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M.Sc THESIS

ON

PHYTOCHEMICAL INVESTIGATION OF *Olea europaea* STEM BARK AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITIES

BY

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JIMMA, ETHIOPIA

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A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES, JIMMA UNIVERSITY FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE (M.Sc) IN CHEMISTRY (GENERAL)

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Abbreviation and Acronyms

ATCC	American Type Culture Collection
CC	Column Chromatography
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
NMR	Nuclear Magnetic Resonance Spectroscopy
TLC	Thin Layer Chromatography
WHO	World Health Organization

Abstract

Medicinal plants play a significant role in the treatment of different diseases. The *Olea europaea subsp. cuspidata* is known by vernacular names "*Ejersa"* in Afaan Oromo and "*Weyira"* in Amharic, is one of the medicinal plants used by communities for the treatment of headaches, urinary tract /bladder/ infections, febrifuge and tapeworm; however, the phytochemicals and the biological activities of this plant have not exhaustively been reported. Therefore, the objective of this study was to isolate and characterize bioactive natural products from the stem bark of *Olea europaea subsp. cuspidata*. The air dried plant material was extracted sequentially with petroleum ether, chloroform, acetone and methanol at room temperature by cold maceration method. The antibacterial and antifungal activities of the extracts were evaluated against four pathogenic bacterial strains (*B. cereus* ATCC 10876*, S. aureus* ATCC 25923*, E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and two fungal strains (*Fuzarium spp.* and *S. cerviceas*) using agar disc diffusion method. The chloroform extracts showed good activity with the inhibition zone diameter of 8-10 mm against tested bacteria and marginal antifungal activity. Following its good activity, the chloroform extract was subjected to column chromatography for isolation of compounds, elution was carried using petroleum ether with increasing amounts of ethyl acetate and 298 fractions were obtained. Based on TLC profile, 10 fractions (F 232-241) obtained from crude by using 36% up to 32% petroleum ether in ethyl acetate were combined. For further purification, the combined fractions were subjected into Sephadex LH-20, eluted using 50% chloroform in methanol, and one pure compound was isolated. The structure of the isolated compound was characterized by spectroscopic techniques such as ^{1}H-NMR, ^{13}C-NMR and DEPT-135 NMR. The isolated compound is found to be (2-methylbenzofuran-6-yl)methanol and it show activities against all the tested strains, with the highest activity was observed against *B. cereus* and *S. aureus* bacterial strains. Devising alternative method of extraction as well as further isolation and characterization of bioactive compounds from this plant are recommended.

Key words: Medicinal plants, *Olea europaea subsp. cuspidata,* phytochemicals, antimicrobial activity, disc diffusion.

1. Introduction

1.1 Background of the study

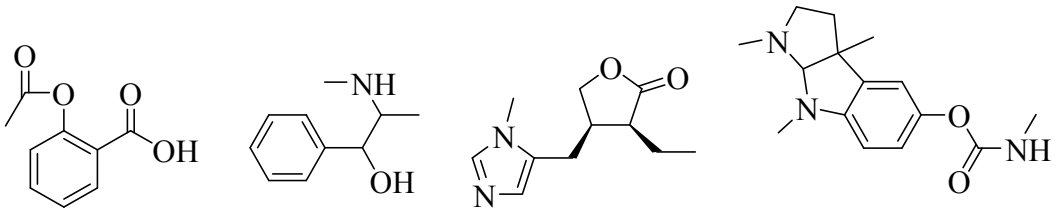
Plants produce a huge variety of natural products with highly diverse structures. These products are commonly termed as secondary metabolites in contrast to the primary metabolites, which are essential for plant growth and development. Secondary metabolites were formerly regarded as waste products without physiological function for the plant [1]. However, these natural products fulfill important functions in the interaction between plants and their biotic and abiotic environment. For example, secondary metabolites can serve as defense compounds against herbivores and pathogens, as flower pigments that attract pollinators, or as hormones or signal molecules. In addition to their physiological function in plants, natural products also have a strong impact on human culture and have been used throughout human history as condiments, pigments, and pharmaceuticals. Humans exploit natural products as sources of drugs, flavoring agents, fragrances and for a wide range of other applications [1].

Natural product molecules originated from plants, microorganisms, and animals has had an irreplaceable role throughout the last 200 years in treating and preventing diseases, and continue to serve as important leads in modern drug discovery [2, 3]. A significant proportion of the natural products used as drugs are derived from terrestrial plants, which offer an invaluable and still incompletely exhausted resource for this purpose [2]. In addition, profound ethnomedical knowledge based on the use of medicinal plants by humans has been accumulated for thousands of years. In the last few decades, pharmaceutical research on plants have been facilitated by the development of relevant technologies including new isolation methods, more sensitive spectroscopic techniques for structural determination, as well as specific high-throughput bioassay systems [2].

Medicinal plants has been used as a major source of drugs for thousands of years in human history and even today, they are basis of the systematic traditional medicine practices in many countries all over the world [4]. A huge number of these plant species have been used in treating numerous ailments for decades [5, 6]. In Africa, the use of traditional medicine dates back 4,000 years ago before the use of orthodox medicine [7]. According to the WHO, traditional medicine

remains the primary healthcare system for an estimated 80% of the population in Africa, because of its affordability and accessibility [8, 9].

Plant fragments such as leaves, barks, roots, flowers and seeds can be used as source of traditional remedies [10]. These can be prepared not only from a single plant but a combination of plant concoctions [11], aiding in ailments such as influenza, arthritis, heart burn, kidney infections, high blood pressure, etc [10]. They have also contributed to the management of epidemic diseases such as HIV/AIDS [12], malaria [13] and diabetes [8]. Their therapeutic potential is due to the existence of phytochemicals, which comprise of tannins, alkaloids, flavonoids, essential oils and chemical compounds established as subordinate metabolites in plants [6]. It has been reported that at least 25% of commercial drugs are derivatives from plants [8], such as aspirin (**1**) (anti pain) [6], ephedrine (**2**) (bronchodilator), pilocarpine (**3**) (parasympathomimetic), and physostigmine (**4**) (cholinesterase inhibitor) are still being widely used today [14] and various others are analogues made by chemical synthesis fabricated from isolated compound from plants [15].



1 **2** **3** **4**

Figure 1. Commercial drugs derivate from plants

Microbial have the ability to cause an infection both on and in man's body. Infections caused by microbial can be prevented, managed and treated through antimicrobial. An antimicrobial is an agent that kills microorganisms or stops their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotics are used against bacteria and antifungal are used against fungi. When microbial are exposed to an antimicrobial, they are sensitive to what cause the inhibition of their growth, division and death, and they can remain unaffected or resistant. The medicinal properties of plants can be attributed to secondary metabolites [16]. The *in vitro* experiments showed that plants produce a great number of secondary metabolites that have antimicrobial activity [17].

There has been a sudden growth in the interest of studying and using medicinal plants, which have led to the isolation of active chemical compounds for therapeutic significance [18]. The *Olea europaea subsp. cuspidata* plant was used extensively to treat various diseases traditionally. However, the data are small regarding the safety, quality and efficiency of the plants used in traditional medicine [5]. Therefore, the present study was aimed to investigate the phytochemical constituents and evaluation of antimicrobial activity from medicinal plant *Olea europaea subsp. cuspidata* stem bark.

1.2 Statement of the problem

Plants have been consumed in medicine to treat infectious diseases and to improve human's health. Traditionally, many plants with medicinal features are used to treat bacterial pathogens [19]. In both developed and developing countries, plant materials, which are the main sources of natural products have a variety of antibiotic resistant bacteria and fewer negative impacts [20].

The *Olea europaea* subsp. *cuspidata* had been used in traditional medicines for many years. It was used in hemorrhages treatment and fevers as a metabolism inducer and bile flow stimulator [21]. It was also used as astringent, antiseptic and a general tonic [21, 22].

In our countries some people uses *Olea europaea subsp. cuspidata* traditionally for medicinal purposes. The leaf parts of the plant is used for treatments of health issues such as backaches or headaches, eye infections, urinary tract infections, hypotensive and febrifuge [23]. A decoction of grated root and scraped bark is taken to treat urinary and bladder infections, tapeworm, ascariasis and diarrhea [24, 25]. Bark decoctions are also used for the treatment of dermatitis, itches and rashes [26]. The extract of olive leaves was also reported to contain a strong antibacterial and antifungal action [21]. Even though the plants have different medicinal values and antimicrobial effects, the isolation of the compound, structural elucidation and identification of the bioactive compounds have not been done in detail. Considering the few available phytochemical study on the leaf parts of the plant, the present study was focused on exhaustive phytochemical investigation and the evaluation of the bioactive molecules for their antimicrobial activity from the stem bark of *Olea europaea subsp. cuspidata.*

1.3 Objectives of the study

1.3.1 General objective

The main objective of this study was to investigate phytochemical composition and antimicrobial activities of the stem bark of *Olea europaea subsp. cuspidata.*

1.3.2 Specific objectives

i. To evaluate antimicrobial activities of the crude extracts and isolated compounds from the stem bark *of Olea europaea subsp. cuspidata* against four strains of bacteria *(Bacillus cereus, Staphylococcus aureus, Escherichia coli,* a*nd Pseudomonas aeruginosa*) and two strains of fungi *(Fusarium spp* and *S. cerviceas*) using agar disc diffusion method.

ii. To isolate compounds from the stem bark of the *Olea europaea subsp. cuspidata* by using column chromatography.

iii. To characterize the isolated compound(s) using NMR spectroscopic techniques (^{1}H, ^{13}C and DEPT-135).

1.4 Significance of the study

The treatment of infectious diseases is an important and challenging problem because of a combination of factors including emerging infectious diseases and the increasing number of multi-drug resistant microbial pathogens.

Therefore, findings of this study would:

i. Provide information about the chemical profile of the stem bark of *Olea europaea subsp. cuspidata* that is responsible for antimicrobial activities.

ii. Produce candidate compounds for antimicrobial activities.

2. Review of Related Literature

2.1 Botanical information of *Olea europaea* subsp. *cuspidata*

The *Olea europaea subsp. cuspidata* belongs to the family *Oleaceae*, a family of dicotyledons [27], containing 600 species in 25 genera, and some genera are wide and arise in several continents [28]. Its genus *Olea* comprises 30 species in which species of the family are trees, shrubs or woody climbers including the olive tree [27, 28]. The *Olea* genus has six subspecies namely: *cuspidata, laperrinei, maroccana, cerasiformis, guanchica* and *europaea* [29].

The *Olea europaea subsp. cuspidata* is a native for South Africa, from which it spread through the Middle East, Pakistan, India and China. In the nineteenth century, it was introduced to the Australian territory for economic purposes [30, 31]. The *Olea europaea* subsp. *laperrinei* is restricted to the massifs of central - southern Sahara and eastern Sahel [29, 32]. The *Olea europaea* subsp. *maroccana* is located in the South - west of Morocco, in the western part of the High Atlas [29]. Wild olive populations present in the Canary Islands are ascribed to the species *Olea europaea* subsp. *cerasiformis*. A recent genetic study concluded that populations of Madeira and the Canary Islands were genetically separate enough as to be separated into distinct subspecies; therefore, the Canarian wild pass was renamed *Olea europaea* subsp. *guanchica* [29]. This subspecies is present throughout the islands forming part of transition forests or thermophiles [29]. The *Olea europaea subsp. europaea* is a typical tree of the Mediterranean regions and it contributed to the Mediterranean forest [33]. The African species of *Olea europaea*, previously acknowledged as *Olea africana subsp. cuspidata*, defined as *Olea europaea* subsp. *africana* (Mill) in the early 1980 [7, 23]. In Africa, it is commonly known as the *wild olive* [23].

In Ehiopia, *Olea europaea subsp. cuspidata* is known by vernacular names "*Ejersa"* in Afaan Oromo and "*Weyira"* in Amharic. It widely grows in natural forests in many parts of Ethiopia. In Ethiopia, the species is widely grown in fields and church areas [34]. It distributed in Africa, Arabia, Himalayas and Southwest Asia [34, 35]. In non-native area such as Australia, is an aggressive weed [36].

The plant is an evergreen tree usually growing 2-15 m in height [31]. It is a tree growing at an altitude of 1250-3100 meters above sea level [37]. The leaves oppositely arranged with entire

margins that are often recurved. The flowers are borne in small clusters at the ends of the branches or in the leaf forks and it occurs mostly during spring. The plant species reproduced mainly by seed and the seeds are dispersed by air as well as when the fruit are eaten by birds and other animals [30, 31].

Figure 2. *Olea europaea subsp. cuspidata* tree (photography taken from study area, Dembi)

2.2 Ethnomedicinal information of *Olea europaea* subsp. *cuspidata*

The natural olive leaf extract have become popular as commercial herbal medicines marketed as having anti-ageing, immune stimulant and antibiotic properties. The main active compound in olive leaf responsible for antioxidant, antimicrobial, hypolipidemic and especially hypotensive activities is the well-known bitter principle of olives, the secoiridoid called oleuropein (**6**) [38] and they are also functioned as a hypotensive, emollient, febrifuge, styptic [23], taenifuge and fumigant [24, 39].

The leaf of *Olea europaea subsp. cuspidata* is used as treatments for health issues such as backaches or headaches, eye infections, sore throat, urinary tract infections and kidney problems. In fact, the leaves of the tree were effectively used for the treatment of malaria in 1854 [21]. Dried fruit powder mixed with oil and rubbed into aching joints [24, 40].

A leaf decoction is gargled to treat diphtheria and other sore throats [24]. A decoction of grated root and scraped bark is taken to treat urinary and bladder infections, tapeworm, ascariasis, diarrhea, intestinal infections and headaches [24, 25]. Root decoctions are used against headache, influenza, fever and rheumatism [24]. Bark decoction is used to treat an itching rash [41], internally and topically for the treatment of dermatitis, itches and rashes [26], helminthiasis, asthma, rheumatism and lumbago [42]. Stem is used in soup to treat backache and painful joints [43].

2.3 Phytochemical constituents of *Olea europaea subsp. cuspidata*

Phytochemicals are various biologically active compounds that occur naturally in plants, which provide potential medicinal benefits for humans. These chemicals accumulate in several parts of the plant including the flower, stems, bark, seed, roots and leaves [44]. Phytochemical screening of the African wild olive has led to the separation of subclass of phenolic compounds including phenols [45], flavonoids [46], triterpenoids [23, 47], coumarins [48] and others.

i. Phenolic compounds

Phenols are a class of chemical compounds consisting of a hydroxyl functional group (-OH) attached to an aromatic phenolic group. The position and number of hydroxyl groups on the phenol group are related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity [19]. Plant phenols are aromatic secondary metabolites that occur in the plant [45]. Some of the phenolic compounds that occur in *Olea europaea subsp. cuspidata* plant includes verbascoside (**5**), oleuropein (**6**), rutin (**7**), luteolin 7-O-glucoside (**8**), apigenin 7-O-glucoside (**9**), luteolin 4'-O-glucoside (**10**), caffeic acid (**11**), tyrosol (**12**), hydroxytyrosol (**13**) and 3, 4-dihydroxy-benzeneacetic acid (1**4**) [21, 45].

Coumarins are large class of phenolic compounds in plants containing 1, 2-benzopyrone [49, 50]. Some of the coumarins compounds that occur in the *Olea europaea subsp. cuspidata* plant are esculin (**15**) and scopolin (**16**) [48, 51].

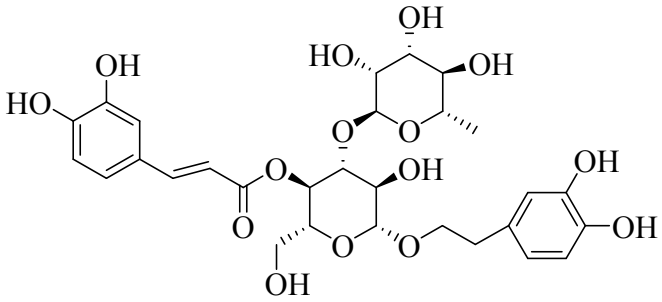
ii. Triterpenoids

Terpenes are a large and varied class of organic compounds built up from isoprene subunits, while the terpenoids are oxygen-containing analogues of the terpenes. Triterpenoids are group of natural products including steroids widely dispersed in plants [23, 47]. Some of these compounds isolated from *Olea europaea subsp. cuspidata* plant include oleanolic acid (**17**), erythrodiol (**18**), ursolic acid (**19**) and uvaol (**20**). Some of steroid compounds isolated from the plant is β-sitosterol (**21**) [23, 47].

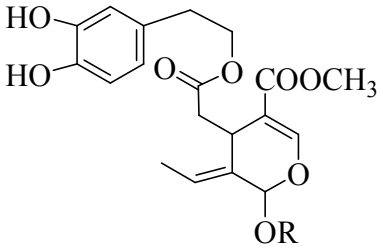
iii. Miscellaneous

Moreover, compounds such as 8-((2*R*, 3S)-3-octyloxiran-2-yl)octanoic acid (**22**), di-n-octyl phthalate (**23**), dibutyl phthalate (**24**), (*E*)-methyl octadec-9-enoate (**25**), oleic acid (**26**) and 1-[7'-methylbenzofuran-2'-carbonyl]-3-ethylazulene (**27**) are isolated from the plant [52].

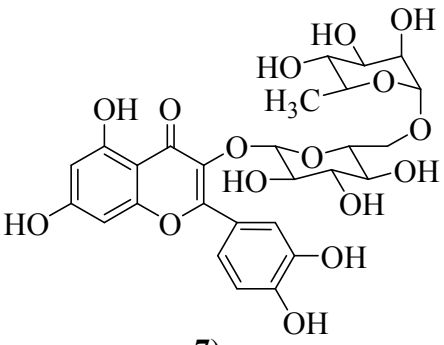
Compounds such as oleuropein (**6**), hydroxytyrosol (**13**), oleanolic acid (**17**), erythrodiol (**18**), ursolic acid (**19**), uvaol (**20**) [45, 47], 3, 4-dihydroxy-benzeneacetic acid (1**4**), di-n-octyl phthalate (**23**) and 1-[7'-methylbenzofuran-2'-carbonyl]-3-ethylazulene (**27**) are isolated from leave of *Olea europaea* [52]. Compounds such as esculin (**15**), scopolin (**16**), β-sitosterol (**21**), dibutyl phthalate (**24**) and oleic acid (**26**) are isolated from bark of *Olea europaea* [51, 52]. Compounds such as tyrosol (**12**), 8-((2*R*, 3S)-3-octyloxiran-2-yl)octanoic acid (**22**) and (*E*)-methyl octadec-9-enoate (**25**) are isolated from both bark and leave of *Olea europaea* [52].



5)



6) R = Glu



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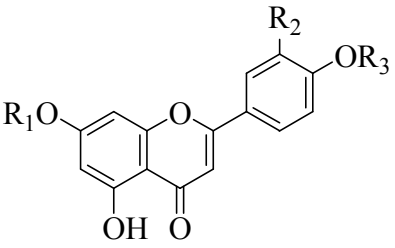
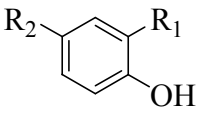
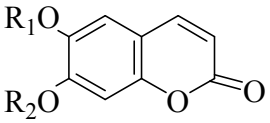


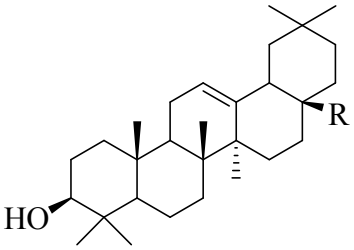
8) R_1 = Glu, R_2 = OH, R_3 = H
9) R_1 = Glu, R_2 = H, R_3 = H
10) R_1 = H, R_2 = OH, R_3 = Glu



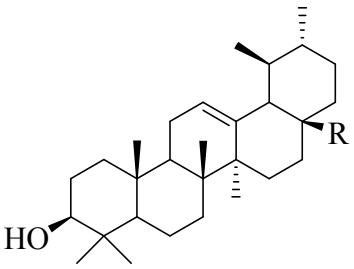
11) R_1 = OH, R_2 = CH=CHCOOH
12) R_1 = H, R_2 = CH_2CH_2OH
13) R_1 = OH, R_2 = CH_2CH_2OH
14) R_1 = OH, R_2 = CH_2COOH



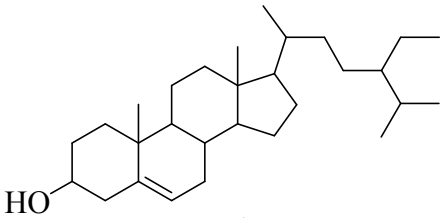
15) R_1 = Glu, R_2 = H
16) R_1 = CH3, R_2 = Glu



17) R = COOH
18) R = CH_2OH

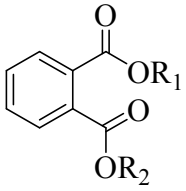


19) R = COOH
20) R = CH_2OH

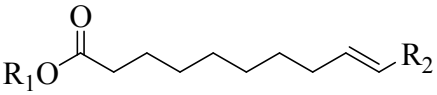


21)

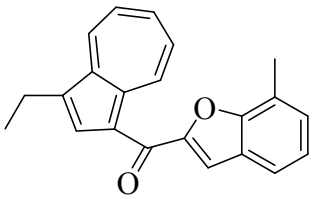
22) R_1 = $(CH_2)_7CH_3$,
R_2 = $(CH_2)_7COOH$



23) R_1 and R_2 = $(CH_2)_7CH_3$
24) R_1 and R_2 = $(CH_2)_3CH_3$



25) R_1 = CH_3, R_2 = $(CH_2)_7CH_3$
26) R_1 = H, R_2 = $(CH_2)_7CH_3$



27)

Figure 3. Compounds isolated from *Olea europaea subsp. cuspidata.*

2.4 Biological activity of *Olea europaea subsp. cuspidata*

The ethanolic extract of olive leaves was reported to contain a strong antibacterial and antifungal action [21, 53]. The antibacterial and antifungal actions of olive leaves are due to the phenolic compounds such as verbascoside (**5**), oleuropein (**6**) and caffeic acid (**11**) [21]. The triterpenoid compound oleanolic acid (**17**) is a biologically active pentacyclic triterpenoid with pharmacologic activities such as anticancer, hepatoprotective effects, antioxidant and anti-inflammatory [54]. Ursolic acid (**19**) is biologically used as an antioxidant, anticancer and anti-inflammatory chemical [55]. Erythrodiol (**18**) and uvaol (**20**) are used as antimalarial, antifungal, antileishmanial, antibacterial and anti-inflammatory activities [47]. Coumarins have received attention in the therapeutic fields such as chemotherapy, multiple sclerosis and organ transplants [56]. The antimicrobial activities of the plant extracts were tested against different pathogenic bacteria using agar well diffusion method [57]. The aqueous extracts of the plant showed lower antibacterial activities while the ethanolic extract of *Olea europaea subsp. cuspidata* was the most active extracts against different pathogenic bacteria [53]. For all the tested Gram-positive and Gram-negative bacteria the bacterial index for ethanol extract of *Olea europaea subsp. cuspidata* was > chloroform extract > acetone extract > methanol extract. This means the ethanolic plant extract exhibited the maximum zone of growth inhibition against some tested bacteria compared to other extracts [53, 58]. The Gram-positive bacteria used are such as *Staphylococcus aureus, Staphylococcus saprophyticus and Streptococcus pyogenes.* The Gram-negative bacteria used are such as *Escherichia coli, Klebsilla pneumonia, Pseudomonas aeruginosa, Salmonella sp. and Serrratia marcescens* [53].

3. Material and Methods

3.1 Chemicals

Chemicals that were used for this study includes methanol, ethyl acetate, acetone, chloroform, petroleum ether (60-80 ^{o}C) and silica gel 60-120 mesh for gradient column elution and extraction. Dimethyl sulfoxide (DMSO), Muller Hinton agar and nutrient agar as culture media, standard antibiotic drug (Gentamycin) and antifungal drug (Miconazole) were used during antimicrobial activity test. A deuterated chloroform ($CDCl_3$) solvent was used for recording NMR spectra. All the chemicals and reagents used were high-grade purity and analytical grade.

3.2 Apparatus and Equipments

Apparatus that were used for this study includes pestle and mortar, weighing balance, rotary evaporator (Labo Rota 4000, Heidolph Instrument), TLC plates, glass columns for column chromatography, UV chamber (UV-Tec) and Bruker advance 400 MHz NMR spectrometer.

3.3 Plant material collection and preparation

The stem bark of *Olea europaea subsp. cuspidata* was collected from South Western Ethiopia, Oromia Regional State, Buno Bedele Zone, Didesa district, Dembi kebele about 430 km away from Addis Ababa in July 2018. The collected plant part was washed under running tap water to remove associated debris, and air-dried under shade. The air dried sample was ground to suitable size using pestle and mortar to improve the subsequent extraction.

3.4 Extraction of plant material

A 1.2 kg of the powdered stem bark of *Olea europaea subsp. cuspidata* was soaked twice for 24 hours each at room temperature by using petroleum ether (2 x 2 L), chloroform (2 x 2 L), acetone (2 x 2 L) and once for methanol (1 x 2 L) sequentially by cold maceration method. The extracts obtained were filtered using cotton plug followed by Whatman filter paper and concentrated using rotary evaporator. The resulting semidried extracts were further dried and weighed. The resulting extracts were kept in desiccators until tested for their antimicrobial activities. Percentage yield of crude extracts were calculated by:

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

3.5 Isolation of compounds

Column chromatography was used to fractionate the most active gradient extract. The silica gel was activated at 105 ^{o}C for 1 h. First column was packed with 200 g silica gel (60-120 mesh size) impregnated using petroleum ether. Then the chloroform crude extract (15 g) adsorbed in silica gel (15 g) was applied to the column and elution was carried using petroleum ether with increasing amounts of ethyl acetate, 100:0, 98:2, 96:4, 94:6, 92:8, 90:10 up to 0:100 respectively. Binary solvent were used because, the best separation was obtained on TLC by this solvent. A 100 mL of solvent were used during each step and 298 fractions were obtained. For further purification, the combined fractions (F 232-241) were subjected into Sephadex LH-20 and eluted using 50% chloroform in methanol.

3.6 Antimicrobial activity tests

i. Test strains and culture

Four bacteria strains: two Gram positive (*Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923) and two Gram negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two strains of fungi: *Fusarium spp* and *S. cerviceas* were used for antibacterial and antifungal evaluation of both crude extract and isolated compounds. All are standard strains and all biological activity tests were carried at Biology Department, Microbiology Research Laboratory, Jimma University. The antibacterial and antifungal activity tests were carried after sub-culturing in nutrient agar at 37 ^{o}C.

ii. Antibacterial activity tests

Agar disc diffusion method was used to evaluate the antibacterial activities of both crude extracts and compound isolated from the plant. The bacteria stock cultures were maintained on the nutrient agar. The stock solution was prepared by taking 0.2 g of each of crude extract of petroleum ether, chloroform, acetone and methanol in 1 mL of DMSO. Then, sterile 6 mm filter paper discs were soaked in each stock solution of crude extract (200 mg/mL). Gentamycin and DMSO were also included as positive and negative controls, respectively. The discs were then placed on a cultured glass plate at equal distance to one another to avoid overlap of zones of growth inhibitions. Antibacterial activities of plant extract against bacterial strains were

determined after incubation of the plates for 24 h at 37 ºC, by measuring the diameter of zone of growth inhibition in mm using transparent ruler [59, 60].

iii. Antifungal activity tests

Agar disc diffusion method was also used to evaluate the antifungal activities of both crude extracts and compound isolated from the plant. Miconazole and DMSO were used as positive and negative controls, respectively. Antifungal activities were determined after incubation of the plates for 72 h at 37 ºC, by measuring the diameter of zone of growth inhibition in mm using transparent ruler [59, 60].

3.7 Characterization of the isolated compounds

Structure elucidation of isolated compound was processed by Bruker advance 400 MHz NMR spectrometer (^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra) using $CDCl_3$ as solvent. Chemical shifts were reported in ppm. All spectroscopic analysis were carried out at the Department of Chemistry, Addis Ababa University.

4. Result and Discussion

4.1 Extraction yield of crude extracts

The stem bark of *Olea europaea subsp. cuspidata* was collected, washed under running tap water, air-dried under shade and ground to suitable size using pestle and mortar to improve the subsequent extraction. The crude extracts were carried sequentially and as the polarity of extracting solvent increase, the mass (percentage yield) of the crude extract increase. The stem bark of *Olea europaea subsp. cuspidata* gave relatively higher yield of acetone extract (29.04 g, 2.42%) black crude and the least with petroleum ether extract (1.87 g, 0.16%) (Table 1). This result showed that most secondary metabolites in the stem bark of *Olea europaea subsp. cuspidata* are polar components.

Table 1. Percentage yield of the crude extracts obtained from sequential extraction of *Olea europaea* stem bark

Type of extract	Mass of the crude extract (g)	Percentage yield
Petroleum ether	1.87	0.16
Chloroform	16.43	1.37
Acetone	29.04	2.42
Methanol	17.84	1.49

4.2 Compounds isolated

The chloroform crude extract (15 g) adsorbed in silica gel (15 g) was applied to column chromatography and elution was carried using petroleum ether with increasing amounts of ethyl acetate. Binary solvent were used for column because, the best separation was obtained on TLC by this solvent. A total of 298 fractions were collected and some of these were combined based on TLC profile. Among these pooled fractions (F 232-241) obtained from 36% petroleum ether in ethyl acetate up to 32% petroleum ether in ethyl acetate were subjected to Sephadex LH-20 chromatography eluted using 50% chloroform in methanol. Then, 21 fractions collected each with 10 mL. Among these fractions 18-21 were combined and yield compound **1** (19.9 mg, 0.13%).

4.3 Characterization of the isolated compounds

Compound **1** was obtained as a brown amorphous solid (19.9 mg) with R_f value of 0.68 in petroleum ether: ethyl acetate (34:66). Structural elucidation of isolated compound was performed by using the spectroscopic data (^{1}H-NMR, ^{13}C-NMR, and DEPT-135) obtained in this study (Table 2, Appendix 1, 2 and 3).

From its, ^{1}H-NMR spectra (400 MHz, $CDCl_3$) (Appendix 1**)** chemical shift showed singlet peak at δ 0.90 ppm representing highly shielded methylene attached to hydroxyl proton, singlet peak at δ 1.27 ppm for one methyl protons, singlet peak at δ 3.97 ppm for hydroxyl proton, at δ 6.29-7.63 ppm for four methine proton, indicating the presence of aromatic ring system (Table 2).

The ^{13}C-NMR spectra (400 MHz, $CDCl_3$) of this compound (Appendix 2) displayed 10 carbons. The signal at δ 111.5-161.4 ppm indicated the presence of aromatic ring. From the ^{13}C-NMR and DEPT-135 spectra (400 MHz, $CDCl_3$) of this compound (Appendix 2 and 3) peak at δ 149.7 ppm, 111.5 ppm, 144.0 ppm and 161.4 ppm indicated the presence of quaternary carbon atoms of fused aromatic ring. The peak at δ 29.7 ppm and 56.4 ppm indicated the presence of methyl carbon and methylene carbon bearing hydroxyl group respectively. The peak at δ 103.2 ppm, 107.5 ppm, 113.4 ppm and 143.3 ppm indicated the presence of methine carbon (CH) in the structure (Table 2).

Table 2. ^{1}H, ^{13}C and DEPT -135 NMR Spectroscopic data for compound **1**

Observed ^{1}H, ^{13}C and DEPT -135 (400 MHz, $CDCl_3$)							
Proton	δ^{1}_{H}	J_{HH} (Hz)	m	Description	Position	$\delta^{13}C$	δ DEPT-135
H-9 (CH_2)	0.90	-	*s*	OH (1H)	C-1	-	-
H-8	1.27	-	*s*	CH_3 (3H)	C-2	149.7	C-
H-9 (OH)	3.97	-	*s*	CH_2 (2H)	C-3	103.2	CH-
H-5	6.29	8.8	*dd*	CH (1H)	C-3a	111.5	C-
H-4	6.93	26.8	*d*	CH (1H)	C-4	113.4	CH-
H-7	6.86	-	*s*	CH (1H)	C-5	143.3	CH-
H-3	7.63	-	*s*	CH (1H)	C-6	144.0	C-
					C-7	107.5	CH-
					C-7a	161.4	C-
					C-8	29.7	CH_3-
					C-9	56.4	CH_2-

Key: m = multiplicity, J_{HH} = Coupling, δ = Chemical shift in ppm, *s* - siglet, *d*- doublet, *dd* - doublet of doublet

Based on the above spectroscopic data (^{1}H, ^{13}C and DEPT-135 NMR), the molecular formula of compound **1** was predicted as $C_{10}H_{10}O_2$ and its structure was proposed as (2-methylbenzofuran-6-yl)methanol (Figure 4).

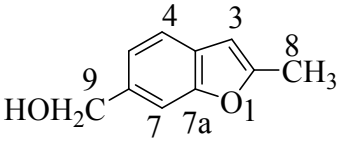


Figure 4. The proposed structure of compound **1**

The compound containing benzofuran ring, 1-[7'-methylbenzofuran-2'-carbonyl]-3-ethylazulene (**27**), was isolated from ethanolic extract of leave of *Olea species* [53]. Similarly, the results of this study also indicated that benzofuran derivative compound was isolated from the chloroform extract of stem bark of *Olea europaea* (Figure 4).

4.4 Result for antibacterial and antifungal activities

The crude extract of *Olea europaea* and the isolated compound **1** were tested against four bacterial strains (*B. cereus, S. aureus, E. coli* and *P. aeruginosa*) and two fungal strains (*Fuzarium spp.* and *S. cerviceas*). All the crude extracts and the isolated compound **1** were showed good activity against all tested bacterial and fungal strains (Table 3, Appendix 4 and 5). The zone of growth inhibition for all the crude extracts against bacterial strains and fungal strains are described (Table 3, Appendix 4).

The antimicrobial analysis showed the mean of diameter of zone of growth inhibition for petroleum ether extract 10.5 mm, 9.5 mm, chloroform extract 9 mm, 8.5 mm, acetone extract 7.75 mm, 7 mm and methanol extract 7.25 mm, 7 mm on bacterial and fungal strains respectively (Table 3, Appendix 4).

Table 3. Antibacterial and antifungal activities of stem bark of *Olea europaea* crude extracts, isolated compound and references

Microbial	Strains	Diameter of zone of growth inhibition (mm)							
		Crude extracts				Isolated Cpd	Controls		
		PE	Ch	Ac	Me	Cpd 1	DMSO	G	M
Bacteria	*B. cereus*	10	9	8	7	11	NI	25	NI
	S. aureus	13	10	8	8	11	NI	20	NI
	E. coli	9	8	7	7	10	NI	18	NI
	P. aeruginosa	10	9	8	7	8	NI	20	NI
Fungi	*Fuzarium spp.*	9	8	7	7	7	NI	NI	14
	S. cerviceas	10	9	7	7	8	NI	NI	19

Key: PE-Petroleum ether, Ch-Chloroform, Ac-Acetone, Me-Methanol, Cpd 1-Compound 1, DMSO-Dimethyl sulfoxide, G-Gentamycin, M-Miconazole, NI-No inhibition

Petroleum ether plant extract exhibited the maximum zone of growth inhibition against all tested bacteria and fungi compared to other extracts and all crude extracts were more active on Gram positive bacteria *S. aureus* (Table 3).

This results were in agreement with reported from leaf of *Olea europaea subsp. cuspidata* has the broad antibacterial activity against both Gram-positive and Gram-negative bacteria [59, 60]. Similarly, the results of this study also indicated that the stem bark of *Olea europaea* crude extract had similar activities on the tested bacterial and fungal strains (Table 3, Appendix 4).

The isolated compound **1** has good activity on all tested bacterial strain and fungi (Table 3, Appendix 5). This is due to the presence of different functional groups (alcohol, benzene ring and heterocyclic ether) in the compound, which contribute to the activity observed (Figure 4).

5. Conclusion and Recommendation

5.1 Conclusion

The stem bark of *Olea europaea subsp. cuspidata* was collected from Buno Bedele Zone, Didesa district, Dembi area and dried powdered sample was extracted sequentially using cold maceration technique. The antibacterial and antifungal activities of the crude extracts were evaluated against four pathogenic bacterial strains and two fungal strains using agar disc diffusion method. Results showed that the crude extracts of stem bark of *Olea europaea subsp. cuspidata* have antibacterial and antifungal activity against four bacterial strains and two fungi (Table 3).

From most active chloroform extract, one pure compound, (2-methylbenzofuran-6-yl)methanol was isolated based on the bioassay guided and TLC profile fraction on column chromatography (Figure 4). The isolated pure compound has good activity on both bacteria and fungi strains (Table 3).

Therefore, the *in vitro* antibacterial and antifungal activity test results of crude extracts and the isolated compound has revealed that the stem bark of *Olea europaea subsp. cuspidata* to be the source of bioactive compounds that could be used as antimicrobials.

5.2 Recommendation

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

- Further isolation and characterization of bioactive compounds from petroleum ether, acetone and methanol extracts.
- Further investigation on its antibacterial activities on other bacterial strains, antifungal activities on other fungus and anti-plasmodium activities.

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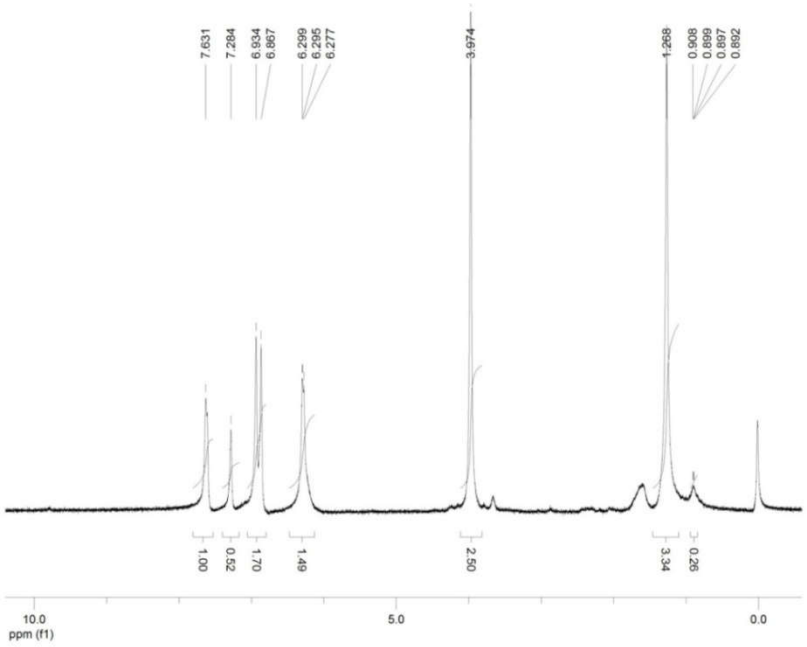
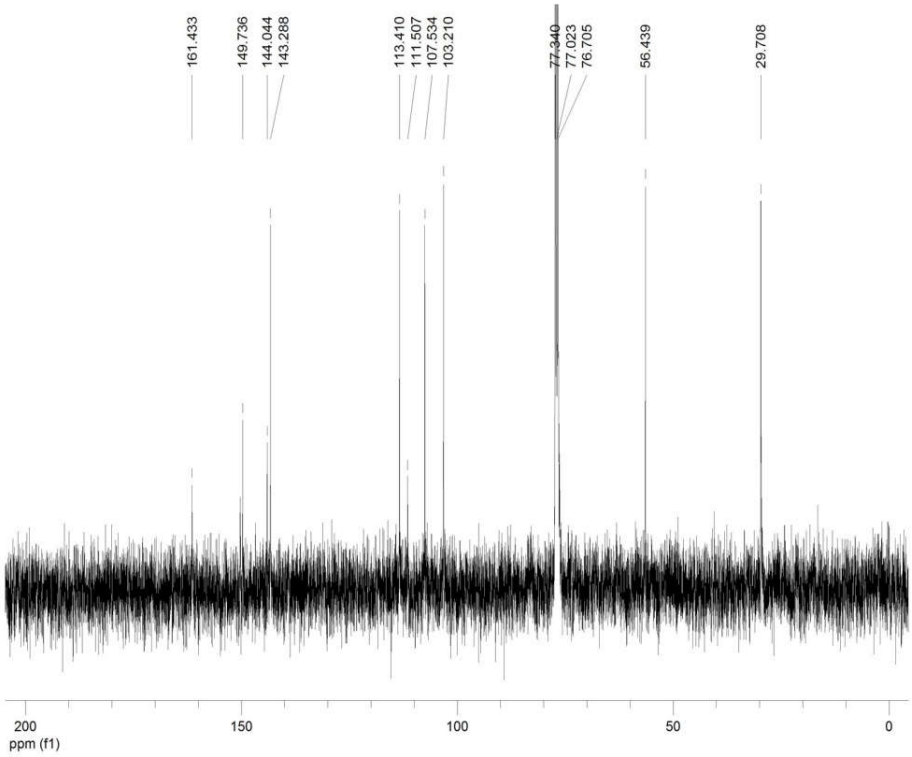
Appendixes

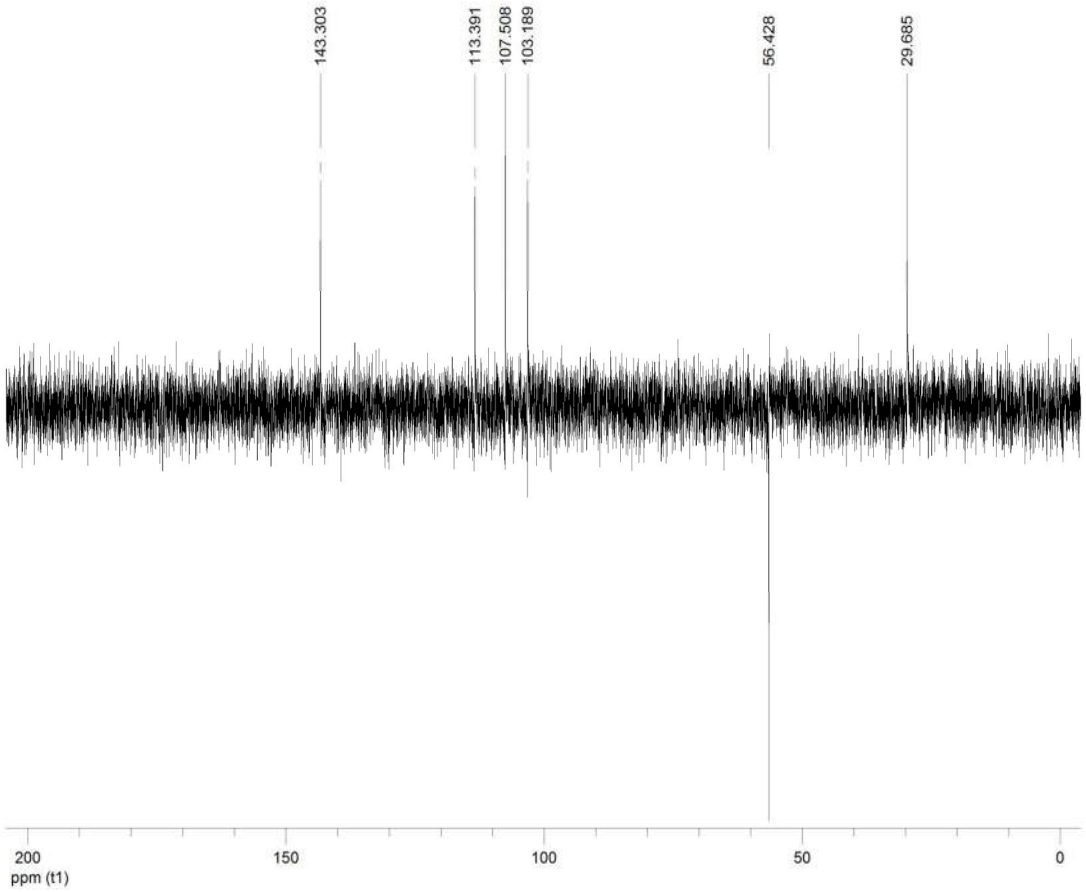
Appendix 1. ^{1}H NMR spectrum of compound **1** in $CDCl_3$



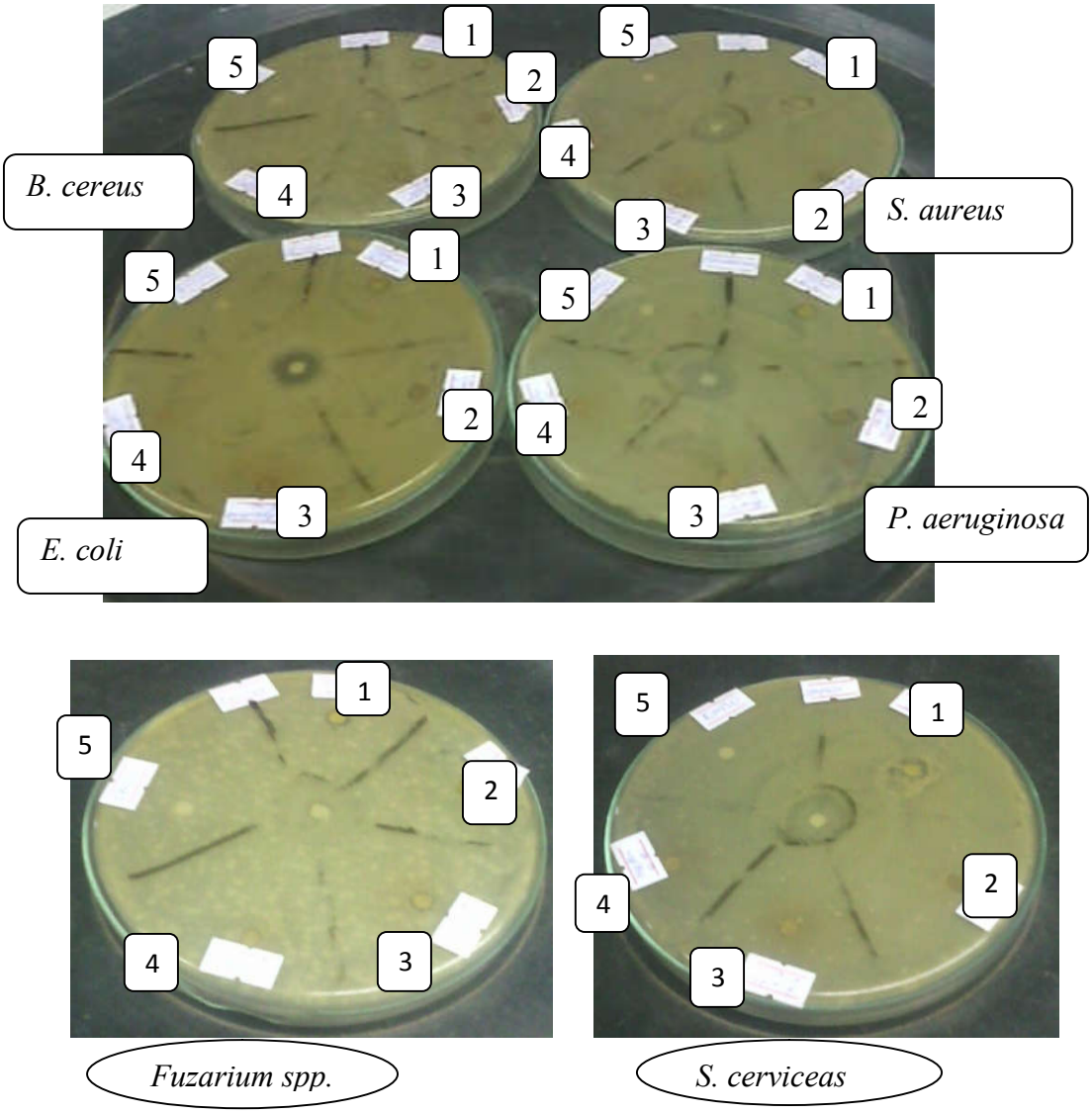
Appendix 2. ^{13}C NMR spectrum of compound 1 in $CDCl_3$



Appendix 3. DEPT-135 spectrum of compound 1 in $CDCl_3$

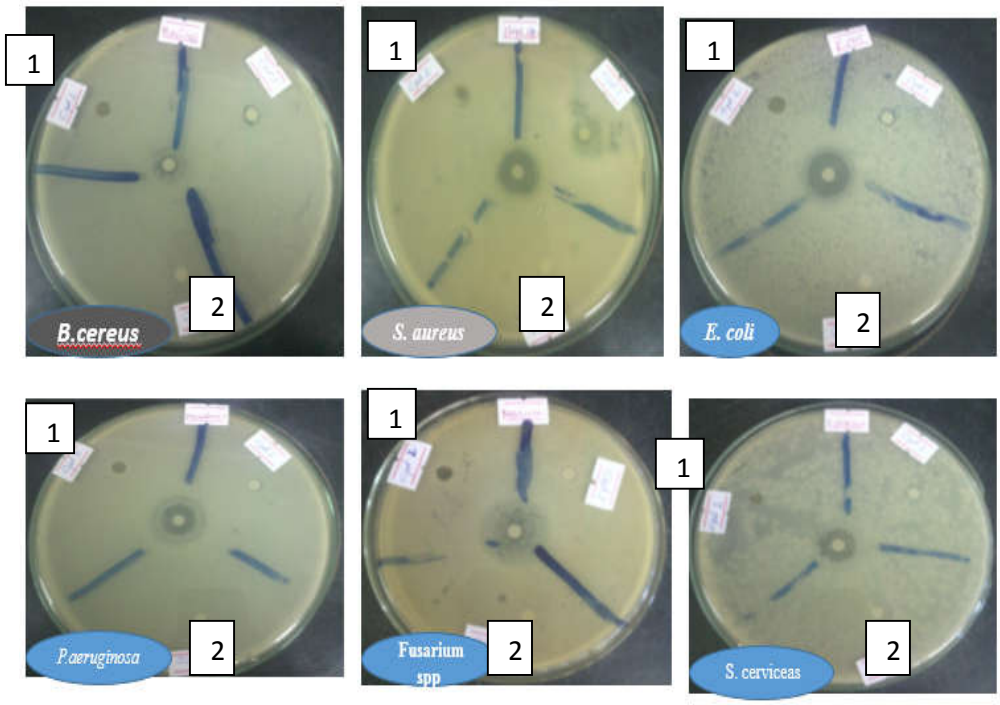


Appendix 4. Bioassay result of crude extract on bacterail strains and fungus



Key: 1- Petroleum ether, 2- Chloroform, 3- Acetone, 4- Methanol, 5- DMSO

Appendix 5. Bioassay result of isolated compound on bacterail strains and fungus



Key: 1- Compound 1, 2- DMSO