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Research article

GC–MS-Based Chemical Profiling and Antibacterial Activity of *Thymus vulgaris* L. Essential Oil Against Carbapenem-Resistant *Pseudomonas aeruginosa*

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ABSTRACT

Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) is a major therapeutic challenge because its biofilm contributes to antimicrobial tolerance. This study aims to determine the chemical composition and antibacterial effect of *Thymus vulgaris* L. essential oil against CRPA and to evaluate the relationship between bacterial response to carbapenem and biofilm formation. The essential oil was prepared from dried *T. vulgaris* flowers by steam distillation and characterized using gas chromatography-mass spectrometry (GC-MS). The isolates of *P. aeruginosa* were isolated from 312 burn-wound specimens and identified using biochemical methods and VITEK 2 technology. Kirby-Bauer method was used to test carbapenem susceptibility of bacterial isolates. Crystal violet microtiter-plate (polystyrene) assay was used to quantify biofilm formation. The microdilution assay was used to measure the minimum inhibitory concentration (MIC) of *T. vulgaris* essential oil against four CRPA isolates that produced the highest biofilm biomass. Forty-six compounds were identified by GC-MS. The main compounds were carvacrol (29.95%), thujanol/terpinen-4-ol-type alcohol (14.55%), γ-terpinene (8.15%), p-cymene (5.34%), and terpinen-4-ol (4.46%), indicating a carvacrol chemotype. Out of 55 isolates of *P. aeruginosa*, resistance rates were 25.5% for ertapenem, 16.4% for doripenem and meropenem, and 9.1% for imipenem. Ten isolates were CRPA, of which 70% exhibited moderate-to-strong biofilm formation. Imipenem susceptibility showed a significant inverse correlation with biofilm biomass ($r_s = -0.312$, $P = 0.0312$). *T. vulgaris* essential oil inhibited all four selected multidrug-resistant isolates, and the MICs ranged from 1/320 to 1/1280; the highest activity was observed against Pa25 (1/1280). *T. vulgaris* essential oil, which is rich in carvacrol, demonstrated antibacterial activity against all CRPA isolates and may warrant further investigation as a potential adjunctive antimicrobial strategy.

Keywords: Biofilm formation; Carbapenem-resistant *Pseudomonas aeruginosa;* GC-MS; Thymus vulgaris L. essential oil.

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1. INTRODUCTION

The resistant to antibiotics is one of the most serious threats to global public health. Multidrug-resistant Gram-negative bacteria pose a serious challenge to infectious disease management and treatment [1]. *Pseudomonas aeruginosa* is an important opportunistic pathogen responsible for different healthcare-associated infections, especially in patients with compromised host defenses, prolonged hospital stays, burns, and medical procedures [2]. This pathogen acquires resistance factors, making its treatment options difficult. Because of its natural resistance mechanisms, such as low outer-membrane permea-

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permeability, multidrug efflux systems and enzymes that deactivate antibiotics, carbapenems are among the most commonly used drugs to treat infections caused by multidrug-resistant *P. aeruginosa*. Nowadays, the emergence of carbapenem-resistant *P. aeruginosa* has made these infections harder to treat and has reduced treatment options and prognosis [3].

The increasing occurrence of carbapenem-resistant *P. aeruginosa* (CRPA) emphasizes the need to discover antimicrobial approaches that work against resistant organisms and can replace or supplement traditional antibiotics [4]. Natural materials, particularly medicinal and aromatic plants, are increasingly being studied as sources of antimicrobial substances. Essential oils consist of a complex of volatile secondary metabolites, mainly monoterpenes and sesquiterpenes, and cause antimicrobial effects by affecting bacterial membranes, such as changing membrane permeability, causing intracellular components to leak, and disturbing vital cellular activity. Their combined action can also help them act against resistant bacteria [5].

Thymus vulgaris L. (common thyme) is an aromatic plant and a member of the Lamiaceae family. It is recognized for its biologically active essential oil. Earlier studies have attributed the antimicrobial properties of thyme essential oil largely to phenolic monoterpenes and other volatile constituents, although its chemical composition can vary considerably with geographical origin, cultivation conditions, plant maturity, extraction procedures, and environmental factors [6]. Establishing the chemical fingerprint of a particular essential oil preparation is important for understanding and evaluating its biological activity and mechanical of action of this essential oil [7].

In spite of increased interest in the antimicrobial effect of *T. vulgaris* essential oil, studies addressing this essential oil's activity against clinically important CRPA are scarce in the literature, especially when chemical composition and antibacterial efficacy are evaluated together. Thus, the current study aimed to illustrate the volatile chemical constituents of *T. vulgaris* L. essential oil using gas chromatography–mass spectrometry (GC–MS). This will be done to check the antibacterial activity of *T. vulgaris* L. essential oil against CRPA isolates. The objective of this study is to clarify the antimicrobial potential of *T. vulgaris* essential oil of combining chemical profiling with phenotypic antibacterial assessment of the essential oil. This will help generate the scientific data needed for subsequent studies on plant-based compounds as alternative agents for tackling difficult-to-treat bacterial pathogens.

2. MATERIALS AND METHODS

2.1. Isolation of Essential Oils.

The standard method of Khalaf and Abed (2021) was followed to extract the essential oils from *Thymus vulgaris*. Flowering plant samples of *T. vulgaris* flowers were procured from the local market in Baghdad, Iraq. The plant materials were identified and authenticated by a plant taxonomist at the Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq. No voucher specimen was deposited. The flowers were cleaned, washed with sterile tap water, dried at room temperature and stored under clean conditions until use. The essential oils of these plants were prepared by drying the flowers. Two hundred fifty grams of dried flowers were distilled by steam distillation (Cleavenger) using DMSO (Dimethyl Sulfoxide, Fluke, UK). The prepared essential oils were dried at 45 °C and kept at 4º C until used [8].

2.2. Gas Chromatography-Mass Spectrometry

Samples were analyzed by gas chromatography using an HP6890 instrument coupled with an HP 5973 mass spectrometer. The gas chromatograph was equipped with a split-splitless injector and a Factor FourTM VF-35ms 5% phenyl-methylpolysiloxane capillary column (30 m, 0.25 mm, 0.25 µm film thickness). Gas chromatography conditions included a temperature range of 50 to 250°C at 40°C/min, with a solvent delay of 5 min. The injector was maintained at 250°C. The carrier gas was helium at a flow of 1.0 mL/min, and the injected volume in splitless mode was 1 µL. The MS conditions were as follows: ionization energy, 70 eV; quadrupole temperature, 100°C; scanning velocity, 1.6 scan/s; mass range, 40-500 amu. Calculation of the percentage composition of the volatile compounds has been done. The qualitative analysis was performed with the percentage area of each peak of the sample compound. The mass spectrum of every compound has been compared with the mass spectrum obtained from NIST 98 Mass Spectrum Library [9].

2.3. Bacterial Isolation and Identification

Wound swabs were collected from 312 inpatients with infected burn wounds at three main teaching hospitals in Baghdad, Iraq (Shahid Ghazi al Harery Surgical, Baghdad Teaching Hospital, and Al Kindi General Teaching Hospital). The specimens were collected in sterile containers. Patients were not allowed to receive antibiotic treatment within 3 days of sample collection [10]. All patients signed the consent form before sample collection. The ethical principles of the Declaration of Helsinki were followed during the collection of clinical specimens. The specimens were inoculated onto different culture media such as MacConkey agar (HiMedia, Mumbai, India) and Cetrimide Agar (HiMedia) and incubated for 18 h at 37°C. Biochemical tests were performed (oxidase, catalase, and Citrate utilization tests). The VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) with the ID-GNB card was used for identification [10].

2.4. Kirby-Bauer Method

To check the level of resistance to imipenem (IPM, 10 µg, HiMedia, India), meropenem (MEM, 10 µg, HiMedia, India), doripenem (DOR, 10 µg, HiMedia, India) and ertapenem (ETP, 10 µg, HiMedia, India) in *P. aeruginosa*, the Kirby-Bauer agar disk diffusion method was utilized. The overnight grown culture of bacteria in Mueller-Hinton broth (MHB) was washed twice in phosphate buffered saline (0.1 M; pH 7.2) and adjusted to 0.5 McFarland standard for its inoculation on Mueller-Hinton agar (MHA), corresponding to 10^8 CFU/mL. After placing the antibiotics discs on the surface, plates were incubated at 37ºC for 24 h. The zones of inhibition were recorded manually in mm. Zones of inhibition were characterized in total diameter (including 6 mm diameter of disk) as per the CLSI guidelines. The isolates were further classified as susceptible (S), intermediate (I) or resistant (R) according to CLSI breakpoints [11].

2.5. MICs of *T. vulgaris* Essential Oil

In this experiment, the broth microdilution method was used to measure the MICs of *T. vulgaris* essential oil against CRPA. Double-fold serial dilutions of *T. vulgaris* essential oil were prepared in Mueller–Hinton broth (MHB, HiMedia, India), in a U-shaped microtiter plate. Five microliters of standard bacterial inoculum were added to each well. The bacterial suspension was prepared by washing the overnight growth of CRPA with

sterile normal saline and centrifuging at 6,500 g for 10 min (Microfuge, Eppendorf, Germany). The optical density (OD) of the bacterial suspension was adjusted to 0.2 at 600 nm (OD_{600}) (corresponding to 10^8 CFU/mL) using a UV–Vis spectrophotometer (BioSpectrometer, Eppendorf, Hamburg, Germany). The final bacterial count in each well was 4.76 x 10^6 CFU/mL. After gentle shaking, the plates were incubated at 37 °C for 18 h, and MICs were determined by measuring the lowest dilution of essential oil that completely inhibited visible bacterial growth. Three controls were used. First control: MHB and bacterial isolate; second control: wells of MHB only; and third control: double dilutions of essential oil only [12].

2.6. Biofilm Formation Assay

The biofilm formation of isolates of *P. aeruginosa* was quantified using a crystal violet microtiter plate method run on polystyrene 96-well plates (flat bottom). In short, 100 µL of Tryptic Soy Broth (0.25 g/L glucose, HiMedia, India) with 5 µL of test culture (0.5 McFarland) was held at 37°C for 24 hours. The wells were rinsed, fixed at 65°C for 60 minutes, treated with 0.4% crystal violet for 20 minutes, rinsed, and decolorized with anhydrous ethanol. Absorbance at 590 nm (OD590) was determined using a microplate reader (BioTek 800 TS, Winooski, USA). Each test was run in triplicate, and the classification of biofilm was done on the basis of cutoff OD values [10].

2.7. Statistical and data analysis

Data analysis was conducted using SPSS Version 21 (IBM Corp., NY). A t-test was used to assess group differences. Data were expressed as mean ± standard deviation (SD). The type of microorganism and the amount of essential oil were used as factors in the full factorial model. Post hoc tests for each amount of essential oil were conducted using Tukey's HSD method to compare effects across different types of microorganisms.

3. RESULTS

3.1. Chemical Profile of *T. vulgaris* Essential Oil

GC-MS analysis of *T. vulgaris* essential oil showed a carvacrol-type profile. The most abundant compounds (>2% area percentages) included carvacrol (29.95%), thujanol/terpinen-4-ol type bicyclic monoterpene alcohol (14.55%), γ-terpinene (8.15%), p-cymene (5.34%), a second thujanol-type alcohol (4.46%), another thujanol isomer (4.04%), α-pinene (3.31%) and α-terpineol (2.90%). The essential oil also contained caryophyllene (1.90%) as a dominant sesquiterpene. As such, being extremely rich in carvacrol as well as its direct biosynthetic precursors γ-terpinene and p-cymene, the oil could be classified as a carvacrol chemotype of *T. vulgaris*.

A total of 46 compounds were detected using gas chromatography-mass spectrometry covering the 5–63 min period (Table A1, supplement file 1). The chromatogram had a clean graph consisting of three main peaks at Rt 33.02 min (carvacrol, 29.95%), Rt 22.13 min (some kind of alcohol thujanol or terpinen-4-ol, 14.55%) and Rt 19.54 min (γ-terpinene, 8.15%), which made up the greater part of the total area of their peaks. A number of monoterpene hydrocarbons (α-pinene, sabinene/thujene isomers, terpinen-4-ol, α-terpineol) appeared between 13 and 31 min, while the few sesquiterpene compounds caryophyllene and aromadendrene appeared from 36 minutes on. A few late-eluting peaks after 43 min (oleic acid, methyl linoleate, 1-octadecene) belonged to co-extracted lipid compounds rather than to terpenes. Some of the early peaks (Rt < 8.5 min: cyclohexane, heptane, octane; Rt 15.23 min: tetraethyl silicate) were actually leftover solvent residues as well as column background (not the constituents of essential oil) and thus, not taken into account in the analysis.

Table 1. Full chromatography-mass spectrometry (GC-MS) peak list for the essential oil of *T. vulgaris*, ranked by elution order (retention time). Compound names correspond to the top NIST/Wiley (W10N14.L) library match for each peak; asterisked entries are considered solvent/background artefacts rather than oil constituents.

Peak	Rt (min)	Area (%)	Tentative identification	CAS No.
1	5.339	0.94	Cyclohexane	110-82-7
2	5.790	0.17	Heptane	142-82-5
3	6.396	0.59	Methylcyclohexane	108-87-2
4	7.231	0.45	2-Methylheptane	592-27-8
5	7.619	1.36	Toluene	108-88-3
6	7.802	0.48	cis-1,3-Dimethylcyclohexane	638-04-0
7	8.214	0.64	Octane	111-65-9
8	11.214	1.24	m-Xylene	1330-20-7
9	11.946	0.21	o-Xylene	95-47-6
10	13.277	2.17	alpha-Phellandrene	1529-99-3
11	13.643	0.85	alpha-Pinene (2-Pinene)	80-56-8
12	15.015	0.57	1-Ethyl-3-methylbenzene	620-14-4
13	15.232	0.50	Tetraethyl silicate *	78-10-4
14	15.444	0.93	4(10)-Thujene (sabinene-type)	3387-41-5
15	16.078	1.22	beta-Myrcene	123-35-3
16	16.627	0.98	1,2,4-Trimethylbenzene	95-63-6
17	17.530	0.36	alpha-Terpinolene-type (cyclohexene deriv.)	586-62-9
18	17.987	5.34	p-Cymene	99-87-6
19	18.273	1.69	2-Thujene	28634-89-1
20	19.536	8.15	gamma-Terpinene	99-85-4
21	20.507	4.04	Terpinen-4-ol isomer (thujanol)	17699-16-0
22	21.896	0.31	Linalool	78-70-6
23	22.130	14.55	Terpinen-4-ol / thujanol-type alcohol	17699-16-0
24	22.816	0.35	1,2,4,5-Tetramethylbenzene	95-93-2
25	23.245	0.41	1-Methyl-4-isopropyl-2-cyclohexen-1-ol	29803-82-5
26	24.188	0.42	4-Isopropyl-1-methyl-3-cyclohexen-1-ol	586-82-3
27	25.960	4.46	Terpinen-4-ol	562-74-3
28	26.857	2.90	alpha-Terpineol	98-55-5
29	28.583	0.30	Linalyl acetate	115-95-7
30	28.720	0.27	Cyclofenchene	488-97-1
31	28.880	0.39	Carvone-type cyclohexenone	99-49-0
32	30.406	0.56	Bornyl acetate	5655-61-8
33	30.961	3.31	alpha-Pinene (isomer)/tricyclene-type	80-56-8
34	31.795	0.88	Isopropyl-methyl dioxatricyclooctane	1619-26-7
35	32.601	0.64	Thymol	89-83-8
36	33.024	29.95	Carvacrol	499-75-2
37	36.744	1.90	trans-Caryophyllene (beta-Caryophyllene)	87-44-5
38	37.556	0.77	Aromadendrene	489-39-4
39	43.723	0.45	Oleic acid	112-80-1
40	46.209	0.61	Bicyclic sesquiterpene alcohol	13744-15-5
41	46.712	0.44	ar-Turmerone	532-65-0
42	50.210	0.40	1-Octadecene	112-88-9
43	53.450	0.21	Sesquiterpenoid oxirane deriv.	2000804-23-8
44	54.833	0.63	2-(2-Butenyl)-3-methyl-2-cyclopenten-1-one	17190-71-5
45	55.651	1.49	(E)-2,6-Dimethylocta-2,5,7-trien-4-one	33746-72-4
46	62.858	0.54	Methyl linoleate (9,12-octadecadienoate)	2462-85-3

3.2. Antibiotic Susceptibility Profile

Among the total of fifty-five clinical isolates of *P. aeruginosa* tested for their susceptibility to four carbapenems: doripenem (DOR), meropenem (MER), imipenem (IMP), and ertapenem

(ETP), the detected rates of resistance were 16.4% (9/55); 16.4% (9/55); 9.1% (5/55); and 25.5% (14/55) for doripenem, meropenem, imipenem, and ertapenem, in respectively (figure 1). Among the tested biofilm-forming isolates, ten isolates (18.2%) showed resistance to at least one of the carbapenems (doripenem, meropenem, imipenem, and ertapenem): Pa1, Pa2, Pa8, Pa11, Pa25, Pa37, Pa38, Pa39, Pa40, Pa48. Five of these strains showed resistance to all carbapenems (Pa25, Pa37, Pa38, Pa39, and Pa48). For two isolates, only single carbapenem resistance was observed (Pa8 against doripenem; Pa11 against meropenem).

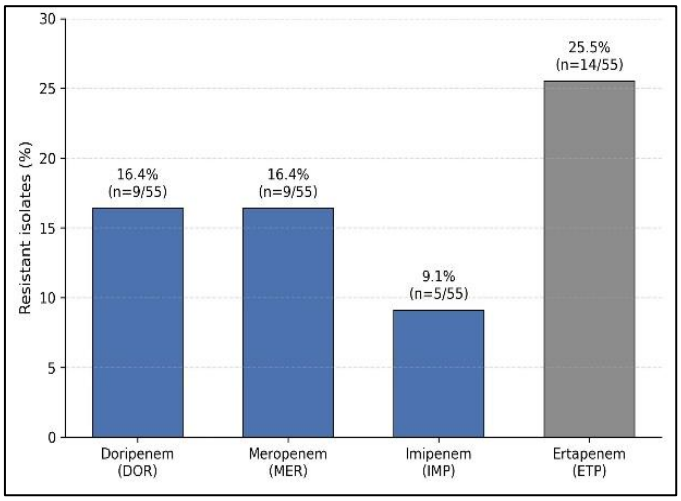


Fig. 1. Antibiotic resistance rates of fifty-five isolates of *P. aeruginosa* for doripenem (DOR), meropenem (MER), imipenem (IMP), and ertapenem (ETP),

3.3. CRPA and Biofilm Formation

Among ten CRPA isolates, biofilm phenotyping showed that three isolates were strong biofilm producers (30.0%), four were moderate biofilm producers (40.0%), two were weak biofilm producers (20.0%), and one was a non-biofilm producer. The results showed that 70% of carbapenem-resistant isolates were moderate to strong biofilm producers (Fig. 2).

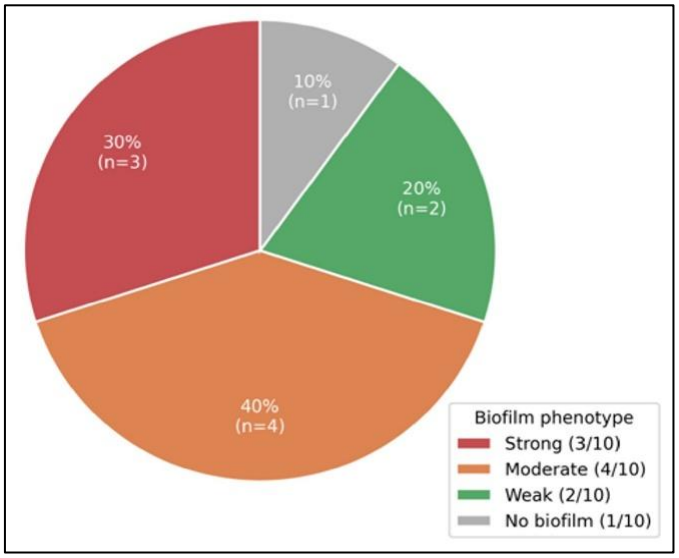


Fig. 2. Biofilm phenotype distribution among the 10 isolates of carbapenem-resistant *P. aeruginosa* (CRPA).

Quantitative biofilm biomass (mean of OD_{590nm} − blank) for each of the carbapenem-resistant isolates, as well as their susceptibility to carbapenems, is presented in Table 2 and in Figure 3.

Table 2. Carbapenem antibiotic resistance [R: resistant, numerical values of inhibition zone diameter in millimeters (mm)] and biofilm formation biomass of 10 clinical isolates of carbapenem-resistant *P. aeruginosa* (CRPA).

Isolate	DOR	MER	IMP	Biofilm (OD)	Category
Pa1	R	R	12	0.3192	Moderate
Pa2	R	R	14	1.0046	Strong
Pa11	R	R	18	0.6396	Strong
Pa8	R	26	48	0.1810	Weak
Pa39	R	R	R	0.0238	No biofilm
Pa38	R	R	R	0.3896	Moderate
Pa25	R	R	R	0.4554	Moderate
Pa40	R	R	9	0.2946	Moderate
Pa48	R	R	R	0.2144	Weak
Pa37	R	R	R	0.7552	Strong

Biofilm biomass varied from 0.0238 (Pa39, resistant to carbapenems but unable to produce biofilms) to 1.0046 (Pa2, strong biofilm producer). The average biofilm biomass in carbapenem-resistant isolates was 0.428, while that in non-resistant isolates was measured at 0.272, with no statistically significant difference (Mann-Whitney U = 295, p = 0.129).

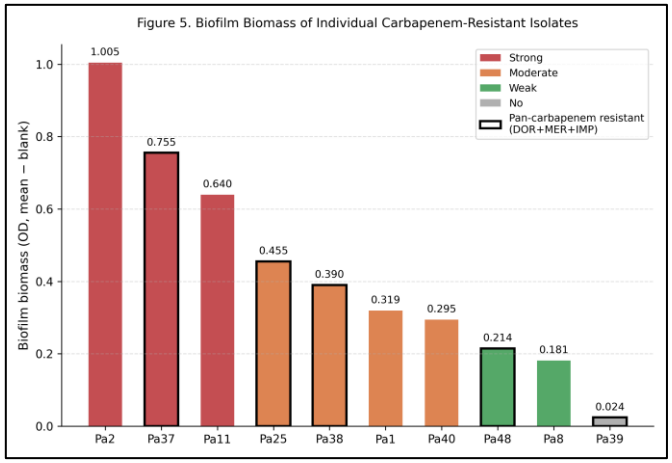


Fig. 3. Biofilm formation of 10 CRPA isolates, ranked from highest to lowest and color-coded by biofilm category. Isolates whose columns are outlined in black were resistant to all carbapenems.

3.4. Correlation between Antibiotic Response and Biofilm Formation

3.4.1. Continuous Inhibition-Zone correlation

In the current study, Spearman correlation between inhibition-zone diameter (continuous variable, isolates with numerical zones only) and quantitative biofilm formation demonstrated a significant correlation for imipenem (rs: -0.312, P: 0.0312, n: 48): smaller imipenem inhibition zones (such as decreased susceptibility) were associated with greater biofilm biomass. The results showed that doripenem showed a similar finding but a non-significant negative correlation (r_s: -0.261, P: 0.087, n: 44), as did meropenem (r_s:-0.192, P: 0.211, n: 44). Ertapenem showed no relationship with biofilm formation (r_s:+0.066, P: 0.75, n: 25) (Figure 4).

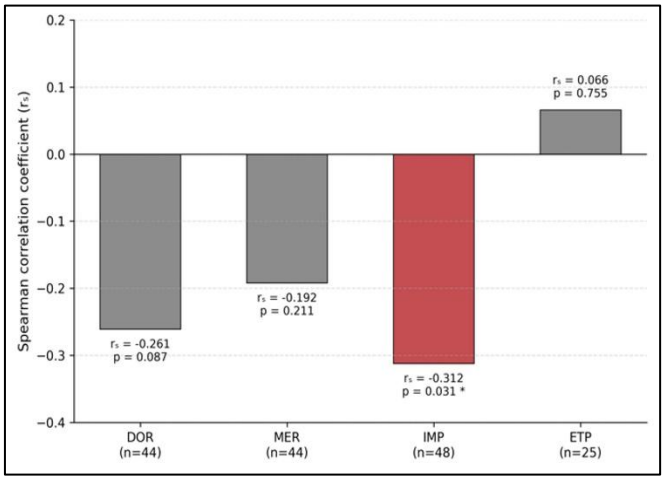


Fig 4. Correlation (Spearman correlation coefficient) between inhibition-zone diameter and biofilm formation for carbapenems [doripenem (DOR), meropenem (MER), imipenem (IMP), and ertapenem (ETP)]. Only IMP showed statistical significance (P<0.05).

3.4.2. Biofilm of resistant and non-resistant CRPA

When groups of isolates were identified according to their resistance classification, resistant isolates had an overall higher mean biofilm biomass than non-resistant isolates for all four antibiotics, but none of these groups showed a significant difference from each other (Figure 5, Table 3). The biggest difference was found with meropenem (0.455 vs. 0.270; +69%; p = 0.078), which was not significant.

Table 3. Mean biofilm formation in resistant vs. non-resistant isolates of carbapenem-resistant *P. aeruginosa* (CRPA) for each of the four carbapenem antibiotics.

Antibiotic	R isolates	Mean biofilm (R)	Mean biofilm (non-R)	*p*-value
DOR	9/55 (16.4%)	0.404	0.280	0.290
MER	9/55 (16.4%)	0.455	0.270	0.078
IMP	5/55 (9.1%)	0.368	0.293	0.540
ETP	14/55 (25.5%)	0.405	0.264	0.156

3.5. Antibacterial Activity of T. vulgaris Essential Oil Against CRPA

The minimum inhibitory concentration (MIC) of *T. vulgaris* essential oil was determined for four isolates of CRPA that displayed resistance to all carbapenems and had the greatest biofilm-forming ability compared with other isolated single biofilm producers. *T. vulgaris* oil was found to have the ability to inhibit growth of all four isolates at dilution values of 1/320 – 1/1280, with the lowest MIC (highest efficacy value of 1/1280) being recorded for the strain Pa25 (Table 4).

4. DISCUSSION

Carbapenem resistance has increased in recent years, making the management of bacterial infectious diseases, especially in immunocompromised patients, a significant challenge for physicians [13]. Thus, the establishment of alternative strategies that depend on the use of safe plant extracts is highly recommended. The current study provided details about the chemical nature of *T. vulgaris* essential oil and its potential to fight against carbapenem-resistant *P. aeruginosa*, which has biofilm-forming ability.

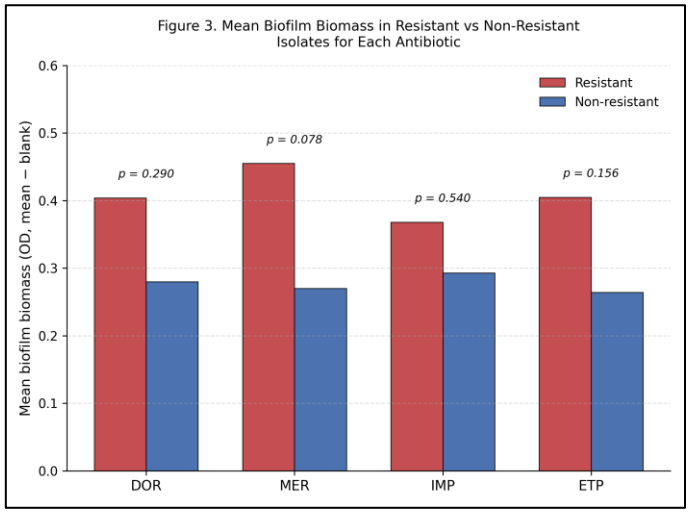


Fig 5. Biofilm biomass of resistant vs. non-resistant isolates of carbapenem-resistant *P. aeruginosa* (CRPA) for each of the four carbapenem antibiotics, with corresponding *p*-values.

In the present study, GC-MS analysis confirmed that *T. vulgaris* oil has a chemotype characterized by the dominance of carvacrol, found alongside its precursor compounds, γ-terpinene and p-cymene, as well as the important compound thujanol/terpinen-4-ol. This proves to be entirely in agreement with the established variability of *T. vulgaris* oil composition based on provenance and conditions of cultivation as numerous chemotypes of *T. vulgaris* essential oil are known, from carvacrol to thymol, p-cymene or camphene based on the population that served as a source. For example, carvacrol was reported at 78.83% and other studies found p-cymene or thymol to be the leading compound [14]. The effect of carvacrol in dealing with bacteria is usually associated with its hydrophobic nature that imparts the ability to integrate with the lipid bilayer of a bacterial membrane and disrupt its functioning by causing the damage to porins and efflux systems related to resistance to antimicrobial substances [14]. The previous study highlighted the effects of thymol and carvacrol on *P. aeruginosa*. Both compounds were shown to destroy its cells and affect the permeability of the inner and outer membranes [15].

Table 4. Minimum inhibitory concentration (MIC) of *T. vulgaris* essential oil against four of carbapenem-resistant *P. aeruginosa* (CRPA).

No.	Isolate	MIC of *T. vulgaris* essential oil
1	Pa39	1/320
2	Pa38	1/640
3	Pa25	1/1280
4	Pa37	1/320

The resistance to carbapenems in the present study (9.1-25.5%) is in agreement with what has already been accepted in the literature about the mechanism of carbapenem resistance in *P. aeruginosa*. Imipenem resistance has been directly linked to either the loss of or decreased levels of *OprD* gene, the specific

porin that enables the entry of carbapenems into the periplasm, which may be further complicated by the actions of AmpC β-lactamase [16, 17]. In contrast, breakthrough in resistance against meropenem and doripenem happens through their overproduction of the efflux pump in addition to the loss of the porins [18]. The findings of several studies relying on Western blotting indicate that most strains resistant to imipenem and meropenem have no OprD at all, thus confirming that loss of porin is the primary mechanism of resistance to the drugs in the class [16]. The high level of resistance to ertapenem in this study is somewhat expected because, unlike group 2 carbapenems (imipenem, meropenem, doripenem), it shows very weak activity against *Pseudomonas* because it is subject to efflux and specific permeation barriers [19]. This may explain the lack of correlation between ertapenem resistance and biofilm formation.

Here, the correlation between biofilm formation and carbapenem resistance, although proven significant in the case of imipenem only, reflects conflicting evidence in the literature: some works indeed report significant associations between biofilm production and imipenem resistance [20], while some others that used correlation analysis across resistant phenotypes found weak, non-significant correlation [21]; a pattern of significance that is similar to what was observed in the data used for the present analysis. This inconsistency indicates that the development of biofilm and the resistance to carbapenems are relevant but distinct processes. There differences may be attributed to genetic varability among isolates, differential regulation of quorum-sensing systems, extracellular polymeric substance production, and variations in resistance mechanisms, including OprD proein experession, efflux pump activity, and beta lactamase production, which may independently influence there phenotypes [22, 23]

The continued effectiveness of *T. vulgaris* oil in preventing biofilm formation of highly biofilm-producing isolates resistant to carbapenems could be attributed to carvacrol's ability to alter quorum-sensing-regulated biofilm structure in *P. aeruginosa* by interacting with the membrane and receptors [22]. Still, the impact of carvacrol is dependent on the strain as one of the studies showed that while carvacrol was able to prevent biofilm formation in some pathogen types, it was ineffective against one strain of *P. aeruginosa* [24]. This means that the antimicrobial effectiveness of carvacrol in *P. aeruginosa* needs to be investigated because of the available reports indicating that the anti-biofilm activity of the oil was successful in combination with carbapenems, such as imipenem, in relation to other strains resistant to carbapenems [14].

There are several limitations in the design of this study. It used only four CRPA isolates in the antibacterial tests, limiting the generalizability of the results. Moreover, the in vitro conditions used in the study do not replicate clinical situations, and the mechanisms responsible for the observed effects were not validated experimentally. Furthermore, the study used only one batch of essential oil, and its individual components were not tested. The future project will be designed to overcome all limitations raised in the current study.

5. CONCLUSION

Based on the results of this study, the strains of *P. aeruginosa* resistant to carbapenems had a significant biofilm formation ability, although the difference between susceptible and resistant strains was not statistically meaningful. A significant inverse correlation was also found between susceptibility to imipenem and biofilm biomass, suggesting a possible relationship between decreased susceptibility and increased biofilm-forming ability. The essential oils of *T. vulgaris* with carvacrol-rich chemotype exhibited antibacterial effects on very resistant strains of CRPA with MIC ranging from 1/320 to 1/1280. These findings of this study support further study of *T. vulgaris* as a potential additional antibacterial agent.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

The current study was conducted following approval from the Ministry of Health, Baghdad, Iraq (Reference number 1502, Date: 22, 10, 2025).

AI Declaration

During the preparation of this work, the authors used Claude AI and Gemini Pro to refine the academic tone and improve the readability of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Data availability

Data will be made available on request.

6. REFERENCES

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