aJIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCE

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF CHEMISTRY

M.Sc. THESIS

ON

# EVALUATION OF FIXED OIL FROM SEEDS OF *MAESA LANCEOLATA* FOR PHYTOCHEMICAL CONSTITUENTS, PHYSICOCHEMICAL CHARACTERISTICSAND BIOLOGICAL ACTIVITY

SEPTEMBER, 2017

JIMMA, ETHIOPIA

# EVALUATION OF FIXED OIL FROM SEEDS OF *MAESA LANCEOLATA* FOR PHYTOCHMICAL CONSTITUENTS, PHYSICOCHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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SEPTEMBER, 2017

JIMMA ETHIOPIA

**Title: -** Evaluation of fixed oil from seeds of *Maesa lanceolata* for phytochmical constituents, physicochemical characterization and biological activity.

**Declaration**

I, the undersigned, declare that this thesis is my original work, not presented for any degree in any universities, and that all the sources used for it are duly acknowledged.

_____ _____ _____

Name Signature

Date

**Confirmation and Approval**

This thesis has been submitted for examination with our approval as a thesis advisors.

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Finally I am thankful to my friend Yehoalework Wondewosen for her help in rearing maize weevil, Mesert Chemedesa for his help in collecting bed bug, Worku Getaneh for his help collecting sample,Abebe Diro, Mengesha Jenberei preparatory school, Hikerm Habtamu, Mulu Kegn, Nitsuh Mehare and My family Solome Adame and Hailegbreal Sewagegn.

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## Acronyms

| | |
|---|---|
| AOAC | Association of official analytical chemist |
| DMSO | Dimethylsulfoxide |
| MLO | *Maesa lanceolata oil* |
| NMR | Nuclear magnetic resonance |
| OAc | Acetoxy |
| OMe | Methoxy |
| TBu | t-butyl |
| TLC | Thin-layer chromatography |

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# ABSTRACT

Plants produce fixed oils with some lipophilic secondary metabolites that possess diverse applications in food/feed, industry, medicine and agriculture. *Maesa lanceolata* is one of such plant in Ethiopia with its seeds known to bear fixed oils and used for greasing clay made pan while baking "Injera".

The present study reports the yield, physicochemical properties, phytochemical constituents of *M. lanceolata* seed oil. Report on biological activities (insecticidal & antimicrobial) and acid-base indicator potential of crude oil and major compounds isolated were also included.

The oil was extracted by soaking the seeds in petroleum ether for five days and the isolated oil was subjected to physicochemical and phytochemical analysis employing standard test protocols available including AOAC. Pure compound isolation and characterization was carried employing chromatographic separation of crude oil, physical and spectral analysis of isolated compounds. Serial concentration range (0.3125 % to 5 %) of the crude oil and isolated compounds were evaluated for biological activates on three insect pests (Maize weevil, *Sitophilus zeamais*; Termite, *Odontotermes formosus* and Bed bug, *Cimex lectularius* employing no-choice assay) & culture of five pathogenic microorganisms (*Bacillus cerus, Staphylococcus aureus, Escherichia coli, Aspargilus niger* and *Fusarium spp* in *vitro* using *disc diffusion* test method). Positive and negative controls were included in each test. The acid-base indicator potential of crude oil and major fraction isolated was also evaluated using simple titration method using Phenolphthalein for comparison.

The seed has 30.4% oil yield. Physicochemical tests performed gave acid value (0.8±0.15), peroxide value (0.375±0.1) and saponification value (106.59±0.57). Preliminary Phytochemical tests performed on the oil indicate presence of terpenoids, quinines and alkaloids. Insecticidal activities carried using the crude oil and the major fraction isolated (MLO-4) confirm both to possess concentration dependent activities on all pests tested. 100% mortality was recorded for 5.00 % test samples (*M. lanceolata* oil /MLO-4) at 9/ 6; 3½/ 3½ and 3/- hrs against Maize weevil, worker termite and bed bug respectively. The antimicrobial tests carried confirm the crude oil and its fractions to have effect on all tested microorganisms except *Escherichia coli* and *Aspargillus niger* and the crude oil had better activity than its fractions. The Inhibition zones recorded (in mm) for susceptible organisms for the crude oil and its major fraction (MLO-3,

(MLO-4) respectively were 9 mm, 12 mm 14 mm and (for *Bacillus cerus*), crude oil and MLO-2 14 mm & 10 mm, (for *Staphylococcus aureus*) and 11, 9, 11and8 (MLO, MLO-2, MLO-3 andMLO-4) respectively. The crude oil and its major fraction (MLO-4) both have shown sharp end point color change (from yellow to purple) almost similar to endpoint for Phenolphthalein.

Column chromatographic separation of the crude oil led to isolation of MLO-2 (Rf 0.66). Based on spectral data ($^{1}$HNMR, $^{13}$C and DEPT) and melting point data of MLO-2 we propose this compound to be an isomeric mixture of monohydrated alkylbenzoquinone (2-Acetoxy-5-hydroxy-6-methyl-3-tridecyl-1, 4- benzoquinone and 5-Acetoxy-2-hydroxy-6-methyl-3-tridecyl-1,4-benzoquinone).

# CHAPTER 1

# 1.0. INTRODUCTION

## 1.1. Background of the study

Fixed oils are plant derived non-volatile liquids with thick, viscous consistency and mostly with yellow color and characteristic odor. Fixed oils may occur in various parts of the plant mainly in fruits, seeds and nuts. Seeds contain larger quantities of oils than other parts and they are usual sources of fixed oils [1]. Several species of plants are known to contain fixed oils. Some of these include Palm, Cotton, Sunflower, Niger, Canola, Jatropha, Soy, Pea nut and Sesame.[2]

Chemically fixed oils are composed typically of triglyceride consisting of other lipophilic constituents such as phospholipids, sphingolipids, waxes, lipid vitamins and lipophilic phytochemicals. They are mostly extracted using organic solvents such as hexane/petroleum ether or mechanically by expression [3].

Fixed oils possess diverse application in food/feed, industry, medicine and agriculture [4]. Plant oils contain chemicals that play role in food flavor, provide energy and to supplement important nutrients for body and thus are common food staffs recommended. Industrially fixed oils are important raw materials during manufacturing soaps, paints, varnishes, pharmaceuticals (as stimulant, cathartic, lubricant, emollient in cosmetics, solvent in preparation of certain injections and laxative)[5]. Some fixed oils might have potential as bio fuels/ biodiesel and hydraulic fluid [6]

Many fixed oils and components isolated from such as free fatty acids and other phytochemicals were investigated for diverse biological activities (antioxidant, antibacterial, antifungal, insecticidal, antiparasitic etc...[7] [8] [9] [10] activities and most were confirmed to have medicinal potential. Epidemiological and clinical studies carried on plant oil also show potential of fixed oils reduces the risk of Alzheimer's disease, stroke, inflammation and certain type of cancer [5].

## 1.2. Statement of the problem

Cereals are the dominant source of nutrition especially in developing and developed countries of sub-Sahara Africa and south East Asia where one-third of the world population exist [11]. Among the cereals; rice, wheat and maize constitute about 85%of total global production. Maize is an important cereal crop in Africa serving as source of food, feed and industrial raw material.

Pre/post harvest loss of crops constituents a great constraint to the realization of food security worldwide. Pests are the primary causes of pre/post harvest loss for maize grains in storage and field. Among several pests that attack maize in storage and field are insect (weevils and termites), soil nematodes and fungi (Aspergillus, Fusarium and Penincllium) are the most serious in Southwest Ethiopia. [7] [11].

Infectious diseases caused by pathogenic micro-organisms (such as bacteria)[12][13][14] and bedbug infestations [15][16]are a serious threat for human health in Southwest Ethiopia. The condition is getting worse with hot and humid environment dominant in this areas.

To overcome problems pests and microorganisms pose, different control methods have been developed worldwide including use of commercially available synthetic pesticides and antibiotics. But many of these products have problems associated including high cost, development of target resistance, environmental pollution and toxic effect on non-targeted organisms and direct user [17] [18].These problems all erg development of new products that are less coasty and with acceptable or no toxicity best from natural source such as plants.

*Maesa lanceolata* is a plant widely distributed in Ethiopia and is known for its fire woods, timber, oil bearing seeds and all parts used for traditional managing of wide range of human and cattle ailments[19][20]. Its seeds are used for greasing clay made pan while baking “Injera”. The plant was also investigated for diverse biological activities (antioxidant, antibacterial, antifungal, insecticidal, antiparasitic…[21][22][23][24].activities and most confirmed its potential medicinal role.

Phytochemicals analysis was carried on the plant also confirm presence of alkaloids, tannins, phenolics, saponins glycoside and terpenoids [10][25][26][27].

But studies on carried fixed oil content, physicochemical properties of fixed oil, some biological activities (antibacterial, antifungal,insecticidal) and acid-base indicator potential of seed oil and fractions of Maesa *lanceolata* seed was not carried.

Therefore in this study attempt was made isolate fixed oil from dried seed of *Maesa lanceolata* , evaluate its physicochemical characteristics, phytochemical constituents, biological activities and

acid–base indicator potential of the crude oil and compounds isolated employing standard test protocols**.**

## 1.3. Objective of the study

### 1.3.1. General objective

- To investigate fixed oil composition physicochemical/phytochemicals analysis and biological activity of *Maesa lanceolata* seed oil.

### 1.3.2. Specific Objective

- To determine the percentage oil content of seed of *Maesa lanceolata*.
- To determine the physicochemical characteristics of the seed oil employing standard test procedures.
- To carry preliminary phytochemicals analysis of the seed oil using standard procedure.
- To isolate pure compounds from the oil of *Maesa lanceolata* seeds using chromatography techniques.
- To characterize pure compounds isolated employing physical data (M.pt) and NMR data generated.
- To investigate the biological activities (anti-bacterial, anti-fungal and insecticidal) of crude oil and compounds isolated against standard reference test organisms *in vitro* employing standard test protocols.
- To investigate potential of seed oil and compounds isolated as an acid-base indicator using volumetric titration

## 1.4. Significant of the study

This work is aimed to investigate fixed oil composition physcochemical/
phytochemical characteristics and biological activity of *Maesa lanceolata* seed oil.

Findings of this study would help to:

- Recommend potential application of seed oil of *Masa lanceolata*
- Identify new phyto compounds for possible drug development
- Confirm use of *Maesa lanceolata* as local medicinal plant

# CHAPTER 2

## 2.0. Literature Review

## 2.1. Botanical Information

### 2.1.1. The genus *Maesa*

The genus *Maesa* belongs to family *Maesacea* and consists of 150 species with scramblers, shrubs and small trees distributed throughout the Old World tropics. Members of the genus are commonly found in secondary and disturbed habitats, but are also in the under storey of primary lowland and montane tropical forest. [20] *Maesa* was first recorded in the Philippines by de Candolle (1841). [28]. *M.balansae,M.tenra,M.laxiflora,M.japonica and M.lanceolata* are some species of *Maesa.*

Maesa lanceolata is one of Maesa spececies.[19] *Maesa lanceolata* is a very variable species as it can be a straggling shrub, 2 to3 m tall, or a small tree with a single stem up to 9 m tall, or a rounded bushy tree with branches almost at ground level. Leaves spirally arranged, lance – shaped, 40 – 180 X 25 = 130mm, tapering to pointed tips, sometimes with blunt or round tips, simple pale or dark green, with toothed margins. The midrib and lateral veins are conspicuously raised below. Sometimes the leaves are large and thick and resemble those of curtisia dentata, the assegi, and are often mistake for this plant them, but those the leaves of C. dentata, opposite not alternate, as those of the false assegai[19].

The bark is usually smooth and brown, flowers are minute bisexual, sweetly scented white or yellow, in many – flowered sprays in the axils of leaves and at the ends of branches. Flowering in spring (peak in October), but it has been observed flower in on and off throughout the year. Matured fruit are usually found in March, usually crowded in dense sprays, are creamy – white, edible berries that have remnants of calyx lobes at top 3–6mm.[19][20]

(A) (B) (C)

Figure 1: Picture of *Maesa lanceolata* Whole part (A), Inflorescence (B) and matured fruits(C) taken from its natural habitat. (Picture by Wubayehu S.)

The white, sweetly scented flowers are typical of those which are pollinated by night-flying insects such as moths and birds.[19]. It grows well in moist and wet with The altitude ranging from 1350-3000m including Central and East Africa, especially Ethiopia and Kenya. In Ethiopia it occurs in gallery forest, dry evergreen forest margin, woodland and on mountain slopes with Acacia, Carissa and Euclea. [19]

## 2.2. Phytochemical constituent of Maesa lanceolata

Phytohemicas are mainly the secondary metabolites offering medicinal attribute to the plants. Plant constitutes have become an important source of active natural products which differ widely in terms of their structure and biological properties. Recent research demonstrates that many phytochemicals can also protect human against disease. The class of compounds isolated from *Maesa lanceolata* includes flavonoids, terpenoids, anthraquinones, benzoquinones with long aliphatic side chains and along chain aliphatic ketone, triterpenoid saponins, and benzophenons. [25][26][30][31][32]

### 2.2.1. Quinones

Quinones are large class of compounds endowed with rich and fascinating chemistry.1,4-benzoquinone or p-benzoquinone is the basic structure of quinonoid compounds. They are widely distributed in the natural world being found in bacteria, plants and arthropods and hence quinones are ubiquitous to living system. [29]

Quinones play pivotal role in biological functions including oxidative phosphorylation and electron transfer. Their role as electron transfer agents in primary metabolic process like photosynthesis and respiration is vital to human life. *Maesanin*, 2,5 - dinydroxy - 3 - (nonadec – 14 - enyl) -benzoquinon, lanciaquinon and alkylated hydroxy benzoquinones are obtained from the fruits of *Maesa lanceolata*[33]. Maesanin, 2 - hydroxyl - 5 Methoxy - 3 -(10’ -pentadecenyl) -1,4 -berzoquinon is a natural p -benzoquinonpossess pronounced biological activities including non-specific immunostimulation, lipoxygnase inhabitation.[33]

(1-7) Alkylatedbenzoquinones ),[33][34] (8) 2,5dihydroxy3(nonadec-14-eny) benzoquinone,[33] (9) Maesanin and (10) Maesanol [34]are from fruits and Lanciaquinon (11) and Ardisiaquinone (12) from leaves of *Maesa lanceolata*.[34]

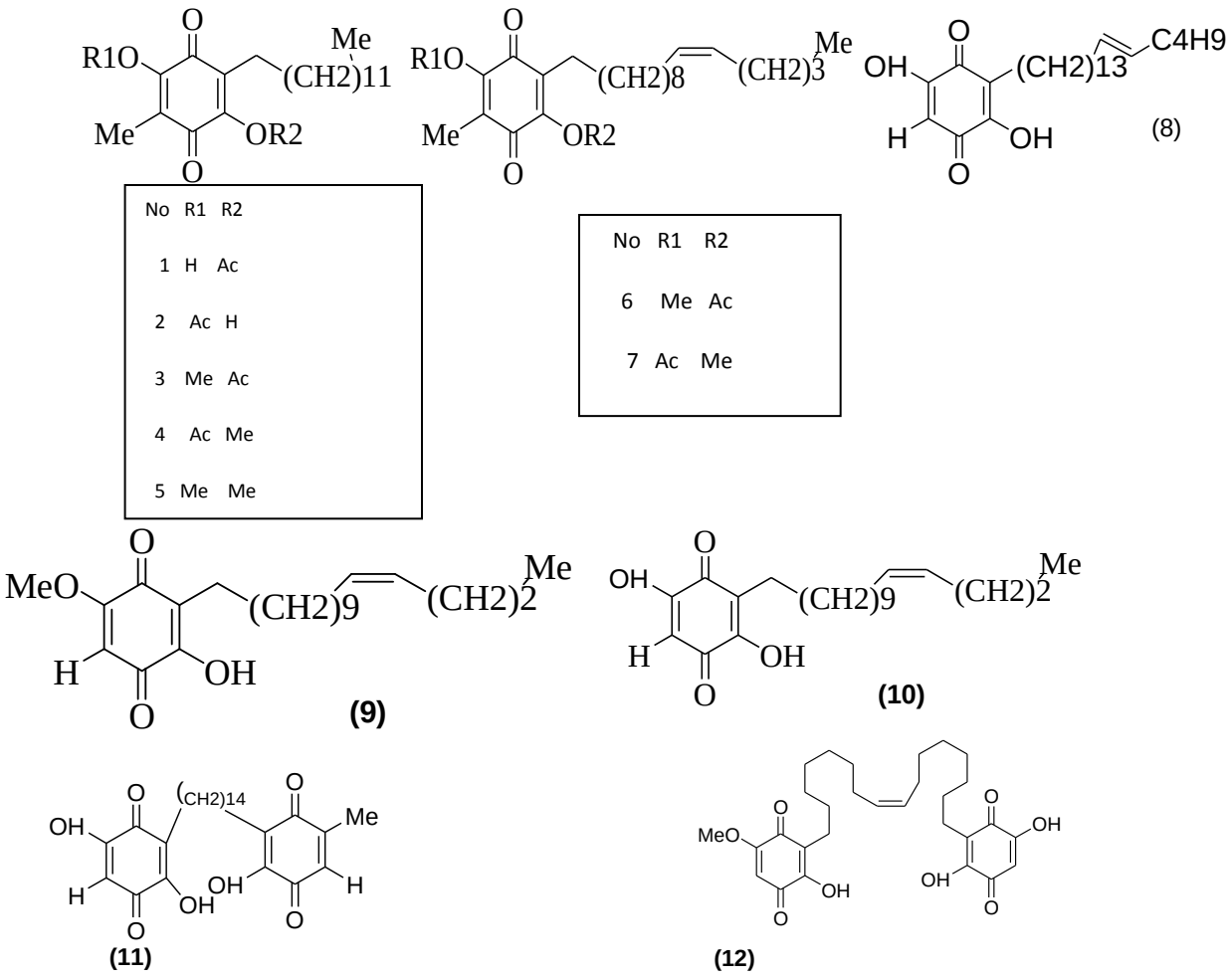
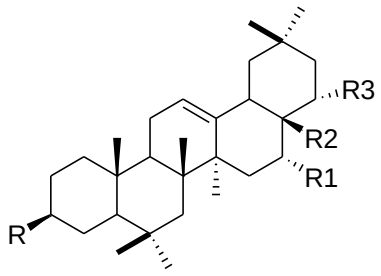



Figure 2: Compounds obtained from *Maesa lanceolata* fruit and leaves

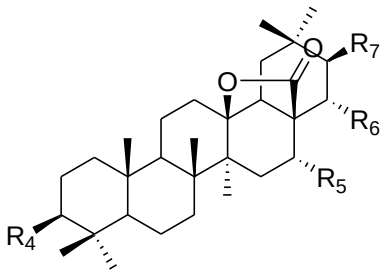
2.2.2. Triterpenoid saponins

Triterpenoid saponins are a large class of structurally diverse and biologically active specialized metabolites produced by numerous plant species. These amphipathic glycosides consist of a hydrophobic backbone or sapogenin with one or more hydrophilic sugar chains attached to it. Triterpenoid saponins display a wide range of commercial applications in the agricultural, food, cosmetic, and pharmaceutical sectors as pesticides, preservatives, surfactants, adjuvants, antimicrobial, anti-inflammatory, and anticancer agents. [31]

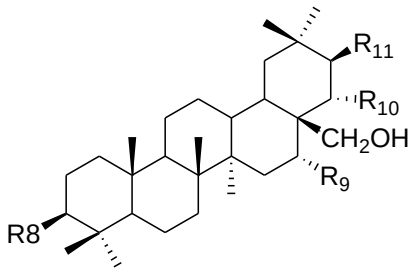
Triterpenesaponins (1-8) obtained from fruit *Maesa lanceolata* [31]




| N0 | R | R1 | R2 | R3 |
|---|---|---|---|---|
| 13 | O-gic | O | $CH_2OH$ | H |
| 14 | O-glc | OH | $CH_2OH$ | H |
| 15 | O-glc | OH | $CH_2OH$ | OH |
| 16 | O-ara. | OH | CHO | OAc |




| No | R4 | R5 | R6 | R7 |
|---|---|---|---|---|
| 17 | O-glc | OH | OAc | OH |
| 18 | O-glc | OAc | OAc | O-CO- |

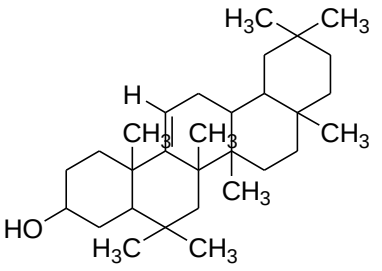



| No | R8 | R9 | R10 | R11 |
|---|---|---|---|---|
| 19 | O-xyl | OH | OH | O-CO C (CH3)=CHCH3 |
| 20 | O-ara | OH | OAc | O-CO(CH3)=CHCH3 |

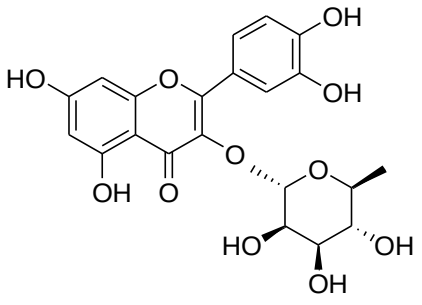
Figure 3: Triterpene saponins obtained from *Maesa lanceolata*

### 2.2.3. Miscellaneous compounds

Myrisene (21) and quercitirin (22) were also isolated from leaves [32] and seeds [31] respectively of *Maesa lanceolata.*




(21)




(22)

(22)

Figure 4: Myrisene from leaves [32] and quercitirin from seeds [31] of of *Maesa lanceolata*

## 2.3. Bio synthesis of quinon

Lipid brnzoquinone suppresses the growth of alarge number of plant species,but it most active onsmall-seed plants.The primary mechanism of actionof this secondary metabolite is not fully established

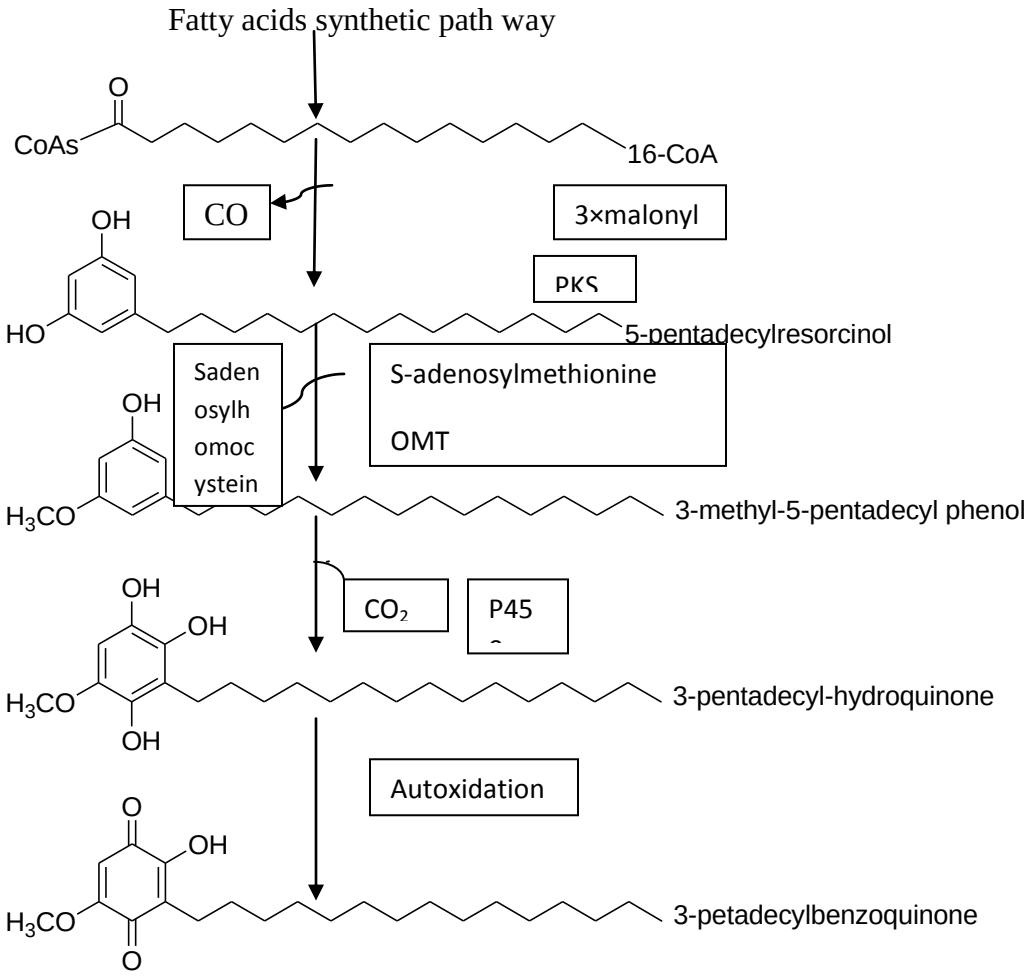



Figure5.Biosynthesis of benzoquinone

## 2.4. Use of Maesa lanceolata plant

Maesa species are used traditional medicine in many countries.Maesa plants could,however,also play a role in modern medicine, for example in cure of cancer or Leishmanisis[36]

The seed and fruits of *Maesa lanceolata* have been used traditionally to treat arthritis and anti-helmenthic in human. The common method of seed and fruit preparation for medicinal use is

seeds and fruits are ground in to a fine powder and boil in water or milk then sieve medical purposes for malaria [34].

Leaves, roots and fruits of *Maese lanceolata* have been used to treat and manage most of the aliments and conditions, including Flu, anti halmenthic, appetizer, stomach ache, sexually transmitted disease (e.g. syphilis and gonorrhea), malaria, arthritis, against bacterial infection in small intestine and viral infections in the liver.[34][37][32] And it is also well known for fishing by Shinasha and Gumuze tribes in Benshangul gumuze region.

## 2.5. Plant products as natural acid –base indicators

Indicator is a substance that has a different color on acid and alkaline. They are usually weak acids or bases when dissolved in water dissociate slightly and form ions. Common indicators used in laboratories are phenolphthalein, methyl orange, methyl red, methyl blue and litmus. There are many natural acid-base indicators that can be obtained from flowers, fruits and vegetables. The plant pigments known as anthacyanins are responsible for many of the red, blue and violet colors seen in plants.[38] It exhibit different colors in acidic and basic medium and this substance give sharp distinct and stable color change on change of acid to alkaline .thus it may be used as acid base indicators in volumetric analysis.[39]

The term volumetric analysis refers to quantitative chemical analysis carried out by determining the volume of solution of accurately known concentration which is required to react qualitatively with a measured volume of a solution of the substance to be determined. The objective of volumetric analysis is to determine the equivalent quantity of the other substance required for neutralization. The point at which complete neutralization is achieved is called the end point or the equivalent point. Commercial indicators are expensive; some of them have toxic effects on users and causes environmental pollution. For this reason there has been increasing interest in searching for alternative source of indicators from natural source. [40]

## 2.6. Harmful Organisms, Effects caused by them and modalities available for their management

### 2.6.1. Insect pest

Insect pests are major constrain on crop production, especially in developing countries, due to the growing concerns over health hazards, environmental pollution which leaves toxic residues

in soil, water and food and negative effect on no- targeted organisms and this lead to ecological imbalance and developmentoffungicidalresistantstrains[17].

### 2.6.1.1. Maize Weevil (Sitophilus zeamais)

The maize weevil (*Sitophilus zeamais*) is a species of beetle in the family Curculionidae. It can be found in numerous tropical areas around the world, especially in locations where maize is grown.[41] Maize weevil, *Sitophilus zeamais* , is a major pest of stored maize and of cob maize prior to harvest.[41] Infestations initiated in the standing crop may further develop in storage as the grain dries whether stored as cobs or bulk grain. It may also infest other cereals if the moisture content is moderate or high. This species attacks both standing crops and stored cereal products, including wheat, rice, sorghum and cottonseed.[42] The maize weevil also infests other types of stored, processed cereal products such as pasta, cassava,[42**]** and various coarse, milled grains. It has even been known to attack fruit while in storage, such as apples. [43]

Apart from the indirect effects, arising from the production of heat by the insects, the major effect of infestation by the maize weevil *Sitophilus spp*. is the damage to grain by feeding activities of the adults and the development of immature stages within the grain. This not only reduces the grain quality but also produces a considerable amount of grain dust mixed with frass.[41] Early detection of infestation is difficult. As *S. zeamais* larvae feed on the interior of individual grains, often leaving only the hulls, a flour-like grain dust, mixed with frass is evident. Infested grains contain holes through which adults have emerged.

A possible indication of infestation is grain, when placed in water, floating to the surface.[41] In large stores of grain, an increase in temperature may be detected. The most obvious sign of infestation is the emergence of adults.

One study recorded, 5 weeks after infestation the emergence of 100 adults per kg per day.[42] Complete development time for the life cycle of *S. zeamais* averaged 36 days (range 33-45) at $27 \pm 1^0$C and 69 ± 3% RH. Maximum daily rate of fecundity (6.7 eggs per female in 24 hours), duration of development, and number of progeny produced were optimal at $30^o$C and the lower limit for development from egg to adult weevils was $15.6^o$C and the upper limit was $32.5^o$Cat75%RH. [42]

### 2.6.1.2. Termites (Odontotermes formosus)

Termites are social insects comprise the order isopteran. The individuals are differentiated into various morphological forms or castes which exhibit division of labor performing different biological functions and which live in highly organized and integrated units, societies or colonies. They differ from hymenoptera social insects(ants ,bee ,warps)in that they are hem boles, their castes are usually bisexual and they have no sub social groups[44].Termites are devasting insects pests which lead to sever soil degradation by reducing vegetation and leaving the soil surfaces and barren and exposed to the elements of erosion[45].The consequence of termites attack reduces farm productivity and increases land degradation[46].In general agricultural production is very difficult in termite infested areas. The crops are attacked while they are standing in field and the soil are compacted and difficult to plough ,this in turn resulting in lower production ,low income and famine in the rural society of the area. As result farmers are forced to leave their farm lands and exposed to sever poverty.

### 2.6.1.3. Bedbugs (Cimex lectularius)

Bed bugs are parasitic insects of the cimicidal family that feed exclusively on blood.[47]*Cimex lectularius,* the common bed bug, is the best known as it prefers to feed on human blood and the species best adapted to human environments.[48]

It is found in temperate climates throughout the world.[49] They obtain all the additional moisture they need from water vapor in the surrounding air.[50] Bed bugs are attracted to their hosts primarily by carbon dioxide, secondarily by warmth, and also by certain chemicals.[51] Bedbugs prefer exposed skin, preferably the face, neck, and arms of a sleeping person. Although under certain cool conditions adult bed bugs can live for over a year without feeding,[52] under typically warm conditions they try to feed at five- to ten-day intervals, and adults can survive for about five months without food.[52]

Bed bugs can survive a wide range of temperatures and atmospheric compositions. [47] Below 16.1 °C, adults enter semi hibernation and can survive longer; they can survive for at least five days at −10 °C, but die after 15 minutes of exposure to −32 °C.They show high desiccation tolerance, surviving low humidity and a 35–40 °C range even with loss of one-third of body weight**.** The thermal death point for *C. lectular*ius is 45 °C; all stages of life are killed by 7 minutes of exposure to 46 °C. [47]

Bedbugs, while significant social problem, do not transmit disease to humans .However, bed bug bites can cause red ,raised itchy lesion on the skin that may take up to 14 days to develop and allergic reactions to the bites have been reported.[15] Scratching bug bites can also lead to secondary skin infections. Some individuals report significant psychological distress, disruption of sleep, nervousness agitation when dealing with bed bug infestation. [16]

### 2.6.2. Infectious Agents

#### 2.6.2.1. Bacteria

Infection disease is the number one among all causes of death. Accounting approximately one lady all deaths throughout the world .About 50-75% of hospital deaths are reported due to infections. [37] these number are still increasing due to development of resistance in microorganisms to the existing first line drug.Scientists from divergent fields are investigaring plants with a new age for their antimicrobial usefulness and as an alternative source to existing drugs.Plants with variety of chemical constituents offer apromising source of new antimicrobial agent with general as well as specific activity.[37]

#### 2.6.2.2. Fungi

Fungi are the major cause of plant diseases and are responsible for large scale harvest failures in crops like maize and other cereals all over the world. [53][8] The fungi genera typically found in stored grains are Aspergillus, Penicilliom,fusarium and some xerophytic species, several of them with capabilities of producing toxins.[9] Chemical control of fungal pathogens has been help of an increase of crop yield. However, usage of these chemical products is being discouraged due to the resultant environmental pollution which leaves toxic residues in soil, water and food. Some chemicals are also harmful to non target organisms and this lead to ecological balance and development of fungicidal resistant strain. [9]

# CHAPTER 3

## 3.0. Materials and Methods

## 3.1. Apparatus and chemicals

### 3.1.1. Apparatus

The apparatus which was used in the experiments laborota evaporator 400, Magnetic stirrer, filter paper, round bottom flask, electrical beam balance, hot plate, volumetric flask, test tube, burette, oven, beaker, TLC plates,. TLC tank and lid, pipette,500ml glass jar, reflux condenser, measuring cylinder, peteridishes, plastic box ,100ml volumetric flask,50ml volumetric flask, cotton cloth

### 3.1.2. Chemicals

Analytical and reagent grade chemicals are used 1%starch indicator ,glacial acetic acid, acetone (LOBAChemie)agar,Chloroform(LOBAChemieIndia),diethylether,ethanolabsolute99.8%(FDR), ethylacetate(LOBAChemieIndia),hydrochloricacid,methanol(LOBAChemieIndia),petroleumether(LOBAChemieIndia),phenolphthalein,potassiumhydroxide,potassiumiodide,sodiumhydroxideandsodiumthiosulphate,maize,cotton,water,boricacid,malathion,mancozeb80%wp(CoromandelInternationalLtd.) and gentamicin (Pharmaceuticals pvt. Ltd.)

## 3.2. Sample collection and preservation

The seed of *Maesa lanceolata* used for this study was obtained from the fruit of the plant. Mature and healthy seeds of *Maesa lanceolata*plant was selected and collected from Benishangul gumuze region Metekel zone Wonbera district with altitude 2769 m. The seed was cleaned (undesired materials) removed and shade dried at room temperature ($25^{o}$C) for 30 days and oil was extracted and used for isolation, evaluation of phytochemicals & biological activity.

## 3.3. Extraction of oil

1 kg of dried seed was ground in to fine powder and socked with 2 liters of petroleum ether in 5000 ml of round bottom flask. The maceration was carried out for 24 hrs with intermittent agitation .The extracted portion was filtered with the aid of cotton cloth and the marc (the residual material) was re macerated successively five times with petroleum ether.

Finally, all the filtrates were collected and concentrated using rotary evaporator at 60 $^0$C. The same procedure was carried out for the second batch and TLC of each batch was examined using different solvent system. The volume of extracted oil was recorded; the percentage was determined and stored at 4$^o$Cuntil required use.

## 3.4. Isolation of Compounds

50g of Silica gel was deactivated for 2 hrs at 1050Cin ovenand50g of extracted oil was absorbed and 269g of silica gel mixed with 8g of Oxalic acid (C2H2O4.2H2O) and deactivate to facilitate the polar compound elution and it is used in packing column chromatography. **Isolation was carried out using column chromatography and gradient elution method was used. The column was eluted first with 200ml of petroleum ether then with petroleum ether: ethyl acetate ratio .The elution solvent system (ratio) was 100:00-78:22and the total amount taken for each ratio was 200ml. Each beaker contains 25-30 ml and all fractions were optimized using TLC and collected in one beaker those have the same polarity.**

The second column chromatography was packed to purify the chosen fraction (100 %, 99:1and 98:2) .It has 30g mass, viscous solid and orange color. The sample was absorbed with 30g and packed with 150g of silica gel. The column was purified first with petroleum ether then with petroleum ether: acetone ratio by using gradient elution method. The elution solvent system (ratio) was 100:00-89:11 and the total amount taken for each ratio was 100ml. All fractions were optimized using TLC and collected in one beaker those have the same polarity each beaker contains 20-25 ml.

## 3.5. Physicochemical analysis

The AOAC method of analysis was employed in the determination of physicochemical properties of the oil. The chemical properties of the oil determined include acid, peroxide and saponification value. [1]

### 3.5.1. Determination of acid value

25ml of diethyl ether and 25 ml of ethanol was mixed in 250 ml beaker. The resulting mixture was added to 10 g of oil in a 250 ml conical flask and a few drops of phenolphthalein were added

to the mixture. The mixture was titrated with 0.1 M NaOH to the end point with consistent shaking for which a dark pink color was observed and the volume of 0.1 M NaOH(Vo) was noted. Acid value (Av) was calculated as

Av= $\frac{VO}{Wo}$

Where Vo- ml of 0.1M NaOH

WO- sample weight

### 3.5.2. Determination of saponification value

2g of oil extract was weighed in to a conical flask 250 ml of 0.1N ethanolic potassium hydroxide was added. The content which was constantly stirred allowed to boil gently for 60min. a reflux condenser was placed on the flask containing the mixture and a few drops of phenol phthalein indicator was added to the warm solution and then titrated with 0.5 MHCl to the end point until the pink colour of indicator just disappeared.The volume of HCl and blank solution was recorded , 0.1 N ethanolic potassium solution was taken as blank solution.

The expression for saponification value (SV) is given by

SV=56.1N (S-B/m)

Where S-Sample titer volume

B – Blank titer volume

N - Actual normality of HCl used

m – Mass of the sample

### 3.5.3. Determination of peroxide value

To 1g of the oil sample 1g of potassium iodide and 20 ml of solvent mixture (glacial acetic acid/ chloroform 2:1 by volume) was added and the mixture was boiled for one minute. The hot solution was poured in to a flask containing 20 ml of 5 % potassium iodide. A few drops of starch solution were added to the mixture and the latter was titrated with 0.25 N sodium thiosulphate. The peroxide value was determined as follows

$$Pv = \frac{SN\ 10^3}{W}$$

Where, S – ml of $Na_2 S_2O_3$

N – Normality of $Na_2 S_2O_3$

W – Weight of oil sample (g)

## 3.6. Phytochemical Analysis

The qualitative screening of bioactive component in petroleumether extracted oil was carried out standard method.[10][27] Qualitative test methods were employed to evaluate for the presence of saponins, alkaloids, tannins, flavonoids, terpenoids, glycosides and quinones. All tests were done in duplicates.

### Saponins

In test tub 0.5 mg of extract oil was added and diluted with 20 ml distill water and agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam indicates the presence of saponin.

### Alkaloids

i. 1ml of oil extract was added in test tub and 3 drops of Wagner's reagent added the presence of alkaloids indicated by the formation of reddish brown precipitate.

ii. 1ml of oil extract was added in test tub and3 drops of Mayer's reagent was added the presence of alkaloids indicated by the formation of creamish yellow precipitate

### Tannins

i. In test tub 1 ml 5% ferric chloride ($Fecl_3$) was added to solvent free 0.5mg extract. The presence of tannin is indicated by the formation bluish black or greenish precipitate.

ii. 1 % lead acetate was added to the test solution. The presence of tannins is indicated by the formation of yellow precipitate.

### Flavonoids

i. In test tube 1ml of test solution, a few drop of dilute sodium hydroxide (NaOH) was added, an intense yellow colour was produced in the plant extract which becomes colorless on addition of few drops of dilute acid indicates the presence of flavonoid.

ii. A few drops of NaOH and HCl added to test solution. The presence of flavonoid indicated by the formation of yellow/orange color

## Terpenoids

In a test tube 5 ml of test solution 2ml of chloroform and 3ml of concentrated sulphuric acid ($H_2SO_4$) was added to form a layer. The yellow color in lower layer indicates the presence of terpenoids.

## Glycosides

In test tube 1mg of oil was dissolved in water and then aqueous 0.5mlNaOH solution was added formation of yellow color indicates the presence of glycoside

## Quinones

In test tube a small amount of seed oil was treated with con.HCl andobserved the formation of yellow precipitate (or coloration**).**

## 3.7. Test for natural acid-base indicator potential

The indicators were prepared using 1mg of extracted oil was diluted in 10 ml of acetone and 1mg of phenolphthalein was diluted in 10 ml of water and In two100ml beakers 10 ml of 0.1M HCl was added and 3 drops of phenolphthalein and 3 drops of crude oil was added respectively and the color observed was recorded .The solution was titrated with 0.1M of NaOH until the color change was observed and the volume of NaOH consumed was recorded when each drop of titrate change the color of the solution. In the similar step was carried for MLO-4.

## 3.8. Organism culture preparation and Bioassay procedures

## 3.8.1. Insecticidal activities

### *3.8.1.1. Evaluation of Maesa lanceolata seed oil in the control of Maize weevil, Sitophlus Zeamais (Matschulsky)*

#### i. Rearing of S. Zeamais

The initial generation of S. Zeamais was obtained from maize store culture of Jimma Markato super Market stores with maize grains. The established S.Zeamais was reared on 5 Kg maize grains in bucket covered with cotton cloth in home at $27^{o}$c and 50 –70 RH in Jimma for 30 days.

ii. Bio-assay procedure

The oil extract and its fraction(MLO-4) were weighed and incorporate in to 100ml of volumetric flask to prepare serial dilution of concentration of 0.3125, 0.6250, 1.2500, 2.5000and 5.0000 %.The prepared concentrations and 25 g healthy disinfected maize grain seeds will put in500ml glass jars and mixed with each concentration. The jar contents (treatment maize grain) was shaken thoroughly for about five minutes to ensure uniform distribution of the oil and allowed to evaporate for 12 h. Then, 20 early emerged adults of almost same aged male and female S.Zeamais was collected from the previously reared culture of insects and induced in to jar. After introduction of the predetermined adult insects in to experimental jar. Adult mortality percentage was experimented as described below.

iii. Adult Mortality Test

Adult mortality was assessed on 3, 6, 9, 12, and 24 hrs after exposure of S.Zmeamais to the treatments. Adult was considered dead when gently probed with sharp objects and there were no responses. Percent adult mortality was determined as using the following formula

$$(\%)\text{Mortality} = \frac{No\ of\ dead\ insects}{Total\ number\ of\ insects}\ X\ 100$$

### *3.8.1.2. Insecticidal activities of Maesa lanceolata seed oil against termites*

**i. Termite collections and establishment for test**

Population of *Odontotermes* formosus

will be collected from Jimma University. Termite mounds were dug up from tree and soil containing termites were put on plastic sheets. Termite populations were collected from the plastic sheets using brush and placed in plastic boxes (polyethylene plastic box). Wooden plants (termites preferred feed) will be added to the plastic boxes as feed for the termites. Then the top parts of the plastic boxes will be covered with cotton cloth that allow air ventilation in and out easily but preventing the escape of the termites.

Moistened wad of cotton was placed in to the plastic boxes to maintain the required moisture level (more than 60 %) for the survival of termites placed in cool and dark area until needed for

the experiments. Continuously dry wooden materials will be provided to termite population and the plastic box was inspected for maintenance of the required moisture level.

**ii. Bioassay procedures for extracted oil toxicity**

The prepared oil and MLO-4 were weighed and incorporate in to flask at concentration of 0.3125, 0.6250, 1.2500, 2.5000 and 5.0000 %.What man No 1 filter paper of 9 cm diameter and was placed in peteridishes and treated separately with 1ml of the oil each of 5 concentrations allowed to evaporate for 12 hr and Moistened wad of cotton was placed in peteri dishes. Twenty collected worker (15) and solder (5) termites was randomly selected from stock population and kept in to the petridishes containing the treated filter papers. In all experiments 5% boric acid and acetone served as standard check and negative control, respectively.

All the treated petridishes were placed on dark place to simulate the dark galleries of termites. The treatment (5 concentrations) will be replicated three times. Mortality of termite will be recorded on 30 min,1,1:30,2,2:30,3,3:30,4,4:30,5,5:30,and 6 hrs after treatment application. The experiment will be conducted under laboratory conditions (25 + $3^{o}$c and 60 – 70% Rh).

Live and dead worker and solder macro terms spp. were counted and percent mortality was calculated according to the following equation.

$$\textbf{percent Mortality} = \frac{\textbf{No of dead termite}}{\textbf{Total Numberof termte}} \textbf{ X 100}\%$$

### *3.8.1. 3.Insecticidal activities of Maesa lanceolata seed oil against Bedbug*

i. Bedbug collections and establishment for test

Population of bedbug was collected from Jimma university dormitories. Bedbug populations were collected using cotton cloth and placed in plastic bottle (polyethylene plastic bottle.)

**ii. Bioassay procedures for extracted oil toxicity**

The prepared oil was weighed and incorporate in to flask at concentration of 0.3125, 0.6250, 1.2500, 2.5000 and 5.0000 %.What man No 1 filter paper of 9 cm diameter and was placed in peteridishes and treated separately with 1ml of the oil each of 5 concentrations was placed in peteri dishes and allowed to evaporate for 12hr.

Twenty collected bed bugs randomly selected from stock population and kept in to the petridishes containing the treated filter papers. In all experiments 5% boric acid and acetone served as standard check and negative control, respectively. Mortality of bedbug was recorded

on 1,2and 3 days after treatment application. Live and dead bedbugs were counted and percent mortality was calculated according to the following equation.

$$\textbf{percent Mortality} = \frac{\textbf{No pf dead bedbug}}{\textbf{Total Numberof bedbug}} \textbf{ X 100}\%$$

## 3.8.2. Anti bacterial and Anti fungal assay (disc diffusion assay)

### *3.8.2.1. Preparation of culture organism*

All the identified bacterial and fungal species were from Jimma University Natural Sciences College Department of Biology Microbiology Research laboratory.

In aseptic condition microorganisms( three strains of bacteria were used two of them were gram positive bacteria Bacillus cereus and Staphylococcus aureus and one gram negative bacteria Escherichia Coli), two fungi namely, Aspergillus niger and Fusarium were sub cultured in freshly made Muller agar .

Measure 120ml of $H_2O$ into 250ml flask and add 4.56gm of Muller agar and shake then heat the medium until completely dissolved and autoclave the medium at120$^oC$ disperse (pour) in to sterilized plate in the laminar flow bench then cheeked the sterility test for24hrs.Aseptically transferred 0.1ml of 24hrs cultured bacteria and fungi on solidified medium and spread by use spreading glass rod. After spreading three bacteria and two fungi on each medium put the immersed disc in oil, its fractions and standard for both bacteria and fungi aseptically and incubated at 37$^oC$ for 24 hrs and note and measure the inhibition zone.

## 3.8.2.2. Preparation of oil and its fraction for anti bacterial and anti fungal activity test

100mg of Extracted oil (MLO) and its fractions (MLO-2, MLO-3and MLO-4) were dissolved each in 1ml of DMSO. after completely dissolved the samples and solvent immersed the sterile disc in to each dissolved samples which contain beaker for few minutes and transfer the immersed discs on pre solidified and spread bacteria and fungi plate. Gentamicin used as for bacteria and 100mg of Mancozeb was dissolved in1ml of DMSO for fugal positive control (pc) respectively, while DMSO was used as negative control (NC).

### *3.8.2.3. Anti bacterial activity*

Following 24 hours of incubation of the test plates the clear zones were measured using a ruler. This was done by measuring the entire diameter of the clear zone and the results were recorded.

**i. Measuring the activity index**

The inhibitory effects of the phytochemical compound extract was calculated and compared by measuring the activity index.[27] This was done by using the following formula.

$$\text{Activity Index (AI)} = \frac{\text{zone inhibition of extract}}{\text{zone of inhibition of antibiotic}}$$

## 3.8.2.4. Antifungal activity

The antifungal activity of the oil and its fractions was assessed daily for three consecutive days through the measurement of diameter of inhabitation zones in millimeter using a ontransparent ruler. The experiment was aseptically conducted in triplicates to minimize errors and ensure consistency of all findings.

**i. Determination of relative zone ofinhibition**

The relative inhabitation zone of the extracted oil and its fractions compared to the positive control was calculated according to as illustrated below [27]

$$\text{Activity Index (AI)} = \frac{\text{zone inhibition of extract}}{\text{zone of inhibition of antibiotic}}$$

## CHAPTER 4

# 4.0. Results and Discussion

## 4.1. Physicochemical test data

The physical properties of the oil are deep red color and its stabilities at room temperature. The percentage oil yield from *M.lanceolata* seed is 30.40. Relative density of the oil obtained is o.75; this indicates that *M.lanceolata* oil is high. The higher mass of oil would higher energy available for work out put per unit volume. [5] and less than water.

**Table 1:physical properties of the oil**

| Characteristics | Result |
|---|---|
| Seed oil yield (%) | 30.40 |
| Relative density | 0.75 |
| Color | Deep red |
| Physical state at room temperature | Viscous liquid |
| Odor | Pleasant |

**Table 2:.Chemical characteristic of *M.lanceolata* seed oil**

| Parameter | Value |
|---|---|
| Acid | 0.8±0.15 |
| Peroxide value( meq/kg) | 0.375±0.1 |
| Saponification value(mgKOH | 106.59±0.57 |

From table 2 Acid value 0.8, the value obtained is an indicator of that oil cannot easily go rancid.. Acid and per oxide indexes are parameters that demonstrated the quality of the oil. [5]

The peroxide (pv) is an index of primary oxidation status of oils that information about the concentration of hydro peroxide (primary oxidation products), which are unstable and easily can decompose secondary oxidation products such as ketones and aldehydes. These low per oxide value increase stability of the oil for long time storage due to a low level of oxidative and lipolytic activities.The peroxide valuefor M.lanceolata seed was found to be0.375meq/kg.This value is relatively low compared with the value of other oil of wild plants. High peroxide value is associated with high rain cidity rate.Thus, with this fact ,the low per oxide value obtained from the oil is simplyan indication of the oil less liable to raincidity at room temperature. [55]

The saponification value is a measure of the alkali reactive groups in fats and oils and predicating the type of glycerides in an oil sample, which gives an idea of approximate chain length of the oil and was found to be 106±0.55KOHg-1 .The oil with a saponification value of 200mgKOHg-1 and above is regarded as high molecular weight fatty acid oil and used in making of soap.Saponification value is measure of the equivalent weight of acid present and therforeit is an indicator of purity.This type of oil with saponification value of 106mgKOHg-1,is ot a very candidate in soap makingindustries. However,the oilcan subjected to reefing process inorder to find place in soap making industries and to be as emulsifiers.[55]

## 4.2. Phytochemical Analysis

Phytochemical analysis of petroleum oil extract of *M.lanceolata* seed revealed that the oil extract has a potential source of bioactive components, such as quinones, alkaloids and terpinoids the presence of these substances in the investigated plant accounts for its use fullness as medicinal plant .The plant can be useful for natural indicator, antimicrobial and anti insecticidal. Information obtained is used to facilitate quantitative estimation and qualitative separation of constituents from seed.

Table 3:phytochemical screening test performed on oil extract of M.lanceolata plant

| Alkaloid | Saponin | Tannin | Qunones | Terpinoids | Glycosides |
|---|---|---|---|---|---|
| + | – | – | + | + | – |

## 4.3. Isolation of Compounds

The column was eluted first with petroleum ether then with petroleum ether: ethyl acetate ratio. Each beaker contains 25-30 ml and all fractions were optimized using TLC and collected in one beaker those have the same polarity.100 % (1-7), 99:1(1-7) and98:2(1-7) have the same polarity and separated component are observed and chosen for the second column. 97:3(1-7), 96:4(1-7), 95:5(1-7) and 94:6(1-7), have the same polarity and observed more than three components .93:7(1-7), 92:8(1-7)), 91:9(1-7)and 90:10(1-7) have the same polarity 89:11(1-7),88:12(1-7),87:13(1-7)and 86:14(1-7)have the same polarity, it is gum substance and more than four components are observed .85:15(1-7) ,84:16(1-7)and 83:17(1-7)have the same polarity, more than two components and small amount.82:18(1-7),81:19(1-7)and80:20(1-7) have the same polarity and yellow crystalline solid compound(MLO-2) is eluted.79:21(1-7) and 78:23(1-7)have not observed any spot on TLC(fractionated component).

The second column chromatography was packed to purify the chosen fraction (100 %, 99:1and 98:2) .It has 30g mass, viscous solid and orange color. The sample was absorbed with 30g and packed with 150g of silica gel. The column was purified first with petroleum ether then with petroleum ether: acetone ratio by using gradient elution method.

Each beaker contains 20-25 ml and all fractions were optimized using TLC and collected in one beaker those have the same polarity. 100 % (1-3) elute has no component observed100% (5-6) and 99:1(1-2) have the same polarity and white crystalline solid (MLO-1) component is eluted, Components 99:1(3-4)and 98:2(1-4)have the same polarity, viscose solid and more than two components observed,97:3(1-4) have the same polarity, orange viscose solid (MLO-3) substance is obtained,96:4(1-4),95:5(1-4)and94:6(1-4) have the same polarity, orange viscose solid (MLO-4)obtained,93:3(1-4)and 92:8(1-4) have the same polarity ,small amount and more than two components observed and 91:9(1-4),90:10(1-4) and 89:11(1-4)have the same polarity, small amount and has more than one component.

## 4.4.Characterization of compounds isolated

Isolated compound was characterized using NMR and Acetone d-6 solvent in Addis Ababa University Department of ChemistryNMR Laboratory.

In previous report, column chromatography of the n-hexane extract of M.lanceolata fruits, using hexane –EtOAc yields two fractions ,each consisting of mixture of isomeric mono hydroxyl alkyl benzoquinones,(1+2) and (3+4),in yields of 0.03% and o.o13%,respectively. These two compounds were not amenable to further separation and their benzoquinone components were only separable as their methyl ethers. The melting point of isomeric mixture of (1+2) is101-$102^0$C, yellow color and Rf 0.60. [56]

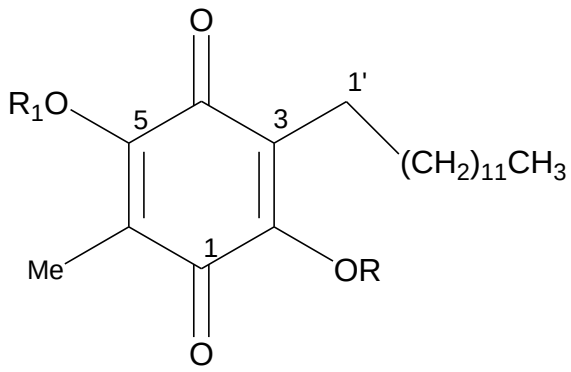
Column Chromatography of Petroleum ether extract of *M.laceolata* seed oil, Using Petroleum ether:ethylacetate, resulted in the isolation of MLO-2 it has melting point 102-$104^0$C,crystalline solid, Rf value is 0.66 and $^1$H and $^{13}$C-NMR data of a compound is given below .

**Table 4: $^{13}$ C NMR data of compound MLO-2 in comparison with reported data (1+2).**

| Carbon number | Multiplicity( DPT) | $^{13}$ C-NMR *MLO-2* | $^{13}$C-NMR Reported compound | *Remark* |
|---|---|---|---|---|
| 1 | C | 180.35,183.32 | 180.5,183.3 | Quaternary carbon |
| 2 | C | 149.63,152.71 | 150.3,151.0 | Quaternary carbon |
| 3 | C | 132.57,128.92 | 132.7,128.7 | Quaternary carbon |
| 4 | C | 180.18,182.94 | | Quaternary carbon |
| 5 | C | 149.83,152.84 | 151.1,150.3 | Quaternary carbon |
| 6 | C | 115.06,119.43 | 115.8,120.1 | Quaternary carbon |
| 1 | $CH_2$ | 22.26,22.95 | 22.7,23.3 | Methylene |
| 2 | CH2 | 28.02,28.41 | 28.1,28.2 | Methylene |
| 12 | CH2 | 19.41,19.36 | 22.6,22.6 | Methylene |
| 13 | $CH_3$ | 13.56, | 14.0,14.0 | Methyl |
| 6-Me | $CH_3$ | 7.88,6.98 | 7.8,8.7 | Methyl |
| OAc | C | 167.78,167.50 | 167.8,168 | Quaternary carbon |
| | $CH_3$ | 19.43,19.36 | 20,9,20.9 | Methyl |
| Other C | 2(6$CH_2$) | 28.60-33.31 | 29.2-31.9 | Methylene |

The proton decoupled $^{13}C$ NMR Spectrum (both 1D$^{13}C$-NMR and DEPT) Showed the signals of $C_{38}$ atoms (table 4) of fourteen Quaternary carbon 2 (C-1,C-2,C-3,C-4,C-5,C-6 and C-OAc),eighteen methylene 2(C-1_C-12) and six methyl 2(C-13,C-14 and C-15).In DEPT Spectrum, the data are collected in such way that the resulting signal is either positive ($CH_3$)or negative ($CH_2$) depending on the number of protons attached.

Accordingly, eighteen signals are pointed down (negative) indicating that there are eighteen methylene groups in MLO-2and six signals pointing upward (positive) indicating six carbons attached with three hydrogen atoms. Based on the information obtained, its melting point, color, Rf value and NMR data the most likely structure of isolated compound (MLO2) is found to isomeric mixture of monohydroxy alkaylbenzoquinone(2- hydroxy -5-Acetoxy -6-methyl-3-tridecyl-1,4-benzoquinone).




| No | R | R1 |
|---|---|---|
| 1 | H | Ac |
| 2 | Ac | H |

Figure 5: Isomeric mixture of monohydroxy alkaylated benzoquions

4.5. **Test results for potential acid- base indicator**

The result obtained in titrations was due to the presence of bioactive substance, sharp color change occurred at end points of the titration. Neutralization point obtained by *M.lanceolata* seed oil and *MLO-4* were much closed with equivalence point obtained by standard indicator (phenolphthalein).This represent usefulness of *M.lanceolata* oil and MLO-4 as indicator in acid base titration. Titrate and titrant with indicators show sharp and intense color change at the equivalence point that is at neutralization.

Table 5: Comparison of Phenolphthalein, *Maesa lanceolata* oil and isolated compound (MLO4)

| Titrant | Indicators | Colour | Titrate | Colour | Volume of titrate recorded at equivalence point |
|---|---|---|---|---|---|
| 0.1MHCl | Phenolphthalein | Colourless | 0.1MNaOH | Pink | 10.3±0.2 |
| 0.1MHCl | Maesa lanceolata seed oil | Yellow | 0.1MNaOH | Pink | 10.2±0.2 |
| 0.1MHCl | MLO-4 | Yellow | 0.1MNaOH | Pink | 10.1±0.2 |

## 4.6. Insecticidal activity test results

Insecticidal activity test of the oil extract and its fraction (MLO-4) on insects (S.Zeamais, termite and bed bug) is presented in table below. Adult mortality significantly increased with increase in concentration and hour of exposure on three different insect species.

The highest value of 100% mortality of Maize weevil was observed in the treatment of oil extract 5.0000% on 9 hours, This followed by 2.5000%, 1.2500%, 0.6250% and 0.3125%.However on MLO-4 treatment the highest value of 100%mortality observed on 6 hours The oil extract and its fraction on application, covered the outer layer of the grains (there by serving as food poison to the adult insects). The mortality of Maize weevil has been graphically represented in figure.

Table 6: Effect of seed oil on adult Maize Weevils ( Sitophilus zeamais)

| Plant extract | Concentration(%,v/w) | Mean mortality(%) at 3_12hrs post treatment | | | |
|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 |
| Maesalanceolata crude extract(Oil) | 0.3125 | 7.50 | 22.50 | 40.50 | 55.50 |
| | 0.6250 | 10.00 | 35.00 | 60.00 | 75.00 |
| | 1.2500 | 17.50 | 42.50 | 50.00 | 82.50 |
| | 2.5000 | 20.00 | 50.00 | 80.00 | 90.00 |
| | 5.0000 | 27.50 | 82.50 | 100.00 | |
| | Standard (Malathion5%) | 52.00 | 92.50 | 100.00 | |
| | Control(solvent-treated) | 2.50 | - | - | - |

**The mortality of Maize weevil has been graphically represent in figure**

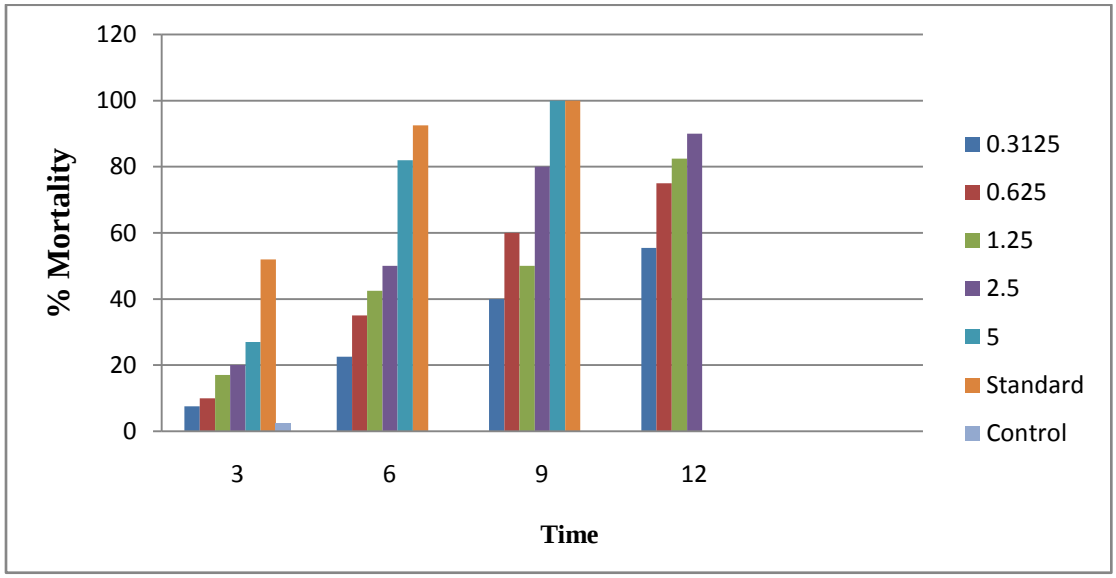



Figure 6: Plot of percent mortality of maize weevil time with different concentration of *M.lanceolata* seed o

Table 7: Effect of *MLO-4* on Maize Weevils ( Sitophilus zeamais)

| Plant extract | Concentration(%,v/w) | Mean mortality(%) at3-9hrs post treatment | | |
|---|---|---|---|---|
| | | 3 | 6 | 9 |
| MLO-4 | 0.3125 | 20.00 | 60.00 | 80.00 |
| | 0.6250 | 20.00 | 60.00 | 90.00 |
| | 1.2500 | 40.00 | 80.00 | 100.00 |
| | 2.5000 | 65.00 | 100.00 | - |
| | 5.0000 | 90.00 | 100.00 | - |

| | | | | |
|---|---|---|---|---|
| | Standard (Malathion5%) | 100.00 | - | - |
| | Control(solvent)treated | 0.00 | 0.00 | 0.00 |

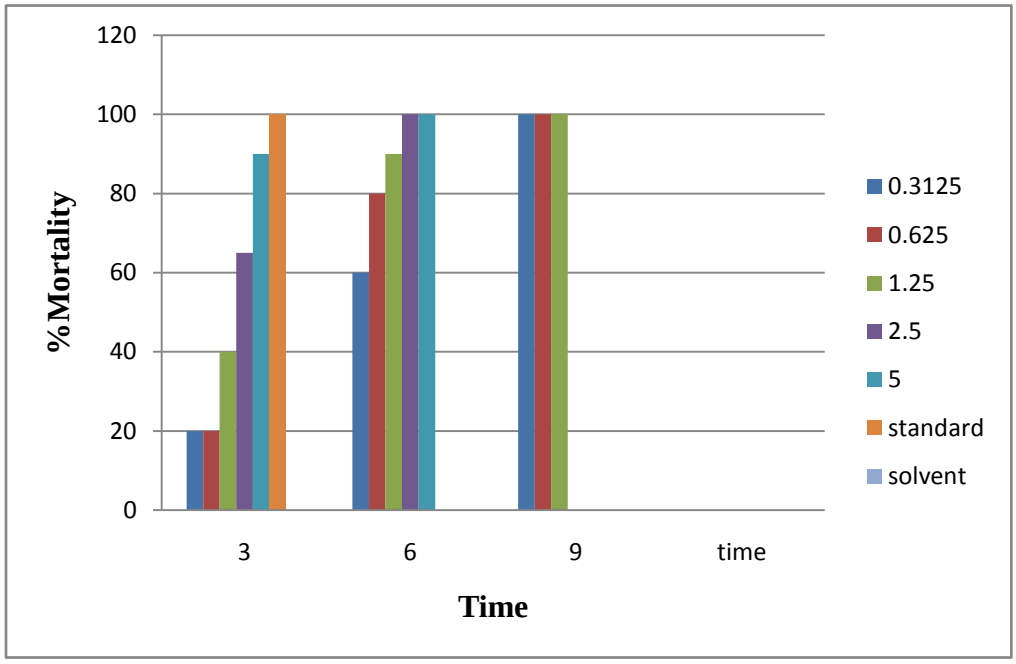



Figure 7: Plot of percent mortality of maize weevil time with different concentration of MLO-4.

The highest of 100% mortality of worker termite was observed in the treatment of oil extract 5% on 1:30 hrs and 0.3125% on4:00 hr, but80% mortality of solder termites was on 4:00 hr in the treatment of 5% and 20% in the 0.3125% treatment.100%mortality of worker and Solder termites highest on 1:30 hrs in the treatment MLO-4 of 5.0000% followed by 2.5000, 1.2500, 0.6250 and 0.3125%

**Table 8**: Effect of seed oil on adult termite

| Time(30min_4 hrs)post treatment | | Mean mortality (%) with concentration (v/w %) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0.3125 | 0.6250 | 1.2500 | 2.5000 | 5.0000 | Standard (Boric acid %) | Control(Solvent treated |
| 30min | W | 13.33 | 13.33 | 33.33 | 40.00 | 46.66 | 13.33 | 0.00 |
| | S | - | - | - | - | - | - | 0.00 |
| 1:00 | W | 33.33 | 33.33 | 53.33 | 66.66 | 73.33 | 33.33 | 0.00 |
| | S | - | - | | - | 40.00 | 20.00 | 0.00 |
| 1:30 | W | 46.66 | 46.66 | 66.66 | 73.33 | 100.00 | 53.33 | 0.00 |
| | S | - | - | 20.00 | 20.00 | 40.00 | 40.00 | 0.00 |
| 2:00 | W | 60.00 | 60.00 | 73.33 | 86.66 | - | 66.66 | 0.00 |
| | S | - | - | 20.00 | 40.00 | 60.00 | 40.00 | 0.00 |
| 2:30 | W | 73.33 | 73.00 | 86.66 | 100.00 | - | 73.33 | 0.00 |
| | S | - | 20.00 | 40.00 | 40.00 | 60.00 | 40.00 | 0.00 |
| 3:00 | W | 86.66 | 93.33 | 100.00 | - | - | 73.33 | 0.00 |
| | S | - | 20.00 | 40.00 | 40.00 | 60.00 | 60.00 | 0.00 |
| 3:30 | W | 93.33 | 100.00 | - | - | - | 80.00 | 0.00 |
| | S | 20.00 | 20.00 | 40.00 | 60.00 | 60.00 | 80.00 | 0.00 |
| 4:00 | W | 100.00 | - | - | - | - | 80.00 | 0.00 |
| | S | 20.00 | 20.00 | 40.00 | 60.00 | 80.00 | 80.00 | 0.00 |

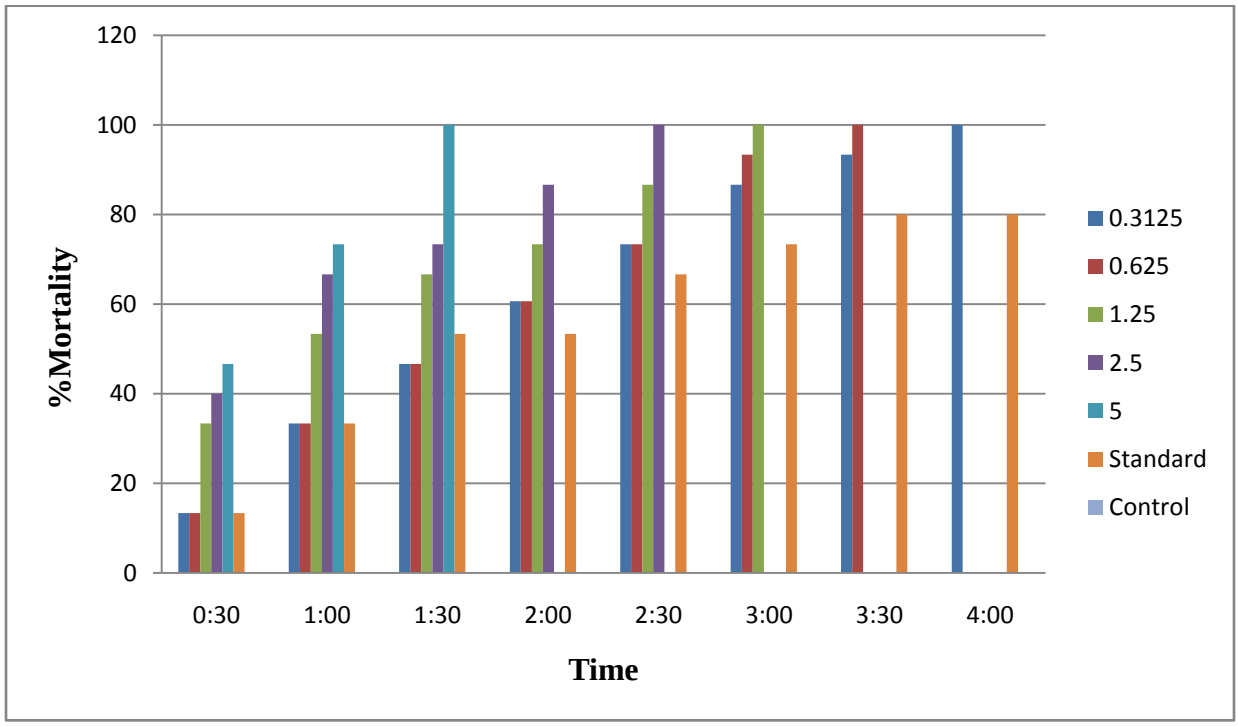



Figure 8: Plot of percent mortality of worker termite time with different concentration of seed oil

Table 9: the effect of MLO-4 on termite

| (30min_4hrs)post treatment | | Mean mortality (%) with concentration (v/w %) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0.3125 | 0.6250 | 1.2500 | 2.500 | 5.0000 | Standard (5%Malathion) | Control(Solvent treated) |
| 30min. | W | 20.00 | 33.33 | 36.66 | 56.66 | 66.66 | 86.66 | 0.00 |
| | S | 0.00 | 0.00 | 20.00 | 20.00 | 40.00 | 60.00 | 0.00 |
| 1:00 | W | 40.00 | 53.33 | 56.66 | 63.33 | 93.33 | 100.00 | 0.00 |
| | S | 0.00 | 20.00 | 20.00 | 40.00 | 80.00 | 80.00 | 0.00 |
| 1:30 | W | 53.33 | 66.66 | 73.33 | 86.66 | 100.00 | - | 0.00 |
| | S | 20.00 | 40.00 | 40.00 | 60.00 | 100.00 | 100.00 | 0.00 |
| 2:00 | W | 60.00 | 73.33 | 86.66 | 93.33 | - | - | 0.00 |
| | S | 40.00 | 40.00 | 60.00 | 80.00 | - | - | 0.00 |
| 2:30 | W | 73.33 | 80.00 | 93.33 | 100.00 | - | - | 0.00 |
| | S | 60.00 | 60.00 | 60.00 | 100.00 | - | - | 0.00 |
| 3:00 | W | 86.66 | 93.33 | 100.00 | - | - | - | 0.00 |
| | S | 60.00 | 60.00 | 80.00 | - | - | - | 0.00 |

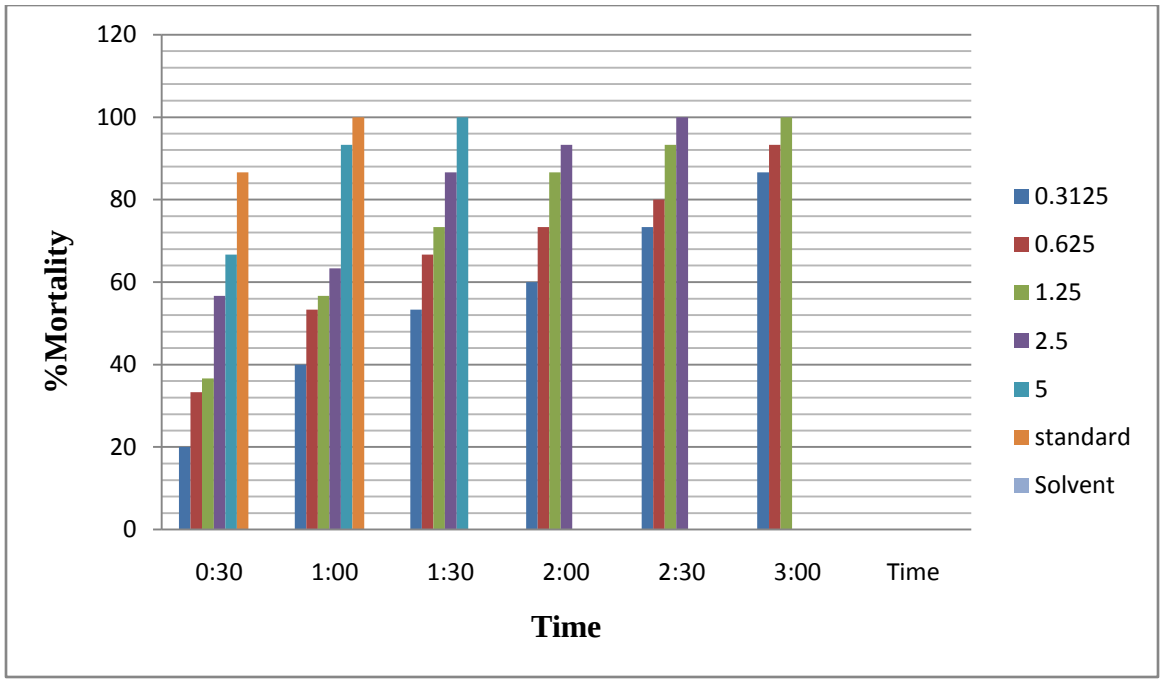



Figure 9: Plot of percent mortality of worker termite time with different concentration of MLO-4

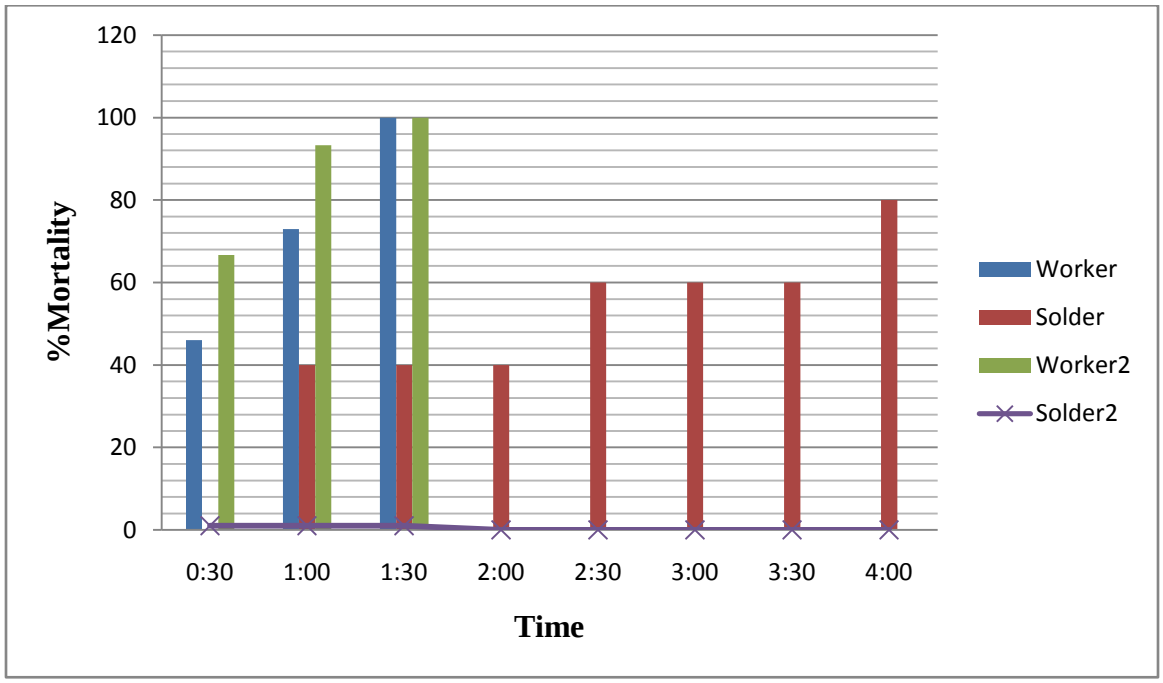



Figure 10: Plot of percent mortality comparison of worker and solder of termite time with 5.00% concentration of seed oil and MLO-4

The highest 100% mortality of bed bug observed on 3 days in the treatment of 5%and 75%on 4 days in the small treatment(concentration).

Table 10: Effect of seed oil on Bedbug

| Plant extract | Concentration(%,v/w) | Mean mortality(%) at 1_4 days post treatment | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| Maesalanceolata crude extract(Oil) | 0.3125 | 35.00 | 45.00 | 60.00 | 75.00 |
| | 0.6250 | 40.00 | 50.00 | 65.00 | 85.00 |
| | 1.2500 | 55.00 | 70.00 | 85.00 | 90.00 |
| | 2.5000 | 60.00 | 75.00 | 85.00 | 95.00 |
| | 5.0000 | 80.00 | 90.00 | 100.00 | |
| | Standard z(Boric acid 5%) | 10.00 | 15.00 | 30.00 | 35.00 |
| | Control(solvent-treated) | 0.00 | 0.00 | 0.00 | 0.00 |

Each value is the mean of three replicate.

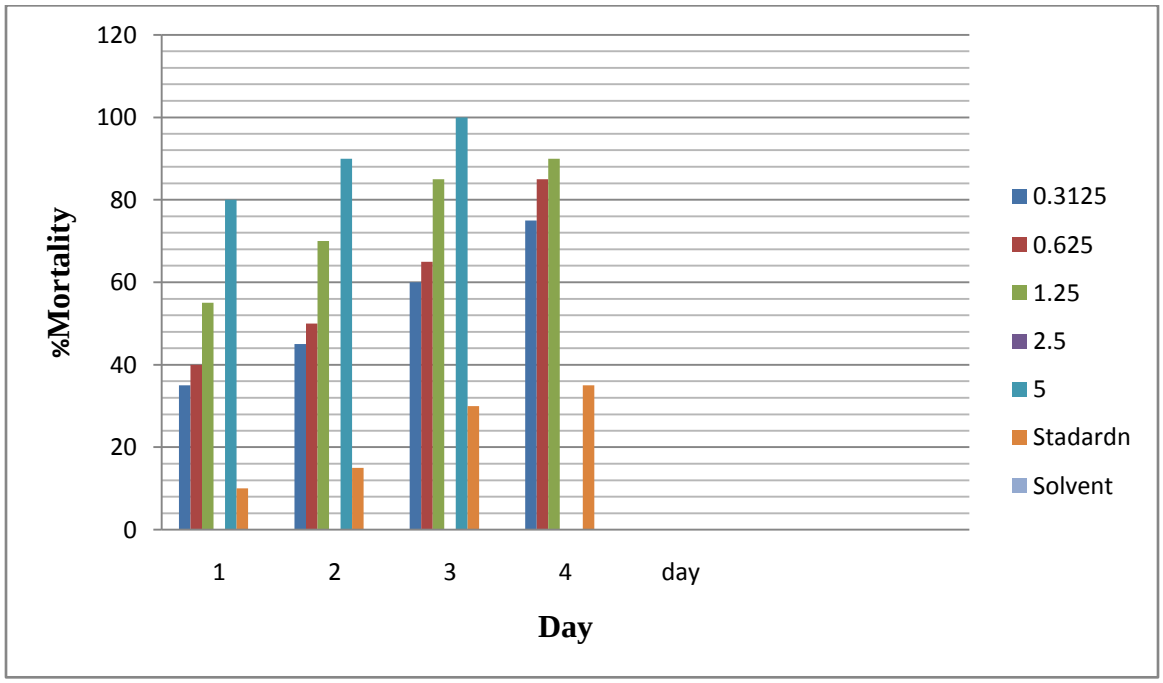



Figure 11: Plot of percent mortality of bed bug day with different concentration of seed oil

## 4.7. Antibacterial activity test results

Antibacterial activity test of seed oil and its three fractions were performed using disc diffusion method .The zone of inhibition were measured in millimeters (mm) and compared the zone of inhibition of antibiotic (gentamicin) which was used as positive control. Antibacterial activity of seed oil and its fractions with positive control has been shown in the table 11 and the comparison of zone of inhibition graphically represent in figure.

Table 11shows the comparative results between the seed oil, its different fractions and the positive control against Bacillus cereus, Staphylococcus aureums and Escherichia coli

Table 11: Anti bacterial activity of the oil extract and its fractions

| Organisms | zone of inhibition (in mm) | | | | | |
|---|---|---|---|---|---|---|
| | MLO | MLO-2 | MLO-3 | MLO-4 | Gentamicin | DMSO |
| Bacillus cereus | 9 | NA | 12 | 8 | 18 | NA |
| Staphylococcus aureus | 14 | 10 | NA | NA | 18 | NA |
| Escherichia coli | NA | NA | NA | NA | 18 | NA |

NA-not active

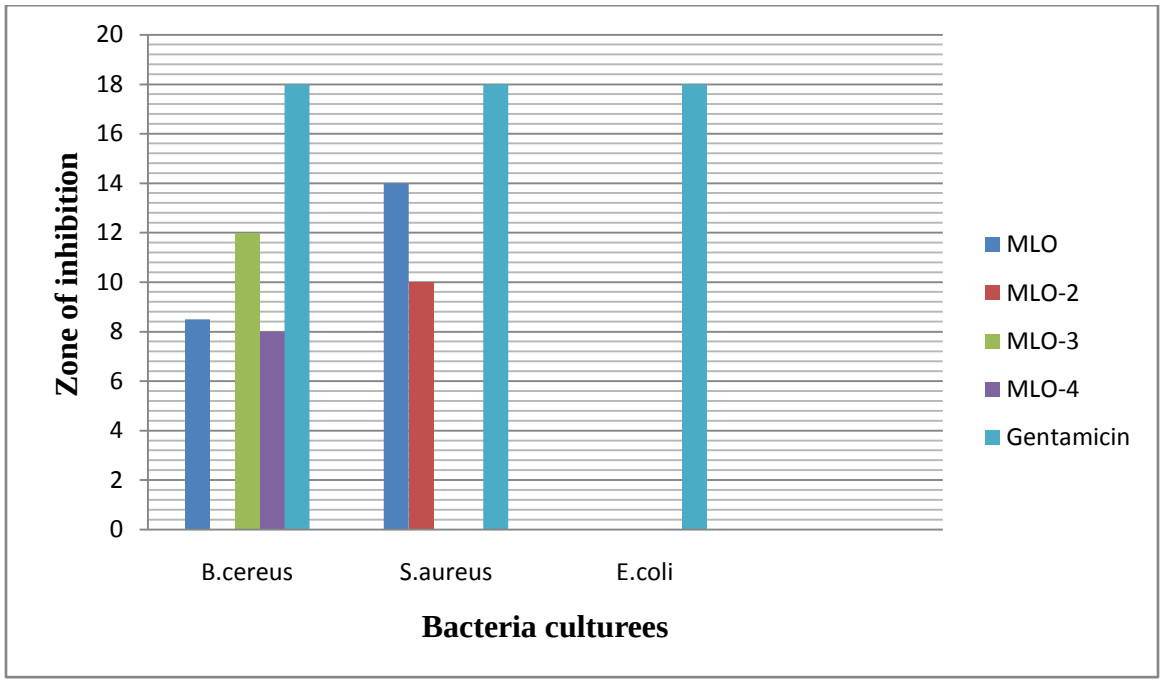



Figure 12: Zone of inhibition of seed oil, its fractions and antibiotics

Activity index of the seed oil and its fractions

Activity index was found out using the following formula

$$\text{Activity Index (AI)} = \frac{zone\ inhibition\ of\ sample}{\text{zone of inhibition of antibiotic}}$$

Table 12: Activity index of seed oil and its fractions

| Organism | MLO | MLO-2 | MLO-3 | MLO-4 |
|---|---|---|---|---|
| B.cereus | 0.5 | NI | 0.66 | 0.44 |
| S.aureus | 0.77 | 0.55 | NI | NI |
| E.coli | NI | NI | NI | NI |

Note-NI not inhibitory

## 4.8. Antifungal activity test results

Antifungal activity test of seed oil and its three fractions were performed using disc diffusion method .The zone of inhibition were measured in millimeters (mm) and compared the zone of inhibition of antibiotic (mancozeb) which was used as positive control. Antifungal activity of seed oil and its fractions with positive control has been shown in the table 11 and the comparison of zone of inhibition graphically represent in figure.

Table 13: Zone of inhibition of seed oil and its fractions against Fusarium .

| extracts | Inhibition zone(inmm)And activity index | |
|---|---|---|
| | Inhibition zone | Activity index |
| MLO | 11 | 0.92 |
| MLO-2 | 9 | 0.75 |
| MLO-3 | 11 | 0.92 |
| MLO-4 | 8 | 0,66 |
| Mancozeb | 12 | - |
| DMSO | NI | - |

Note-NI not inhibitory

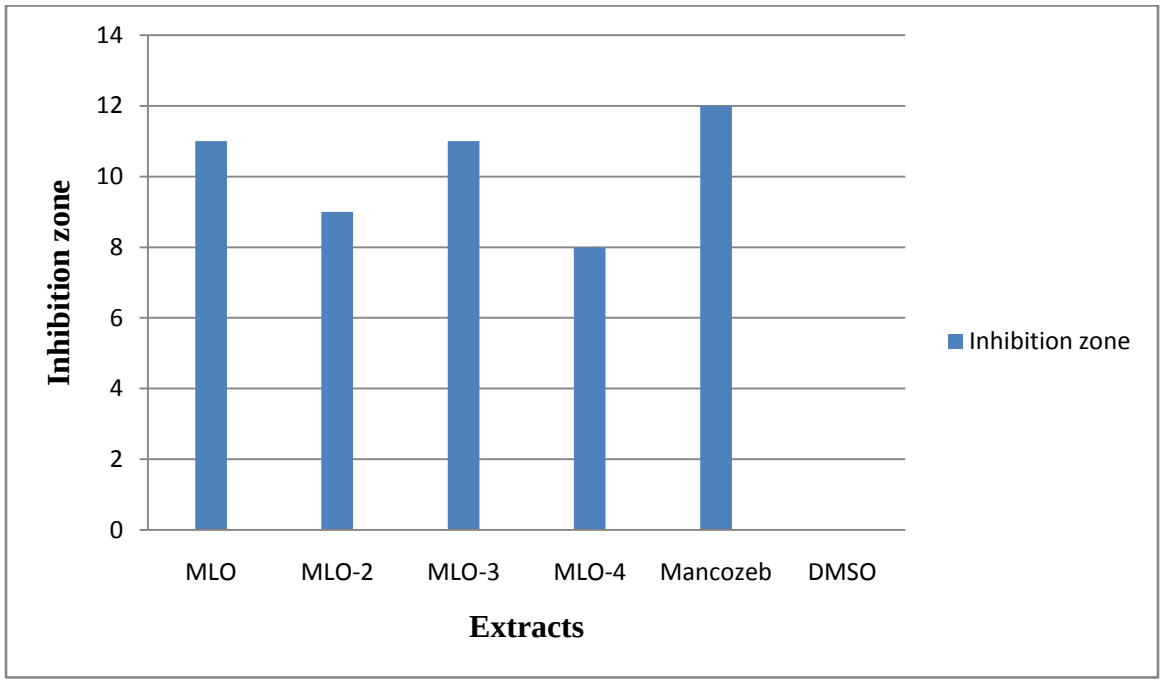



Figure 13: Zone inhibition inhibition of seed oil and its fractions against fusarum spp

# CHAPTER 5

## 5.0. Conclusion and recommendation

Fixed oils possess divers' application in food/feed, medicine, industry, agricultur and fuel. The extraction and characterization of *M.lanceolata* seed oil results obtained from the various tests and analysis carried out on M.lanceolata seed oil, it showed that oil can be extracted from the

*M.lanceolata seeds*. The percentage oil content is high and suggests that its extraction on commercial scale is possible and economical. The physico chemical parameter of M.lanceolata seed oil indicates this oil can be used in liquid soap production, detergents and shampoo industries. Because oil exhibit low saponification value and low free fatty acid indicates that oil have low deteriorating rate and high ediblity. This makes the oil good in quality and higher in molecular mass.

The oil extracted has potential source of bioactive components, such as quinones, alkaloids, terpinoids and flavonoids. The plant may be used for insecticidal, antibacterial, antifungal and an indicator. The result obtained in acid base titrations lead to as conclude that in strong acid-strong base titration was found to be more significant over standard indicator as it gives sharp color change at equivalence point showed that phenolphthalein indicator replaced successfully by M.lanceolata seed oil, it is simple, accurate ,economical , precise and prepared easily.

The proposed oil indicator can be used as substitute to synthetic (phenolphthalein) indicator. Based on Bronsted-Loweryacid base theory the proton from the M.lanceolata seed oil received by $^{-}OH$ ions thus causing color change.

The seed oil produces different chemical as secondary metabolites and used as controlling against insects, bacteria, and fungi. In the present study the petroleum extract of M.lanceolata oil and its fractions show significant insecticidal activity against maize weevil, termites and bed bug, anti bacterial and anti fungal activity against bacillus cereus and stphylcocous aureaus and afusarium respectively

## Recommendation

Further studies would still required for better understanding of especially the chemistry of *M lanceolata* seed NMR (2D),GM-MS,MS,LC-MS and elementary analyzer. The researchers believe that field level would needed to further validate and reproduce anti bacterial, anti fungal and insecticidal potential *of M.lanceolata* seed.

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## Annex I Figures show research activities

1. Mature seed of *Maesa lanceolata* plant from Benisangul gumuze region

2 .Sample collection

3. *Maesa lanceolata* seed

4. Grinded seed socked with petroleum ether and filtrate

5. Filtrate was concentrated by rotary evaporator


6. Extracted *Maesa lanceolata* oil

7. Optimization of seed oil in different ratio of petroleum ether to ethyl acetate

8. Seed oil observed with silica gel

9. Process of isolation of compounds using column chromatography




10. Fractionated compound to be purified

11. Purification process

12. Color change of phenolphthalein and MLO-4 in the presence of acid and base

A B A B

HCl before titration after titration

13. Color change of phenolphthalein and seed oil in the presence of acid and base

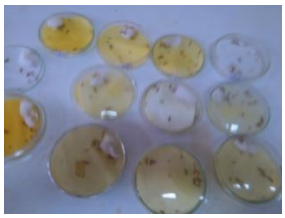
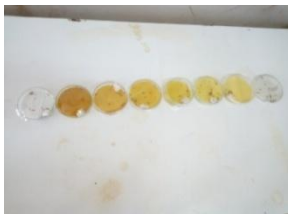
A B A B

HCl before titration after titration

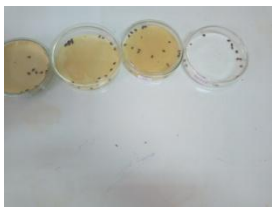
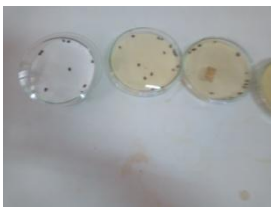
13. Rearing of *S.zeamais*


14. Mortality test for S.zeamais


15 .Mortality test for termites

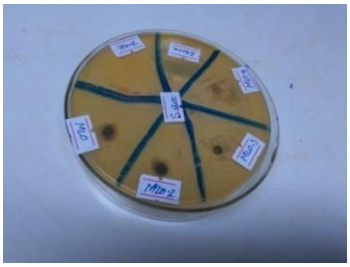



16 .Mortality test for bed bug

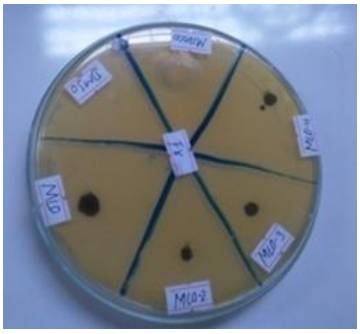
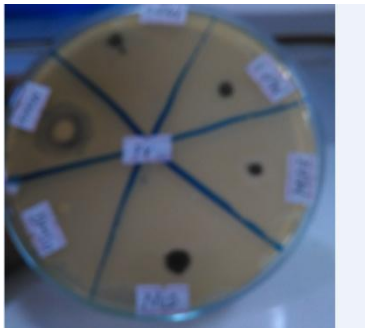




17. Anti bacterial and anti fungal activity of the oil extract and its fractions



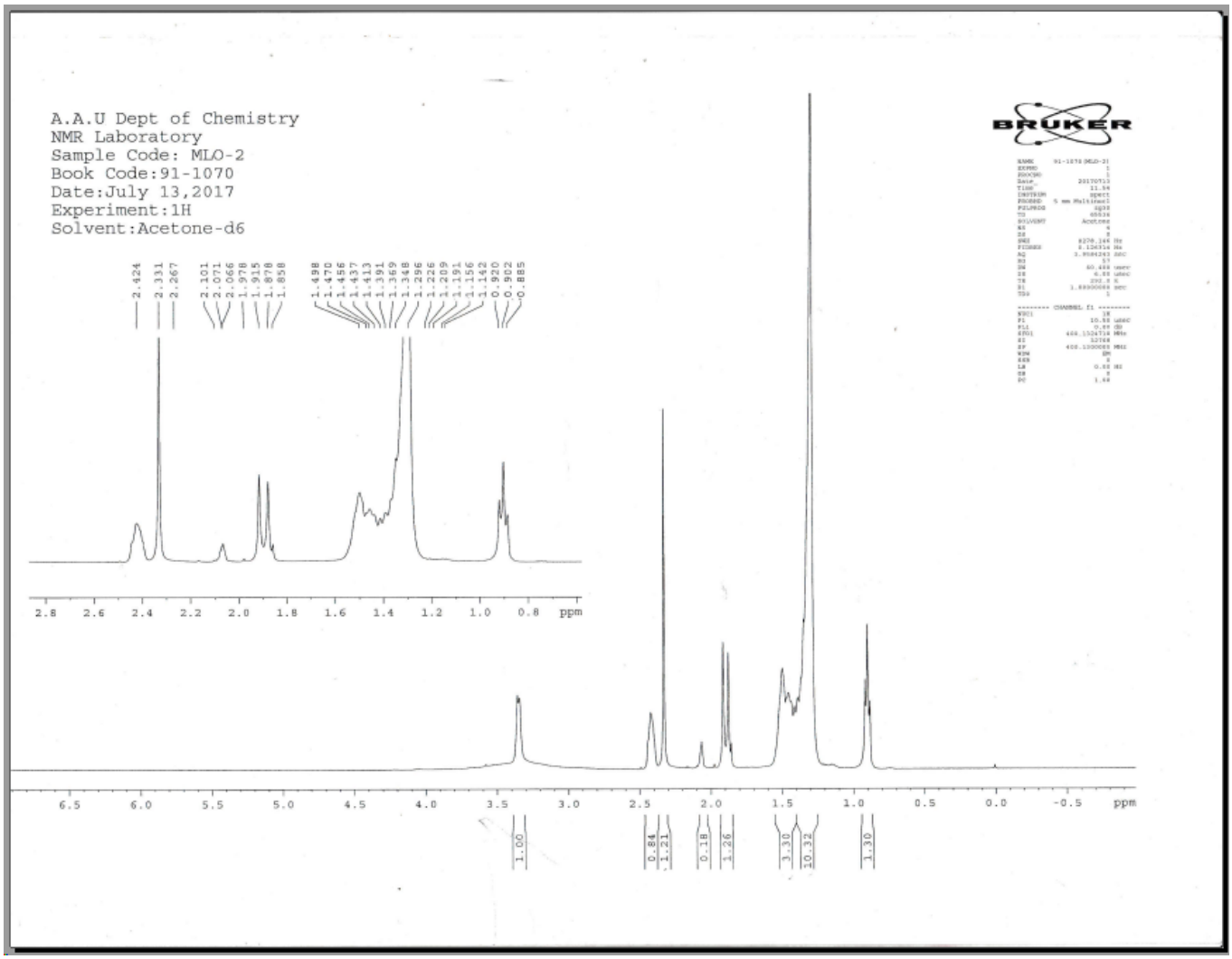


18. Anti fungal activity of the oil extract and its fractions

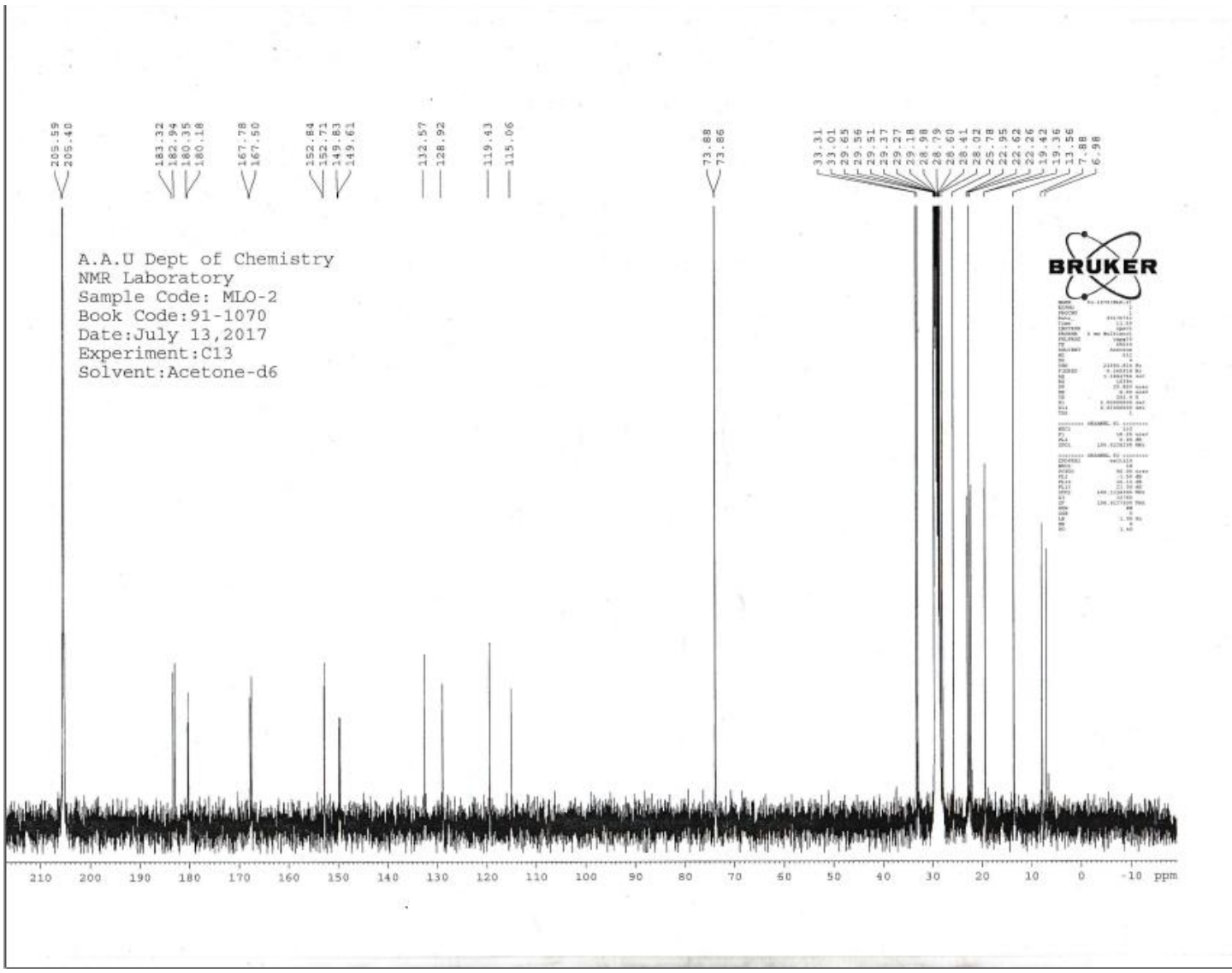



Annex II NMR data

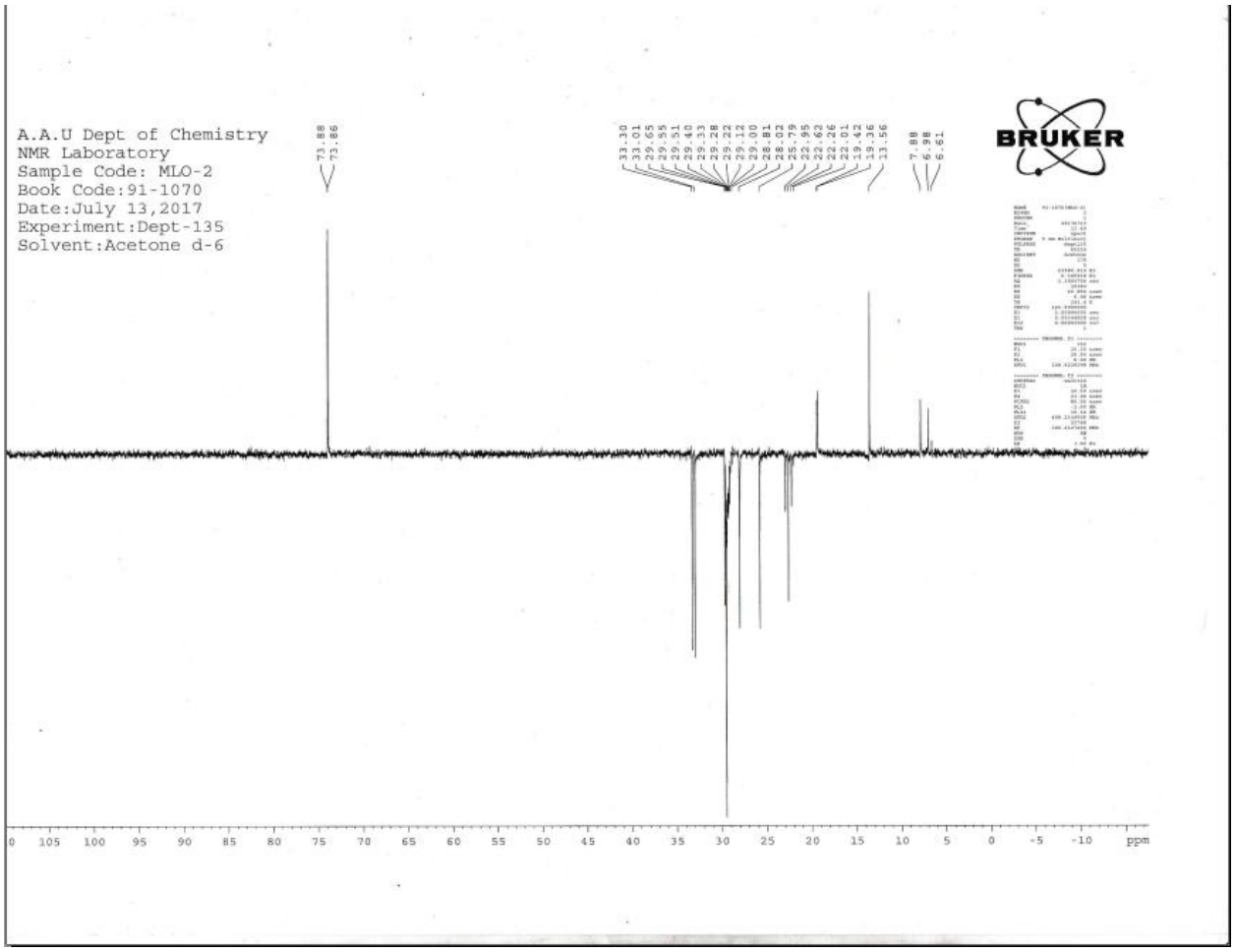
1.[1]H Chemical shift of MLO-2




**2.** [13]C Chemical shift of MLO-2




3. DPT-135 of MLO-2


A.A.U Dept of Chemistry
NMR Laboratory
Sample Code: MLO-2
Book Code:91-1070
Date:July 13,2017
Experiment:Dept-135
Solvent:Acetone d-6
73.88
73.86
33.30
33.01
29.65
29.55
29.51
29.40
29.33
29.28
29.22
29.12
29.00
28.81
28.02
25.79
22.95
22.62
22.26
22.01
19.42
19.36
13.56
7.88
6.88
6.51
BRUKER
105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0 -5 -10 ppm