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Antifungal Substances from Essential Oils

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By

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List of Abbreviations

ALNAP: African Laboratory for Natural Products

EO: Essential Oil

MIC: Minimum Inhibitory Concentration

NMR: Nuclear Magnetic Resonance

DEPT: Distortionless Enhancement by Polarization

COSY: Homonuclear Correlation Spectroscopy

HMBC: Heteronuclear Multi Bond Correlation

HSQC: Heteronuclear Spin Quantum Correlation

GC: Gas Chromatography

GC/MS: Gas Chromatography/Mass Spectrometry

CC: Column Chromatography

2D: Two Dimensional

FID: Flame Emission Detector

Abstract

In vitro study of the antifungal activity of essential oils, plant extracts with MeOH : $CHCl_3$, pure compounds and, condensed smoke extracts were conducted using agar disc diffusion method. Out of these 14 showed strong, 13 medium and 23 showed no activities to the fungal pathogen of *Aspergillus niger*. The essential oil of *Origanum vulgare* (oregano), *Thymus schimperi* (Tosign), *Cymbopogon nardus* (Citronella), *Cymbopogon citratus* (Lemongrass), *Trachyspermum ammi* (Nech azmud) and *Eucalyptus citriodora* showed strong inhibition zones compared to the essential oils and extracts. The GC and GC/MS analysis of essential oils revealed that *O. vulgare* contained thymol (56%) and carvacrol (43.5%), *T. schimperi* contained thymol (56.5%), linalool (18.5%), and carvacrol (8.9%) and *T. ammi* contained γ-terpinene (42%), thymol (26.2%), *p*-cymene (26.8%), and β-pinene (2.8%).

1. Introduction

Skin diseases are the most common form of infection occurring in people of all ages. Skin disorders due to its ugliness and associated hardships are among the ailments that are difficult to get accustomed to especially when located in parts of the body that are difficult to conceal like the face. Most of the treatments for skin infection take a long time to show their effects. The problem becomes more worrisome if the ailment does not respond to skin disorder treatments.

It has been estimated that skin diseases account for 34% of all occupational diseases [38]. In a study, it was revealed that skin diseases constitute 6.3% of the total number of the patients who attend medical care [15]. The management of skin diseases is becoming a priority due to the association of skin opportunistic infections with HIV/AIDS. Estimates show that 92% of HIV infected individuals have skin disease complications [22].

The important groups among these skin pathogens are the fungi [16]. Furthermore, in the last few years, the numbers of immune suppressed and immune compromised patients, who frequently develop opportunistic, systemic and superficial fungal infections have increased dramatically [30, 33]. This is mainly due to the non-availability of effective antifungal drugs for systemic fungal infections and the toxicity of available drugs [35]. It is therefore important to actively seek effective antifungal agents from natural and other sources.

1.1 Microbial Diseases of the Skin

1.1.1 Fungal Diseases

Fungus is a simple organism that lacks the green pigment chlorophyll, and yet it is regarded as a plant. It includes yeasts, rusts, moulds and mushrooms. Fungi live either on dead or decayed organic matter which is known as saprophytes, or they live on the body of plants and animals as parasites [1].

Mycosis is skin infection of humans and animals usually caused by fungi [4]. The infection could be superficial or subcutaneous.

The superficial infections are worldwide in their distribution and affect hair, nails and the outer layers of the skin [6, 42]. Dermatophytosis, "pityriasis versicolor" and "cutaneous candidiasis" are good example of superficial infections [42]. Dermatophytosis is the infection of keratinized structures, such as the hair, nails, and the outer layer of the skin, and the genera of fungi termed as dermatophytes [32, 42]. "Pityriasis versicolor" is a chronic superficial fungal infection of the skin that affects the trunk and caused by the yeast *Malassezia furfur* [43]. "Cutaneous candidiasis" is an infection of the skin that is caused by the yeast like fungus called *Candida albicans* and it can be acute or chronic in nature [43].

Subcutaneous infections mainly occur in the tropics and subtropics [43]. These infections are more serious than superficial mycosis because it extends into tissue and sometimes into the adjacent structures such as bones and organs [43]. The infection includes subcutaneous candidiasis, sporotrichosis, and aspergillosis. Sporotrichosis is a subcutaneous fungal infection caused by the dimorphic fungus *Sporothrix schenckii*, which can be isolated from decayed vegetable matter such as plant debris, leaves, and woods. Aspergillosis is also subcutaneous infection which is caused by inhalation of the spores of *Aspergillus* species [39].

1.2 Antifungal Activity of Some Medicinal Plants

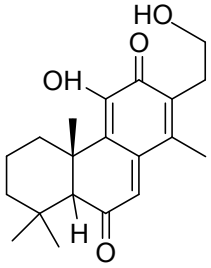
Traditional medicinal resources, especially plants, have been found to be playing a major role in managing dermatological conditions [21]. In Ethiopia, traditional remedies represent not only part of the struggle of the people to fulfill their essential drug needs but also they are integral components of the cultural beliefs and attitudes of the people [3].

The acceptance and recognition of herbal medicine is increasing day by day. One of the important reasons for the increasing of interest is the awareness of natural remedies being more efficient and less harmful than synthetic drugs [21].

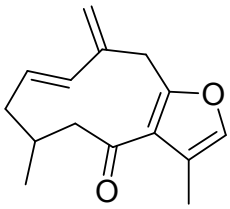
Medicinal plants are important because they are the only means of health care of millions of peoples; they are also source of new pharmaceuticals [26]. Natural products, either as pure

compounds or as standardized plant extracts, provide good opportunities for the discovery of new drugs [26].

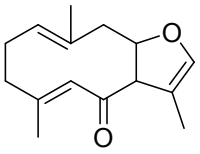
A very potent antifungal agent, the quinone methide diterpene (**1**) type was isolated from the root bark of *Bobgunnia madagascariensis*. This compound showed strong antifungal properties towards human pathogenic fungi, in particular the yeast *Candida albicans* (MIC: 0.19 μg/mL) as well as against other *Candida* species. The activity of this new natural product was found to be more potent than that of amphotericin B and fluconazole (MIC: 0.5 μg/mL) [25]. From the hexane extract of *Commiphora erythraea* resin five furanosesquiterpenoids were isolated and compounds **2** and **3** were reported to possess high antifungal activity [19]. As per this report, compound **2** showed 24.7, 49.3, 23.4 mm growth inhibition zones of the three plant's fungal pathogens of *Alternaria solani, Fusarium culmorum* and *Phytophtora cryptogea*, respectively, and compound **3** showed 80.7, 82.4, and 76.5 mm growth inhibition zones for the same fungal pathogens, respectively, with the dose of 3000 ppm per disc. The other species of *Commiphora* is myrrh (*Commiphora myrrha*) used in traditional medicine, particularly by women who fumigate their bodies for health care. The main compounds of Myrrh are furanodiene **4**, furanoeudesm-1, 3-diene **5**, isofuranogermacrene **6**, etc., [44].



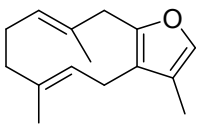
Quinone methide diterpene **1**



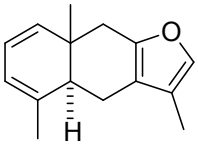
Furanogermacradienone **2**



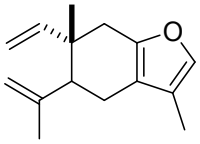
Furanodienone **3**



Furanodiene **4**



Furanoeudesm-1, 3-diene **5**



Isofuranogermacrene **6**

1.3 The Importance of Essential Oils as Potential Sources of Antifungal Substances

Currently there is a limited number of effective conventional antifungal drugs [34]. Researchers therefore seek new, effective, and safe antifungal agents from natural products. Essential oils are among the promising group of natural products that are potential sources of active antifungal agents.

Essential oils are good candidates due to their natural origin safety to people and to the environment. They are also considered as low risk for the development of microbial resistance since they are mixtures of compounds which may possess different mechanisms of antimicrobial activities [13]. For instance the oil of *Melaleuca alternifolia*, commonly known as tea tree oil, has been used medicinally in Australia for its antimicrobial and anti-inflammatory properties. It is mostly used in the manufacturing of antiseptic agents, cosmetics, and germicides. The major components of tea tree oil, terpinene-4-ol **7**, is a clear liquid with mobile consistency and a distinct odor, and has been shown to effectively treat dandruff and oral candidiasis in clinical trials [40]. The other components of tea tree oil are 1,8-cineole **(8)**, γ-terpinene **(9)**, α-pinene **(10)**, β-pinene **(11)** and terpinolene **(12)** [45].

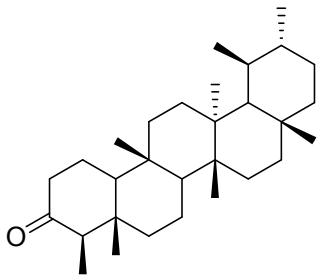
Spices and herbs such as *Thymus schimperi* (Thyme), *Cinnamomum zeylanicum* (Cinnamon), *Eucalyptus citriodora*, *Origanum vulgaris* (Oregano), *Cymbopogon nardus* (Citronella) and *Trachyspermum ammi* (Bishop's weed), etc., are essential oil plants commonly grown in various parts of Ethiopia. At the same time they are extensively used by local people as food preservatives and cure for various diseases [18].

1.4 Review of the Literature for Selected Medicinal Plants with Antifungal Activities

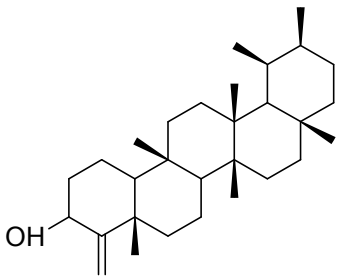
The Genus *Cymbopogon*

The genus *Cymbopogon* is an important member of aromatic grasses, belonging to the Poaceae family comprising 140 species. Many members of this genus are widely distributed and cultivated in tropical and subtropical regions in the world, and more than 52 species occur in Africa [5, 24, 11]**.**

Members of this genus yield large amount of essential oils with a wide array of aroma chemicals like citral A **13**, citral B **14**, geraniol **15**, geranyl acetate **16**, bisabolol **17**, citronellal **18**, citronellol **19**, piperitone **20**, ocimene **21**, methyl eugenol **22**, etc. Citral-containing lemongrass oil takes a prominent place among the most widely consumed aroma chemicals in the world [24, 23]. The essential oils of some *Cymbopogon* species showed remarkable antibacterial and antifungal activities by inhibiting the growth of fungal and bacterial species, for example *C. nardus*, *C. pendulus*, *C. citratus* and *C. olivieri*. Other constituents of *Cymbopogon* species are non-volatile terpenoids and flavonoids. Two triterpeniods, a ketone named Cymbopogone **23** and an alcohol Cymbopogonol **24** were isolated from *C. citratus* [14, 23].



Cymbopogone **23**



Cymbopogonol **24**

Cymbopogon citratus

C. citratus, Lomi Sar (Amh.), Lemongrass (Eng.), is a perennial plant with sturdy stems and broad aromatic leaves, with characteristic lemon-like smell. Citral A **13** and citral B **14** are the two components of a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial and neral, respectively, which also serve as chemical markers for the species [18, 11]. Lemongrass oil and its components, citral A **13** and B **14** were reported as antifungal agents for skin diseases of cutaneous candidiasis and dermatomycosis [11].

Cymbopogon nardus

C. nardus is a perennial grass, up to 2.5 m tall, 1-2 cm in diameter, leaf sheaths reddish purple at base, smooth, glabrous, leaf blades dark green or dark brown and cultivated in Southeast Asia. The essential oil from *C. nardus* is known as citronella oil, and has been traditionally used as household fumigant and for fragrance of soaps and cosmetics [28].

The Genus Eucalyptus

Eucalyptus is a large genus belonging to the family Mytraceae, containing more than 700 species, most of them native to Australia [2, 9].

The common oil yielding *Eucalyptus* species include: lemon-scented eucalyptus (*E. citriodora*), Tasmanian blue gum (*E. globulus*), blue mallee (*E. polybractea*), and red gum (*E. camaldulensis*). Essential oils from the species are among the world's top traded oils and oil extracted from *E. citriodora* is one of the world's major oil in terms of trade volume. The essential oil from *Eucalyptus* species possesses wide range of biological activity including anti-bacterial, fungicidal, insecticidal and herbicidal [9].

Eucalyptus citriodora

Amharic (Shito Bahir zaf); *Arabic* (Kafur Limuni); English (spotted gum, lemon-scented gum, lemon-scented eucalypt, lemon gum); it is a large, evergreen tree, 24-40 m in height; tall, straight trunk, 60-130 cm in diameter; open, graceful crown of drooping foliage; bark smooth, white,

powdery, sometimes pink, red or blue-grey, on large trunks dark or grey and shaggy, it is widely planted in tropical and South Africa [2, 31, 7]**.**

The volatile oil from lemon scented eucalyptus and its major constituent, the monoterpene citronellal **18,** possess a wide spectrum of fungicidal activity and inhibit the growth of fungal pathogens [9]. The oil also contains citronellol **19**, limonene **31**, *p*-cymene **28** and isopulegol **25**.

The Genus *Thymus*

The genus *Thymus* (Lamiaceae) includes about 350 species worldwide and is mainly originated from Mediterranean region of the temperate zones [27, 21]. *Thymus* species are known as medicinal plants because of their biological and pharmacological properties. In traditional medicine, the leaves and flowering parts of the species are widely used as tonic and herbal tea, flavouring agents, antiseptic, and carminative as well as treating colds [36].

Thymus vulgaris (Lamiaceae) is the common thyme of European gardens and is cultivated on large scale throughout the world for culinary, cosmetic, and medicinal purposes. It exhibits antimicrobial effects, ascribed mainly to the high content of phenolic constituents such as thymol **26** and carvacrol **27** [12].

The two species, *T. schimperi* and *T. serrulatus*, locally known as "Tosign", are endemic to Ethiopia [12]. They are growing on highlands, edges of roads, in open grassland, on bare rocks and on slopes, between 2200-4000 m altitudes.

Thymus schimperi

Thymus schimperi is a perennial herb, woody at the base, 5 to 40 cm high, crowded inflorescence with pink corollas and ovate to elliptic leaves with entire margins. The name thyme, which is derived from Greek, refers to courage and sacrifice [18].

T. schimperi (Lamiaceae), Tosign (Amh.), abyssinian thyme (Eng.), occurs in the wild at high altitudes such as in Bale and Debre Sina mountains. It is an endemic species known only from

Ethiopia. The main constituents of the volatile oil are *p*-cymene **28**, γ-terpinene **9**, thymol **26** and carvacrol **27** [18, 7, 12].

The Genus Origanum

The genus *Origanum* comprises 43 species with the Lamiaceae family, mainly distributed in the Eastern Mediterranean region. Due to their high content of essential oil, species of the genus have traditionally been collected for centuries, for the flavouring of traditional dishes as well as for several purposes in traditional medicine [20]. The essential oils of most of the species have antibacterial, antifungal, antioxidant, and germicidal properties.

Origanum vulgare

Origanum vulgare (Lamiaceae)*,* Oregano (Eng.), is an aromatic perennial herb of up to 0.9 m in height. It is found in Europe to Central Asia, Mediterranean region and the Middle East [41].

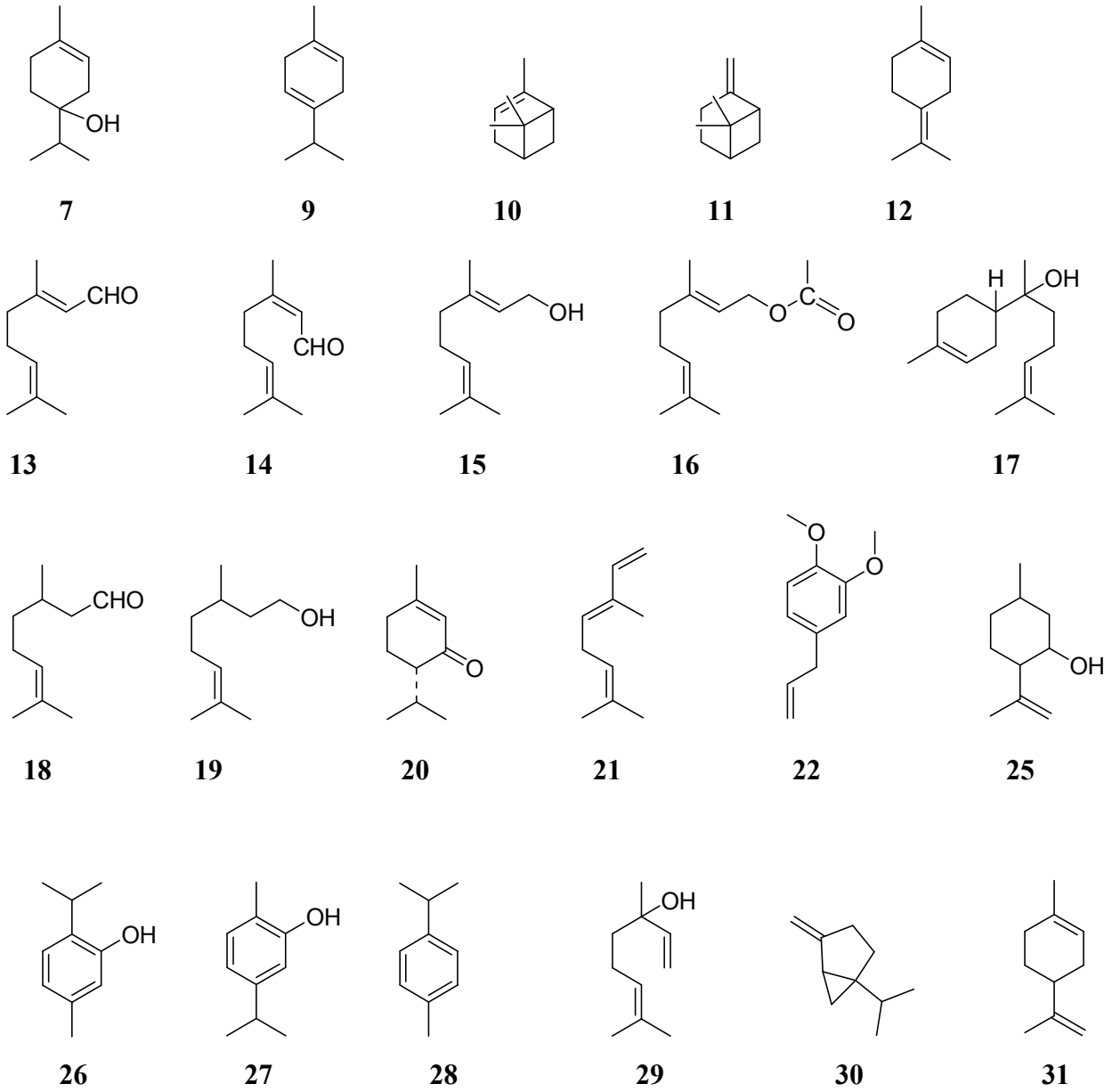
The essential oil from oregano plants is characterized by particular biological importance. It is known for its antibacterial and antifungal activities, antispasmodic effects, and is composed of carvacrol **14** as the dominant component, followed by γ-terpinene **9**, *p*-cymene **28**, linalool **29**, thymol **26**, terpinen-4-ol **7** and sabinene **30** [8, 18]

The Genus *Trachyspermum*

Trachyspermum ammi

Ajwain (*Trachyspermum ammi*) in India belonging to family Apiaceae, Nech Azmud (Amh.), Bishop's weed (Eng.), its other names are ajwan, carom, or Ethiopian cumin [5]. It is slightly branched annual or perennial herb, 90-160 cm high. The origin of this species is not known, but it is indigenous to Egypt and Ethiopia. It is also cultivated in North Africa, India, other Asian countries and in some parts of Europe. The fruits of *T. ammi* have an aromatic smell and a pungent test [18].

The essential oil of *T. ammi* exhibited broad fungal toxicity against all tested fungi, such as *Aspergillus niger*, *Fusarium moniliforme* and *Curvularia lunata*, as absolute mycelial zone of inhibition was obtained at a 6 μL dose of the oil. Analysis of ajwain essential oil showed that thymol **26** was found to be a major component along with *p*-cymene **28**, γ-terpinene **9**, β-pinene **11** and terpinen-4-ol **7** [37]**.**



7 **9** **10** **11** **12**

13 **14** **15** **16** **17**

18 **19** **20** **21** **22** **25**

26 **27** **28** **29** **30** **31**

Structures of monoterpene isolated from six species (*T. schimperi* (Thyme), *C. citratus* (Lemongrass), *E. citriodora*, *O. vulgaris* (Oregano), *C. nardus* (Citronella) and *T. ammi* (Bishop's weed))

2. Objectives

The main objectives of this study was to determine the relative activities of essential oils and extracts of some selected medicinal plants with the intention of identifying natural products to treat fungal infections. Bioassay guided isolation of components of selected essential oils will be used to arrive at the compounds responsible for the observed activity.

3. Results and Discussion

3.1 Screening of Antifungal Substances using Agar Disc Diffusion Method

The in vitro antifungal activities of more than fifty plants were screened by using agar disc diffusion method and the results are shown in Tables 1, 2, 3, and 4. Agar disc diffusion is a method in which the fungal suspension is spread on the surface of the agar in the plate either by swabbing or by mixing together with the agar media. Then, 5 to 10 μL dose of the test materials are applied on filter paper discs, placing them directly onto the agar surface. Then, the test material diffuses out of the disc onto the agar. This is called agar disc diffusion method and is a simple way of establishing whether a test substance is antifungal or not.

In this project work a total of fifty samples were tested by the above method. The results showed 14 to be strongly active (12 EOs, 2 pure compounds), 13 of medium activity (9 EOs, 4 condensed smoke extracts) and 23 (10 EOs, 10 $CHCl_3$/MeOH (1:1) extracts, 2 condensed smoke extracts, 1 water distillate of thyme) were not active at all against the fungal pathogen, *Aspergillus niger*. Of the 50 samples, 31 were essential oils, 2 were pure compounds, 1 was the water distillate of thyme, 10 were $CHCl_3$/MeOH (1:1) extracts and 6 were condensed smoke extracted with chloroform. The maximum antifungal activity was shown by essential oils of *Origanum vulgare* (Oregano) and *Thymus schimperi* (Tosign), followed by *Cymbopogon nardus* (Citronella), *Cymbopogon citratus* (Lemongrass), *Trachyspermum ammi* (Nech azmud), *Eucalyptus citriodora* see Table 5.

Table 1: Some essential oils that exhibited strong activity on *A. niger*

S/N	Botanical name, Common English name, Local name	Activity
1	*Cinnamomum zeylanicum*, Cinnamon, Kerefa	+++
2	*Eucalyptus citriodora*, Citriodora, Shito zaf	+++
3	*Cymbopogon nardus*, Citronella	+++
4	*Cymbopogon citratus*, Lemongrass, Lomi sar	++
5	*Cymbopogon martini*, Palmarosa, Tej sar	+++

6	*Thymus schimperi*, Ethiopian thyme, Tosign	+++
7	*Thymus vulgaris*, European thyme	+++
8	*Lippia adoensis*, Koseret	+++
9	*Trachyspermum ammi*, Bishop's weed, Nech azmud	+++
10	*Origanum vulgare*, Oregano	+++
11	*Syzygium aromaticum*, Clove from China	+++
12	*Syzygium aromaticum*, Clove from India	++

+++ = indicates strong effect on the fungal pathogen, ++ = moderate effect on the fungal pathogen, and + = weaker effect on the fungal pathogen.

Table 2: Some essential oils that showed small inhibition zone on *A. niger* fungal pathogen

S/N	Botanical name (Common English name, Local name)	Activity
1	*Commiphora myrrha* (Myrrh, Kerbe)	+
2	*Juniperus procera* (Tid)	+
3	*Origanum majorana (* Sweet marjoram)	+
4	*Boswellia rivae* (Frankincense (Ogaden Type))	+
5	*Tagetes minuta* (Geme)	+
6	*Lavandula angustifolia* (Lavender)	+
7	*Lippia multiflora*	+
8	*Rosmarinus officinalis* (Rosemary, Tibis kitel)	+
9	*Melaleuca alternifolia* (Tea tree)	+

The smoke extracts of six plant materials were done using fumigation experiment in which the plant sample was put onto burning charcoal and the smoke was covered with jar and trapped with chloroform and then each extract was bioassay tested and out of them four showed small inhibition zone (Table 3).

Table 3: Some smoke extracts that showed small inhibition zone on the fungal pathogen

S/N	Botanical name (Common English name, Local name)	Activity
1	*Commiphora myrrha* (Myrrh, Kerbe)	+
2	- (Wogert)	+
3	*Olea europaea* (Olive, Woyira)	+
4	- (Woyiba)	+

Extracts of several plants were prepared by soaking with $CHCl_3$/MeOH (1:1) and dried to get crude extract. The antifungal activities of each of the resulting crude extracts were screened using agar disc diffusion method. All of them did not show any inhibition zone to the fungal pathogen.

Table 4: Some essential oils of plants that showed no inhibition zone on *A. niger* with botanical name, common English name and local name.

S/N	Botanical name (Common English name, Local name)
1	*Eucalyptus globulus* (White Eucalyptus , Nech Bahir zaf)
2	*Commiphora guidotti (* Opoponax,)
3	*Artemisia abyssinica* (Chikugn)
4	*Artemisia annua* (Sweet Wormwood,)
5	*Artemisia afra* (Chikugn)
6	*Artemisia rehan* (Artiti)
7	*Foeniculum vulgare* (Fennel, Ensilal)
8	*Boswellia neglecta* (Frankincense, Borena Etan)
9	*Boswellia Papyrifera* (Frankincense, Tigray Etan)
10	*Myrtus communis* (Myrtle, Ades)

In addition to this, pure carvacrol and pure thymol possessed strong inhibition zones, whereas the water distillate of thyme and the smoke extracts of *Echinops kebericho* (kebericho) and *Otostegia integrifolia* (tinjute) exhibited no inhibition zones on the fungal pathogen.

3.2 Bioassay Guided Isolation of Active Components from Essential oils

The bioassay test was done for several plants in vitro on the *A. niger* fungal pathogen using agar disc diffusion method. Out of the total bioassay tested samples the five most active essential oils were selected for further study (Table 5).

Table 5: Antifungal activity of some selected EO using agar disc diffusion method

Essential oils (10 µL/disc)	Inhibition zone in mm
Thymus schimperi	36
Trachyspermum ammi	10
Cymbopogon nardus	30
Cymbopogon citratus	18
Origanum vulgare	60
Flucytosine (Standard)	-
Itraconazol (10 mg/0.2 mL)	9

The diameter of zones of inhibition were measured in mm including the filter paper disc and it was compared with positive control of Flucytosine or Itraconazol which are drugs used for the treatment of *Aspergillus* infection.

3.2.1 Essential oil from *Trachyspermum ammi* (Nech azmud)

The essential oil from the seeds of *T. ammi* was hydrodistilled using Clevenger apparatus which gave 4% w/w of pale yellow aroma. The crude EO showed antifungal activity on the *A. niger* fungal pathogen, and the MIC was determined to be 5 µL/disc.

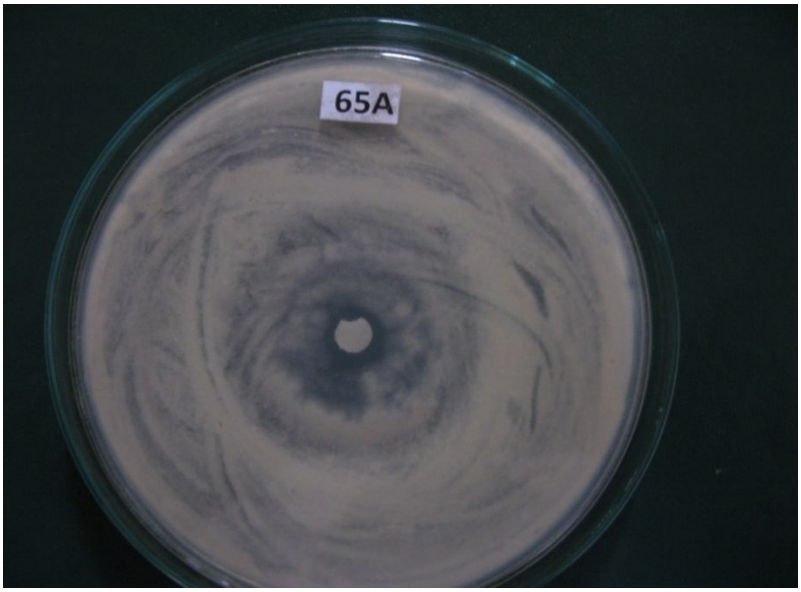


Fig.1 Growth inhibition zone of *Trachyspermum ammi* EO on *Aspergillus niger*

Furthermore, the oil was subjected to GC and GC-MS analysis and correlated with standards in the Wiley library. The result obtained indicated that the major components of *T. ammi* were γ-terpinene (42%), thymol (26.2%), *p*-cymene (26.8%), and β-pinene (2.8%) as presented in Table 6

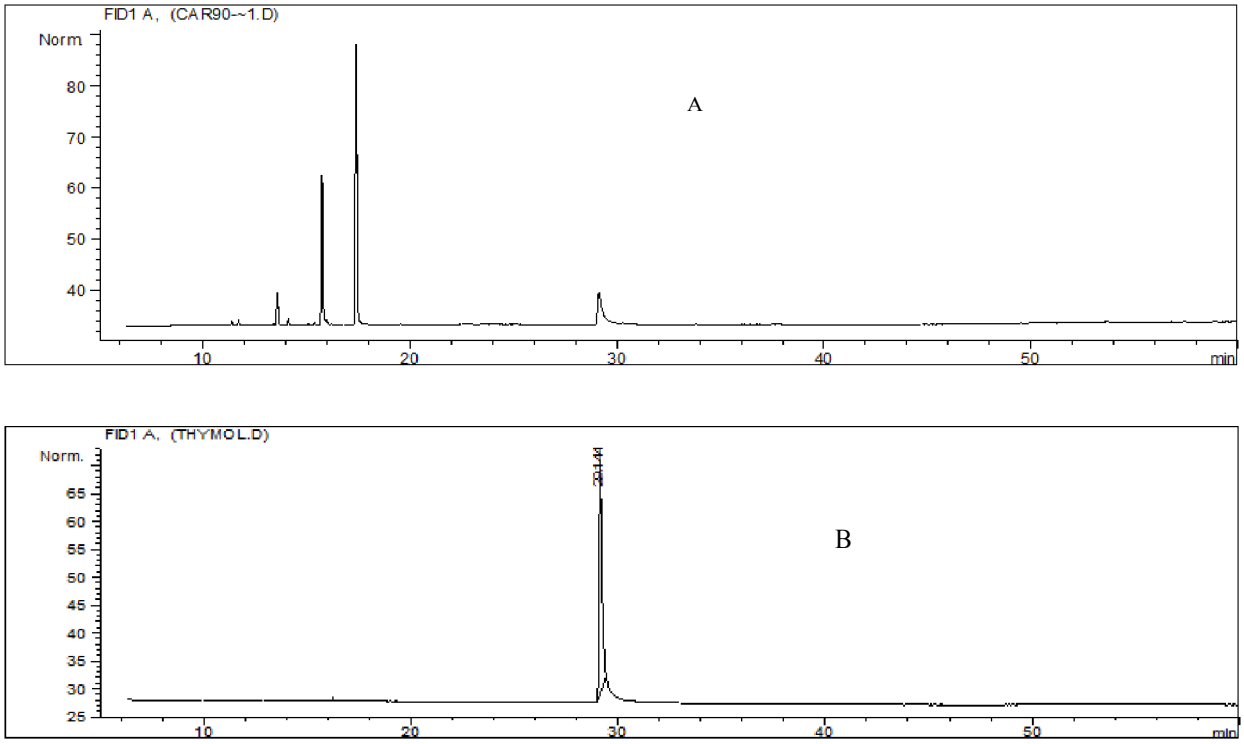


Fig. 2 GC of *T. ammi* EO (A) and pure thymol (B)

Table 6: Chemical composition of essential oil from *T. ammi* using GC/MS

Peak #	Compounds	Percentage of total oil	Retention time (min.)	Peak % max.
1	α-Thujene	0.203	11.35	0.48
2	α-Pinene	0.338	11.65	0.81
3	β-Pinene	2.77	13.76	6.6
4	Myrcene	0.49	14.49	1.17
5	α-Terpinene	0.202	15.75	0.48
6	*p*-Cymene	26.75	16.42	63.67
7	γ-Terpinene	42	18.29	100
8	-	0.33	23.62	0.79
9	Thymol	26.15	29.94	62.25
Total		99.233%		

* Compounds are listed in the order of elution on GC/MS

Besides, an attempt was made to fractionate the EO sample. In line with this, 700 mg of *T. ammi* EO was subjected to CC and 16 fractions were collected. By using their TLC result, similar spots were combined and bioassayed. From this, one active fraction was obtained and showed a diameter of 36 mm inhibition zone at 10 μL/disc on the fungal pathogen. And then, the resulting active fraction was analyzed using instrumental analysis and elucidated. The GC, ^{1}H NMR (Fig. 3) and ^{13}C NMR (Appendix-1) data of the compound was compared with pure thymol **27** and showed quite similar.

The ^{1}H NMR (400 MHz) in $CDCl_3/CCl_4$ spectrum (Fig. 3) showed doublet peak at δ 1.3 ppm indicating the signal of two symmetric methyl protons of the isopropyl group, singlet peak at δ 2.3 ppm for the primary methyl proton attached to the aromatic carbon, septet peak at δ 3.3 ppm for the methine proton attached to isopropyl carbon, singlet peak at δ 5.2 ppm indicating the hydroxyl proton. The aromatic methine proton appeared at δ 7.2 (1H, d, J = 8 Hz), 6.8 (1H, d, J = 8 Hz) and 6.6 (1H, d, J = 0.4 Hz). The ^{13}C NMR (400 MHz) spectrum in $CDCl_3/CCl_4$ showed three quaternary aromatic carbons at δ 152.6, 131.5 and 136.3 ppm. This was observed from the DEPT-135 spectrum (Appendix-2), since these three carbon signals were absent. In addition to this, the compound contains signals at δ 21, 22.8, 26.7, 116, 121.6 and 126.2 ppm as indicated in ^{13}C and DEPT-135 NMR spectrum.

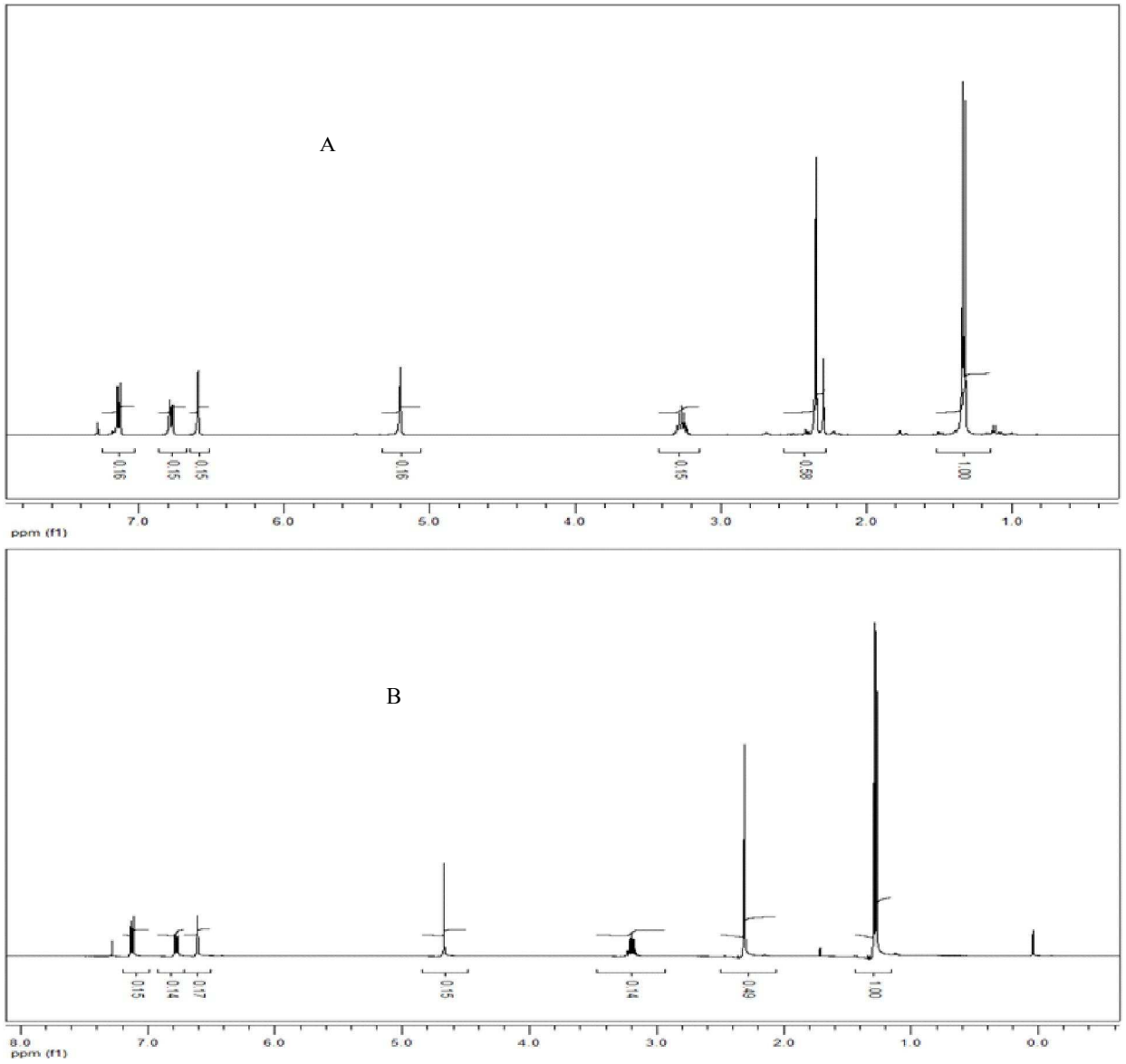


Fig. 3 ^{1}H NMR spectrum of thymol isolated from *Trachyspermum ammi* EO (A) and reference thymol (B)

3.2.2 Essential oil from *Thymus schimperi* (Tosign)

The dried leaves of *T. schimperi* were hydrodistilled by Clevenger apparatus to yield 0.6% w/w of essential oil. The crude EO of *T. schimperi* showed strong activity against *A. niger* fungal pathogen. This was observed after applying 10 µL/disc of the oil, which completely inhibited up

to a diameter of 36 mm. Furthermore, its minimum inhibitory concentration (MIC) was estimated to be lower than 5 μL/disc.

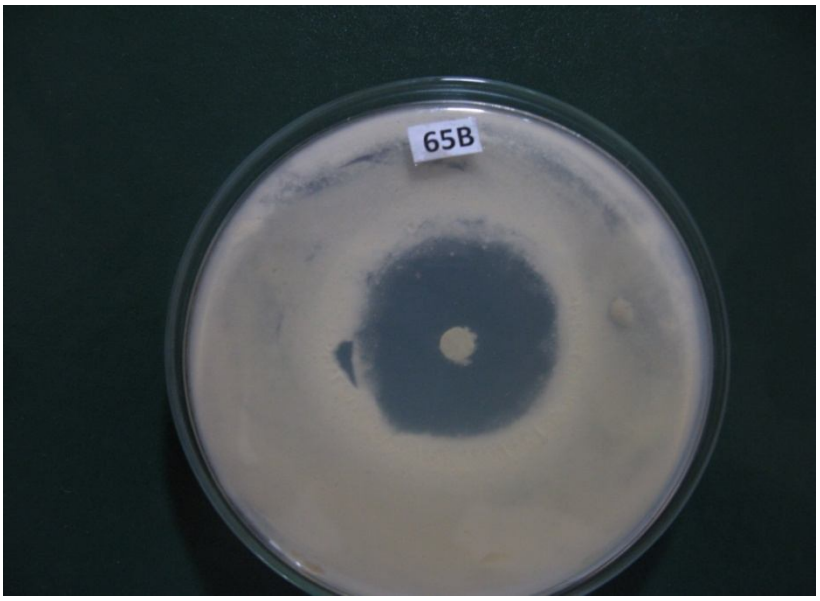


Fig. 4 Growth inhibition zone of *Thymus schimperi* on *Aspergillus niger*

The essential oil of thyme is dominated by its thymol content. GC and GC/MS result indicates that (Table 7) the major components were thymol (56.5%), linalool (18.5%), and carvacrol (8.9%) were identified by correlating it with the data found in Wiley library. The result we found fits with *T. schimperi* that was collected from Dinshu (Bale province) (Dagne, E., *et al*., 1998). Since the major components of thyme oil were the most active against the fungal pathogen as shown in Table 5, they are also taken as the marker compound to most of the active essential oils in our study.

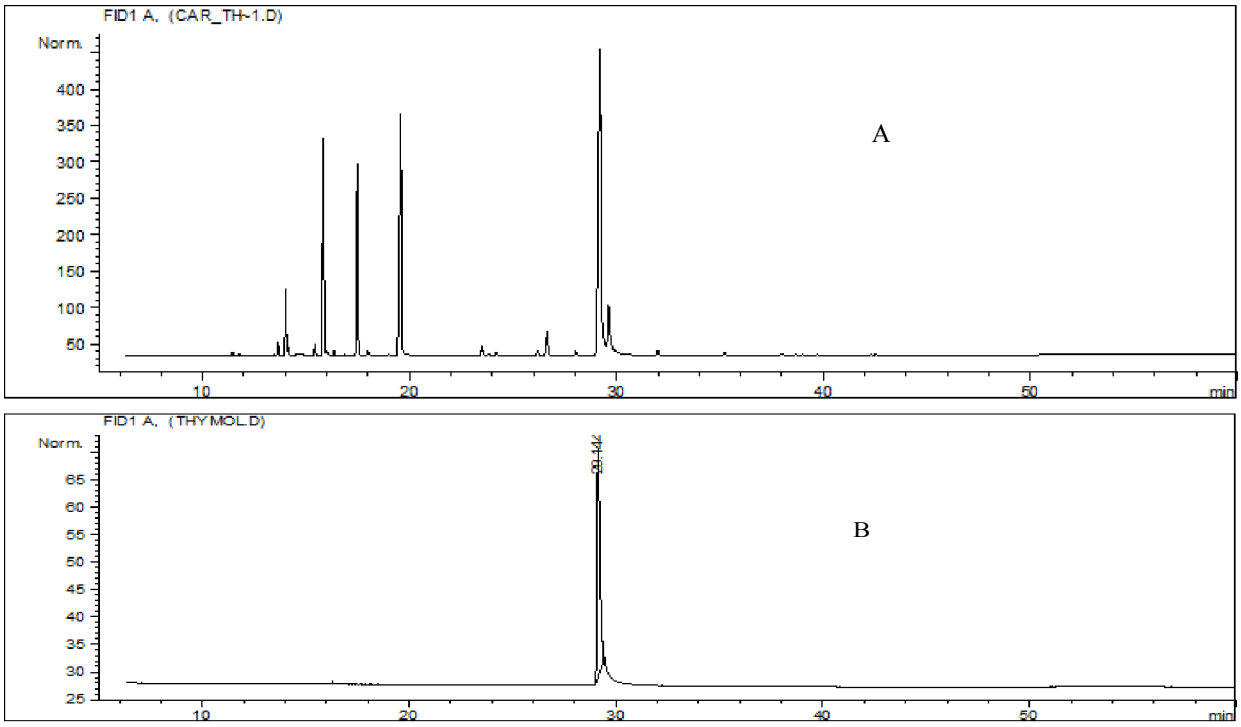


Fig. 5 GC of *T. schimperi* EO (A) and ref. thymol (B)

Table 7: Chemical composition of essential oil from *T. schimperi* using GC/MS

Peak #	Compounds	Percentage of total oil	Retention time (min.)	Peak % max.
1	3-Octanone	1.67	14.36	2.96
2	-	0.399	14.76	0.71
3	*p*-Cymene	8.25	16.24	14.59
4	γ-Terpinene	1.95	17.95	3.45
5	Linalool	18.45	20.32	32.64
6	Terpinene-4-ol	0.78	24.08	1.37
7	-	2.54	27.31	4.49
8	Thymol	56.52	29.94	100
9	Carvacrol	8.93	30.26	15.8
Total		99.48%		

3.2.3 Essential oil from *Origanum vulgare* (Oregano)

The essential oil of oregano was hydrodistilled from the leaves by using Clevenger apparatus and yielded 0.2% w/w aroma oil. The EO of *O. vulgare* showed strong activity against the fungal pathogen (Fig.), and which have high inhibition zone compared to the other oils, this is because the oil contain large amount of phenolic compounds.

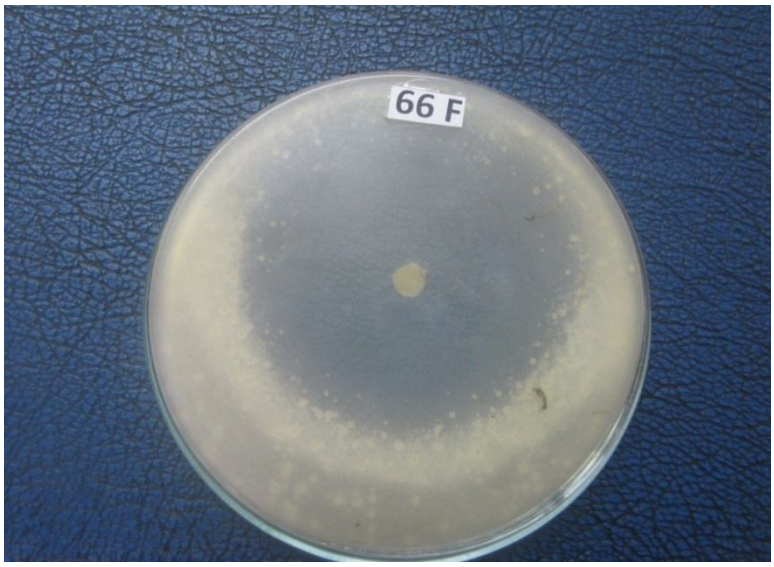


Fig. 6 Growth inhibition zone of *Origanum vulgare* on *Aspergillus niger*

The components were analyzed by GC and GC/MS (Table 8). Accordingly, the oil contains thymol and carvacrol as the major constituents and account for 99% of the total percentage in the oil. Fig. 7 shows the GC of oregano compared with that of pure thymol.

Table 8: Chemical composition of essential oil from *O. vulgare*

Peak #	Compounds	Percentage of total oil	Retention time (min.)	Peak % max.
1	-	0.601	27.35	1.08
2	Thymol	55.9	29.90	100
3	Carvacrol	43.5	30.40	77.86
Total		100%		

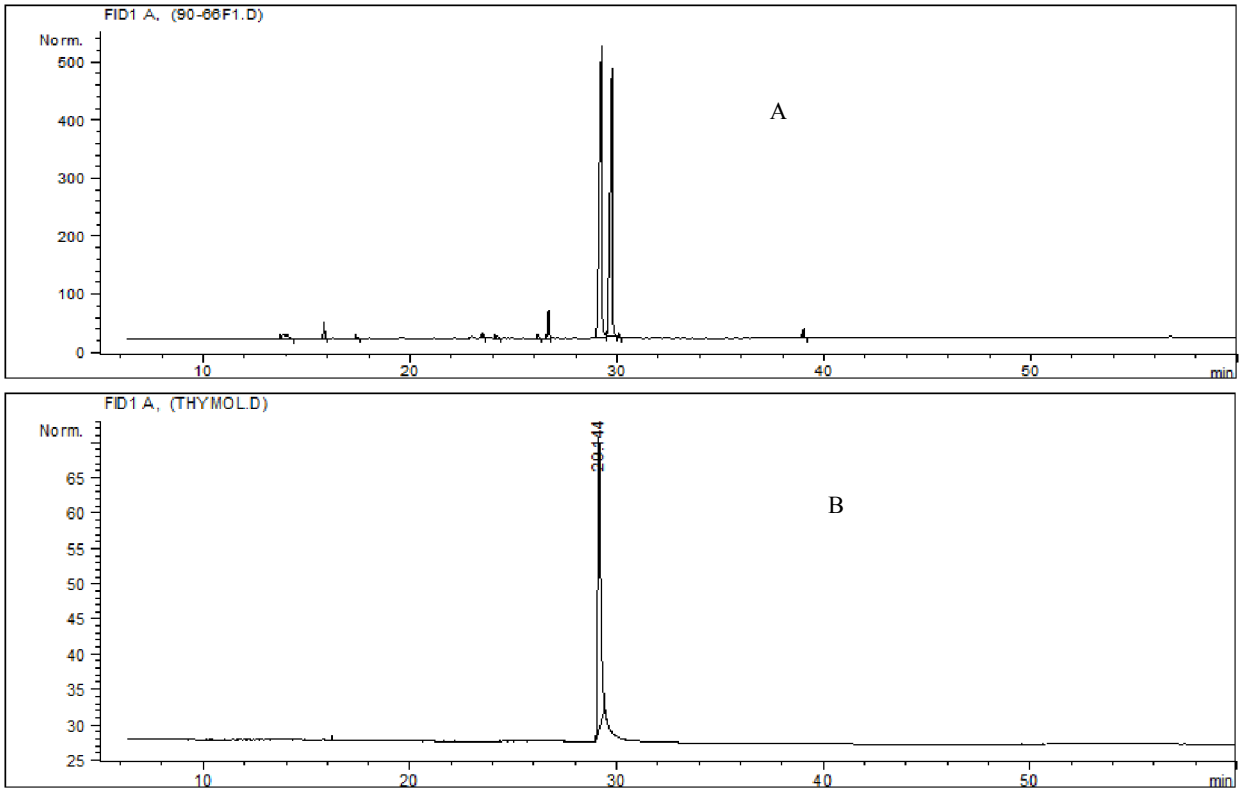


Fig. 7 GC of *O. vulgare* EO (A) and reference thymol (B)

3.2.4 Essential oil from *Cymbopogon nardus* (Citronella)

The chemical composition of the crude oil was determined by GC and GC/MS which indicates that monoterpenes predominated in the oil. Besides, the TLC result of the crude oil was derivatized with Brady's reagent gave yellow color which showed that the presence of carbonyl compounds in citronella EO. Besides this, citronella oil was exhibited strong inhibition zone on the *A. niger* fungal pathogen with diameter of 30 mm (10 µL/disc) (Table 5).

Further fractionation of citronella essential oil with 100% hexane and 100% ethyl acetate gave four fractions. Ethyl acetate fraction, fraction-3 and fraction-4 were found to be active against the fungal pathogen whereas the hexane fraction showed no activity.

The GC/MS analysis of the active fraction of citronella essential oil revealed that the two compounds named as cis-citral (neral) and trans-citral (geranial) were identified in Table 9.

Table 9: Chemical Composition of Active Fraction of Citronella EO

Peak #	Compounds	Percentage of total oil	Retention time (min.)	Peak % max.
1	Neral	41.88	27.3	72
2	Geranial	58.13	28.8	100

3.2.5 Essential oil from *Cymbopogon citratus* (Lemongrass)

The oils of lemongrass were brought from South Africa, Rwanda, and India have strong activity against the fungal pathogen. Among the three essential oils the one from South Africa comparatively showed strong inhibition zone of 18 mm (10 µL/disc) against *A. niger*.

The chemical analysis of the crude oil from S. Africa was determined by GC (Fig. 5)

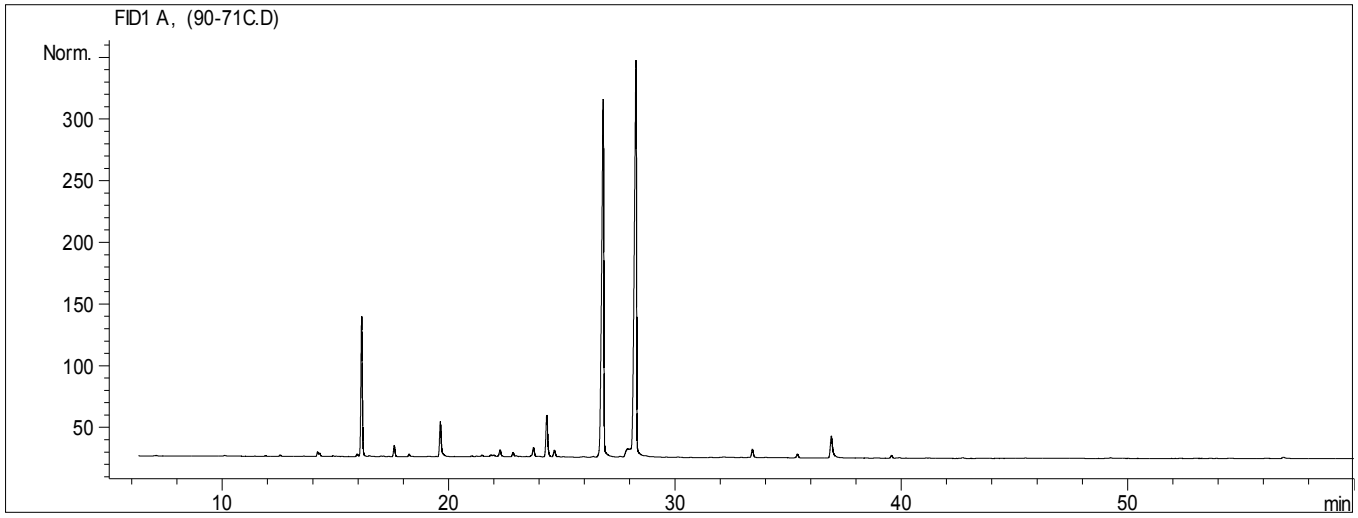


Fig. 8 GC of *Cymbopogon citratus* essential oil

3.3 Antifungal Activity of Commercial Thymol and Carvacrol

Thymol and carvacrol are the two isomeric monoterpenes of phenols appears as the main components in many volatile phenolic essential oils (thyme, savory and oregano) of Lamiaceae family plants [29]. Studying their antifungal activities and distinguishing them by using

instrumental method is also part of this work since they showed good antimicrobial and they are the marker compound to most of the essential oil in this work.

In this study, the minimum inhibitory concentration (MIC) of pure thymol was determined by agar diffusion method, the value ranges from 3 to 10 μmol/disc (10 to 30 μL/disc) against the fungal pathogen of *A. niger*. Carvacrol exhibited strong fungal growth inhibition zone at 66.7 μmol/disc (10 μL/disc).

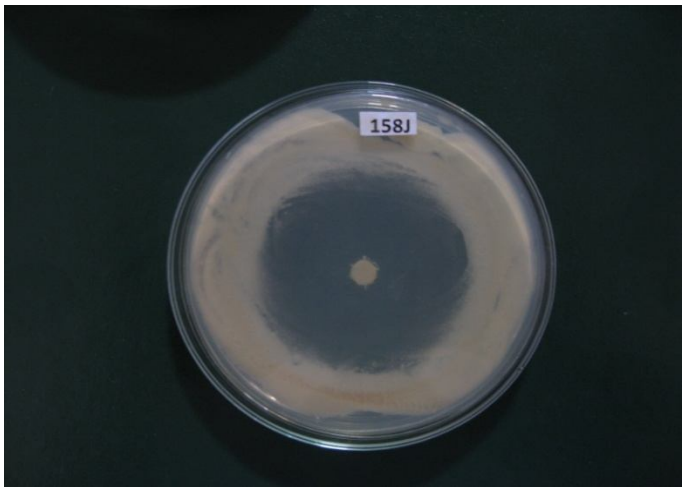


Fig. 9 Growth inhibition zone of pure carvacrol on *Aspergillus niger*

3.4 Analysis of Thymol and Carvacrol

The physical and chemical properties of commercially obtained thymol **(27)** and carvacrol **(28)** were compared. Thymol is a white crystalline phenolic compound which melts at 52^{o}C, soluble in hexane and alcohol. The compound was analyzed using GC and UV and obtained with the retention time of 29.91 min. and max. absorbance at wavelength of 276.2 nm (Appendix-3) respectively, and the mass spectrum (Appendix-4) showed a molecular ion peaks of m/z 150, 135 (base peak), 115, 107, 91, 77, 65, 51. Its TLC showed red color when derivatized with vanillin-H_2SO_4 reagent.

Commercial carvacrol: phenolic, yellowish oil, its solubility and molecular ion peak fragmentation (Appendix-9) resembled thymol, UV absorbed at λ = 276.4 nm (Appendix-10), and GC (R_T in min. 30.4).

Furthermore, the 1D and 2D NMR spectrum of thymol and carvacrol were obtained using 400 MHz NMR spectroscopy.

The ^{13}C NMR (Appendix-5) in $CDCl_3/CCl_4$ (Table 10) of pure thymol contained signals due to three quaternary aromatic carbons at δ 152.5 (C-1), 131 (C-2) and 136.6 (C-5) in addition to the signals of the groups observed in the ^{1}H NMR.

The aromatic methines of thymol appeared in ^{1}H NMR spectrum (Table 10) at δ 7.2 (1H, d, J = 7.6 Hz, H-3), 6.8 (1H, d, J = 8 Hz, H-4) and 6.6 (1H, d, J = 0.8 Hz, H-6)

The ^{1}H, ^{13}C NMR spectrum (Appendix-8) in $CDCl_3/CCl_4$ of pure carvacrol showed similar to pure thymol's spectrum except the position of hydroxyl group (Table 10). As indicated in spectrum, the aromatic proton appeared at δ 7.2 (1H, d, J = 7.6 Hz, H-3), δ 6.8 (1H, dd, J = 1.6 Hz, H-4) and at δ 6.7 (1H, d, J = 1.6 Hz), the tertiary methine proton appeared at δ 2.9 (1H, septet, H-8).

Table 10: ^{1}H and ^{13}C Analysis of Pure Thymol & Carvacrol

	Thymol		Carvacrol	
Atom. no.	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1	-	152.5	-	153.6
2	-	131.3	-	121
3	7.2 (1H, d, J = 7.6 Hz)	126.3	7.2 (1H, d, J = 7.6 Hz)	131
4	6.8 (1H, d, J = 8 Hz)	121.7	6.8 (1H, dd, J = 1.6 Hz)	119
5	-	136.6	-	148.5
6	6.6 (1H, d, J = 0.8 Hz)	116	6.7 (1H, d, J = 1.6 Hz)	113
7	3.2 (1H, septet)	26.7	2.3 (3H, s)	15.5
8	1.3 (3H, d)	22.7	2.9 (1H, septet)	33.8
8'	"	"	-	-
9	2.3 (3H, s)	21	1.3 (3H, d)	24
9'	-	-	"	"

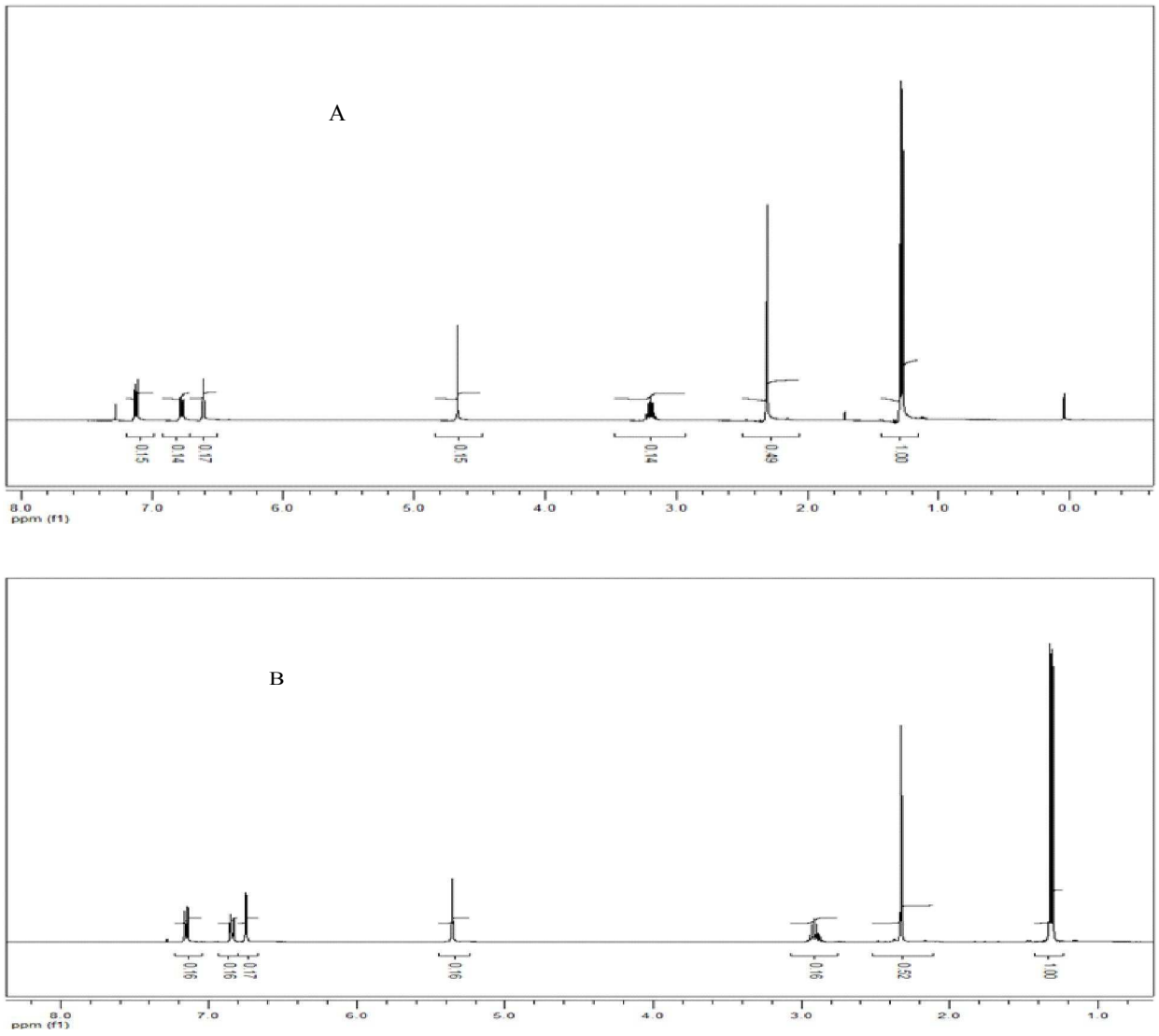


Fig.10 ^{1}H NMR spectrum of pure thymol (A) and pure carvacrol (B)

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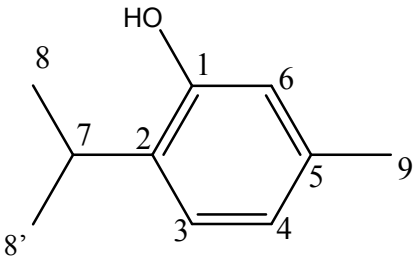
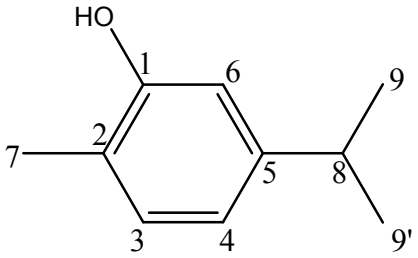
3.4.1 Distinguishing thymol and carvacrol by using 2D NMR techniques



Thymol

5- Isopropyl -2- methyl phenol

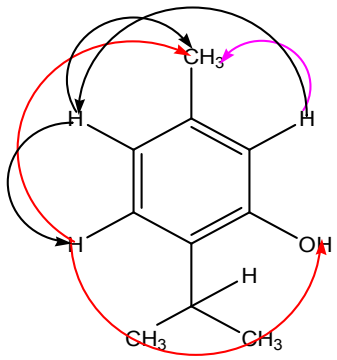


Carvacrol

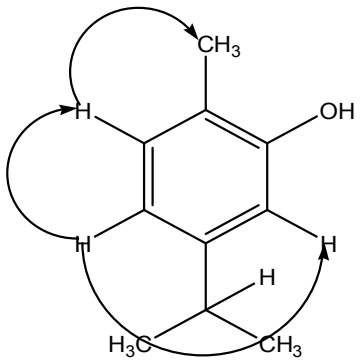
2- Isopropyl-5-methyl phenol

Table 11: ^{1}H-^{1}H COSY Correlations of thymol and carvacrol

Thymol		Carvacrol	
Hydrogen	Correlated with	Hydrogen	Correlated with
H-3	H-4	H-3	H-4
H-4	H-6	H-4	H-6
H-7	H_3-8	H-8	H_6-9
H-3	H_3-9	H-3	H_3-7
H-4	H_3-9		
H-6	H_3-9		
H-3	O-H		



Thymol



Carvacrol

Comparison of thymol and carvacrol were made using COSY (^{1}H-^{1}H) spectroscopy. As revealed in the above structure the colored arrows used in the correlation of thymol was not observed in carvacrol which can be used to distinguish one from the other.

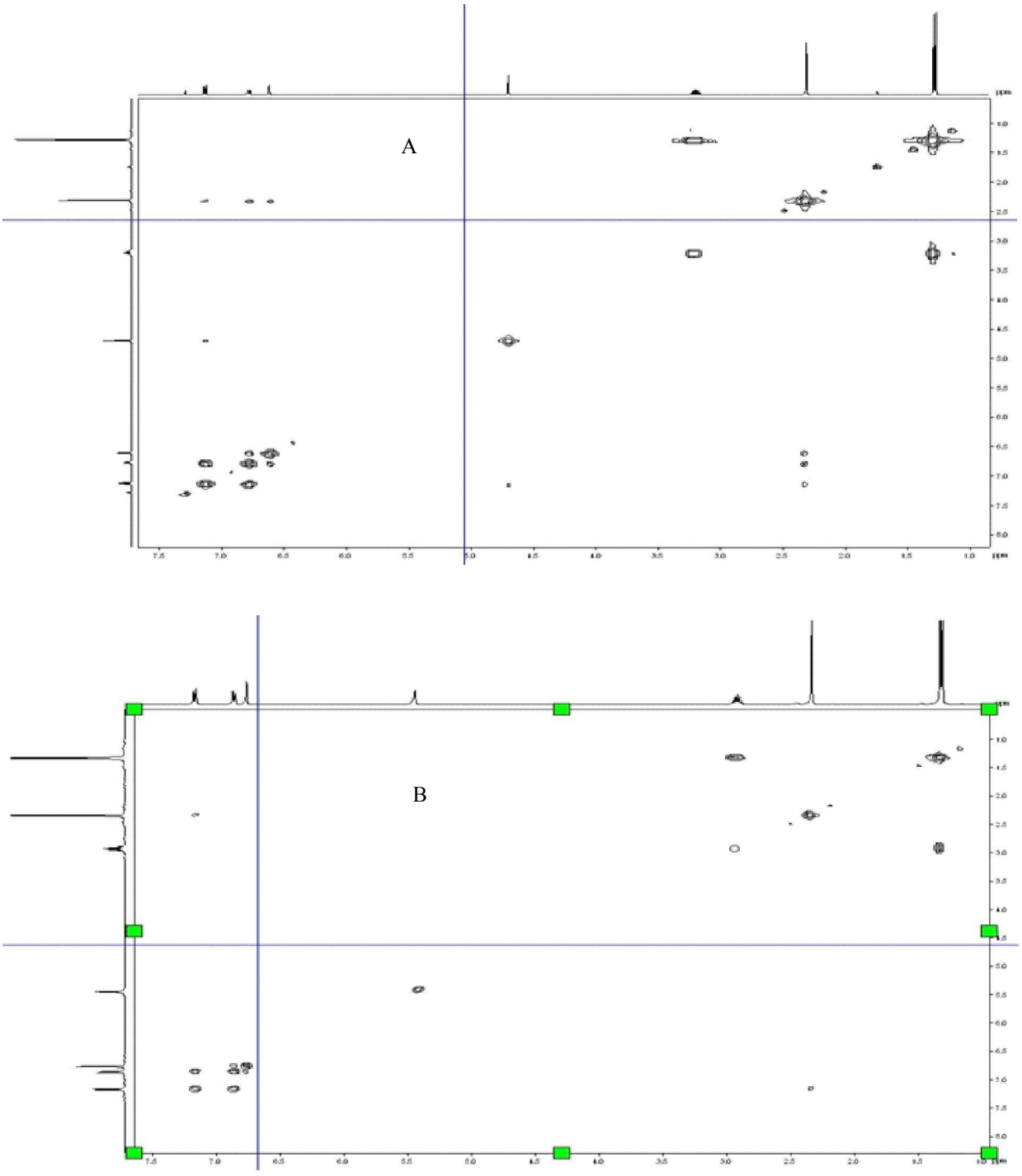
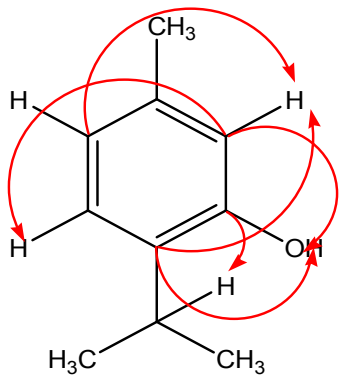


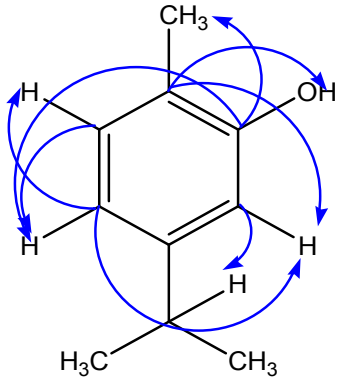
Fig.11 COSY NMR Spectrum of pure thymol (A) and pure carvacrol (B)

Table 12: HMBC (^{13}C- ^{1}H) Correlations of pure thymol and pure carvacrol.

Thymol		Carvacrol	
Carbon	Correlated with	Carbon	Correlated with
C-1	H-7, H-6, H-3, OH	C-1	H-6, H-3, H_3-7
C-2	H_3-8, H-7, H-6, H-4, OH, H_3-9	C-2	H-4, H-6, H_3-7
C-3	H-7	C-3	H_3-7
C-4	H_3-9, H-6	C-4	H-6, H-8
C-5	H_3-9, H-3	C-5	H-3, H_3-9', H-8, H-7
C-6	H_3-9, H-4, H-3, OH	C-6	H-4, H-8,H_3-7
C-7	H_3-8, H-3	C-8	H-4, H-6, H_3-9
C-8	H_3-8, H-7	C-9	H-8, H_3-9



Thymol



Carvacrol

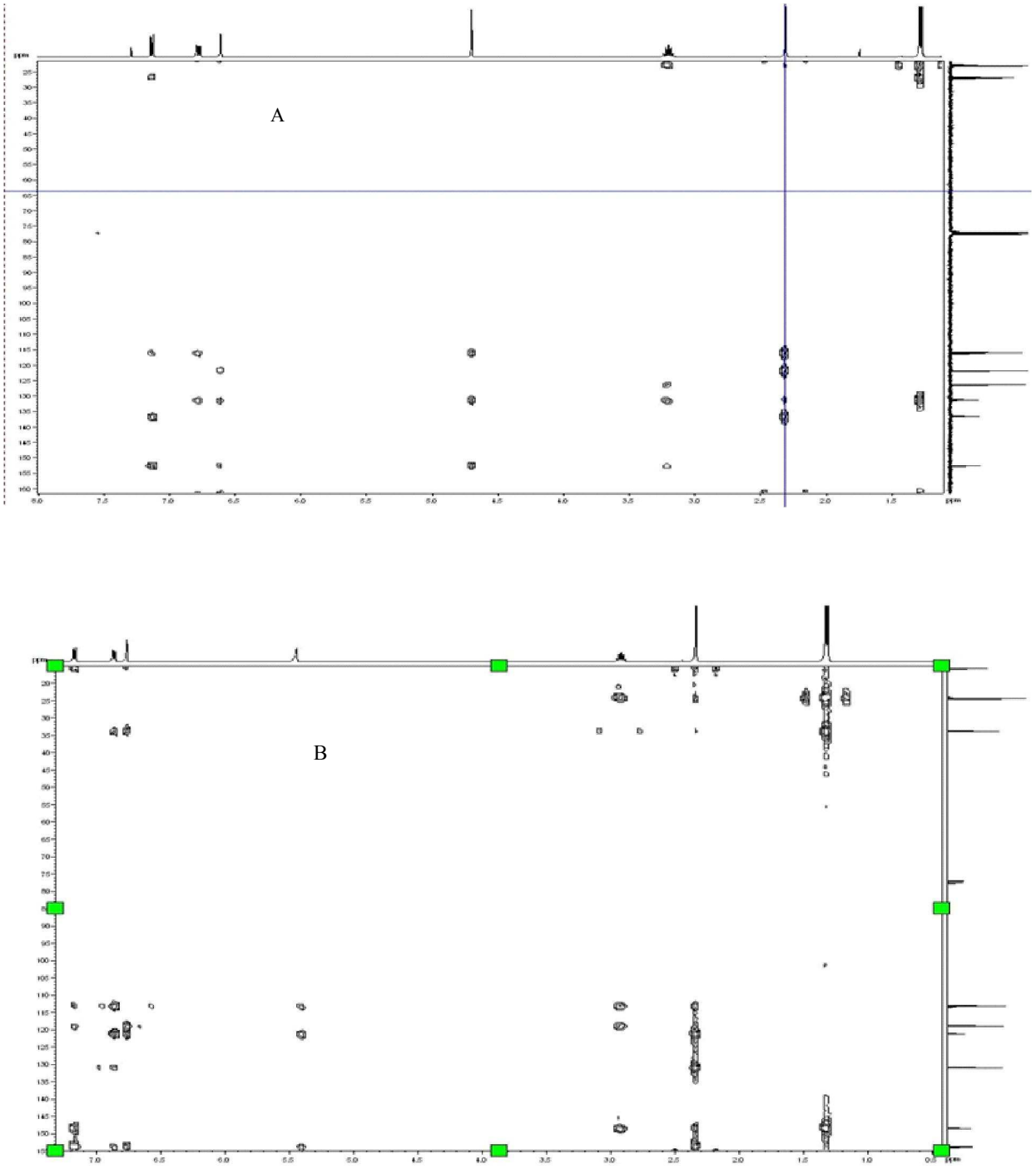


Fig. 12 HMBC NMR Spectrum of pure thymol (A) and pure carvacrol (B)

Table-13 HSQC Spectrum of pure thymol and pure carvacrol

Thymol		Carvacrol	
Carbon	Correlated with	Carbon	Correlated with
C-3	1H	C-3	1H
C-4	1H	C-4	1H
C-6	1H	C-6	1H
C-7	1H	C-7	3H
C-8	3H	C-8	1H
C-9	3H	C-9	3H

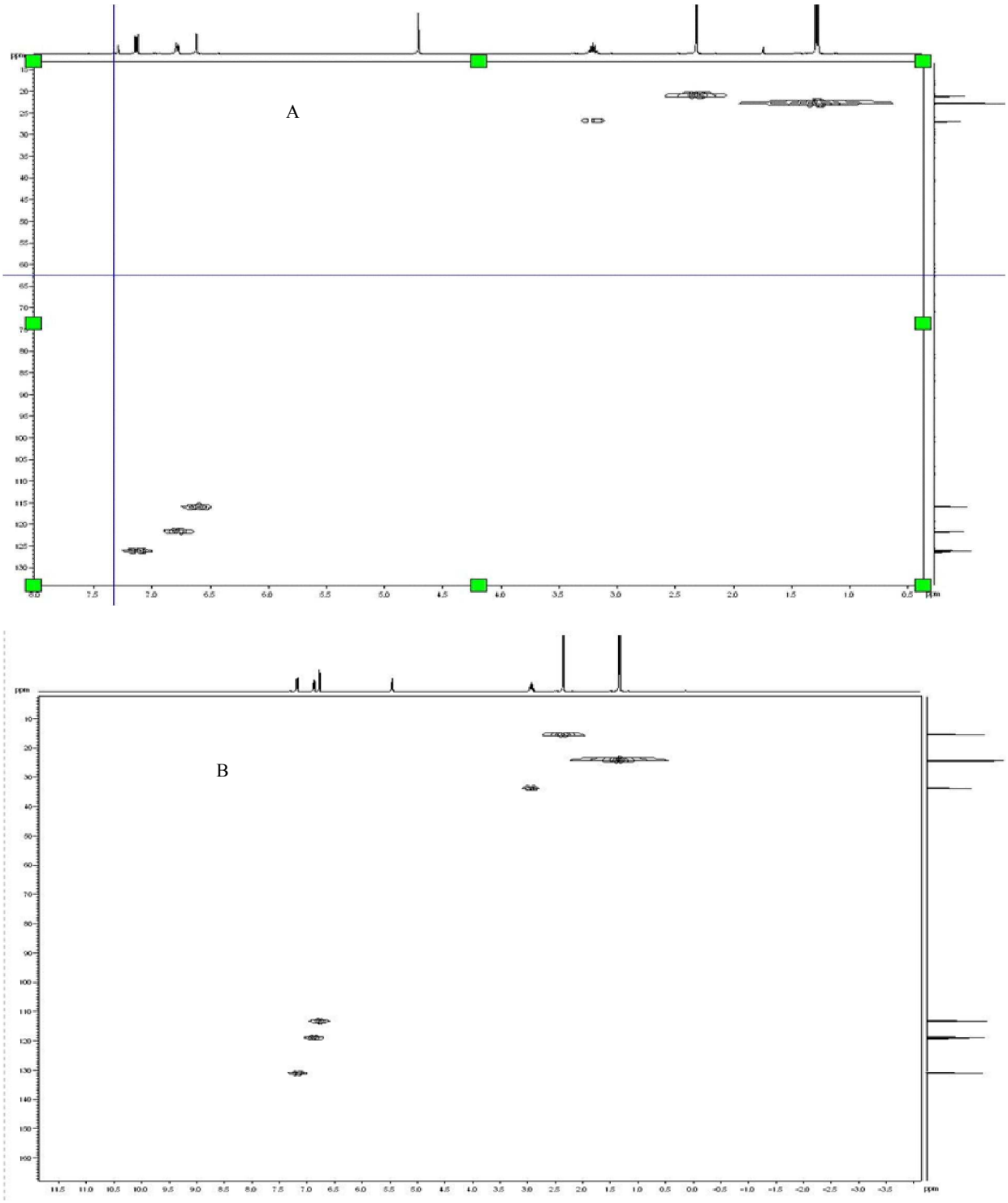


Fig.13 HSQC Spectrum of pure thymol (A) and pure carvacrol (B)

4 Experimental

4.1 Materials and Methods

Clevenger apparatus, Rotary evaporator, digital measuring balance, TLC plates (pre-coated aluminum sheet, 20 X 20 cm, silica gel 60 F_{254}), 254 nm UV lamp, column, silica gel (230-400 Mesh), UV-Visible spectrometer, NMR machine (Bruker Advance 400 MHz NMR spectrometer), HP 6890 GC, 5890 HP GC/MS, Potato Dextrose Agar media (PDA), *Aspergillus niger* fungal pathogen, Autoclave sterilizer, vortex, Incubator, organic solvents, and chemicals etc., were used during this work.

4.2 Plant materials

More than fifty different plant materials were collected from medicinal market Merkto, ALNAP garden and others were brought by Prof. Ermias Dagne.

4.3 Isolation and Analysis of Essential oils

The seeds and leaves of plant materials were dried, powdered and (100-200 g) were hydro distilled using Clevenger apparatus for 1.5 h. The oil remaining in the aqueous phase was extracted using ethyl acetate, dried with sodium sulphate, filtered and the solvent evaporated to dryness with rotary evaporator, weighed and stored with sealed glass vial till bioassay test.

The chemical compositions were analyzed by using a model Hewlett-Packard HP 6890 GC series equipped with FID and HP-5 capillary column (cross linked 5% diph., 95% dimethyl polysiloxan, 30 m X 0.32 mm i.d. X 0.25 μm film thickness). The oven temperature was programmed at 50-210^{o}C at a rate of 3^{o}C /min using N_2 as carrier gas. The injector and detector temperature were 210 and 270^{o}C respectively. The GC/MS analysis of oil samples were done by using 5890 HP GC/MS instrument equipped with a fused silica capillary column (50 m X 0.25 mm, film thickness 0.25 mm). The oven temperature was programmed from 50^{o}C up to 240^{o}C at 3^{o}C /min. and helium used as carrier gas. Identification of components of volatile oils was based on comparison of their retention times with standards, and its computer matching with the Wiley libraries.

4.4 In vitro Antifungal Assay

The antifungal activity of the essential oils against *Aspergillus niger* was determined using agar disc diffusion method, which are normally used as a preliminary screening for essential oils and extracts. The strain *A. niger* was obtained from Addis Ababa University, Biology Department, Mycology Laboratory.

Potato Dextrose Agar (PDA) which is a mixture of glucose, potato and distilled water was used as growing medium for the fungi. It was prepared in the ratio of 500 g of PDA dissolved in 12.8 L distilled water, and 4 mL distilled water in separate vial were autoclaved at 120^{o}C for 3 h and left away from autoclave material to room temperature for about 50-55^{o}C to cool. To the 4 mL sterile distilled water the fungi were added using spatula and homogenized with vortex and mixed with media, between 20-25 mL the solution was dispensed into sterile petri dish and set aside. After solidified, sterile filter paper disc (Whatman no.1, 5 mm in diameter) impregnated with 5-10 µL/disc of each crude essential oil and their active fractions were placed over the middle of the plates already seeded test organism. The plates were incubated at 37^{o}C for 48-72 h, at the end of the incubation period; the plates were evaluated for the presence or absence of microbial growth. The MIC was determined as the lowest concentration of oils and components inhibiting the visible growth of organism in the plates.

4.5 Fractionation of Essential Oils

About (500-700 mg) of essential oils were subjected to column chromatography using a column packed with 8 g silica gel and eluted with 50 mL hexane and 50 mL ethyl acetate/50 mL of dichloromethane. The eluted samples were analyzed by TLC using dichloromethane/hexane (2:1) as solvent system, dried with sodium sulphate, filtered and evaporated in a Buchi rotary evaporator, weighed and kept in fridge with vial till bioassay test.

Fractionation of *Trachyspermum ammi* with column chromatography: 700 mg of *T. ammi* was subjected to CC and the result obtained is presented as follows:

Fractions	Solvents system	Weight of fractions	Labelled as (90-	Bioactivity
1-5	100% Hexane	329 mg	65 fr-1	Not active
6-10	100% DCM	130 mg	66 fr-2	Active
11-16	"	78 mg	66 fr-3	Active

Fractionation of citronella (*Cymbopogon nardus*) essential oil using CC: 700 mg of citronella oil was subjected to CC and eluted with 100% hexane and 100% ethyl acetate as follow:

Fractions	Solvent system	Weight of fractions (mg)	Labelled as (90-	Bioactivity
1	100% of Hexane	5	67 fr-1	Not active
2	"	80	67 fr-2	Not active
3	"	152	67 fr-3	Partially active
4	100% of Ethyl acetate	450	67 fr-4	Active

Fractionation of lemongrass (*Cymbopogon citratus*) with CC: 100 mg of lemongrass oil was subjected to CC and eluted with 100% hexane and ethyl acetate as follow:

Fractions	Solvent system	Weight of fractions (mg)	Labelled as (90-	Bioactivity
1	100% Hexane	-	75 fr-1	-
2	"	-	75 fr-2	-
3	100% Ethyl acetate	50	75 fr-3	Active
4	"	40	75 fr-4	Active

4.6 Preparation of Brady's Reagent

DNPH (2,4-dinitrophenylhydrazine) was dissolved in ethanol at elevated temperature, and filtered while it is still hot. The solution was allowed to cool, and then the crystal was separated from the solution. Then 30 mg of the recrystallized DNPH, 3 drops of H_2SO_4 and 2.5 mL of ethanol were mixed in 10 mL volumetric flask and $CHCl_3$ was added to get 3 mg/mL solution of DNPH. 1 mL was taken from the labelled stock solution using 2 mL measuring pipette and $CHCl_3$ was added up to the mark in order to get 0.3 mg/mL solution of Brady's reagent and the solution was kept in the refrigerator.

The TLC results of essential oil derivatized with diluted 0.3 mg/mL of Brady's reagent were used for carbonyl test.

5 Conclusions and Recommendation

The results revealed that the antifungal activity of essential oils were observed in decreasing order of *Origanum vulgare* (oregano), *Thymus schimperi* (Tosign), *Cymbopogon nardus* (Citronella), *Cymbopogon citratus* (Lemongrass), *Trachyspermum ammi* (Nech azmud), and *Eucalyptus citriodora*.

Origanum vulgare and *Thymus schimperi* showed strong inhibition zone with a diameter of 60 mm and 36 mm, respectively, against the fungal pathogen of *Aspergillus niger* by agar disc diffusion method. This is due to the presence of thymol and carvacrol as observed from GC and GC/MS analysis. These essential oils can also be used as potential candidates for preparation of fungal drug formulations and thus may be useful in the treatment of different kinds of dermatophytes in both humans and animals.

Thymol isolated from *Trachyspermum ammi* showed strong inhibition zone against the fungal pathogen with a diameter of 36 mm (10 μL/disc) clear inhibition zone.

Further studies are required to know the effect of these essential oils in experimental animals (in vivo) and to establish if they could be safely used as antifungal agent against dermatophytes and other infectious diseases with their corresponding isolated components.

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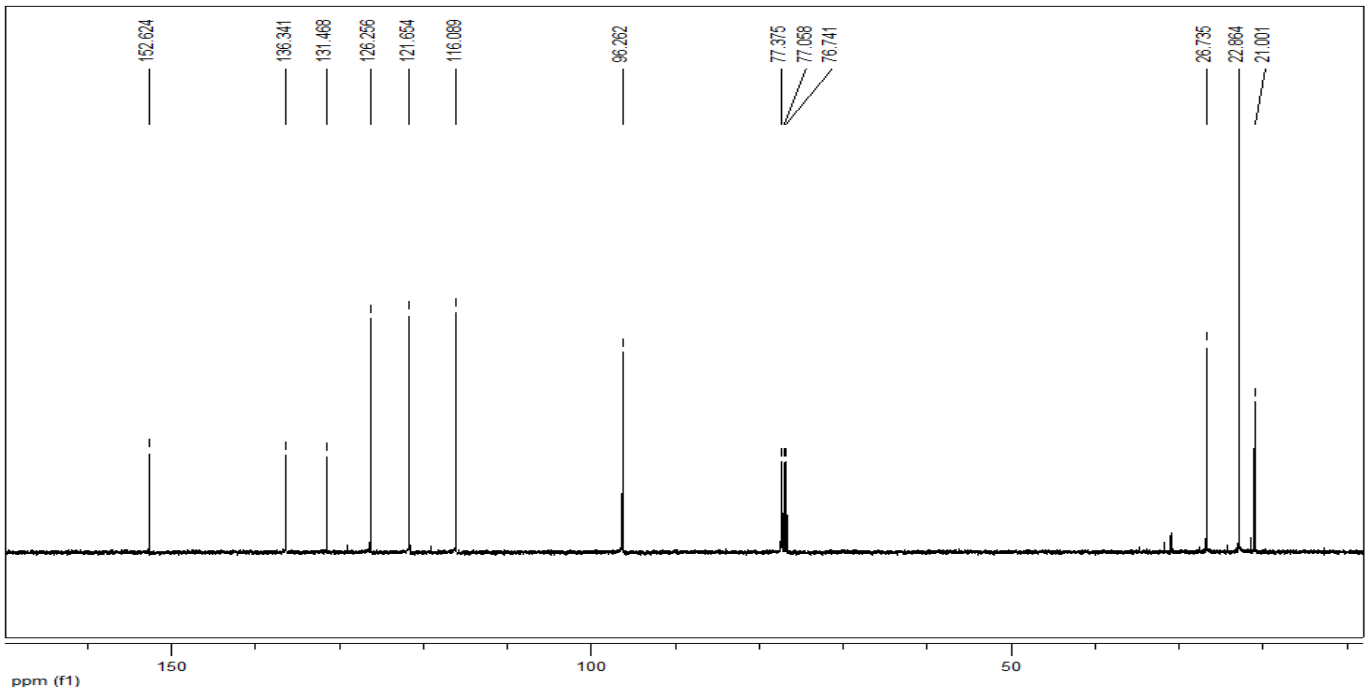
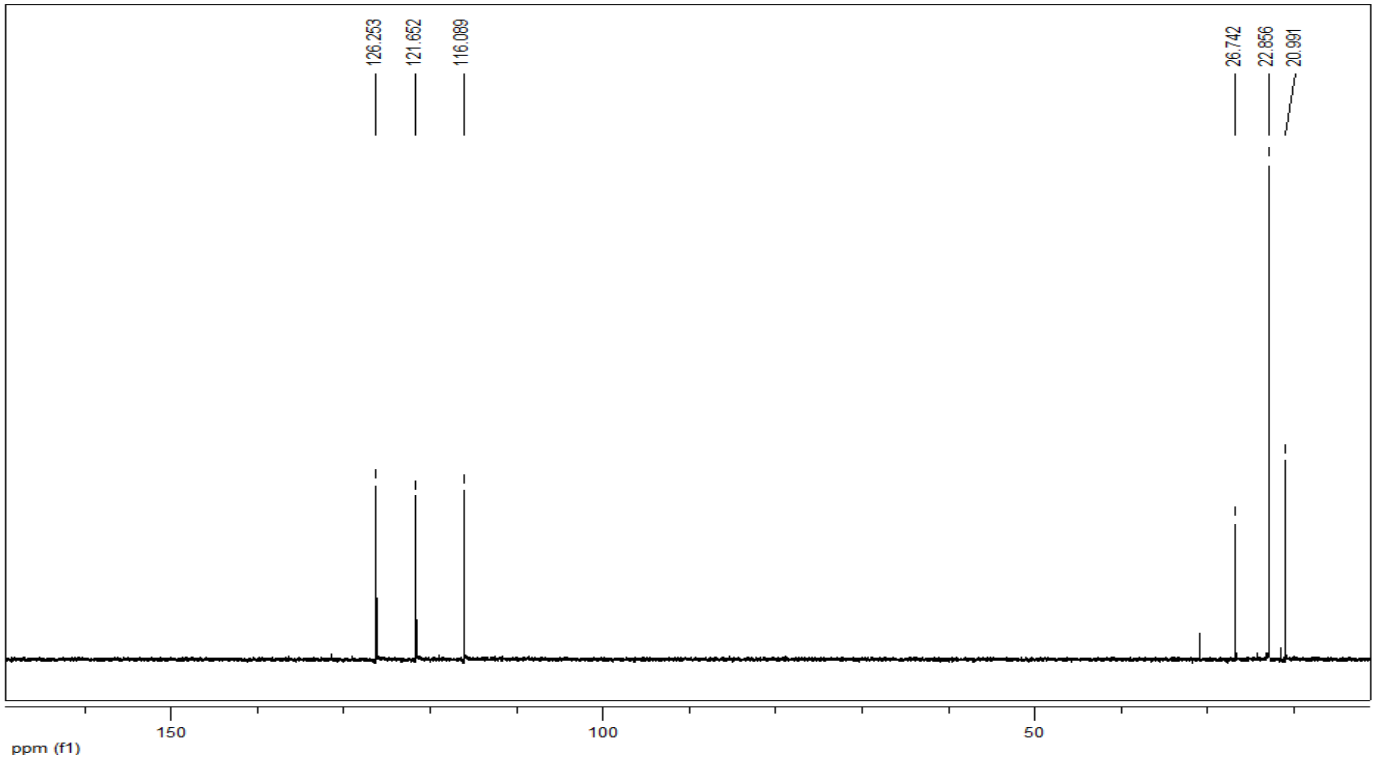
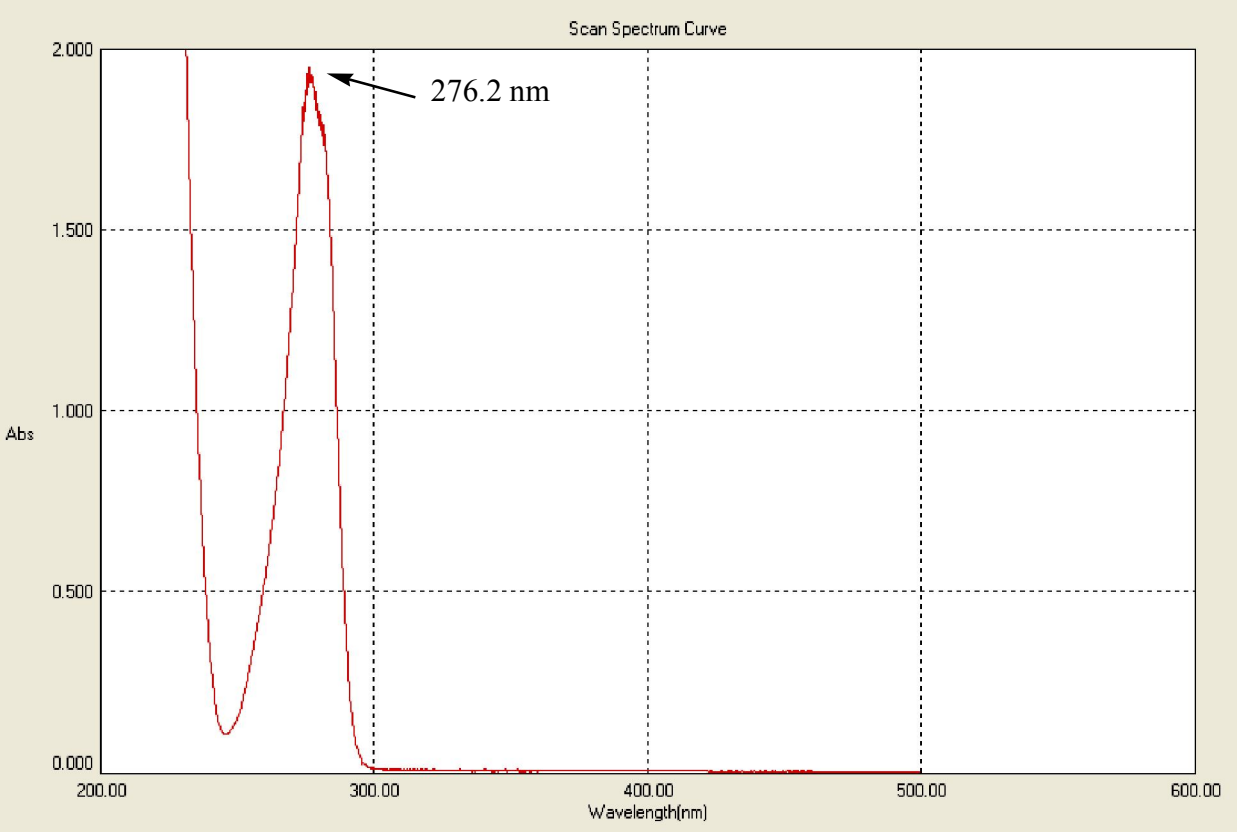
Appendices



Appendix-1 ^{13}C NMR spectrum of thymol isolated from Trachyspermum ammi EO



Appendix-2 DEPT-135 NMR spectrum of thymol isolated from Trachyspermum ammi EO



Appendix-3 UV-Visible spectrum of pure thymol

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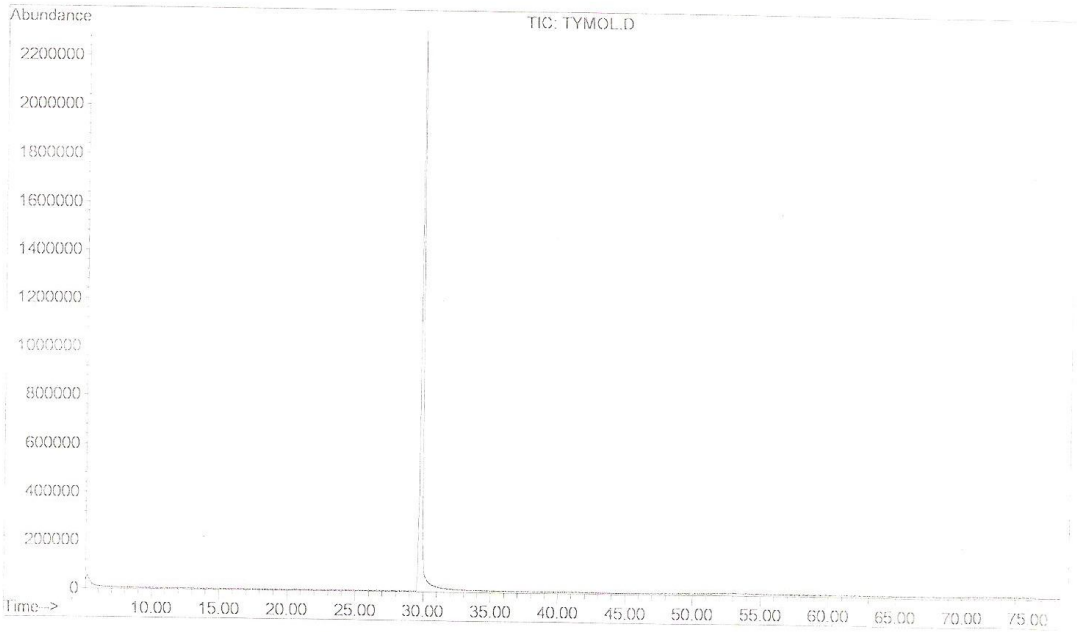
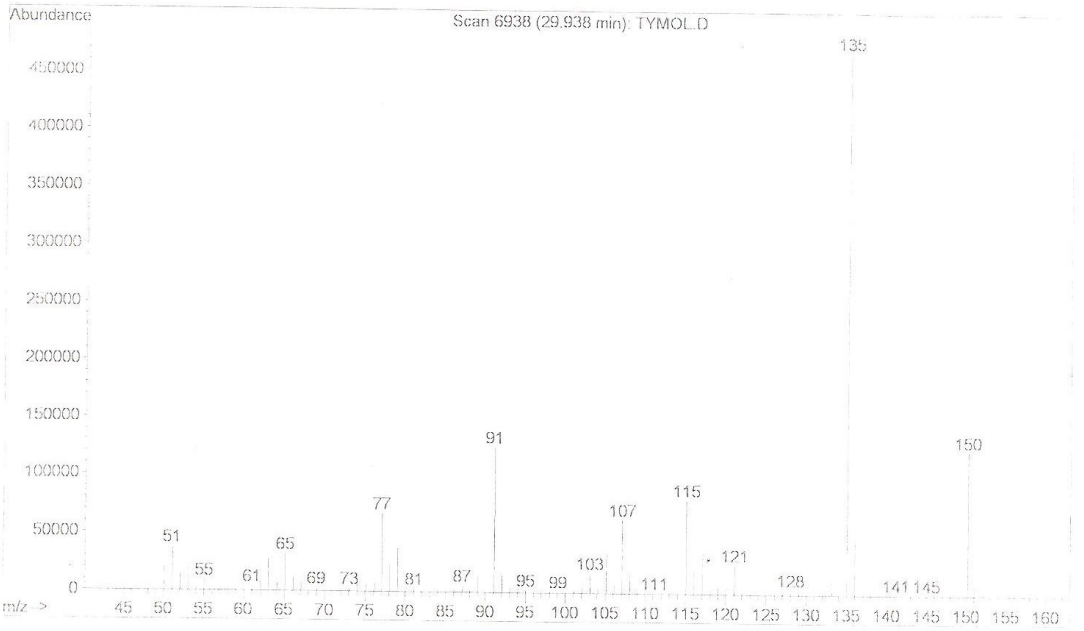
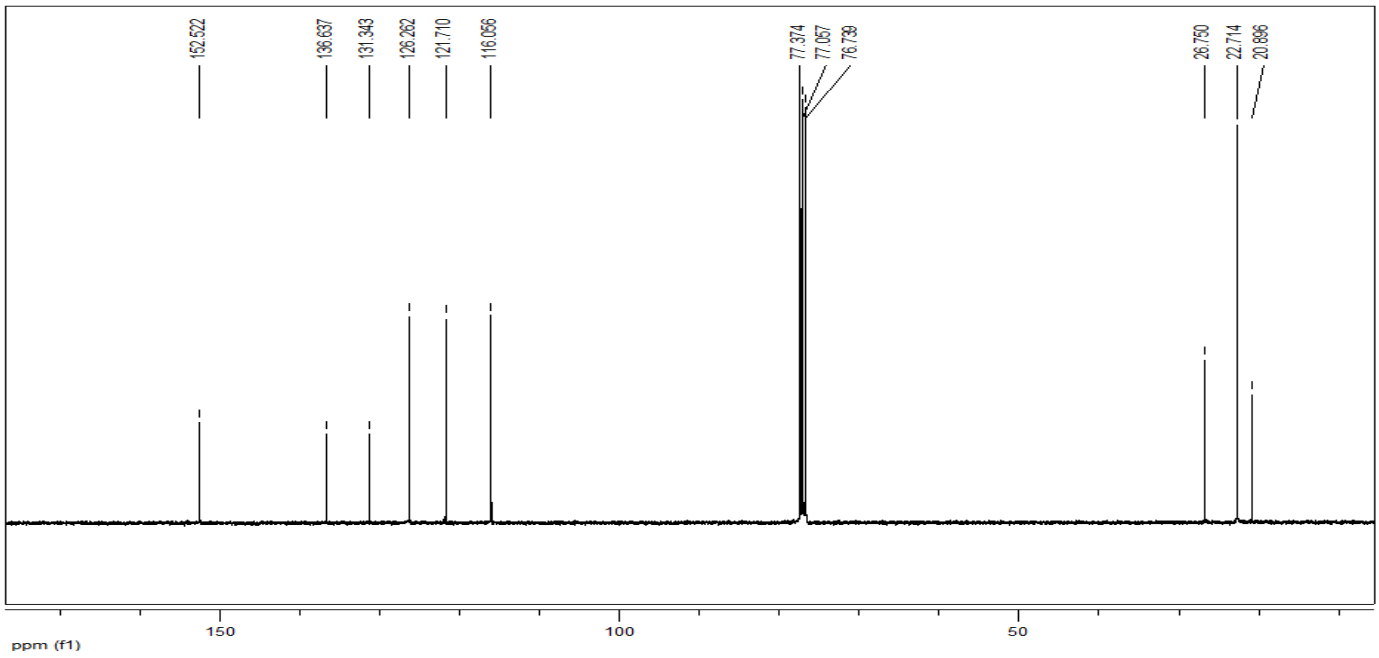
Instrument : GC/MS Ins
Sample Name: tymol
Misc Info :
Vial Number: 1

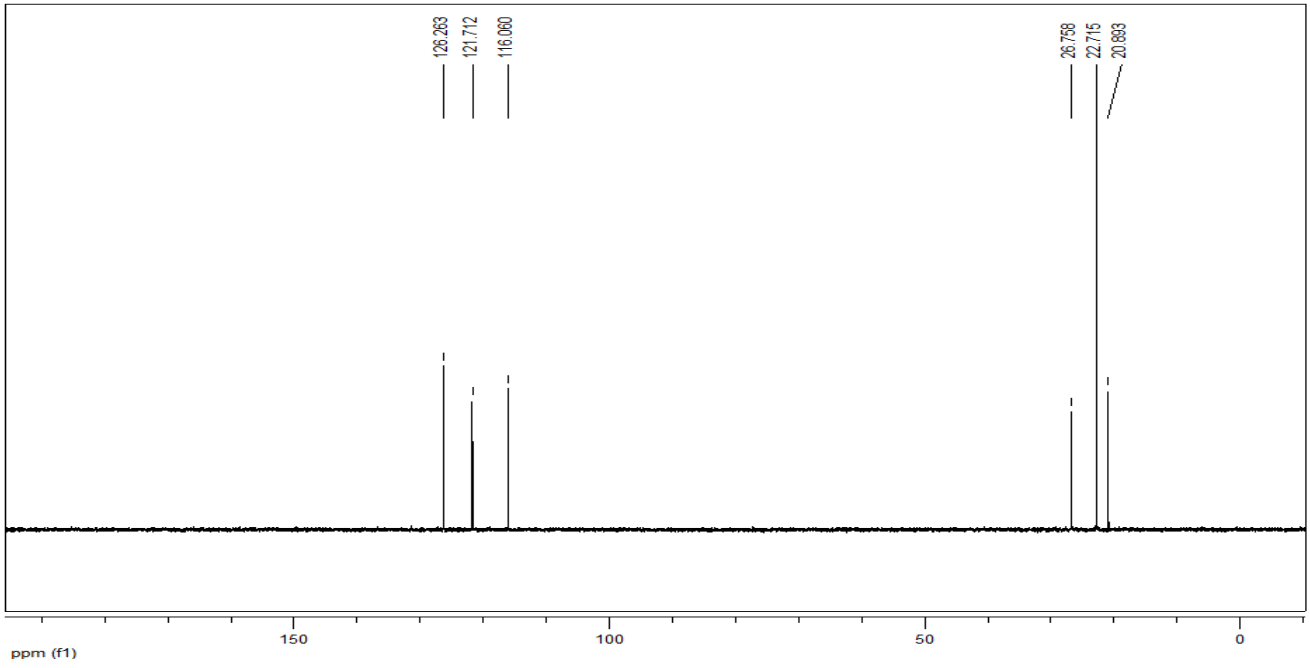




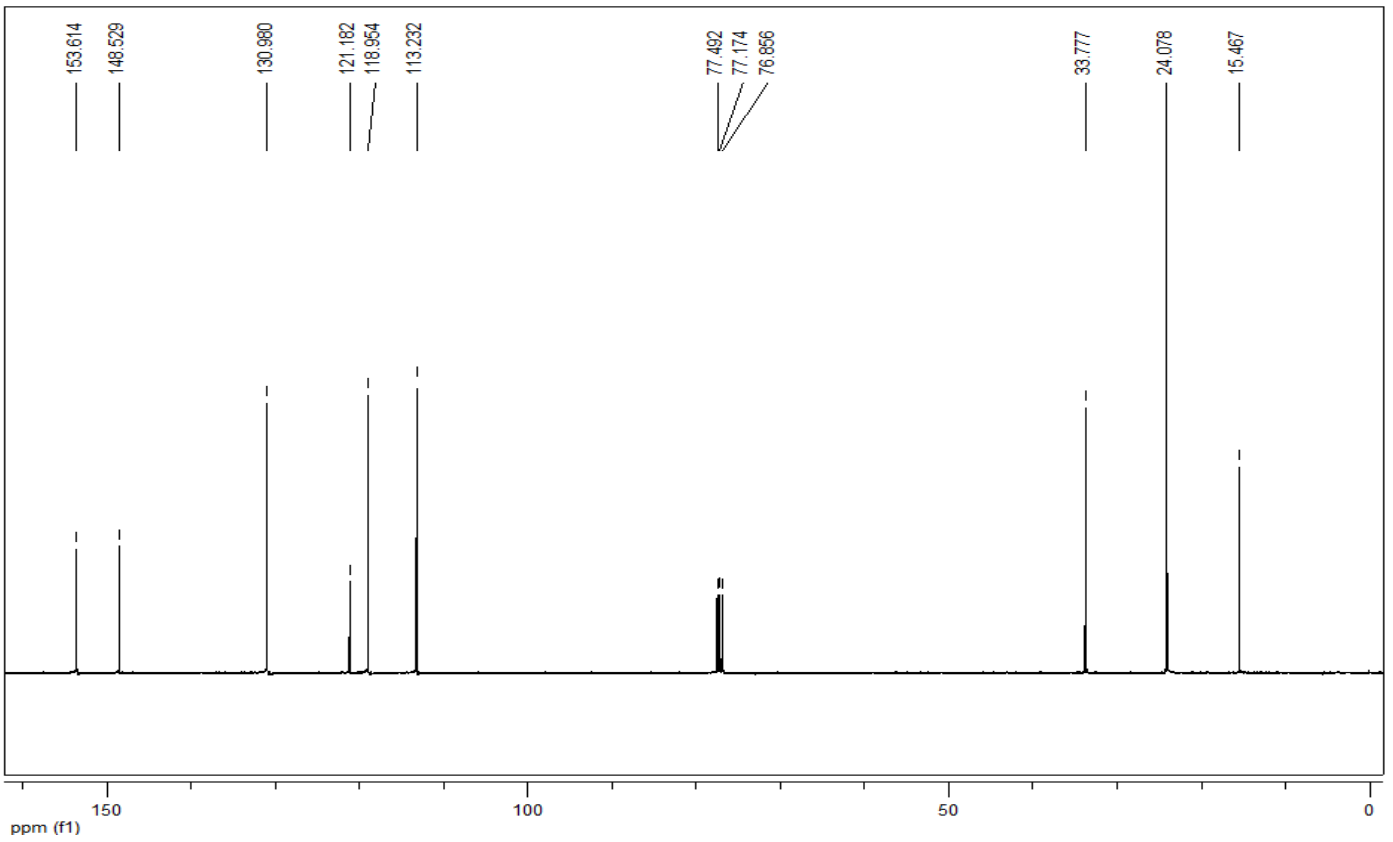
Appendix-4



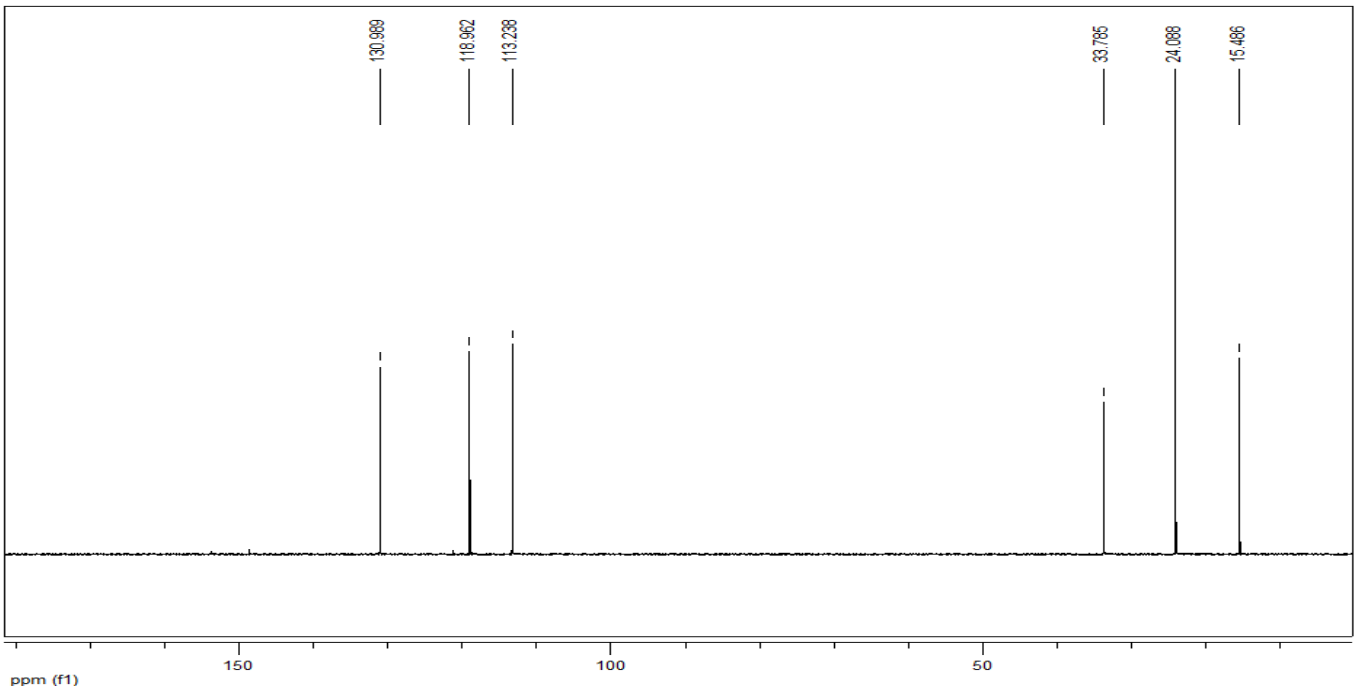
Appendix -5 ^{13}C NMR spectrum of pure thymol (400 MHz NMR)



Appendix-6 DEPT-135 NMR spectrum of pure thymol (400 MHz)



Appendix-7 ^{13}C NMR spectrum of pure carvacrol (400 MHz NMR)



Appendix-8 DEPT-135 spectrum of pure carvacrol (400 MHz NMR)

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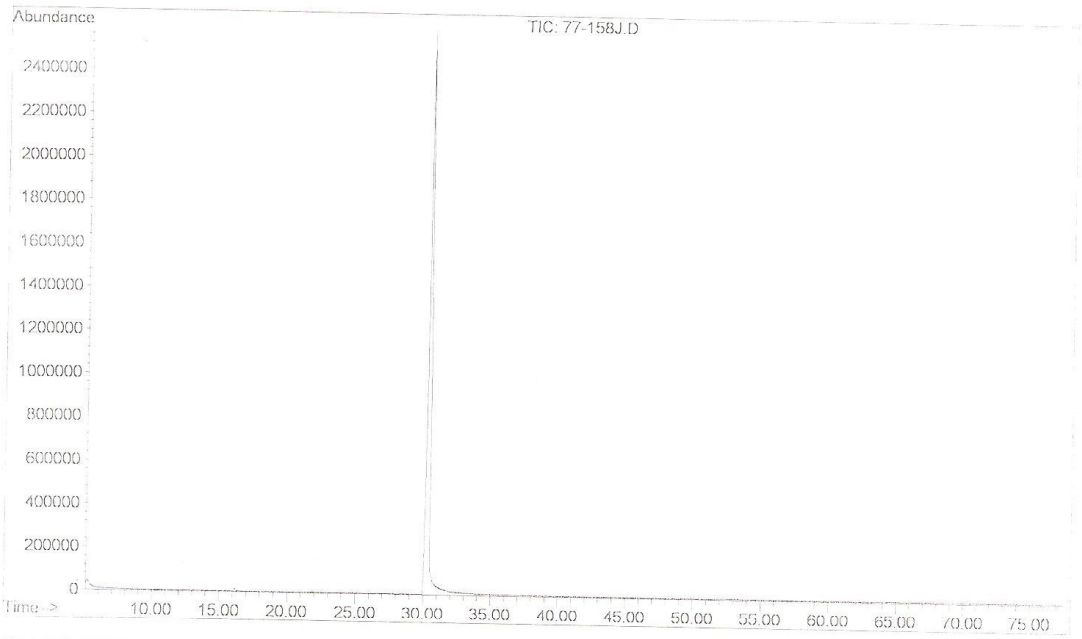
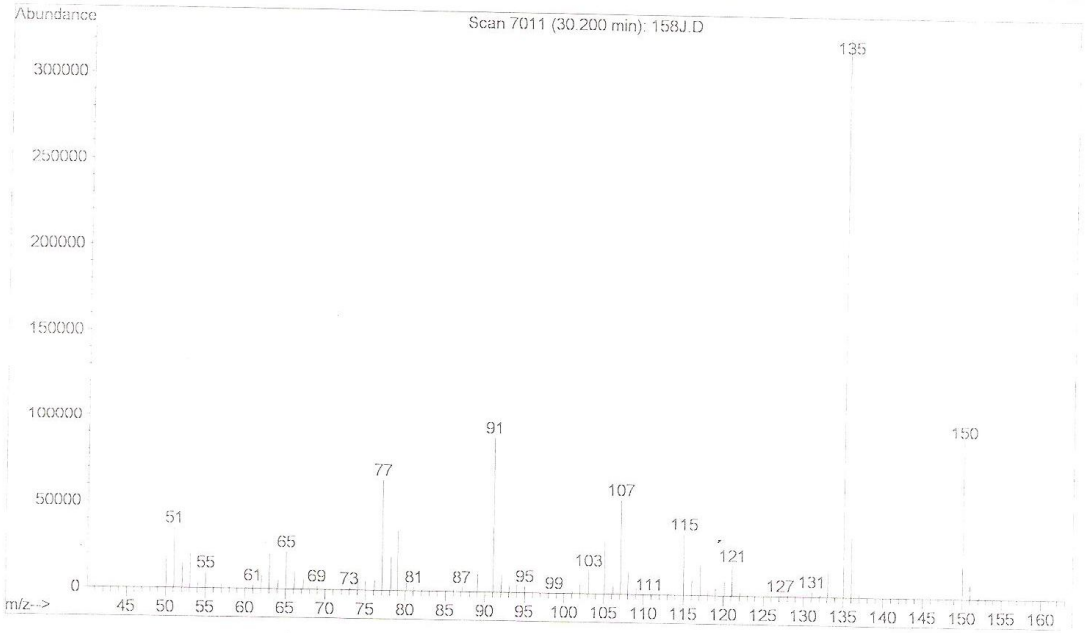
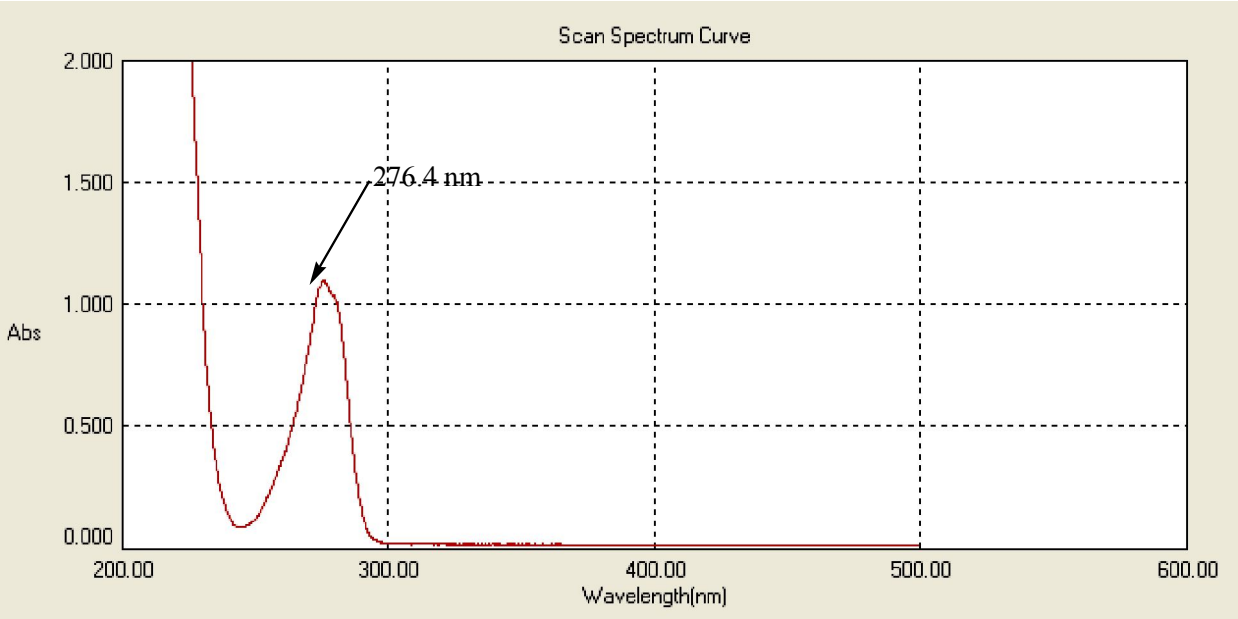
Instrument : GC/MS Ins
Sample Name: 77-158J
Misc Info :
Vial Number: 1





Appendix-9



Appendix-10 UV-Visible spectrum of pure carvacrol

Declaration

I, the undersigned, declared that this thesis work is my original work and has not been presented for a degree in any other University and all the sources of materials used for this thesis work has been duly cited.

Name: Shewaye Lakew

Signature: _____

This thesis has been submitted for examination with approval as University Advisors

	Signature
Prof. Ermias Dagne (Advisor)	_____
Dr. Dawit Abate (Co-Advisor)	_____

Place and Date of submission: Department of Chemistry, Addis Ababa University

June 2011