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DEPARTMENT OF CHEMISTRY

ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM ROOT EXTRACTS OF *Helinus* m*ystacinus*

BY

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JIMMA, ETHIOPIA

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ABBREVIATIONS

DMSO…… Dimethylsulfoxide

$CDCl_3$……. Deuterated Chloroform

TLC……. Thin Layer Chromatography

^{1}H-NMR…….. Proton Nuclear Magnetic Resonance Spectroscopy

^{13}C-NMR…… Carbon-13 Nuclear Magnetic Resonance Spectroscopy

IR……. Infrared spectroscopy

DEPT…..…… Distortionless Enhancement by Polarization Transfer

UV…… Ultraviolet

CFU……..… Colony Forming Unit

HM …… Helinus mystacinus

ABSTRACT

The study was focused on isolation and characterization of bioactive compounds from methanol and 80% methanol/H_2O extracts of *Helinus mystacinus* (root). The fresh plant material was collected from Jren district, Jimma town and shed dried in laboratory, the dried plant part crushed was ground. The powdered plant sample was extracted with methanol and 80% methanol and yield 2.4% and 1.4% crude extracts respectively. The biological activity of the extracts were done using standard pathogenic organisms such as *staphylococcus aureus(OSM 7346),, Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (DSMZ 1117) and Salmonella typhimurium (ATCC 13311)*. Methanol extracts which relatively showed better antibacterial activity than 80% methanol/H_2O subjected to column chromatographic separation of its components using mixture of chloroform: ethyl acetate as eluting solvent. Four compounds (**HM I, HM II, HM III** and **HM IV**) were isolated among these only **HM II** was fully characterized based on physical property, spectroscopic data(IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135) and in comparison with literature reports. Compound **HM II** identified as be linoleic acid. The antibacterial activity test result of these isolated compounds showed that they were active on all bacterial strains than the crude extracts. Therefore, further study is recommended to isolate and characterize more bioactive compounds from *Helinus mystacinus.*

1. INTRODUCTION

1.1 Background Information

The study of plants is vital because they are a fundamental part of life on Earth, which generates the oxygen, food, fibres, fuel and medicine that allow humans and other higher life forms to exist. A good understanding of plants is crucial to the future of human societies as they allow us to produce food to feed an expanding population, understand fundamental life processes, produce medicine and materials to treat diseases and other ailments understand environmental changes more clearly. The number of higher plant species (angiosperms and gymnosperms) on this planet is estimated at 250,000 [1], with a lower level at 215,000 and an upper level as high as 500,000 [2]. Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically. 25% of modern medicines are made from plants first used traditionally. Out of total medicines prescribed by physicians, 30% of them are herbal medicines[3]. Medicinal plants have been used as traditional treatments for various human diseases for thousands of years in many parts of the world. According to WHO medicinal plants would be the best source to obtain a variety of drugs. It has been confirmed by WHO that herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries[4].

Plant constituents comprise a wide variety of organic substances that are formed and accumulated by plants. They include Primary metabolites which are produced by primary metabolism The primary metabolites such as carbohydrates, proteins, fats, and nucleic acids, are essential for life and are commonly present in all organisms in large amounts. Secondary metabolites**,** also known as natural products, are those products of metabolism that are not essential for normal growth, development or reproduction of an organism. Each plant family, genus, and species produces a characteristic mix of these secondary metabolites, and they can sometimes be used as taxonomic characters in classifying plants. Occasionally, two plants are found to have identical physical aspects which botanists use for classification, but different in the secondary metabolites they produce[5]. Secondary metabolites are grouped into classes based on similar structures, biosynthetic pathways, or the kinds of plants that make them. The largest such classes are the alkaloids, terpenoids, flavonods, essential oils, phenols, saponins and glycosides.

Humans use many of these compounds as medicines due to their variable biological activities. They may be produced in the plant as: defense against predators, detoxifying agents and attract pollinators. They include most of the pharmacologically active natural plant products and usually produced in small quantity in the plant. The natural products derived from medicinal plants have proven to be an abundant source of bioactive compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals. With respect to diseases caused by microorganisms, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antiviral agents, has lead to the discovery of novel anti-infective chemicals[6]. Developing novel bio active molecules is never ending process to cater the need of society include human and animals. Traditional medicinal plants are the source of lead bioactive molecules and for drug discovery programme.

Ethiopia has a flora that is extremely rich in its diversity. It is therefore not surprising that some of these plants have chemical compounds of therapeutic value that may be used in the treatment of major diseases such as HIV/AIDS, malaria, cancer, etc[7]. The use of traditional medicine is still wide spread in Ethiopia, and its acceptability, availability and popularity is no doubt as about 90% of the populations use it for health care needs [8]. Medicinal plants occur throughout the country's diverse high land and low land areas but, little work has so far been done in the country to properly document and promote the associated knowledge of the bioactive compounds found in the medicinal plants[9].

Hence, the present study will be help full in such a way that to investigate the chemical principle existing in medicinal plant called *Helinus mystacinus* which was claimed its root traditionally as anti malarial agent and abdominal pain in rural parts of Ethiopia[10]. In line with this the present study was focus on extraction, isolation, biological activity test and structural elucidation of methanol and 80% methanol/H_2O root extracts of *Helinus mystacinus.* Phytochemical screening was done on the root extracts of the plant *Helinus mystacinus* that used three solvent systems for extraction and the results indicated that the methanol extract was rich in secondary metabolites like Terpenoids, flavonoids, alkaloids, Saponins and glycosides. From the chloroform extract two pure compounds were isolated and characterized namely benzoic acid and betulinic acid[11].

1.2. Statement of the Problem

In Ethiopia various plants are commonly used in disease treatment by local healers. An estimated 80% of the Ethiopian population relies on traditional medicine. Socio-cultural appeal, accessibility, affordability, and effectiveness against a number of health problems seem to promote its widespread use[25].

The acceptance of traditional medicine as an alternative form of health care and the development of bacterial resistance to the available synthetic drug have led to investigate active antibacterial drug from natural products. From the literature review the *in vitro* anti-bacterial activity study of root extracts of *Helinus mystacinus* were found to have significant *in vitro* antibacterial activities, against most of the test bacteria and the phytochemical screening indicates the presence of alkaloids, flavonoids, terpenoides, saponons and glycosides in the methanolic extract[11]. To the best of our knowledge isolation and characterization of methanol extract of the plant was not previously carried out**.**

In order to fill the mentioned gap the current study designed for isolation and characterization of medicinal plant known as *Helinus mystacinus* which is used traditionally by Shinasha people for anti malaria and anti abdominal pain and used to treat burning in rural part of north western Ethiopia local healers and to identify compounds which are antibacterial effect against some bacterial species.

1.3. Objectives of the Study

1.3.1. General objective

- ❖ The main objective of this study is to isolate and characterize bioactive compounds from the root extract of *Helinus mystacinus*.

1.3.2. Specific objectives

- Extract the roots of *Helinus mystacinus* using methanol and aqueous methanol.
- Isolate bioactive molecules from root extracts of *Helinus mystacinus* using chromatographic techniques.
- Characterize the compounds isolated using spectroscopic techniques (IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135).
- Evaluate antimicrobial activities of the crude and isolated compound using *in vitro* test method

1.4. Significance of the Study

The treatment of infectious diseases still remains an important and challenging problem because of a combination of factors including emerging infectious diseases and the increasing number of multi-drug resistant microbial pathogens. Thus, this study expected to yield lead compounds for the development of pharmaceutical products, to give information about photochemical investigation of the root extract and to make use of the important information for further isolation and purification,

2. REVIEW OF LITERATURE

2.1. Botanical Description, Habitat and Distribution of *Helinus mystacinus*

Synonyms*: Helinus mystachinus* forma *pilosiusculus*; *Helinus mystachinus* forma *tomentosus* Vernacular name, Esat Abered (Amharic); Homachesa (Afan Oromo); Hidda Hoomoo(Afan Oromo): Qemida (Tigringa). *Helinus mystacinus* (Figure 1a) is the species under the Rhamnaceae family. It is a woody climber to 10 m or more, petiole 2-23 mm, lamina ovate or elliptic to orbicular, 1.5-6.5 x 1-5.5 cm, sepals 2 mm long, petals white to yellowish green, 1.5-2 mm long; filaments 1.5-2 mm, fruit ellipsoid to subglobose, 5-9 mm long, with numerous acute glandular tubercles and pilose[12]. Forest margins and forest clearings, secondary forest and shrub, wetter types of wood land, wooded grassland and bush land, abandoned cultivations. The Rhamnaceae are mainly trees or shrubs, sometimes twining or climbing, comprising about 55 genera and 900 species. *Ziziphus, Rhamnus, Ceanothus* and are the chief genera of this family[13]. In Ethiopia there are 8 general and 15 species. The plant is distributed throughout Ethiopia, Uganda, Somalia and other East African countries Africa to Nepal[12]. This family has a worldwide distribution but is more common in the tropical and subtropical regions of the world.

Figure 1. Ariel parts of *Helinus mystacinus*

Figure 2. Crushed roots of *Helinus mystacinus*

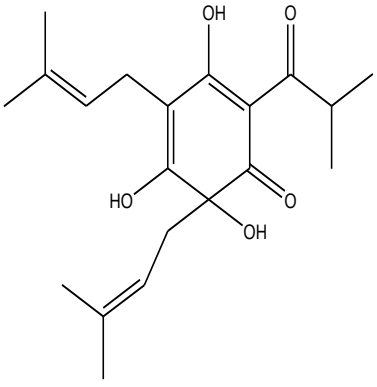
2.2. Medicinal Importance of the Rhamnaceae Family

Several species of Rhamnaceae notably *Zizyphus jujube* fruits have biological effects, such as anticancer, anti-inflammatory, antiobesity, immune stimulating, antioxidant, hepatoprotective, and gastrointestinal protective activities and inhibition of foam cell formation in macrophage[14]. Root of *Zizyphus glabrata* are used in making dyes and inks, photographic and used as astringents. It has a wide range of biological activities, including antioxidant, anti-inflammatory[15], anti-bacterial[16], against various Gram-positive and antifungal [17] activity against various fungus, and anticancer activities[18]. Gallic acid showed *in vitro* anti-HSV-1 (*Herpes simplex* Virus type1) and Human Immunodeficiency Virus (HIV-1) activity[19].

2.3. Natural products isolated from plant family Rhamanceae

2.3.1. *Rhamnus Prinoides*

Pytochemical study on *Rhamnus prinoides* 'Gesho', family Rhamnaceae isolation and characterization of different classes of compounds (Figure 3) such as flavonoids and anthraquinone, Cohmlone (**1**), Humulone (**2**), Adhumulone (**3**), Colupulone (**4**), Lupulone (**5**), Adhupulone (**6),** Quercetin (**7**), Kaempferol (**8**), and one anthraquinones compound (**9**) were isolated and characterized from the leaves of *Rhamnus prinoide*[20].



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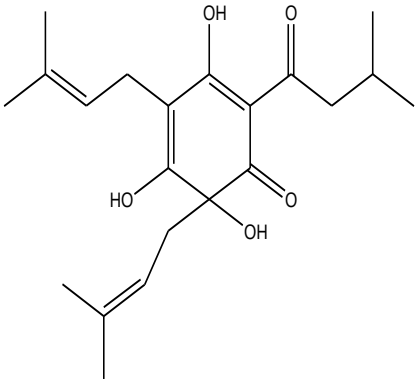

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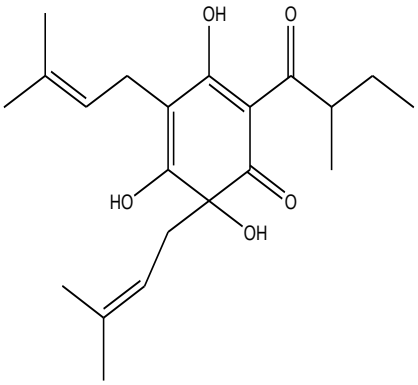

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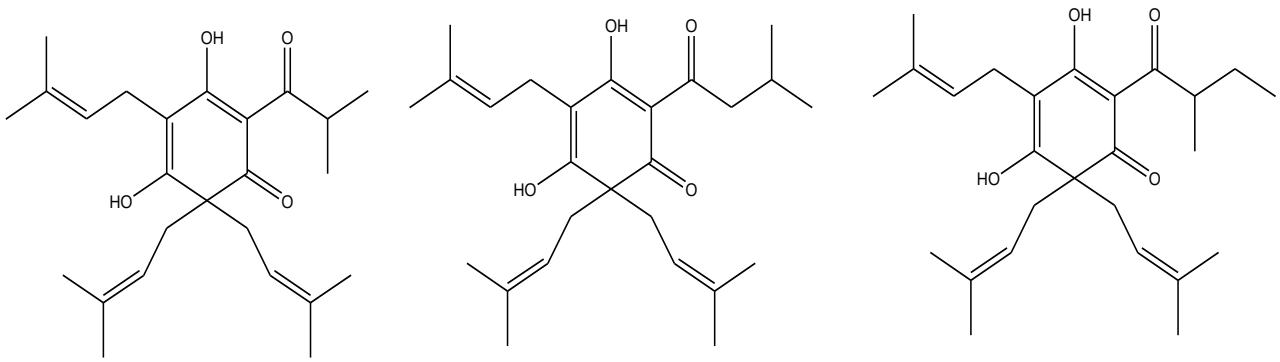

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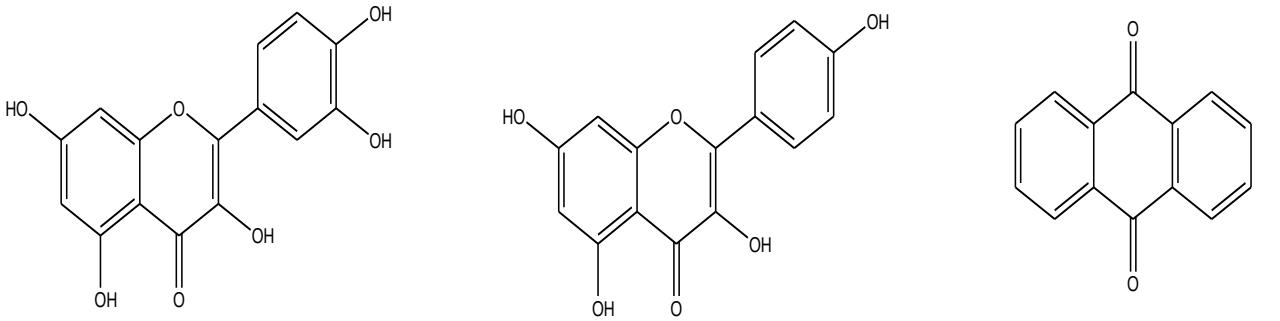
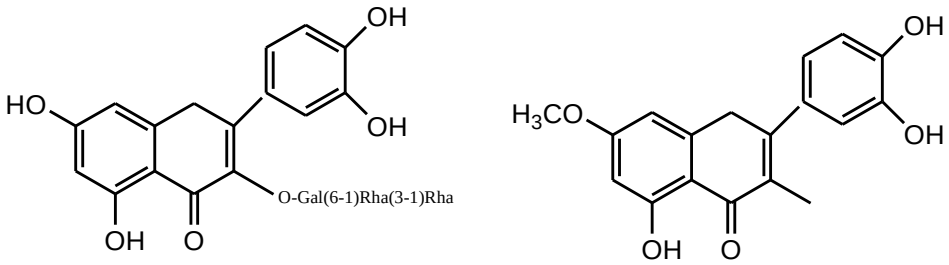


7 **8** **9**

Figure 3. Isolation and characterization of different classes of compounds from *Rhamnus prinoides*

2.3.2. *Rhamnus Alaternus*

Another study shows some flavonoid enriched extracts from *Rhamnus alaternus* leaves (Rhamnaceae**)** has resulted in three flavonoids(Figure 4) kaempferol-3-o-isorhamninoside (**10**), rhamnocitrin-3-o-isorhamninoside (**11**) and keampferol(**12**)[21].



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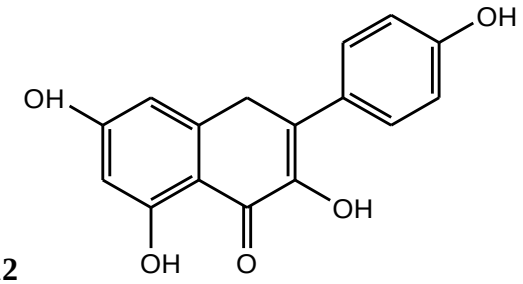

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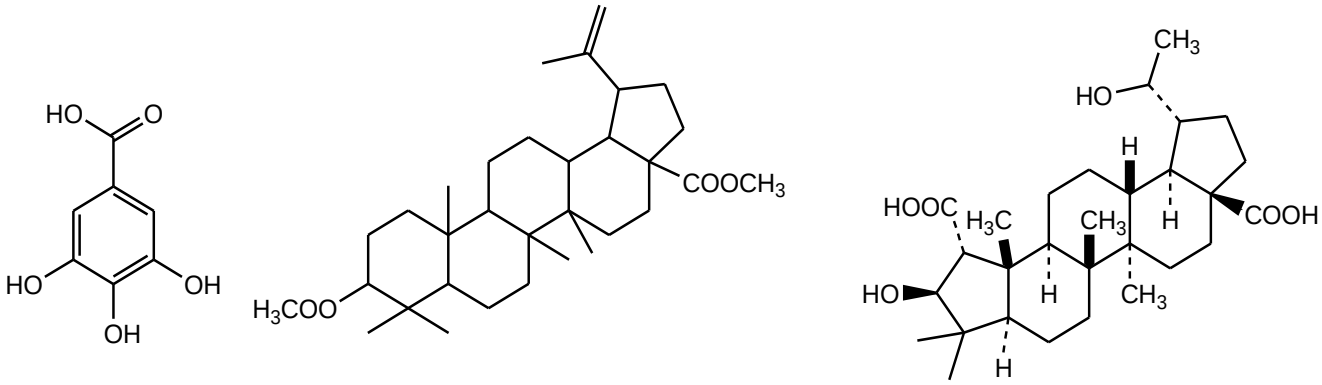
Figure 4. Flavonoid enriched extracts from *Rhamnus alaternus* leaves

2.3.3. *Zizyphus glabrata*

The methanol extract of roots of *Zizyphus glabrata* (Rhamnaceae) yielded (Figure 5) Gallic acid (**13**) and Butalinic acid (**14**) [22] and from stem of this plant a triterpenoids, ceanothic acid (**15**) were isolated[23].

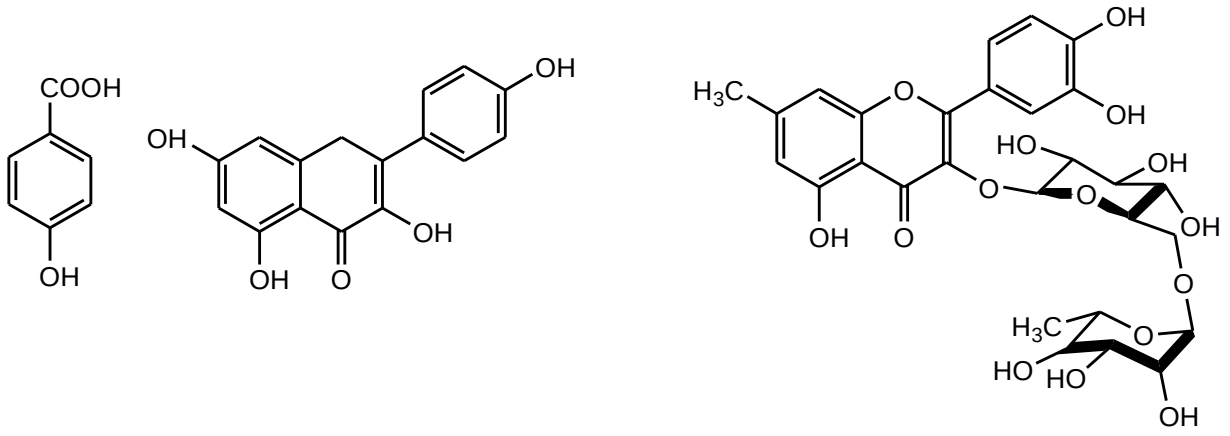


13 **14** **15**

Figure 5. Isolated and characterized compounds from *Zizyphus glabrata* (Rhamnaceae)

2.4.4. Zizyphus Spina-christi

Pytochemical study on *Zizyphus Spina-christi*, family Rhamnaceae phenolic compounds (Figure 6) such as p-hydroxybenzoic acid (**16**), Kaempferol(**17)** and Rutin(**18**) were isolated from the seeds of *Zizyphus spina-christi* for the first time[24].



16 **17** **18**

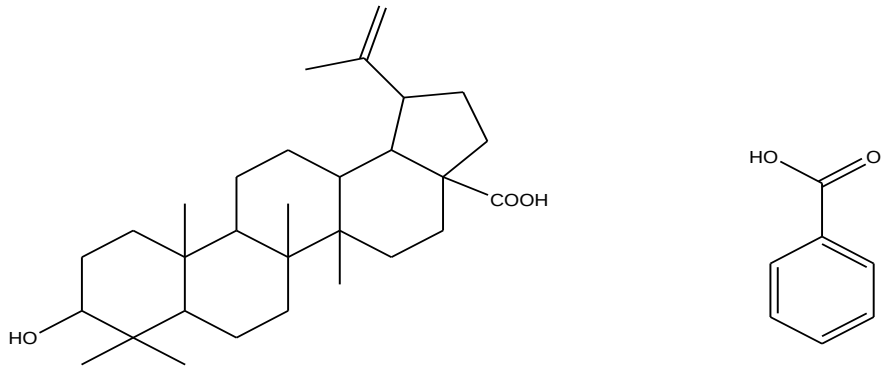
Figure 6. Compounds isolated from seed of *Zizyphus spina-christi* (Rhamnaceae)

2.4. Natural products isolated from *Helinus mystacinus*

Scientific research into medicinal plants has led to the identification of many valuable compounds through isolation, identification, purification, characterization and standardization of active components of medicinal plants. Nature has made available plants which can be used for the cure of various diseases that continually plague mankind. Among these plants *Helinus mystacinus* has been used in Shinasha for its medicinal value to treat diseases (antimalaria and abdominal pain) and to treat burning in north western part of Ethiopia[12]. Traditionally, in northwest Ethiopia, used to treat burning, dressing with crushed fresh leaves of *Helinus mystacinus*[42]. In west Ethiopia (East Welega zone), also used to prevent infection of external parasite[43].

Phytochemical screening was done on the root extracts of the plant *Helinus mystacinus* that used four solvent systems(petroleum ether, chloroform, ethyl acetate and methanol) for extraction and the results indicated that the plant contains different types of natural products (alkaloids, tannins, terpenoids, flavonoids, saponins, glycosides and anthraquinones. The study described that the

methanol extract was rich in secondary metabolites like Terpenoids, flavonoids, alkaloids, Saponins and glycosides. From the chloroform root extract (Figure 7) two pure compounds were isolated and characterized namely Betulinic acid (**19**) and benzoic acid (**20**).

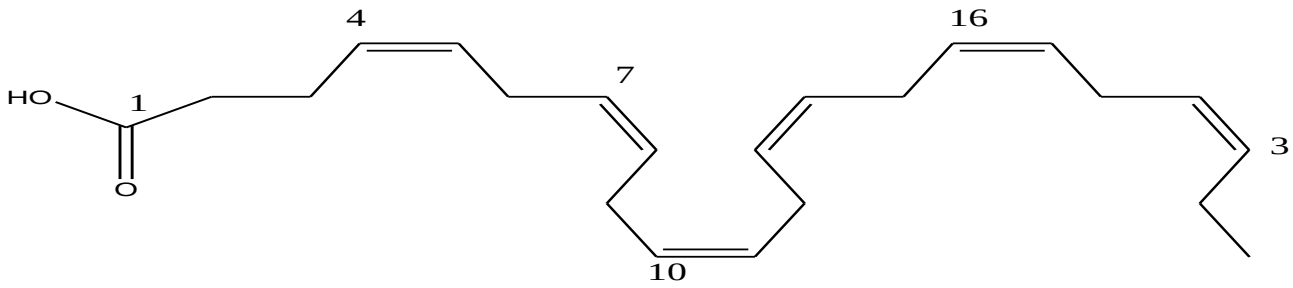


19 **20**

Figure 7. Compounds isolated and characterized from root of *Helinus mystacinus*

2.5. Essential Fatty Acids

Essential fatty acids (EFAs) are the "good fats because of their claimed positive health benefits. They are essential to human health, but cannot be synthesized by the body and therefore must be obtained through diet. There are two types of essential fatty acids: Omega-3s and Omega-6s. There are three major types of omega 3 fatty acids that are ingested in foods and used by the body[36]. The omega-3 family includes (Figure 8) alpha-linolenic acid,), docosahexaenoic acid (**21**) and eicosapentaenoic acid (**22**)[37]. Linoleic acid is the primary Omega-6 fatty acid. These essential fatty acids have shown to play a fundamental role in several physiological functions. The omega-6 family includes cis-linoleic acid, linoleic acid, and gamma-linolenic acid (GLA). Cis-linoleic acid is found in certain plants and vegetable oils. Linoleic acid is found in most plants and vegetable oils. The properties and uses of omega-3 and omega-6 fatty acids overlap to a great degree. Both enhance the body's own anti-inflammatory process, acting like cortisone, cardioprotective and anticancer properties. They play in the body's manufacture of prostaglandins, hormone-like substances that are produced and used by all cells. In addition, fatty acids serve as structural parts of cell membranes, and therefore help protect the cells from invading toxins, bacteria, viruses, carcinogens, heart disease, cancer, arthritis, allergies, and various immunological disorders[38].



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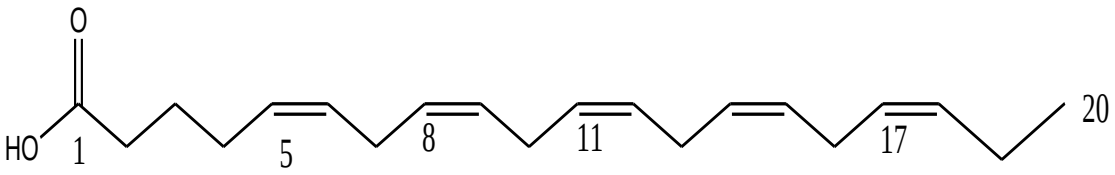

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Figure 8. The structures of some omega-3 and omega-6 fatty acids

3. MATERIALS AND METHODS

3.1. Materials and Chemicals

In this study analytical grades chemicals ethyl acetate, methanol and chloroform (supplied by Sigma Aldrich Chemicals Co. Ltd.) and distilled water where used as solvent. Silica gel (60-120 mesh size), Pre-coated TLC (silica gel), UV (UVitec 254 and 365 nm) and iodine chamber used for extraction and chromatographic analysis. Shaker (Grant GLS400) and rota vapor (Heidolph, Laborota 4000 UK) were used for shaking and solvent evaporation respectively. DMSO was used for dissolving the extracts for antibacterial activity test. Standard antibioti disc Gentamycin was used as positive controls. Mueller Hinton agar and nutrient broth were used for the biological activity test. Bacterial strain *Staphylococcus aureus (*OSM 7346), *Escherichia coli (*ATCC 25922), *Pseudomonas aeruginosa* (DSMZ 1117) and *Salmonella thyphimurium* (ATCC 13311) were obtained from Biology Department, Jimma University. Spectral recording were done using IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 at the Department of Chemistry, Addis Ababa University.

3.2. Collection and Preparation of Plant Material

The fresh plant material, root part of *Helinus mystacinus* was collected from Jiren district, Jimma town, Oromia region, south west Ethiopia in February 2014. The plant material was identified by Dr. Kitessa Hundera, Botanist of Department of Biology, Jimma University and voucher number Acc. 002 was given and specimen of *Helinus mystacinus* was deposited in the herbarium. The collected plant material (2.5Kg) was washed with water, air-dried under shade in laboratory at room temperature chopped into small pieces and then powdered using a milling machine at Department of post-harvest management, Jimma University.

3.3. Extraction of *Helinus mystacinus*

The powdered root of *Helinus mystacinus* (1 Kg) was soaked thoroughly and extracted with methanol (5 L) for 72 hours, then the residual plant material was extracted with aqueous methanol (4 L)(80% methanol/distilled water) for 72 hours using maceration technique with constant and continuous shaking. The extract obtained was filtered using cotton plug followed by Whatman No 1 and concentrated by using a Rota vapor at average temperature of 40^{0}C. Each crude extract was weighed and placed in refrigerator below 4 ^{o}C till used for the biological

activity test. The resulting extracts were tested for their antibacterial activities and the methanol extract shown better than that of aqueous methanol extract. So, the methanol extract was selected for column chromatography isolation of its components.

3.4. Isolation of Compounds

Appropriate solvent was selected for elution after carrying out TLC analysis of the extracts, accordingly chloroform: ethyl acetate combination of different polarity was found to be suitable for elution of column. Due to its better antibacterial activity against the tested bacterial strains methanol extract was selected and 7.5 g of methanol extract was absorbed on 15 g of silica gel and then loaded on to previously packed column with 100 g activated silica gel in chloroform (100%). The column was eluted with chloroform and ethyl acetate combination of increasing polarity. [100:0, 98:2, 96:4, … 0:100.] A total of 274 fractions each with 25 ml were collected and the solvent was evaporated using rota vapor. Fractions with similar TLC profiles were combined together and concentrated using rota vapor. Spots developed were visualized under UV light at 254 and 365 nm and iodine chamber. The isolated pure compounds obtained were identified using spectral (^{1}H-NMR, ^{13}C-NMR, DEPT-135 and IR) analyses.

3.5. Test Strains

Antibacterial activity of root extracts of *Helinus mystacinus* was investigated against bacterial strains Staphylococcus aureus (OSM 7346), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (DSMZ 1117) and Salmonella thyphimurium (ATCC 13311) using standard procedures[26]. The stock bacterial cultures were maintained at 4 °C on slants of nutrient agar. Active cultures for the experiments was prepared by transferring a loop full of bacterial cells from the stock cultures to test tubes containing 5 ml Mueller-Hinton broth that was incubated for 24 hrs at 37 °C. A cell suspension of each organism equivalent for McFarland 0.5 turbidity standard was obtained by preparing 1% V/V of H_2SO_4 and 1% W/V $BaCl_2$ then 95.5 ml of 1% V/V of H_2SO_4 mixed with 0.5 ml of 1% $BaCl_2$W/V for comparison of the turbidity to a cell suspension of each organism in order to have a suspension containing approximately 1-2 x 10^8 CFU ml^{-1}. The bacteria were spread over solid Mueller Hinton agar plates with a sterile swab and each crude extract 50 µL of 100 mg ml^{-1} concentration dissolved in DMSO was impregnated on Whatmann No 1 filter paper disc (diameter 6 mm) using micropipette. At the same time Gentamicine was used as positive control and DMSO for negative control then the plates left for

5 minute till the extract diffuse in the medium and then incubated at 37^{o}C for 24 hrs. After 24 hrs of incubation zone of inhibition in diameter was measured using ruler and mean was recorded.

4. RESULTS AND DISCUSSION

4.1. Extraction Yield Root of *Helinus mystacinus*

Air dried root of *Helinus mystacinus* were extracted with methanol and aqueous methanol (80 % methanol/water) (section 3.3). The resulting methanol and aqueous methanol root extracts of *Helinus mystacinus* was weighted and the yield was calculated as given below (Table 1).

Table 1. Yield of the root crude extracts of *Helinus mystacinus*

Solvent	Weight of the crude extracts	% yield of the extract
Methanol	24 g	2.4 %
Aqueous methanol	14 g	1.4 %

The yield showed that methanol extracts give more yield than aqueous methanol extracts, this might indicated as the amount of solvent increases the amount of yield obtained increases.

4.2. Antibacterial Activity of the Crude Extracts

The crude extracts were dried under reduced pressure, and resulted 24 g and 14 g of crude methanol and aqueous methanol extracts respectively. Antibacterial activity of the extracts was tested against the test bacterial strains.

Table 2. Evaluation of antibacterial inhibition zones (in millimeter) for crude extracts of root of *Helinus mystacinus (*100 mg/ml), DMSO as a solvent

Strains	Methanol extract	Aqueous methanol extract	Gentamycin(10µg)	DMSO
Salamonella thypimurium	22	18	20	NI
Staphylococcus aureus	17	15	19	NI
Escherichia coli	15	10	19	NI
Pseudomonas aeruginosa	18	17	20	NI

NI = Not inhibitory

In DMSO no any zone of inhibition observed. The result obtained from antibacterial activity test of the crude extracts indicated that, the crude extracts were relatively lower than that of gentamycin. However the methanol extracts were better than that of Gentamycine especially on

S. thyphimurium bacterial strain. As observed in Table 2 above the activity of methanol extract were found to be relatively superior to that of aqueous methanol extract on all bacterial strains (Appendix 7). Based on the TLC profile and preliminary antibacterial activities test, the methanol extract was selected for isolation using column chromatographic technique.

4.3. Purification of Crude Methanol Extracts By Column Chromatography

As the herbal drugs contain so many chemical compounds, it is essential to single out those responsible for therapeutic effect to be called as active constituents[30]. Plant extracts usually occur as a combination of various types of bioactive compounds or photochemical with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is a common practice to isolate bioactive compounds using different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC. The pure compounds are then used for the determination of structure and biological activity[31]. Based on the observed data of antibacterial activity tests of the crude extracts given above (Section 4.2, Table 2), the methanol crude extract was then subjected to isolation process using different combinations of chloroform and ethyl acetate as eluent with increasing polarity. As shown on figure 6 from methanol extract four compounds labeled from **HM I-HM IV** was isolated using column chromatography with increasing polarity of chloroform and ethyl acetate solvent combinations.

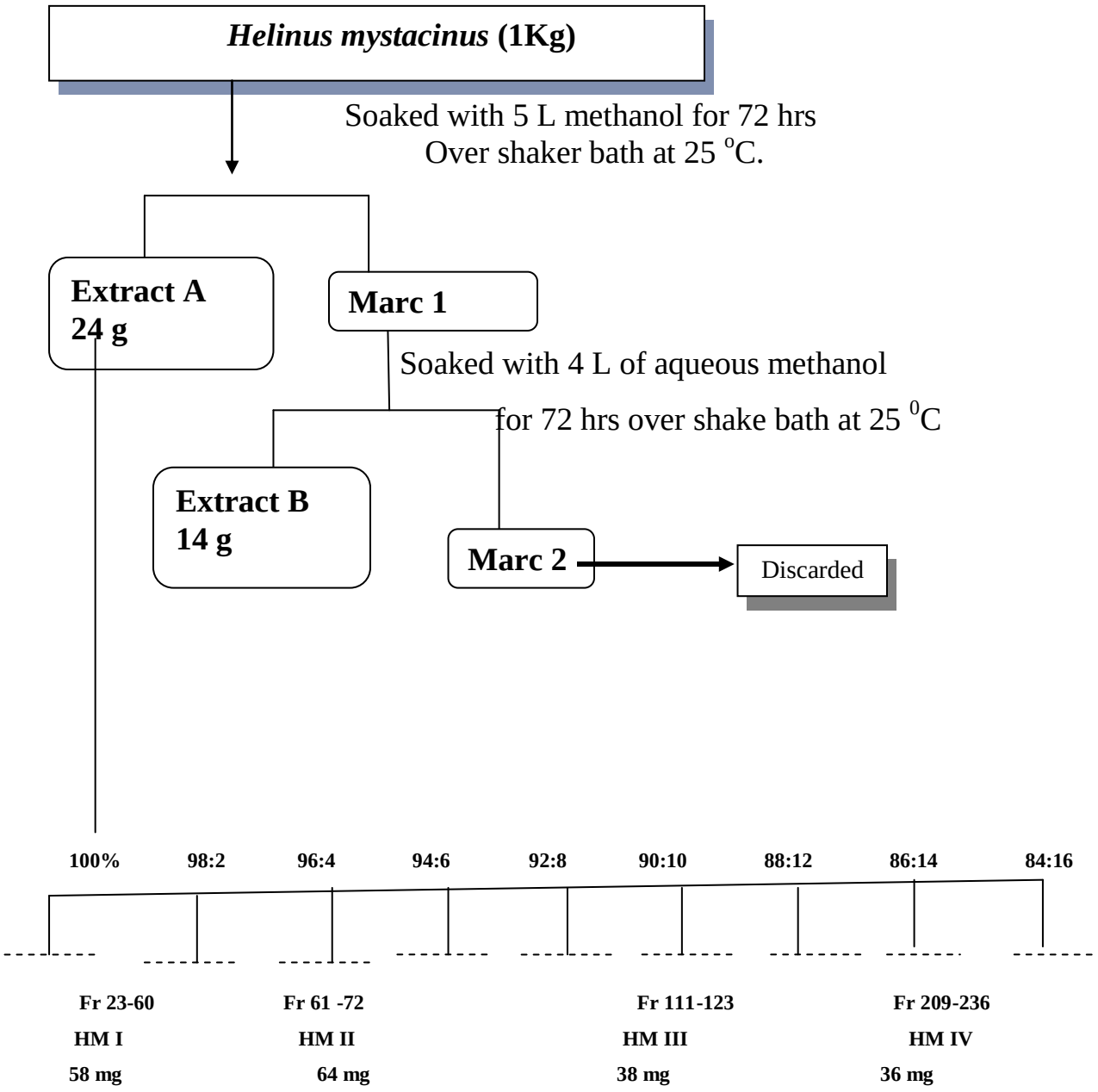

Figure 9. General procedures followed in the extraction and isolation of compounds from *Helinus mystacinus* root

Fractions 23-60 eluted with chloroform (100 %) were combined based on their TLC profiles by examining under UV light (254 nm and 365 nm) and iodine chamber. Concentrating of these fractions gave 58 mg of white crystalline powder coded as **HM I**. Its $\mathbf{R_f}$ value was determined as 0.49 in 98:2 chloroform-ethyl acetate solvent system. Combination of fractions 61- 72 eluted with chloroform: ethyl acetate (96:4). The compound was labeled as **HM II** (64 mg) and its $\mathbf{R_f}$ value was determined as 0.56 in 80:20 chloroform: ethyl acetate solvent system. The third compound labeled as **HM III** (was obtained by combining fractions 111-123 which gave 38 mg yellowish solid and its R_f value was obtained as 0.50 in 75:25 chloroform: ethyl acetate solvent system. The fourth compound labeled as **HM IV** (36 mg) has a yellowish-pink color was obtained by combination of fractions 209-236 and its R_f value was determined as 0.51 in 60:40 chloroform: ethyl acetate solvent system.

4.4. Structural Elucidation of the Isolated Compounds

Structural elucidation of compounds **HM II** and **HM IV** compounds was done based on the spectral data obtained from ^{1}H-NMR, ^{13}C-NMR, DEPT-135, and IR spectra. **HM I** and **HM III** were not characterized because of their low yield which is not sufficient for spectral analysis.

4.4.1. Structural Elucidation of HM II

Compound **HM II** was isolated as colorless oily liquid with $\mathbf{R_f}$ value of 0.56 in chloroform and ethyl acetate (80:20).The IR spectrum of the Compound (Appendix 1) indicated a broad absorption band in the range of 2500 – 3500 cm^{-1} which represent carboxylic group while the strong band at 1694 cm^{-1} represent the presence of carbonyl group. The stretching vibration band above 2950 cm^{-1} represents C-H stretch of alkenes whereas the bands at 2950 cm^{-1} and below 2950 cm^{-1} indicate C-H stretching of methylene and methyl groups, respectively. The absorption bands at 1458 cm^{-1} showed the C-H bending of CH_2 and the band at 1218 cm^{-1} represents C-H bending of CH_3 and the absorption band at 1024 cm-1 corresponds to C-C stretching.

In the ^{1}H-NMR spectrum of compound **HM II** (Appendix 2) the peak at δ 10.83 represented to proton of carboxylic acid; the multiplets at δ 5.37 - δ 5 .35 assigned for olefinic protons of carbon 9, carbon 10, carbon 12 and carbon 13 respectively; triplets at δ 2.78 and δ 2.76 represented to proton of carbon adjacent to the carboxylic group. Multiplets at δ 2.35 and 2.07 represented to proton of carbon adjacent to olefinic carbon. The proton signals at δ 1.64, 1.33

and 1.27 were due to methylene protons. Triplet peak at δ 0.89 represents to methyl protons (C-18). Based on the ^{1}H-NMR, ^{13}C-NMR and IR spectral data compound **HM II** could be unsaturated fatty acid and the proposed structure linoleic acid

From the ^{13}C-NMR and DEPT-135 spectra of compound **HM II** (Appendix 3 and 4) one peak at δ 178.4 indicate quaternary carbon atoms of carbonyl carbons of carboxylic group. The peaks at δ 127.88 (C-9), 128.26 (C-10), 132.79 (C-12) and 132.87 (C-13) indicated presence of C=C bonds; the peak present between δ 33.89 and 22.70 stands for aliphatic methylene carbon (-CH_2) whereas the peak at δ 14.08 indicate the presence of methyl (-CH_3) in the structure (C-18). The above IR and NMR spectral were found to be consistent with the reported data of linoleic acid[32, 33]. From the chemical test of the compound treating with sodium bicarbonate ($NaHCO_3$) in order to confirm the presence of carboxylic acid functional group resulted the formation of effervescence which indicates evolution of carbon dioxide. Moreover, the compound was treated with potassium permanganate ($KMnO_4$) and the result revealed the violate color of permanganate changed to brown colour which confirms the presence of double bond in the compound **HM II** Therefore, from the aforementioned spectroscopic data and chemical test the chemical structure of **HM II** was proposed to be similar with the chemical structure of linoleic acid (Figure7). This is the first report of the compound from the roots of *Helinus mystacinus*. The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 data of compound **HM II (**linoleic acid) are given in Table 4.

Table 3. ^{13}C-NMR, DEPT and ^{1}H-NMR data of **HM II** in comparison with reported data of linoleic acid[32, 33].

C No	δ ^{13}C-NMR (**HM II**)	δ ^{13}C - NMR(Reported Data)*	δ ^{1}H-NMR (**HM II**)	δ^{1}H-NMR (reported data)*	δ DEPT (**HM II**)	Nature of the carbon
1.	178.4	179.1	-	-	-	C
2.	33.89	33.8	2.78, 2.76	2.77, 2.75	33.88	CH_2
3.	25.38	24.7	1.64	1.65	28.61	CH_2
4.	27.95	29.4	1.32	1.25	25.38	CH_2
5.	29.18	29.4	1.32	1.25	29.18	CH_2
6.	29.18	29.4	1.32	1.25	29.18	CH_2
7.	29.18	29.4	1.32	1.25	29.24	CH_2
8.	31.49	31.48	2.35	1.3	31.48	CH_2
9.	127.88	128.2	5.35	5.32	127.87	CH
10.	128.26	128.2	5.35	5.32	127.87	CH
11.	31.49	31,48	2.35	2.34	25.38	CH_2
12.	132.79	130.5	5.37	5.32	132.78	CH
13.	132.87	130.5	5.37	5.32	132.86	CH
14.	31.49	27.2	1.61	1.58	31.48	CH_2
15.	29.25	29.4	1.32	1.3	29.24	CH_2
16.	28.97	29.4	1.32	1.25	28.96	CH_2
17.	24.67	27.2	1.32	1.25	25.38	CH_2
18.	14.02	14.1	0.89	0.89	14.02	CH_3

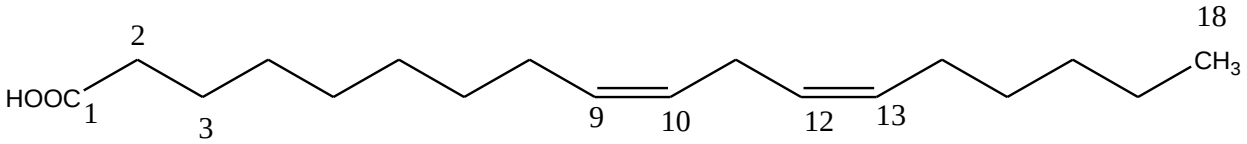

Figure 10. The proposed structure of **HM II**

4.4.2. Structural Elucidation of HM IV (Partial characterization)

Compound **HM IV** was obtained from combined fractions 209-236, yielding (36 mg of light yellow oily liquid) and its R_f value was determined as 0.42 in 60:40 chloroform-ethyl acetate solvent system. The IR (KBr) spectrum of compound **HM IV** (Appendix 5) showed that there is a broad band seen at 3431 cm^{-1-} which s the presence of –OH group. There is no broad band seen between 3400-2500 cm^{-1} to be associated to carboxylic acid –OH group this confirms the absence of carboxylic group. The bands seen around 2928 cm^{-1} indicates the presence of alkyl – CH_3, CH_2, and CH stretching. The absorption band at 1519 cm^{-1} is attributed to C= C double bond in the structure where as 1453 cm^{-1} show the C-H bending of CH_2 and the band at 1251 cm^{-1} represents C-H bending of CH_3 and the absorption band around 1100 cm^{-1} corresponds to C-C stretching. ^{1}H-NMR spectra of compound **HM IV** (Appendix 5) shows peaks at δ 0.89 and δ 1.27 attributed to CH_3 hydrogen's where as the peaks at δ 1. 27 and δ 1.32 represents CH_2 While δ 1.58 and δ 1.61 represents methine hydrogen (CH). The proton signals appeared at δ 1.6-2.6 are assigned to proton attached to carbon adjacent to double bond (allylic hydrogen) therefore δ 2.34-2.5 was attributed to peaks of allylic protons. The proton signals at δ 2.7-4.1 peaks shows the presence of methoxy protons. Signals at δ 5- 6.5 indicates the presence of allylic protons in the structure of the compound. ^{13}C-NMR and DEPT-135 of compound **HM IV** (Appendix 5 and Appendix 6). The carbon signals between δ 132.87-128.34 indicate the presence of carbon atoms of terminal alkenes with –CH and $–CH_2$ respectively. From DEPT-135 spectral data the compound contain more $–CH_3$ and $–CH_2$ group. The compound has no quaternary carbons observed. Thus, from the observed spectral data, the compound most likely aliphatic unsaturated substituted organic compound. However, due to lack of 2D-NMR and MS spectroscopic data, the structure of the compound (**HM IV**) was not characterized.

4.5. Summary of Spectral data of the Isolated Compounds

HM II (colorless oily liquid, 58mg); **IR** (KBr) υ_{max} cm^{-1} 753, 1024, 1218, 1458, 1694, 2950 and broad band in the region 3500-2500,(Appendix 1) **^{1}H-NMR** (400MHz, $CDCl_3$) : δ 0.89 (3H, H-18), δ 1.32-1.33 (m,14H, (H-(4-7) and H- (15-17)) x 2), δ 1.638 (m, 4H, (H-(3 AND 14)X 2, δ δ 2.78 and 2.76 (2H, H-2), δ 2.03-δ 2.35 (m, 4H, (H-(8 and 11) x 2), δ 5.35 - δ 5.37 (m, 4H, H-9 H-10, H-12 and H-13), δ 10.83 (s, 1H, H-O) (Appendix 2). **^{13}C- NMR** (100 MHz, $CDCl_3$): δ 14.02 (C-18), δ 24.61 (C-17), δ 25.38 (C-3), δ 27.95 (C-4), δ 28.97 (C-16), δ 29.25 (C-15), δ

31.49 (C-8 ,C-11 and C-14), δ 29.18 (C-(5-7)) δ 127.88 (C-9), δ 128.26 (C-10), δ 132.79 and 132.87 (C-12 and C-13)respectively, δ 33.89 (C-2) and δ 178.4 (C-1) (Appendix 3).

5.6. Evaluation of Antibacterial Activity of Isolated Compounds

The antibacterial activates of the isolated compounds from methanol extract of *Helinus mystacinus* plant extract were determined using disk diffusion method (Section 5.5). In the study, standard bacterial strains Staphylococcus aureus (OSM 7346), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (DSMZ1117) and Salmonella thyphimurium (ATCC 13311) were used. Each of the bacterial strains was streaked on the Muller - Hinton agar plates using a sterile swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation. 100 mg/ml isolated compounds were impregnated by 50µL extract on 6 mm diameter sterile disk the test was carried out by dissolving the isolated compounds in DMSO. The DMSO which was used as a solvent to dissolve the isolated compounds was used as negative control during the test. Gentamycin 10µg was used as a positive control and the diameter of zone of inhibitions reported in all cases include the diameter of the disks. The resulting diameter of the zones of inhibition was measured and the results were recorded in mm.

Table 4. In vitro antibacterial activity of the isolated compounds *Helinus mystacinus (*100 mg/ml), DMSO as asolvent

Strains	Isolated compounds				Gentamycin(10µg)	DMSO
	HM I	HM II	HM III	HM IV		
Salamonella thypimurium	12	24	14.5	17	20	NI
Staphylococcus aureus	11	19	14	15	19	NI
Escherichia coli	13	15	13	13	19	NI
Pseudomonas aeruginosa	14	23	12	16	20	NI

NI = Not inhibitory

The isolated compounds show antibacterial activity over all bacterial strains. **HM II** showed higher inhibition over Gentamycin on *Salamonella thypimurium* and *Pseudomonas aeruginosa* and **HM IV** on all strains. Long-chain unsaturated fatty acids show antibacterial activity and are

the key ingredients of antimicrobial food additives. Fatty acids are widely occurring in natural fats and dietary oils and they play an important role as nutritious substances and metabolites in living organisms[36, 37]. Many fatty acids are known to have antibacterial and antifungal properties [38]. From the current study confirmed that linoleic acid possesses antibacterial activity against the four bacterial strains under the study. From the preliminary antibacterial activity test of the gradient crude methanol extract showed comparative activity as reference drug (Gentamicine 10 µg) against *Staphylococcus aureus* and *Salmonella thyphimurium*. Compound HM II also showed better activity against these strains. This result was consistent with the reported antibacterial activity of linoleic acid. The antibacterial activity of long-chain unsaturated fatty acids has been well known for many years. Fatty acids fucation as the key ingredients of antimicrobial food additives which inhibit the growth of unwanted microorganism. Additionally, the long-chain unsaturated fatty acids such as linoleic and oleic acids are bactericidal to important pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus*[34] In recent years, it has been reported that they also exhibited antimicrobial activity against *Bacillus subtilis, Listeria monocytogenes, Staphylococcus aureus and Pseudomonas aeruginosa*[35] (Appendix 9).

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Traditional medicine plays significant uses for the society, research and development should give more attention to traditional health practices in order to maximize carry in the health sector. *Helinus mystacinus* is the plant belongs to the family Rhamnaceae. This plant traditionally clamed for its medicinal value (anti malarial and abdominal pain) in Ethiopia. In this study four compounds were isolated from the methanol (root) and one compound was characterized as linoleic acid. The study reviled that bioactive compounds were identified from the plant.

In the current study, the antibacterial activity of *Helinus mystacinus* was assessed extracting the root with increasing solvent polarity (methanol and aqueous methanol). The result showed that the activity of the crude extracts were relatively less than to that of gentamycine reference drug but the methanol extracts were better than aqueous methanol extracts on bacterial strain. Two compounds: compound **HM II** and compound **HM IV** (partially characterized), were isolated from methanol root extracts of *Helinus mystacinus.* Compound **HM II** was isolated for the first time from this plant. The isolated compound was characterized using spectroscopic data (IR and NMR), chemical properties as well as in comparison with literature reports. Compound **HM II** is active on all bacterial strains.

5.2. Recommendations

Further tests are needed to evaluate activities of the compounds against other bacterial species to explore all possibilities, to evaluate potential of the isolated compounds as lead in the development of antibacterial agents. Hence, much more Phytochemical and biological study should be carried out on the plant in future.

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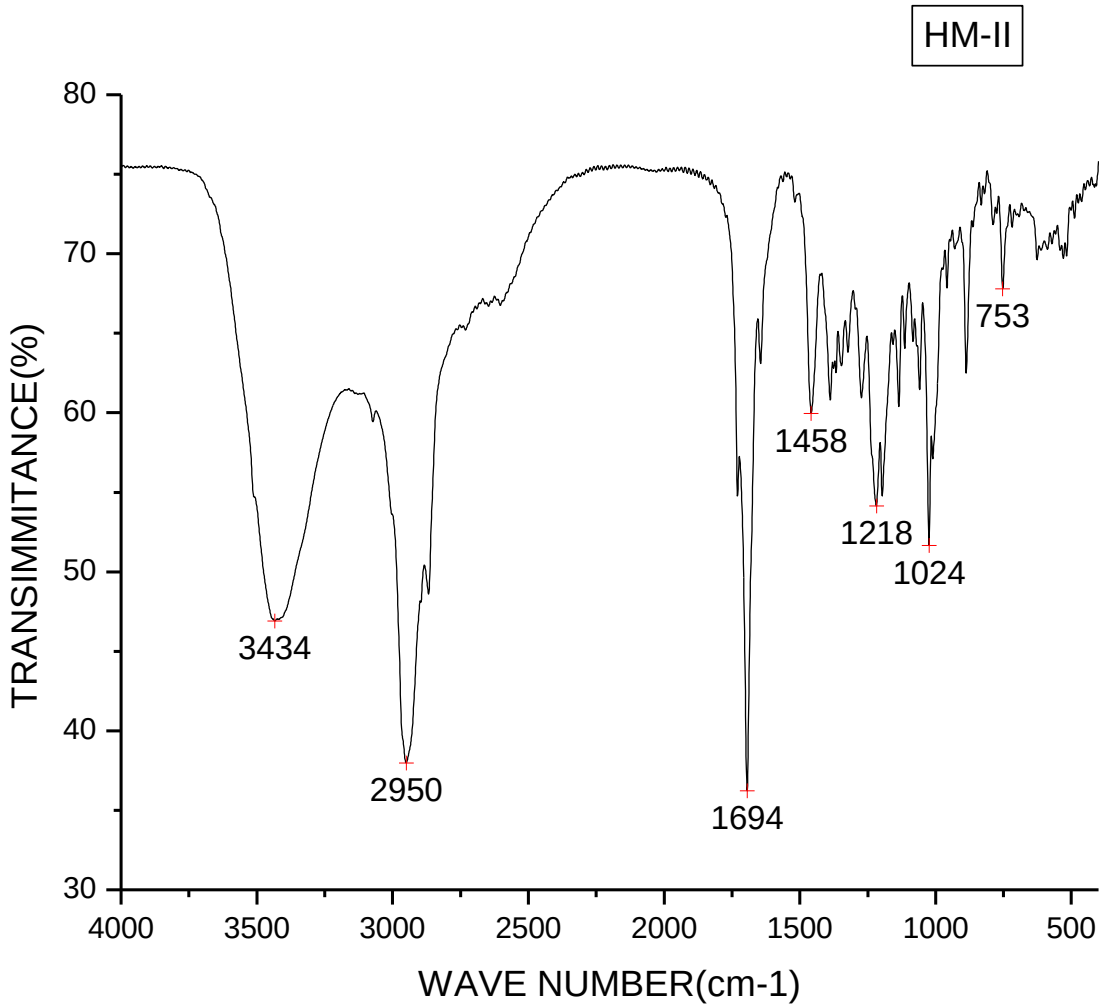
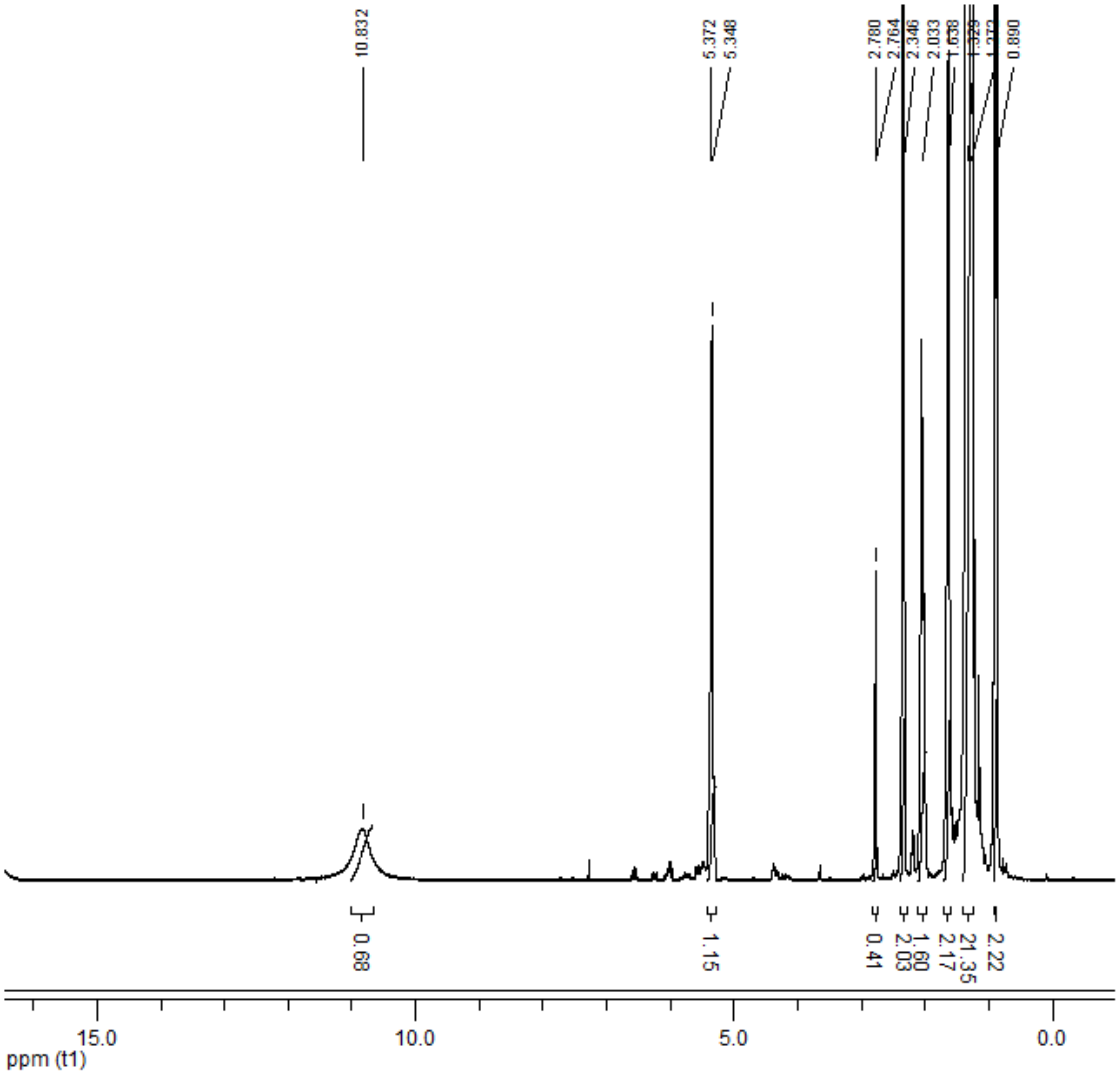
Appendices

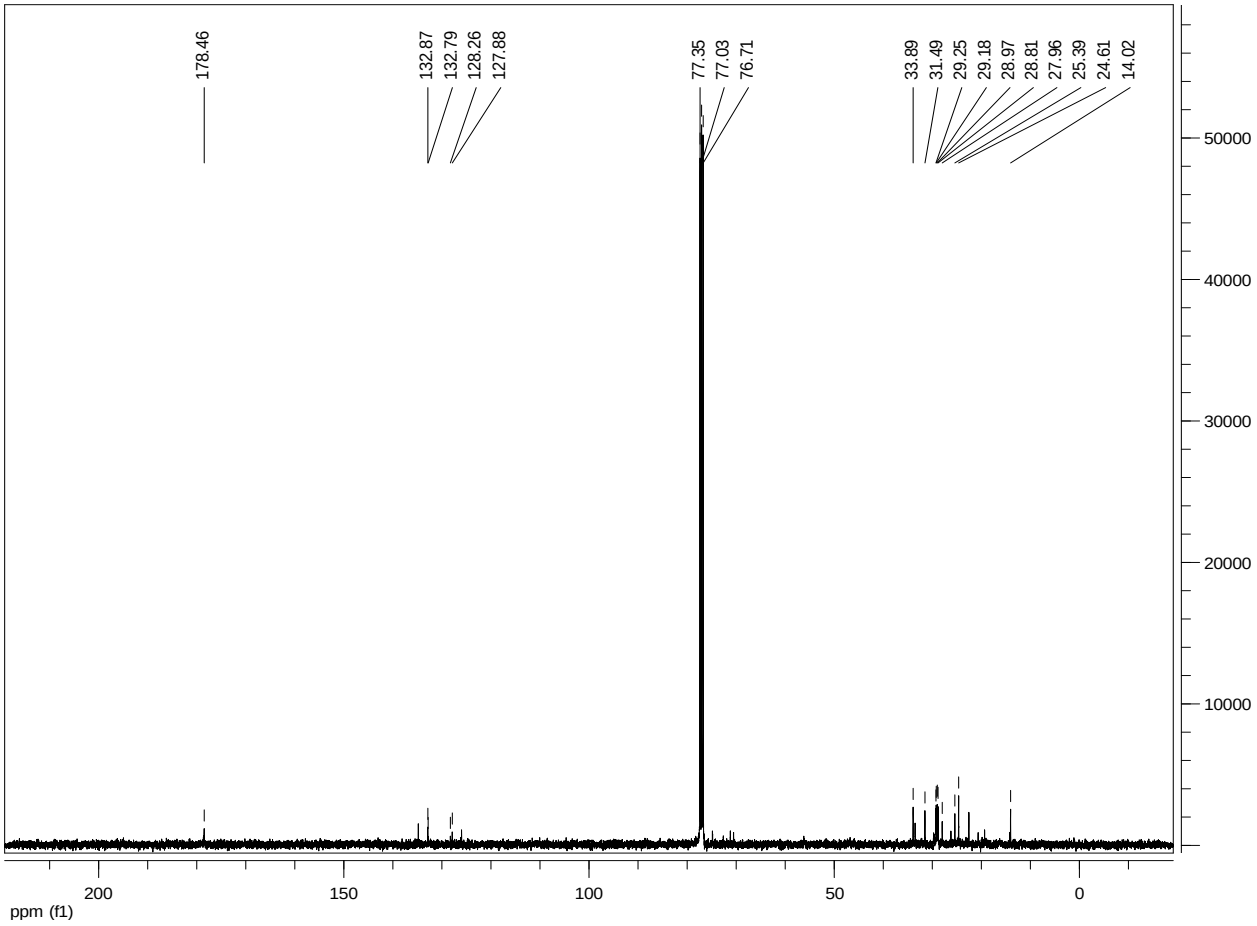
Appendix 1 IR spectrum of **HM II** in $CDCl_3$



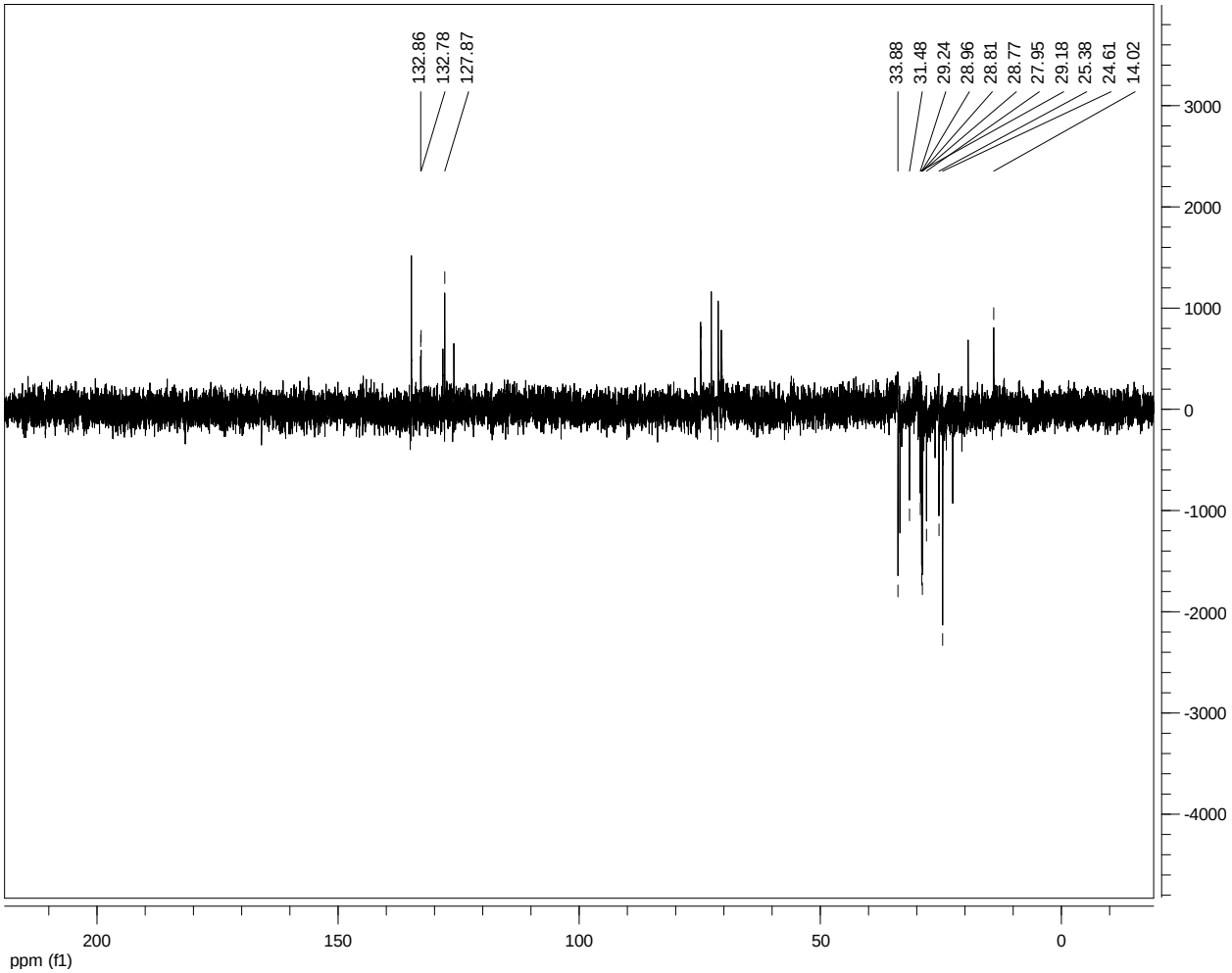
Appendix. 2 ^{1}H-NMR spectrum of **HM II** in $CDCl_3$



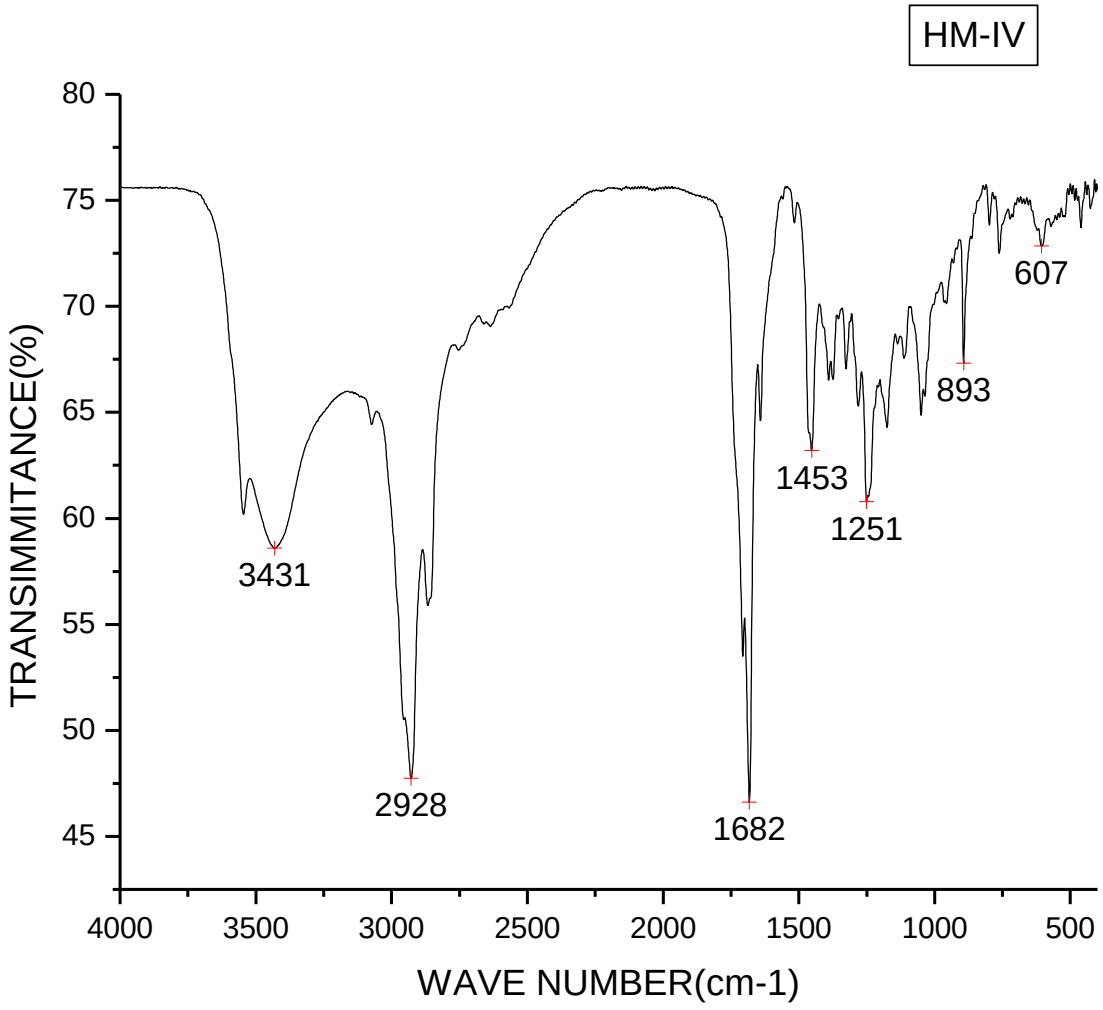
Appendix 3 ^{13}C-NMR spectrum of **HM II** in $CDCl_3$



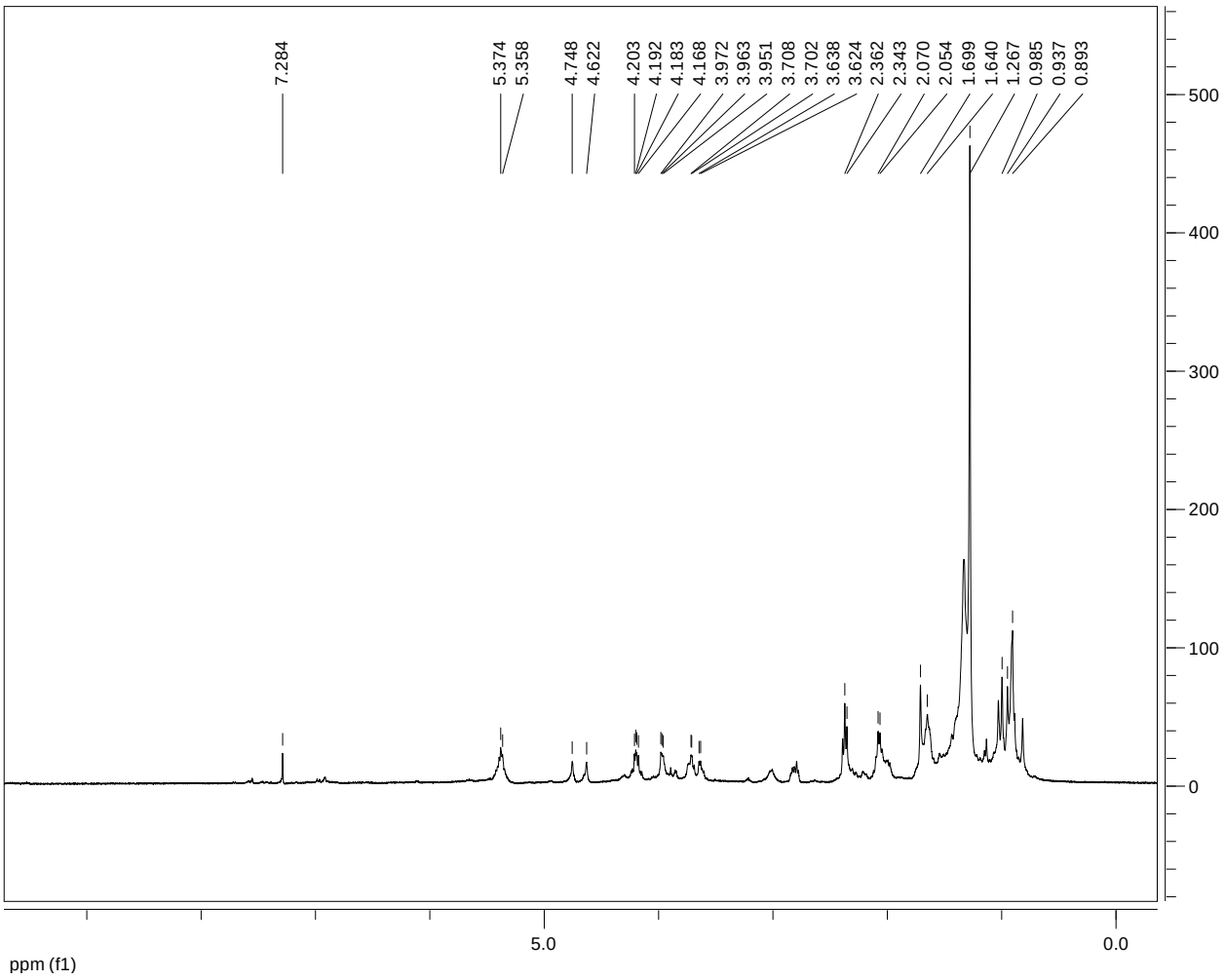
Appendix 4 DEPT-135 Spectrum of **HM II** in$CDCl_3$



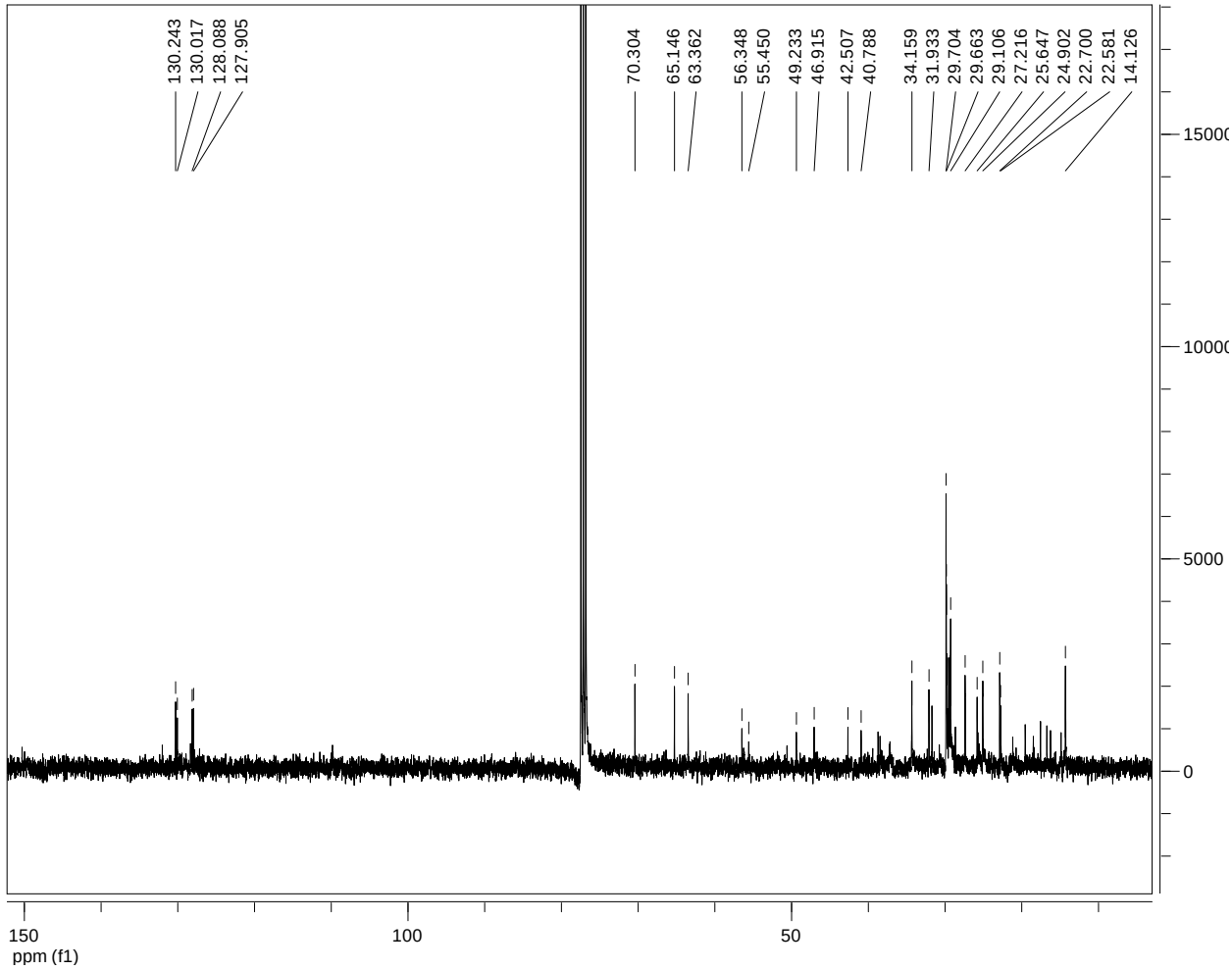
Appendix 5 IR spectrum of **HM IV** in $CDCl_3$



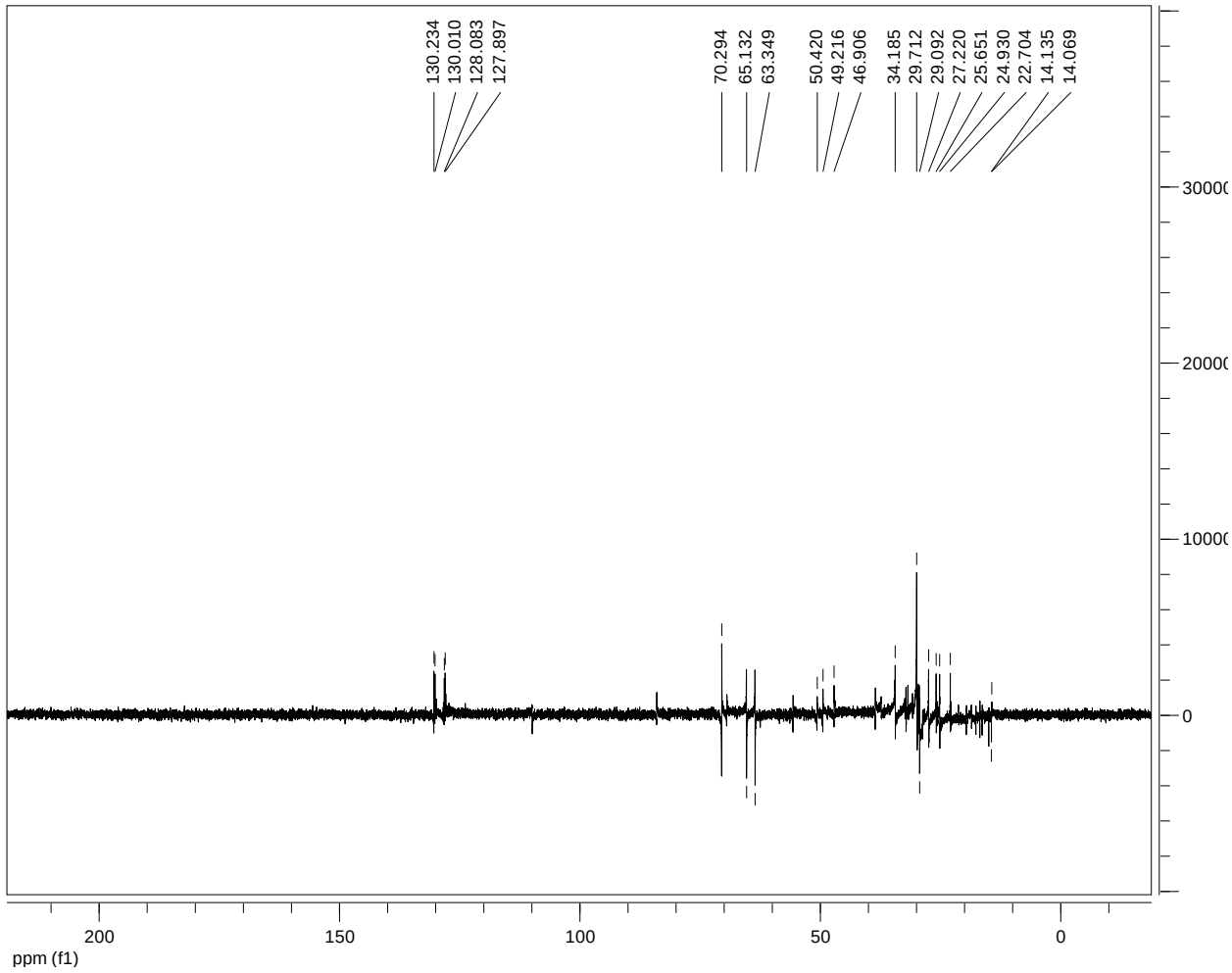
Appendix 6 1H-NMR Spectrum of **HM IV** in $CDCl_3$



Appendix 7 ^{13}C-NMR Spectrum of **HM IV** in $CDCl_3$

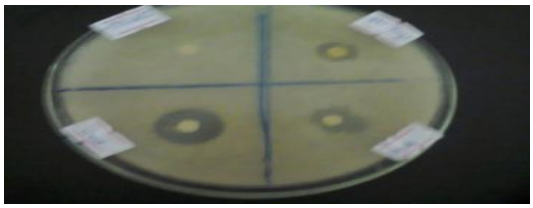


Appendix 8 DEPT-135 Spectrum of **HM IV** in $CDCl_3$

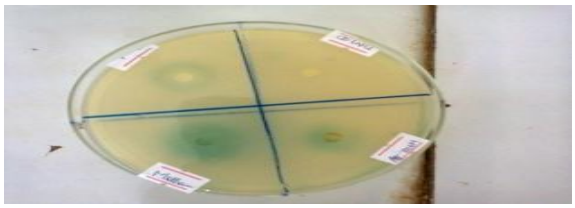


APPENDIX 9 Pictures antibacterial activity of extracts and isolated compound

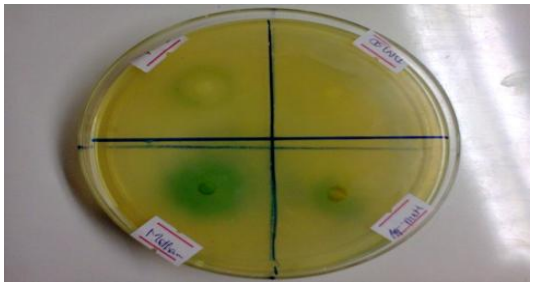
Pictures antibacterial activity of extracts



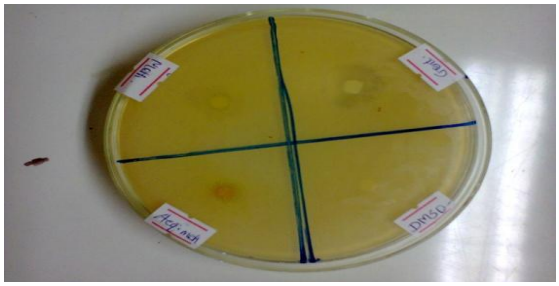
S.aureus



p.aurginosea

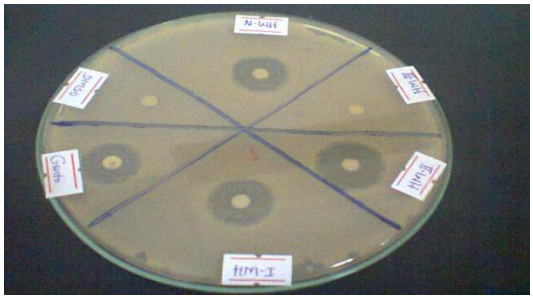


E.coli

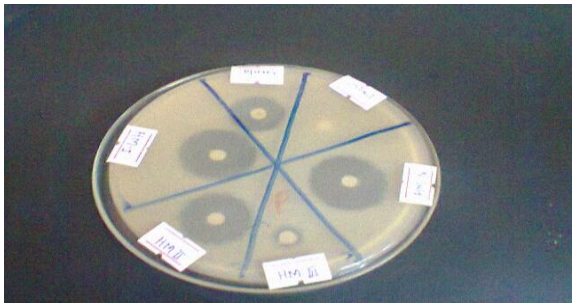


S.typhi

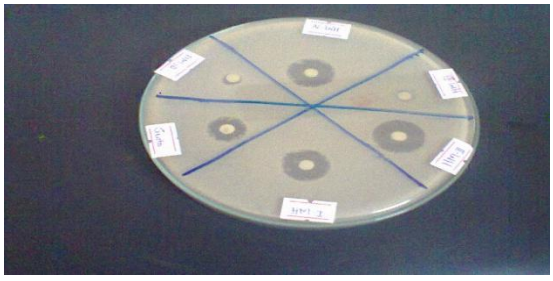
Pictures of antibacterial activites of isolated compounds



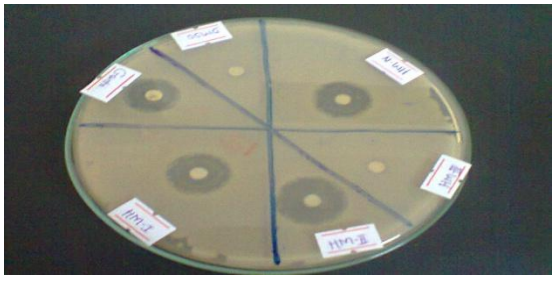
S.aureus



p.aurginosea



S.typhi



E.coli