3 was appeared as a triplet of doublet at δ 3.529 ppm. At δ 5.197 ppm and at δ 5.378 ppm corresponds to a peak in the form of a single in the region of the ethylene protons suggesting the presence of three protons. The proton NMR showed the proton of H-3 appeared as a multiplet at δ 3.529 ppm and revealed the existence of signals for Olefinic proton at δ5.067(m), 5.197 (m), 5.378 (m), and 2.323(m). Angular methyl proton at 0.69(s), 0.80(s) and 1.02(s) corresponds to C18 and C19 proton respectively. The value at 19.064ppm corresponds to angular carbon atom (C19)138.404 ppm for C-20 and 129.341ppm for C-21. Spectra show twenty nine carbon signal including six methyls, nine methylenes, eleven methane and three quaternary carbons. The alkene carbons appeared at 140.94, 138.40, 129.34 and 121.13ppm

Table 5: 1H-NMR chemical shifts of compound 2 in $CDCl_3$ (δ400MHZ)

Proton	Observed data (400MHZ,in CDCl3) δ	Reported data(400MHZ,in $CDCl_3$) δ
H-18	0.71(3H,s)	0.7(3H,s)
H-19	1.03(3H,s)	1.1(3H,s)
H-3	3.53(m,1H)	3.52(M,1H)
H-6	5.36(1H,d)	5.38(1H,d)
H-22	5.15 (1H,dd)	5.1(1H,dd)
H-23	5.02(1H,dd)	5.1(1H,dd)

^{13}C NMR spectrum of Compound (**2**) has given signal at 140.9 and 121.1ppm for C5=C6 double bond respectively, 71.9for C3 β-hydroxyl group 19.0 and 12.0 for angular methyl carbon atoms for C19 and C18 respectively .138.4 ppm for C-22 and 129.3ppm for C-23. The C5, C6, C22 and

C23 appeared to be alkene carbons. The similarity of 13C spectral data of compound 2 with published data confirmed as -2 compound is stigmasterol (dien-3-beta-ol).



2

Figure 4: The chemical structure of compound 2.(stigmasterol

Table 6: Comparison of ^{13}CNMR and DEPT-135 chemical shifts of compound2 with that of stigmasterol in $CDCl_3$

Carbon	^{13}C-NMR(δ) Observed data	DEPT-135 (multiplicity)	Reported value for cpd2	Nature of carbon Reference[26,27]
1	37.28	CH_2	37.3	CH_2
2	28.92	CH_2	28.9	CH_2
3	71.81	CH	71.8	CH
4	42.30	CH_2	42.3	CH_2
5	140.75	C	140.7	C
6	121.71	CH	121.7	CH
7	31.90	CH_2	31.9	CH_2
8	31.66	CH	31.9	CH
9	50.14	CH	50.2	CH

10	39.68	C	39.7	C
11	24.37	CH_2	24.4	CH_2
12	29.18	CH_2	29.7	CH_2
13	39.78	C	39.7	C
14	56.77	CH	56.7	CH
15	25.41	CH_2	25.5	CH_2
16	28.25	CH_2	29.7	CH_2
17	56.06	CH	56.0	CH
18	12.27	CH_3	12.1	CH_3
19	12.07	CH_3	12.2	CH_3
20	40.50	CH	40.5	CH
21	21.22	CH_3	21.2	CH_3
22	138.32	CH	139.3	CH
23	129.28	CH	129.3	CH
24	51.24	CH	51.3	CH
25	32.42	CH	321.9	CH
26	19.82	CH_3	19.4	CH_3
27	19.02	CH_3	19.0	CH_3
28	29.16	CH_2	29.7	CH_2
29	11.88	CH_3	12	CH_3

. **Spectral data of compound 2**

^{1}HNMR: δ 5.24 (m, 1H, H-6), δ 4.57 (s, 1H), δ 4.14 (s, 1H), 3.20 (tdd,OH,H-3), δ 1.23 (s, 3H), δ 1.19 (s, 3H), δ 1.06 (s, 3H), δ 0.98 (s, 3H), δ 0.91 (s, 3H), δ

13C NMR: δ 140.8 (C-22), δ 130.1 (C-5), δ 129.1 (C-23), δ 121(C-6), δ 71.6 (C-3), δ 56.1 (C-4), δ 55.1 (C-5), δ 52.2(C-24), δ 0.10 (C-17), δ 43.8 (C-9), δ 41.2 (C-13), δ 39.4 (C-10), δ 37.7 (C-10), δ 33.4 (C-20), δ 31.7 (C-25), δ 29.1 (C-21), δ 28.1 (C-23), δ 25.1 (C-12), δ 21.8 (C-11, C-25, C-26), δ 15.1 (C-29), δ 12.8(C-27).

4.3.3 Evaluation of the results of antibacterial and antifungal activity.

The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs in millimeter with the ruler and the results of antibacterial activity and fungus activities was recorded as shown below.

Table 7 : Zone growth of inhibition of the extracts and isolated compounds in concentration of 5 ml crude extracts and 5 Ml of isolated compounds in mm.

Crude extracts and isolated	*S. aureus*	*S. typhe*	*E.coli*	*B.subtilus*	*Candida albicum*	*Fusarium spp*
Petroleum	7	7.2	7.0	7.0	7.0	6.7
Chloroform	NI	6.7	NI	NI	7.1	NI
Acetone	7.9	7.1	7.3	6.0	7.0	7.0
Methanol	7.2	6.0	NI	6.0	NI	NI
Compound 1	6.7	7.0	6.8	6.8	7.0	NI
Compound 2	7.6	7.2	7.6	7.1	7.1	7.0
Chloramphenicol	8.2	9.8	9.0	8.3	*	*
Ketoconazol	*	*	*	*	8.0	7.8
DMSO	NI	NI	NI	NI	NI	NI

NI=no inhibition * =not applied

As shown on the Table 7:- acetone extract showed the greatest activity in both Gram positive and Gram negative bacteria. It has high potential against *s. typhe,, E. coli, and S. aureus* by showing inhibition zone of 7.1, 7.3, and 7.9 mm respectively. Petroleum ether extract showed greater inhibition zone against *E. coli, s.typhe* and *S. Aureus* by showing inhibition zone of 7,7.2 and 7 mm respectively.

On the other hand, the chloroform and methanol extracts showed moderate activity against *s. typhe* and *S.Aureus* by showing inhibition zone of 6.7 and 7.2 mm respectively. chloroform extract cannot inhibit the other three bacterias *S. Aureus, E. coli* and *B.subtilus.* The methanol extract also inhibited only *S.aureus* bacteria and became functionless on the other three bacterias.

In the case of fungus strains Acetone extract shows greater inhibition zone than the other three extracts. It shows 7mm inhibition zones both for *Candida albicum* and *Fuzariu spp.* On the other hand petroleum extract showed moderate inhibition zone on the two funguses,7 and 6.7 mm respectively. Chloroform extract shows 7.1 mm inhibition zone towards *Candida albicum* fungus but become functionless towards *Fuzarium spp* and Methanol extract cannot inhibit both *Candida albicum* and *Fuzarium spp.* Generally Acetone extract shows greater efficiency towards bacteria and funguses, chloroform extract records low killing efficiency on both types of microorganism.

Compound **2** (stigmasterol) shows greater ability than compound 1 (1-tricosanol) towards to both types of organisms. Compound 2 shows greater inhibition zone on *S.typhe* and *B.Subtillus,* 7.2 and 7.1 mm respectively. But it records equal inhibition zone on the two bacterias, *S. Aureus* and *E. coli* and inhibits 6.6 mm for both. Compound 1 shows greater inhibition zone on *S.typhe* and low inhibition zone on *S. Aureus.* But it records equal activity on the two other bacterias *B. Subtillus,* and *E.coli,*6.8 mm inhibition zone for both.

In the case of fungus strains compound 2 shows larger inhibition zone, 7.1 and 7.0 mm for *Candida albicum* and *Fuzarium spp* respectively. But compound 1 inhibits only *Candid*a *albicum* with 7 mm inhibition zone and no inhibition zone was recorded on *Fuzarium spp* with compound 1. (6 mm)

Generally petroleum extract showed moderate activity against all the tested strains, compared to other extracts and isolated compounds. This could be due to interaction of petroleum with nonpolar organic molecules.

Acetone extract shows greater efficiency towards bacteria and funguses due to the presence of antioxidant bioactive compounds (saponins, tannins, and flavonoids) [18,19]. Flavonoids are synthesized by the plants in for the case of anti microbial infection in nature. This activity is probably due to ability of flavonoids to form complex with outercell wall and soluble proteins .

5. Conclusion and Recommendation

5.1 Conclusion

Vernonia biafrae is the plant belongs to the family *Asteracea*. *T*his plant is traditionally claimed for its medicinal value against tooth ache in Ethiopia. However thorough search of literature revealed that there were few attempts on phytochemical investigation on *vernonia biafrae.* The present investigation revealed that the extract of *vernonia biafrae* have potent antimicrobial activity which explains its use in traditional system of medicines. These four extract and isolated compounds were analyzed for their antimicrobial activity against four bacterial strains and two fungus strains. The antimicrobial analysis showed that the acetone extract was the most active followed by petroleum, chloroform and methanol extracts. Compound **2** showed effective antibacterial activity where as compound 1showed moderate antimicrobial activity. Based on the ^{1}H NMR, ^{13}C NMR and DEPT-135 comparison of literature reports compound 1 is identified as 1-tricosanol and compound 2 as stigmasterol .Both compounds are isolated from this plant for the first time. Therefore further investigations are recommended on the plant for the isolation of promising compounds and also detailed studies to be carried out on their biological potentials and novel drug leads.

5.2 Recommendation

Bacterial diseases are several causes for mortality and morbidity in all regions of the world due to the resistance of antibiotics. Plants have been used as a primary source of medicine in the traditional healthcare systems around the world due to the presence of bioactive secondary metabolities. Both the crude extracts and isolated compounds showed positive inhibition zones on bacteria strains and fungus strains. This showed that *Vernonia biafrae* contain biologically active compounds. Therefore further studies should be done on this plant to produce anti bacterial and antifungal drugs. On this research only the chloroform extracts were used for isolation of compounds, I recommend that the rest extracts should be checked to obtain other bioactive compounds.

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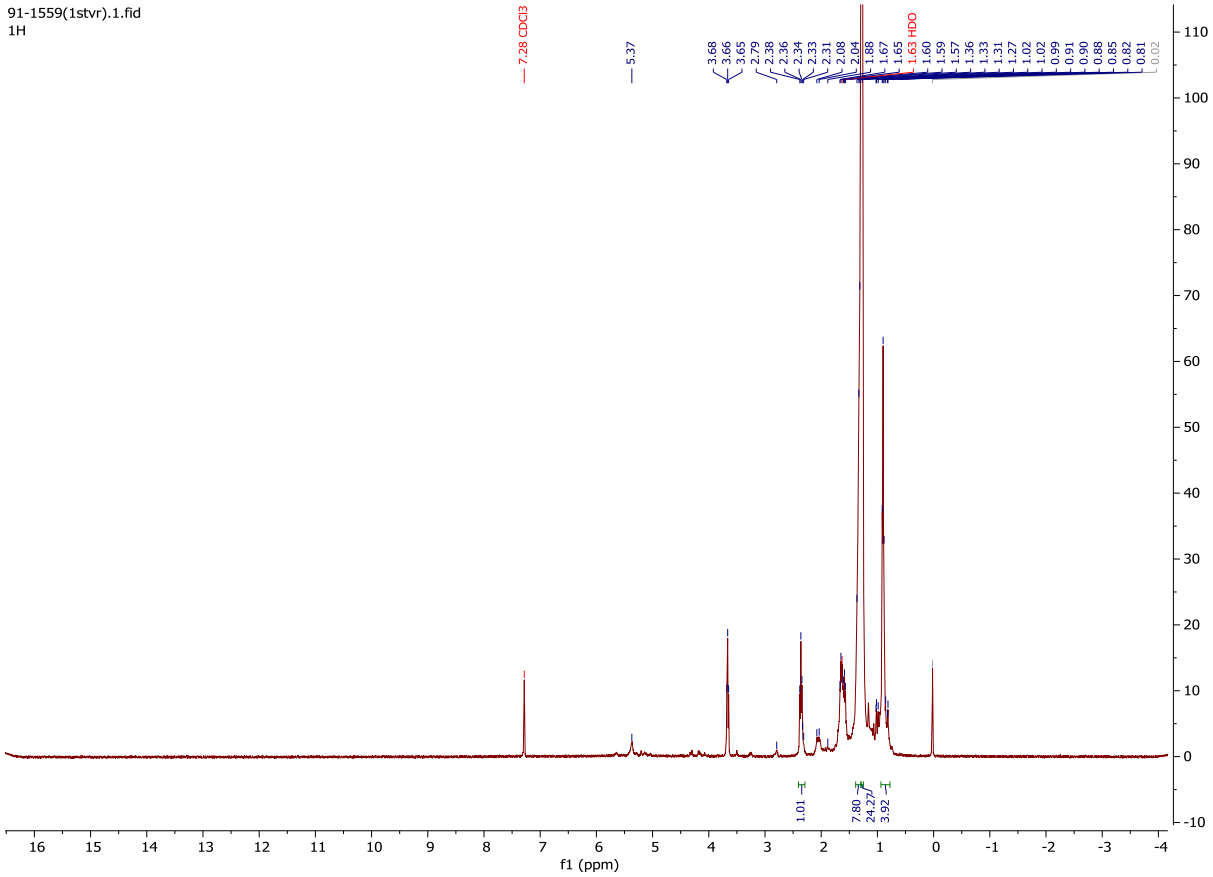
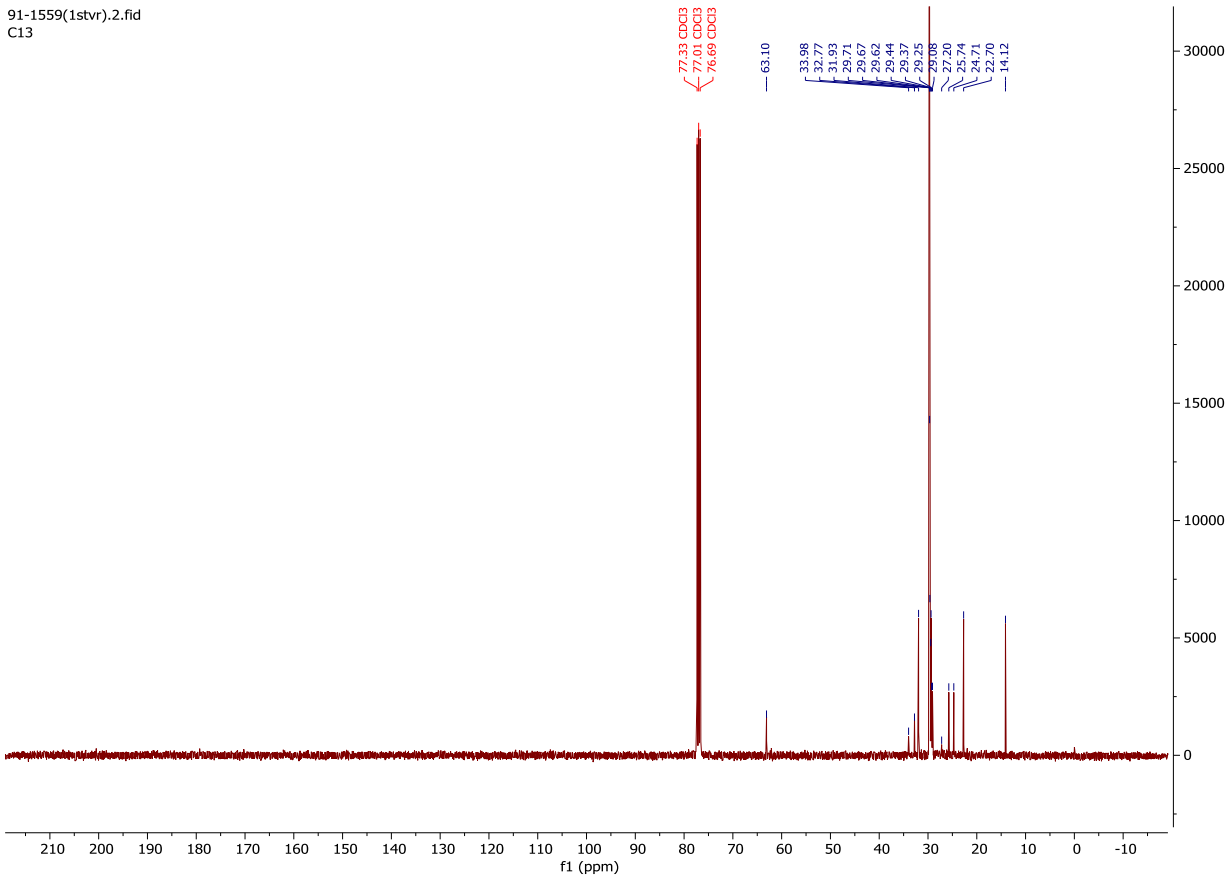
Appendixes

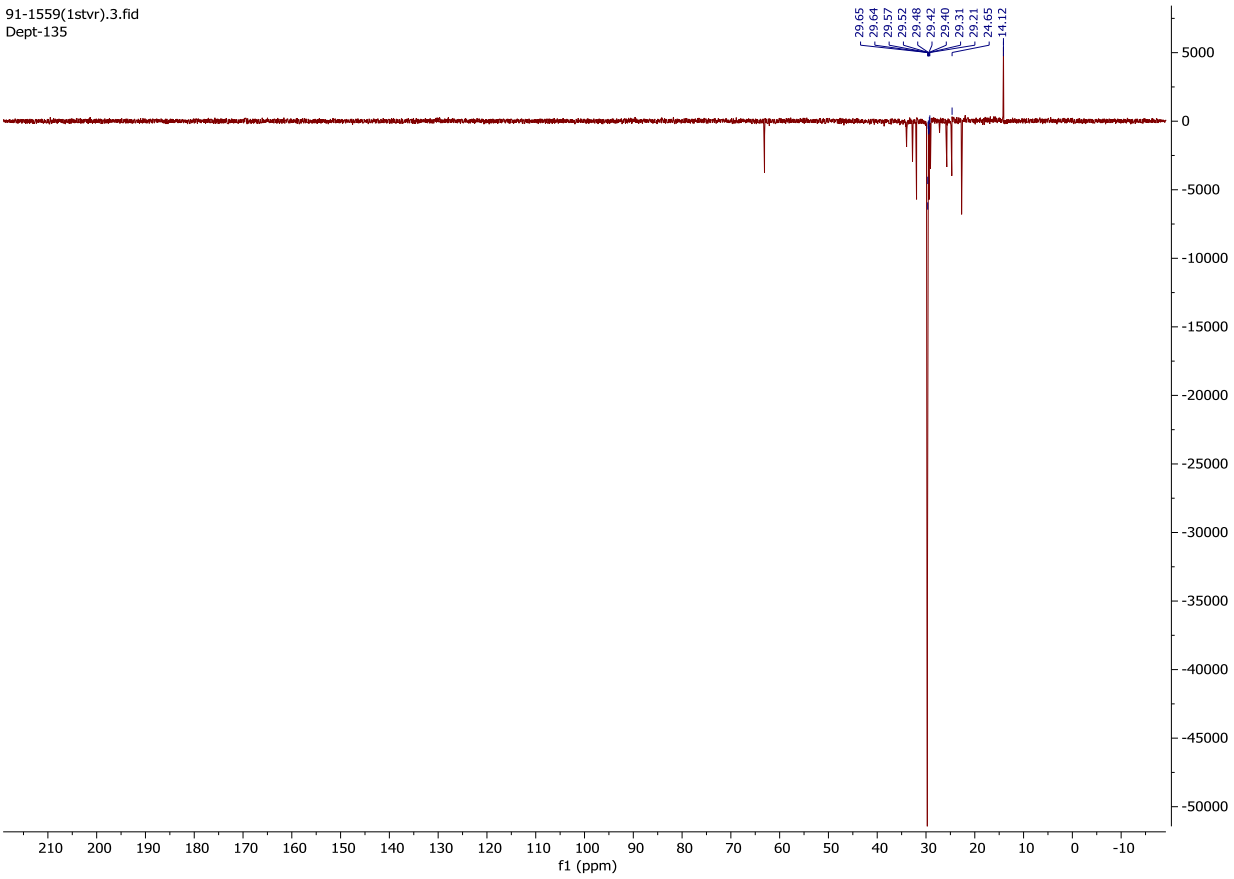
Appendix1: The ^{1}HNMR Spectrum of compound-1



Appendix 2: ^{13}CNMR spectrum of compound -1



Appendix 3: DEPT-135 Spectrum of compound -1



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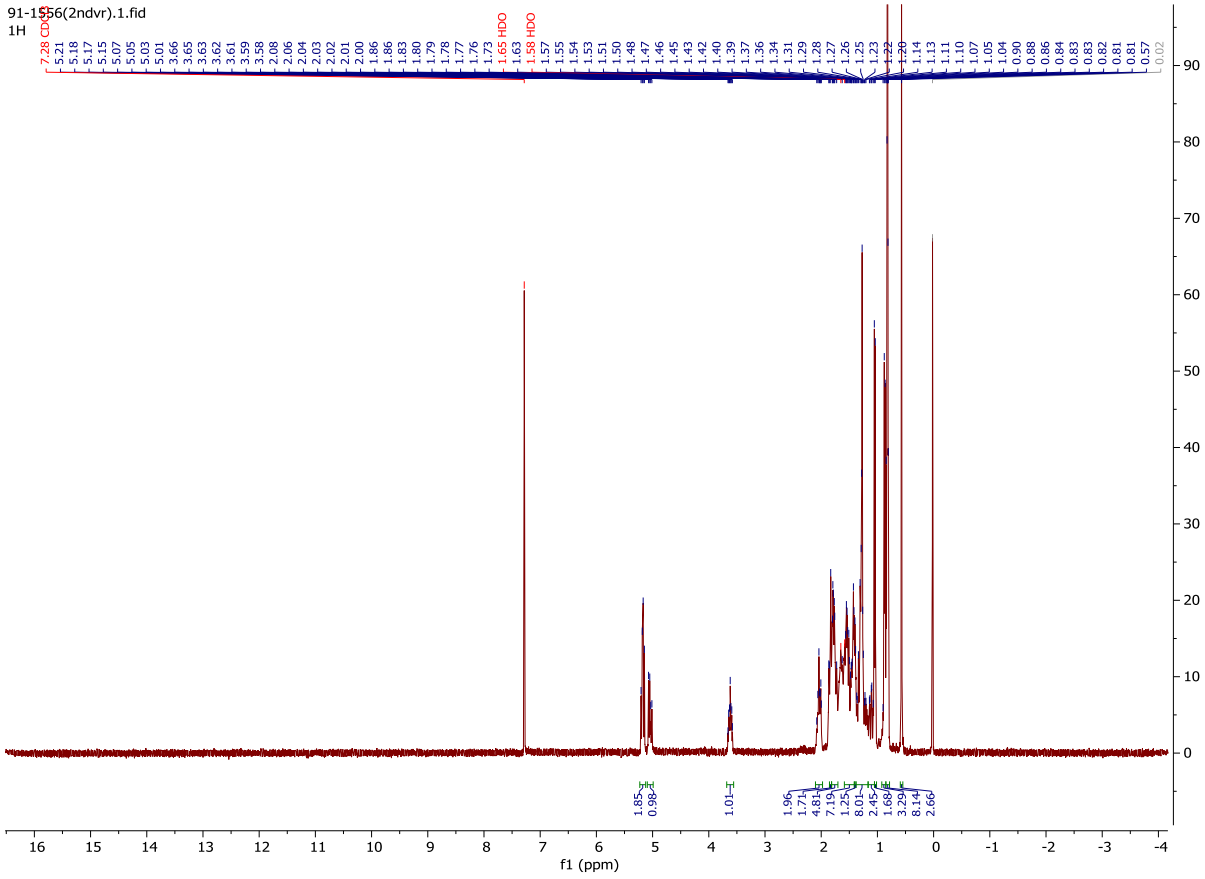
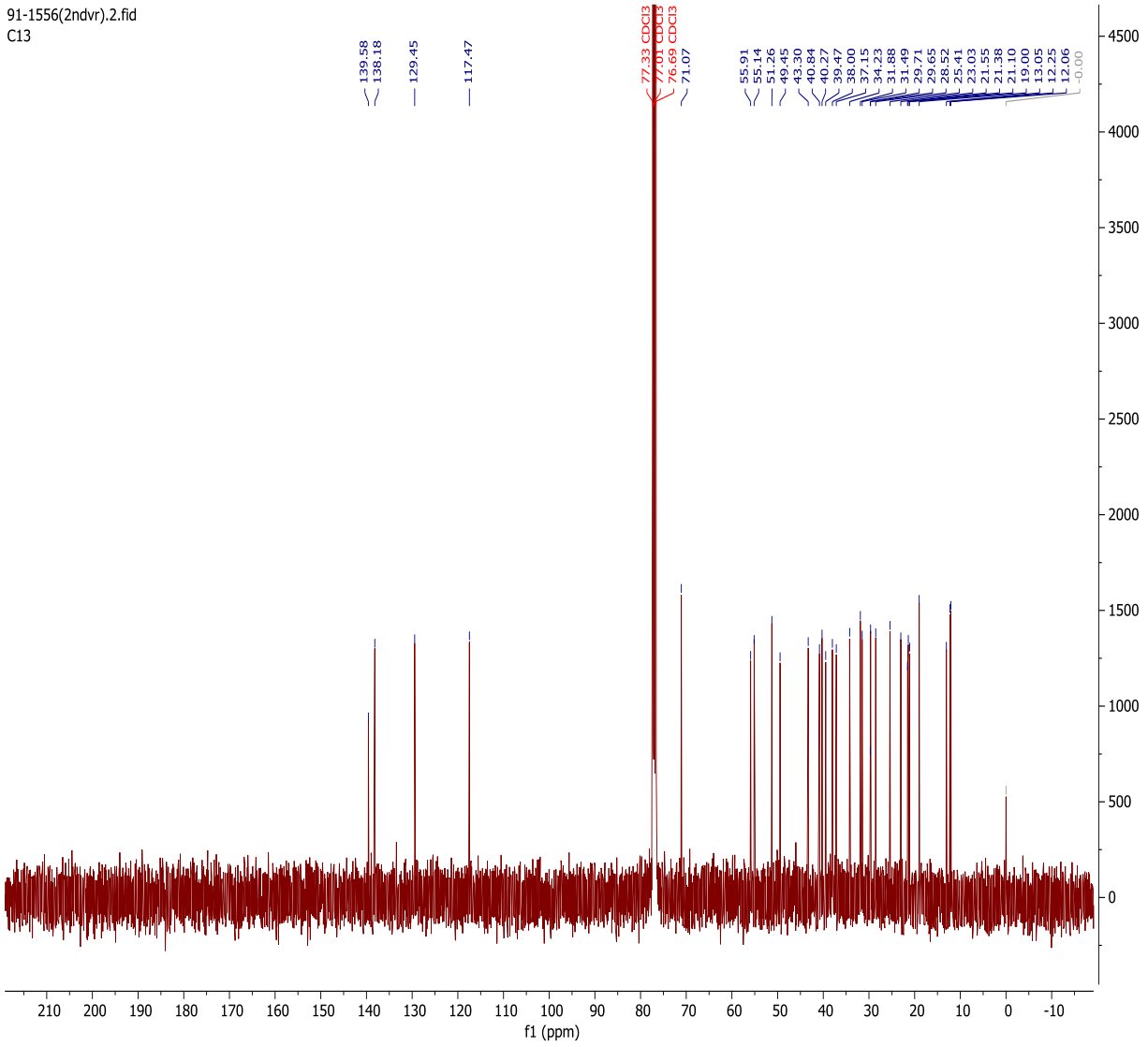
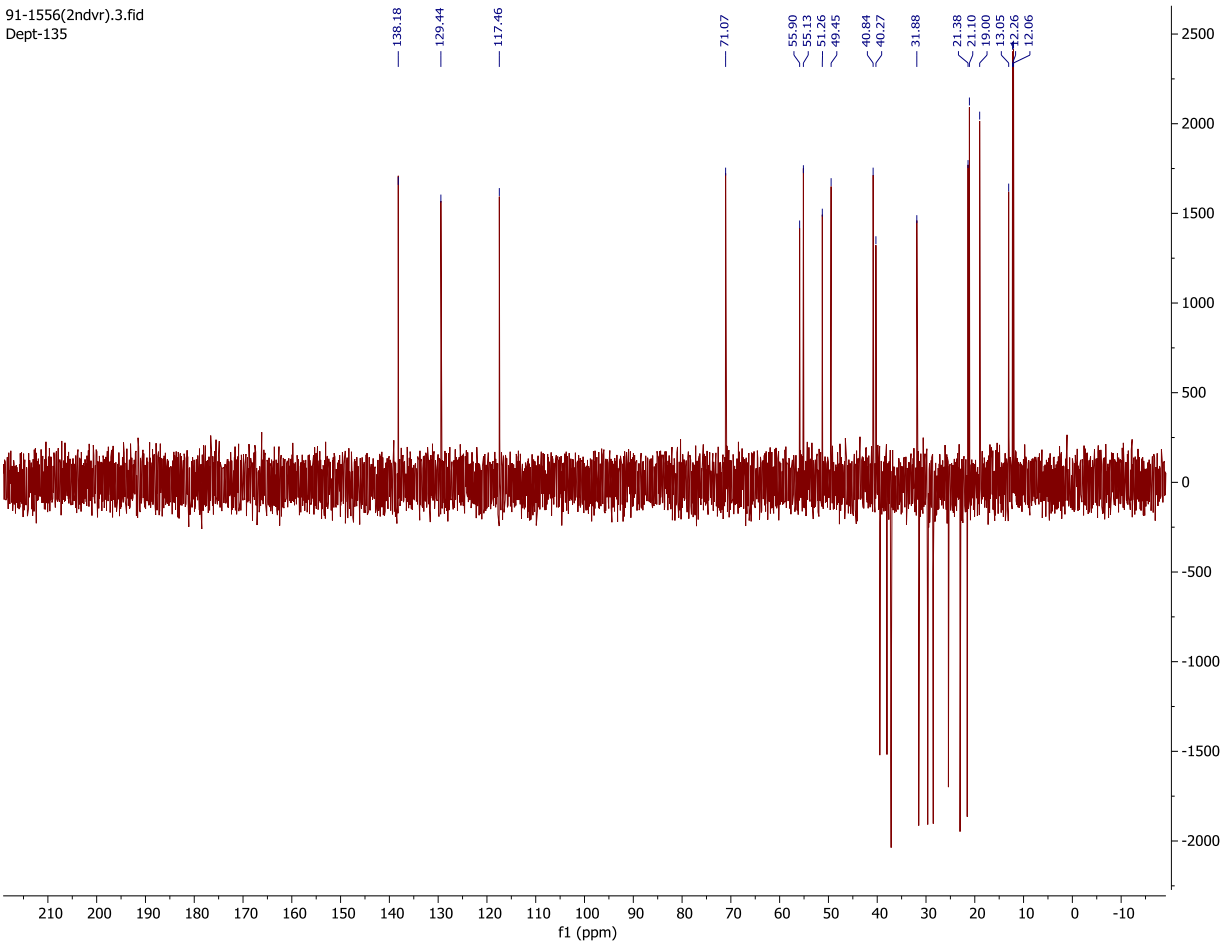
Appendix 4:[1]HNMR spectra of compound-2



Appendix 5: ^{13}C NMR spectra of compound-2

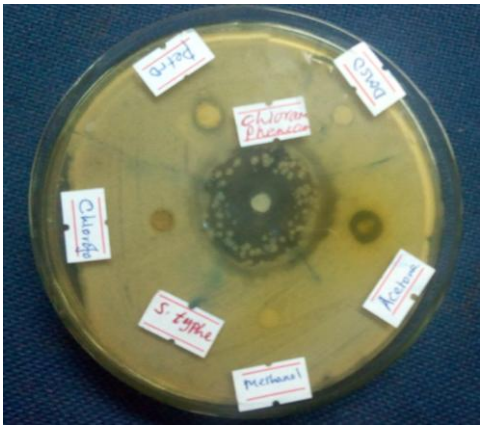


Appendix 6: DEPT-135 Spectra of compound -2

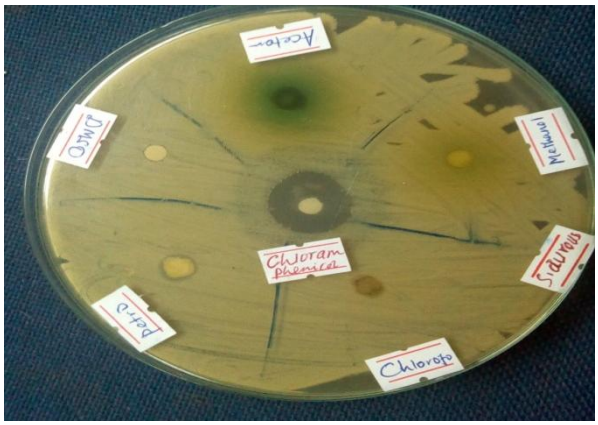


Appendix 7: Antibacterial tests for crude extract

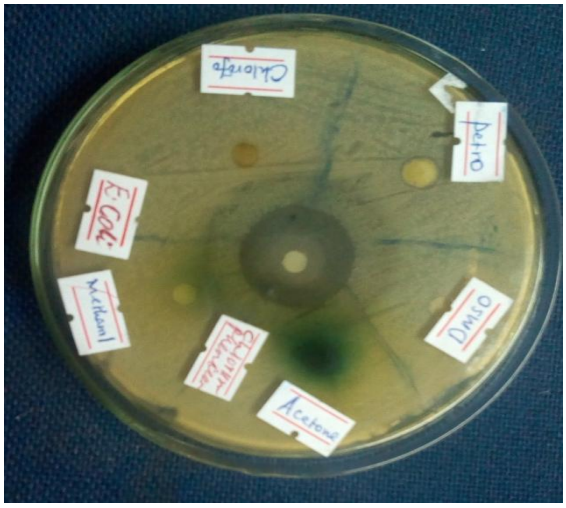
1.*Salmonella typhe;*



2. *Staphylococcus aureus*



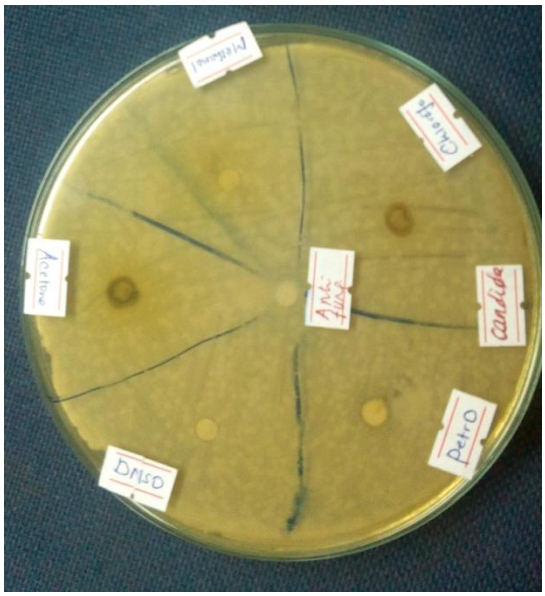
3.*E.coli*



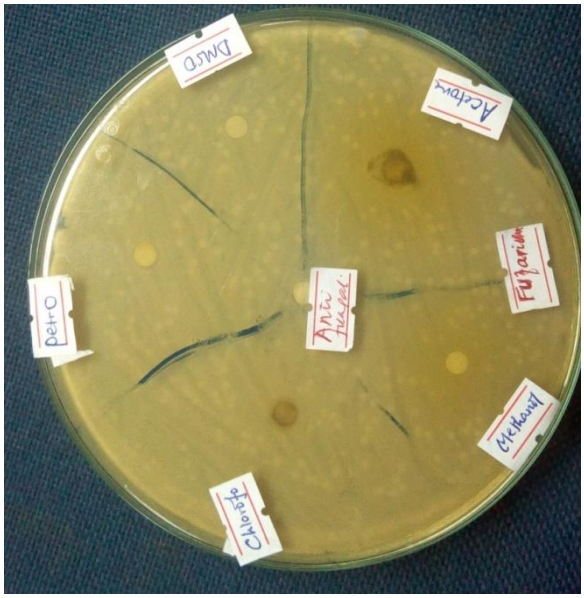
4. *Bacillus.subtilus*

Appendix 8:Antifungal test for crude extracts

1.*Candida albicum*



2.Fuzarium spp



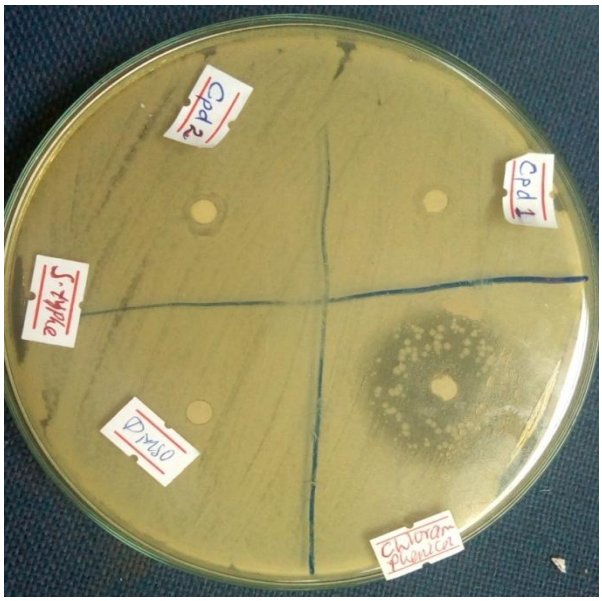
Appendix 9: Antifungal test for isolated compounds

1.*Candidaalbicum*

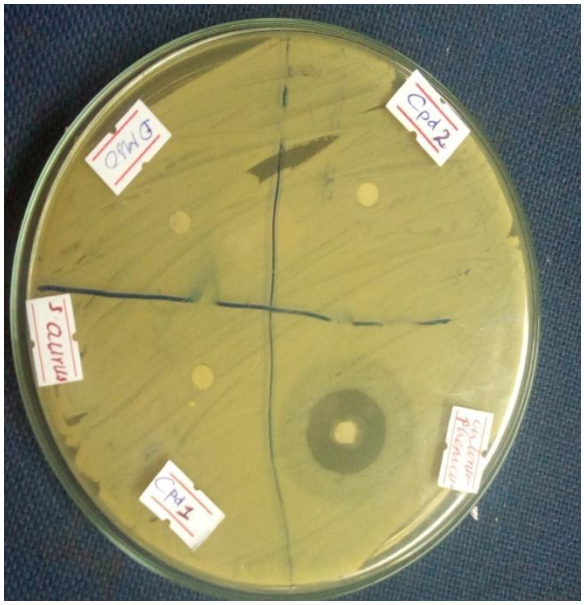
2.Fuzarium spp`

Appendix 10: Antibacterial bacterial test for isolated compounds

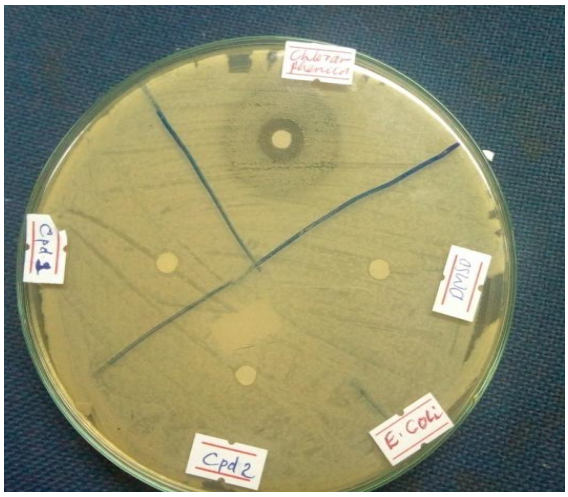
1. *salmonella typhe*



2. *Staphylococcus aureus*



2. *E.coli*



4. *Bacillus.subtilus*