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Antibacterial activity of ethanolic extracts of *Thymus vulgaris* and *Cinnamomum camphora* on human pathogenic bacteria.

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Abstract

Medicinal Various chemical compounds produced by herbs have primary medicinal uses, particularly in treating bacterial diseases, such as nosocomial infections. *Thymus Vulgaris Cinnamomum camphora* ethanolic extracts were investigated for their antibacterial activity against three hazardous bacteria: *Staphylococcus aureus*, *Acinetobacter baumannii complex*, and *Klebsiella pneumoniae*. The three dangerous bacteria—*Staphylococcus aureus*, *Acinetobacter baumannii complex*, and *Klebsiella pneumo*niae—were detected utilizing the 16rRNA gene extracted from clinical specimens (pneumonia, sputum, urine). Two Egyptian plant extracts, ethanolic (*Thymus vulgaris*) and the other *Cinnamomum camphora*, were tested in vitro for antibacterial activity. The *Thymus Vulgaris* extract showed main inhibition diameters of 27, 30.3, and 20.6 mm against three human pathogenic bacteria, while the *Cinnamomum camphora* extract showed main inhibition diameters of 25, 27.6, and 18.3 mm. The gas chromatography-mass spectrometry (GC-MS) examination of the *Thymus Vulgaris Cinnamomum camphora* extracts indicated the presence of several terpene compounds. The main ingredient (in this order: cis-vaccenic acid, otadecanoic acid, cis-13-eicosenoic acid, erucic acid, oleic acid, 13-docosenoic acid, isochlapin B, thymol, epiplobol).

Key words: *Acinetobacter baumannii complex, Staphylococcus aureus, Cinnamomum camphora, Klebsiella pneumoniae, Thymus vulgaris*

1. Introduction

The quest for more effective drugs and strategies to prevent antimicrobial resistance has been pushed by the ever-increasing incidence of bacterial resistance among hazardous germs, which is sometimes caused by the inappropriate or abuse of antibiotics.

(Raghunat, et al.,2017). Additionally, there is a lack of monitoring data on a recent upsurge in biofilm-associated diseases in people.

(Huh et al.,2011 and Swain et al.,2014) Regarding nosocomial community-based bacteremias, *Staphylococcus aureus* is still a big player. Bacteremia usually occurs when an infection on the skin gets into the circulation. The patient runs the danger of endocarditis and other metastatic consequences of germs entering circulation **(Boyce et al.,1997).** The --resistant *Acinetobacter baumannii complex* has been highlighted as a significant priority by the World Health Organization (WHO) on their worldwide priority list of antibiotic-resistant bacteria. This list is used to guide drug research development efforts. *Acinetobacter baumannii*, which is resistant to ampicillin, has been associated with various hospital-acquired infections, including pneumonia, bloodstream infections, wound infections, and UTIs, especially in critically sick patients during intensive care unit (ICU) stays.

(Ayobami, et al.,2019). Isolation rates in clinical samples of *Klebsiella pneumoniae*, the type species of the genus, are about 85% **(Wyres et al.,2020)**. The ESKAPE category of organisms comprises the infamously deadly resistant-to-antibiotic clinical diseases *Klebsiella pneumonia, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter species.*

(Navon-Venezia, et al.,2017). In the past, people used the *Thymus vulgari*s to burn poisonings caused by scorpion snakes. The antibacterial characteristics of this substance have made it extensively used in traditional medicine **(Khalilnezhad et al.,2015; Mohammadi et al.,2019)**—some of the physiologically active chemicals found in the water-based *Thymus. Vulgaris* extract include, 2.5-O-Methylene-D-mannitol, xylitol, caffeic acid, gallic acid, chlorogenic acid, quinoline, decahydro-1,7-dimethyl **(Umer, et al.,2024)**. of the most popular fragrant species used for afforestation in China is *Cinnamomum camphora*, an evergreen species in the Lauraceae family. It is a prominent species in the subtropical broad-leaved forest. Essential oils in *C.*

camphora's roots, bark, leaves, and fruits have antimicrobial, anti-inflammatory, and insect-repellent effects **(Ying et al.,2016)**. , eucalyptol, sabinene, α-terpineol, caryophyllene, nerolidol, α-pinene, camphor, and β-pinene were the main components of the C. camphor essential oil **(Wenting et al.,2019)**.

2. Material Methods

Medicinal plants collection:-

Fresh leaves *of Thymus vulgaris were* purchased from Egyptian commercial markets, while *cinnamon camphor* leaves were sourced from Benha, Qalyubia, Egypt. The plant samples obtained were taken to the microbiological laboratory at Benha University's faculty for further analysis.

Contaminating microbes: Based on the samples taken at the Microbiology Immunology Department of Benha University Hospital, three different bacterial isolates were identified: *Staphylococcus aureus, Acinetobacter baumannii complex, Klebsiella pneumoniae*. Isolates were identified via molecular analysis. Proteomic Quantification Tests As a first step in diagnosing bacterial infection, the 16srRNA gene was found in every sample (**Xu et al.,2004)**. Table 1 displays the primers. Table 1 shows the results of the subsequent steps for identifying several bacteria, including *Acinetobacter baumannii complex, Klebsiella pneumonia*, and *Staphylococcus aureus*. Every sample was subjected to PCR using a particular combination that included the following elements: To make 25 µL of the final volume, add 1X Specific PCR buffer, 0.4 mM dNTPs mix, 0.7 mM MgCl2, 1.6 M primers, a unit of Taq polymerase enzyme, and 2 µL DNA to sterile distilled water. The PCR conditions were as follows: Thirty cycles of denaturation at 94°C for 45 seconds, annealing at 48°C, 61°C, 53°C, and 50°C for 45 seconds, extension at 72 °C for 45 seconds, and finally, a final extension at 72 °C for 7 minutes are applied to the 16srRNA gene, *Acinetobacter baumannii complex, Klebsiella pneumonia*, and *Staphylococcus aureus* genes. The internal positive control was used in the PCR experiments.

.**Which Primers Were Employed for This Research:**

Primer	Sequence (5'→3')	PCR Product Size (bp)	References
16S rRNA-F	CCTCTCAGACCAGTTA	250	(Anbazhagan, et al.,2011)
16S rRNA-R	CCTAACACATGCAAGTCGA		
Acinetobacter baumannii-F	TAATGCTTTGATCGGCCTTG	353	(Khorsi, et al.,2015)
Acinetobacter baumannii-R	TGGATTGCACTTCATCTTGG		
Klebsiella pneumonia-F	ATGCGTTATATTCGCCTGTG	865	(Babini, et al.,2000)
Klebsiella pneumonia-R	GTTAGCGTTGCCAGTGCTCG		
Staphylococcus aureus-F	TCAGCAAATGCATCACAAACAG	287	(CRLAR,et al.,2009)
Staphylococcus aureus-R	CGTAAATGCACTTGCTTCAGG		

Preparation of plant extracts:-

The study made use of these two medicinal plants. After cleaning with filtered water from the tap, the leaves of the plants were laid aside to dry for three days in the shade at room temperature. The plant's dried leaves were mashed into a powder using a high-powered blender. After that, they may remain in the dry bags until extraction, which occurs at room temperature. Ten grams of dried powdered plant material were immersed in eighty percent ethanol in a sterile conical flask for hydro-alcoholic extraction and swirled continuously for forty-eight hours. The supernatant was collected and concentrated under vacuum at temperatures below forty degrees Celsius using a Heidolph, VE-11 rota evaporator after eight layers of muslin cloth centrifugation at 5000 rpm for 10 minutes.

With this strategy, we were able to reduce the stock solutions in half, as indicated in the reference. **(Lokhande, et al.,2007)** stored in sterile bottles with labels until required, then frozen at four ºC (**Aneja etal.,2009)**

Antibacterial activity of ethanolic plant extracts:-

Plant performs activity testing using the healthy diffusion technique: adding 1 ml of inoculum solution to each sterile petri dish, and about 20 ml of autoclaved nutritional agar was added and allowed to harden. Following this, a sterile metallic pipette was used to create holes in the seeded agar that were 6 mm in diameter. The seeded medium had 50 µl of plant crude extract added to each well. Incubating the for 48 hours at 37 ºC, the millimeters (mm) of inhibitory zones were measured **(Aneja et al.,1996)**.

Effects of varying amounts of *Thymus vulgaris, Cinnamomum camphora* ethanolic extract on three distinct types of bacteria for antimicrobial activity. These values are the average of three measurements in millimeters In a solid medium:

Adding 1 ml of inoculum solution to each sterile petri dish, about 20 ml of autoclaved nutritional agar was allowed to harden. Following this, a sterile metallic pipette was used to create holes in the seeded agar that were 6 mm in diameter. Twenty, forty, sixty, eighty, hundred microliters of crude extracts from each plant were applied to every well on the seeded medium. They were incubated at 37 ºC for 48 hours. The millimeters (mm) were used to measure the inhibitory zones produced **(Dhiman et al.,2011)**.

***Thymus vulgaris* extract and *Cinnamomum camphora* extract have minimum bactericidal concentrations (MBC) and minimum inhibitory concentrations (MICs).**

In broth medium: The MIC values were determined via microdilution. Approximately 10 µl of each bacterial culture (108 CFU/ml) was mixed individually with 10 mL of nutrients).Six sterile test tubes were arranged in two rows, each extract in one row. Two hundred milligrams of dried extract were added to 0.4 ml of dimethyl sulfoxide DMSO for a 500 mg/ml concentration. Then, 100 µl was employed, totaling 250 mg. After that, there was a serial dilution of the extract in each tube to obtain concentrations of 125, 62.5, 31.25, 15.62, and 7.81 mg ml−1, respectively. Briefly, 100 µl of various samples were placed into the test tubes separately, containing 9 ml of the standardized solution of tested bacteria (108 CFU/ml), while the control sample contained bacterial suspension alone. After then incubated at 37 ºC for 48 h. After that, the MIC values were computed by evaluating the turbidity in each tube. The MIC was estimated as the lowest plant crude extract concentration inhibiting bacterial growth **(Das et al.,2016).** The minimum bactericidal concentration (MBC) was calculated as the concentration that killed 99.9% of the bacteria **(Basri et al.,2005).** Nutrient soup injected with microorganisms was employed as a positive control, whereas nutrient broth was used as a negative control.

DETERMINATION OF BACTERICIDAL CONCENTRATION (MBC):-

The following is how the MBC of various plant extracts was assessed against the chosen bacterial strains: 100 µL of each non-growing MIC tube was subcultured on nutrient agar, were incubated at 37 °C for 24 hours, and the growth was examined incubation. We used the lowest concentration that failed to produce colonies of the bacteria we examined as our MBC **(Dhiman et al., 2011).**

Gas-chromatography-mass spectrometry GC/MS analysis:-

A TG-5MS direct capillary column of 30 m x 0.25 mm x 0.25 µm film thickness and a GC-TSQ mass spectrometer made by Thermo Scientific in Austin, TX, USA, were used to

identify the chemical composition of your samples. From 50°C to 250°C, the column oven was heated at a five °C/min rate for 2 minutes. After that, it was heated to 300°C at a rate of 30°C/min for another 2 minutes. Maintaining temperatures of 270 and 260°C, respectively, was done on the injector MS transfer lines. Helium was a carrier gas at a constant flow rate of 1 ml/min. A 4-minute solvent delay was automatically inserted into 1 µl diluted samples using the Autosampler AS1300 and GC in split mode. Full scan mode was utilized to collect EI mass spectra in the m/z 50-650 range at ionization voltages of 70 eV. A temperature of 200 °C was applied to the ion source. By comparing their mass spectra to those in the WILEY 09 NIST 14 databases, we could determine the components it was consuming. **(AbdEl-Kareem, et al.,2016).**

3. Results discussion

Three The Microbiology-Immunology Department at Benha University Hospital identified bacterial strains (1P;2S;3U) from urine, sputum, and pus samples. The 16s rRNA gene sequencing method was used to identify these isolates. The Microbiology Immunology Department at Benha University Hospital identified bacterial strains (1P;2S;3U) from urine, sputum, and pus samples. The 16s rRNA gene sequencing method was used to identify these isolates.

Molecular identification of Bacterial isolates 1P,2S, 3U:

Three bacterial isolates—1S, 2P, 3U—were chosen for identification using 16srRNA because they exhibit resistance to 85 percent of twenty different antibiotics. as well as *Klebsiella pneumoniae* strain YM2023, OR673717, Acinetobacter *baumannii strain* YM2023, OR673716, *Staphylococcus aureus* strain YM2023, OR673718.

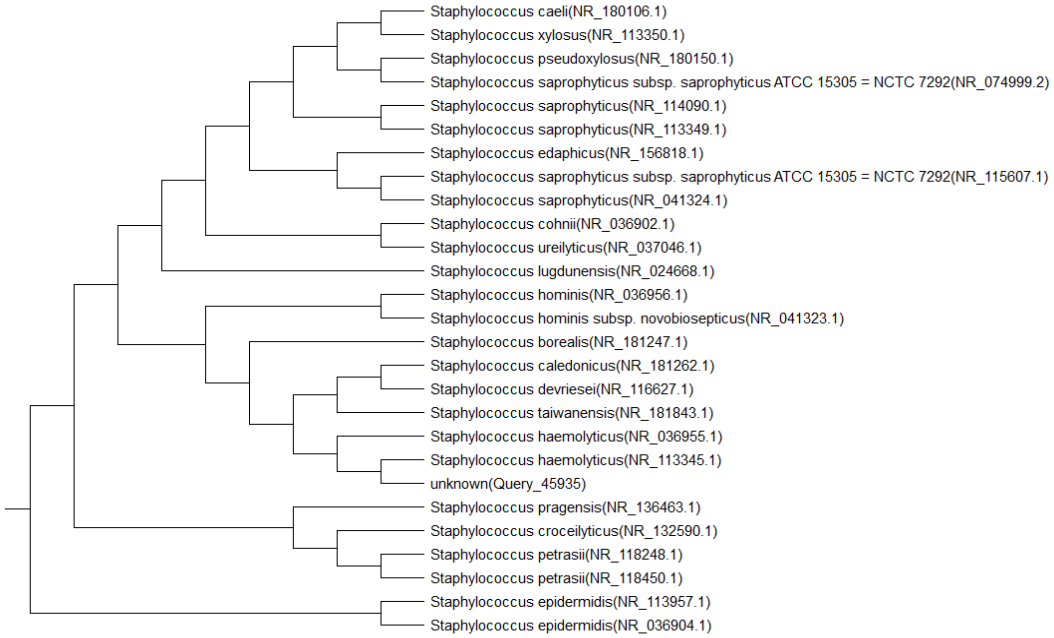

Figure 1:*Staphylococcus aureus* is phylogenetic .

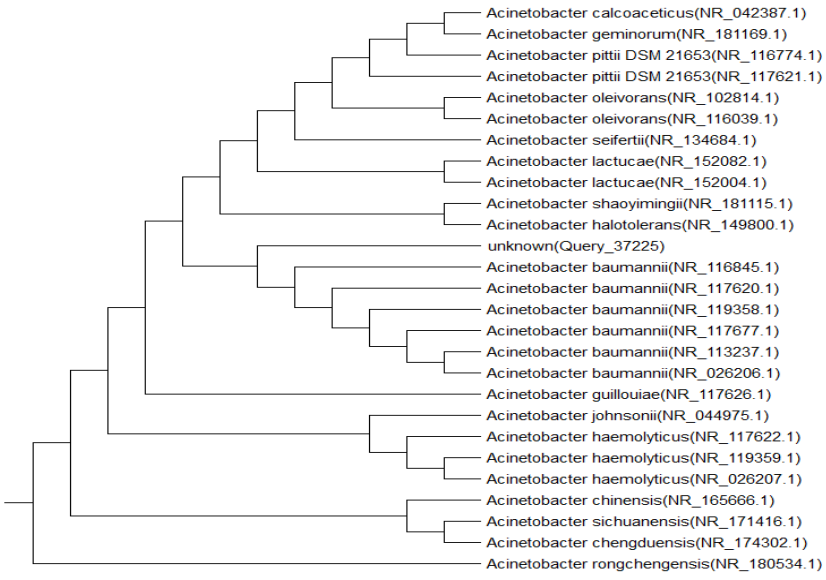

Figure 2: *Acinetobacter baumannii* phylogenetic tree.

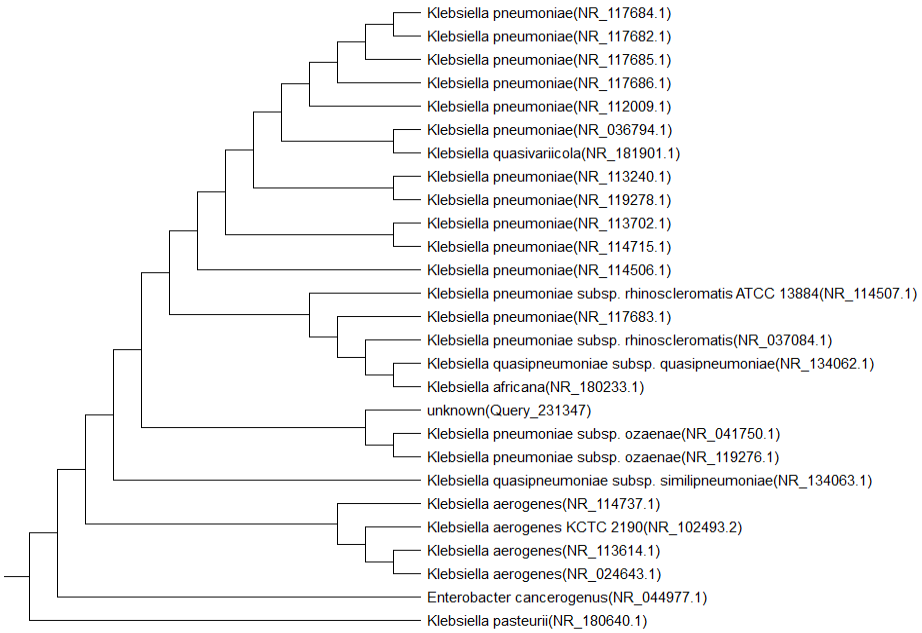

Figure 3: The phylogenetic of *Klebsiella pneumoniae* is shown in .

Table (2): The antibacterial effects of three harmful bacteria on medicinal plant extracts in an ethanolic format.

Plant extract	Mean diameter of inhition zone(mm), original diameter (5mm)		
	Staphylococcus aureus(1p)	***Acinetobacter baumannii complex(2S)***	***Klebsiella pneumonia (3U)***
Thymus vulgaris	27	30.3	20.6
Cinnamomum camphora	25.0	27.6	18.3

According to the data in the table, the ethanolic extract of *Thymus vulgaris* had a strong antibacterial effect against *Acinetobacter baumannii complex(2S)* (30.3 mm), *Staphylococcus aureus(1p)* (27 mm), *Klebsiella pneumonia (3U)* (20.6 mm). Then, the ethanolic extract of *Cinnamomum camphora* exhibited a similar level of effectiveness against the same bacteria, with a concentration of 27.6 mm, 25 mm, and 18.3 mm, respectively. A spray comprising essential oils of *Rosmarinus officinalis, Eucalyptus citriodora, Mentha x piperita, Origanum syriacum*, and *Eucalyptus globulus* was studied in a randomized, double-blind controlled experiment to determine its efficacy in treating urinary tract infections (URTIs).

Thirty-four patients in the study group sprayed themselves four times daily with this spray for three days. Then, patients' most distressing symptoms, such as a sore throat, hoarseness, or cough, were

evaluated for any changes. Twenty minutes after spray application, those in the experimental group reported more symptom relief than those in the control group. After three days of therapy, neither group's symptoms were significantly worse than the other. These findings led the authors to postulate that this spray's action on the upper respiratory tract is localized rather than systemic **(Ben-Arye et al.,2011).**

Table (3):Effects of varying amounts of *Thymus vulgaris* ethanolic extract on three distinct types of bacteria for antimicrobial activity. These values are the average of three measurements in millimeters.

Concentration of *Thymus vulgaris* extract / Bacterial species	The mean diameter of inhibition z(mm)				
	20	**40**	**60**	**80**	**100**
Staphylococcus aureus(1p)	19	20	22	25	27
Acinetobacter baumannii complex(2S)	18	19	22	25	29
Klebsiella pneumonia (3U)	18	19	20	21	21

The antibacterial effects of *Thymus vulgaris* at various concentrations

Table 3 shows that all concentrations (20, 40, 60, 80, 100 µg/ml) were very effective against the three harmful bacterial strains. The bacteriostatic activity against the bacteria tested was considerable for all the essential oils of *thyme* that were evaluated. Against gram-positive bacteria, this effect was more pronounced. When tested against the microbial species, the oil extracted from fully bloomed *thyme* proved the most efficient inhibitor. Additionally, direct contact testing revealed that the oils had potent antibacterial properties **(MARILENA et al., 1999).**

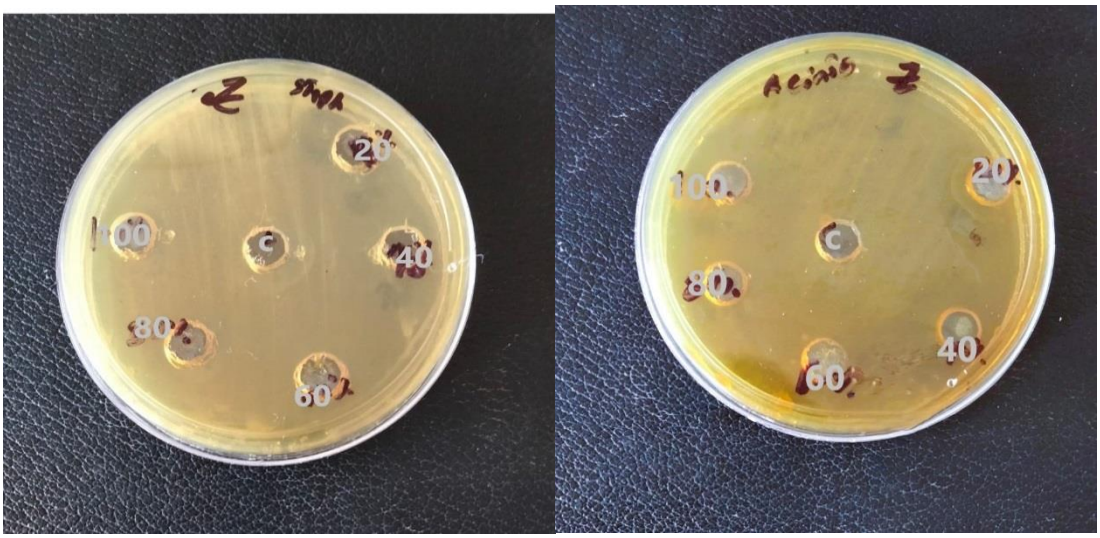

Figure 4: Antibacterial assay of *Thymus vulgaris* against *Staphylococcus aureus* and *Acinetobacter baumannii complex(2S)* at different concentrations (1=20, 2=40, 3=60, 4=80 and 5= 100 µg/ml).

Table(4):Evaluation of three bacterial species' susceptibility to antimicrobial effects of *Cinnamomum camphora* ethanolic extract at varying doses. These values are the average of three measurements in millimeters.

Concentration of *Thymus vulgaris* extract / Bacterial species	The mean diameter of inhibition z(mm)				
	20	**40**	**60**	**80**	**100**
Staphylococcus aureus(1p)	0	0	15	18	23
Acinetobacter baumannii complex(2S)	15	17	21	24	25
Klebsiella pneumonia (3U)	0	0	17	18	19

Cinnamomum camphora antimicrobial activity at various concentrations For *Acinetobacter baumannii complex (2S)*, all concentrations (20,40,60,80,100 µg/ml) had vigorous antibacterial activity. However, for *Staphylococcus aureus (1p) Klebsiella pneumonia (3U)*, only concentrations (60,80,100) demonstrated this activity, as shown in Table (4). The antibacterial activity inhibitory concentration (MIC) of C. camphora EO was investigated. The most sensitive bacteria to *C. camphor EO were P. aeruginosa and E. coli* ATCC25922, respectively. *C. camphor EO* inhibited formation swarming movement without influencing *C. violaceum* growth and significantly reduced violacein production biomass in *C. violaceum*, according to QS inhibitory activity tests (Balciunaitiene **et al.,2021)**. The maximum inhibition rates for these processes were 63% and 77.64%, respectively.

Table 5: Values of MIC for different ethanol extracts against. *staphylococcus aureus(1p), Acinetobacter baumannii complex (2S), Klebsiella pneumonia (3U)*.

Bacteria	*Thymus vulgaris* extract		*Cinnamomum camphora* extract	
	MIC (mg/ml)	MBC	MIC (mg/ml)	MBC
Staphylococcus aureus(1p)	**7.81**	**7.81**	**62.5**	**62.5**
Acinetobacter baumannii complex(2S)	**7.81**	**7.81**	**15.6**	**15.6**
Klebsiella pneumonia (3U)	**7.81**	**7.81**	**31.25**	**31.25**

As shown in Table (5), *Thymus Vulgaris* has antibacterial solid activity with MIC value against *Staphylococcus aureus(1p), Acinetobacter baumannii complex(2S), Klebsiella pneumonia (3U)* (7.81) but *Cinnamomum camphora* extracts show moderate antibacterial activity with MIC value against *Staphylococcus aureus(1p), Acinetobacter baumannii complex(2S) Klebsiella pneumonia (3U)* (62.5),(15.6), (31.25). **(Friedman et al.,2004)** have also found that cinnamon oil shows antibacterial action against numerous species.

Caffeic acid, gallic acid, and chlorogenic acid were the most abundant phenolics measured. A prior investigation of extracts from T. vulgaris also found that they had high concentrations of phenolic flavonoid components **(Friedman et al.,2004)**. Gas chromatography-mass spectrometry (GC-MS) revealed a multitude of terpene chemicals, among 23 other chemical components, in the essential oil of C. camphor leaves. With relative concentrations of 51.57% and 22.07%, eucalyptol was the predominant component, respectively. **(Wenting, et al.,2019).**

Table (6): GC/MS identifications of significant components of *Cinnamomum camphora* *Thymus vulgaris*

No.	List of identified components	Chemical structure	*Thymus Vulgaris*	*Cinnamomum camphora*	Known function
1	**cis-vaccenic acid**	**C18H34O2**	+	+	It acts as an antiviral essential fetal hemoglobin inducer.
2	**Otadecanoic acid**	**C18H34O2**	+	+	commercially in the preparation of oleates lotions as a pharmaceutical solvent
3	**cis-13-Eicosenoic acid**	**C20H38O2**	+	+	Used in research development, it has been observed to increase triglyceride accumulation in cells at specific concentrations.
4	**Erucic acid**	**C22H42O2**	+	+	Produce behenic acid, which is used in plasticizers, lubricants, and stabilizers in the plastic, pharmaceutical, and food industries.
5	**Oleic acid**	C18H34O2	+	+	Flavoring Agents
6	**13-Docosenoic acid**	C22H42O2	+	+	the preparation of surfactants, lubricants, plasticizers, softeners, waterproofing agents, detergents
7	**Isochlapin B**	**C19H22O6**	+	+	It was identified as one of the volatile organic compounds (VOCs) that decreased following surgery.
8	**thymol**	**C10H14O**	+	**nd**	Thymol is an active component in many home cleansers. It is also found in mouthwashes, acne treatments, insect repellents, and fungicides.
9	**Epiglobulol**	**C15H26O**	**nd**	+	significant components of essential oil, which possesses considerable contact toxicity against three stored-product insects

For components that were discovered, it means "nd."

4. Conclusion:

This report details the isolates of three human pathogenic bacteria from Egypt: *Klebsiella pneumoniae (3U), Acinetobacter baumannii complex (2S),* and *Staphylococcus aureus (1p)*. We synthesized an ethanolic extract of *Cinnamomum camphora Thymus vulgaris* to combat some clinical isolates resistant to many drugs. Both are considered suitable replacements for antibiotics in treating nosocomial pathogenic bacteria. Eight compounds, accounting for all of the substances in the inquiry, were discovered in the GC-MS analysis. The energy needed to transport from a deprotonated molecule is much lower than that of hydrogen, as seen in Table 6.

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