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Investigation of Antibacterial and Anti-biofilm Activity of *Thymbra spicata* Essential Oil on Multidrug- Resistant *Pseudomonas aeruginosa* Strains

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Abstract. *Thymbra spicata* has been used traditionally as herbal tea for the treatment of some diseases such as asthma, bronchitis, respiratory, coughs, and sore throat infection in Anatolia. In the current study, the essential oil of *T. spicata* (EOTS) were investigated for their antimicrobial, anti-biofilm activity and chemical composition. The chemical composition of *Thymbra spicata* was analyzed by gas chromatography-mass spectrometry (GC-MS). Antimicrobial and anti-biofilm properties were determined using broth microdilution methods. According to the results of the present study, the GC-MS profile highlights that the mains compounds were found grandisol 21.99%, cadinol 6.45%, lavandulol acetate 4.39%. The antibacterial activity of the EOTS was evaluated by the micro-dilution method against multidrug resistant *Pseudomonas aeruginosa*. EOTS produced inhibitory effects against the tested strains. It inhibited 11 clinical isolates of against *P. aeruginosa* with Minimum Inhibitory Concentration (MIC) values in the range of 1.56-12.5 µl/mL. Minimum Biofilm Inhibitory Concentration (MBIC) value was found to be range of 3.12-25 (µl/mL) while the Minimum Biofilm Eradication Concentration (MBEC) value was found to be 6.25-25 (µl/mL). MIC value of the EOTS was applied onto the bacterial biofilm formations. It was seen that biofilm metabolism was reduced by 2.0-51.6 %. The findings of this study show that EOTS has antibacterial and anti-biofilm activity against *P. aeruginosa* isolates. Thus, essential oil of *T. spicata* may be useful a potential source for the treatment of multidrug resistant *P. aeruginosa* infections and biofilms.

Keywords**:** *Thymbra spicata, Pseudomonas aeruginosa,* Antibacterial activity, Anti-biofilm activity.

Thymbra spicata Esansiyel Yağının Çok İlaç Dirençli *Pseudomonas aeruginosa* Suşlarına Antibakteriyel ve Antibiyofilm Etkisinin Araştırılması

Özet. *Thymbra spicata* Anadolu'da astım, bronşit, solunum, öksürük ve boğaz ağrısı enfeksiyonu gibi bazı hastalıkların tedavisi için geleneksel olarak bitki çayı olarak kullanılmaktadır. Bu çalışmada, *T. spicata* esansiyel yağının (EOTS) antimikrobiyal, anti-biyofilm aktivitesi ve kimyasal bileşimi araştırılmıştır. *T. spicata*' nın kimyasal bileşimi, gaz kromatografisi-kütle spektrometresi (GC-MS) ile analiz edilmiştir. Antimikrobiyal ve anti-biyofilm özellikleri, broth mikro-dilüsyon yöntemleri kullanılarak belirlenmiştir. Bu çalışmanın sonuçlarına göre, GC-MS profili, ana bileşiklerin % 21,99 grandisol,% 6.45 cadinol,% 4.39 lavandulol asetat olduğu görülmektedir. EOTS antibakteriyel aktivitesi, çoklu ilaç dirençli *P. aeruginosa*'ya karşı mikro-dilüsyon yöntemi ile değerlendirilmiştir. EOTS'un test edilen suşlara karşı inhibitör etkiler ürettiği görülmektedir. Esansiyel yağ, 11 adet *P. aeruginosa* klinik izolatı, 1.56-12.5 µl / mL aralığında Minimum inhibitör Konsantrasyon (MIC) değerleri ile inhibe etmiştir. Minimum Biyofilm İnhibitör Konsantrasyon (MBIC) değeri 3.12-25 (µl / mL), Minimum Biyofilm Eradikasyon Konsantrasyonu (MBEC) değeri ise 6.25-25 (µl / mL) olarak bulunmuştur. EOTS' nin MIC değeri bakteri biyofilm oluşumlarına uygulanmıştır. Biyofilm metabolizmasının % 2.0-51.6 arasında azaldığı görülmüştür. Bu çalışmanın bulguları, EOTS'un *P. aeruginosa*

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izolatlarına karşı antibakteriyel ve anti-biyofilm aktivitesi olduğunu göstermektedir. Bu nedenle, *T. spicata* esansiyel yağı çok ilaca dirençli *P. aeruginosa* enfeksiyonları ve biyofilmlerin tedavisi için kullanılabilir potansiyel bir maddedir.

***Anahtar Kelimeler*:** *Thymbra spicata*, *Pseudomonas aeruginosa*, Antibakteriyel aktivite, Antibiyofilm aktivite.

1. INTRODUCTION

Pathogenic microorganisms pose a great risk in terms of environment and public health due to the infections they cause. Also, the resistance mechanisms developed by these microorganisms against antimicrobial agents cause important problems in fighting against infections. One of the factors involved in the formation of antimicrobial resistance is the polymeric matrix structures called biofilm, which the microorganisms create by adsorbing to the biotic and abiotic surfaces that let them survive in various environmental conditions [1].

The formation of biofilm occurs in three stages, namely adsorbent, development, and spread. Microorganisms adsorbed owing to their adhesive matrix molecules, fibrinogen, fibronectin, and collagen structures allow the biofilm to mature through cell division and extracellular polymeric matrix production. Although the composition of the biofilm matrix varies among strains, it usually consists of host factors, polysaccharide, protein and extracellular DNA [2].

Implanted medical devices and equipment help to solve important problems in the modern health field, however, the microorganisms developing owing to the biofilm formation on their surfaces significantly affect the morbidity and mortality of the patients. The sessile microorganisms within the biofilm are reported to be much more resistant to antimicrobial agents owing to the biofilm matrix and its phenotypic characteristics. Biofilm structures are a continuous source of infection and cause cross-contamination. Therefore, it is important to understand the environmental conditions and mechanisms controlling biofilm formation in order to reduce the microbiological risk [3-4].

Pseudomonas aeruginosa is an opportunistic pathogenic bacterium causing biofilm-associated chronic infections. Depending on the formation of biofilm, the sensitivity of the cells adsorbed to the polymer-based matrix against antimicrobials and host immune defenses decreases. Also, the cells which spread around by detaching from the biofilm population cause an increase in infection and make the treatment difficult. Increasing resistance problem against antimicrobials and biofilm structures produced by microorganisms raised the importance of studies for finding new therapeutic agents. In this respect, plant extracts and the effects of their components on resistant microorganisms and their biofilm structures draw the attention of many researchers [5].

Thymbra spicata L. (Lamiaceae), which is known as “kekik” in Turkey. *T. spicata*, grow naturally in Turkey's flora. [6]. *T. spicata* has been used as the herbal tea for the treatment of some diseases such as asthma, colic, bronchitis, respiratory, coughs. and sore throat infection. It is also used as a spice in the food industry for flavoring. Previous studies have reported that essential oils of *T. spicata* and major components such as carvacrol and thymol show significant antimicrobial and antioxidant activities. [7-8].

The aim of this study was to determine antimicrobial and anti-biofilm activity of essential oil of *T. spicata* against multidrug resistant strains of *P. aeruginosa*. The results obtained are considered to contribute to the literature and the studies for developing alternative agents against the microbial resistance problem which increases every passing day.

2. MATERIALS AND METHODS

2.1. Extraction of essential oil of plant material

T. spicata aerial parts were collected in June 2017 in Manisa City, Akhisar and Gördes counties flora in the Aegean Region of Turkey. The samples of plant dried in the laboratory. *T. spicata* was authenticated and identified by a qualified botanist in Süleyman Demirel University, in Isparta. The essential oil of *T. spicata* was extracted by hydrodistillation method using the Clevenger apparatus after the aerial parts of *T. spicata* were ground.

2.2. Microorganisms

Pseudomonas aeruginosa strains were isolated and identified in the Microbiology Laboratory of Cumhuriyet University Practice and Research Hospital between 2016 and 2017. *Pseudomonas aeruginosa* isolates were identified using the matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) automated microbiology system.

2.3. Analysis and identification of *T. spicata* leaf extract

Chemical composition of essential oil of *T. spicata* (EOTS) was determined according to the method applied by Aksit et al. [9]. GC-MS analyzes were performed on a Perkin-Elmer Clarus 500 Series GC system in 50: 1 split mode equipped with a flame ionization detector (FID) and a BPX-5 apolar capillary column equipped with a mass spectrometer. The injector and FID were operated at 250 ° C. Standard ingredients were present for most of the essential oil components and Kovats retention indices (RIs) were determined for all components. The relative peak area percentages of the compounds were calculated based on FID data.

2.4. Evaluation of antibacterial activity

The broth microdilution method used to determine the antibacterial activity of EOTS was done in line with the CLSI recommendation [10]. This method was used to evaluate the minimum inhibitory concentration (MIC) of EOTS against multi-drug resistant *P. aeruginosa* strains.

The suspensions of the bacteria were adjusted to the standard turbidity of 0.5 McFarland. The essential oil was dissolved in nutrient broth (NB) containing Tween 20 (0.5 %). Serial two-fold dilutions were prepared with NB at a concentrations range of 50-1.56 µl/mL. After incubation at 37 ° C for 24 hours, a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA) was used to measure the optical density (OD) of the wells at 570 nm. At the end of incubation, the plate's absorbance remains the same or decreases in the MIC determined as the lowest concentration of EOTS compared to the initial reading.

2.5. Evaluation of anti-biofilm activity

2.5.1. Biofilm inhibitory assay

The minimum biofilm inhibitory concentration (MBIC) is called the minimum concentration at which agents are protected from microbial biofilm [11]. The microtiter plate method was used in order to determine the anti-biofilm effect of EOTS against *P.aeruginosa* [12]. Overnight cultures of *P.aeruginosa* strains in the TSB (Tryptic Soy Broth) medium with the containing of 2 % (w/v) glucose were prepared as 10^8 CFU/ mL and then dispensed 100 µL into with per each test well. Then 100 µL of different EOTS (100-1.56 µL / mL) concentration was distributed to each well. Positive control includes cell cultures without EOTS addition when the negative control contains only TSB. The supernatant was poured off and each well was rinsed three times with Phosphate-buffered saline (PBS) after incubation for 24 h at 37 °C. The plates were dried at room temperature during 30-40 min. The wells were stained with crystal violet 0.1% (w/v) for 15 minutes and washed with distilled water. The crystal violet in the wells were then dissolved in 95% ethanol and the OD of wells was read in a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA) at 570 nm. The MBIC of EOTS was determined as where the absorbance was less to or equal than the negative control. The test was performed in

triplicate and was taken as an average of three readings.

2.5.2. Biofilm eradication assay

The minimum biofilm eradication concentration (MBEC) is the minimum concentration that can disrupt the microbial biofilm formation. Two hundred microliters of each strain (10^8 CFU/mL) was inoculated into wells of 96-well microtiter plate. The plates were incubated for 48 h at 37 °C. Non-adherent bacteria and medium were removed by aspiration using a micropipette after washing with PBS. Serial dilutions of EOTS (100-1.56 µl/mL) were distributed into wells. Plates were incubated at 37 °C for 24 h and the wells were stained with crystal violet 0.1 % (w/v) after washed with PBS. Biofilm formation without essential oil was used as positive control [13]. Concentration of the existing biofilm formations from the treated wells was accepted as MBEC.

2.6. Biofilm metabolism assay

The bacterial viability within the biofilm was quantified using the tetrazolium salt XTT reduction test [14]. After biofilm formation at 37 °C for 48 h. The wells were washed twice with distilled water and planktonic bacteria were removed. EOTS diluted in TSB medium with the containing of 2 % (w/v) glucose with 0.5% Tween 20 (v/v), concentrations of MIC was applied to the biofilm formed in 96-well plates. TSB with Tween 20 (0.5%) was used as control. After incubation at 37 °C for 24 h, TSB and non adherent bacteria were removed by washing with distilled water. 200 µl XTT was added to each well for 5 h incubation. The plate was incubated at room temperature in darkness for 30 min. The cell viability in biofilm was determined by reading in a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA) at 450 nm.

The percentage of biofilm metabolism was calculated as in Eq 1.

Biofilm eradication (%) = [(Optical Density growth control – Optical Density sample) / Optical Density growth control] x100 ….2

2.7. Statistical analysis

All experiments were done in triplicate. Results were expressed in the form of the arithmetic mean ± standard deviation (x ± SD). OneWay variance analysis (ANOVA) and post-hoc Tukey analysis were used to reveal relationships between groups. Differences were considered significant for $p < 0.05$.

3. RESULTS AND DISCUSSION

The clinical isolates of *P.aeruginosa* strains were found resistant to the antibiotics such as amikacin, aztreonam, ceftazidime, gentamicin, imipenem, meropenem, netilmicin, piperacillin/tazobactam.

3.1. Chemical composition of EOTS

The composition of the EOTS is presented in Table 1. Forty-three compounds were identified in the EOTS. Essential oil contained a complex mixture of compounds. The main components of the essential oil were determined grandisol 21.99%, cadinol 6.45%, lavandulol acetate 4.39%. Previous studies showed that the major components of extracts and essential oils obtained through different methods from the plant of *Thymbra spicata* collected from different geographical regions were quite different from each other [15-17].

Baytok et al. [18] detected carvacrol (66.86%), p-cymene (12.18%), γ-terpinene (10.73%), and thymol (2.77%). In another study by Al Hafi et al. [19] 60.90% carvacrol and 14.16% p-cymene were detected in the plants collected from Lebanon. The major components of the essential oil of *T. spicata* collected from Turkey by Unlü et al. [15] were found to be carvacrol (60.39%), gamma-terpinene (12.95%), and p-cymene (9.61%). In another study conducted in Turkey carvacrol, p-cymene, a-terpinene, gamma-terpinene, beta-myrcene, and trans-caryophyllene were found to be the most common components of the chemical composition of the plant [16]. According to the results of previous studies, the presence of major components were non similar to our results.

Table 1. Components of essential oil of *Thymbra spicata.*

	Rt[a]	(%)[b]	Compound
1	11,691	0,49	α-Pinene
2	13,474	0,75	Yomogi alcohol
3	14,538	1,51	p-Cymene
4	14,828	1,87	Eucalyptol
5	15,63	2,56	Artemisia ketone
6	16,16	0,40	α-Methyl-α-[4-methyl-3-pentenyl]oxiranemethanol
7	16,415	0,19	Artemisia alcohol
8	16,982	1,21	Linalool
9	17,161	0,34	3,7-Octadiene-2,6-diol, 2,6-dimethyl-
10	17,29	0,85	Chrysanthenone
11	17,812	1,99	cis-Verbenol
12	17,948	0,77	Isophorone
13	18,089	2,91	Chrysanthenone
14	18,395	0,40	1,3,3-Trimethylcyclohex-1-ene-4-carboxaldehyde
15	18,639	0,99	3(10)-Caren-4-ol
16	18,768	0,83	Verbenol
17	19,338	2,06	Lavandulol
18	19,653	0,72	Linalool oxide pyranoside
19	19,913	0,67	4-Terpineol
20	20,14	0,71	5-Caranol
21	20,336	0,31	α-Terpineol
22	21,229	21,99	Grandisol
23	21,771	0,59	Bicyclo[3.1.1]hept-2-en-4-ol, 2,6,6-trimethyl-, acetate
24	22,102	1,22	1,5,5-Trimethyl-6-methylene-cyclohexene
25	22,667	4,02	Bicyclo[3.1.1]hept-2-en-4-ol, 2,6,6-trimethyl-, acetate
26	23,176	0,58	Spiro[4.5]dec-6-en-8-one, 1,7-dimethyl-4-(1-methylethyl)-
27	23,428	4,39	Lavandulol acetate
28	23,66	0,48	Verbenone
29	23,976	0,61	Carvacrol
30	24,319	1,45	Ascaridole
31	24,654	0,57	Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl
32	24,838	2,18	1,3-Cyclopentadiene, 5,5-dimethyl-1-ethyl-
33	25,37	4,94	Grandisol
34	25,892	0,91	Eugenol
35	26,321	1,62	Verbenol
36	26,989	1,61	Verbenone
37	27,673	0,52	lavandulyl acetate
38	28,276	0,67	p-mentha-1(7),8-dien-2-ol
39	29,093	0,28	Limonen-6-ol, pivalate
40	29,254	0,21	α-Himachalene
41	29,718	0,71	1,5,9-Trimethyl cyclododecatriene
42	30,374	1,62	Neryl (S)-2-methylbutanoate
43	34,505	6,45	δ-Cadinol

Rt[a]: retention time**;** (%)[b]: relative percentage obtained from peak area

3.5. Antibacterial activity

The antibacterial activity of the EOTS was evaluated by the micro-dilution method against *P. aeruginosa.* EOTS produced inhibitory effects against the tested strains. Minimum Inhibitory Concentration (MIC) values are summarized in Table 2. It inhibited 11 clinical isolates of *P.aeruginosa* with MIC values in the range of 1.56-12.5 µl/mL. Many researchers stated in their studies that essential oils were active against pathogenic microorganisms. Due to their hydrophobic characteristics, essential oils act by enabling the breakdown of cell membranes and mitochondrial lipids [20].

Previous studies demonstrated that the extracts and essential oils obtained from the plant of *T. spicata*

collected from different geographical regions had antimicrobial activity against many bacteria and fungi [15,16, 21]. Sarac et al. [21] emphasized that the essential oil of *T. spicata* was active against *C. albicans* in their study on the antimicrobial activity of the essential oil of *T.spicata.* Unlü et al. [15] reported that the essential oil of the plant collected from Turkey was quite effective on most microorganisms, particularly the *Candida* species.

In our study, the essential oil obtained from *T. spicata* collected from Manisa City, Akhisar and Gördes counties flora located in the Aegean Region of Turkey was found to be effective on the clinical isolates of *P. aeruginosa*.

3.6. Biofilm formation and Anti-biofilm Activity

The studies conducted indicated that nosocomial infections secondary to *P. aeruginosa* biofilm became a quite important problem. It was reported that sessile microorganisms within biofilm could be more resistant to antimicrobial agents compared to free planktonic cells [22]. The inhibition and eradication effect of EOTS on biofilm formation of *P. aeruginosa* clinical isolates was investigated in our study. According to the results obtained, the MBIC value was found to be 3.12 (µl/mL) in one isolate, 6.25 (µl/mL) in two isolates, 12.5 (µl/mL) in six isolates, and 25 (µl/mL) in two isolates while the MBEC value was found to be 6.25 (µl/mL) in three isolates, 12.5 (µl/mL) in five isolates, and 25 (µl/mL) in three isolates. Although there are publications related to the antimicrobial activity of the EOTS, not much is known about its anti-biofilm activity.

3.7. Reduction in biofilm metabolism

MIC value of the *T. spicata* essential oil was applied onto the bacterial biofilm formations that emerged after the 48h incubation. It was seen that the EOTS damaged in the biofilm metabolism at the MIC value by 2.0-51.6 % (Table 2).

Table 2. Antimicrobial and anti-biofilm activities of essential oil of *Thymbra spicata* against clinical isolates of multidrug resistant *Pseudomonas aeruginosa.*

Strain	MIC(µl/mL)	MBIC (µl/mL)	MBEC (µl/mL)	Reduction in biofilm formation on MIC (%)
1	6.25	12.5	12.5	47.3±1.5
2	1.56	6.25	6.25	19.3±2.5
3	12.5	6.25	6.25	48.3±1.1
4	3.12	12.5	12.5	51.6±2.5
5	6.25	12.5	12.5	32.0±1.0
6	12.5	25	12.5	9.3±0.5
7	3.12	12.5	12.5	2.0±1.0
8	12.5	12.5	25	21.3±1.1
9	1.56	3.12	6.25	17.3±2.8
10	12.5	12.5	25	28.0±1.0
11	12.5	25	25	31.6±2.3

MIC, MBC, MBIC, MBEC: minimum inhibitory concentration, minimum bactericidal concentration, minimum biofilm inhibitory concentration, minimum biofilm eradication concentration, respectively.

4. CONCLUSION

The findings of this study show that *Thymbra spicata* essential oil has strong antimicrobial and antibiofilm activity against Multidrug- resistant *Pseudomonas aeruginosa* strains. Thus, it is a potential agent for the treatment of MDR *P. aeruginosa* infections.

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Conflict of interest

No conflict of interest associated with this work

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