

**Isolation, Identification  
and Biological Activity of  
Volatile Oil Extracted From  
Local Thyme**

**A Thesis  
Submitted to the College of Science  
Babylon University in Partial Fulfillment of the  
Requirements for the Degree of Master of Science in  
Chemistry**

**By  
Amal Sabah Abdullah  
Baghdad University  
B.Sc. 1991**

September /2005 A.D

Shaaban/ 1426 H.

## *Certification*

We certify that this thesis was prepared by **Amal Sabah Abdullah** under our supervision at the Department of Chemistry, College of Science, Babylon University, as a partial requirements for the degree of **Master of Science in Chemistry**.

Signature:

Name: **Dr. Ikbal S. Al-Sheibany**

Title: Professor

Address: Baghdad University, College of  
Education/ Ibn- Al-Haitham.

Date:            /            /2005

Signature:

Name: **Dr. Kasim H. Kadhim**

Title: Assistant Professor

Address: Babylon University, College of  
Science.

Date:            /            /2005

In the view of the available recommendation, I forward this thesis for debated by the examination committee.

Signature:

Dr. **Kasim H. Kadhim**

Head of Chemistry Department

Date: /

/2005

### **Examination Committee Certification**

We certify that we have read this thesis, and as an examination committee, we examined the student **Amal Sabah Abdullah** on its contents, and that in our opinion it is adequate for the partial fulfillment of the requirements of **Master Degree in Chemistry**.

Signature:

Name: Dr. Intisar A. Al-Timimy

Title: Assiastant Professor

Date: / /2005  
(Chairman)

Signature:

Name: Dr. A.A. Abd Al-Jalil

Title: Assiastant Professor

Date: / /2005  
(Member)

Signature:

Name: Dr. Sadiq J. Baqir

Title: Lecturer

Signature:

Name: Ikbal S. Al-Sheibany

Title: Professor

Date:     /     /2005  
          (Member)

Date:     /     /2005  
          (Supervisor)

Signature:

Name: Dr. Dr. Kasim

H. Kadhim

Title:     Assiastant

Professor

Date:     /     /2005  
          (Supervisor)

**Approved by the University Committee  
of Postgraduate Studies**

Zamily

of Science

Signature:

Name: Oda M. Al-

Title: Professor

Dean of the College

Date:     /     /2005

—

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2005

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TO MY FAMILY...

بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِیْمِ  
وَآیةٌ لَهُمُ الْاَرْضُ  
الْمِیْتَةُ اَحْيٰیْنَهَا  
وَ اَخْرَجْنَا مِنْهَا  
حَبًا فَمِنْهُ  
یَاْكُلُوْنَ  
صَدَقَ اللّٰهُ الْعَظِیْمُ

سورة یس (32)

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## LIST OF ABBREVIATIONS AND SYMBOLS

B.C.	Before Christ
GC	Gas Chromatography
MS	Mass Spectrometry
HD	Hydrodistillation
SFE	Supercritical Fluid Extraction
LH	Lutinizing Hormone
FSH	Follicular Stimulating Hormone
UV-Vis	Ultraviolet and Visible
TLC	Thin-Layer Chromatography
HPLC	High-Performance Liquid Chromatography
DMSO	Dimethyl Sulphoxide
R <sub>f</sub>	Retardation value
IR	Infrared spectra
t <sub>R</sub>	Retention time
Gr+Ve	Gram – positive
Gr-Ve	Gram – negative

# ***Introduction***

## ***1.1 General Introduction:-***

Finding healing power in plants is an ancient idea. People on all continents have long applied poultice and imbibed infusions of hundreds, if not thousands, of indigenous plants, dating back to prehistory. There is evidence that Neanderthals living 60.000 years ago in present-day Iraq used plants such as hollyhock; these plants are still widely used in ethnomedicine around the world<sup>(1,2)</sup>.

The Chinese have ancient herbal traditions that can be found and recorded 2000 years B.C. Hippocrates of Greece, known as the “father of medicine”, prescribed perfume fumigation's and fomentations. But perhaps the most famous and richest association, the first aromatic associations, dates back to Egyptian papyrus manuscripts written 2800 years B.C.<sup>(3)</sup>.

Around 3000 B.C. the Sumerians used the herbal and medicinal plants for therapeutic purposes<sup>(4)</sup>. They were using thyme as a medicinal ingredient, and the Egyptians included it among the herbs and spices used in mummification. The Greeks used thyme as a temple incense ( the word thyme comes from a Greek word meaning “to fumigate”), and both they and the Romans praised its healing virtues<sup>(5)</sup>.

In old Babylon the laws of Hammurabi (1728-1686) B.C. state “ the therapeutic uses of many plants and the special law for doctors who fail in treatment of their patient”<sup>(4)</sup>.

Treatment with plants continues till the 19th century when chemical science progresses and Herbalist time began. Then chemists started to extract active compounds from different plants, like atropine from *Atropa belladonna* and ephedrine from *Ephedra alata*<sup>(6)</sup>.

Many crude drugs are used medically because of their volatile oils content; however, in numerous cases, the volatile oils separated from the drugs are used as drugs themselves. Similarly, various crude drugs are powdered and are employed as spices and condiments (anise, clove, pepper and sage)<sup>(7)</sup>.

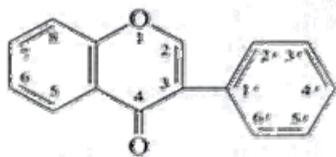
Any plant in fact is a complete pharmacy containing active compounds in different percentages, for example Onion (*Allium cepa*) contains digestive compounds and others which regulate burning, also it contains volatile oils with special smell and this oil is powerful antiseptic, besides, *onion* contains salts that strengthen the hairfollicles. Also Arac (*Salvadora persica*), the leaves contain trimethylamine alkaloid, while the seeds contain volatile oils and resin and a few of silica. Its roots were used in the treatment of ancylostoma, also its leaves were used as analgesics for these urinary tract; its sticks were used as tooth brush tree.

Spinach (*Spinacea oleracea*) contains high percentage of minerals as iron, iodine and calcium. It contains vitamins as A, K, C and folic acid; it also contains saponins concentration in plant roots and the

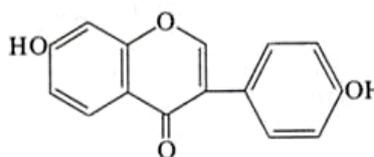
plant was used in the treatment of anaemia because it contains iron and folic acid; also it helps to decrease sugar in the blood. While the fruitage of Crist's thorn (*Zizyphus spina-christi*) contains a high percentage of vitamin C, in ethnomedicine, the cool wood mixed with vinger was used in the treatment of snake bite, and the boiled leaves were used as anti-diarrheal and anti-helmenthics and the fruitage was used for fever and as laxative. It is also is used for the treatment of measles.

Seeds of Soya bean (*Glycine hispid*) contain a high percentage of oil (19-22%) and a high percentge of protein (40%), also it contains isoflavones(1) like Daidzein(2) which have effect on estrogen and also it contains lecithin, saponins, vitamin E, B and carotens. The Soya bean is considered as the most important plant in the world and a meat substitution and a diet for diabetic patients because it contains sugar which is difficult in digestion and metabolism. Garden radish (*Raphanus sativus*) has a great effect on liver and neural disease, and the boiled leaves were used as diuretic and laxative, also the thick fibers of Garden radish help in food absorption and decrease the cholestrol in blood and decrease the possibility of the increase of hypertension<sup>(6)</sup>.

In the law of Avicenna about thyme ((it is softening solution beneficial in pelvic pain and toothache and cure redundant gum; its oil is beneficial for liver and stomach and cidal for intestinal worms))<sup>(8)</sup>.



Isoflavones(1)



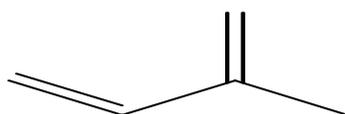
Daidzein(2)

## 1.2 Review of Literature

### 1.2.1 Terpenoids

The terpenoids form a group of compounds the majority of which occur in the plant kingdom; a few terpenoids have been obtained from other sources. The simpler mono- and sesqui-terpenoids are the chief constituents of the essential oils; these are the volatile oils obtained from the sap and tissues of certain plants and trees. The essential oils have been used in perfumery from the earliest times. The di- and tri-terpenoids which are not steam volatile, are obtained from plant and tree gums and resins. The tetraterpenoids form a group of compounds known as the carotenoids. Rubber is the most important polyterpenoid.

Most natural terpenoids hydrocarbons have the molecular formula  $(C_5H_8)_n$  and the value of  $n$  is used as a basis for classification; this is known as the isoprene unit(3). These units arise from acetate via mevalonic acid and are branched chain, 5-carbon unit containing two unsaturated bonds.



Isoprene(3)

The isoprene units in natural terpenoids were joined ‘head to tail’ (the head being the branched end of isoprene).

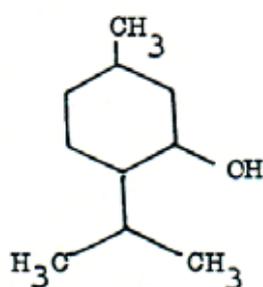
<u>Number of carbon atoms</u>	<u>Class</u>
(i) 10	Monoterpenes $C_{10}H_{16}$
(ii) 15	Sesquiterpenes $C_{15}H_{24}$
(iii) 20	Diterpenes $C_{20}H_{32}$
(iv) 30	Triterpenes $C_{30}H_{48}$
(v) 40	Tetraterpenes $C_{40}H_{64}$
(vi) > 40	Polyterpenes $(C_5H_8)_n$

Monoterpenes like Menthol(4) together with sesquiterpenes like Bisabolans(5) and diterpenes like Cembrene(6) form the majority of essential oils<sup>(9)</sup>. Terpenoids differ from fatty acids in that they are cyclic compounds containing extensive branching; Terpenoids or terpenoids are active against bacteria<sup>(10-12)</sup>, fungi<sup>(13,14)</sup>, viruses<sup>(15-17)</sup>, and protozoa<sup>(18,19)</sup>.

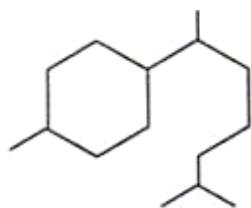
Essential oils have wide and varying industrial applications for the flavoring of food, beverages, candy and sugar confectionary, in tobacco, in the creating of fragrances and for cosmetics. They are also used for medical purposes. Food scientists have found the Terpenoids

present in essential oil plants to be useful in the control of *Listeria monocytogenes*<sup>(20)</sup>.

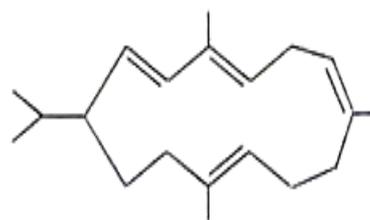
In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria<sup>(21)</sup>.



Menthol(4)



Bisabolane(5)



Cembrene (6)

### 1.2.2 Volatile Oils

Volatile oils are colourless liquids consisting of mixtures of saturated or unsaturated cyclic compounds<sup>(22)</sup>. The constituents of the oils are mainly monoterpenes and sesquiterpenes; it is estimated that there are more than 1000 monoterpenes and 3000 sesquiterpenes structures<sup>(23)</sup>. There are four general methods for the extraction of essential oils:-

1. Expression.
2. Steam distillation.

3. Extraction with volatile solvent.
4. Resorption in purified fats<sup>(24)</sup>.

Depending on the plant family, volatile oils may occur in specialized secretory structure such as glandular hairs (Labiatae), modified parenchymal cells (Piperaceae), and oil-tubes called vittae (Umbelliferae)<sup>(6)</sup>.

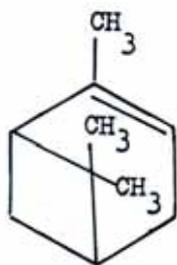
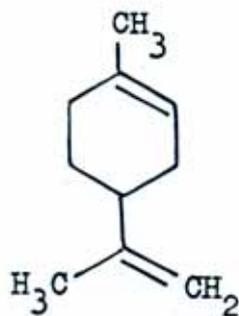
Several points of differentiation exist between volatile oils and fixed oils. Volatile oils can be distilled from their natural sources; they do not consist of glyceryl esters of fatty acids. Hence, they do not have a permanent grease spot on paper and cannot become rancid as do the fixed oils, but instead, on exposure to light and air, they oxidize and resinify. Finally they differ entirely in both chemical and physical properties from fixed oil<sup>(25)</sup>.

### ***1.2.2.1 Chemistry of Volatile Oils***

The mixture compounds of volatile oils may be separated in various ways:-

1. Low temperatures.
2. Fractional distillation.
3. Fractional crystallization.
4. Different forms of chromatography.
5. Removal by chemical action.

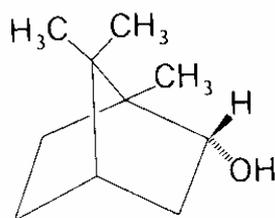
The divisions in which volatile oils and volatile oil-containing drugs are placed as in the followings:

1. Hydrocarbon volatile oils. $\alpha$ -pinene(7)

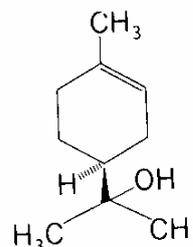
d-limonene(8)



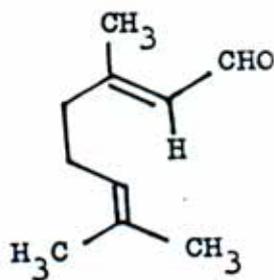
p-cymene(9)

2. Alcohol volatile oils.

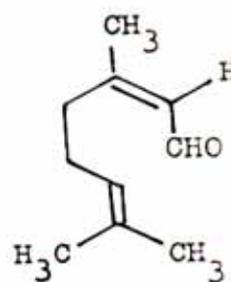
Borneol(10)



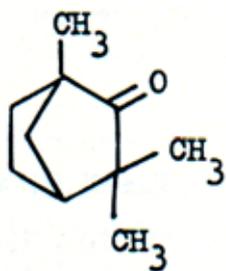
Terpineol(11)

3. Aldehyde volatile oils.

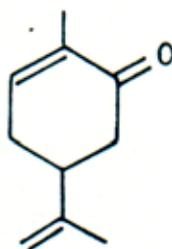
Geranial(12)



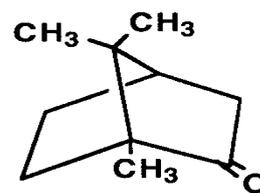
Neral(13)

4. Ketone volatile oils.

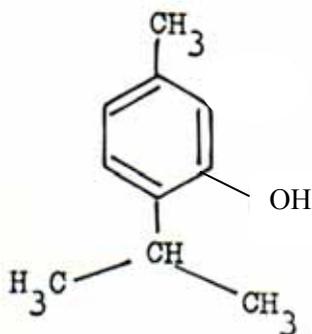
Fenchone(14)



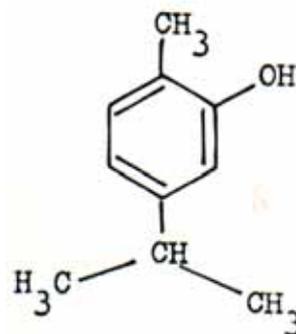
Carvone(15)



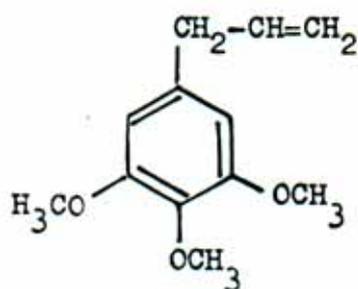
Camphor(16)

5. Phenol volatile oils.

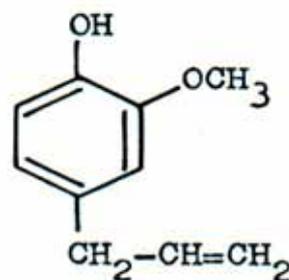
Thymol(17)



Carvacrol(18)

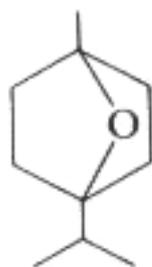
6. Phenolic ethers volatile oils.

Elemicin (19)



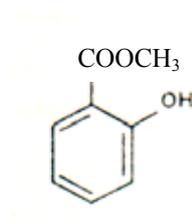
Eugenol(20)

### 7. Oxides volatile oils.



1,4-Cineoil(21)

### 8. Esters volatile oils.



Methyl salicylate(22)

Volatile oils are used for their therapeutic action, for flavouring (e.g. oil of lemon), in perfumery (e.g. oil of rose) or as starting materials for the synthesis of other compounds (e.g. oil of turpentine). For therapeutic purposes they are administered as inhalation (e.g. eucalyptus oil), orally (e.g. peppermint oil), as gargles and mouthwashes (e.g. thymol).

The constituents of many volatile oils are stated to interfere with respiration and electron transport in a variety of bacteria, hence accounting for their use in food preservation and in cosmetic preparations<sup>(7)</sup>.

## ***1.2.3 Factors that Affect the Quantitative and Qualitative Properties of Volatile Oils:-***

### ***1.2. 3.1 Environmental Factors:***

Environmental factors affect quantitative and qualitative properties of extracted volatile oils. Rustaiyan et al<sup>(26)</sup> had chemically studied 3 species of herba thyme ( *Thymus ktschyanus*, *Thymus pubescens* and *Thymus carmanicus*) collected from different areas of Iran and they extract volatile oils from each species by hydrodistillation for 3h. The oils of all 3 species are rich in monoterpene, phenols, thymol at amount (38.0%, 37.9%, and 40.8%) and carvacrol at amount (14.2%, 14.1%, and 24.8%).

Recent studies<sup>(27)</sup>: the essential oils of four Turkish wild-growing Labiatae herbs, the essential oils, hydrodistilled from flowering plants of (*Salvia crybtantha*, *Saturea cuueifolia*, *Thymbra spicata*, *Thymus cilicicus*), were analysed by gas chromatography(GC) and GC-mass spectrometry(MS). 33 compounds were identified in *Thymbra spicata* essential oils, the main components were p-cymene 22.1%, gamma-terpinen 21.4%, carvacrol 21.1% and thymol 11.8%. The essential oil of *Thymus cilicicus*, which contained 35 compounds, was rich in alpha-terpineol 16.4% and camphor 9.7%.

The watering level affecting *Thymus hymalis* was studied<sup>(28)</sup> and it was found that winter harvesting showed high concentrations in thymol percentage, in spring this thymus species needs a greater water supply to a chive the same amount of thymol in the essential oil. From this, it was concluded that winter harvesting could be used for the

extraction of the essential oil, with a low level of the water supply, whereas spring harvesting could be employed for the collection of leaves as a food condiment.

Baranauskine et al<sup>(29)</sup> studied the influence of nitrogen fertilizers on the yield of the production and composition of the essential oil and some other chemical characteristics of thyme; they found that there was no effect of nitrogen fertilizers on the chemical characteristic. It was determined that the nitrogen fertilizers influence the amount of nitrates which has its highest level in the second-year-first-harvest.

### ***1.2. 3.2 Stages of Plant:***

Al- Shahatt<sup>(30)</sup> states that quantity and contents of terpenoids oil results from herba thyme increasing its value with growth, and reaching its maximum at flowering and seeding. Granger<sup>(31)</sup> emphasized that the types and percentage of compounds of volatile oils differ according to the stage of level operant during the season of flowering as in *Thyme herba barona* .

Cabo et al<sup>(32)</sup> studied the best time for harvest *Thymus hyemalis* in terms of essential oil yield and concentration of various components (April 1981 to March 1982); it was found that the yield and composition of the oil changed from month to month. The highest percentage of oil was found during July: 0.58% and the lowest in December: 0.15 %. During the months of the lowest temperature and fewest hours of sunlight there is an obvious decrease in essential oil production.

### ***1.2.3.3 Effect of Stimulation Method on the Amount and Type of Volatile Oils***

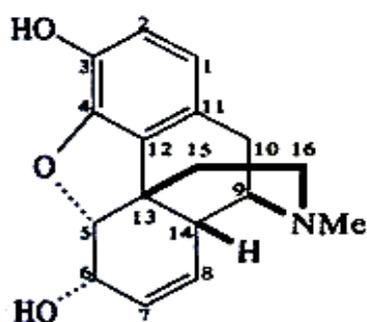
Extractions of thyme by two methods was carried out by Slavco et al<sup>(33)</sup>; the compounds extracted by supercritical fluid extraction (SFE) with carbon dioxide and hydrodistillation (HD) were identified by GC and GC-MS. The difference in the aroma of the products was quite noticeable and can be attributed to qualitative and quantitative differences in the extracts and hydrodistillates. Predominance of aromatic alcohols carvacrol and thymol and their biosynthetic precursors p-cymene and gama-terpinene was revealed in both SFE extracts and HD oil.

Ihssan<sup>(34)</sup> explained the effects of milling on essential oil yield of *Mentha spicata*, using the whole leaf which result in increasing the yield of oil .Similar results have been seen by Serap et al<sup>(35)</sup> where they studied the effect of milling , and found dried thyme way ground samples gave a low amount of essential oil and could be due to the loss of aroma compounds during the grinding process. The SFE of aroma compound from *Thymbra Spicata* was studied by Sonsuzer et al<sup>(36)</sup>; they found that this method gave less amount of essential oil than the steam distillation.

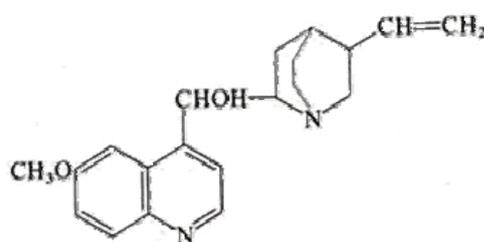
## 1.2.4 Another Major Groups of Antimicrobial Compounds from Plant

### 1.2.4.1 Alkaloids

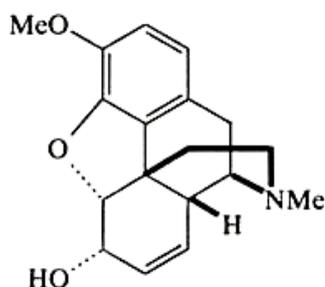
Typical alkaloids are natural plants confirming a basic organic nitrogenous compounds. The first medically useful example of an alkaloids is morphine(23), isolated in 1805 from the opium poppy (*papaver somniferum*). Alkaloids have been found to have microbiocidal effects as quinine(24) which acts as antimalarial drug, codeine(25) as antitussive drug and papaverine(26) as antispasmodic; the major antidiarrheal effect is probably due to their effects on transit time in the small intestine<sup>(37)</sup>.



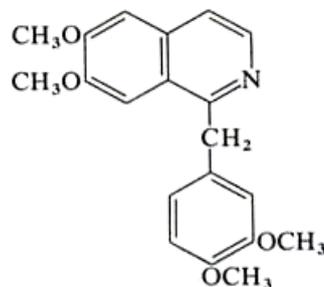
Morphine(23)



Quinine(24)



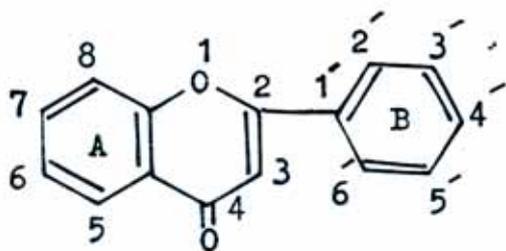
Codeine(25)



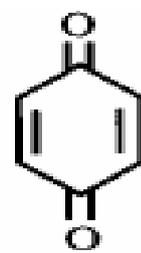
Papaverine(26)

### 1.2.4.2 Flavonoids

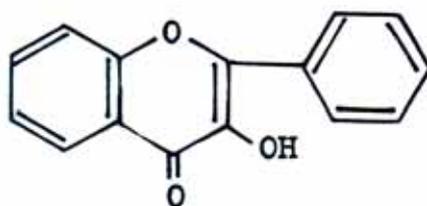
Flavones(27) are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones(28)). When hydrogen atom on C<sub>3</sub> is replaced by hydroxyl group, this gives the first class of flavonol(29)<sup>(38)</sup>. Since they are known to be synthesized by plants in response to microbial infection<sup>(39)</sup>. Their activity is probably due to their ability to complex with extracellular and soluble proteins, and also may be complex with bacterial cell walls<sup>(40)</sup>.



Flavone(27)



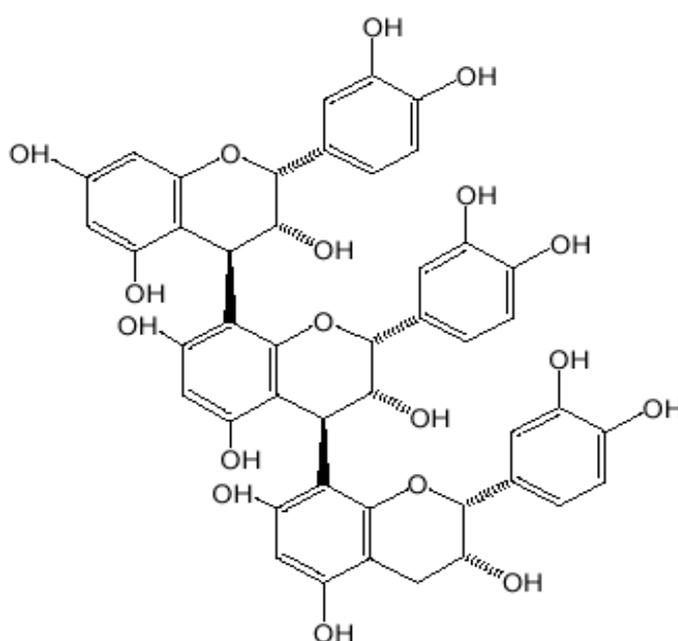
Quinone(28)



Flavonol (29)

### 1.2.4.3 Tannins

Tannins are derivatives of complex polyphenols of either galloyl polyester or proanthocyanidin(30) which are widely distributed in the vegetable kingdom<sup>(41)</sup>. Their molecular weights range from 500 to 3,000 gm/mol<sup>(42)</sup>, and they are found in almost every plant part: bark, wood, leaves, fruits, and roots<sup>(43)</sup>. Tannins in plants inhibit insect growth<sup>(44)</sup> and disrupt digestive events in ruminants<sup>(45)</sup>.



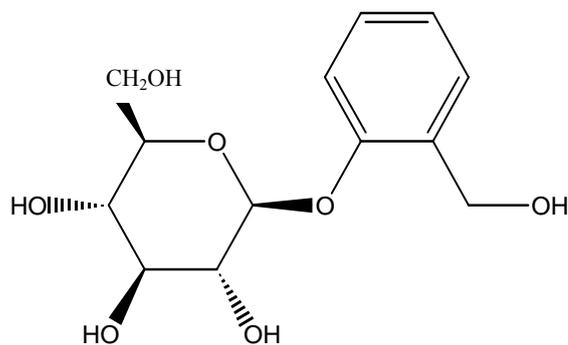
Procyanidin C-1 condensed tannin(30)

### 1.2.4.4 Glycosides

Glycosides are compounds that yield one or more sugars among the products of hydrolysis.

Chemically, the glycosides such as salicin(31) are acetals in which the hydroxyl of the sugar is condensed with a hydroxyl group of the

non-sugar component, and the secondary hydroxyl is condensed within the sugar molecule itself to form an oxide ring<sup>(7)</sup>.

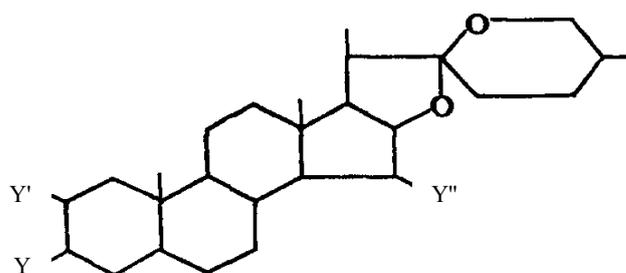


Salicin(31)

### 1.2.4.5 Saponins

The term saponin is applied to a group of natural products which have in common the property of foaming when shaken with water. They are widely distributed in the vegetable kingdom and have been reported to be present in at least 500 genera of plants. Chemically, saponins are glycosides which yield on hydrolysis :

(a) one or more sugar units and (b) sugar free glycones which are derived from polycyclic ring systems and are commonly referred to as sapogenins such as digitogenin(32)<sup>(46)</sup>.



Digitogenin(Y, Y', Y''-OH)(32)

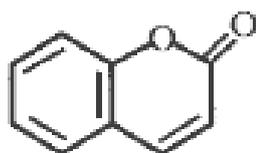
### 1.2.4.6 Resins, Gum-resins

The term 'resin' is applied to more or less solid, amorphous products of complex chemical nature. On heating they soften and finally melt. They are insoluble in water and usually insoluble in petroleum spirit but dissolve more or less completely in alcohol, chloroform and ether. Chemically, resins are complex mixtures of resin acids, resin alcohols (resinols), resin phenols (resinotannols), resin esters and chemically inert compounds known as resenes<sup>(25)</sup>.

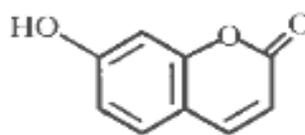
Gums are materials which have the property of water absorption and transformation to sticky materials, these materials are extracted by plants on bark surface rather in continuous way on when the plant is wounded, and it is thought to originate from cellulose or starch due to metabolism<sup>(6)</sup>.

### 1.2.4.7 Coumarins

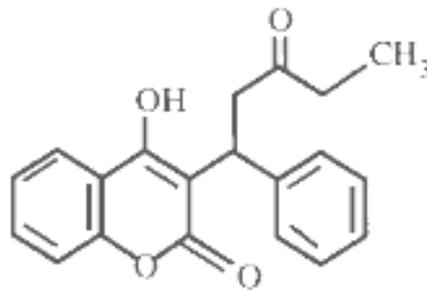
Coumarin (33), 7-hydroxycoumarin (34) and warfarin (35) are phenolic substances made of fused benzene with  $\alpha$ -pyrone rings<sup>(47)</sup>. They are responsible for the characteristic odor of hay<sup>(48)</sup>. Their fame has come mainly from their antithrombotic<sup>(49)</sup>, anti-inflammatory<sup>(50)</sup> and vasodilatory<sup>(51)</sup> activities.



Coumarin(33)



7-Hydroxycoumarin(34)



Warfarin(35)

### ***1.3 Definition of Plant***

**Pharmacopeial name** : Thymi herba.

**Synonyms**: Lamiaceae are also known as Labiatae family.

**Other names**: Common thyme, farigola, garden thyme, herba timi, herba thymi, mother of thyme, red thyme, rubbed thyme, thick leaf thyme, thym, Thymian, timi, tomillo, black thyme (Thymbra), Jatra(in kurdish), and za'ater ( in Arabic)<sup>(52-55)</sup>.

### ***1.4 Description of Plant***

An aromatic perennial sub-shrub, 20–30 cm in height, with ascending, quadrangular, greyish brown to purplish brown lignified and twisted stems bearing oblong-lanceolate to ovate-lanceolate greyish green leaves that are pubescent on the lower surface. The flowers have a pubescent calyx and a bilobate, pinkish or whitish, corolla and are borne in verticillasters. The fruit consists of 4 brown ovoid nutlets<sup>(56)</sup>.

## 1.5 Where Found

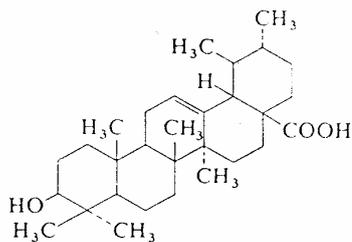
Thyme is a wild plant that grows on dry banks and heaths. It grows wild on hillsides in Greece and throughout the Mediterranean. It is now found throughout Asia Minor, Algeria, Turkey, Tunisia, China and central Europe. Its oil is mainly produced in Spain but also in France, Morocco, Algeria, Germany, and the USA<sup>(7,55)</sup>, and it is found in the north of Iraq but is not widely spread.

## 1.6 Chemical Composition of the Plant

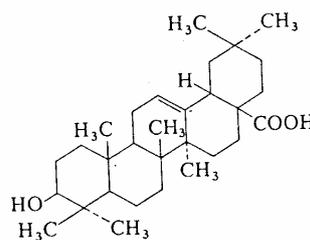
Herba thyme contains 1-2.5% volatile oil<sup>(56)</sup>, phenolic material about 55%<sup>(53,57)</sup> but not less than 0.5% phenols<sup>(56)</sup>; the major constituent of *Thymbra spicata* was carvacrol 75.5%<sup>(58)</sup>, while in *Thymus vulgaris* the chief components of volatile oil are thymol 20-55%, carvacrol 1-10%<sup>(25)</sup>, and Flavonoids<sup>(59)</sup>.

Triterpenes include many others, ursolic acid(36) 2%, oleanolic acid(37) 0.5%, caffeic acid(38) derivatives rosmarinic acid<sup>(56)</sup>, saponins and resin<sup>(60)</sup>, while Tannins 1% and gum<sup>(4)</sup>.

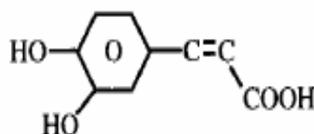
Smith<sup>(61)</sup> found that the seeds of thyme contain about 13% hydroxy fatty acid. In 1998<sup>(62)</sup> it was found that 10% of the seeds are resins, gums and tannins, while the foreign organic matter is about 2%, and acid insoluble matter is about 4%.



Ursolic acid(36)



Oleanolic acid(37)



Caffeic acid(38)

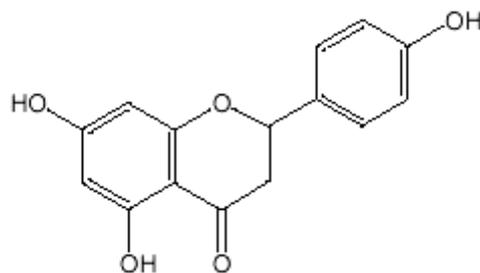
## 1.7 Medical Importance of Thyme

Thyme (Labiatae) is commonly used as a cough-drug. Liquid thyme extract is a fundamental part of many galenical preparations with antitussive action<sup>(21)</sup>. The spasmolytic and antitussive activity of thyme has been most often attributed to the phenolic constituent, thymol, which forms a large percentage of the volatile oil<sup>(56)</sup>.

Thyme is considered as oregano plant that is used in the treatment of maldigestion, chronic gastritis, chronic bronchitis, whooping cough, bronchial asthma, mouthwash and diarrhea<sup>(59)</sup>.

Barberan et al<sup>(63)</sup> isolated and identified the compounds of Flavonoids of thyme plant in the genus *Thymbra*.

Recently Jussi<sup>(64)</sup> isolated three species of Flavonoid: Flavones(27), Flavonols(29) and Flavanones(39).



Flavanone(39)

Francisco et al<sup>(65)</sup> studied the chemotaxonomic applications of Flavones, that has been found in species of the taxonomically related, *Mentha*, *Thymbra*, *Thymus* and *Origanum*. It has been reported<sup>(60)</sup> that preliminary phytochemical screening of the thyme herb confirms the presence of different active compounds like volatile oils, glucosides, alkaloids, Flavonoids, saponins, tannins and resins.

*Thymbra* is used in the treatment of respiratory diseases and as expectorant and bronchodilators. Also thyme oil has been used in making perfumes and dental pastes and as gurgling fluid for the mouth and pharynx and in the treatment of gum inflammation. It enters in analgesic and antihelmenthic formula, and it helps in wound healing<sup>(66)</sup>. Alcohol extract of these compounds is used in treatment of seborrhic eczema mixed with other plants<sup>(67)</sup>.

Thyme was used in the treatment of psoriasis and seborrhic eczema by using a mixture of 80-90% of alcohol extract from *Metricaria chamomilla*, *Urtica dioica*, *Arnica eupatoria*, resorcinol mono

acetate, *Arnica lapp*, *Thyme*, salicylic acid and added to the aqueous alcohol from Na-P-aminosalicylate and glycerol<sup>(68)</sup>.

It was found that alcoholic aqueous and acetic extracts from the *Thyme*, *Peppermint*, *Cardamom*, *Cinnamon* had high efficiency in preventing the growth of all skin fungi<sup>(69)</sup>. Thyme oil is considered as antioxidant because it contains a large quantities of carvacrol and thymol<sup>(70)</sup>.

An interesting researches about using thyme oil as antioxidant has increased, because thyme oil when oxidized during oxidation gives no effect on oil plant taste<sup>(71)</sup>.

Thyme contains plant oestrogen (phytoestrogen). This is a weak oestrogen that can substitute human oestrogen, and as the large quantities of oestrogen can cause breast cancer in females, plant oestrogen is considered to be as a good substitute for natural oestrogen<sup>(72)</sup>.

### ***1.8 The Antimicrobial Activity:***

The antimicrobial activity of plant oils and extracts has been recognized for many years<sup>(73)</sup>. Because of its antiseptic, antispasmodic and antimicrobial properties it is also used for medicinal purposes<sup>(74)</sup>.

In recent years, several reports have been published concerning the composition or the biological properties of thyme essential oils<sup>(75-82)</sup>

these studies have emphasized the existence of marked chemical differences among oils extracted from different species or varieties. These variations are likely to influence the antimicrobial activity of the oil and generally a function of three factors: genetically determined properties, the age of the plant and the environment.

### ***1.9 Toxicity of Thyme***

Thyme consists of a large percentage of phenolic materials about 55% of its constituents, thymol and carvacrol are the most important of them. These isomers are identical in chemical structure regarding number and kind of atoms but differ in atomic arrangement and chemical characters.

In spite of the chemical difference between the two compounds carvacrol is considered to be more toxic than thymol <sup>(83)</sup>.

Thymol ethers and carvacrol ethers cause acute toxicity when experimented on dogs with lethal outcome because of arterial hypotension and these compounds are accumulated in lungs, liver and kidneys<sup>(84)</sup>.

On the other hand thyme dust is capable of inducing occupational airborne contact dermatitis, but no specific etiologic factor has been identified<sup>(85)</sup>. Watt<sup>(86)</sup> found that thyme was not preferred to be used in case of hypertension due to some side effects.

Caffeic acid(38) present in thyme; was found to have antigonadotrophic effect so it decreases fertility in both sexes through depressing the secretion of pituitary hormones Lutinizing Hormone (LH) and Folkcular Stimulating Hormone (FSH)<sup>(87)</sup>.

### ***1.10 Aims of the Study***

1. Extraction of the volatile oils from local Thyme (*Thymbra spicata*) grown in the north of Iraq.
2. Qualitative identification and quantitative evaluation of some organic active compounds in the extracted oil.
3. Qualitative identification of some active medical compounds present in thyme like Alkaloids, Flavonoids, Tannins, Glycosides, Saponins, Resins and Coumarins.
4. Studying the antimicrobial activities of the extracted oil on some common species of bacteria and one type of fungi.

## Experimental Part

### 2.1 Chemicals

The chemicals used in this work are listed in Table (2-1).

Table (2-1) Chemicals used in this study

Compound (analar)	Company	Source
Anhydrous Sodium Sulphate	BDH	England
Acetonitrile	Fluka	Switzerland
Benzene	Fluka	Switzerland
Carbon tetrachloride	Fluka	Switzerland
Diethyl ether	BDH	England
Dimethyl sulphoxide	BDH	England
Ethanol 70% & 95%	BDH	England
Ethyl acetate	Fluka	Switzerland
Ethylene dichloride	Fluka	Switzerland
Ferric chloride	Fluka	Switzerland
Glacial acetic acid	Fluka	Switzerland
Hydrochloric acid	BDH	England
Iodine	BDH	England
Lead acetate	Fluka	Switzerland
Mercury chloride	Fluka	Switzerland

Nitric acid	BDH	England
Potassium hydroxide	BDH	England
Potassium Iodide	Difco	USA
Picric acid	BDH	England
Perchloric acid	BDH	England
Sodium hydroxide	BDH	England
Sulphuric acid	Fluka	Switzerland
Toluene	BDH	England

## 2.2 Instruments

The following instruments have been used in this work:

1. Digital pH-Meter, Knick (England).
2. Digital balance, Sartoris, BP3015 (Germany).
3. Oven, Gallenkamp, (England).
4. FT-IR 8300-Schimadzu, single beam, laser path, (Japan).  
KBr disc, Al-Nahrain University.
5. High-Performance Liquid Chromatography (HPLC),  
Waters, (Netherlands), Ministry of Health/National Center  
for Drug Control and Researches.
6. Gas Chromatography GC14A, Schimadzu, (Japan), Ibn  
Seena company.
7. Incubator, Gallenkamp, (England).

8. Muffle Furnace, Gallenkamp, (England).
9. Flameless atomic Absorption Spectrophotometer, Shimadzu, (Japan), Ibn Seena company.
10. Clavenger apparatus, Quickfit, (England).
11. Refractometer, Zeiss, Pye Unicam (Germany).
12. Magnetic stirrer, Gallenkamp, (England).
13. UV-Visible Spectrometer, Cintra (5) GBC Scientific Equipment ( England), Babylon University.

## ***2.3 Collection of Plant***

The dry plant Thyme was obtained from the Ministry of Health/ Center of Herbal Medicine. The plant was identified by "herbeliuem college of science" Baghdad University. It was air-dried, and packed in plastic containers until used.

Thyme species *Thymbra spicata* Figure (2-1) is widely grown in the north of Iraq<sup>(88,89)</sup>. The collection of the plant was at the spring harvesting.

## ***2.4 Plant Extraction***

### ***2.4.1 Ethanolic Extract***

One hundred grams of the dried plant (leaves and flowers), Figure (2-2) were placed in 1000 ml round-bottomed flask, 250 ml of 70% ethanol was added the mixture was refluxed for 6 h, then cooled and filtered off, the filtrate was kept at 4°C temperature until used for the chemical testing<sup>(90)</sup>.



Figure (2-1) Plant Thyme ( *Thymbra Spicata*)



Figure (2-2) Leaves and flowers of ( *Thymbra Spicata*)

### ***2.4.2 Aqueous Extract***

One hundred grams of dried plant (leaves and flowers) were placed in 1000 ml round-bottomed flask, and 200 ml of distilled water was added, mixed for 1h with magnetic stirrer at room temperature, then left for 24 h. On filtration a brownish-green liquid was obtained. All the chemical tests took place on the filtrate<sup>(91)</sup>.

### ***2.4.3 Extraction of Volatile Oils by Steam distillation***

One hundred grams of the dried plant (leaves and flowers) were placed in 1000 ml round-bottomed flask, 300 ml of distilled water was added, the distillation process was carried out for 7 h ; the extracted solution was transferred to the separatory funnel, 50 ml of diethyl ether was added, the organic layer was separated; dried with anhydrous sodium sulphate, filtration and removal of solvent gave oil which was stored in dark vials at 4°C temperature until used<sup>(92)</sup>.

### ***2.4.4 Extraction of Volatile Oils by Using Clavenger apparatus***

The determination of volatile oil in plant is carried out by steam distillation in the apparatus shown in Figure (3-3). One hundred grams of dried plant (leaves and flowers) were placed

in 1000 ml round-bottomed flask, and distilled water was added in a ratio of (5:1) (water: plant). Remove the stopper (K') and run water into the orifice (K) until it overflows at (B). Heat the flask until the ebullition begins and continue the distillation at a rate that leaves the lower of the condenser cold at the point (G). At the end of the specified time (3 h), discontinue heating and after at least 5 minutes read off the volume of oily layer in the graduated portion of the tube. The yield of volatile oils which was separated from the aqueous phase with diethyl ether and then dried with anhydrous sodium sulphate, filtration and removal of the solvent gave oil which was stored in dark vials at 4°C<sup>(93)</sup>.

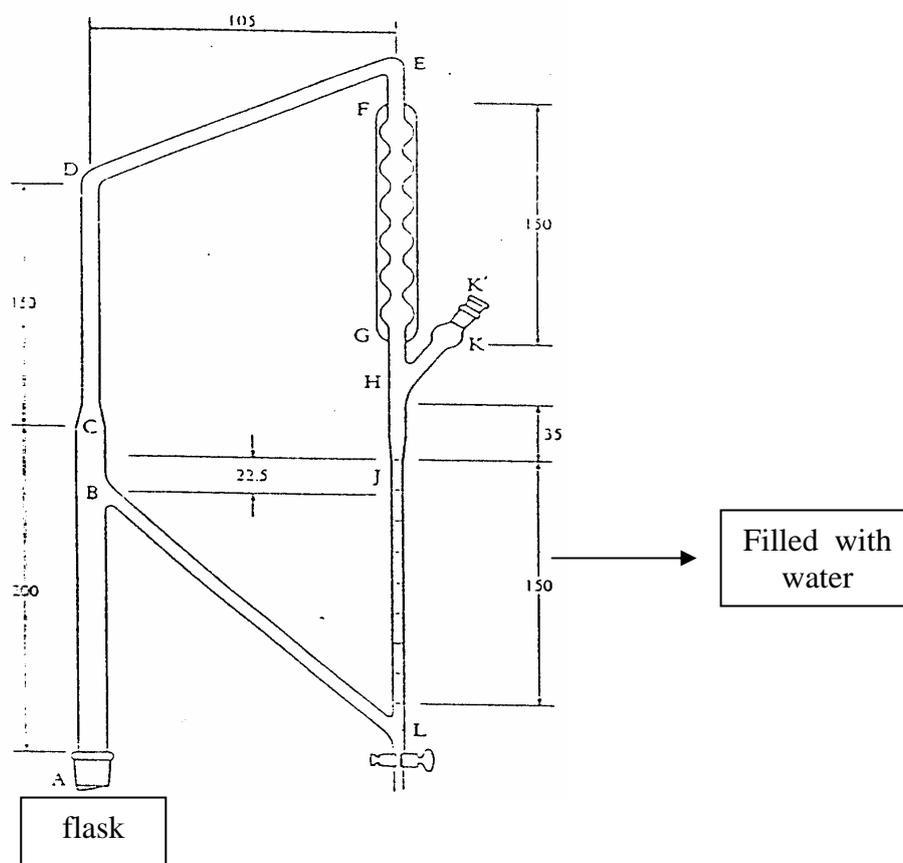


Figure (2-3) Clavenger apparatus

## 2.5 Chemical Testing

### 2.5.1 pH measurement<sup>(95)</sup>

10 gm of plant powder was stirred with 50 ml distilled water for 10 minutes, the mixture was filtered off and the pH value of the filtrate was determined by using pH-meter; it was 5.45.

### 2.5.2 Test for Tannins<sup>(95)</sup>

10 gm of the plant powder was boiled with 50 ml distilled water for 5 minutes, the mixture was filtered off and left to cool at room temperature. Two methods were adopted to indicate the presence of tannins:

- To 10 ml of the filtrate 1% lead acetate was added, white gelatin precipitated, this confirms the presence of tannins.
- 1% Ferric chloride was added to 10 ml of the filtrate, greenish blue colour appears; this confirms the presence of tannins.

Tannins + 1% Lead acetate  $\longrightarrow$  white gelatin precipitate

Tannins + 1% Ferric chloride  $\longrightarrow$  white gelatin precipitate

### 2.5.3 Test for Glycosides<sup>(95)</sup>

5 ml of aqueous extract of the plant is either mixed with 5 ml of Fehling's solution heating the mixture for 10 minutes on water bath, or mixed with 5 ml of Benedict's solution the red

precipitate appearance in both tests is an evidence for the presence of Glycosides.

Glycosides + Fehlinic reagent  $\longrightarrow$  red precipitate

Glycosides + Benedict reagent  $\longrightarrow$  red precipitate

### ***2.5.4 Test for Resins<sup>(95)</sup>***

50 ml of ethanolic extract was mixed with 100 ml of acidified distilled water; turbidity of solution refers to the presence of Resins compounds.

### ***2.5.5 Test for Saponins<sup>(95)</sup>***

**A.** The aqueous solution of the plant powder was agitated hardly in the test tube, the formed bulky foam refers to the Saponins when it stays for a long time.

**B.** 3 ml of mercury (II) chloride solution was added to 5 ml of the aqueous extract, white precipitate was formed which refers to the Saponins.

Saponins + HgCl<sub>2</sub>  $\longrightarrow$  white precipitate

### ***2.5.6 Test for Alkaloids<sup>(96)</sup>***

#### **A. Preparation of reagents<sup>(94)</sup>**

- Wagner test: 20 gm of KI was added to 12.7 gm of I<sub>2</sub> in 1000 ml distilled water.

- Mayer test: 13.5 gm of  $\text{HgCl}_2$  and 50 gm of KI were dissolved in 1000 ml distilled water.

### **B. Testing**

10 ml of aqueous extract was acidified by a few drops of 4% HCl solution; 1ml was taken from the solution and placed in watch glass then the above reagent was added;

Wagner reagent: No red precipitate was formed, which indicates the absence of alkaloids.

Mayer reagent: No white precipitate was formed, which indicates the absence of alkaloids.

Picric acid: No yellow precipitate was formed, which indicates the absence of alkaloids.

Alkaloids + Wagner reagent  $\longrightarrow$  red precipitate

Alkaloids + Mayer reagent  $\longrightarrow$  white precipitate

Alkaloids + Picric acid  $\longrightarrow$  yellow precipitate

### **2.5.7 Test for Flavonids<sup>(97)</sup>**

10 ml of 70% ethanol was mixed with 10 ml of 50% potassium hydroxide. 5 ml of ethanolic extract was mixed with 5 ml of the above solution, the colour turned yellow; which indicates the presence of Flavonids.

Flavonids + ( 70% ethanol/ 50%KOH)  $\longrightarrow$  yellow colour

### **2.5.8 Test for Coumarins<sup>(98)</sup>**

The ethanolic extract was placed in a test tube, covered by a filter paper moistured with diluted NaOH which was placed in a boiling water bath for few minuets. The filter paper turned yellowish green, when placed under the radiation of UV-Vis source, which indicates the refers to the presence of Coumarins.

## **2.6 Determination of the Major Component of Thyme**

### **2.6.1 Total ash**

3 gm of the ground air-dried material, accurately weighed, was placed in a crucible (usually of platinum or silica). It was ignited by gradually increasing heat to 500-600°C and maintained at this temperature for 3 h in a muffle furnace until it is white; to insure the absence of carbon; after cooling in a desiccator the total ash was accurately weighted and it was 9.77%<sup>(99)</sup>.

### **2.6.2 Acid-insoluble ash**

To the evaporating dish containing the total ash, 25 ml of concentrated HCl, and 5 ml of hot water was added; this was coverd with a watch glass. Insoluble material was collected on a shless filter-paper and washed with hot water. This material was

transferred to the original crucible, dried on a hot-plate and ignited to the constant weight. Cooled in a suitable desiccator for 30 minutes, the content of acid-insoluble ash was weighted accurately, and it was 3%<sup>(99)</sup>.

### ***2.6.3 Heavy metals (Cadmium and Lead)***

0.25 gm of air-dried plant material was accurately weighted and placed into a cleaned silica crucible, 1ml of the digestion mixture (2 parts of concentrated nitric acid and 1 part of concentrated perchloric acid) was added; the crucible was covered and placed in an oven with a controlled temperature and time regulator.

The solution was heated slowly to 100°C and maintained at this temperature for 3 h ; then the temperature raised to 120°C and maintained at this temperature for 2 h after that the temperature was raised very slowly to 240°C, and maintained so for 4 h. The remaining dry inorganic residue was dissolved in 2.5 ml of concentrated nitric acid and used for the determination of heavy metals. The contents of Lead and Cadmium were determined by flameless atomic absorption spectrophotometer. The result gaived was 0.003 mg/Kg for Cadmium and 0.008 mg/Kg for Lead<sup>(99)</sup>.

## ***2.7 Physical Properties***

### ***2.7.1 Moisture***

In fresh plant, the moisture was determined by drying it at room temperature and calculating the difference between the dried weight and the moistured weight. The moisture percentage was 6.8%.

### ***2.7.2 Refractive Index***

The refractive index of the extracted volatile oil is determined by using refractometer apparatus at room temperature (25° C). The refractive index was 1.510.

## ***2.8 Isolation of Thymol [5-methyl-2-isopropyl phenol] in Volatile oils***

Thymol component was isolated from the volatile oil<sup>(100)</sup> obtained from Clavenger extract as follow:-

5 ml from the natural oil was treated with 15 ml of 5% sodium hydroxide solution, the aqueous solution of sodium thymol salt being separated, the product was treated with 10 ml of 4% hydrochloric acid solution, on cooling white crystal was separated, filtration and drying at room temperature, gave white crystal melting point 49°C that (48-52°C) in literature<sup>(101)</sup>.

### ***2.8.1 Colour Reaction of Thymol***

- a) Few thymol crystals dissolved in 1 ml glacial acetic acid, to this solution 6 drops of concentrated sulphuric acid and 1 drop of concentrated nitric acid were added; a bluish green colour was developed.
- b) When thymol dissolved in concentrated sulphuric acid, it forms thymol sulfonate which produces a violet colour with ferric chloride.
- c) It does not give any coloration when treated with ferric chloride in concentrated alcohol solution<sup>(101)</sup>.

## ***2.9 Preparation of Carvacrol [2-methyl-5-isopropyl phenol]***

Carvacrol was prepared according to the method mentioned by Finar<sup>(102)</sup>. Where five parts (0.630 gm) of camphor were mixed with one part (0.126 gm) of Iodine and 15 ml of  $\text{CCl}_4$ ; the mixture was heated gently for 10 minutes, filtration gave a yellow compound which gave one spot on TLC.

## ***2.10 Identification Study***

### ***2.10.1 Thin-Layer Chromatography (TLC)***

For testing the essential component of volatile oil, the following solvents have been used as mobile phase<sup>(103-104)</sup>:

- EtOAc:C<sub>2</sub>H<sub>4</sub>Cl<sub>2</sub> (1.0:9.0)
- EtOAc:Toluene (0.7:9.3)
- EtOAc:Toluene (0.5:9.5)
- EtOAc:C<sub>6</sub>H<sub>6</sub> (1.0:9.0)

Silica gas as a coating material of about 0.25 mm crystal diameter was used to make the preparative layer chromatography of the dimension (8 cm X 2 cm) and the thickness of 1 mm. Mixture of (5:95) volumes ratio of ethyl acetate : toluene was used as an eluent; the chromatogram shows five zones, belonging to camphor, carvacrol, thymol, p-cymene and an unknown compound; each zone was separated by scratching, the spot dissolving in ether, filtration and removal of the solvent. The desired compounds were get and identified by IR and UV.

### ***2.10.2 High-Performance Liquid***

#### ***Chromatography (HPLC)***

Solinas et al<sup>(105)</sup> methods have been followed to test the presence of thymol and carvacrol in the volatile oil, that has been extracted from thyme, by using HPLC apparatus model Waters 2795 auto injector: column 250X4.6 mm I.D., packed with microbondapak C<sub>18</sub> (water Assoc.) with U.V detector at

wave length of 283 nm, the mobile phase used was acetonitrile-water (40:60) with a flow-rate of 1.5 ml/ min were used.

The compounds separated at retention time  $t_R$  13.769 minutes was carvacrol and at  $t_R$  15.77 minutes was thymol.

### **2.10.3 Gas Chromatography (GC)**

The identification of the compounds in the essential oil was done according to Serap et al<sup>(35)</sup> methods, using GC14A instrument equipped with a flame ionization detector. A thermon-600T (50m X 0.25 mmID) of fused silica capillary column coated with 0.25  $\mu$ m layer of macrogol 20,000 was used. The carrier gas was nitrogen at a flow rate of 2 ml/min.; both the injection and detector temperature were 250°C. The oven temperature was kept at 70°C for 10 min., programmed to rise up to 180°C at a rate of 2°C/min., and then kept constant at 180°C for 30 min<sup>(35)</sup>. The essential oil components were identified by comparing their retention times with those of authentic samples.

The retention time of thymol is 3.913 minutes at 0.381%, p-cymene is 8.976 minutes at 5.748%, camphor is 22.249 minutes at 0.456% and the retention time of carvacrol is 46.076 minutes at 29.500% in the volatile oil obtained from Clavenger method.

## 2.11 Determination of Antimicrobial Activity:-

Antimicrobial activity of essential oils were determined using (well method)<sup>(106)</sup>. The test organism (isolates) obtained from the Biotechnology Department/ Baghdad University were: (*Staphylococcus aureus*, *Streptococcus pyogene*, *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans*).

Antimicrobial activity testing are as follows:

1. The isolate under study is grown on nutrient broth at 37°C for 18 h.
2. An inoculum of 0.1 ml of the nutrient broth was spread on nutrient agar using glass spreads to get even spreading, then, the plate was incubated at 37°C for 10 minute; this was done in duplicate.
3. After the plate had dried up, 5 mm wells were done in the agar using steril cork borer, the agar disc was discarded, then 0.1 ml of the oil extract diluted with dimethyl sulphoxide (DMSO); (50% v/v) was put in each well; the plates were incubated at 37°C for 18 h after which the inhibition zone was measured and compared with the control.

It has been reported that the concentrations of essential oils (10% v/v) and (20% v/v) have no antimicrobial activities<sup>(60)</sup>. The result was taken for every kind of bacteria after being repeated three times. The diameters of the inhibitory zones were measured.

## ***Results and Discussion***

### ***3.1 Chemical Testing***

It is now possible to draw the following conclusions:-

The chemical test has shown that the thyme species *Thymbra spicata* contains active medical components such as; glycosides, tannins, resins, coumarins, saponins and flavonoids. The present work revealed the absence of alkaloids, the pH value of the aqueous extract was acidic (pH=5.45). These are in agreement with other researchers' finding confirming the presence of flavonoids<sup>(107,108)</sup>, saponins<sup>(45)</sup>, glycosides<sup>(109)</sup>, resins<sup>(110)</sup>, coumarins<sup>(25)</sup> and alkaloids<sup>(111)</sup>.

The present work shows the chemical analysis of active ingredients of the plant Table(3-1).

Table (3-1) The chemical tests of compounds for *Thymbra spicata*.

Compounds	Reagent	Detection	Results
Tannins	1%Lead acetate	White gelatin precipitate	+
	1%Ferric chloride	Greenish blue colour	+
Glycosides	Fehling's reagent	Red precipitate	+
	Benedict's reagent	Red precipitate	+
Saponins	No reagent	Large foam is formed on agitation of aqueous solution	+
	Mercury II chloride	White precipitate	+
Alkaloids	Wagner reagent	Red precipitate	-
	Mayer reagent	White precipitate	-
	Picric acid	Yellow precipitate	-
Resins	Ethanol	Turbidity	+
Flavonoids	Ethanol + NaOH	Yellow colour	+
Coumarins	NaOH then UV source	Greenish blue colour	+
pH	pH-meter	5.45	acidic

The presence of all these active medical compounds in parts of the plant have many medical and therapeutic benefits and this accounts for the common uses of thyme in old medicine since Egyptian papyrus and Sumerians until now.

### 3.2 Characteristic Features

Analysis of general characters of thyme *Thymbra spicata* shown in Table (3-2), gives the following results: the moisture percentage was 6.8% and total ash was 9.77%, while the acid insoluble ash was 3%<sup>(57,112)</sup>. The refractive index was 1.510<sup>(104)</sup>.

Analysis of heavy metals of thyme shows that it contains Cadmium and Lead in the amounts of 0.003, 0.008 mg/Kg respectively.

Table (3-2) General characteristics of *Thymbra spicata*

Component	Specification <sup>(101)</sup>	Present work
Moisture	10%	6.8%
Total ash	15%	9.77%
Acid insoluble ash	3%	3%
Refractive index of volatile oils extract	1.490-1.500	1.510
Heavy metals Cd, Pb	Cd 0.3 mg/Kg Pb 10 mg/Kg	0.003 mg/Kg 0.008 mg/Kg

The difference in the results from specification can be ascribed to the: type and percentage of chemical components of Iraqi thyme compared with other countries which is probably due to the season of collection and to the nature of the plant. In addition to that there are other factors such as genotype, chemotype, geographical origin. Environmental and agricultural conditions can all influence the composition of the final natural product of the plant.

### ***3.3 Extraction***

The volatile oil which has been collected by steam distillation is more favorite than other methods. This is due to the uniform temperature 50°C which preserves natural odors. Using high temperature not only affects the volatile oil content of the plant, but it also affects on the other constituents. So complete distillation is necessary to obtain all the components.

By using 100 gm of dried plant, the yield obtained by Clavenger was 3%, while only 1.2% oils were obtained from steam distillation method. So the Clavenger method is considered to be a better method for extracting the volatile oil.

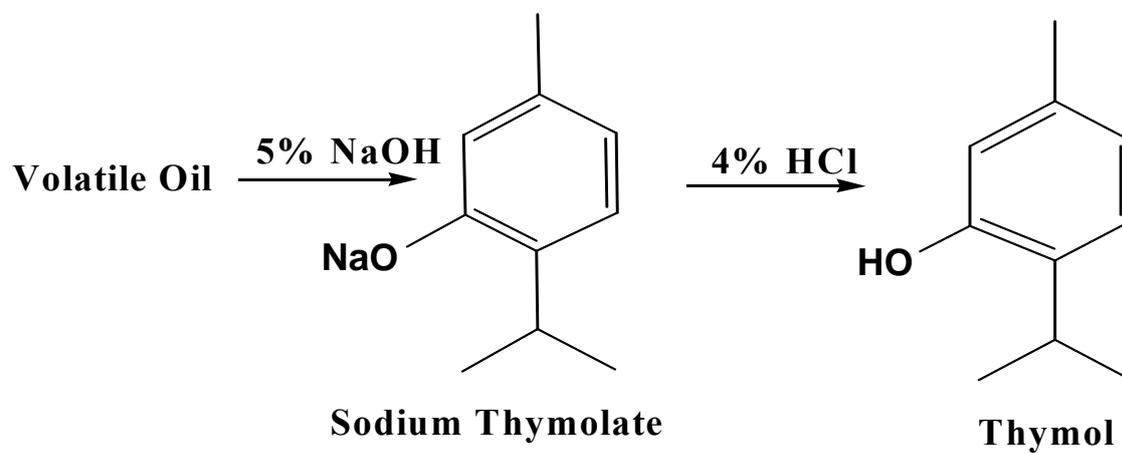
### ***3.4 Identification of Active Components in Volatile Oils in *Thymbra spicata****

Complete identification of the compounds of volatile oil depends on measuring other properties and comparing these data with those in the literature. Unknown plant compound can usually be identified by melting point, boiling point, and retention factors. Spectral analysis of ultra-violet (UV) and infra-red (IR), besides other analysis, gas chromatography (GC) and high performance liquid chromatography (HPLC) give more information data. A known plant can usually be identified by the above methods. If authentic material is not available, a careful comparison with literature data may suffice for its identification<sup>(113)</sup>.

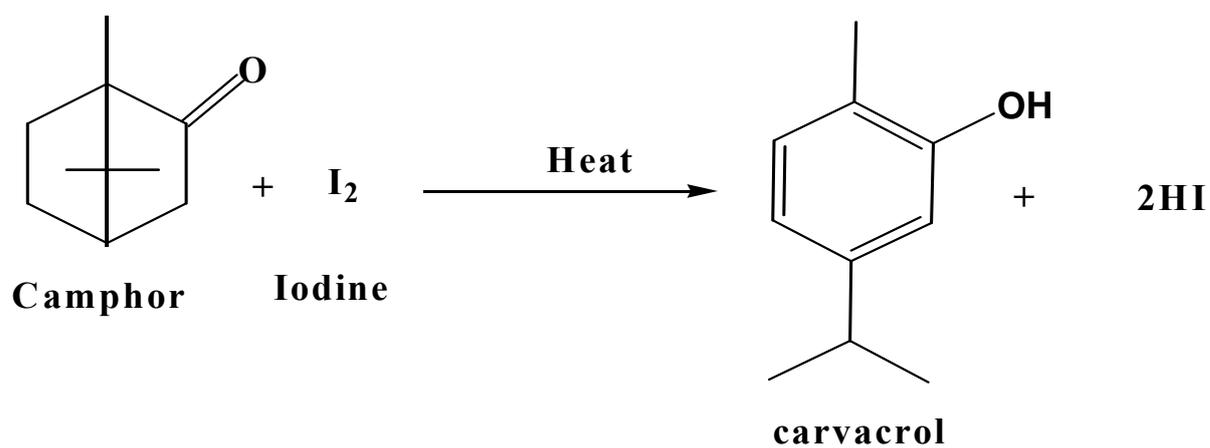
The extraction of Thymol is affected by treating the thyme oil with solution of sodium hydroxide. This alkali dissolves the thymol, the undissolved oil (terpenes, etc.) rises to the surface. The alkaline thymol compound is treated with hydrochloric acid and on cooling white crystals are separated, equation (1).

Carvacrol is prepared by the prolonged heating of five parts of camphor with one part of iodine for 10 minutes, equation (2). The reaction of concentrated alcohol solution of carvacrol with 1% ferric chloride gives a green colour while thymol does not give any coloration.

Eq...1



Eq...2



### 3.4.1 Thin-Layer Chromatography (TLC)

Of the many chromatographic methods presently available, the thin-layer chromatography has become widely adopted for the rapid and positive analysis of essential oil. TLC also provides semi-quantitative information on the chief active constituents of oil. The identity or non-identity of compounds can be established by comparison with the chromatogram of the standard compound. Unknown commercial compounds can be more easily identified by comparison with the visual record in the (TLC) atlas<sup>(103)</sup>. Preparative Layer Chromatography (TLC) is carried out using thick (up to 1 mm) layers of adsorbent and using ether as solvent.

The results of the volatile oil extracted by Clavenger methods from thyme by using TLC contain p-cymene at value ( $R_f=0.96$ ), thymol at value ( $R_f =0.87$ ), carvacrol at value ( $R_f =0.67$ ), unknown compound at value ( $R_f =0.55$ ) and camphor at value ( $R_f =0.25$ ). The volatile oil that extracted by steam distillation contain p-cymene, thymol, carvacrol and camphor at the same value, while the unknown compound give value ( $R_f =0.28$ ), that shown in Figure(3-1). The identification depend on corresponding  $R_f$  value with standard compound under the same conditions.

The best eluent for separation of active compounds of volatile oil is mixture of 5 volumes of ethyl acetate and 95 volumes of

toluene, the chromatogram shows five zones, and this system is suitable for the analysis.



Figure(3-1) The TLC chromatogram of active organic compounds in volatile oil.

### 3.4.2 Infrared Spectra ( IR)

The most important diagnostic features of the IR spectra are as follows:

IR spectrum for thymol shown in Figure (3-2), exhibits a broad band appearing at 3250-3200  $\text{cm}^{-1}$  assigned to the stretching vibration of (OH) phenolic group, in the region 3010  $\text{cm}^{-1}$  is due to stretching of C-H aromatic, 2962-2867  $\text{cm}^{-1}$  due to stretching of  $\text{CH}_3$  group, 1458-1419  $\text{cm}^{-1}$  due to the C=C aromatic ring stretching. In the region 1380-1340  $\text{cm}^{-1}$  due to the O-H in-plane bending vibration, 1286  $\text{cm}^{-1}$  due to isopropyl group region, a strong band 1244  $\text{cm}^{-1}$  due to C-O stretching in phenol, 800  $\text{cm}^{-1}$  out-of-plane due to the aromatic C-H bending.

The IR spectrum of carvacrol Figure (3-3) revealed a clear broad strong band in the region 3382  $\text{cm}^{-1}$  due to stretching vibration of phenolic O-H group. The presence of (OH) absorption band was a clear proof and a good indication for the success of preparation reaction. 3020  $\text{cm}^{-1}$  due to C-H aromatic ring stretching, 2960-2868  $\text{cm}^{-1}$  due to C-H stretching branched alkane, 1585-1458  $\text{cm}^{-1}$  due to C=C aromatic ring stretching band, 1458-1421  $\text{cm}^{-1}$  due to the O-H bending vibration, the peak at 1359  $\text{cm}^{-1}$  refers to the isopropyl group, a strong band in 1251  $\text{cm}^{-1}$  is due to C-O stretching, while at 800  $\text{cm}^{-1}$  is due to aromatic C-H bending .

The IR spectrum of camphor ( bicyclo [2,2,1]1,7,7-trimethyl-2-one) heptane Figure (3-4) shows a band at  $2950\text{ cm}^{-1}$  for a stretching vibration of  $\text{CH}_3$ , the band at  $1750\text{ cm}^{-1}$  is attributed to  $\text{C}=\text{O}$  stretching, the region  $1390\text{-}1375\text{ cm}^{-1}$  due to  $\text{C}-(\text{CH}_3)_2$  band, and at  $750\text{ cm}^{-1}$  is due to aromatic C-H bending.

The most prominent and informative bands in the spectrum of the aromatic hydrocarbon p-cymene [1-isopropyl-4-methylbenzene] shown in Figure (3-5),  $3049\text{-}3018\text{ cm}^{-1}$  due to aromatic C-H stretching band,  $2965\text{-}2869\text{ cm}^{-1}$  due to C-H stretching methyl group,  $1514\text{ cm}^{-1}$  due to  $\text{C}=\text{C}$  aromatic ring stretching,  $1390\text{-}1370\text{ cm}^{-1}$  due to  $\text{CH}_3$  symmetrical bending.  $1056\text{ cm}^{-1}$  for in-plane bending C-H band,  $813\text{ cm}^{-1}$  at low frequency range due to C-H out-of-plane bending<sup>(114)</sup>.

It is important to mention here that IR spectrum of the isolated thymol and prepared carvacrol shows a similar spectrum with thymol and carvacrol that obtained from preparative (TLC).

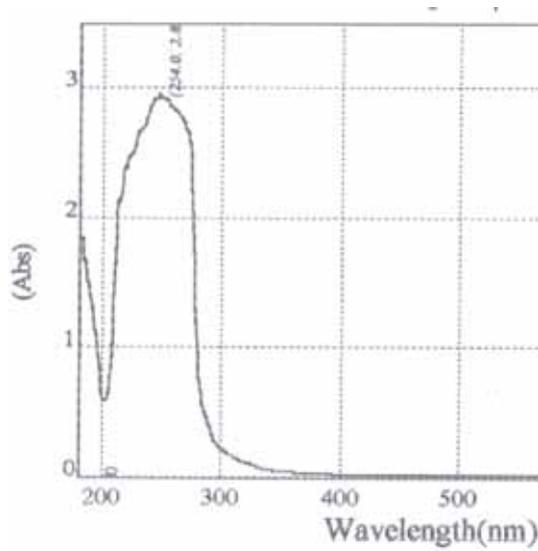
### 3.4.3 Ultraviolet and Visible Spectroscopy

#### (UV-Visible)

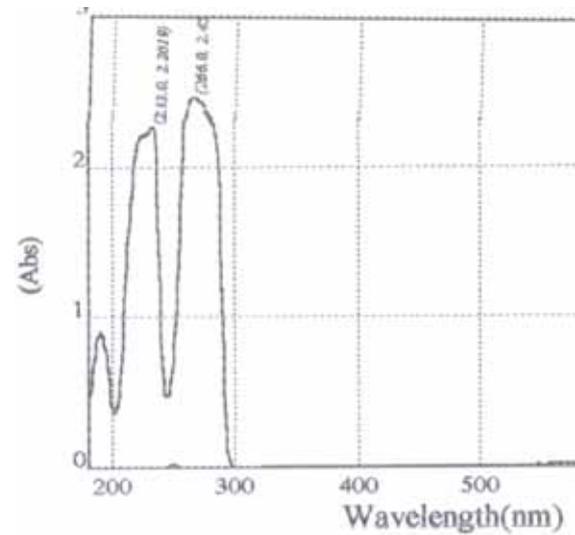
The electronic spectra of the four compounds that were obtained from preparative (TLC) were recorded using 95% ethanol in the range of (200-400) nm. Figure(3-6) and Table (3-5) show the spectra and the absorption of all different compounds, the available result has been found to be superimposed with authentic sample. P-cymene showed  $\lambda_{\max}$  at 254 nm and this is due to the  $\pi$ - $\pi^*$  transitions. In thymol and carvacrol compounds showed  $\lambda_{\max}$  at 233 nm and 266 nm for thymol, and  $\lambda_{\max}$  at 228 nm and 274 nm for carvacrol; this is due to  $n$ - $\pi^*$  and  $\pi$ - $\pi^*$  transitions. Saturated ketone camphor shows  $\lambda_{\max}$  at 285 nm due to  $n$ - $\pi^*$  transition.

Table (3-5) The results of UV-Visible spectra to the organic active compounds obtained from volatile oil.

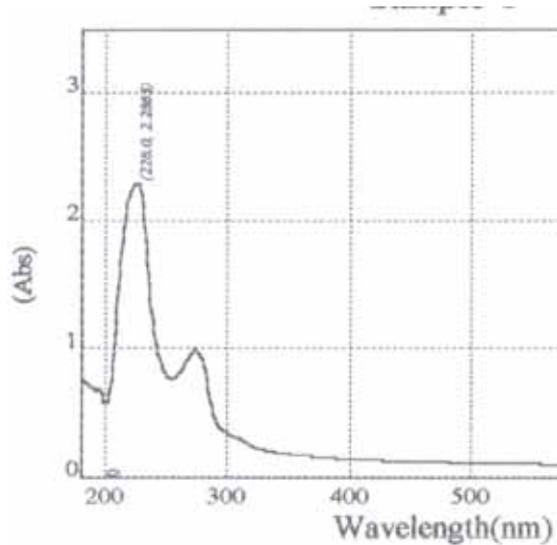
Compounds	$\lambda_{\max}$ (nm)
P-Cymene	254
Thymol	233 266
Carvacrol	228 274
Camphor	285



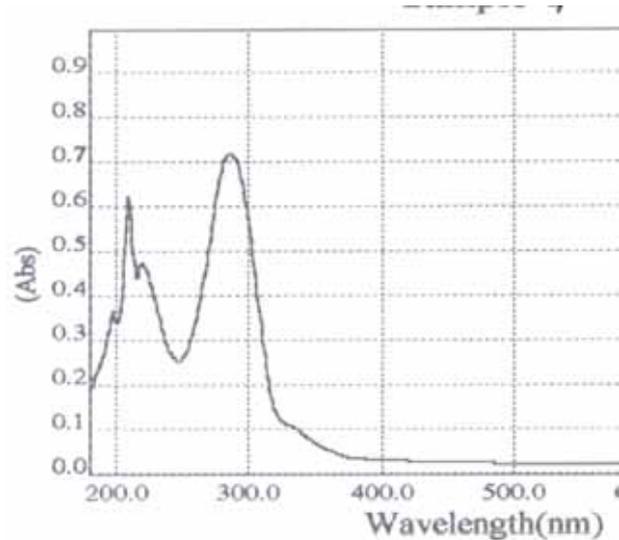
P-Cymene



Thymol



Carvacrol



Camphor

Figure(3-6) The UV spectra of active organic compounds in volatile oil.

### 3.4.4 Gas Chromatography (GC)

The gas chromatography of the thyme oil Figure (3-7) obtained from Clavenger method showed that the retention time of thymol is 3.913 minutes of 0.381%, p-cymene is 8.976 minutes of 5.748%, camphor is 22.249 minutes of 0.456% and that of carvacrol is 46.076 minutes of 29.5%. The main component was carvacrol, followed by a lower amounts of p-cymene, camphor and thymol.

The essential oil components were identified by comparing their relative retention times with those of authentic samples. Table (3-6) shows the results of GC analysis. A total of 4 components have been identified. The unidentified peaks besides many other peaks appeared, which could not be identified due to the lack of authentic materials.

Table (3-6) Quantitative and qualitative composition of extracted *Thymbra* volatile oil obtained from Clavenger method.

Components	Retention time(min.)	w/w%
Thymol	3.913	0.381
P-Cymene	8.976	5.748
Camphor	22.249	0.456
Carvacrol	46.076	29.500

### ***3.4.5 High-Performance Liquid Chromatography (HPLC)***

The analysis by HPLC showed the presence of carvacrol and thymol as shown in Table (3-7). The concentration of solutions was 200 ppm of oil in methanol..

The major compound was carvacrol at retention time  $t_R=13.769$  minutes and the minor was thymol at  $t_R=15.770$  minutes in volatile oil extracted from Clavenger method, while the major compound was thymol at  $t_R=15.678$  and the minor was carvacrol at  $t_R=13.770$  in volatile oil extracted from the steam distillation.

The height of the peaks shown in Figure (3-8) indicates that thymol is present in a very small amount 0.28% in comparison with carvacrol 26.7% in the oils obtained by Clavenger method, while the results were reversed when using the oils obtained by steam distillation; which show the presence of thymol in 19.29% and carvacrol in 6.49% Figure (3-9). This value indicates that the way of extraction is still responsible for this result. The above available environmental methods were not sensitive enough to detect all the minor compounds like camphor and p-cymene.

Table (3-7) Retention times and the percentage of carvacrol and thymol of Clavenger method and steam distillation.

Compound	Clavenger method			Steam distillation		
	t <sub>R</sub> (min.)	Height ( $\mu$ v)	%	t <sub>R</sub> (min.)	Height ( $\mu$ v)	%
Carvacrol	13.769	10253	26.7	13.77	27596	6.49
Thymol	15.77	910	0.28	15.678	87130	19.29

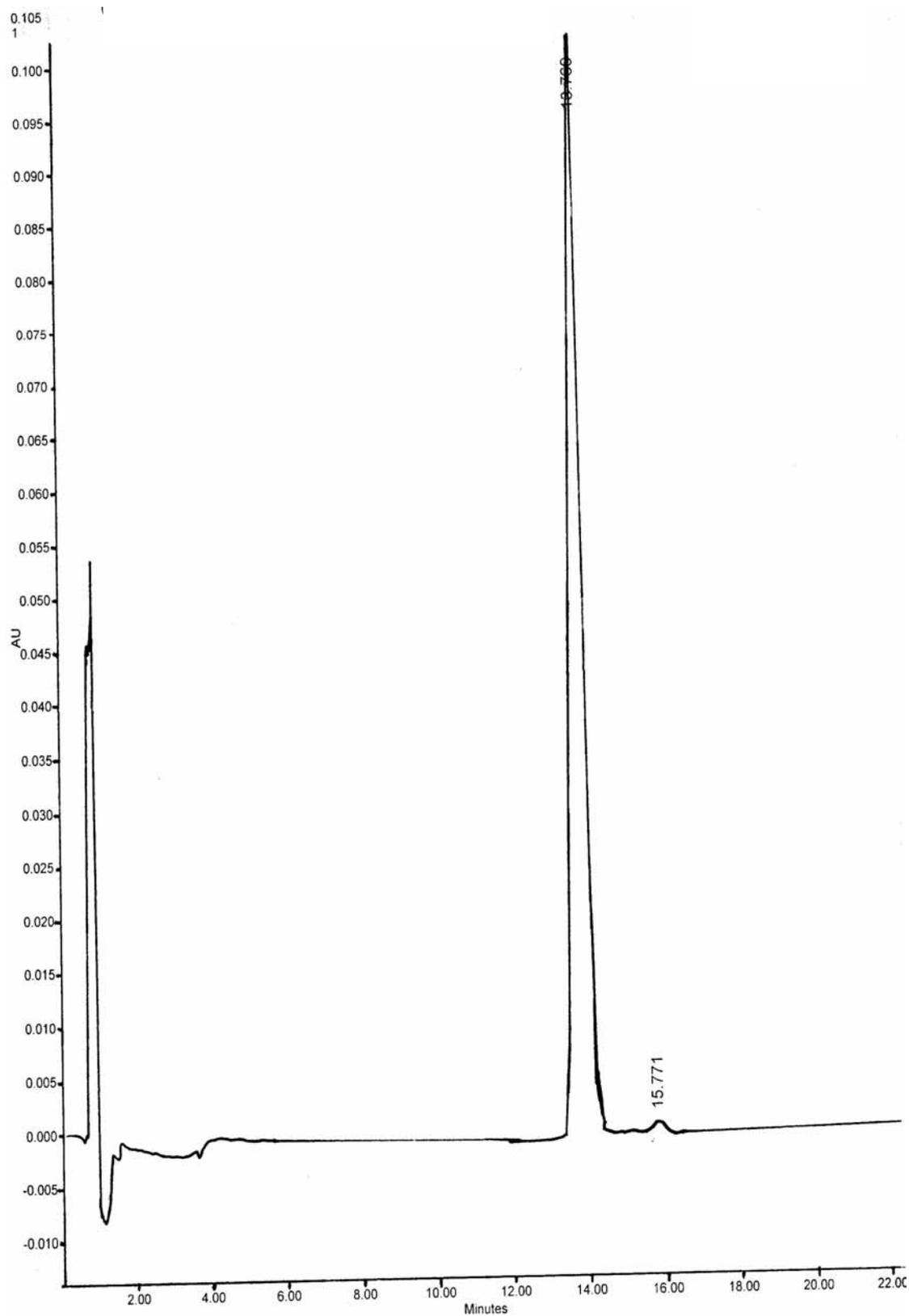
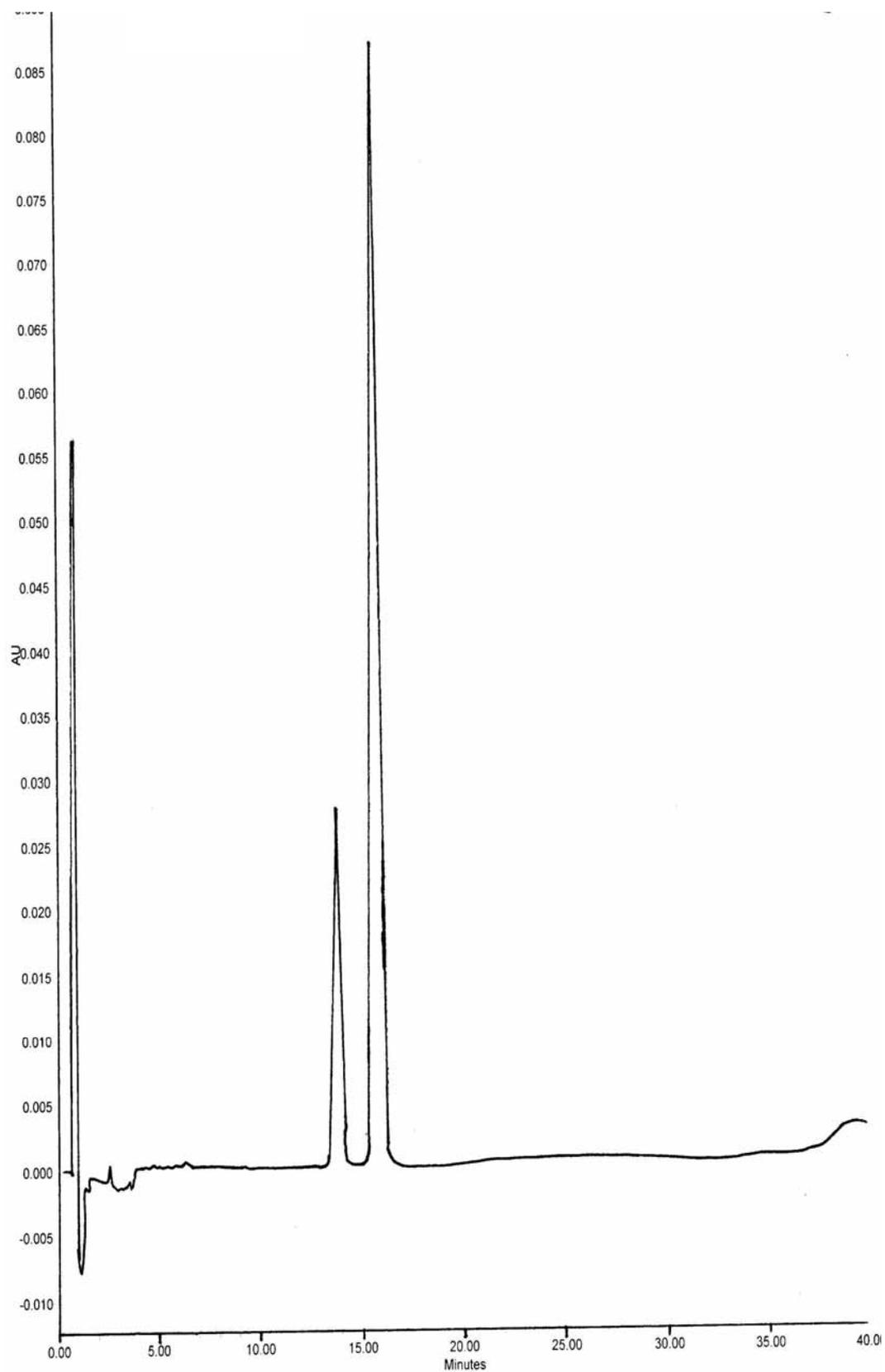


Figure (3-8) HPLC for the oils obtained from Clavenger method



Figure(3-9) HPLC for oils obtained from Steam distillation

### ***3.5 Investigation of the Antimicrobial Activity***

Antibiotics are sometimes associated with adverse effects that include hypersensitivity. Therefore, the search for new antimicrobial substances exhibiting minimal side effects from natural sources is needed.

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted to prove such efficiency<sup>(115-123)</sup>.

Relatively little work has been done on the phytochemistry of local thyme in recent years. The only other study concerning the effect of this plant was conducted by Jenan<sup>(124)</sup> which used the cold alcoholic extract of *Thymbra spicata* to prepare ointment in the treatment of ringworm in calves naturally infected. Al-Makzumi<sup>(125)</sup> studied the effect of cold water extract and hot ethanolic extract of *Thymbra spicata* on bacteria *staphylococcus aureus* and *pseudomonas aeruginosa* isolated from patients affected with Boils.

The antibacterial activity compared between the volatile oils obtained from the steam distillation and Clavenger methods, has been investigated by the well method at the concentration of (50% v/v). It has been reported that the concentration of essential oils of thyme at (10% v/v) and (20% v/v) has no antimicrobial activity<sup>(60)</sup>.

The following bacteria were used in all experiments:

1. *Staphylococcus aureus.* (Gr. +Ve)
2. *Streptococcus pyogenes* (Gr. +Ve)
3. *Escherichia coli.* (Gr. -Ve)
4. *Klebsiela pneumoniae.* (Gr. -Ve)

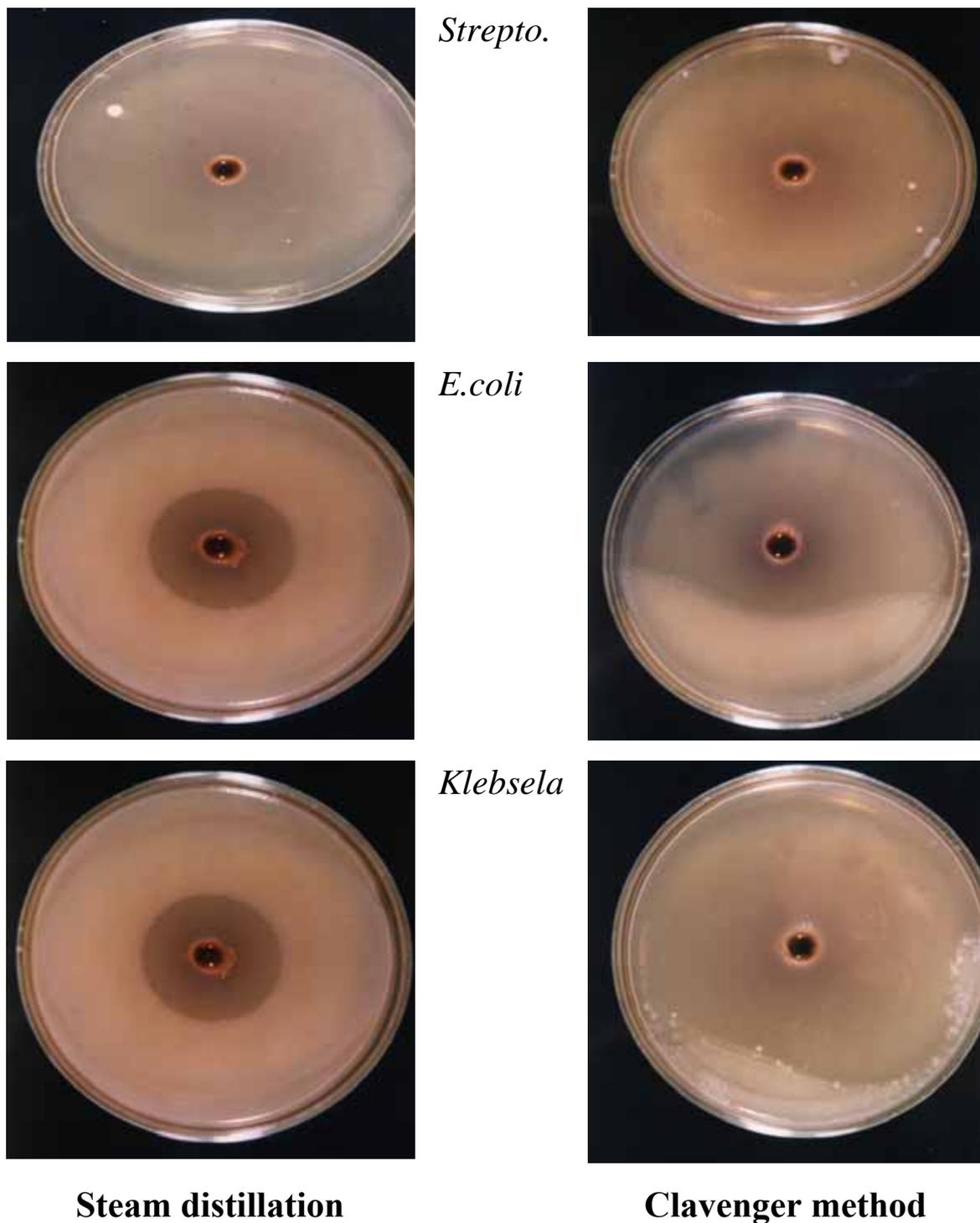
and only one type of fungi is used which is *Candida albicans*.

Four types of selective bacteria (2 Gram-negative and 2 Gram-positive) and one fungi have been used in this study for the detection of the effect of volatile oil of thyme on it, due to the pathological effect that is caused by this microorganisms on human and animals and on feed decay<sup>(126-128)</sup>. The sizes of inhibition of culture are summarized in Table (3-8) which shows the differences between the two types of volatile oils of the thyme used.

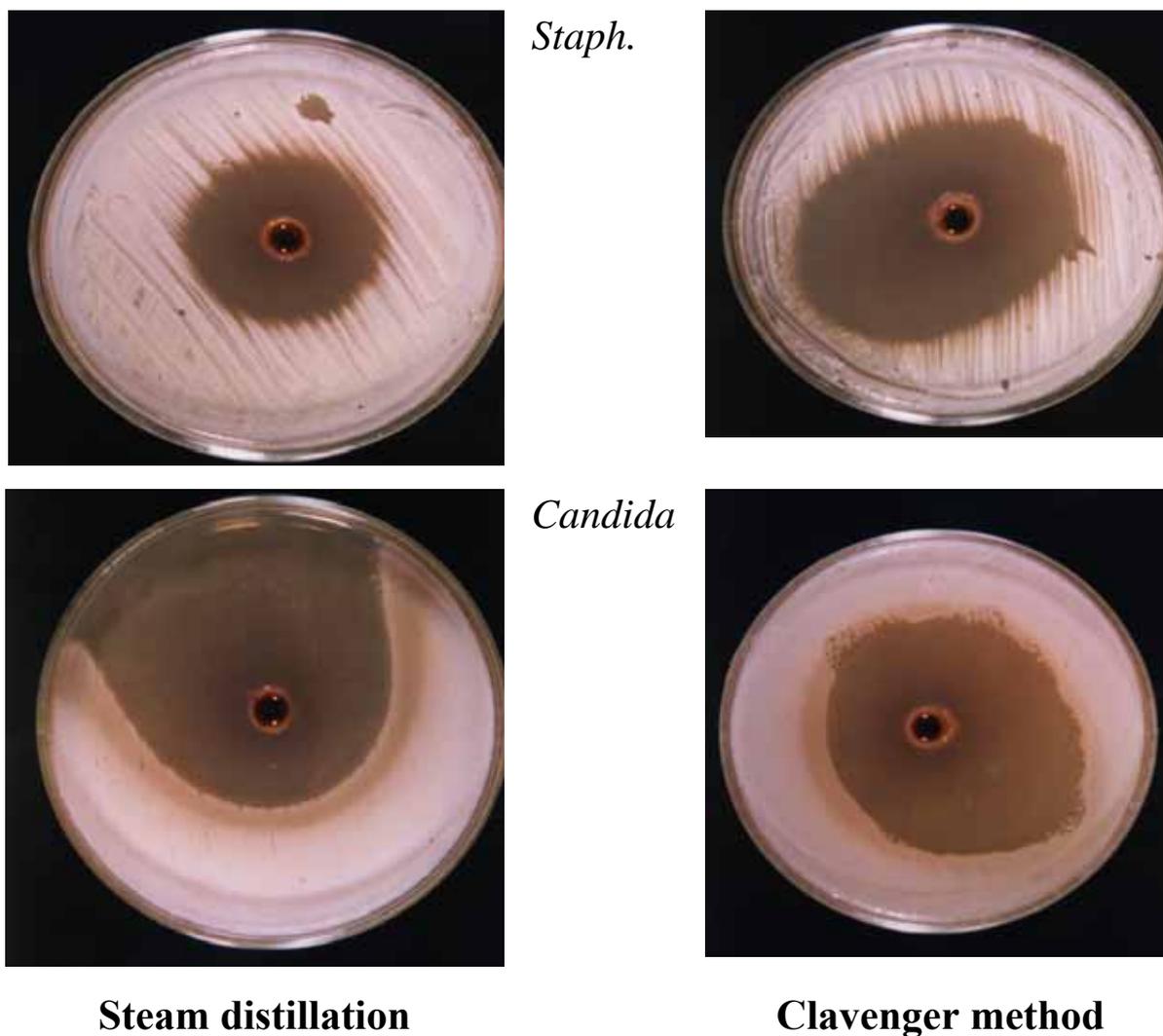
From the above data and Figure (3-10), the antimicrobial activity has been concluded. The Gram-negative (*E.coli*) gave inhibition zone (69,28)mm, Gram-negative bacteria (*Klebsila*) gave inhibition zone (81,31)mm, while the Gram-positive bacteria (*Staph.*) gave inhibition zone (54,35)mm, Gram-positive (*Strepto.*) inhibition zone was full field in petery dish, and fungil (*candida*) gave inhibition zone (52,57)mm from the oils obtained from Clavenger method and steam distillation method respectively. Thymol and carvacrol are considered as the most effective compounds which caused total inhibition.

Table (3-8) Effects of extracted volatile oil on the microbial activity of some bacteria.

Microorganism	Inhibition zone (mm)	
	Clavenger method	steam distillation
<i>E.coli</i> (Gr.-)	69	28
<i>Klebsila</i> (Gr.-)	81	31
<i>Staph.</i> (Gr.+)	54	35
<i>Strepto.</i> (Gr.+)	Full field	Full field
<i>Candida</i> (fungi)	52	57



Figure(3-10) Effects of extracted volatile oils on the microbial activity of some types of bacteria.



Figure(3-10) continue

Table (3-8) shows the exhibited similar levels of antimicrobial activity, but the oils extracted from Clavenger method appeared to be more efficient, due to the higher percentage of carvacrol compound at a high percentage than thymol. One of the most important findings in this investigation is that the Gram-positive bacteria are known to be more susceptible to the essential oils than Gram-negative.

That difference in the results of exhibition zones was explained by the results of identification tests of active components in each oil; the carvacrol percentage was more than of thymol in volatile oil obtained from Clavenger method, and this indicates that the type of extracted oil has a great role in biological effect, because it could be easier in external therapy than that of other extracts or with food as a protective material. The antiseptic, antioxidative and food-preservative properties of thyme oil are considered to be due to oil components such as thymol, carvacrol, p-cymene and camphor.

Because of its high phenol content, thyme oil has antiseptic properties<sup>(129)</sup>, and for this reason it has been concluded that the extracted volatile oil can be considered as antibiotic that had some degree of low toxicity, it is prohibited to be administered to be taken orally, but it has been widely used externally such as in the treatment of skin diseases like eczema.

## **3.6 Conclusions and Future Work**

### **3.6.1 Conclusions**

The present work has shown that the volatile oil extracted from Iraqi thyme by steam distillation and Clavenger methods contains the active organic compounds which are: carvacrol, thymol, p-cymene and camphor. These compounds are qualitatively identified and quantitatively evaluated; the oil extracted by Clavenger method contains higher amount of carvacrol.

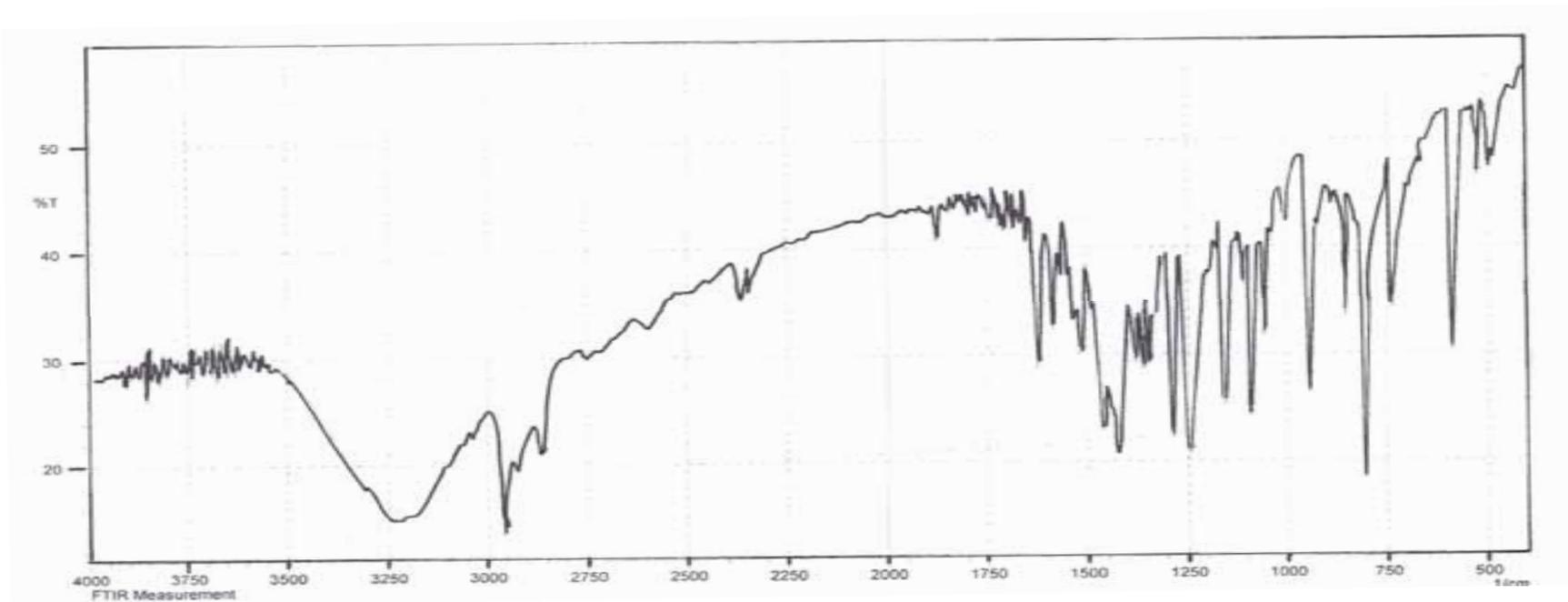
The active medical compounds proved to be present in *Thymbra spicata* are: Flavonoids, Tannins, Glycosides, Saponins, Resines and Coumarins. The study has revealed the absence of Alkaloids.

Antimicrobial activities of volatile oil obtained by the two methods were examined on four species of bacteria and one species of fungi us . It has been found that the volatile oil possesses antimicrobial activity against some Gram-positive and Gram-negative organisms, and fungi, where the activity is mainly attributed to thymol and carvacrol.

From the results of the antibacterial activity we can confirm the possibility of using the volatile oil as food preservative to prevent the growth of foodborn bacteria.

### ***3.6.2 Recommended Future Work***

1. Identification of other expected active compounds in volatile oil which have medical importance that has not been identified in volatile oil now such as:  $\alpha$ -pinene, borneol, and geraniol due to the absence of standards.
2. Quantitative evaluation for other active ingredients such as Flavonoids, Tannins and Glycosides which present in the whole plant.



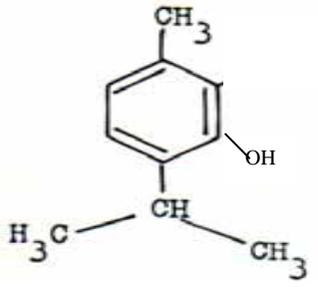
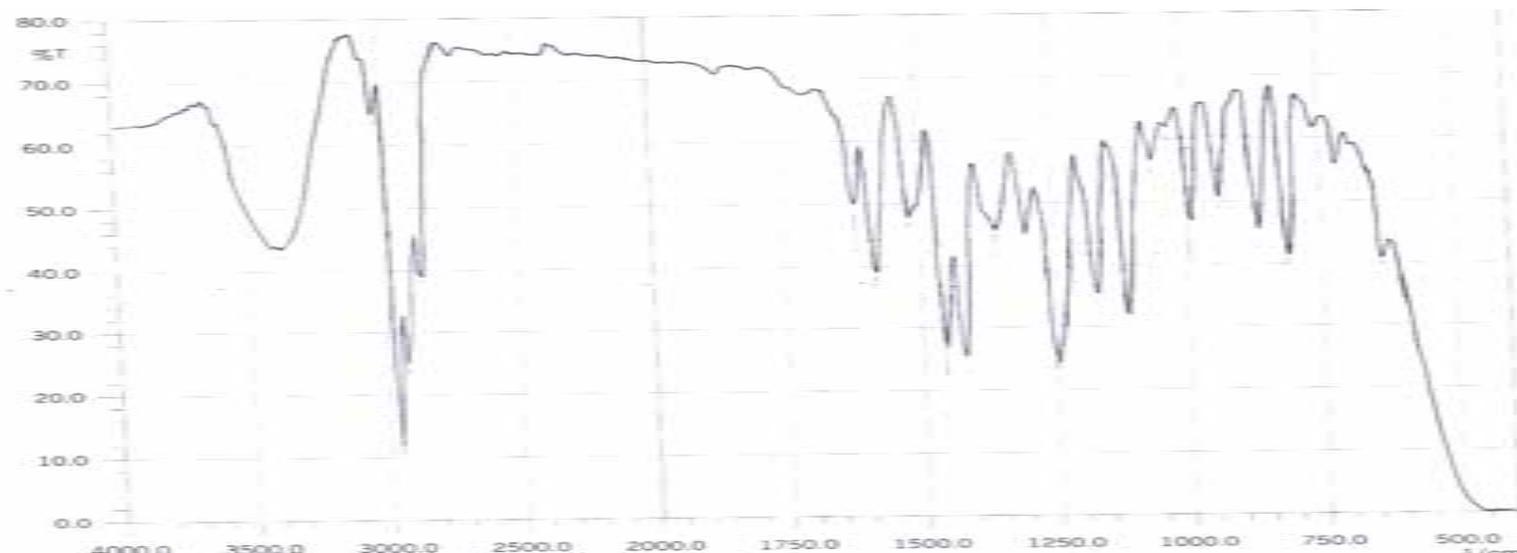
	3250-3200 $\text{cm}^{-1}$ st. O-H	1380-1340 $\text{cm}^{-1}$ bend. O-H
	3010 $\text{cm}^{-1}$ st. C-H aromatic	1286 $\text{cm}^{-1}$ st. $-\text{CH}(\text{CH}_3)_2$
	2962-2867 $\text{cm}^{-1}$ st. $-\text{CH}_3$	1244 $\text{cm}^{-1}$ st. C-O
	1458-1419 $\text{cm}^{-1}$ st. C=C aromatic ring	800 $\text{cm}^{-1}$ bend. C-H

Figure (3-2) IR spectrum of Thymol



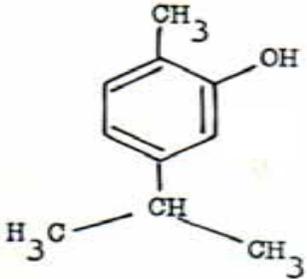
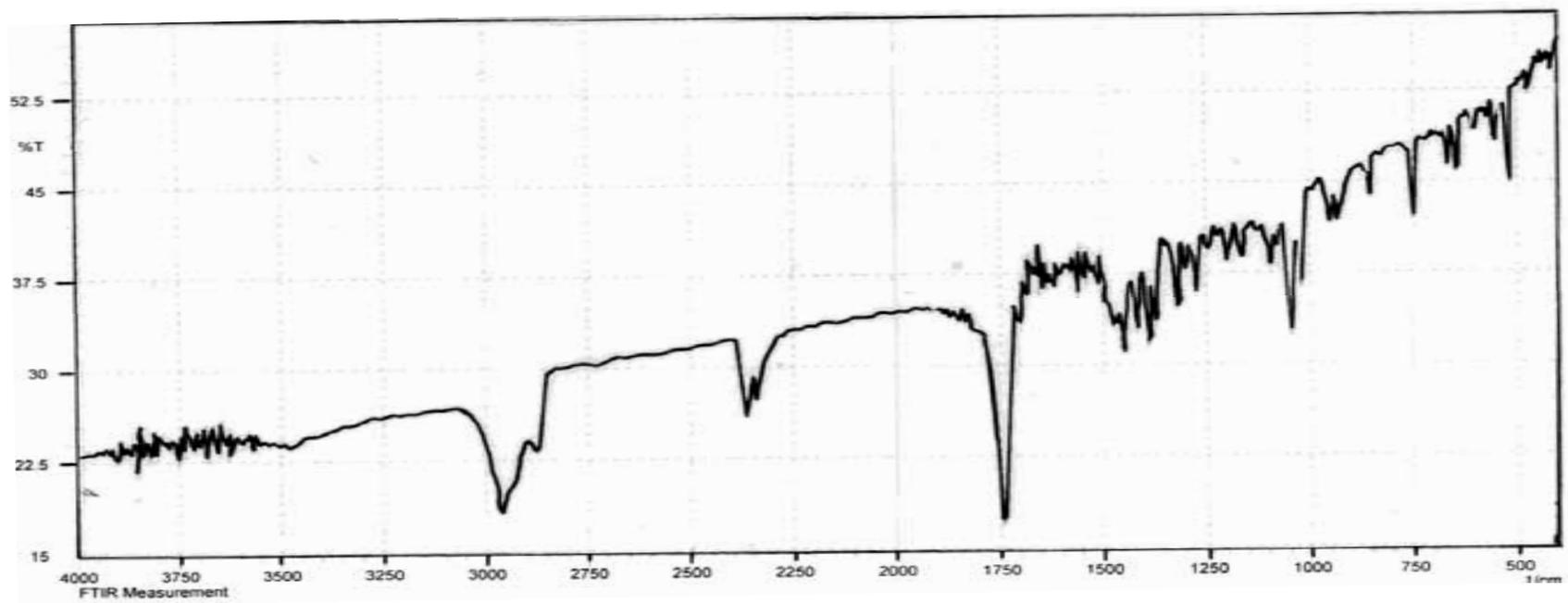
	3382 cm <sup>-1</sup> st. OH	1458-1421 cm <sup>-1</sup> bend. OH
	3020 cm <sup>-1</sup> st. C-H aromatic	1359 cm <sup>-1</sup> st. -CH(CH <sub>3</sub> ) <sub>2</sub>
	2960-2868 cm <sup>-1</sup> st. C-H alkane	1251 cm <sup>-1</sup> st. C-O
	1585-1458 cm <sup>-1</sup> st. C=C aromatic ring	800 cm <sup>-1</sup> bend. C-H

Figure (3-3) IR spectrum of Carvacrol



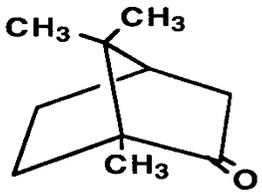
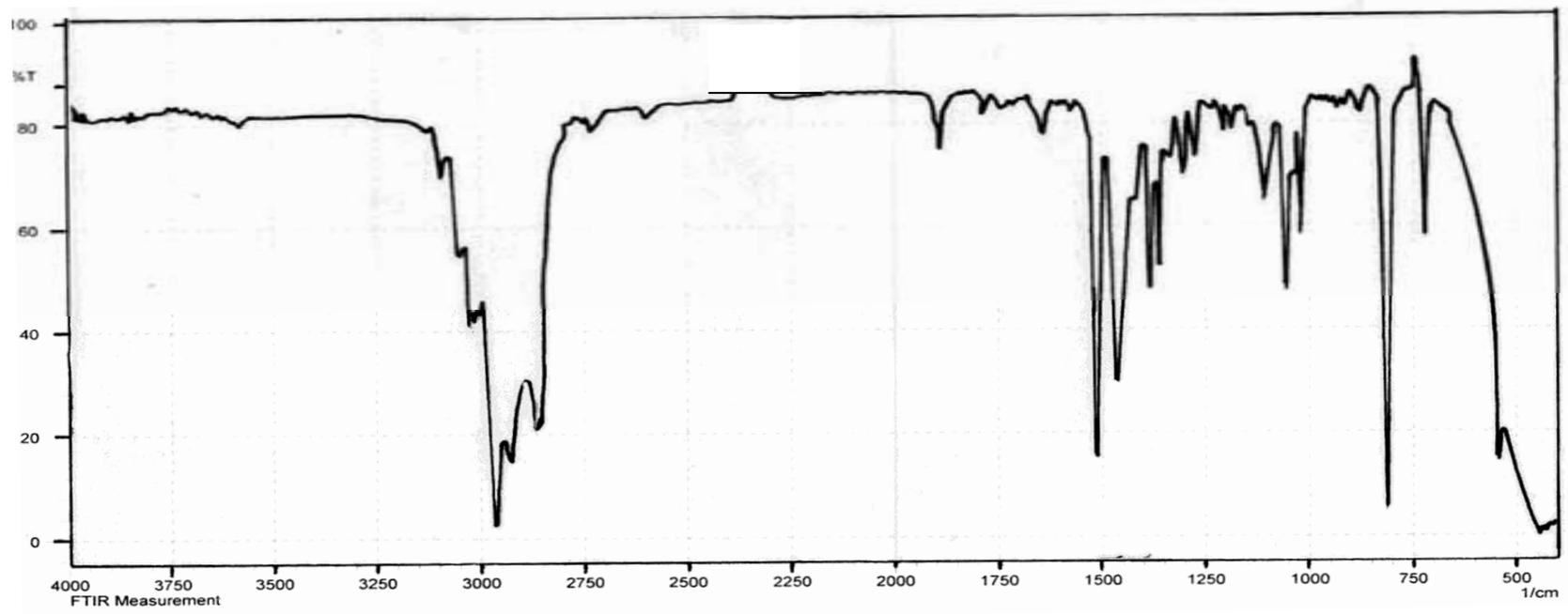
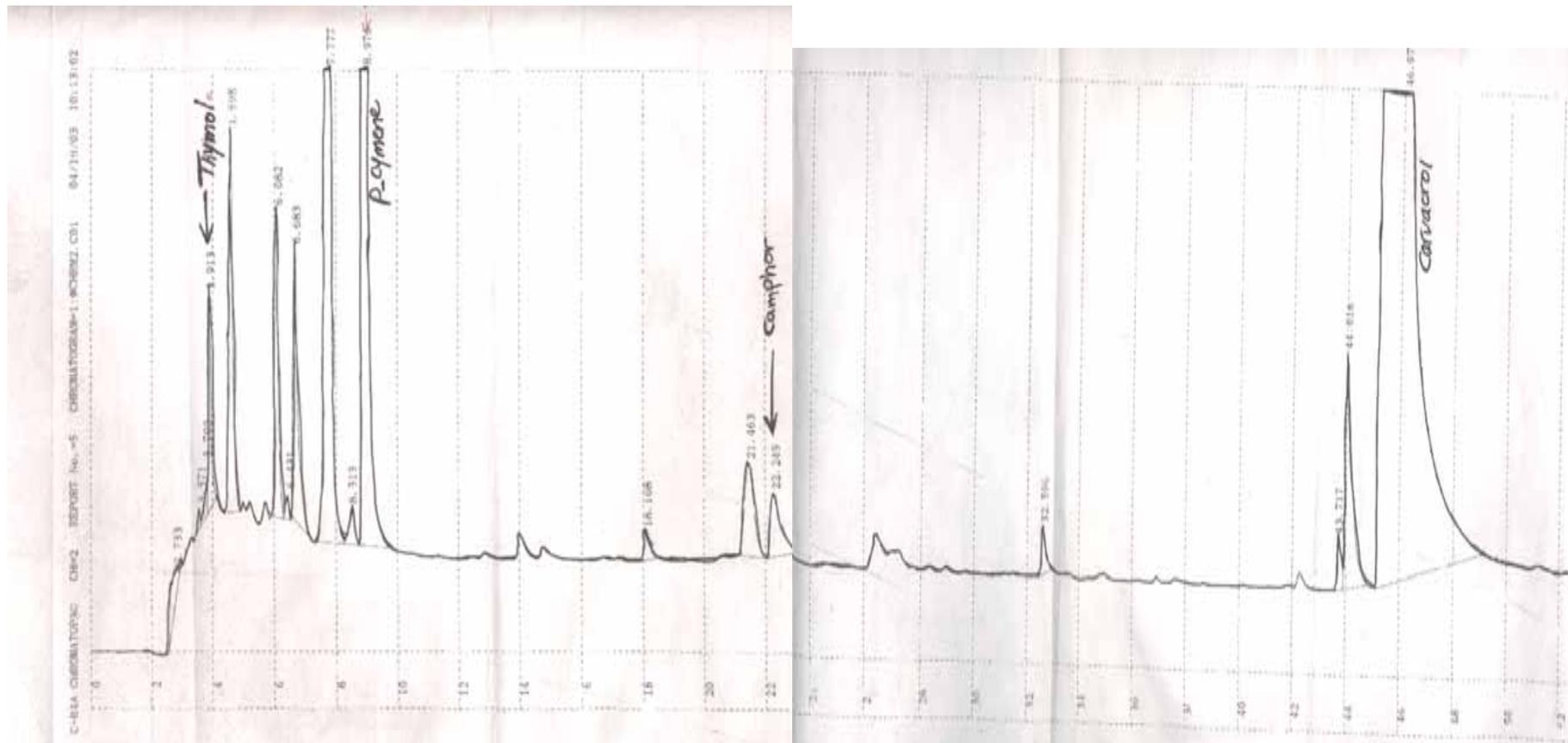
	2950 $\text{cm}^{-1}$ st. $-\text{CH}_3$	1390-1375 $\text{cm}^{-1}$ st. $-\text{CH}(\text{CH}_3)_2$
	1750 $\text{cm}^{-1}$ st. $\text{C}=\text{O}$	750 $\text{cm}^{-1}$ bend. $\text{C}-\text{H}$

Figure (3-4) IR spectrum of Camphor



	3049-3018 $\text{cm}^{-1}$ st. C-H aromatic	1390-1370 $\text{cm}^{-1}$ bend. -CH <sub>3</sub>
	2965-2869 $\text{cm}^{-1}$ st. CH <sub>3</sub>	1056 $\text{cm}^{-1}$ bend. C-H
	1514 $\text{cm}^{-1}$ st. C=C aromatic ring	813 $\text{cm}^{-1}$ bend. C-H

Figure (3-5) IR spectrum of P-Cymene



Figure(3-7) Gas chromatogram of extracted volatile oil

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## *Summary*

This study was done on Thyme plant species (*Thymbra spicata*) which widely grown in the north of Iraq. The plant was identified by herbarium of College of Science/ Baghdad University. The study includes chemical identification and biological investigations.

The preliminary phytochemical studies revealed the presence of several constituents which possess medical and biological activities such as: Flavonoids, Tannins, Glycosides, Saponins, Resines and Coumarin derivatives. Also the study revealed the absence of Alkaloids.

The aqueous extract of the plant was acidic (pH=5.45). The analysis of plant showed that the moisture reached 6.8%, the percentage of ash was 9.77%, and acid insoluble ash was 3%; and the refractive index of the extracted oil was 1.510, its containment of heavy metals like Cadmium and Lead in the amount of 0.003, 0.008 mg/Kg respectively.

The extract of volatile oil obtained from *Thymbra spicata* by using Clavenger method was compared with the volatile oil obtained by steam distillation; the oil yield by Clavenger method was 3 % while from steam distillation was 1.2%, and the oils found to contain: carvacrol, thymol, p-cymene and camphor in 29.5%, 0.381%, 5.748% and 0.456% respectively. Thymol obtained from volatile oil has been isolated. Carvacrol was prepared from thermal fusion of camphor with iodine.

The active organic chemical compounds in the oil extract from the two methods, were identified by Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Infrared (IR) and Ultraviolet (UV).

The biological activity of the volatile oils *Thymbra spicata* were studied by examining some Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogene*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) and one fungi (*Candida albicans*).

The oils extracted by Clavenger method showed more activity against the selected microorganism in vitro; this activity is mainly attributed to thymol and carvacrol.

:

*Thymbra spicata*

/

(pH=5.45)

9.77%

6.80%

.1.510

3%

0.003, 0.008 mg/Kg

3%

1.2%

0.456%, 5.748%, 0.381% , 29.5%

HPLC

TLC

.UV

IR

GC

*Staphylococcus aureus*, *Streptococcus pyogene*, *Escherichia coli* and *Klebsiella pneumoniae*  
(*Candida albicans*)