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SCHOOL OF GRADUATE STUDIES

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M.Sc. THESIS

ON

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATION OF ROOT EXTRACT OF *Lawsonia inermis*

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APRIL, 2022

JIMMA, ETHIOPIA

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATION OF ROOT EXTRACT OF *Lawsonia inermis*

A THESIS SUBMITTED TO THE SCHOOL OF GRADUTE STUDIES, JIMMA UNIVERSITY COLLEGE OF NATURAL SCIENCES DEPARTMENT OF CHEMISTRY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

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We, the undersigned, member of the Board of Examiners of the final open defense by **Belay Gadisa** have read and evaluated his/her thesis entitled "PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATION OF ROOT EXTRACT OF *Lawsonia inermis*" and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree Master of Science in **Chemistry**.

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DECLARATION

I hereby declare that, this thesis entitled "**PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATION OF ROOT EXTRACT OF *Lawsonia inermis***" and the work presented in it are my original work and has not been presented for a degree in any other university and that all sources have been appropriately acknowledged.

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I hereby declare that this MSc thesis is my original work and has not been presented for a degree in any university, and all sources of material used for this thesis have been duly acknowledged.

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ABBREVIATIONS AND ACRONYMS

^{13} C NMR	Carbons Nuclear Magnetic Resonance
$CDCl_3$	Deuterated Chloroform
d	Doublet
dd	Double of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsuphoxide
1H NMR	Proton Nuclear Magnetic Resonance
m	Multiplet
nm	Nanometer
Rf	Retention factor
s	Singlet
TLC	Thin Layer Chromatography
IR	Infrared
WHO	World Health Organization

ABSTRACT

Medicinal plants are now more focused than ever because they have the capability of producing many benefits to society indeed to mankind, especially in the line of medicine and pharmaceutical. *Lawsonia inermis* is one of the most commonly known medicinal plants used. However, its phytochemical and biological information is very limited in Ethiopia. The aim of this study is to investigate phytochemical constituents and evaluate antimicrobial activity of the plant. The air dried plant was extracted using chloroform: methanol (1: 1) ratio by maceration at room temperature and gave 31 g. The crude extract 31 g (6.2% yield) was subjected to silica gel Column Chromatography (CC) for fractionation and purification of the compounds. The column was eluted using petroleum ether with increasing polarity of ethyl acetate and methanol. Fractions were merged depending on Rf values. β-sitosterol (C1) was obtained by recrystallization of fractions (F24 to F28) using ethyl acetate and n-hexane. Fractions (F10 to F23) and fraction left without recrystallized from C1 were merged and subjected to silica gel Column Chromatography (CC) for fractionation and purification of the compounds. Finally, fractions (F1 to F5) obtained from merged fractions of F10 to F28 were merged and subjected to a column packed by sephadex for separation of compound β-sitosterol oleate (C2) using chloroform: methanol. The structures of the isolated compounds (C1 & C2) were established using ^{1}H, ^{13}C-NMR, DEPT-135 and IR spectroscopic methods comparison with literature reports. The resulting crude extracts were in vitro assayed against four bacterial strains (*B. cereus, S. aureus, E. coli, & S. typhi*) and one fungal strain (*C. albicans*) using disc diffusion method. The crude extracts showed promising antibacterial activities with zone of inhibition 19.5 ± 0.5 (*S. aureus),* 18.5 ± 0.5 (*C. albicans),* 17.5 ± 0.5 (*E. coli)* and 18 ± 0 (*B. cereus and S. aureus*) at 300 mg/mL by comparison with standard antibiotics zone of inhibition 21.5±0.5 (*S. aureus*), 20±0 (*C. albicans*), 23.5±0.5 (*E. coli*), 23.5 ±0.5 (*B. cereus*) and 22.5±0.5 (*S. typhi*) at 300 mg/mL (Gent.) for bacteria and 200 mg/mL(Clotr.) for fungus. The antimicrobial activity of the extract indicates that the crude extract has good antibacterial and antifungal activities.

Key words: *Lawsonia inermis, root extracts, phytochemicals, antimicrobial,* β-sitosterol, β-sitosterol oleate, medicinal use

1. INTRODUCTION

1.1 Background of the study

Over the centuries societies around the world have developed their own tradition to make sense of medicinal plants and their uses. The wide spread use of herbal remedies and health care preparations gained from ordinarily used traditional herbs and medicinal plants have been elevated due to the occurrence of natural products with medicinal properties [1]. Medicinal plants according to World Health Organization (WHO) defines as herbal preparations made by introducing plant materials to extraction, fractionation, purification, concentration, or other physical or biological processes which may be produced for basis for herbal product or for the immediate consumption [2].

Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological and medicinal activities, higher safety margins and lesser costs [3]. Medicinal plants are now more focused than ever because they have the capability of producing many benefits to society indeed to mankind, especially in the line of medicine and pharmaceutical. Plant parts such as leaves, roots and bark are used for the therapeutic purposes and as well serve as precursors for the synthesis of useful drugs due to their ethno medical importance in nature. The medicinal potentials of these plants could be traceable to the bioactive phytochemical constituents that are responsible for the physiological action on the human body [5]. Substances derived from plants have recently being of great interest due to their versatility. These substances in the plant which enhance their usefulness globally are classified as phytochemicals [6].

Phytochemicals may be derived from different parts of plant including the bark, leaves, flowers, roots, seeds, fruits, rhizomes and others. These major groups of phytochemicals (alkaloids, tannins, flavonoids, phenols etc.) are now widely used to treat degenerative diseases, such as malaria, cancers, diabetes etc. [7]. These phytochemical derived from plants depends on the geographical location, type of plants used and their parts as (leave, bark, steam, seed, root…) and mostly the type and methods of Extraction. *Lawsonia inermis* commonly referred to as hiinnaa (afaan oromoo) is a medicinal plant used in folk medicine to treat various ailments in Ethiopia. Previous ethno pharmacological reports indicate the plant has induced abortion [8], Diuretic,

gonorrhoea and bronchitis [9], Boils and burns [10], Wound healing [11], Dysentery and Wound healing [12-15], Ringworm infection Leaves mixed with coconut oil and flowers of Ashoka tree [16]. The present study was carried out the phytochemical investigation and antimicrobial evaluation of root extract of *lawsonia inermis.*

1.2 Statement of problem

In Ethiopia, traditional medicinal plants are used widely to treat different ailments caused by micro-organisms. Even if they are effective in treating diseases, the phytochemical constituents of most of the plants were unknown. Thus it needs to support by scientific experiment to identify constituents of the plant in searching for new chemically bioactive drugs. *Lawsonia inermis* is one of the most common known medicinal plants used. There are documents concerning the phytochemical analysis & antimicrobial activity of the chemical constituents of the *Lawsonia inermis* plant parts (leaves, whole plant, stem bark, seeds, flowers and roots). Phytochemical analysis results of *Lawsonia inermis* leaves were confirmed that the presence of Glycosides, Phytosterol, Steroidal compounds, Saponins and flavonoids [4] and Saponins, Glycosides, Flavonoids and Steroids present in root of *Lawsonia inermis* [54]. However, the phytochemical derived from plants depends on the geographical location, type of plants used and their parts as (leave, bark, steam, seed, root…etc.) and mostly the type and methods of extraction and biological information of the molecules from the root of *Lawsonia inermis* hasn't been sufficiently reported so far in Ethiopia. Therefore, this research is mainly focused on phytochemical investigation and antimicrobial evaluation of root extract of *lawsonia inermis*.

1.3 Objectives

1.3.1 General objective

- To investigate phytochemical constituents and evaluate antimicrobial activity root extract of *lawsonia inermis*.

1.3.2 Specific objective

- To extract the crude yield from *lawsonia inermis* root using 1:1 (chloroform: methanol) ratio.
- . To isolate the chemical constituents from the crude extracts by using silica gel and sephadex column chromatographic techniques.
- To elucidate the structure of isolated compounds based on spectroscopic methods (^{1}H and ^{13}C) NMR, IR, Dept-135 and comparison with literature data.
- To test the antimicrobial activity of crude extracts of *lawsonia inermis* root by disc diffusion method against two Gram positive bacteria, two Gram negative bacteria and one fungus.

1.4 Significance of the study

The study focused on phytochemical investigation and antimicrobial evaluation of *lawsonia inermis* root extract. The outcome of this study was expected to have the following Significances:

- ✓ Provide the chemical profile of the root of *lawsonia inermis,*
- ✓ Identify some compounds that could be used in the discovery of antimicrobial agents,
- ✓ Validate medicinal application of *lawsonia inermis root* and initiate chemists to further study on the plant.

2. LITERATURE REVIEW

2.1 Botanical & Ecological information on *Lawsonia inermis Linn*.

Lawsonia Inermis (*L. inermis*) is a scientific name of a tall shrub plant commonly known as Henna Arabic: henna; Bengali: mendi, mehedi; English: Egyptian-privet, henna, Jamaica-mignonette, mignonette-tree; French: henné; German: Hennastrauch; Hindi: mehndi; Indonesian: inai, pakarkuku; Portuguese: hésia, hena, alfeneiro; Spanish: alcana, alheña; Swedish: henna; Vietnamese: nhuômmóng taylâmòn. Scientific classification Kingdom: Plantae, Subkingdom: Viridiplantae, Infrakingdom: Streptophyta, Superdivision: Embryophyta, Division: Tracheophyta, Subdivision: Spermatophytina, Class: Magnoliopsida, Superorder: Rosanae, Order: Myrtales, Family: Lythraceae, Genus: Lawsonia, Species: *Lawsonia inermis* [17]. It is believed to have originated in North Africa (Egypt Arid area perhaps Ethiopia) and has naturalized and cultivated in the tropics of America, Egypt, India and part of Middle East. Henna is a large shrub reaching a height of up to 6 meters. It has spreading lateral branches with opposite leaves [18]. Leaves have length of 2–3 cm with 1–2 cm width [21]. Henna shrub is highly branched and has greyish-brown barks (**Fig. 1**) [19]. Is a flowering plant and one of the only two species of the genus *lawsonia*, with the other being *lawsonia odorata.* Flowers are small, numerous, aromatic, white or red coloured with four crumbled petals. The fruits are small, brown globose capsule, opening irregularly and split into four sections with a permanent style. Seeds have typical, pyramidal, hard and thick seed coat with brownish coloration [18-20]. Synonyms *Alcanna spinosa, Casearia multiflora, Lawsonia alba, Lawsonia speciosa, Lawsonia spinosa, Lawsonia* and *Rotantha combretoides* [21].

Figure 1 Picture of *Lawsonia inermis* plant taken from Tokuma Harar Chewaka Woreda Buno Beddele Oromia region by Belay; 27, sept, 2021 GC.

2.2 Traditional Medicinal use of *Lawsonia inermis*

Henna has been used cosmetically and medicinally for over 9,000 years. Henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailments as rheumatoid arthritis, headache, ulcers, diarrheoa, leprosy, fever, leucorrhoea, diabetes, cardiac disease, hepatoprotective and colouring agent. Henna **leaf** has an orange-red dye and leaf paste or powder is widely used for decorating hands, nails and feet with patterns. It is also used as a hair dye. It is used for alleviating jaundice, skin diseases, venereal diseases, smallpox and spermatorrhoea [24]. Henna was used in Medieval Persian, Arab, Turkish and Jewish medicine to treat headaches, skin and teeth diseases, as well as animal bites [22]. In Arab countries, it was still used in folk medicine to treat different skin conditions [23]. Decoction of the flowers is describing as an emmenagogue. Seeds are deodorant and in powered form are good medicine for liver disorders and associated problems. The bark is applied in the form of a decoction to burns and scalds. It is given internally in a variety of affections, such as jaundice, enlargement of the spleen, calculus, as an alternative in leprosy and obstinate skin affections. Root is considered as a potent medicine for gonorrhoea and herpes infection. Root is astringent may be pulped and used for sore eyes. Pulped root may also be applied to the heads of children for boils. Decoction of the root generally in combination with prepared indigo as a powerful abortifacient. The root is supposed to be useful in treatment of hysteria and nervous disorders [24].

Table 1 Ethnomedicinal uses of different parts of *Lawsonia inermis*

Plant parts	Medicinal uses (in/as)
Root	Bitter, depurative, diuretic, emmenagogue, abortifacient, burning sensation, leprosy, skin, skindisease, amenorrhoea, dysmenorrhea and premature graying of hair [25].
Leaves	Bitter, astringent, acrid, diuretic, emetic, edema, expectorant, anodyne, liver tonic, cough, leprosy, falling of hair, anemia, fever, dysentery, boils, inflammations [26-29].
Seeds	Antipyretic, intellect promoting, constipating, intermittent fevers, insanity, amentia, diarrhea, dysentery and gastropathy [25].
Flowers	Cardiotonic, refrigerant, soporific, febrifuge, tonic, cephalalgia, burning sensation, cardiopathy, amentia, insomnia, fever [25].

2.3 Phytochemical constituents of *Lawsonia inermis*

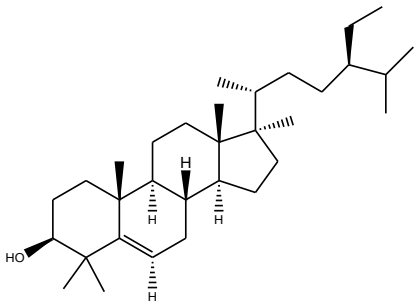
Phytochemicals are group of non-nutrient bioactive compounds founded naturally in plant parts such as flower, buds, roots, leaves, fruits, barks. They are also found in spices, and medicinal plants; and work in conjunction with other plant components as a defensive mechanism for the plants against diseases and many external attacks and they also provide characteristic color, aroma and flavor in plant [30]. The chemical constituents isolated from *L.inermis* are napthoquinone derivatives, phenolic compounds, terpenoids, sterols, aliphatic derivatives, xanthones, coumarin, fatty acids, amino acids and other constituents [31-46]. Some of the phytochemicals are indicated in (table 2) and structure of selected compounds in (**fig.2**).

Table 2 Phytochemicals found in various parts of *lawsonia inermis*

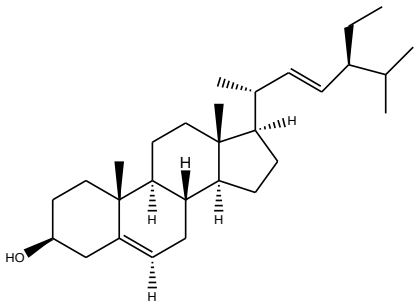
Compounds	plants parts	references
Napthoquinone derivatives		
Lawsone (2-hydroxy 1, 4-naphthoquinone)	Leaves	[31]
1, 3-dihydroxy naphthalene, 1, 4-napthaquinone,		
1, 2-dihydroxy-4-glucosylnaphthalene	Leaves	[32]
Isoplumbagin	stem barks	[33]

Phenolic compounds		
Lawsoniaside (1, 3, 4-trihydroxynaphthalene 1, 4-di-β-D-gluco-pyronoside), Lalioside (2, 3, 4, 6-tetrahydroxyacetoxy-2-β-D-glucopyranoside) Lawsoniaside B (3-(4-O-a-D-glucopyranosyl-3, 5-dimethoxy) phenyl-2E-propenol), syringinoside, daphneside, daphnorin, agrimonolide 6-O-β-D-glucopyranoside, (+)-syringaresinol O-β-D-glucopyranoside, (+)-pinoresinol di-O-β-D-glucopyr an oside, Syringaresinol di-O-β-D-glucopyranoside, isoscutellarin, Hennotannic acid, gallic acid	bark, leaves	[34]
Terpenoids		
3β, 30-dihydroxylup-20(29)-ene (hennadiol), (20S)-3β, 30-dihydroxylupane, Lupeol, 30-*nor*-lupan-3β-ol-20-one, betulin, betulinic acid, lawnermis acid (3β-28β-hydroxy-urs-12, 20-diene-28-oic acid) and its methyl ester	bark, seeds	[35-36]
Sterols		
Lawsaritol (24β-ethycholest-4-en-3β-ol), Stigmasterol and β-sitosterol	Roots, Leaves	[37]
Aliphatic constituents		
3-methyl-nonacosan-1-ol, n-tricontyl n-tridecanoate	Stem bark	[38]
Xanthones		
Laxanthone I (1, 3 dihydroxy-6, 7 dimethoxy xanthone), Laxanthone II (1-hydroxy-3, 6 diacetoxy-7-methoxyxanthone), Laxanthone III (1-hydroxy-6-acetoxy xanthone)	Whole plant	[39]
Coumarins		
Lacoumarin (5-allyoxy-7-hydroxycoumarin)	Whole plant	[40]
Flavonoids		
Apigenin-7-glucoside, apigenin-4-glycoside, luteolin-7-glucoside, Luteolin-3-glucoside	Leaves	[41]
Essential oil		
(Z)-2-hexenol, linalool, α ionone, β ionone, α-terpineol, terpinolene,		

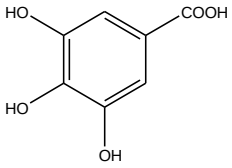
δ-3-carene and γ-terpineol	Leaves	[42-43]
Other chemical constituents		
Glucose, amino acid	Whole plant	[44]
Alkaloids		
Harmine and Harmaline	Seed	[45, 46]



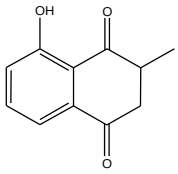
β-sitosterol



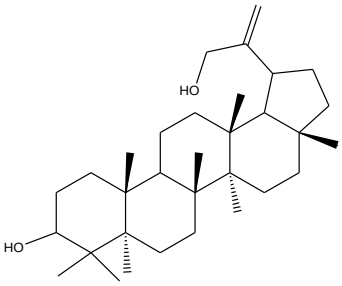
Stigma sterol



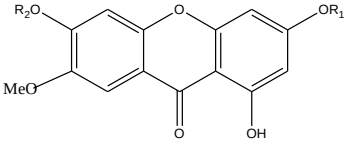
Gallic acid



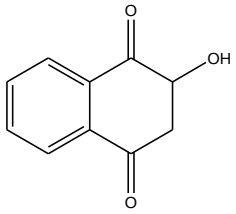
Isoplumbagin



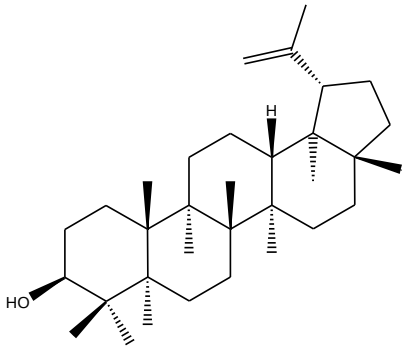
Henna diol



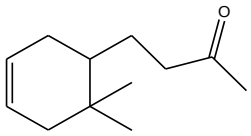
Laxanthone-I, R1 = H and R2 = Me.
Laxanthone-II, R1 = Ac and R2 = Ac.



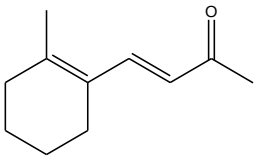
Lawsone



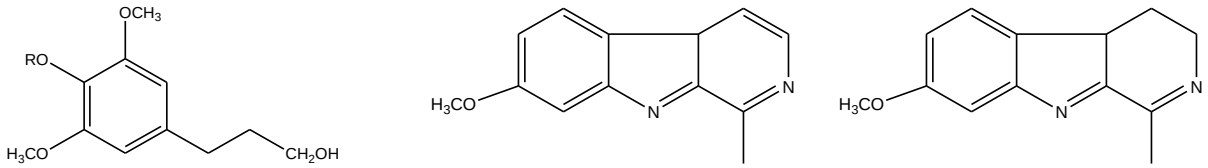
Lupeol



α–Ionone



β–Ionone



Lawsoniaside B, R = α-D-glucopyranoside Harmine Harmaline

Syringinoside, R = β-D-glucopyranosyl - (1, 6)-β-D-glucopyranoside

Figure 2 Structure of some bioactive constituents in *L. inermis*.

2.4 Biological activity of Lawsonia inermis

2.4.1 Antibacterial activity

The antibacterial effects of ethanol, petroleum ether and chloroform extracts of *Lawsonia inermis* leaves were investigated against Gram-positive: *Staphylococcus aureus, Bacillus cereus, Staphylococcus haemolytica, Bacillus subtilis, Bacillus megaterium, Sarcina lutea* and Gram-negative: *Escherichia coli, Klebsiella sp, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhi, Shigella dysenteriae, Shigella shinga, Shigella sonnei and Pseudomonas sp*. [47].

2.4.2 Antinfilamatory

Isoplumbagin and lawsaritol, isolated from root of *L. inermis* L. showed anti- inflammatory activity against Carrageenan induced paw oedema in rats. The compounds phenylbutazone, isoplumbagin and lawsaritol at the oral dose of 100 mg/kg exhibited 61, 60 and 40 percent inhibition in comparison with controls. Isoplumbagin showed significant anti- inflammatory activity similar to that of phenylbutazone [48].

2.4.3 Wound healing

Ethanol extract of the plant (200 mg/kg/day) was used to evaluate the wound healing activity on rats using excision, incision and dead space wound models. Extract of *L. inermis* when compared with the control and reference standard animals: a high rate of wound contraction, a decrease in the period of epithelialization, high skin breaking strength, a significant increase in the granulation tissue weight and hydroxyproline content [49]. The water and chloroform extracts of *L. inermis* (henna plant) leaves was found effective against the growth of microorganisms which

causes burn wound infections [50]. Ethanolic extract of *L.inermis* accelerate the healing process in experimental animals as compared to control animals [51].

2.4.4 Hepatoprotective activity

The hepatoprotective activity of the ethanolic extract of the dried leaves of *L. inermis* and its crude fractions (petroleum ether, ethyl acetate, butanol and butanone fractions) was evaluated against CCl4 induced hepatotoxicity in mice. The ethanolic extract and its fractions reduced the total bilirubin content and SGOT, SGPT and SAL activities, and reduced liver weight compared to LIV-52 (control) [52-53].

2.4.5 Antiparasitic activity

During an ethnopharmacological survey of antiparasitic medicinal plants used in Ivory Coast, 17 plants were identified and collected. Polar, non-polar and alkaloidal extracts of various parts of these species were evaluated *invitro* in an antiparasitic drug screening. Antimalarial, leishmanicidal, trypanocidal, antihelminthiasis and antiscabies activities were determined. Among the selected plants, *L. inermis* L. showed interesting trypanocidal activities [54].

2.4.6 Molluscicidal activity

L. inermis showed significant molluscicidal activity. [55]

2.4.7 Antifungal

Lawsone isolated from the leaves of L. inermis has shown significant antifungal antibiotic effect [56]. Essential oil obtained by hydro-distillation from leaves of L. inermis growing in Iran were analyzed by GC-MS and showed an antifungal activity [35]. Ethanol extract of leaves of L. inermis showed significant antifungal effect against phytopathogenic fungi. Ethanol extract could be used as alternative source of antifungal agents for protection of plants or crops against fungal infection [57].

3. METHODS AND MATERIALS

3.1 Chemicals and apparatus

3.1.1 Chemicals

Chemicals used were analytical reagent: solvents (petroleum ether, chloroform, n-hexane, ethyl acetate, and methanol), acetone for IR, and $CDCl_3$ and tetra methyl silane (TMS) were used for NMR; Standard drug (Gentamicin and Clotrimazole), culture medium (Mueller Hinton agar, nutrient agar) and DMSO were used during antimicrobial test.

3.1.2 Apparatus

The Heidolph LABOROTA 4000 was used to concentrate the filtered extract at 40℃. TLC was run on a 0.25 mm thick layer of silica gel on aluminum plate. Spots were detected by observation under UV light (254 nm and 365 nm) followed by fuming on iodine chamber. Column chromatography was performed on silica gel (60-120 mesh) Merck and sephadex (LH-20). ^{1}HNMR and ^{13}CNMR analysis were recorded on a Bruker 400MHz spectrometer, IR [58].

3.2 **Plant sample material collection and preparation**

The roots of *Lawsonia inermis* was collected from Tokuma Harar district of Cewaka Woreda, Buno Beddele Zone Oromia Regional State and 490 Km from Addis Ababa the capital city of Ethiopia. The study was carried out at Jimma University, Jimma Ethiopia from September 2021 to January 2022. The plant was dig until the base of the root was reach. The axis was used to cut the root. After collection the root sample was immediately separated from the soil particles by washing the roots with pure water cut the root into small pieces with knife, then air dried at room temperature. The dried root was crushed into powder using manual mortar and pestle and finally pounded into fine powder using electric grinder and the powdered root was store for further use (Appendix 1) [59].

3.3 **Extraction of *Lawsonia inermis* root**

The powdered root (500 g) was soaked in 500 mL Erlenmeyer flask with Chloroform: Methanol (1:1) ratio for 5 days totally; first round for three days, second and third rounds for one day for

each. The extract was filtered using whatman No-1 filter paper and concentrated using rotary evaporator (Heidolph LABOROTA 4000) under reduced pressure at 40℃ and air dried at room temperature [60]. The resulting solid extract was further dried and weighed. The resulting solid extract was stored in beaker for further use. Percentage yield of extract was calculated by:

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

3.4 **Isolation of the crude extract of *Lawsonia inermis* root**

The methanol: chloroform crude extract of *Lawsonia inermis* root was subjected to thin layer and high performance thin layer chromatographic studies for the separation and identification of their components [61]. This principle of separation is either partition or adsorption. The constituent which is having more affinity for mobile phase moves with it, while the constituent which is having more affinity for stationery phase gets adsorbed on it. This way various compounds appear as a band on the TLC plate, having different Rf values. The TLC of the crude extract was seen by trial and error with different ratio of petroleum ether: ethyl acetate.

The chloroform: methanol crude extract 31 g was applied to column chromatography already packed with silica gel (60 to 120) mesh size by using petroleum ether. The column was initially eluted with 100 % of petroleum ether twice, and then followed with petroleum ether and ethyl acetate, continued with n-hexane and ethyl acetate mixture in different combination with increasing polarity up to 100% ethyl acetate. Then the elution was continued with 100% ethyl acetate: drops of methanol ratio. From collected fractions; fractions (F24 to F28) were merged based on similar Rf value (appendix11) and recrystallized partially with ethyl acetate and n-hexane and which was left without recrystallization was merged to fractions (F10 to F23) based on similar Rf value. Then merged fraction was applied to column chromatography already packed with silica gel (60 to 120) mesh size by using petroleum ether. The column was eluted with 100% of petroleum ether twice; with increasing gradient of ethyl acetate. Among those fractions (F1 to F5) were merged based on similar Rf value and purified by using sephadex with chloroform to methanol (1:1) ratio.

3.5 **Characterization of Isolated Compounds**

The purity of the isolated compounds was monitored by TLC analysis. However, only two compounds (C1 and C2) were analyzed for structural determination. The isolated pure compounds (C1 and C2) were then characterized by the various spectroscopic techniques namely ^{1}H NMR, ^{13} C NMR and DEPT-135 as well as comparison of their data with data reported in literatures. All the above spectroscopic analysis was carried out at the Department of Chemistry, Addis Ababa University. Additionally, the Infra-Red (IR) of C2 was generated at Department of Chemistry, Jimma University [62-64].

3.6 **Antimicrobial test**

The crude extract was evaluated for in vitro anti-microbial activities. It was tested against four bacterial strains (*Escherichia coli, Bacillus cereus, Staphylococcus aureus and Salmonella typhi)* and one fungus strain *(Candida albicans*) by disc diffusion method. Both bacterial and fungal strains were obtained from Microbiology Laboratory Research, Biology Department, Jimma University. Stocked microbial strains were sub-cultured on mullor hinton agar. Incubation was done for 24 h at 37 °C to obtain freshly growing bacterial and fungal strains. The test solutions were prepared with final stock concentrations of 300 mg/mL, 200 mg/mL, 100 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. Sterile whatman No-4 discs (6mm) were soaked with stock solution of the extract, and Mueller-Hilton agar was poured into sterile Petri dishes and seeded with bacterial suspensions of the pathogenic strains. Positive control was set using standard antibiotic (gentamicin) and antifungal (clotrimazole) drugs while negative controls were set using disc impregnated DMSO solvent. Plates were then inverted and incubated at 37 °C for 24 and 48 h for bacterial and fungal strains. Then clear zone around discs and inhibition zone diameter was measured in millimeter. Antimicrobial activities of crude extract were evaluated in duplicates and zone of inhibition was recorded as mean ± standard deviation [65].

4. RESULTS AND DISCUSSION

4.1 Percentage yield of crude extract

Air dried root of *lawsonia inermis* was extracted with chloroform: methanol (1:1) ratio and was resulted a yield of 31 g dark red crude extract. The percentage yield of the crude extract (6.2%) was obtained. The TLC of the crude extract was seen by trial and error with different ratio of petroleum ether: ethyl acetate (8: 2, 7: 3, 5: 5, and 6: 4). Around four components with Rf (0.83, 0.3542, 0.1 and 0.073) and unmoved component were observed with 60%: 40% (petroleum ether: ethyl acetate).

4.2 Isolation of pure compounds

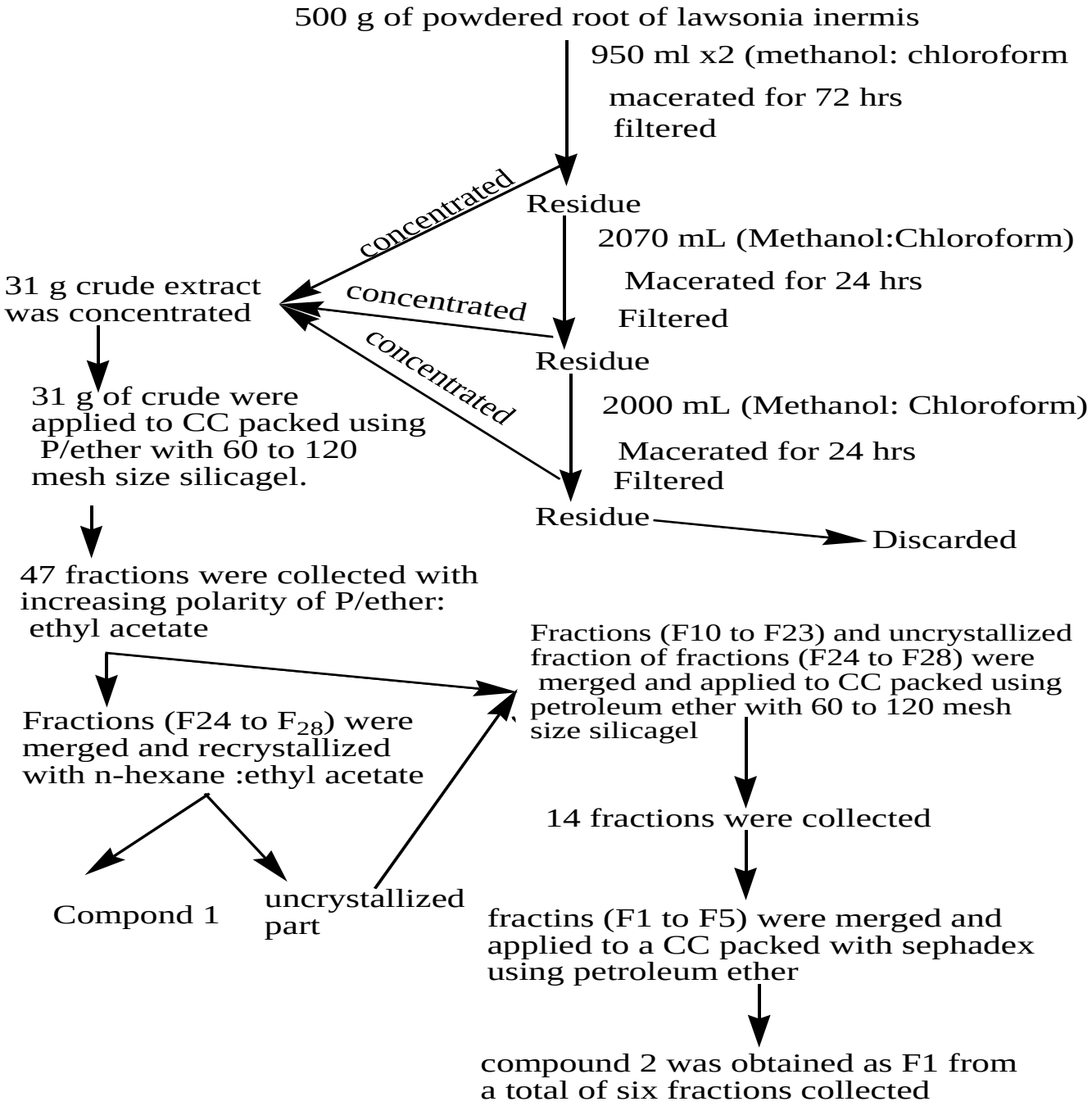


Figure 3 The schematic flow chart of the extraction and isolation of pure compounds

The extract was eluted using of petroleum ether: ethyl acetate and ethyl acetate: methanol using column chromatography and resulted in 47 fractions. Fractions 1-11, were eluted with increasing polarity in the ratio of (100:0, 100:0, 99:1 98:2, 96:4, 97:3, up to 89:11), fractions 12-25 were eluted with n-hexane: ethyl acetate (88: 12 to 73: 27) with increasing of polarity by 1%, fractions 26-30 were eluted with n-hexane: ethyl acetate in the ratio of (70:30,67:33, up to 55: 45) increasing polarity by 3%, fractions 31-40 (50:50, 45:55, up to 0:100) were eluted with n-hexane: ethyl acetate increasing polarity by 5% and the left seven fractions were eluted by 100% ethyl acetate: drops of methanol ratio (100 mL: 6 drops three times, 100 mL: 10 drops, 100 mL: 15 drops, 100 mL: 21drop and 100 mL: 30 drops). Depending on TLC analysis (Rf values) fractions (F1 & F2), F10 to F23, F24 to F28, F29 to F38 and F39 to F45 were merged together to yield 5 major fractions. Fractions (F10 to F23) and left fraction without recrystallization of merged fractions (F24 to F28) were merged and applied to column chromatography already packed with silica gel (60 to 120) mesh size using petroleum ether totally (20 mL: 0 mL, 20 mL: 0 mL, 19 mL: 1 mL to 8 mL: 12 mL). The white-needle crystal (**C1**) 10 mg was obtained by recrystallization with n-hexane and ethyl acetate from merged fractions (F24 to F28) with Rf of 0.6 in (20% Ethyl acetate to 80% Petroleum ether). The yellow oil (**C2**) 180 mg was obtained with Rf of 0.83 in (20% Ethyl acetate to 80% Petroleum ether) from further purification of column chromatography packed with sephadex using chloroform: methanol (1:1) ratio. The result of TLC was showed a single spot component under UV-light at (254 and 365 nm) for both C1 & C2.

4.3 Characterization of the isolated compounds

Structural elucidation of isolated compounds C1 & C2 were performed by using the spectroscopic data ^{1}H NMR (400 MHz), ^{13} C NMR (100 MHz) and DEPT-135 experiments.

4.3.1 Characterization of compound C1

^{1}H NMR ($CDCl_3$, 400 MHz); The ^{1}H NMR spectrums (table 3 & appendix 4) of the compound (ppm) showed the presence of three allyllic signals that appeared one singlet at δ_H 2.31 (s, 2H**),** and one multiplet from 2.07 – 1.94 (m, 2H). Two methyl singlets appeared at 0.7 (s) and 1.02 (s), three methyl overlaps appeared as multiplet from 0.79 – 0.87 (m, 9H) and one methyl as doublet

at 0.93 (d, 3H). The ^{1}H NMR spectrum of this substance also showed one olefinic proton at δ_H 5.37 (s, 1H). Its ^{1}H NMR spectrum showed a proton carbon corresponding to the hydroxyl group which appeared as a multiplet at δ_H 3.60 – 3.51 (m, 1H) [66].

The ^{13}C NMR spectrums (table 3 & appendix 5) also showed twenty-nine carbon signals with the following functionalities: six δC methyl carbons δC 11.99 (C-18), 19.41 (C-19), 18.78 (C-21), 19.84 (C-26), 19.03 (C-27) and 11.87 (C-29); eleven methylene carbons δC 37.25 (C-1), 31.65 (C-2), 42.29 (C-4), 31.46 (C-7), 21.09 (C-11), 39.77 (C-12), 24.31 (C-15), 28.26 (C-16), 33.93 (C-22), 26.03 (C-23),and 23.05 (C-28); nine methine carbons δ_C 71.82 (C-3), 121.74 (C-6), 31.91 (C-8), 50.12 (C-9), 56.76 (C-14), 56.04 (C-17), 36.15 (C-20), 45.82 (C-24) and 29 (C-25) and two quaternary carbons δC 36.50 (C-10), 42.32 (C-13) and one olefinic carbon atom with no proton 140.75 (C-5) were confirmed from corresponding DEPT-135 spectrum. Dept-135 spectrum further showed the presence of twenty six carbon signals with the following functionalities: eleven methylene carbons and six methyl carbons and with three carbons nulled of two quaternary carbons and one olefinic carbon (table 3 and appendix 6). Based on these results and also by comparism with existing literature the C1 spectral peaks are typical of β-sitosterol [66-67].

Table 3 ^{1}H NMR and ^{13}C NMR (100 MHz) Spectroscopic Data for compound C1

Position	Observed ($CDCl_3$)		Reference [66-67]			
	δH (m, J)	δC	δH (m, J)	δC	DEPT 135	Nature of carbon
1		37.25		37.28		CH_2
2		31.65		31.69		CH_2
3	3.55(m, 1H)	71.82	3.52(m)	71.82		CH
4	2.31 (d, 2H)	42.29	2.24 (dd)	42.33		CH_2
5		140.75	-	140.7	-	C
6	5.37 (br,s, 1H)	121.74	5.36 (br, s)	121.72		CH
7	2.07 – 1.94 (m, 2H)	31.46	2.04(m)	31.69		CH_2
8		31.91		31.93		CH

9		50.12		50.17		CH
10		36.50	-	36.52	-	C
11		21.09		21.10		CH_2
12		39.77		39.8		CH_2
13		42.32	-	42.33	-	C
14		56.76		56.79		CH
15		24.31		24.37		CH_2
16		28.26		28.25		CH_2
17		56.04		59.09		CH
18	0.7 (s, 3H)	11.99	0.7 (s)	11.99		CH3
19	1.02 (s, 3H)	19.41	1.03 (s)	19.4		CH3
20	1.28 (m, 1H)	36.15	1.35 (m)	36.52		CH
21	0.93 (d, 3H)	18.78	0.92 (d)	18.79		CH3
22		33.93		33.98		CH_2
23		26.03		26.14		CH_2
24		45.82		45.88		CH
25		29		28.91		CH
26	0.84 (dd, 3H)	19.84	0.84 (d, 3H)	19.80		CH3
27	0.86 (d, 3H)	19.03	0.86 (d)	18.79		CH3
28		23.05		23.10		CH_2
29	0.83 (t, 3H)	11.87	0.85 (t)	11.86		CH3

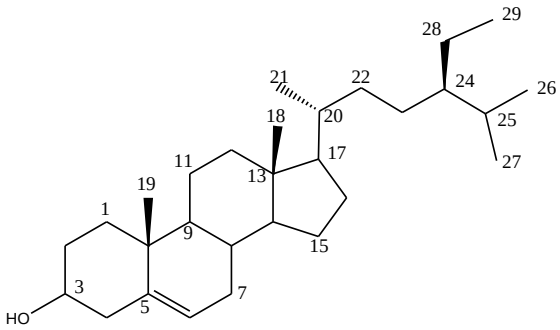


Figure 4 The proposed structure of compound C1 β-sitosterol (24R)-cholest-5-en-3β-ol

The isolated compound sitosterol has been reported that to possess pharmacological activities such as antifungal and antibacterial, antioxidant and antidiabetic properties and also anticancer activities, antihelmitic and antimutagenic activities and anti-inflammatory activity in human systems [68]. β-Sitosterol has been proven to be a safe, nontoxic, effective nutritional supplement and has amazing potential health benefits in many diverse applications including antibacterial activity. Earlier experimental studies have shown that β-sitosterol has antibacterial activity against different bacteria species including *S. aureus* and *E. coli*. According to [69-70] reports, β-sitosterol inhibited the growth of *S. aureus* (17.83 ± 0.58 mm) and *E. coli* (14.5 ± 1.84 mm) and *S. aureus* (13 mm) and *E. coli* (14 mm) respectively. So, the study suggested that the presence of β-sitosterol in chloroform extract of the root bark *Malva parviflora* might contribute to its potency of growth inhibition against tested bacteria.

4.3.2 Characterization of compound C2

Compound C2 180 mg was obtained as yellowish oil with Rf value of 0.7.

The IR (appendix **7**) was showed that there is C-O absorption of medium intensity at 1373 cm^{-1} and 1164 cm^{-1}. Then around 2920 cm^{-1} & 2857 cm^{-1} (m or s) was showed the aliphatic C-H stretch and at 1736 cm^{-1} stretch showed esterification at C-3 and at 724 cm^{-1} C-H bending.

^{1}H NMR (400 MHz, Chloroform-*d*) at δ_H 5.36 (d) showed olefinic groups, at δ_H 2.79 (m) one methylene adjacent to C-O, δ_H at 2.32 (m), 2.05 (s) and 2.01 (m) three allyllic carbons. Then ^{1}H NMR spectrums (ppm) showed the presence of methylene signals δ_H as multiplets at 1.28 (m), methyl signals showed at δ_H 1.03 (m) and at 0.88 (m), one doublet at 0.96 (d) (appendix **7**, **8** and **9**) of the compound.

The ^{13}C NMR spectrums (Appendix **8, 9 and 10**) also showed forty-seven carbon signals listed as follow 173.22, 130.14, 129.98, 129.94, 129.72,129.64 128.06, 128.02, 127.90,125.02, 122.58, 73.64, 68.87, 62.07, 56.67, 45.80, 42.29, 39.74, 38.16, 37.01, 36.57, 36.16, 34.67, 34.17, 34.02, 33.90, 33.77, 32.20, 31.96, 31.85, 31.54, 29.74, 29.57, 29.51, 29.41, 29.37, 29.30, 29.19, 29.12,28.93, 28.27, 27.20, 26.41, 25.62, 25.52, 25.15, 25.05, and 24.87.

The DEPT-135 (Appendix **10**) was showed one carbonyl 173.22 (C-1'), two quaternary carbon atoms 42.29 (C-13) and 36.57 (C-10) and one olefinic 129.64 (C5) were nulled which was confirmed from corresponding Dept-135 spectrum and also showed two olefinic carbon atoms 130.14 (C-9'), 129.88 (C-10'). Dept-135 further showed the presence of twenty-five methylene carbons. Based on these results and also by comparism with existing literature the C2 spectral peaks are typical of beta sitosterol oleate [71].

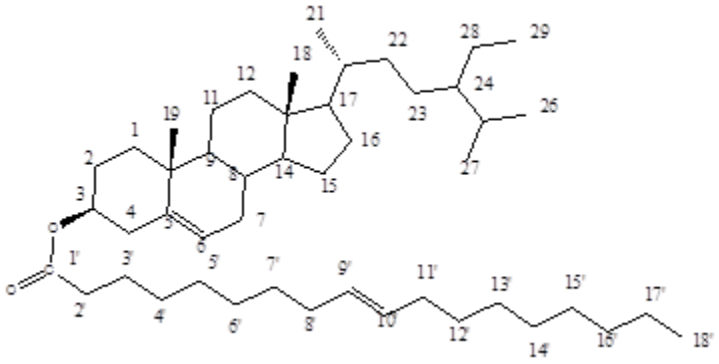


Figure 5 Proposed structure of compound C2 (Sitosterol oleate**)**

4.4 Antimicrobial activity

The crude extract (chloroform: methanol) was screened for its *in vitro* antimicrobial activity using disk diffusion method and showed against four bacterial strains and one fungal strain (Table **4**, and Appendix **2** and **3**).

Table 4 Antimicrobial effect of crude extract of *lawsonia inermis* roots

Zone of inhibition of the crude extract in mm (mean ± SD)										
Strains	Concentration in (mg/mL)							Positive control		N.cont.
								GM	Clotr.	DMSO
	300	200	100	50	25	12.5	6.25	300 mg/mL	200 mg/mL	
B.cerus	18 ±0	15 ± 0.5	13.5 ± 0.5	12.5 ± 0.5	11.5 ± 0.5	10.5 ± 0.5	7 ± 0	23.5 ±0.5	NT	NI
S.typhi	18±0	16.5 ± 0.5	15 ± 0.5	12.5 ± 0.5	10.5 ± 0.5	9± 0	7.5 ± 0.5	22.5±0.5	NT	NI
E.coli	17.5±0.5	15.5± 0.5	13.5 ± 0.5	13± 0	10.5 ± 0.5	9 ± 0	7.5 ± 0.5	23.5±0.5	NT	NI
S.aures	19.5±0.5	17.5± 0.5	15 ± 0.5	12.5 ± 0.5	10.5 ± 0.5	9.5 ± 0.5	7± 0	21.5±0.5	NT	NI
C.albicans	18.5±0.5	17.5± 0.5	15.5 ± 0.5	14.5 ± 0.5	13 ± 0.5	10.5 ± 0.5	7.5 ± 0.5	NT	20±0	NI

Key: Clotr = clotrimazole, SD= standard deviation, GM= gentamicin, N. cont. = negative control, NI = No Inhibition, NT = Not Test

The antimicrobial activity results (Table **4**) showed that the crude extract exhibited good in vitro antibacterial and antifungal activities of related zone of inhibition comparing with the standard antibiotics (gentamicin and clotrimazole) for bacterial and fungal respectively. The maximum zones of inhibition were 19.5±0.5 (for *S.aures),* 18.5±0.5 (for *C.albicans),* 18 ±0 (for *B.cerus* and *S.aures) and* 17.5±0.5 (for *E.coli).* The minimum zones of inhibition were 7, 7.5 mm for both gram-positive and gram-negative bacteria and for fungal strains that were used in the experiment. It was also observed that the chloroform: methanol extract showing that the antibacterial activity degrees of responses at 300 mg/mL concentration as compared to standard antibiotics. The results are also consistent with previous report on antibacterial activities of the ethanol extract of *Lawsonia inermis* root showed antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus sp and Klebsiella pneumoniae*. The ethanol extracts of *Lawsonia inermis* inhibits the bacterial pathogens *Escherichia coli, Staphylococcus arvensis, Staphylococcus epidermidis, Bacillus sp* and *Klebsiella pneumonea* potentially at varying concentration [72].

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, the isolated compounds were characterized as β-sitosterol (24R)-cholest-5-en-3β-ol (C1) and β-sitosterol oleate (C2) by IR, ^{1}H NMR and ^{13}C NMR; are the compounds isolated for the first time from the study plant species. Extracts was found to be more significant and effective suppression on *candida albicans* (18.5±0.5 and 18 mm for crude extract and clotrimazole respectively).The antimicrobial activity of the extract indicates that the crude extract has good antibacterial and antifungal activities.

5.2 Recommendation

Based on the current finding, the following are recommended on the study:

- The structural elucidation of isolated compound should be further relying on 2D NMR spectroscopies.
- Isolated compounds antimicrobial activity test is recommended so as to validate the traditional medicinal use of the plant.
- Additional studies are needed with this plant to evaluate for *in vivo* bioassay.
- The crude TLC showed still couple of unidentified minor compounds. Thus these minor constituents can be isolated using high-tech separation techniques including HPLC techniques.

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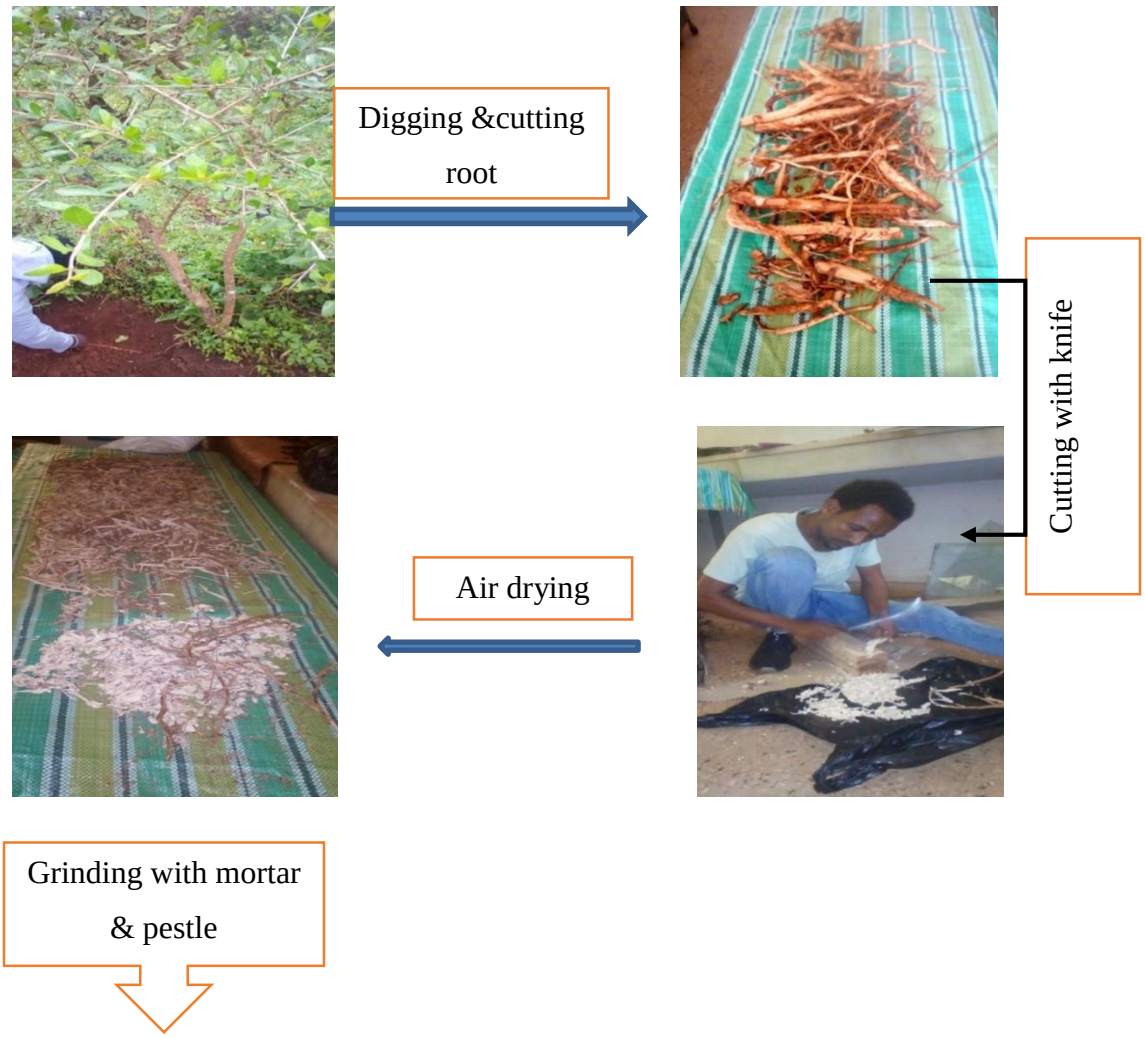
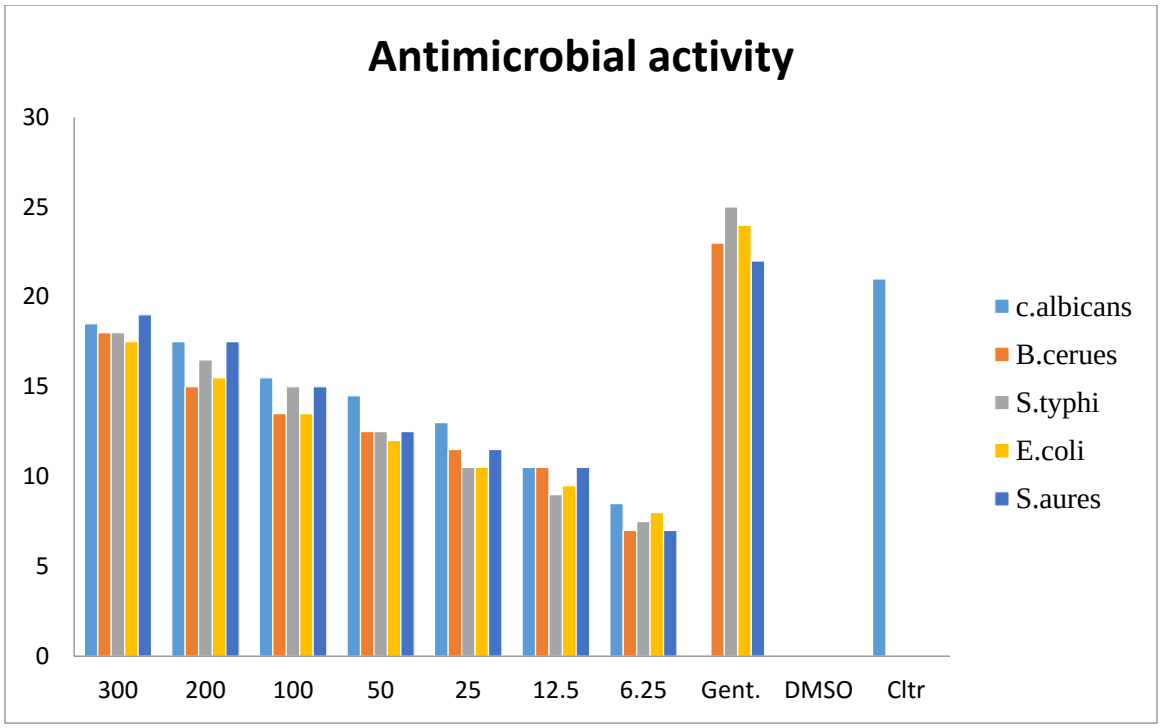
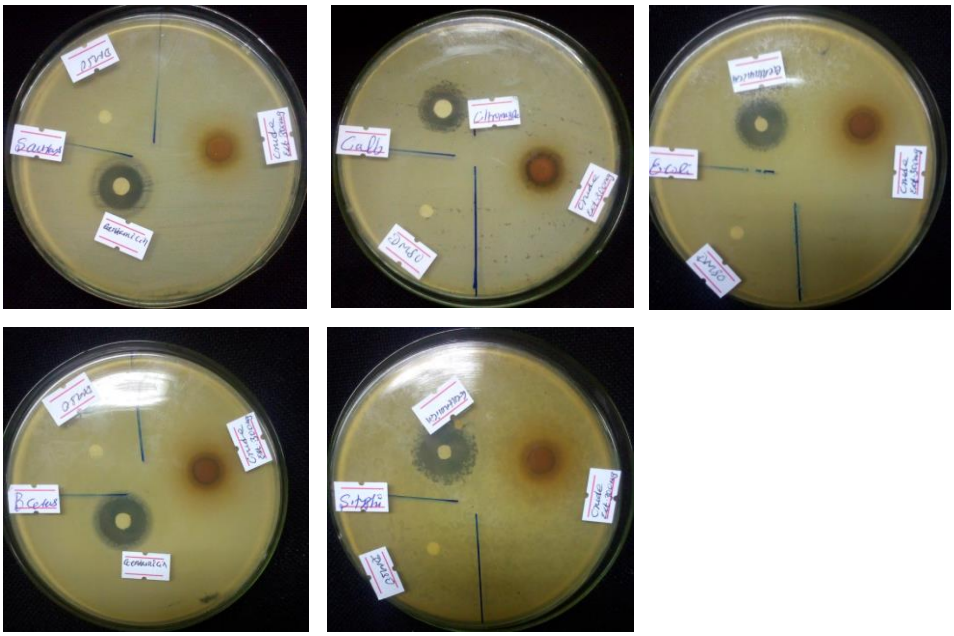
APPENDICES



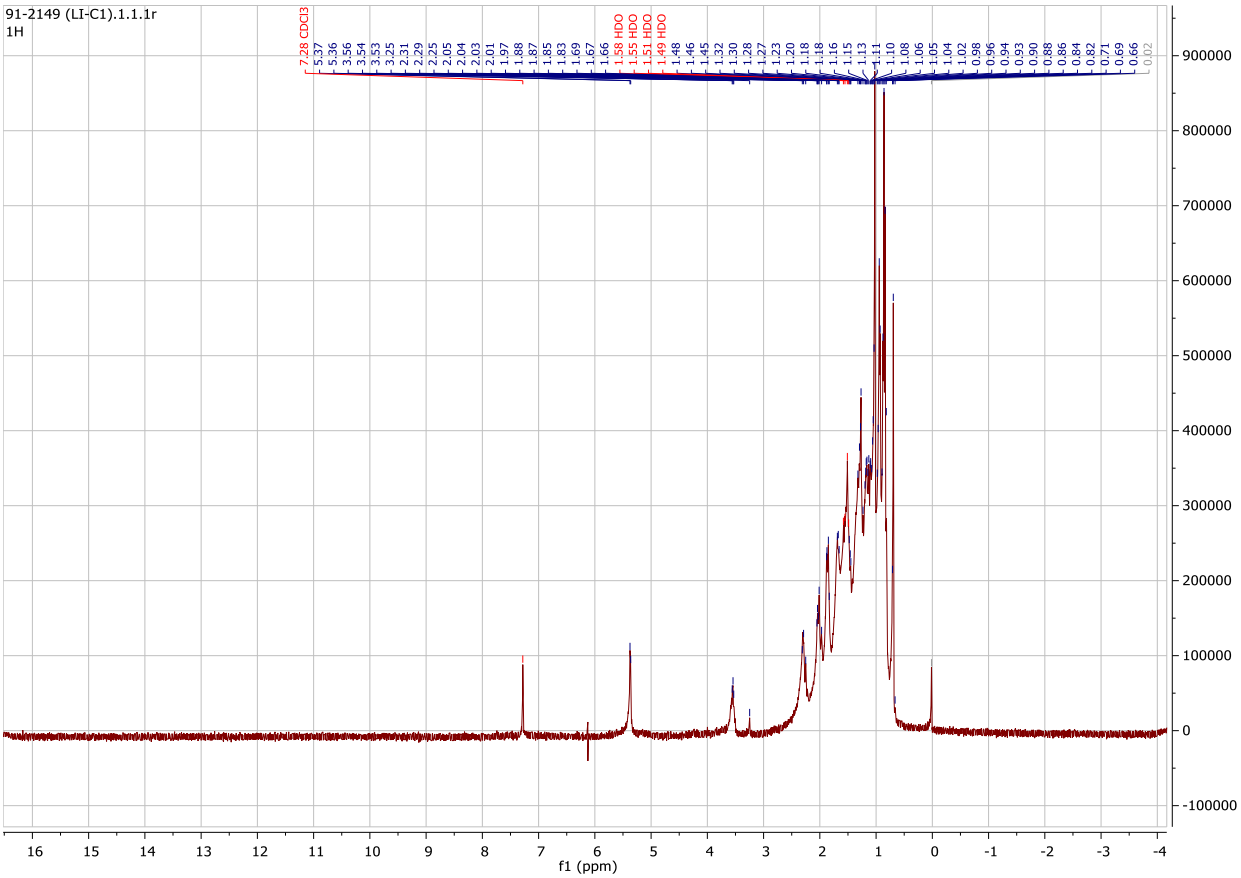
APPendix 1. Sample collection & preparation



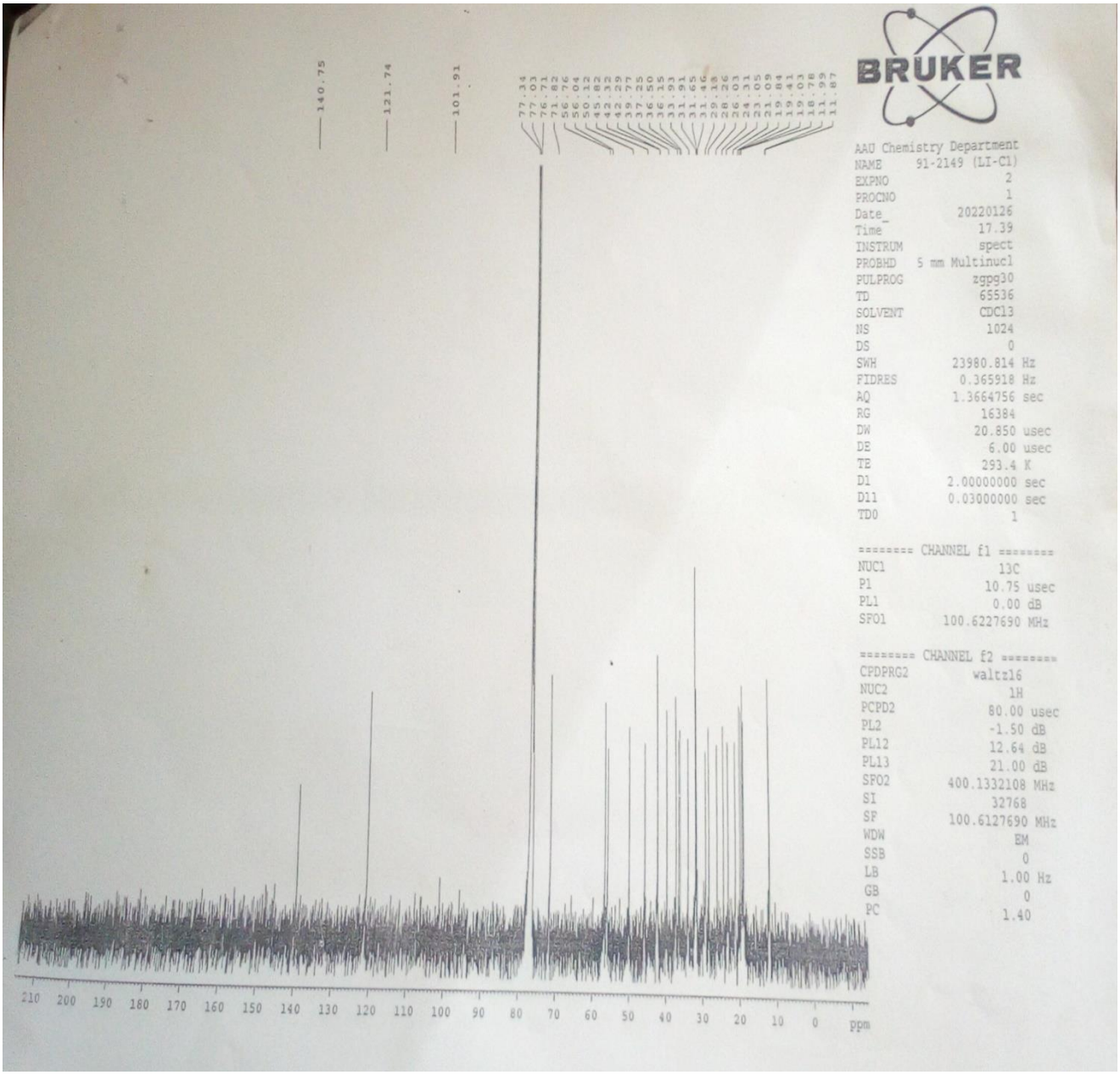
APPendix 2. Graph of mean of MIZ doublet measurements



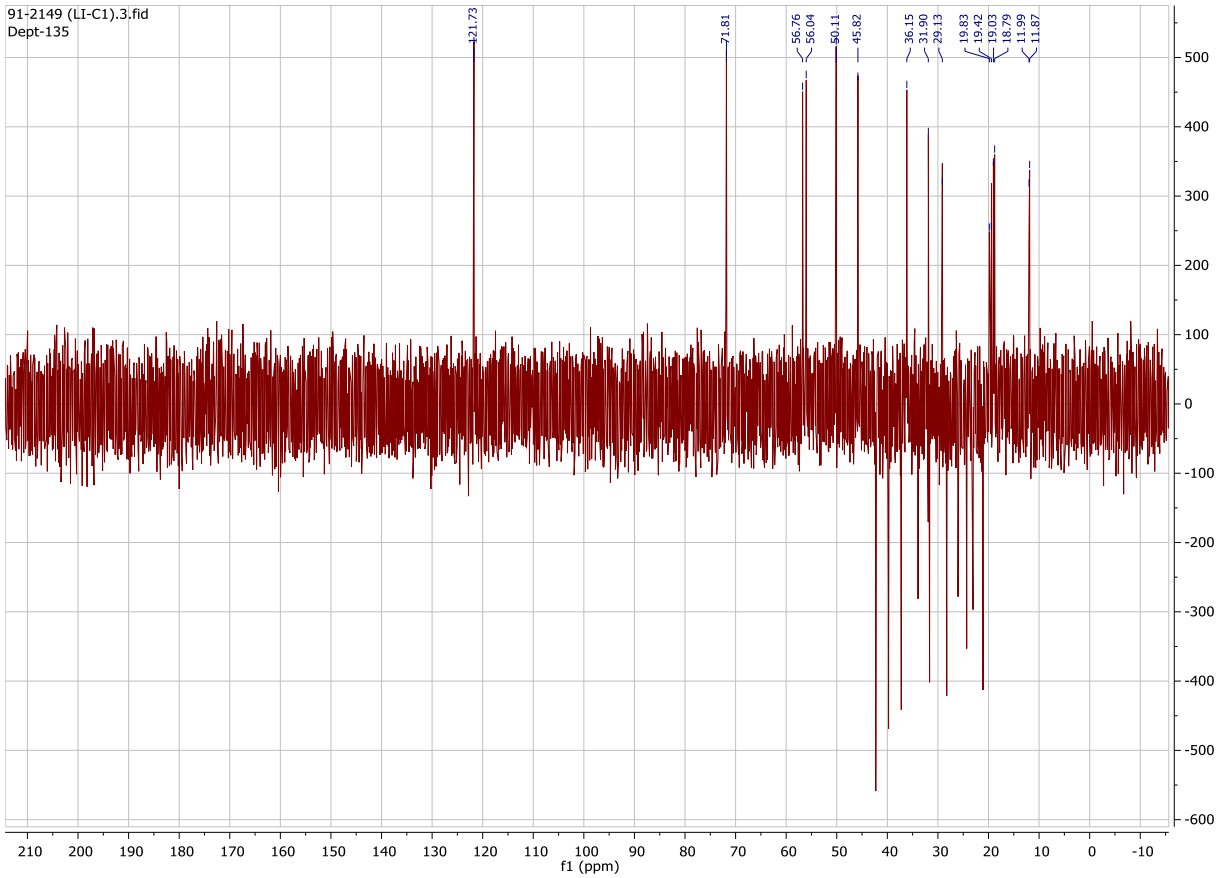
APPendix 3. Zone of growth inhibition of crude extract



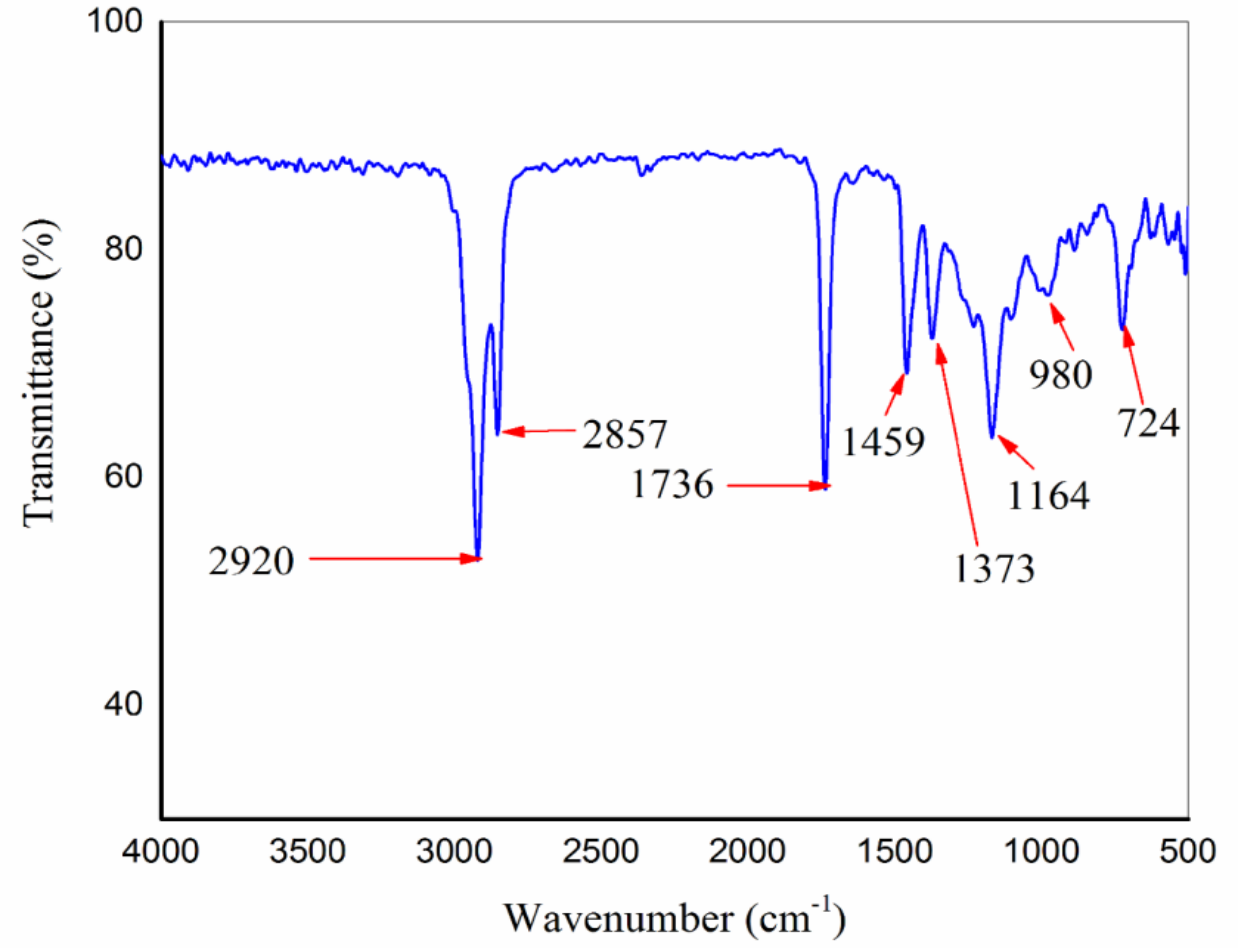
APPendix 4. ^{1}H-NMR spectrum of C1 in $CDCl_3$



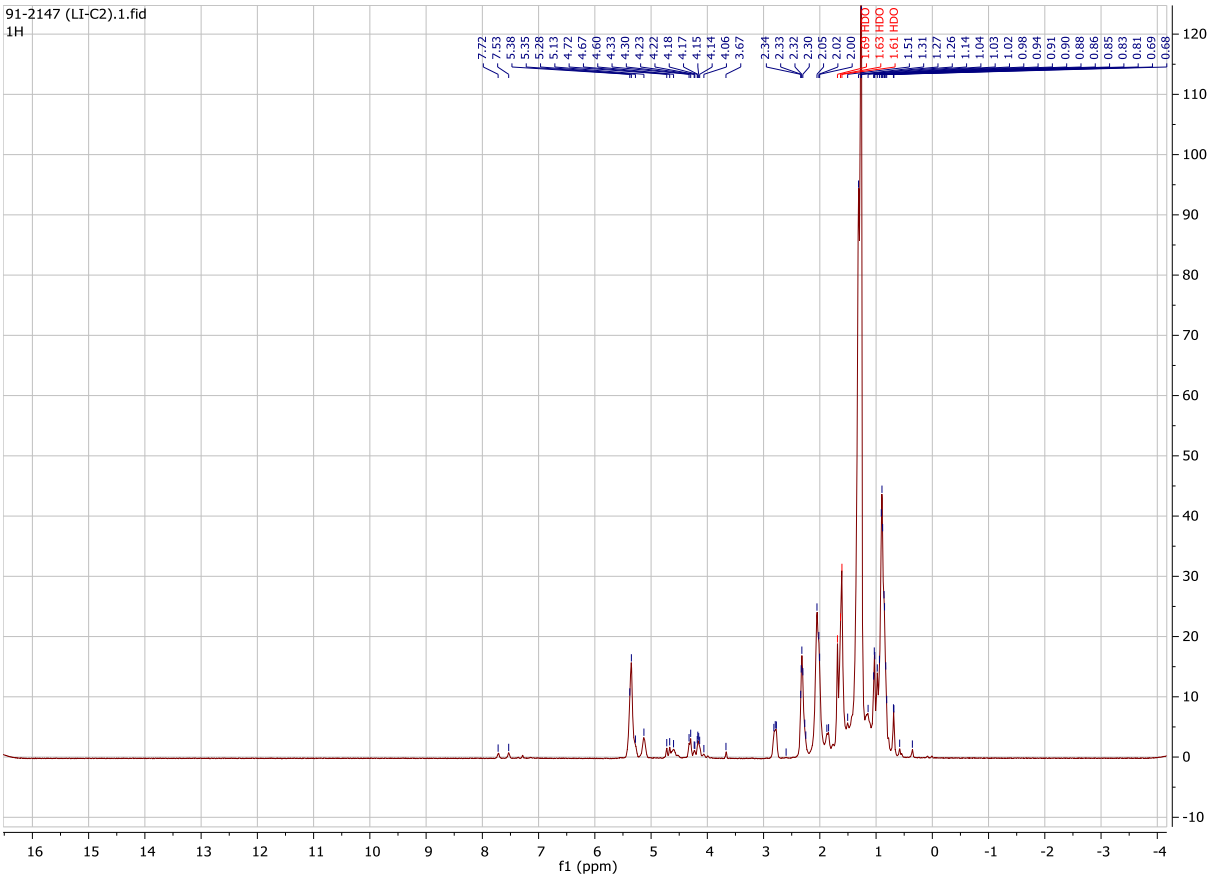
APPendix 5. ^{13} C-NMR spectrum of compound C1 in $CDCl_3$



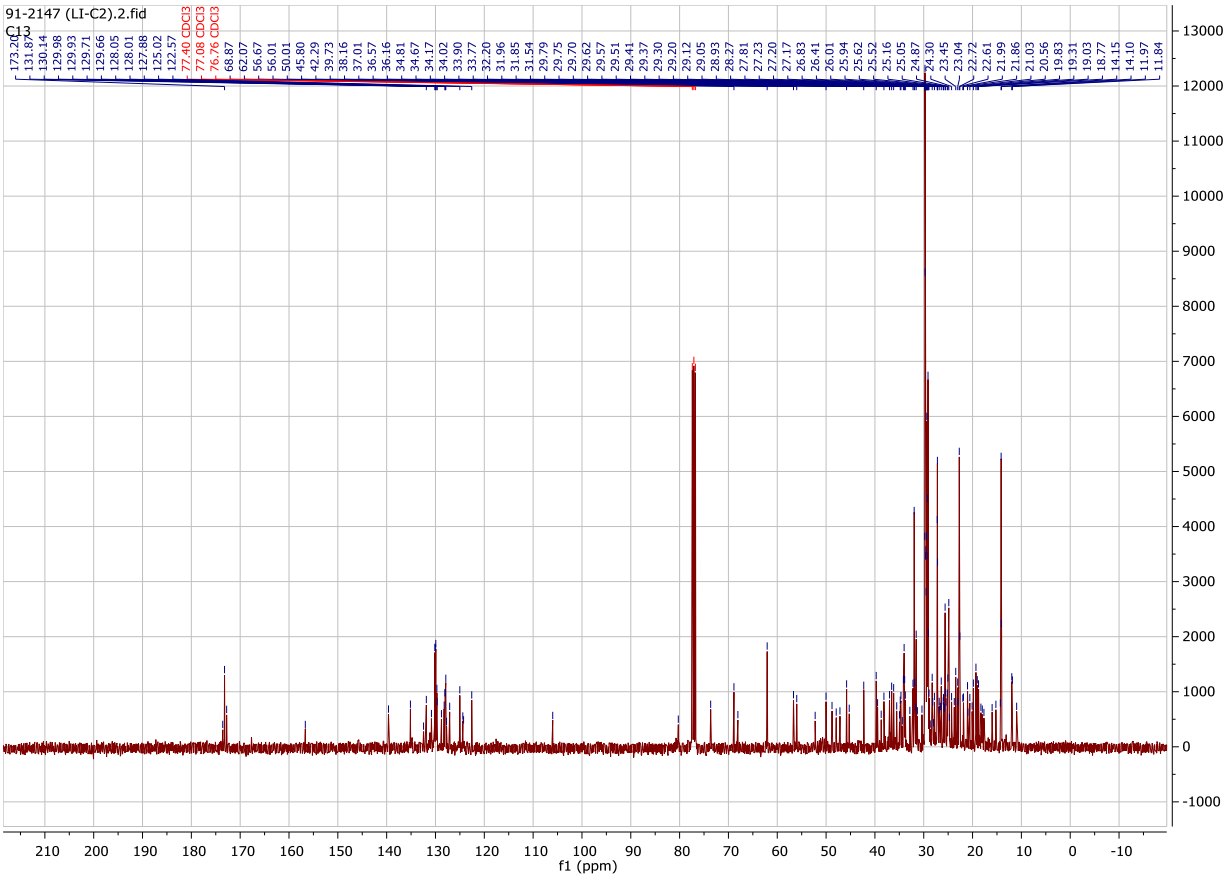
APPendix 6. DEPT-135 spectrum of compound C1 in $CDCl_3$



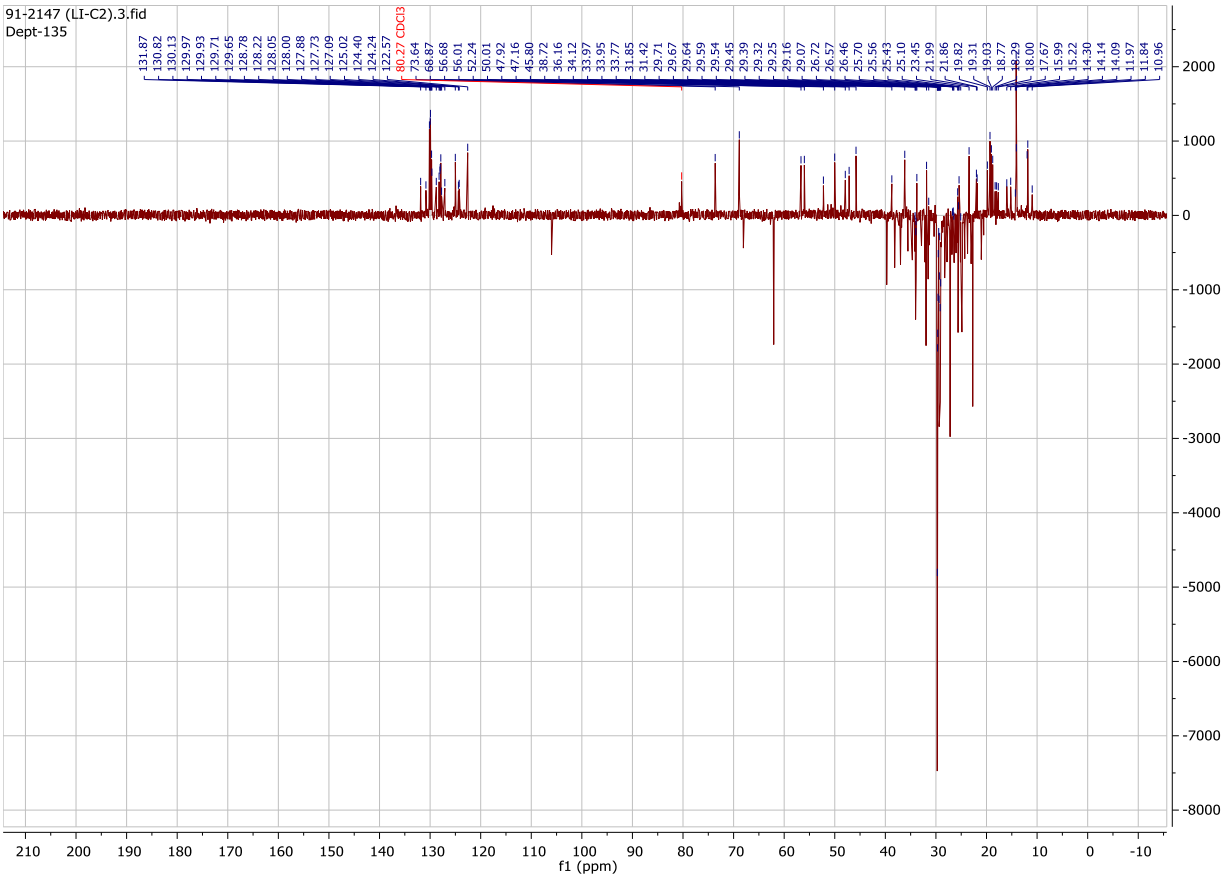
APPendix 7. IR spectrum of compound C2



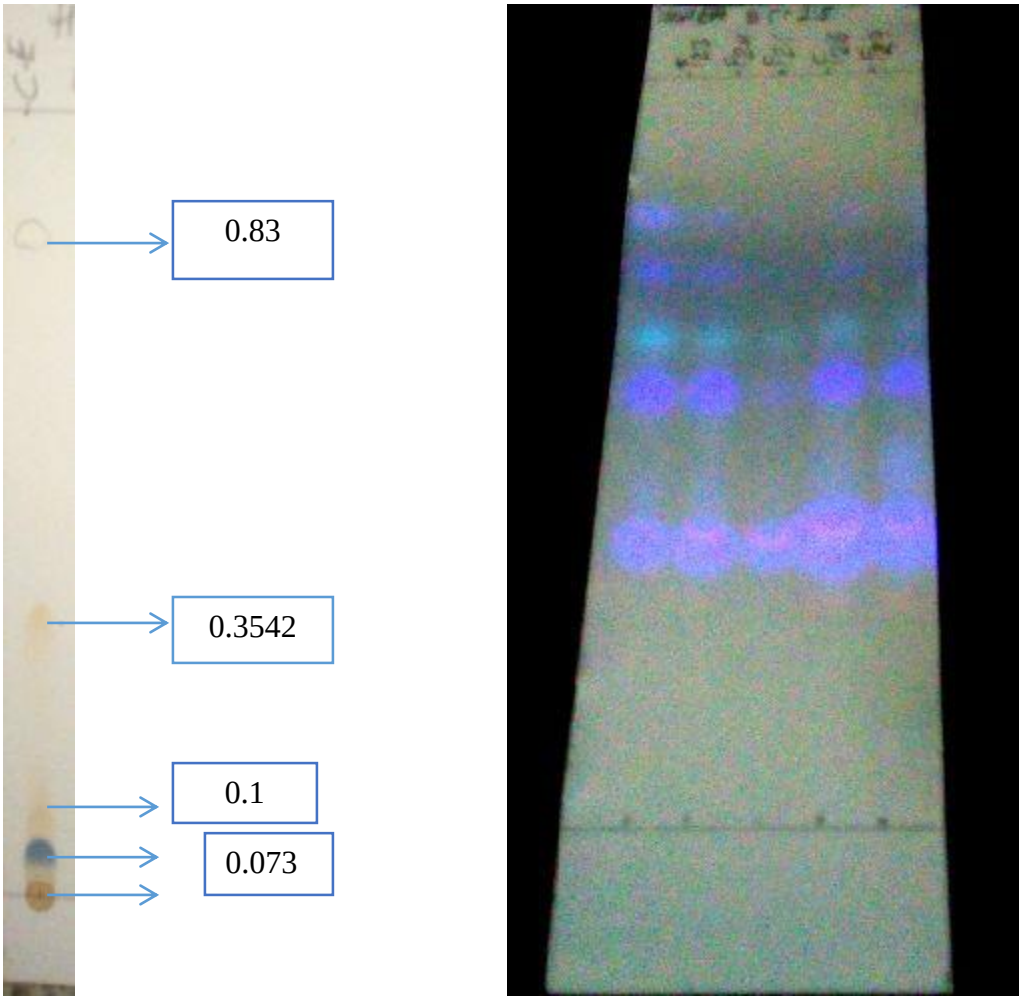
APPendix 8. ^{1}H-NMR spectrum of C2 in $CDCl_3$



APPendix 9. ^{13} C-NMR spectrum of compound C2 in $CDCl_3$



APPendix 10. DEPT-135 spectrum of compound C2 in $CDCl_3$



APPendix 11. The TLC in n-hexane to ethyl acetate (8:2) and (8.5:1.5) of crude extract with its Rf values and fractions F23 to F27 respectively.