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Original article

Study the inhibition effect of oily thyme extract on sensitive and resistant *Staphylococcus aureus* and compare results with standard antibiotic

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ABSTRACT

Background: *Staphylococcus aureus* is one of the most pathogens of the respiratory and intestinal tract, and it is widely spread in hospitals, especially resistant ones. Surprisingly, thyme oil is effective against both sensitive and resistant bacteria. **Aim:** The study aims to find treatments of plant sources to reduce toxic effects and side effects of chemical pharmaceuticals used in therapeutic purposes. **Methods:** The study was carried out on the thyme oil extract that was bought from local markets and classified by the Herbarium of the University of Baghdad, the oil was extracted at Ibn Al-Betar Research Center. Tube Dilution Method was used to determine the minimum inhibitory concentration (MIC) toward *Staphylococcus aureus*. **Results:** the first detection of the functional groups in oil extract of thyme showed that it contains phenols, flavonoids, tannins. Interestingly, phenols are considered basic antioxidant material that is what gives the bactericide effect to thyme oil. The difference in the (MIC) of thyme oil against both bacteria was twice as the (MIC) of sensitive bacteria was at a concentration of 12% and an inhibitory diameter of (15mm) compared to gentamicin (12mm), while the (MIC) of thyme oil against the resistant bacteria was at a concentration of 25% with an inhibitory diameter of (20mm) compared to gentamicin (15mm). In both cases, the results showed that the inhibitory effect of thyme oil exceeds that of the standard antibiotic. **Conclusion**: The thyme oil in different concentrations proved its effectiveness on sensitive or resistant *Staphylococcus aureus* compared to the standard antibiotic.

Introduction

Over the last decades, the drug factories produced tens of antibiotics but unfortunately, resistance against these antibiotics keeps increasing [1, 2]. As a result of arising resistance from the pathogens, it is necessary to develop techniques or methods to control the use of antibiotics and to know the genetic mechanism of the emergence of this type of resistance to find alternative synthetic or natural medicines [2]. Over the centuries, plants have been considered a natural available source to maintain

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human health, as many plants have been used for their antibacterial activity, such as in essential oils [2, 3], which are called volatile oils or ethereal oils, these oils are a liquid obtained from different parts of the plant, which as well as having pharmacological effects, also having antibacterial qualities [4, 5]. In addition to some oils that exhibit antiviral, anti-parasitic, and insecticidal effects, as well [4].

Thyme is a perennial herbaceous plant belonging to the Lamiaceae family. It is spread in the Mediterranean region and the scientific name is Thymus vulgaris. The essential oil of thyme is one of the ten essential volatile oils and has antibacterial, antiseptic [5,6] and anti-parasitic effects, a natural preservative that is added to food, a carminative, anti-rheumatic spasm and skin diseases [6]. Thyme oil contains 55% of phenolic substances and the most important ones are thymol, and carvacrol to them most of its medical benefits were attributed [7].

Scientific classification of thyme: Taxonomic Classification **[8]**

Kingdom	**Plantae**
Class	Magnoliopsida
Order	Lamiales
Family	Lamiaceae
Subfamily	Nepetoideae
Genus	Thymus L.
Species	Thymus vulgaris L.

Gram-positive *Staphylococcus aureus* is clinically one of the most important types of bacteria, as it represents (30%) of the natural flora in the human body, especially in the skin and respiratory system. However, it is considered a human pathogen [9]. What is dangerous about these bacteria is their ability to develop resistance to antibiotics and to multiply rapidly to produce toxins and enzymes that play a role in the spread of them [10].

Gentamicin is an aminoglycoside antibiotic first isolated in 1958, an intermediate salt gentamicin sulfate of the compound's gentamicin (C1a C2 C1). It is considered a broad-spectrum Gram-negative subject, especially pseudomonas aeruginosa type. It is also effective against Gram-positive bacteria. Gentamicin sulfate is widely known for treating localized skin infection, in the form of either a cream or an ointment [11].

Materials and methods

Preparation for thyme oil extract

1-The leaves of the thyme plant were obtained from the local market and classified by the Herbarium of the College of Science / Department of Biology - the University of Baghdad as Thymus vulgaris.

2-leaves of the plant have been washed with water very well to remove all dirt and impurities stuck to them and left to dry in the shade with stirring to prevent rotting from it.

3-The leaves have been crushed with an electric grinder to get a fine powder.

4-A 100g of the powder was taken and put in a Clevenger steam distillation apparatus to extract the volatile oil.

5-A liter of water has been added to the powder in the apparatus.

6-The mixture was left for 6hr. to extract active substances.

7-The extracted oil was isolated and investigated by chemical and biological tests and then a determination of the minimum inhibitory concentration.

Oil of Thyme has been examined using Fourier Transform Infrared Spectroscopy Analysis (FTIR) and gas chromatography-mass spectrometry (GC-MAS) techniques in the Ibn Al-Betar research center.

The active groups and compounds were detected at Ibn Al-Betar Research Center / Plant Extraction Research Division.

Examination of the biological activity of the oil of thyme

- A well diffusion method has been used to test the effectiveness of the oil extract.
- Bacteria were activated in the medium of the nutrient broth
- Two hundred and fifty (250) ml of the mentioned medium was prepared according to the manufacturer's account and sterilized with an autoclave at a temperature of (121) °C for (15) minutes and left to cool at a temperature of (25) °C.
- This medium was then inoculated with 1 ml of the bacterial suspension, prepared by taking 3-4 colonies of bacterial growth after incubation for 18-24 hours.
- Twenty (20ml) medium was poured into each dish, and they were left to cool, and several pits with a diameter of (8 mm) were made for each dish, and

(50) microliters of the extract have been added at concentrations of (25%, 50%, 75%) in addition to the solvent for each pit using (micropipette).

- The biological activity of the extract has been examined at 100% concentration in another dish for comparison as in **figures (2,3).**

Preparation of microbial cultures

The target bacteria (*Staphylococcus aureus* ATCC 6538) was obtained from Ibn Al-Betar Research Center and after confirming its purity by activation in the middle of the nutrient broth and incubated for 24 hours at a temperature of 37 °C, then activated on the blood agar medium and with the same conditions as the previous ones, the bacterial cultures were prepared by transferring (4-5) colonies from the electoral medium to the nutrient broth and incubated at a temperature of 37 °C for (4-5) hours, then the suspension was diluted with a physiological salt solution in comparison with the control tubes. Standard MacFarland tubes No. (0.5) [12,13].

Minimum inhibitory concentration (MIC) assay

The extract was dissolved with a Tween 80 emulsifier [14] to conduct the tube dilution method [12] (it does not have any antibacterial effect on the bacteria under study) [15] and a series of half dilutions of volatile oil was prepared with the following concentrations (50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.7%) consisting of thyme oil and the emulsifying agent that was used as a diluted factor. After pouring in sterile test tubes, they were inoculated with a 100 µl of the bacterial suspension and incubated for 24 hours at a temperature of 37 °C. Then, a (0.1) ml was transferred from each tube and spread by sterile metal diffuser on the surface of a plate containing the nutrient medium and incubated at 37°C for 24 hours to investigate the value of the minimum inhibitory concentration as in **figure (3,4).**

Comparison the effect of the standard antibiotic gentamicin and the oil of thyme on the target bacteria (at the minimum inhibitory concentration)

The comparison model has been made using standard anti-gentamicin tablets at a concentration of (10) µg. The dish was incubated at a temperature of 37 ° C for 24 hours, and the diameter of inhibition was measured with a ruler after the incubation period ended [16], as in the **figure (6).**

Table 1. Fourier transform infrared spectroscopy-(FTIR)

Wave number (cm-1)	Absorption band	Group	Compound
3406	broad	O-H (stretching)	Phenol- OH
2823-2925	medium	C-H(stretching)	Alkane(CH3)
2906-3018	medium	C-H(stretching)	Alkene
1621-1874	weak	C=C(stretching)	Alkene(aromatic Trisubstituted)
1512-1584	medium	C=C	Congugated
1371-1425	medium	O-H (bending)	Phenol

Table 2. Gas Chromatography – Mass Spectroscopy (GC-MAS) analysis.

Compound	Retention Time	Percentage %
cyclopentanol	7.729	5.56
alpha-pinene	9.906	1.94
beta - pinene	11.306	2.34
o - Cymene	12.220	5.31
Gamma- Terpinene	12.765	4.71
Gamma- Terpinene	12.998	2.88
Butanoic acid	14.077	2.67
Octenal	15.244	5.20
Diglycerol	15.788	3.56
Thymol	17.849	5.73
Carvacrol	18.238	4.77
Propan	18.510	2.66
Caryiphyllene oxide	20.766	2.60
Hexadecanoic acid	29.409	3.02

Table 3. The chemical test to extract the thyme oil.

n	The test	Result
1	Tannins Test	+
2	Carbohydrate Test	_
3	Glycosides Test	+
4	Phenols Test	+
5	Resins Test	+
6	Flavonoids Test	+
7	Saponin Test	_
8	Alkaloid Test	+
9	Protein test	_
10	Coumarins Test	+
11	Terpenes test	_
12	Steroids Test	_

Table 4. Shows the biological activity by well diffusion method of thyme oil on *Staphylococcus aureus* at a concentration of (25, 50, 75, 100) %.

Inhibition diameter	Gentamicin	Thyme oil conc.
16 mm	15 mm	25%
34 mm		50%
38 mm		75%
65 mm		100%

Table 5. The values of the minimum inhibitory concentration and the minimum bactericidal concentration before and after resistance induction.

Type of bacteria	MIC	MBC
Staphylococcus aureus before resistance induction	12 %	25 %
Staphylococcus aureus after resistance induction	25 %	50 %

Table 6. Comparison of the biological activity of thyme oil extract before and after resistance induction with standard antibiotic gentamicin.

Bacteria	Inhibition zone diameter(mm)		
	Oil extract%		Positive control mg /ml (Gentamicin)
	before resistance induction	After resistance induction	
Staphylococcus aureus	12 %	25 %	
	15	20	15 , 11

Table 7. Inhibitory effect of thyme oil extract on susceptible and resistant *Staphylococcus aureus* in comparison with the standard antibiotic.

	Inhibition zone diameter(mm)				
Bacteria	**Oil extract %**				**Positive control mg/ml (Gentamicin)**
	25%	50%	75%	100%	
Staphylococcus aureus	16	34	38	65	15

Figure 1. Fourier transform infrared spectroscopy-FTIR) analysis.

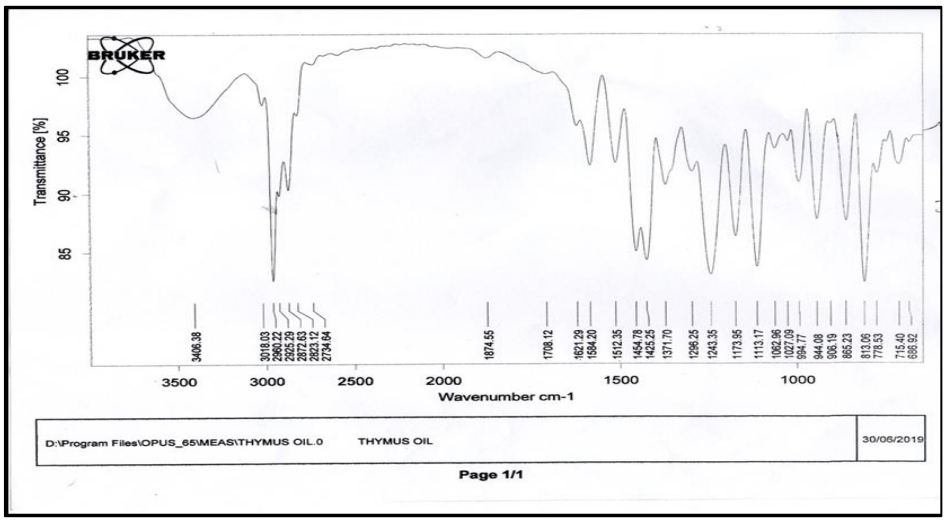

Figure 2. Biological activity of thyme oil at concentration of 100%.

Figure 3. Biological activity of thyme oil at concentration of (25%, 50%, 75%) with the solvent.

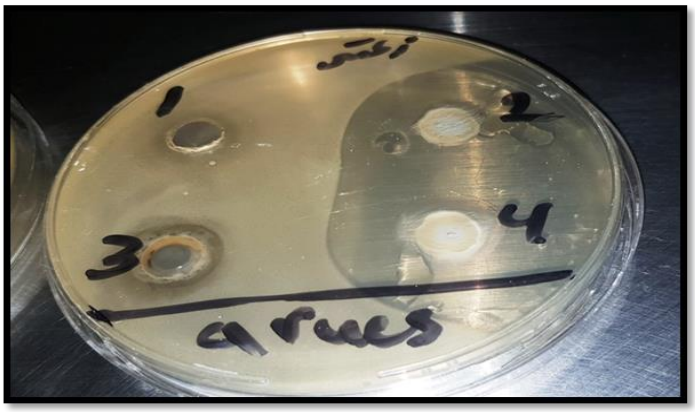

Figure 4. Determination of the value of the minimum inhibition concentration of the thyme oil by tubes dilution method.

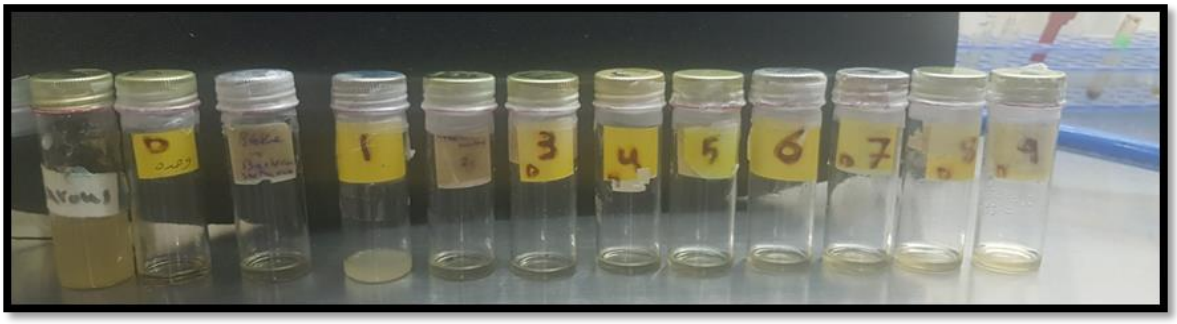

Figure 5. The value of the minimum inhibition concentration after culturing it at culture media with concentrations (1:50%, 2: 25%, 3: 12.5%, 4: 6.25%).

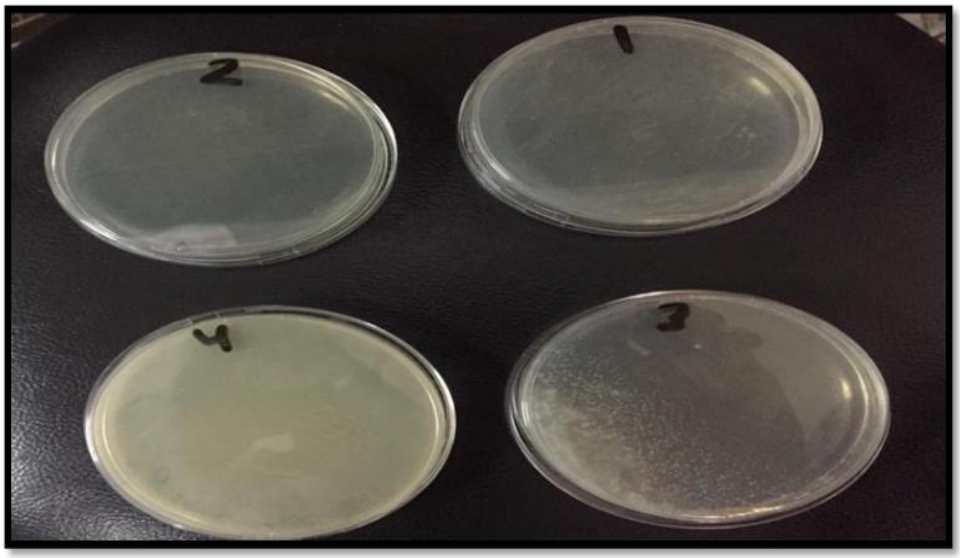

Figure 6. Comparison between the activity of thyme oil with concentration 12.5% (the value of minimum inhibition concentration) and gentamicin antibiotic with concentration of 10 µg.

Figure 7. *Staphylococcus aureus* colonies on the media of blood agar.

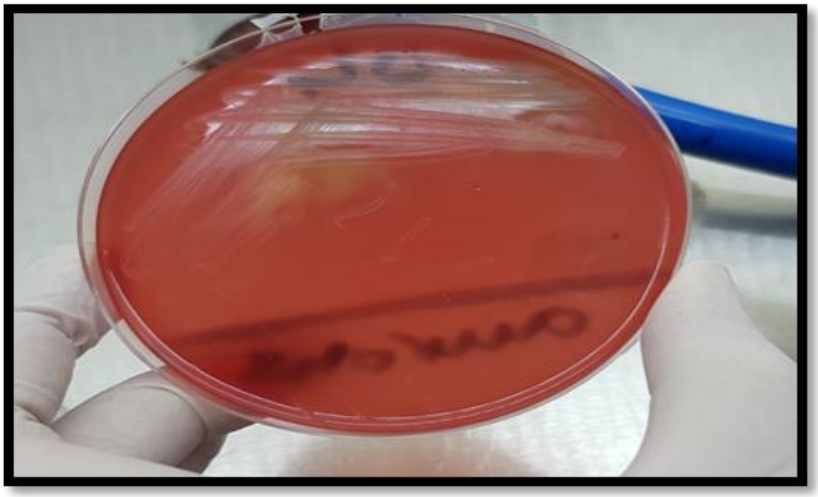

Figure 8. Determination of the minimum inhibitory concentration of thyme oil against *staphylococci* after induction of resistance.

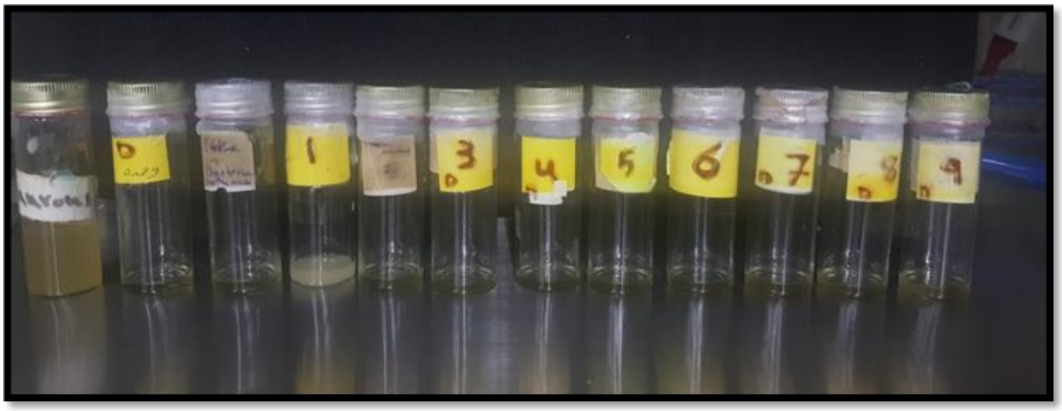

Figure 9. The value of the minimum inhibition concentration after culturing it at culture media with concentrations (1:50%, 2: 25%, 3: 12.5%, 4: 6.25%).

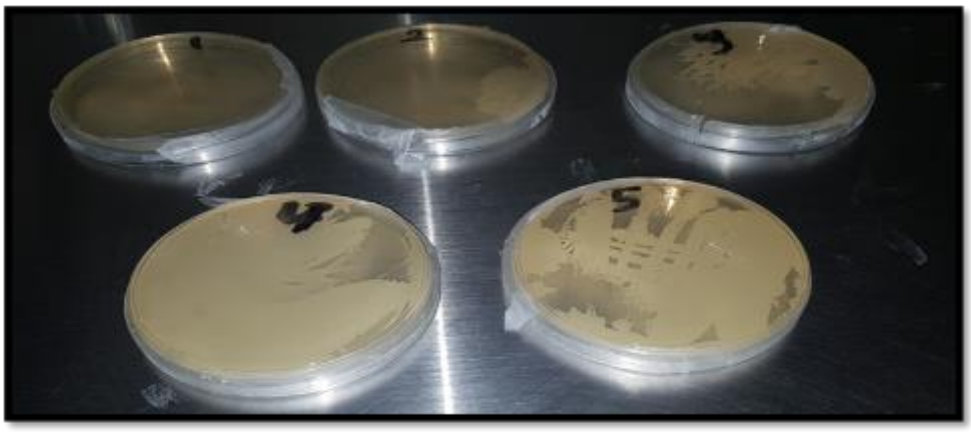

Figure 10. Comparison of biological activity of thyme oil at concentration of 25% (the value of inhibition conc. after resistance induction) with gentamicin.

Discussion

The thyme plant was classified as Thymus vulgaris by the Herbarium of the University of Baghdad. The results of (Fourier transform infrared spectroscopy-FTIR) showed broad absorption bands (340.6 cm-1) as in **table (1), figure (1)** indicating the existence of (OH) functional groups of phenol which can attract hydrogen with an internal bond making the compound unfree, leading to the appearance of the broad peak. Whereas the depth that appeared at the wavelength (2823 cm-1-2925cm-1) was moderate and specific for (C-H) group in (CH3) compound that belongs to alkane. Also, the wavelength of (2960cm-1 – 3018cm-1) was the mild band and specific for (C-H) that belongs to (alkene) compounds that have bond shape as (- C=C-). However, the depth that appeared at the wavelength (1874cm -1 - 1621cm-1) was shallow and specific for the (C=C) group that belongs to (Aromatic tri substituted). The wavelength (1512cm-1 - 1584cm-1) showed moderate depth indicating the existence of (C=C) double bonds, also the (1371cm-1 – 1425cm-1) wave length showed mild depth specific for the hydroxyl group (OH- Bending) for phenol compound agrees to what mentioned at [17,18]..

Table (2) showed clarifies detection by Gas Chromatography – Mass Spectroscopy (GC-MAS) which revealed the exist of the following compounds and the most important of them are Thymol and carvacrol as major compounds which to them pharmacological efficacy was attributed found in the extract, and less of o – Cymene, Terpinene, the similarity of some elements in thyme extract may variable depending on locality, plant origin, biotic factor and climate as it was mentioned at [19,20].

The biological activity of the oil thyme extract on *Staphylococcus aureus* revealed varying inhibition of bacterial growth according to the different concentrations of thyme oil used compared with 10 micrograms of standard antibiotic gentamicin, and this inhibitory ability is due to the chemical composition of thyme oil and its containment of thymol at a rate of (42.7%) and carvacrol, which act on proteins in bacteria by dissolving membranes and lysing bacterial cell wall and weakening their vital activities and thus its death [21], phenol acts strongly on antimicrobial activity , non-aromatic alcohol & terpens that what has been indicated at [19] and in the **table (3)** and as shown in the **figure (2, 3).** The ability of thyme oil to inhibit microbial even with low concentration on the organism that has been tested , this confirm that sometimes the effect of some essential oils on G+ve & G-ve bacteria be active more than antibiotics as in [19] that above 300 mg will give bactericidal effect on all strains tested in their research even on Staphylococcus aureus that has multidrug resistance which involving in some critical hospital infections .This biological activity is due to the chemical composition determining by their genotype and environmental condition [20,22].This result give a notion by using a combination of an essential oil and antibiotic which give an additive or synergistic effect & modification the resistant phenotype to be sensitive in many stains [23]. The efficacy of the essential oil such as thyme oil contain a bioactive compound in which it has inhibitory effect on bacteria cellular structure [24] by increasing the susceptibility to these agent also that may give a notion of using an essential oils as food preservative as these oils can antimicrobial activity even with its low concentration [4, 25]. Thyme showed a bactericidal and bacteriostatic effect as mentioned in [26-29]. Within the effect of an essential oils which known as generally recognized as safe (GRAS) such as: oregano, basil, ginger, thyme, cinnamon [30] and this will give an idea to use these oils as a combination that give either synergistic or additive effect [4] and has an antioxidant effect and act as natural antibacterial due to the phenolic compound which affect either on protein denaturation or enzyme activity that enhances decrease meat product spoilage [29,31]. Thyme consider as medical plant which can increase the effectiveness of intestine [21], other researchers mentioned thyme and other medical plants extract (as a mixture) can also improve productive and physiological trais of chicken broiler and storage meat that treated with theme and menthe extract showed reduction in microbial account, pH and increase their acceptability [31,32].

Table 5 showed that the results of the minimum inhibitory concentration) MIC (12% and the minimum bactericidal concentration) MBC (25% measured by tube dilution method as in figure (4, 5) and that is close to what was mentioned in [17,18]. The resistance of the bacterium was induced by passing it at a concentration lower than the minimum inhibitory concentration and activated on the medium of the blood agar, so it appeared in the form of golden-colored colonies as in the figure (7), The colonies were transferred into the nutrient medium. After the induction of the resistance, the minimum inhibitory concentration was 25%(, while the minimum bactericidal concentration was)50%(as shown in **table (5)** and **figure (8,9).** Eventually, in comparing the efficacy of thyme oil before the induction of resistance at a minimum concentration (12%) and after the resistance induction at (25%) that was (15,20) mm to the efficacy of (10 µg) standard antibiotic gentamicin was (11,15) mm respectively as in **table (6)** and **figure (10)** this agree with [22].

The chemical tests for thyme oil revealed the presence of active substances as in **table (3),** including phenols which consider essential constituents in volatile oil of the thyme, in addition to its containing flavonoids and tannins [21,25] containing like these substances makes the thyme oil widely used in the medical field, as many researchers mentioned [33]. And also, to them was attributed its inhibiting effect on germ growth which enhances the ability to extract active materials from it and examine their pharmacological efficacy and facilitate their use in drug industry fields to create high-efficiency treatments with low toxicity [20,33].

Conclusions

The thyme oil in different concentrations proved its effectiveness on Staphylococcus aureus, which is one of the most prevalent germs from the toxic and cumulative effects that arise from the use of antibiotics over a long period, in addition to its danger in generating a resistant generation of germs when misused. in hospitals and causes many complications at treatment due to its ability to resist most traditional antibiotics.

Thyme oil overpowered this bacterium after the development of resistance compared to the

standard antibiotic, and its effect was similar or slightly greater than that of the standard antibiotic.

Using the oil extract of the thyme plant is in the ranks of safe materials and preparations away.

Recommendations

Using thyme oil in therapeutic formulations alone or in combination with other substances or other antibiotics in order to reduce the concentration of antibiotics and avoid their toxic effects.

Improving the oil extract in the future by mixing it with other extracts or adding substances that enhance its action.

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Conflict of interest

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