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ANTIBACTERIAL ACTIVITIES OF UNSATURATED FATTY ACIDS AND STIGMASTEROL ISOLATED FROM STEM EXTRACTS OF *OCIMUM LAMIIFOLIUM*

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ABSTRACT

Most Ethiopians use medicinal plants/herbs and their products in traditional medicine to treat many human illnesses. *Ocimum lamifolium* is a typical example in this regard. This study aimed to conduct phytochemical analysis and antibacterial activity tests on the stem extracts of this plant. The powder of the stem of the plant (500g) was subjected to solvent extraction in chloroform/methanol (1:1) and methanol successively. This results in 14.5g (2.9%) and 3.5g (0.72%) crude extracts, respectively. Phytochemical screening tests on the extracts revealed the presence of alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins, steroids, tannins and terpenoids. The column chromatographic separation of chloroform/methanol (1:1) extract also resulted in the isolation of Stigmasterol (LM-3), Oleic acid (LM-2) and (5Z, 9Z)-22-methyl-5, 9-tetracosadienoic acid (LM-1). Antibacterial activity tests against *E. coli*, *S. aureus*, *P. aeruginosa* and *S. pyogenes* showed the methanol extract to be relatively more active than chloroform/methanol (1:1) extract. Consistent with literature reports, the isolated compounds (LM-3, LM-2, and LM-1) also showed interesting antibacterial activities against *S. pyogenes* with superior activity exhibited by compound LM-1. *S. aureus* and *S. pyogenes* were susceptible to both isolated compounds and extracts. Though the data were lower than that of the reference drug (Ampicillin), the observed activities could justify the traditional use of the plant to treat different bacterial infections in humans.

KEYWORDS

Ocimum lamiifolium, Antibacterial activity, Phytochemical constituents, Stigmasterol, Oleic acid, (5Z, 9Z)-22-methyl-5 and 9- tetracosadienoic acid.

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INTRODUCTION

Medicinal plants are being used in traditional and complementary medicine worldwide for the treatment, management, or prevention of various human and animal diseases. The absence of serious side effects, social acceptability and accessibility and high cost of modern drugs could be the possible

reasons for the wide utilization of medicinal plants (Marzouk, 2009[1], Das *et al*, 2011[2], Luitael *et al*, 2019[3], Ekor, 2014[4]). The plants are also used as sources of modern drugs. Pilocarpine, Ephedrine, Quinine, Calanolide, Salicylic acid, Benzoxazinone, Costunolide, Tetramethyl-pyrazine and Lovastatin are some the typical examples (Petrovska, 2012[5], Maury *et al*, 2009[6], Box *et al*, 2010[7], Springob and Kutchan, 2009[8], Wei *et al*, 2012[9], Yuan *et al*, 2016[10]).

Medicinal plants that belong to *Ocimum* species are also used for traditional medicine in different parts of the world (Kalita and Kahn, 2013[11], Avetisyan *et al*, 2017[12], Gowda and Prasad, 2019[13], Enegide, 2021[14], Chowdhury *et al*, 2017[15], Paton, 1992[16]). They are used, for instance, for the treatment of various ailments including rheumatism, paralysis, epilepsy, Febrile or high fever, diarrhea, sunstroke, influenza, gonorrhea, mental illness, abdominal pains, colds, coughs, measles, and they also possess antipyretic, antihelmintic, stomatic, antiemetic, and antimalarial effects (Juhar *et al*, 2024[17], Caceres *et al*, 1990[18], Zahran *et al*, 2020[19], Nyarko *et al*, 2002[20]). In general, the plants in this class are herbs, under shrubs, or shrubs containing essential oils of various aromas. The oils are valuable inputs/ingredients in the pharmaceutical, perfumery and food processing industries (Pandy *et al*, 2014[21]). Experimental findings also showed that the oils from the various *Ocimum* species possess biological activities such as antimicrobial, antibacterial and antifungal activities (Girma, 2020)[22] and cytotoxic activities (Fentahun *et al*, 2023)[23]. These properties could be attributed to secondary metabolites such as steroids, tannins, alkaloids, flavonoids and phenolics that are abundant in plants (Lekhak and Sharma, 2009[24], Dian *et al*, 2022[25]). *Ocimum* species are also used in Ethiopia and different parts of the world in traditional medicine (Girma, 2020[22], Kalita and Kahn, 2013[11], Enegide, 2021[14]).

O. lamiifolium is one of the 12 *Ocimum* species (*O. stirbeyi, O. forskolei, O. basilicum, O. americanum, O. ciricinatum, O. jamessi, O. spicatum, O. cufodontii, O. gratissimum, O. urticifolium, O. tricodon, and O. lamiifolium*) growing in Ethiopia (Girma, 2020[22]). It is used for traditional uses in many parts of the world including Ethiopia to treat several human illnesses (Kefe *et al*, 2016[26], Kewessa *et al*, 2015[27], Habtamu, 2024[28], Seid *et al*, 2021[29], Muhidin *et al*, 2021[30]). For instance, the leaf of *O. lamiifolium* is used for the treatment of diarrhea, stomach disorders, and abdominal pains (Pandy *et al*, 2014[21], Tausha *et al*, 2018[31], Abdela *et al*, 2022[32], Abenezer *et al*, 2024[33]), headache, toothache and febrile illness (Ezekwesili *et al*, 2005[34], Chekole, 2017[35], Abenezer *et al*, 2024[33]), cough (Mefine *et al*, 2009[36], Abenezer *et al*, 2024[33]), malaise (Giday *et al*, 2009[37], Abenezer *et al*, 2024[33]) and soreness (Abdela *et al*, 2022[32]). It is also used for the treatment of colds, measles, and eye infections (Maryo and Nemomissa, 2019[38]). Reports also revealed that the plant is traditionally used for the treatment of fungal infections and malaria (Abenezer *et al*, 2024[33], Girma, 2020[22], Tausha *et al*, 2018[31], Belay, 2017, Misganaw and Zemede, 2014[39], Vieira *et al*, 2003[40]).

Reports on essential oils and extracts from the leaves of *O. lamiifolium* and its different parts revealed promising activities such as antidiabetic activities, antimalarial activity (Misganaw and Zemede, 2014[39], Atetegeb *et al*, 2016[41]), antimicrobial activities (Nigus *et al*, 2018[42], Teklit and Said, 2015[43], Melese *et al*, 2019[44]) and anti-inflammatory activities (Woldesellassie *et al*, 2011)[45]. The reported biological/pharmacological activities could be associated with the presence of secondary metabolites such as tannins, sterols/steroids, carbohydrates, glycosides, flavonoids, saponins, terpenoids, and alkaloids (Juhar *et al*, 2024[17], Arika *et al*, 2016[46], Nigus *et al*, 2018[42], Runyoro *et al*, 2010[47], Kifle *et al*, 2002[48]).

The reports also showed the isolation of compounds from the leaves of this plant growing in Ethiopia. Some of the compounds isolated from this plant include ursolic acid (Girma, 2020)[22], apigenin, genkwanin, acacetin, apigenin 7, 4'-dimethyl ether, luteolin, ladanein, 5, 6-dihydroxy-7, 3', 4'-trimethoxyflavone, 5, 7-dihydroxy-6, 4'-dimethoxyflavone, cirsimaritin, salvigenin, cirsiliol,

cirsilineol, eupatorin, 5-hydroxy-6, 7, 3',4'-tetramethoxyflavone (Million and Yirefu, 2016)[49], quercetin 3-*O*-xylosyl (1'''→2'')-galactoside (Grayer, 2002)[50], rosmarinic acid, lithospermic acid, hydroxybenzoic acid, syringic acid, caffeic acid, ferulic acid, cinnamic acid and dihydroxy phenyllactic acid, vanillic acid, p-coumaric acid, hydroxybenzoic acid and sinapic acid reported from different parts of this plant (Teklit and Said, 2015[43], Hakkim *et al*, 2008[51], Etagegnehu, 2019[52], Nur Alisa, 2021[53]**)**. Our exhaustive literature review revealed no reports on phytochemical studies of the stem of *O. lamiiolium* growing in Southern Ethiopia. Therefore, the present study was initiated to carry out extraction and isolation of compounds from the extract of the stem of *O. lamiifolium* and also to perform antibacterial activity tests on the extracts and isolated compounds.

MATERIAL AND METHODS

Collection of plant material

The stem of *O. lamiifolium* (Figure No.1) was collected in December 2022 from Eastern Badawacho District Hadiya Zone, SNNPR, Ethiopia. The area is located in the great East African Rift Valley, 357 km South of Addis Ababa and 200km west of Hawassa. It lies between $7^0$45' N latitude and $38^0$28' E longitude. Authentication of the plant was made by botanist Mr. Reta Regassa, Department of Biology at Hawassa College of Teachers Education, Ethiopia. The collected plant specimen was dried and stored in the Organic Chemistry laboratory room at the Department of Chemistry, Hawassa University.

Preparation of plant material and extraction

The collected plant material was chopped into small pieces and air-dried for one month at room temperature. The dried plant material was ground using a Coffee-grinder Machine (XFYC810) to facilitate the extraction process. About 500g of the powdered stem of *O. lamiifolium* was soaked at room temperature in an Erlymenyer flask containing 4 L of chloroform-methanol (1:1 v/v) and placed on the orbital shaker (at rpm of 281 for 72 hr (Filippo *et al*, 2018)[54]. After 72 hr, the solution was filtered using Whatman filter paper. The filtered solution was concentrated under reduced pressure using a rotary evaporator at a temperature of 40^{o}C. To maximize the yield of extract, the residue was further subjected to extraction using a mixture of solvents chloroform and methanol (1:1 v/v) by shaking it for 72 hrs under the conditions mentioned above. The solution was then filtered and then the residue was soaked in 100% methanol following the same steps outlined below (Scheme No.1). All the solvents used in the experiments were analytical grades. They purchased from Ranchem PLC, Addis Ababa, Ethiopia.

Phytochemical screening tests

The solvents used in the experiment were of medium polarity (chloroform/methanol (1:1)) and high polarity (methanol) to obtain a wide range of phytochemicals and also to increase yields of crude extracts. Such solvent selection was based on the information obtained from literature reports (Nur Alisa, 2021[53], Garba *et al*, 2020[55]). The reports stated that using appropriate solvents for phytochemical extraction is very important. This would help to get phytochemicals with different chemical structures and polarities and with high yields.

Phytochemical screening tests were performed on both extracts using standard procedures reported in the literatures (Alqethami and Aldhebiani, 2021[56], Sawant and Godghate, 2013[57], Pant *et al*, 2017[58], Longbap *et al*, 2018[59], Mohammed *et al*, 2014[60], Khan *et al*, 2011[61]) to identify classes of secondary metabolites such as alkaloids, anthraquinones, flavonoids, phenols, saponins, tannins, terpenoids, steroids and glycosides using preliminary phytochemical analysis.

Test for alkaloids (Dragendroff's Test)

A 2ml solution of crude extract was treated with 1 ml of concentrated HCl. The mixture was then filtered and mixed with a small amount of amyl alcohol at room temperature. A few drops of Dragendroff's reagent (Solution of Potassium Bismuth Iodide) was added to the acid layer and a reddish brown precipitate was observed.

Test for anthraquinones (Ammonia Test)

About 0.3g of the crude extract was boiled with concentrated HCl for few minutes in a water bath and then filtered. The filtrate was allowed to cool and an equal volume of $CHCl_3$ was added to it. A few drops of ammonia were also added to the mixture and heated in a water bath. The formation of rose-pink color was inspected.

Test for flavonoids (Alkaline Reagent Test)

To 2ml solution of crude extract was treated with 2ml of 20% NaOH solution to form an intense yellow color indicating the presence of flavonoids.

Test for glycosides (Killer-Killiani Test)

2ml solution of crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2ml of concentrated H_2SO_4. A brown ring at the interphase indicated the presence of cardiac glycosides.

Test for phenols (Ferric chloride Test)

A 2ml solution of crude extract was treated with 5% ferric chloride solution; the formation of a blue-black color indicated the presence of phenolic compounds.

Test for saponins (Froth Test)

About 0.2g of the extract was shaken with 5ml of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) was shown and indicated the presence of saponins.

Test for steroids (Liebermann-Bur chard Test)

A 0.2g of the extract was mixed with 10ml chloroform. 2ml of this filtrate was taken and 2ml acetic anhydride and concentrated H_2SO_4 was added. A blue-green ring indicates steroids.

Test for tannins (Alcoholic ferric chloride Test)

About 0.3g of the extract was boiled in 5ml of water in a test tube and then filtered. A few drops of 0.1% $FeCl_3$ were added and a blue-black coloration was observed that indicates the presence of tannins.

Test for terpenoids (Salkowski Test)

5ml solution of crude extract was mixed with 2ml of chloroform and carefully added to conc. H_2SO_4 (3ml) to form a layer. A reddish-brown coloration at the interface shows positive results for the presence of terpenoids.

Chromatographic isolation and characterization of compounds

In the experiment, the column chromatographic separation of methanol extract was not successful as the elution resulted in fractions containing mixtures of compounds with closer R_f values as demonstrated by their TLC profiles. Moreover, some of the compounds were obtained in trace amounts. So, focus was given to chloroform/methanol (1:1 v/v) crude extract. About 10g of dried crude extract was adsorbed onto 18g of silica gel. The adsorbed crude extract was then eluted with ethyl acetate in n-hexane with a gradual increase in polarity and using column chromatography (150g silica gel 60-120 mesh size). This successive elution was followed by a collection of fractions (each 50ml). The numbers of spots on TLC plates were visualized using a UV chamber (MO3-4015) at 254 and 365nm. A total of 134 fractions were collected. Depending on their similarity in TLC profiles these fractions were combined regrouped to 16 combined fractions. The combined fractions 8, 11, and 18 were concentrated under reduced pressure using a rotary evaporator (Heidolph, Grant UK, GLS 400)) to obtain a white needle-like compound (labeled as LM-3), greenish-black gummy paste (compound LM-2) and garden green gummy paste (compound LM-1), respectively. The melting points of the isolated compounds were measured using a melting point apparatus (MEL-TEMP, model 120d, Barnstead International, USA). The ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectral analyses were done using Bruker 400 MHz spectrometer, and FTIR spectra were obtained from Perkin Elmer BX infrared spectrometer (400-4000cm^{-1}). All the spectral analyses were carried out at The Department of Chemistry, Addis Ababa University, Addis Ababa, Ethiopia.

Antibacterial activity test result

The strains used in the experiment were two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*. They were obtained from the Department of Biology, Adama Science and Technology

University, Ethiopia. I*n-vitro* antibacterial activities of the stem extracts of *O.lamiifolium* and isolated compounds were evaluated using the disk diffusion method against four bacterial strains by employing standard procedures (Mounyr *et al*, 2016)[62].

RESULTS AND DISCUSSION

In this section of the report, the yields of extracts, phytochemical screening tests, elucidation of isolated compounds, and antibacterial activity tests are discussed briefly.

Yields of extracts

Effectiveness of a given extraction is usually evaluated by the yields of extractives and the presence of biologically active components (Alipieva *et al*, 2010)[63]. Thus, extraction yields (masses of crude extracts) are commonly used as indicators of the effects of the extraction conditions such as solvent polarities (Nur Alisa, 2021[53], Garba *et al*, 2020[55]). In this study, the chloroform/methanol (1:1) and methanol extractions afforded crude extracts of 14.5g (2.9%) and 3.5g (0.72%) yields, respectively (Table No.1). The data showed that out of the two solvent systems, the chloroform/methanol (1:1) extracts resulted in, a relatively, higher yield than methanol extracts. The differences in masses of the crude extracts suggested that the stem has more medium polar secondary metabolites (Srinivasan *et al*, 2001)[64].

$$\text{Percentage yield} = \frac{Mass\ of\ crude\ extract}{Mass\ of\ plant\ material\ used\ for\ extraction} \times 100$$

Phytochemical screening tests

Phytochemical screening not only helps to reveal the constituents of the plant extracts and the ones that predominate over the others but also helps search for bioactive agents that can be used in the synthesis of useful drugs (Okoli *et al*, 2009)[65]. The qualitative phytochemical screening of the extracts was performed to identify the main groups of chemical constituents (glycosides, alkaloids, tannins, saponins, terpenoids, anthraquinones, flavonoids, and phenols) (Table No.2) present in the extracts using the color reactions (Savithramma *et al*, 2011)[66]. The results of the phytochemical screening of the extracts in both chloroform-methanol and methanol extracts are indicated below (Table No.2, Appendix 1). The results were found to be consistent with literature reports that revealed the presence of secondary metabolites (alkaloids, terpenoids, flavonoids, tannins, saponins, flavonoids and phenols) in the leaves of *O.lamiifolium* (Arika *et al*, 2016[46], Nigus *et al*, 2018[42], Sahalie *et al*, 2018[67], Habtamu, 2024[28], Seid *et al*, 2021[29], Muhidin *et al*, 2021[30]). The biological activities of the plant mentioned above could be attributed to the occurrence of these secondary metabolites.

Isolation of compounds

The column chromatographic separation resulted in the isolation of several compounds. However, most of them were obtained in very small (trace) amounts and were not sufficient for characterization and spectroscopic analyses. Three compounds (LM-3, LM-2 and LM-1) were isolated from Chloroform/methanol (1:1 v/v) extract in sufficient amounts. The compound LM-3 (32.7mg) and LM-2 (37.3mg) were isolated from n-hexane/ethyl acetate (85:15%) elution whereas compound LM-1 (38.2mg) from the n-hexane/ethyl acetate (80:20) elution.

Structure elucidation of the isolated compounds

Compound LM-3 was obtained as white needles with an R_f value of 0.62 (15% ethyl acetate in n-hexane) and a melting point of 173-176^{o}C. Analysis of the FTIR spectrum of compound LM-3 (Appendix 2) revealed a broad band at 3436 cm^{-1} indicating the presence of a hydroxyl functional group. The strong band at 2933 cm^{-1} represents the C-H stretch of alkenes whereas the weak band around 2864cm^{-1} could be attributed to the C-H stretching of methyl groups. The observed data suggested that compound LM-3 could be an alcohol with at least one C=C double bond (Filippo *et al*, 2018[54]).

The ^{1}H-NMR (400MHz, $CDCl_3$) spectrum of compound LM-3 (Appendix 3) showed 3 olefinic protons at δ 5.05, δ 5.15 and δ 5.36 indicating the presence of three olefinic protons and the peak at δ3.53 indicate a methin proton on hydroxyl bearing carbon. Moreover, the peaks at δ1.01, 0.71, 0.94,

0.83, 0.81 and 1.08 suggested the presence of six methyl protons. The observed peaks and their patterns signals and comparison of the data with literature reports suggest that compound LM-3 could be most likely a Stigmasterol (Htay *et al*, 2019[68], Chev *et al*, 2018[69], Erwin *et al*, 2020[70]). The ^{13}CNMR data of LM-3 was also found to be consistent with that of Stigmasterol. The ^{13}CNMR ($CDCl_3$, 101 MHz) (Appendix 4) and DEPT-135 spectrum (Appendix 5) of compound LM-3 showed 29 and 26 signals, respectively, that can be assigned to three quaternary carbons, eleven methane, nine methylene, and six methyl carbons. The signals at 140.8, 121.7, 138.3 and 129.3ppm are assigned to C-5, C-6, C-22 and C-23 double bonds, respectively, of stigmasterol-type compound (Table No.4). Angular carbon atom signals (C-19, C-18) were also recognized at 19.8 and 12.1. A signal at 71.7 indicates a methane carbon bearing a hydroxyl group. Thus, the observed FTIR and NMR data of compound LM-3 was found to be consistent with the reported data of Stigmasterol (Figure No.2) (Htay *et al*, 2019[68], Chev *et al*, 2018[69], Erwin *et al*, 2020[70]). The ^{13}C-NMR, DEPT-135 and ^{1}H-NMR data of compound LM-3 and reported data of Stigmasterol (Appendix 6). This compound was isolated for the first time from this plant species. Reports revealed that Stigmasterol is one of the most common plant sterols, found in a variety of natural sources, including vegetable fats or oils from many plants. The findings from previous studies showed various pharmacological activities such as anticancer, anti-osteoarthritis, anti-inflammatory, anti-diabetic, immunomodulatory, antiparasitic, antifungal, antibacterial, antioxidant, and neuroprotective properties (Bakrim *et al*, 2022[71], Tolstoy *et al*, 2003[72]). Therefore, the presence of this compound supports the traditional medicinal use of *O. lamiifolium* for the treatment of bacterial infections, inflammatory diseases, and wound healing.

The compound LM-2 was obtained as a greenish-black gummy paste (37.3mg). Its R_f was determined to be 0.54 in n-hexane: ethyl acetate (85:15%). Analysis of FTIR spectrum of compound LM-2 (Appendix 7) revealed a broad band in the 3500-2500 cm^{-1} region. This could be attributed to the O-H stretching of the carboxylic acid group (-COOH). The bands that appeared at 2856cm^{-1} and 2926cm^{-1} represent symmetric and asymmetric $–CH_2$ stretching vibrations, respectively. The sharp band that appeared at 1704cm^{-1} could be attributed to C=O stretching whereas the band appeared at 1292cm^{-1} to the C-O stretching of the carboxylic group. The spectrum of ^{1}H-NMR of compound LM-2 (Appendix 8) displayed the peaks at δ1.3 and δ1.63 indicating the presence of aliphatic protons (-CH_2) group whereas the peak at δ2.04 could be assigned to the vinylic protons or protons of methylene group that is bonded to C=C bond. The multiplet peaks at δ5.36 could suggest the presence of at least two olefinic protons (H-9 and H-10) in the structure. The signals at δ2.35 and 1.63 indicated CH_2 protons on alpha- and beta-carbons of the carboxyl group, respectively, and the terminal CH_3 group gives a peak at δ0.88. The ^{13}C-NMR ($CDCl_3$, 101 MHz) spectrum of compound LM-2 (Appendix 9) revealed a single peak at δ180.0 that could be attributed to a quaternary carbon atom of carbonyl carbon of carboxylic acid. The peak at δ 34.1 indicates the vinyl carbon atoms. The peaks observed at δ130.0 and δ129.7 (C-9 and C-10) could be attributed to olefinic carbons or indicated the presence of at least one C=C bond in the compound. The other methylene of hydrocarbon chain resonated in the range of δ22.7-31.9 while the terminal methyl group showed the signal at δ14.1 (C-18). Moreover, the absence of a peak at δ180.0 in the DEPT-135 spectrum (Appendix 10) indicated the presence of a quaternary carbon atom in the chain of fatty acid group. All the spectral data (Appendix 8 and 9) obtained from the spectral analyses and comparison with data reported in the literature suggested that compound LM-2 is an oleic acid (Figure 3; Appendix 11) (Ruksilp, 2020[73], Be *et al*, 2009[74], Enrica *et al*, 2020[75]).

Oleic acid (LM-2) is a naturally occurring fatty acid with antibacterial, anti-inflammatory, anticancer, wound healing, and anti-fungal properties and used as an additive to a variety of drug products (Carrillo

and Cavia, 2012[76], Sales-campos *et al*, 2012[77]). This compound was isolated for the first time from this plant. Traditionally, *O. lamiifolium* is used to treat cough, malaria, headache, febrile illness, cold, eye infections, and nose bleeding (Nair *et al*, 2016[78]). Therefore, the occurrence of this compound supports the traditional medicinal use of *O. lamiifolium* as a wound healing, antibacterial, anti-inflammatory, and anti-fungal agent (Nair *et al*, 2016)[78].

Compound LM-1 was isolated as a garden greenish gummy paste (38.2mg) with an R_f value of 0.31 in n-hexane: ethyl acetate (80:20%). In the FTIR spectrum (Appendix 12), a broad band at 3429 cm^{-1} and an absorption band at 1711cm^{-1} indicated the presence of O-H stretching of the carboxylic acid group (-COOH) and stretching of the unsaturated carbonyl group (-C=O), respectively. Two sharp bands appeared at 2850cm^{-1} and 2926cm^{-1} representing aliphatic C-H stretching vibrations (Choudhury *et al*, 1995)[79].

In the ^{1}HNMR spectrum (Appendix 13), the peaks at δ 0.90 and 0.88 could be attributed to methyl groups. The peaks in the range of δ1.10-2.04 could be attributed to long-chain hydrocarbon methylene groups. The strong peaks at δ2.08 and a peak at δ 2.04 are due to methylene protons of carbon at the allylic position whereas the signals in the range of δ 5.36-5.38 could correspond to vinyl protons (Pant *et al*, 2017[58], Longbap *et al*, 2018[59]). The ^{13}CNMR ($CDCl_3$, 101MHz) spectrum of compound LM-1 (Appendix 14) showed a total of 25 signals for carbon atoms. The downfield single peak at δ179.9 could be attributed to a quaternary carbon atom of carbonyl carbon (C=O) of carboxylic acid. The signal at δ14.1 and 19.7 attributed to methyl groups whereas the peak at δ32.8 could be assigned to the methylene carbon atom at α-position to carbonyl group. The bunch of signals in the range of δ24.7-30.1 attributed to methylene carbons in long chain hydrocarbon of fatty acids. The four signals at δ127.9, 130.2, 128.1 and 130.0 in the DEPT-135 spectrum (Appendix 15) indicate an unsaturated (olefinic) carbon region. Moreover, the absence of a peak at 179.9 in the DEPT-135 spectrum indicated the presence of a quaternary carbon atom in the chain of fatty acid group. Based on these spectral data and comparing with literature reports, compound LM-1 was found to be (5Z, 9Z)-22-methyl-5,9-tetracosadienoic acid that has been previously isolated from Sponge *Geodinella Robusta* (Makarieva *et al*, 2002[80]) (Figure No.4, Appendix 16). Unsaturated fatty acids (UFAs) have been reported to improve blood cholesterol levels, ease inflammation, stabilize heart rhythms, and play several other beneficial roles in human health (Mozaffarian *et al*, 2010[81], Makarieva *et al*, 2010[80]). Thus, the findings of our study supported the traditional medicinal use of *O. lamiifolium* for the treatment of different illnesses such as inflammation and heart diseases.

Antibacterial activities of isolated compounds

The antibacterial activities of the crude extracts of *O.lamiifolium* and isolated compounds were evaluated using the disk diffusion method against four bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes*). The diameters corresponding to the zone of inhibition were measured (in mm) for the extract and compounds. The finding showed that the zone inhibition to increase increased when the concentrations when the concentration increased from 200µg/ml to 300µg/ml (Table No.7, Figure No.13 and Figure No.14). In this study, the zones of inhibition of compounds LM-3 (Stigmasterol), LM-2 (Oleic acid) and LM-1 (5Z, 9Z)-22-methyl-5, 9-tetracosadienoic acid) at the concentration of 300µg/ml were found to be 9.5mm, 10.5mm and 11.5mm each for *E. coli*, 9.5mm, 10mm, and 12mm for *S. aureus*, 9mm, 9.5mm, and 11mm for *P. aeruginosa*, 10mm, 11mm and 13.5mm for *S. pyogen*, respectively. The chloroform-methanol (1:1 v/v) extract showed higher antibacterial activity against *S.aureus* (8mm) and *S. pyogenes* (8.5 mm) than *E. coli* (7.5mm) and *P. aeruginosa* (7mm) at the concentration of 300µg/ml (Appendix 17). In this study, methanol extract was found to show the highest inhibition zone against all selected bacterial strains compared to chloroform-methanol (1:1 v/v)

extract. This observation is consistent with literature reports (Teklit and Said, 2015[43], Destaw and Yalemtseha, 2015[82]). *E. coli*, *S. aureus* and *S. pyogenes* were found to be the most susceptible species to both the isolated compounds and extracts. Their inhibition zones were also comparable with that of the standard antibiotics (Ampicillin) with inhibition diameters of 14mm and 15mm, respectively (Table No.3, Figures No.3 and Figure No.4).

The isolated compounds also showed interesting antibacterial activity against *S. pyogenes* with superior activity exhibited by compound LM-1 with a zone of inhibition 13.5mm. Comparing the results with literature reported data, it showed that *E. coli*, *S. aureus* and *S. pyogenes* were susceptible to the plant extracts and compounds. This observation indicates the potential of the extracts and the isolated compounds as antibacterial agents.

The results of the current study, the chloroform/methanol (1:1 v/v) and methanol crude extracts of *O. lamiifolium* stem showed antibacterial activity against *E. coli*, *S. aureus*, *S. pyogenes, and P. aeruginosa*. In contrast, the methanol extract of *O. lamiifolium* leaf didn't show any zone of inhibition against *P. aeruginosa* (Melese *et al*, 2019)[44], but in this study, *P. aeruginosa* showed zone of inhibition of 7mm and 8mm for chloroform/methanol (1:1 v/v) and methanol crude extracts, respectively. The difference in the zone of inhibition among the four tested bacteria depends on the extraction solvent and the bacteria inheritance behavior (Dodiya *et al*, 2015)[83]. Several literature reports revealed the promising antibacterial activities of stigmasterol (LM-3) against a broad spectrum of pathogenic strains belonging to Gram-positive and Gram-negative bacteria (Ibrahim and Yaacob, 2017[84], Odiba *et al*, 2016[85], Ayele *et al*, 2022[86], Yinusa *et al*, 2014[87]). Reports showed that this compound exerts its antibacterial activity by inhibiting protein synthesis and preventing bacterial growth (Saad, 2020)[88].

It has been reported that long-chain unsaturated fatty acids show antibacterial activities with a possible mechanism of action of inhibition of bacterial fatty acid synthesis that results in damaging bacterial cells (Sandro, 2005)[89]. Moreover, their antibacterial activities increase with the increase in the degree of unsaturation (Dilika *et al*, 2000[90], Ghavam *et al*, 2022[91], Singh, 2007[92]). In line with reports, unsaturated fatty acids isolated in this study (LM-2 and LM-1) showed high antibacterial activities with LM-1 (with two double bonds) to be more active than LM-2 (with one double bond) (Table No.3). The antibacterial activity of LM-1 could be attributed to its ability to alter the integrity of the bacterial cell membrane and loss of ribonucleic acid (Andrew and Valerie, 2010[93], Undurti, 2018[94], Mukerjee *et al*, 2021[95]). These interesting antibacterial activities of the extracts and isolated compounds could justify the traditional medicinal use *of the plant (*O. lamiifolium*).*

Table No.1: The Percentage yields of crude extracts

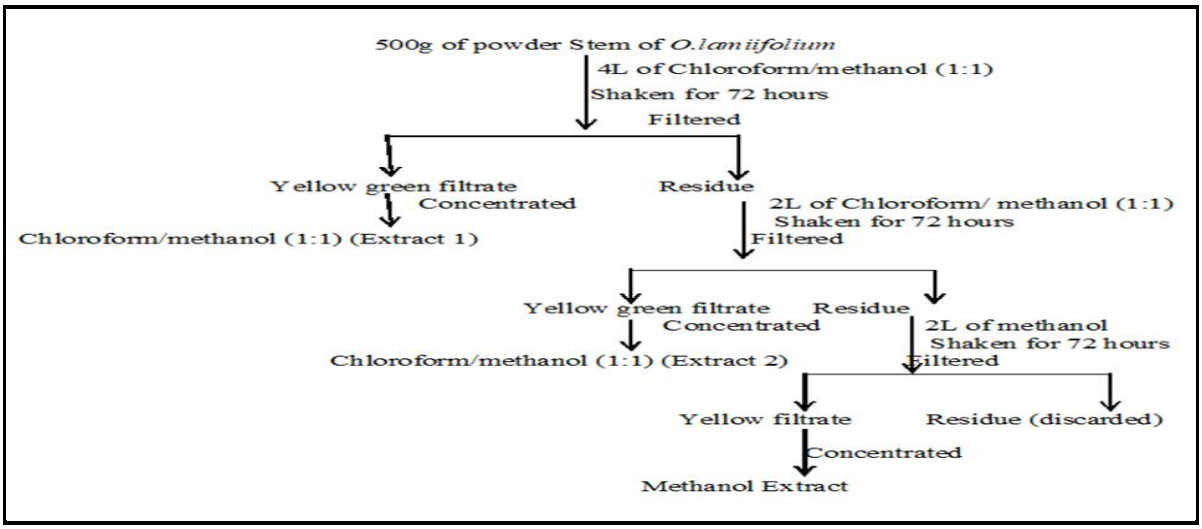
S.No	Solvents used for extraction	Weight of sample (g)	Weight of crude extract (g)	Yield (%)
1	Chloroform/methanol (1:1 v/v)	500	14.5	2.9
2	Methanol	485.5	3.5	0.72

Table No.2: The Phytochemical constituents of *O.lamiifolium* stem extracts

S.No	Phytochemical constituents	Types of tests	Extracts	
			Chloroform/methanol (1:1v/v)	Methanol
1	Alkaloids	Dragendroff's Test	+	-
2	Anthraquinones	Ammonia Test	-	+
3	Flavonoids	Alkaline Reagent Test	+	-
4	Glycosides	Killer-Killiami Test	+	-
5	Phenols	Ferric chloride Test	+	-
6	Saponins	Froth Test	+	+
7	Steroids	Liebermann-Bur chard Test	+	-
8	Tannins	Alcoholic ferric chloride Test	+	-
9	Terpenoids	Salkowski Test	+	-

Table No.3: Antibacterial activities of the plant extracts and isolated compounds

S.No	Samples with Conc.		Bacteria species and zone of inhibitions (in mm)			
			E. coli	***S. aureus***	***P. aeruginosa***	***S. pyogenes***
1	Chloroform/methanol (1:1)	A1=200µg/ml	7	7	6.5	8
		A2= 300µg/ml	7.5	8	7	8.5
2	Methanol extract	B1= 200µg/ml	7.5	8	7	9
		B2= 300µg/ml	8	8.5	8	9
3	Compound LM-3	C1= 200µg/ml	8.5	8.5	8	10
		C2= 300µg/ml	9.5	9.5	9	10
4	Compound LM-2	D1= 200µg/ml	9	9	9	11
		D2= 300µg/ml	10.5	10	9.5	11
5	Compound LM-1	E1= 200µg/ml	10	11	10	12
		E2=300µg/ml	11.5	12	11	13.5
6	Ampicillin		13.5	14	13	15



Scheme No.1: Outline of the procedure employed during the stem extraction of *O. lamiifolium*

Figure No.1: *O. lamiifolium* (a). The aerial part (b) The stem part (Photo by Temesgen B. from Misrak Badewacho Woreda, Hadiya Zone, December 2022)

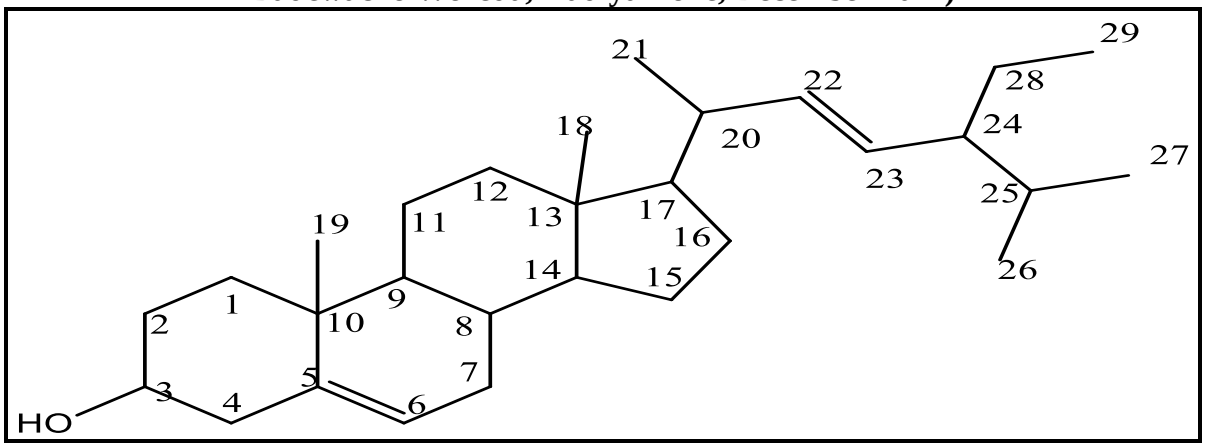


Figure No.2: The proposed chemical structure of compound LM-3 (Stigmasterol)

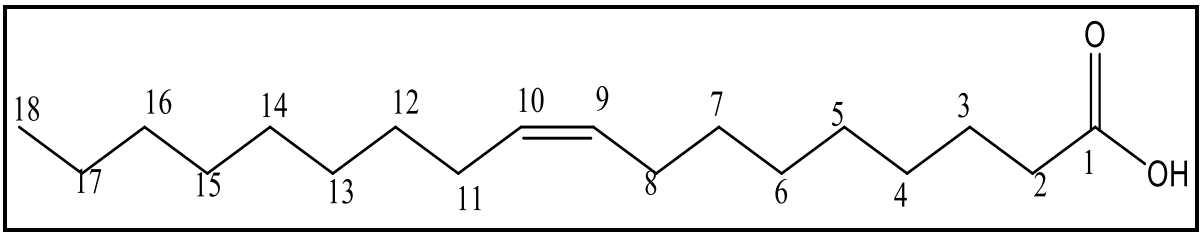


Figure No.3: The proposed chemical structure of compound LM-2 (Oleic acid)

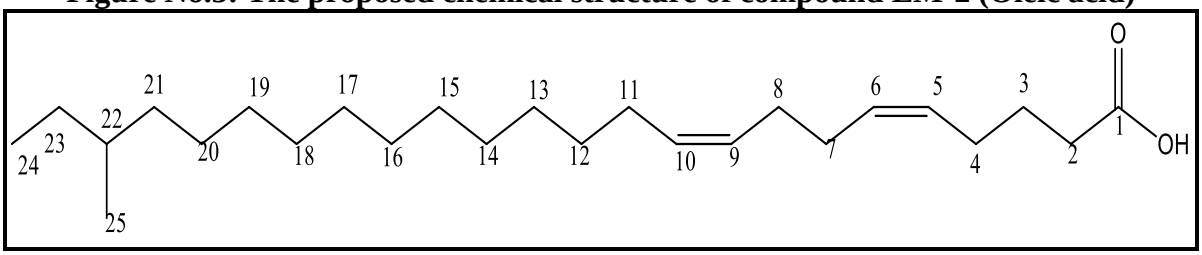


Figure No.4: The proposed chemical structure of compound LM-1((5Z, 9Z)-22-methyl-5, 9-tetracosadienoic acid)

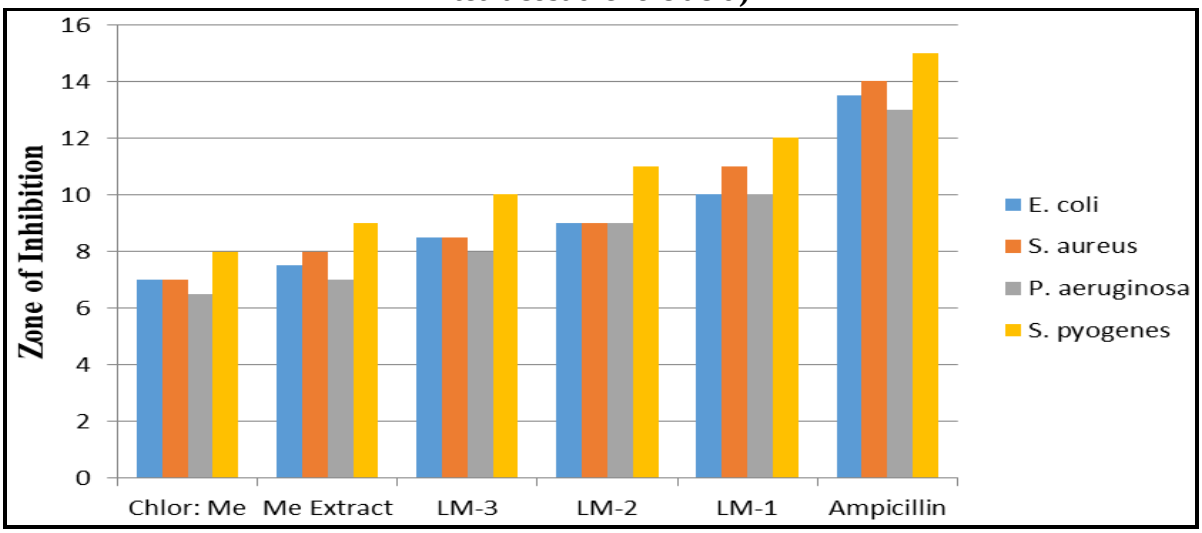


Figure No.5: Antibacterial activities of the extracts and isolated compounds at 200µg/ml

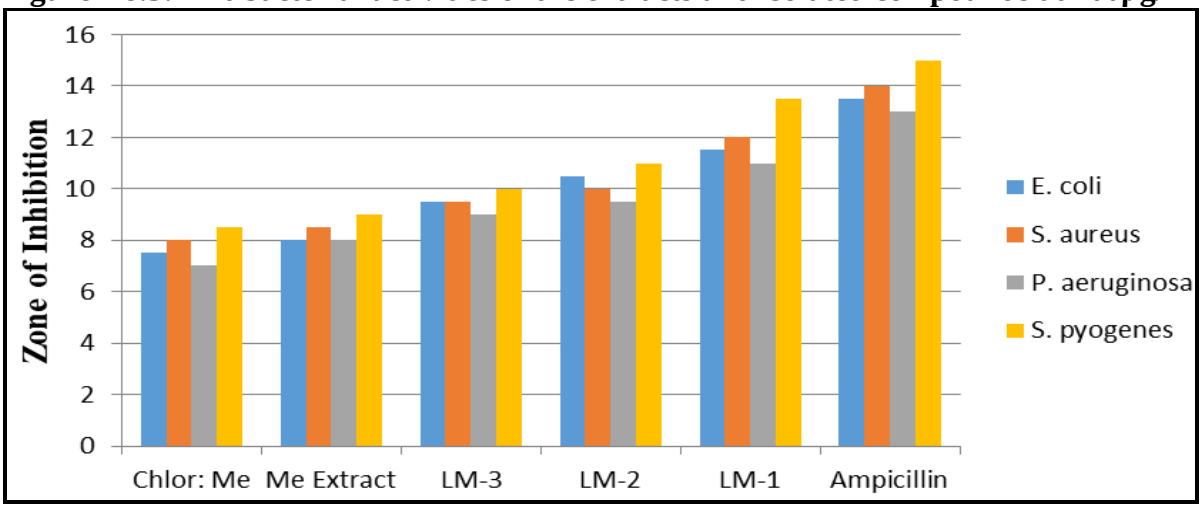
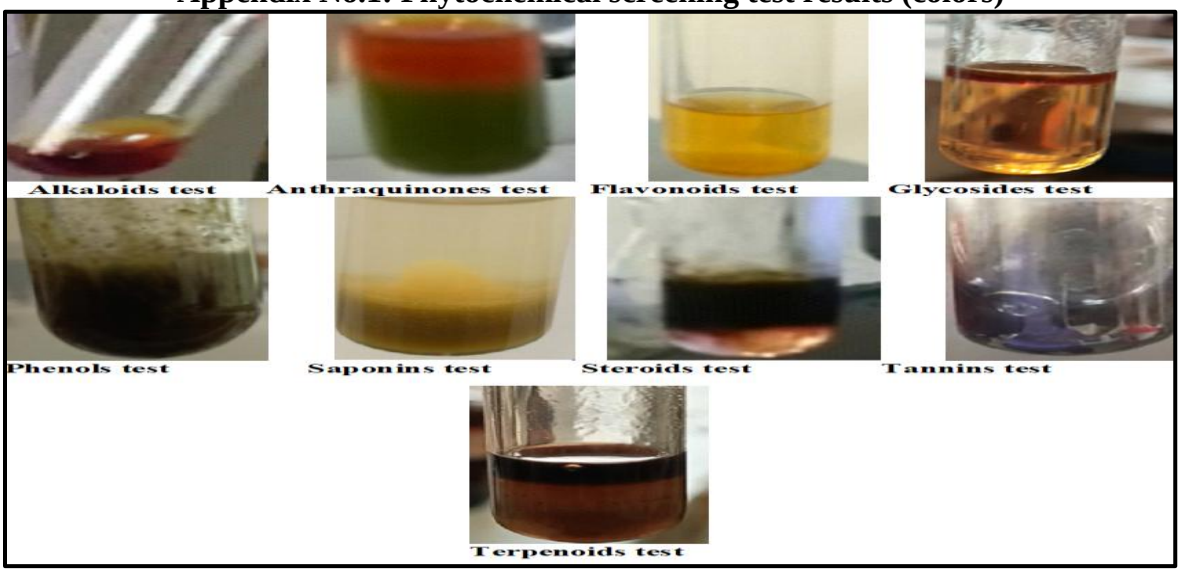
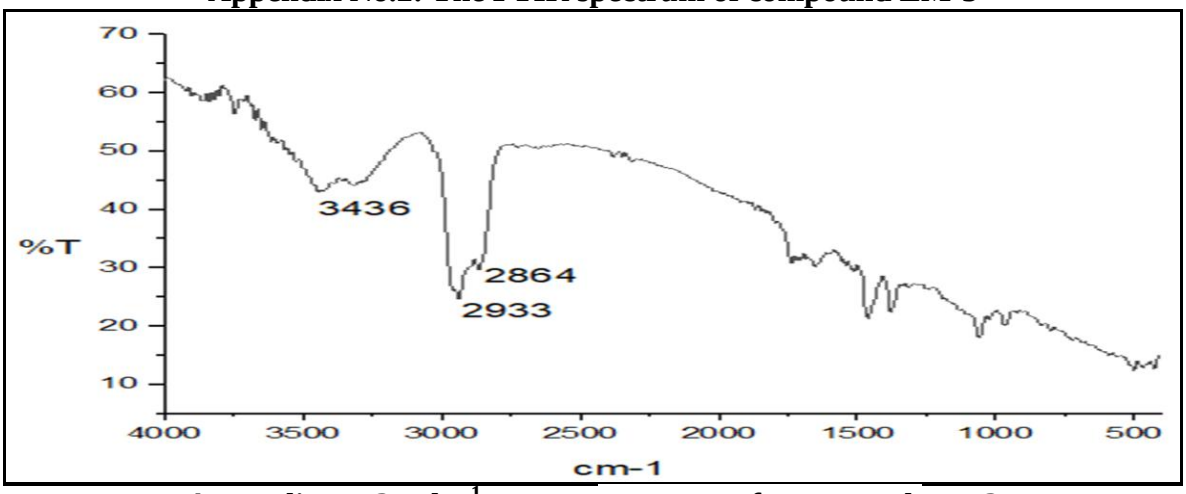


Figure No.6: Antibacterial activities of the extracts and isolated compounds at 300µg/ml

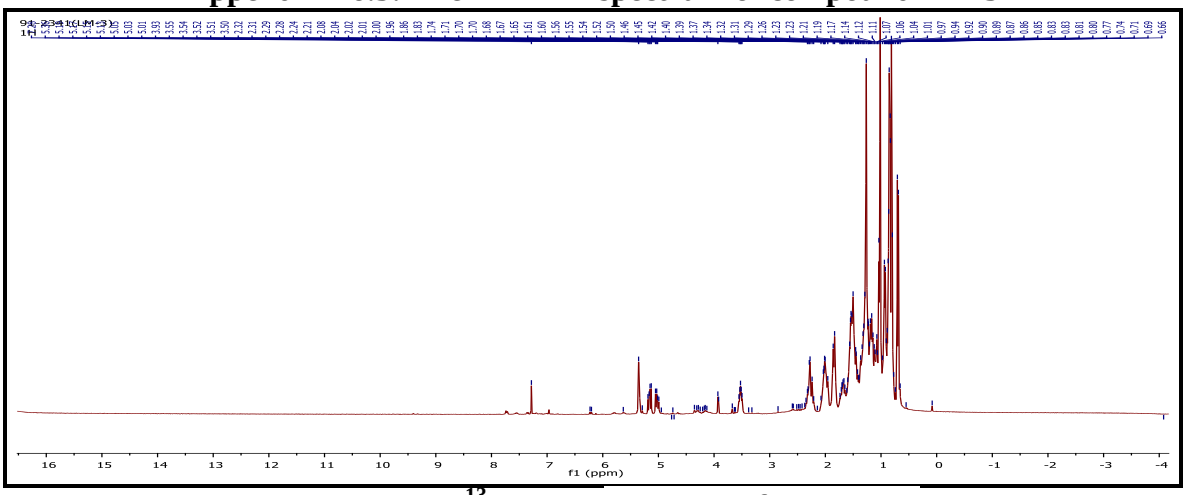
Appendix No.1: Phytochemical screening test results (colors)



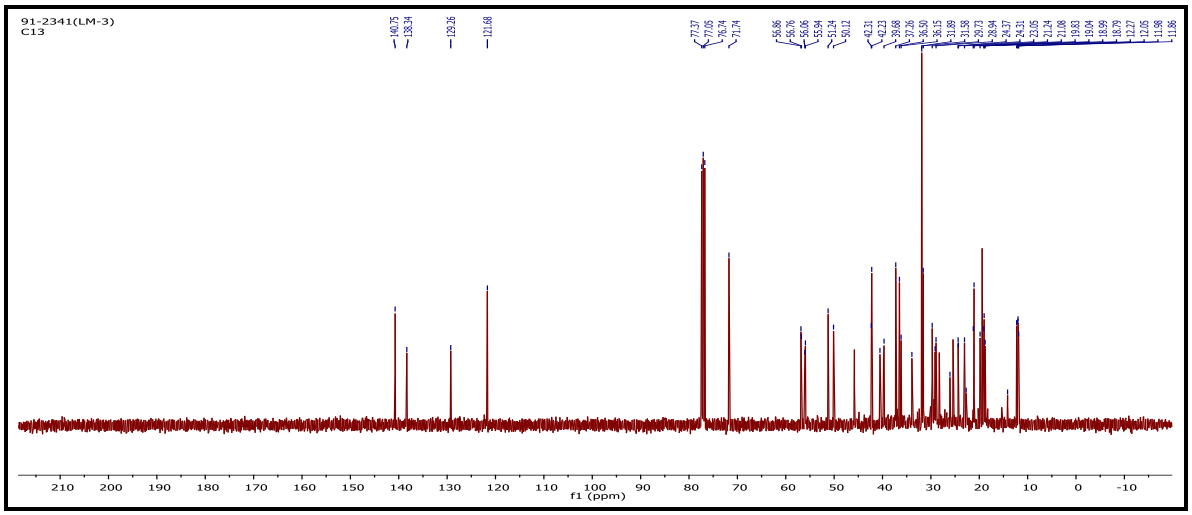
Appendix No.2: The FTIR spectrum of compound LM-3



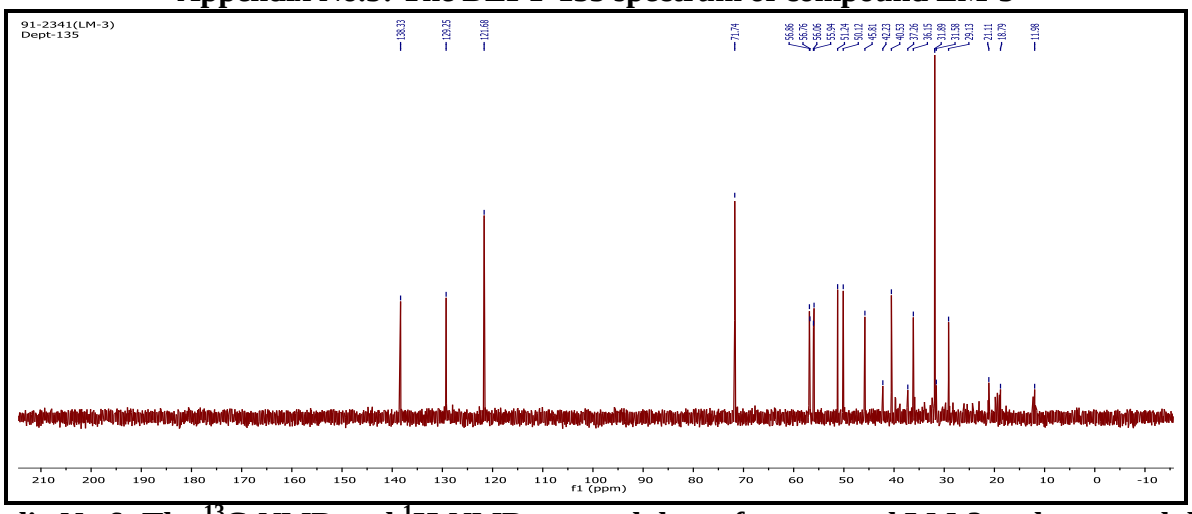
Appendix No.3: The ^{1}H NMR spectrum of compound LM-3



Appendix No.4: The ^{13}C-NMR spectrum of compound LM-3



Appendix No.5: The DEPT-135 spectrum of compound LM-3

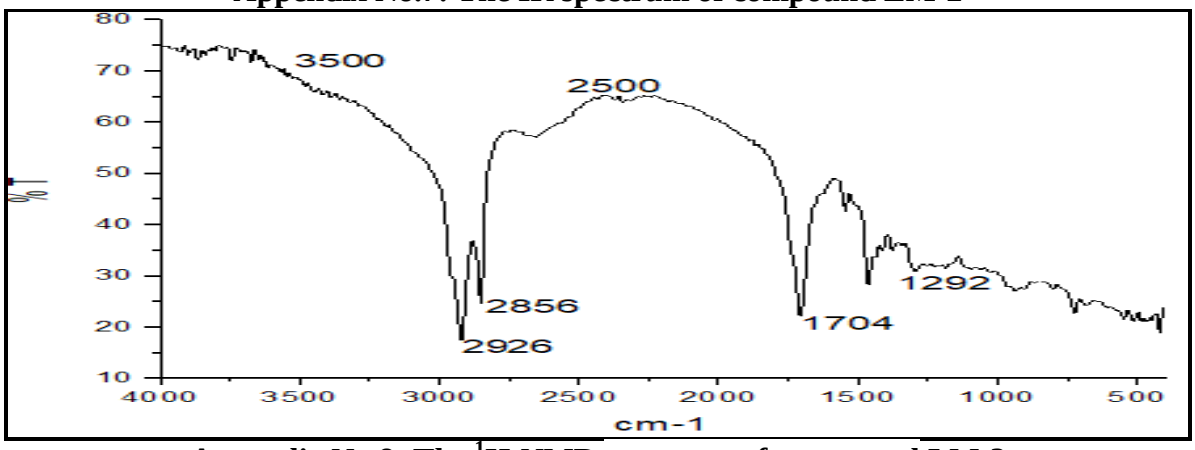


Appendix No.6: The ^{13}C-NMR and ^{1}H-NMR spectral data of compound LM-3 and reported data of NMR data of Stigmasterol (Htay *et al*, 2019, Chev *et al*, 2018, Erwin *et al*, 2020)[67-69]

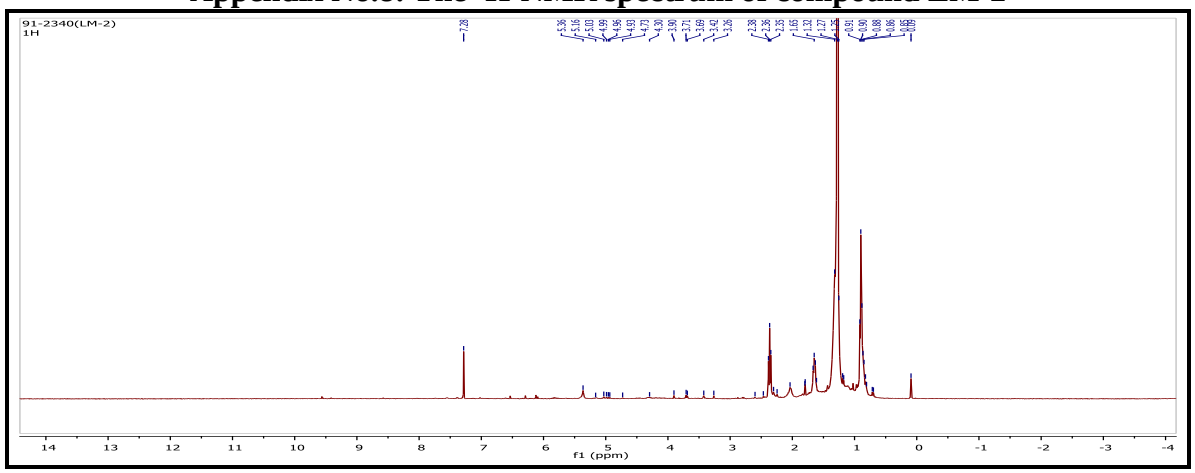
Carbon No.	^{13}C-NMR data of compound LM-3	Reported ^{13}C NMR data of Stigmasterol	^{1}H-NMR data of compound LM-3	Reported ^{1}H NMR data of Stigmasterol	Nature of Carbon
1	37.3	37.3			CH_2
2	31.6	31.6			CH_2
3	71.7	71.7	3.53(m)	3.53(m)	CH
4	42.2	42.2			CH_2
5	140.8	140.8			C=C
6	121.7	121.6	5.36(s)	5.38(s)	C=CH
7	31.9	31.9			CH_2
8	29.7	29.2			CH
9	50.1	50.1			CH
10	36.5	36.5			C
11	21.1	21.1			CH_2

12	39.7	39.6			CH_2
13	42.3	42.1			C
14	56.9	56.9			CH
15	24.4	24.4			CH_2
16	28.9	28.9			CH_2
17	56.8	56.2			CH
18	12.1	12.1	1.01(d)	1.27(d)	CH_3
19	19.8	19.9	0.71(d)	0.74(d)	CH_3
20	40.5	40.5			CH
21	21.2	21.2	0.94(d)	0.91(d)	CH_3
22	138.3	138.3	5.05(m)	5.07(m)	C=C
23	129.3	129.3	5.15(m)	5.14(m)	C=C
24	51.2	51.2			CH
25	36.2	36.2			CH
26	19.0	19.0	0.83(d)	0.84(d)	CH_3
27	18.8	18.8	0.81(d)	0.82(d)	CH_3
28	25.4	25.4			CH_2
29	12.0	12.1	1.08(t)	1.04(t)	CH_3

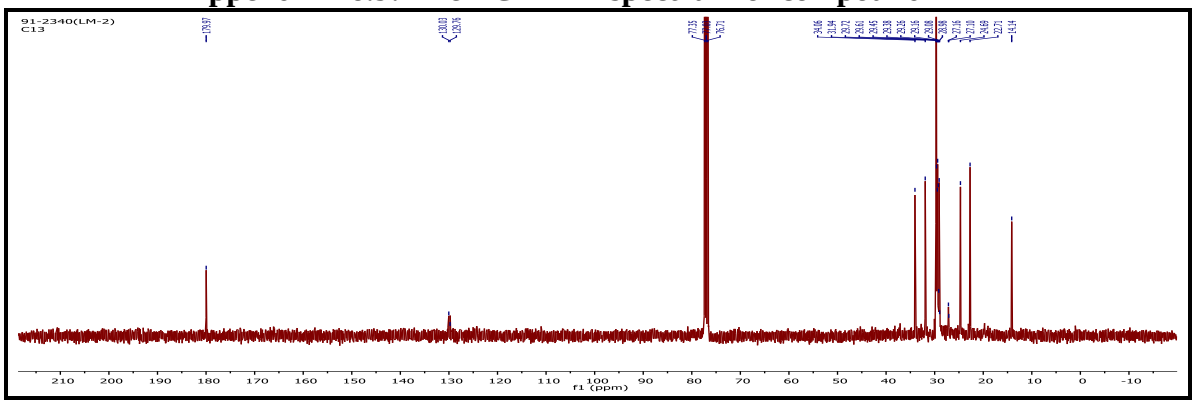
Appendix No.7: The IR spectrum of compound LM-2



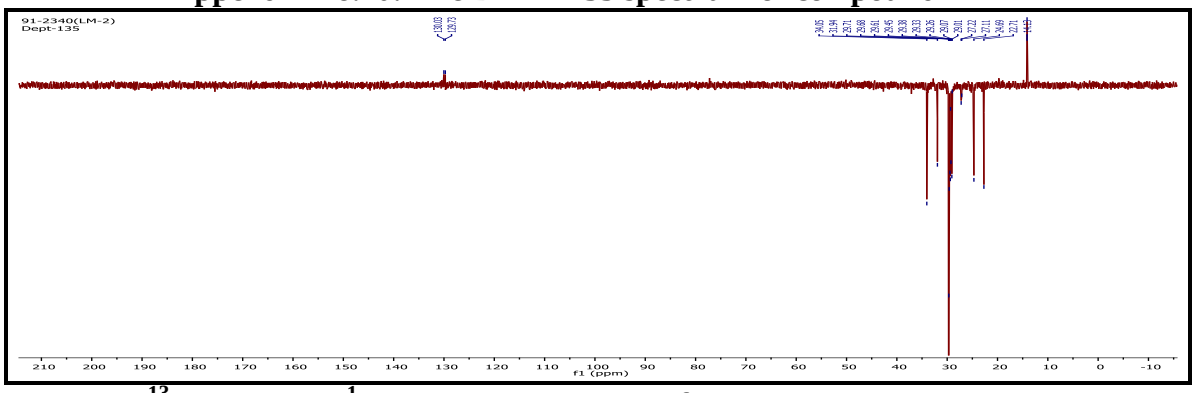
Appendix No.8: The ^{1}H-NMR spectrum of compound LM-2



Appendix No.9: The ^{13}C-NMR spectrum of compound LM-2



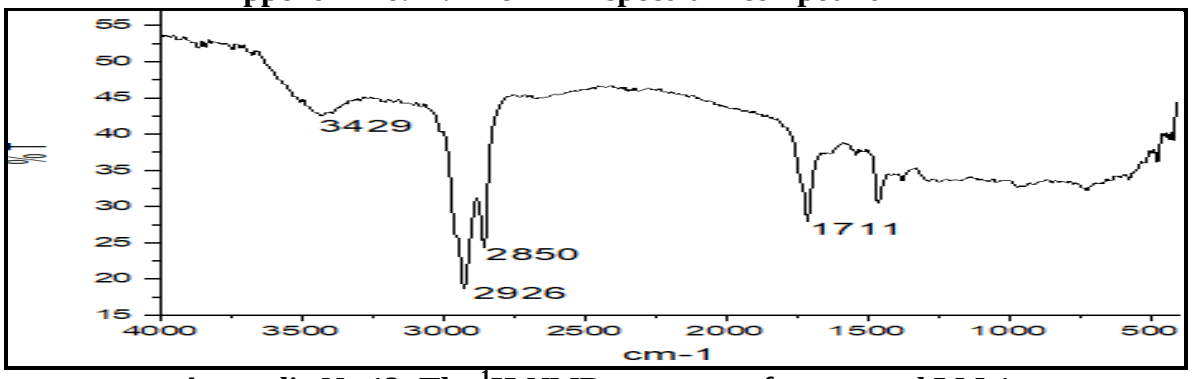
Appendix No.10: The DEPT-135 spectrum of compound LM-2



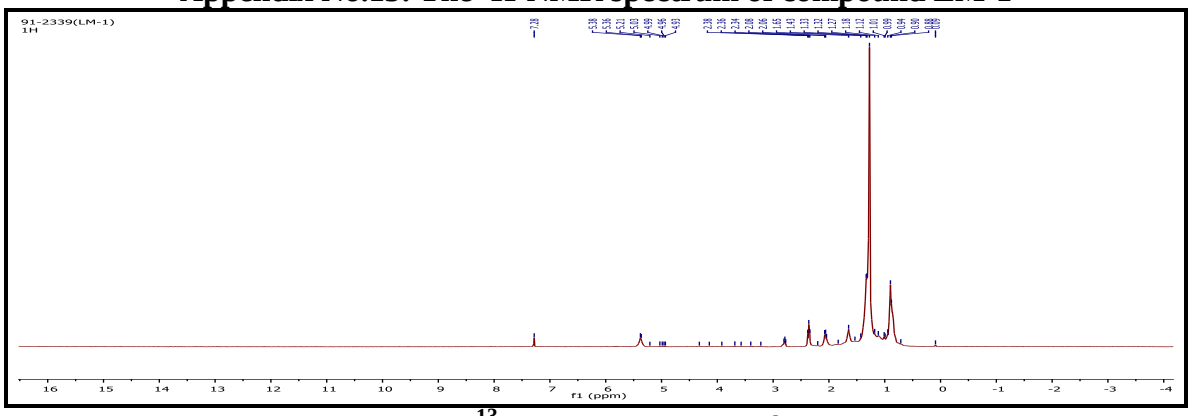
Appendix 11: The ^{13}C-NMR and ^{1}H-NMR spectral data of compound LM-2 and reported NMR data of Oleic acid (Makarieva *et al*, 2002)[79]

Carbon No.	^{13}C NMR data of compound LM-2	Reported ^{13}C NMR data of Oleic acid	^{1}H NMR data of compound LM-2	Reported ^{1}H NMR data of Oleic acid	Nature of Carbon
1	180.0	180.0			C
2	34.1	34.0	2.35(t)	2.35(t)	CH_2
3	24.7	24.7	1.63(m)	1.63(m)	CH_2
4	29.7	29.8			CH_2
5	29.6	29.6			CH_2
6	29.5	29.5			CH_2
7	29.4	29.4			CH_2
8	27.2	27.2	2.04(m)	2.01(m)	CH_2
9	130.0	130.0	5.36(m)	5.34(m)	CH
10	129.7	129.7	5.36(m)	5.34(m)	CH
11	27.1	27.1	2.04(m)	2.01(m)	CH_2
12	29.2	29.2			CH_2
13	29.1	29.1			CH_2
14	29.0	29.0			CH_2
15	29.3	29.3			CH_2
16	31.9	31.9			CH_2
17	22.7	22.7			CH_2
18	14.1	14.1	0.88(t)	0.88(t)	CH_3

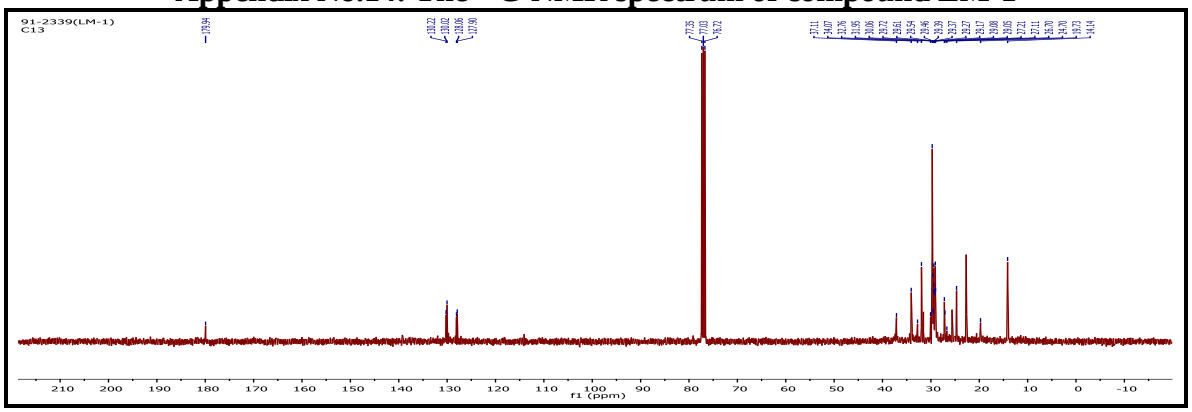
Appendix No.12: The FTIR spectrum compound LM-1



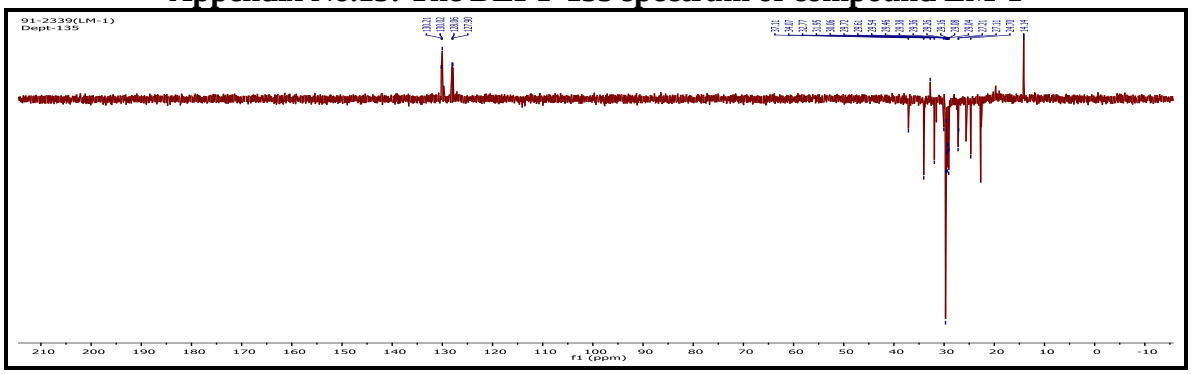
Appendix No.13: The ^{1}H-NMR spectrum of compound LM-1



Appendix No.14: The ^{13}C-NMR spectrum of compound LM-1



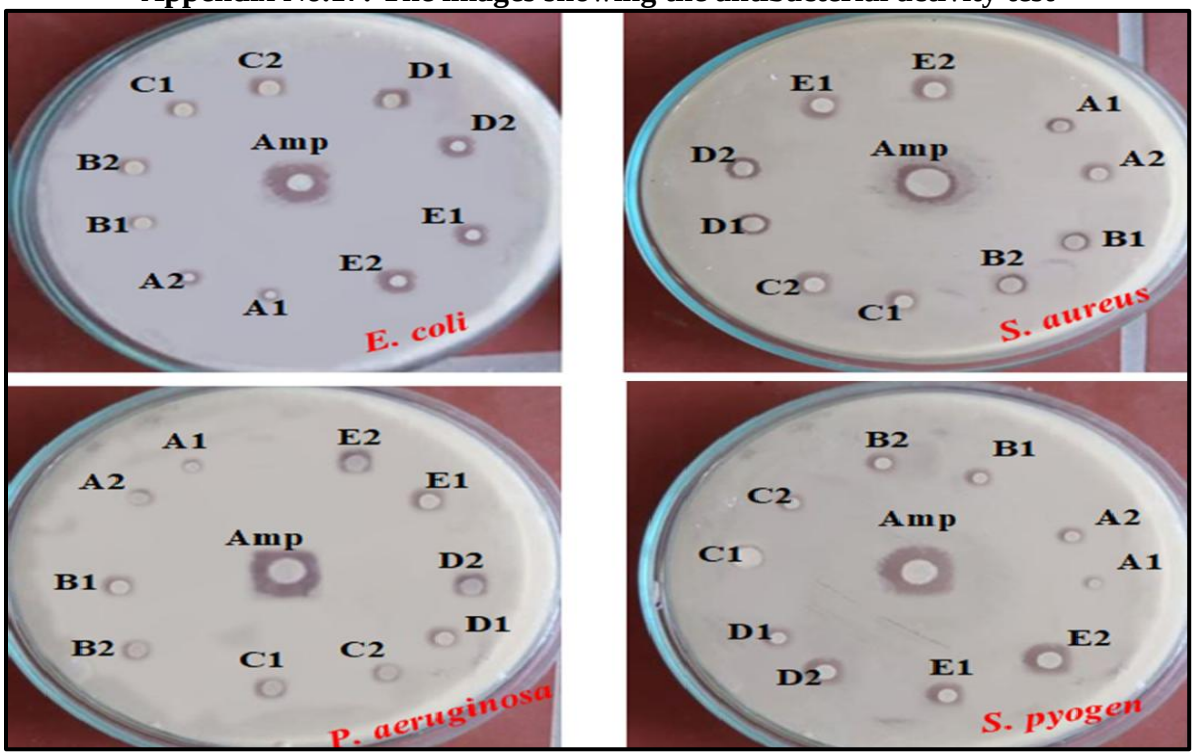
Appendix No.15: The DEPT-135 spectrum of compound LM-1



Appendix No.16: The ^{13}C-NMR and ^{1}H-NMR spectral data of compound LM-1 and reported NMR data of (5Z, 9Z)-22-methyl-5, 9-tetracosadienoic acid (Makarieva *et al*, 2010)[79]

Carbon No.	^{13}C-NMR data of compound LM-1	Reported ^{13}C-NMR data of (5Z, 9Z)-22-methyl-5,9-tetracosadienoic acid	^{1}H-NMR data of compound LM-1	Reported ^{1}H- NMR data of (5Z, Z)-22 methyl-5,9-tetracosadienoic acid	Nature of Carbon
1	179.9	178.7	-	-	C
2	32.8	33.2	2.36(t)	2.36(t)	CH_2
3	24.7	24.6	1.65(m)	1.70(m)	CH_2
4	26.7	26.5	2.08(m)	2.08(m)	CH_2
5	127.9	128.5	5.36-5.38(m)	5.30-5.40(m)	CH
6	130.2	130.6	5.36-5.38(m)	5.30-5.40(m)	CH
7	27.1	27.3	2.08(m)	2.08(m)	CH_2
8	26.7	27.3	2.08(m)	2.08(m)	CH_2
9	128.1	128.9	5.36-5.38(m)	5.30-5.40(m)	CH
10	130.0	130.5	5.36-5.38(m)	5.30-5.40(m)	CH
11	27.2	27.4	2.04(brd)	2.02(brd)	CH_2
12-21	29.1-30.1	29.4-30.1	1.18-1.43(m)	1.20-1.40(m)	CH_2
22	34.1	34.4	1.53(m)	1.51(m)	CH
23	37.1	36.7	1.10(m)	1.15(m)	CH_2
24	14.1	11.4	0.90(t)	0.85(t)	CH_3
25	19.7	19.2	0.88(d)	0.84(d)	CH_3

Appendix No.17: The images showing the antibacterial activity test



CONCLUSION

Phytochemical screening of the stem extract of *O.lamiifolium (*collected from Southern Ethiopia*)* revealed the presence of alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins, steroids, tannins, and terpenoids. The presence of these bioactive constituents is significant as they may account for the traditional use of the species. Column chromatographic separation of chloroform/methanol (1:1) ratio yielded three compounds: Compound LM-1 (Stigmasterol), compound LM-2 (Oleic acid) and compound LM-3 ((5Z, 9Z)-22-methyl-5, 9- tetracosadienoic acid). To the best of our knowledge, this is the first report on the isolation of these compounds from the stem of *O. lamiifolium* in the genus of *Ocimum* from Ethiopia flora.

In vitro test data also showed that antibacterial activities of both isolated compounds and extracts *E.coli, S. aureus*, and *S. pyogenes* to be the most susceptible species among the bacterial species used in the experiment but lower than the inhibitory activity of the reference drug (Ampicillin). The compounds also showed interesting antibacterial activities against *S. pyogenes* with superior activity exhibited by compound LM-1 with a zone of inhibition 13.5 mm. The results were also consistent with that of crude extract and isolated compounds. Therefore, the observed antibacterial activities of extracts and compounds justify the traditional use of the plant for the treatment of bacterial infections. However, similar studies are recommended on crude extracts and compounds isolated using several bacterial species to get new chemical entities that can be used as lead compounds in the discovery of antibacterial agents.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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