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Araştırma Makalesi

Türkiye'nin Maraş İlinden Toplanan *Trigonella mesopotamica* Hub.-Mor. Türünün Farklı Kısımları Üzerine Araştırmalar: Antimikrobiyal ve Antibiyofilm Aktiviteleri

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ÖZ

Bu araştırmada, *Trigonella mesopotamica* Hub.-Mor. tohum, toprak üstü ve tohum kabuğu kısımlarından elde edilen etanol ekstrelerinin antimikrobiyal ve antibiyofilm aktivitelerinin değerlendirilmesi amaçlanmıştır. Antimikrobiyal aktivite, in vitro mikrodilüsyon yöntemi kullanılarak referans bakteri ve mantar suşları üzerinde gerçekleştirilmiştir. Bunlara ek olarak, ekstrelerin *P. aeruginosa*'nın biyofilm oluşumunu engelleme ve önceden oluşturulmuş biyofilmi yok etme potansiyeli kristal viyole yöntemi kullanılarak belirlenmiştir. Ekstreler, referans antimikrobiyallerle karşılaştırıldığında test edilen bakteri ve mayalara karşı orta ila düşük antimikrobiyal aktivite göstermiştir. Ekstreler ayrıca biyofilm oluşumunu inhibe etme ve farklı minimum inhibitör konsantrasyonlarında (MIC) önceden oluşturulmuş biyofilmleri önleme potansiyeli göstermiştir. Tohum ve toprak üstü ekstrelerinin, tohum kabuğu ekstresine kıyasla daha iyi antibiyofilm aktivitesi gösterdiği belirlenmiştir. Özellikle veriler göz önüne alındığında, toprak üstü ekstresinin sub-MIC'de biyofilm oluşumunu %50 oranında azaltması ilgi çekicidir. Bulgular, *T. mesopotamica*'nın farklı kısımlarının özellikle antibiyofilm aktivitesi gösterdiğini kanıtlamaktadır. Literatürde ilk kez bu çalışma ile *T. mesopotamica*'nın antimikrobiyal ve antibiyofilm aktivitesine ilişkin veriler sunulmuştur. Bu nedenle çalışmamız bu türle ilgili literatüre önemli ön veriler sağlamaktadır.

Anahtar kelimeler: *Trigonella mesopotamica*, Antibiyofilm, Antimikrobiyal, Çemenotu, Etanol ekstresi

Studies on Different Parts of *Trigonella mesopotamica* Hub.-Mor. Collected From Maraş Province of Turkey: Antimicrobial and Antibiofilm Activities

ABSTRACT

In this research, *Trigonella mesopotamica* Hub.-Pur. It was aimed to evaluate the antimicrobial and antibiofilm activities of ethanol extracts obtained from seeds, aerial parts, and seed coat parts. In addition, the potential of the extracts to inhibit biofilm formation of *P. aeruginosa* and destroy preformed biofilm was determined using the crystal violet method. The extracts showed moderate to low antimicrobial activity against the tested bacteria and yeasts when compared to reference antimicrobials. The extracts also showed the potential to inhibit biofilm formation and prevent preformed biofilms at different minimum inhibitory concentrations (MIC). It was determined that seed and aerial part extracts showed better antibiofilm activity compared to seed coat parts extract. Especially considering the data, it is interesting that aerial part extract reduces biofilm formation in sub-MIC by 50%. The findings prove that different parts of *T. mesopotamica* especially show antibiofilm activity. For the first time in the literature, data on the antimicrobial and antibiofilm activity of *T. mesopotamica* are presented with this study. Therefore, our study provides important preliminary data to the literature about this species.

Key words: *Trigonella mesopotamica,* Antibiofilm, Antimicrobial, Ethanol extract, Fenugreek

INTRODUCTION

The genus *Trigonella* L. is considered a genus of traditional medicinal plants from the Fabaceae family (Nagulapalli Venkata et al., 2017). The green leaves and seeds of many of the *Trigonella* species have long been used as foodstuffs, vegetables, fodder and medicinal plants in many countries (Kumar et al., 2019) and shows various pharmacological effects such as anti-diabetic, anti-inflammatory, anti-fungal, antioxidant, hypocholesterolemic and hepatoprotective (Zameer et al., 2018). It contains about 135 species widely distributed in dry regions around the Eastern Mediterranean, Western Asia, Southern Europe, Northern and Southern Africa, Southern Australia. Some taxa of the genus are used as food and also in medicine. *Trigonella mesopotamica* Hub.-Mor. is a species belonging to the Section *Cylindricae* Boiss. and is known as "Dicleboyotu" in Turkey. *T. mesopotamica* is an annual herbaceous plant commonly found on stony hills, fallow fields, and roadsides at 300-1500 m (Akan et al., 2020).

Recently, resistance to antibiotics used in the treatment of infections caused by microorganisms has reached very serious levels (Spellberg and Gilbert, 2014). The development of antimicrobial resistance complicates the treatment of infectious diseases and lengthens hospitalization. This situation creates patient safety and cost problems and increases the rates of morbidity and mortality (Morrison and Zembower, 2020; Golkar et al., 2014). Biofilms are organized bacterial communities embedded in the extracellular matrix (ECM), a complex mixture of exopolysaccharides, nucleic acids, proteins, and other compounds (Berlanga and Guerrero, 2016; Melandera et al., 2020). Many microorganisms form biofilms instead of survive and increase their virulence in stressful environments such as nutrient-limited and unsuitable temperature conditions (Bjarnsholt, 2013). The biofilm not only protects microorganisms against adverse environmental conditions but also protects them from the host's immune response (Bjarnsholt, 2013; Grant and Hung, 2013). Therefore, biofilm-forming microorganisms can evade the host's immune response and are 1000 times more resistant to antibiotics compared to planktonic forms (Bjarnsholt, 2013; Melandera et al., 2020). For all these reasons, biofilm is accepted as the main drug target in the treatment of many infections.

Although there are many reports on *Trigonella* L. in the literature (Uras Güngör et al., 2017), there is no study on the antimicrobial and antibiofilm activity of *T. mesopotamica* yet. Therefore, in this study, it was aimed to investigate the antimicrobial and antibiofilm potentials of ethanol extracts obtained from *T. mesopotamica* seeds, aerial parts and seed coats.

MATERIALS and METHODS

Plant Material

T. mesopotamica Hub.-Mor. was collected from Kahramanmaraş (C6 Maraş: Çağlayancerit, 1300-1500 m.) province of Turkey during the seedling period in June 2021 by one of the authors (Ş. S. Uras Güngör). The plant was identified by Assist. Prof. Dr. Ş. Selma URAS GÜNGÖR and Prof. Dr. Ahmet İLÇİM. Herbarium samples of plant material were kept in Mustafa Kemal University, Faculty of Arts and Sciences, Department of Biology Herbarium, Hatay (MKU1754).

Extract Preparation

The powdered air-dried seeds, aerial parts, and seed coats of *T. mesopotamica* were macerated with ethanol at room temperature for three days, then filtered. The solvents were evaporated with the aid of a vacuum evaporator and the extracts were stored at 4°C in the dark until analyzed (Güzel Kara et al., 2021).

Antimicrobial Activity Study

Microbial Strains

Five reference bacterial strains (*Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 35150, and *Bacillus subtilis* ATCC 6633) and three reference yeast strains (*Candida albicans* ATCC 90028, *Candida glabrata* ATCC 15126, and *Candida parapsilosis* ATCC 90018) included in antimicrobial activity studies.

Antimicrobial Activity

Antimicrobial susceptibility testing was performed with minor modifications in the standard microdilution method as previously reported (Uras Güngör and Öksüz, 2022; Woods et al., 1995; Jorgensen and Ferraro, 1998). Briefly, 100 µL of Sabouraud dextrose broth (Merck, Germany) for yeasts, and Mueller-Hinton broth (MHB) (Merck, Germany) for bacteria was dispensed into each well of the 96-well microplate. 100 µL of the stock solution that the extracts prepared in dimethyl sulfoxide (DMSO) at 1000 µg/mL was added to the

first wells. Then, two-fold serial dilutions of the stock solutions of the extracts were prepared in 96-well microplates at concentrations ranging from 500 to 3.90 µg/mL. Microorganism suspension concentrations; for yeasts on Sabouraud dextrose agar (Merck, Germany) for 24 hours at 28°C and for bacteria on Mueller-Hinton agar (Merck, Germany) for 24 hours at 37°C, from stock cultures grown, were adjusted McFarland 0.5 (5x105 CFU/mL). Then, 5 µL of bacteria or yeast suspension was added to each well. Microorganism suspension was not added to some wells to create the medium control well, while only 5 µL of yeast or bacterial suspension was added to some wells without the tested extracts for microbial growth control. The minimum inhibitory concentration (MIC) was determined visually and using a microplate reader (BioTek Inc., USA) at a wavelength of 630 nm. MIC values were evaluated as the lowest concentration at which the tested extracts inhibited the growth of the microorganism. As a reference drug; Ampicillin (Sigma, USA) for bacteria and fluconazole (Sigma, USA) for yeasts were used. It was tested that DMSO had no effect on the growth of microorganisms included in the study and all experiments were repeated 2 times.

Biofilm Tests

Determination of biofilm formation capacity

The biofilm formation of the strains was determined with minor changes in the crystal violet (CV) staining method as previously practiced by our study group (Uras Güngör and Öksüz, 2022; O'Toole, 2011). The biofilm formation was determined by measuring the absorbance at optical density (OD) at 550 nm using a microtiter plate spectrophotometer (BioTek Inc., USA). The OD values of the wells without microorganism inoculum were used as the negative control. Biofilm production capacity was calculated according to the critical OD value (ODc); Biofilm formation negative (-): OD≤ OD; non-biofilm formation (+): ODc < OD ≤ 2 x ODc; moderate biofilm formation (++): 2 x ODc < OD ≤ 4 x ODc; strong biofilm formation (+++): OD> 4 x ODc (Gomes et al., 2019). All tests were performed in duplicate.

Biofilm-Prevention Assay

Biofilm prevention testing of extracts was performed with minor modifications to the CV staining test as previously practiced by our study group (Uras Güngör and Öksüz, 2022; Zhong et al., 2019). The lowest extracts concentration at which biofilm formation was inhibited by at least 50% was defined as the minimum biofilm inhibition concentration ($MBIC_{50}$).

$MBIC_{50}$ value was calculated according to the following equation;

Biofilm inhibition (%) = (A control – A sample / A control) × 100

A control: Absorbance value of wells containing only *P. aeruginosa* strains.

A sample: Absorbance value of wells containing extracts + *P. aeruginosa*.

Biofilm-Eradication Assay

The effect of extracts on preformed biofilm was performed with minor modifications to the CV staining assay as previously practiced by our study group (Uras Güngör and Öksüz, 2022; Zhong et al., 2019). The lowest concentration of the extracts required to destroy at least 50% of the preformed biofilm was defined as the minimum biofilm reduction concentration ($MBRC_{50}$).

$MBRC_{50}$ value was calculated according to the following equation;

Biofilm inhibition (%) = (A control – A sample / A control) × 100

A control: Absorbance value of wells containing only *P. aeruginosa* strains

A sample: Absorbance value of wells containing extracts + *P. aeruginosa*

RESULTS and DISCUSSION

In the present study antimicrobial and antibiofilm activities of the ethanol extract obtained from *T. mesopotamica* were investigated. The yield of seed (MSP 1), aerial part (MSP 2) and seed coat (MSP 3) extracts were determined as 13.35%, 6.1% and 8.2% (w/w).

Antimicrobial Activity

The antimicrobial activities of the extracts against the five bacteria and three yeast strains included in the study are shown in Table 1. It was determined that the extracts inhibited the tested bacteria at concentrations varying between 250-62.5 µg/mL and yeasts at concentrations varying between 125-62.5 µg/mL (Table 1). The extracts showed moderate to low antimicrobial activity compared to the reference antimicrobials fluconazole (for yeasts) and ampicillin (for bacteria). In general, it was determined that had better antimicrobial

activity MSP 1 extract against *E. coli* and MSP 2 extract against *P. aeruginosa* compared to other extracts. When evaluated in terms of the yeasts included in the study, it was determined that all three extracts showed the lowest antimicrobial activity against *C. albicans* compared to other yeasts (Table 1).

Table 1. MIC values (µg/mL) of the tested *Trigonella mesopotamica* seed, aerial part and seed coat extracts

Extract/ Reference antimicrobials	***B. subtilis ATCC 6633***	***P. aeruginosa* ATCC 27853**	***S. aureus* ATCC 29213**	***E. faecalis* ATCC 29212**	***E. coli* ATCC 35150**	***C. albicans* ATCC 90028**	***C. glabrata ATCC 15126***	***C. parapsilosis* ATCC 90018**
MSP-1	125	125	250	125	62.5	125	62.5	125
MSP-2	125	62.5	250	125	125	125	62.5	62.5
MSP-3	125	125	250	250	125	125	62.5	62.5
Ampicillin	1,95	31,25	0,48	0,97	3,90	-	-	-
Fluconazole	-	-	-	-	-	0,12	8	0,24

MSP-1: *T. mesopotamica* seed, MSP-2: *T. mesopotamica* aerial part, MSP-3: *T. mesopotamica* seed coat

Economically and medicinally, the most important species of the genus *Trigonella* L. is *T. foenum-graecum* (fenugreek). Yakout et al., (2013) reported that aqueous-ethanol extract (50%) of fenugreek seed showed antimicrobial activity against *S. aureus*, *B. cereus*, *E. coli*, *P. aeruginosa*, *K. pneumonia* and *S. typhi* with varied level of inhibition. Kadaikunnan et al. (2015), have also demonstrated that ethanol and water extracts from fenugreek seeds exhibited moderate to high antimicrobial activities against the tested bacterial and fungal pathogens. The data in the literature are compatible with our current study. In another study, aqueous-ethanol extract from fenugreek seeds demonstrate to low antimicrobial activity against the tested bacterial and fungal pathogens (Akbaş et al., 2017). Also, a previous study by Al-Timimi (2019), indicated that the ethanol extract of fenugreek seed showed prominent effect on *S. aureus* and *P. aeruginosa*. In the study of Singh et al. (2022) on fenugreek, it was shown that the leaf and seed extracts of fenugreek has significant antimicrobial activity against different microbial pathogens. In our previous study, ethanol extracts from seed, aerial part and seed coat of *T. cylindracea* showed moderate to low antimicrobial activity when compared with reference antimicrobials (Uras Güngör and Öksüz, 2022).

As a result of our literature search, we found that there is no antimicrobial activity study on *T. mesopotamica*. Therefore, our findings provide important preliminary data to the literature in terms of evaluating the antimicrobial activity of *T. mesopotamica* extracts.

Determination of biofilm formation capacity

Biofilm forming capacities of the reference bacterial strains included in the study were determined according to the crystal violet method.

Test results showed that *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 strains strong (+++) and also *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35150, and *Bacillus subtilis* ATCC 6633 formed moderate biofilm (++). In this study, biofilm prevention and eradication tests were performed on the biofilm of *P. aeruginosa* that which is one of the strains with the best biofilm production capacity.

Biofilm prevention and eradication assays

The percentages of biofilm inhibition of *P. aeruginosa* at 0.5X, 1X, and 2X MIC concentrations of the extracts included in the study are shown in Figure 1A. Biofilm prevention test revealed that MSP-1 extract inhibited biofilm formation by 51% at 1X MIC concentration. In addition, it has been proven that MSP-2 extract prevents biofilm by 50% at a concentration of 0.5 MIC. Therefore, the $MBIC_{50}$ of the MSP-1 extract is 125 µg/mL, while the $MBIC_{50}$ of the MSP-2 extract is 31.25 µg/mL. On the other hand, MSP-3 extract could not prevent biofilm formation by 50% at sub-MIC and MIC concentrations. For this reason, there is no $MBIC_{50}$ value. The most striking result of the biofilm prevention test is that MSP-2 extract can prevent biofilm

formation by 50% at sub-MIC (0.5X). Because studies in the literature show that higher drug concentrations may be required for the eradication of biofilm-producing bacterial cells (Hengzhuang et al., 2012).

The rates of inhibition of biofilm formation of *P. aeruginosa* by extracts at 2X, 1X, and 0.5X MIC concentrations by the biofilm eradication test are shown in Figure 1B. Biofilm removal testing revealed that MSP-1 extract was able to reduce preformed biofilm formation by 50% at 2X MIC concentration. It has also been proven that MSP-2 extract reduces preformed biofilm by 50% at 1X MIC concentration. Therefore, the $MBRC_{50}$ of the MSP-1 extract is 250 µg/mL, while the $MBRC_{50}$ of the MSP-2 extract is 62.5 µg/mL. On the other hand, MSP-3 extract failed to reduce preformed biofilm by 50% at 2X, 1X, and 0.5X concentrations. Therefore, there is no $MBRC_{50}$ value.

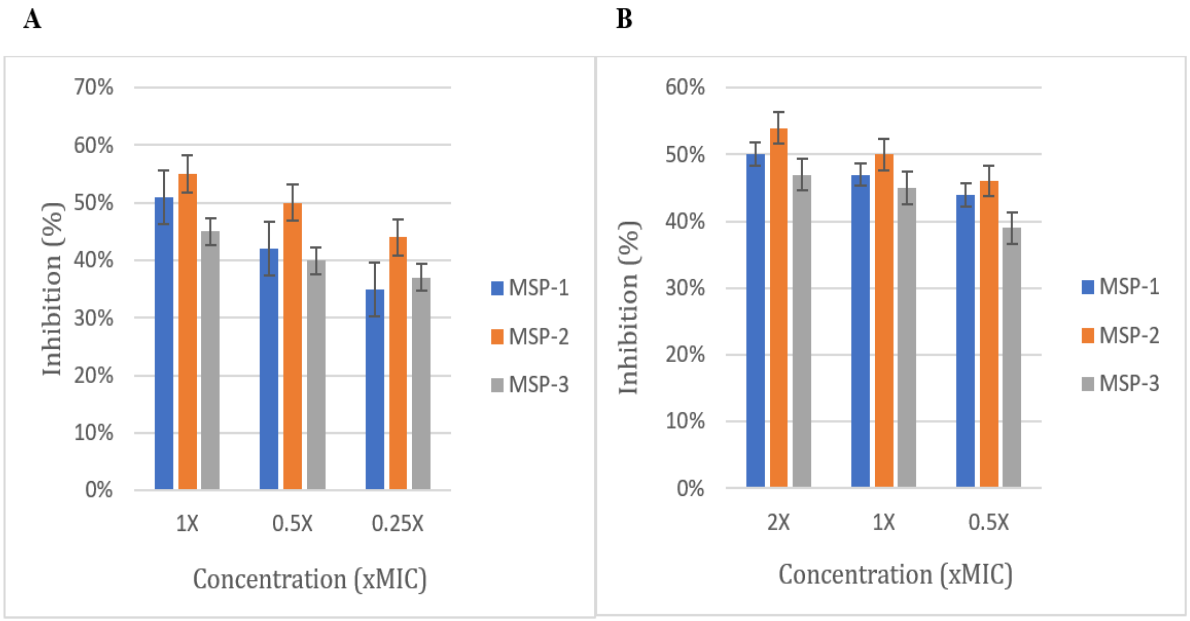

Figure 1. (A) Inhibition (%) of *P. auriginosa* biofilm by *T. mesopotamica* seed (MSP-1), aerial part (MSP-2) and seed coat (MSP-3) extracts at 1X, 0.5X, and 0.25X MIC concentrations. (B) Preformed biofilm inhibition (%) of *T. mesopotamica* MSP-1, MSP-2 and MSP-3 extracts at 0.5X, 1X, and 2X concentrations.

There are limited studies in the literature on the antibiofilm activity of species belonging to the *Trigonella* L. genus. A study on *T. foenum-graecum* showed that seeds of the fenugreek inhibit Quorum sensing (QS) and biofilm formation in *Aeromonas hydrophila* and *P. aeruginosa* with activity associated with caffeine production by the plant (Husain et al., 2015).

In our previous study, we found that ethanol extracts of *T. cylindracea* obtained from seed, aerial part and seed coat have antibiofilm potential. Biofilm tests have shown that seed coat extract can reduce biofilm formation by 50% at subMIC (0.5X). It was also determined that the seed and seed coat extracts were able to reduce the preformed biofilm by 50% above the 2X MIC (Uras Güngör and Öksüz, 2022). No study was found in the literature regarding the antibiofilm activity of the *T. mesopotamica*.

CONCLUSIONS

In this study, antimicrobial and antibiofilm activities of *T. mesopotamica* seeds, aerial parts and seed coats were studied for the first time. Therefore, our study provides important preliminary data to the literature about this species. Our research shows that although the extracts show low and moderate antimicrobial activity, their antibiofilm potential is remarkable. Due to the advantages of natural compounds such as low cost and abundant availability, their use as antimicrobial agents and food stabilizers is increasing day by day. Our findings indicate that *T. mesopotamica* can be a promising resource for the development of new therapeutic agents for various industries. In addition, our study will lead to new research aimed at evaluating the potential of this species as an antibiofilm agent in the treatment of infectious diseases, together with further research such as animal experiments and toxicity tests.

Conflict of Interest Declaration: The authors have no conflict of interest concerned to this work.

Contribution Rate Statement Summary: The authors declare that they have contributed equally to the article.

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