RESEARCH ARTICLE
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Assessment of the Antimicrobial and Antibiofilm Activities of *Trigonella smyrnea* Boiss. Growing in Türkiye

ABSTRACT

Background: The aim of this study is to assess the antimicrobial and antibiofilm capabilities of *Trigonella smyrnea* Boiss. plant species.

Methods: The antimicrobial activity of an ethanol-derived extract from the aerial parts, seeds and seed coat of *Trigonella smyrnea* were tested using the microdilution technique against 5 reference bacteria and three yeast strains. Additionally, the ability of the extracts to inhibit both biofilm formation and pre-formed biofilms produced by *Pseudomonas aeruginosa* was assessed using the crystal violet method.

Results: The utilized samples exhibited mild to moderate antimicrobial activity against the examined bacteria and yeasts, with minimum inhibitory concentration (MIC) values ranging from 250 to 62.5 µg/mL. The aerial parts, seed and seed coat extracts inhibited biofilm formation by approximately 36%, 28%, 40% and 30%, 25%, 33% at MIC/2 and MIC/4 MIC, respectively. In addition, preformed biofilm inhibition rates of aerial parts, seed and seed coat extracts were MIC/2: 11%, 26%, 26%; MIC: 15%, 30%, 31% and in 2×MIC: 31%, 38%, 38%, respectively.

Conclusion: Our data indicates that the extracts have mild to limited antimicrobial activity and moderate potential in combating biofilm formation. Especially when compared to seed extract, aerial parts and seed coat extracts showed better antibiofilm activity. In conclusion, although extracts of this species are promising in terms of antibiofilm activity, these findings should be supported by further study.

Keywords: Antibiofilm activity, antimicrobial activity, ethanolic extracts, *T*rigonella smyrnea.

INTRODUCTION

The increasing prevalence of drug-resistant pathogenic microorganisms concern about appropriate treatments for the infections they cause.[1] Microbial biofilms, described as groups of microorganisms enclosed in a self-produced matrix of organic exopolysaccharides, are known to have significant role in the development of diverse infectious diseases.[2,3] Biofilms, an important virulence factor, can increase resistance to antimicrobials and the host's immune defenses.[4] Therefore, biofilms pose serious problems in the treatment of the infections they are associated with and seriously limit treatment options. Today, there are still very few treatment options available to eliminate biofilm-related infections.[5] For these reasons, the need for new and effective pharmaceutical compounds, especially those of natural origin and without side effects, is greater than ever. In the literature, it has been shown that some plants have potential as anti-biofilm agents as well as being a promising source of antimicrobial compounds.[6-8]

Plants with diverse pharmacological properties have significant value in traditional herbal medicine.[9] The Trigonella L. genus is considered as a group of traditional medicinal plants within the Fabaceae family. It comprises around 135 species, found widely across dry regions in the Southern Europe, Eastern Mediterranean, Western Asia, Southern Australia and Northern and Southern Africa. Some members of this genus are utilized for both dietary and medicinal purposes. In Türkiye, the Trigonella L. genus is classified into 10 sections; *Trigonel-*

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la smyrnea Boiss., belonging to the Cylindricae Section, is commonly referred to as "Efeboyotu" in Türkiye.[10]

While there is extensive literature on Trigonella L., studies exploring the antimicrobial and antibiofilm properties of *T. smyrnea* remain unavailable. Hence, this research aimed to assess the antimicrobial and antibiofilm capabilities of ethanol extracts derived from *T. smyrnea* aerial part, seed and seed coat.

MATERIAL AND METHODS

Plant Material

T. smyrnea Boiss. was collected from Antalya (C2 Antalya: Gömbe, 1150 m.) province in Türkiye during the seedling period by the author of the study, Şerife Selma Uras Güngör. The identification of the plant was conducted by Assoc. Prof. Şerife Selma Uras Güngör, and herbarium samples were preserved at Mustafa Kemal University, Faculty of Arts and Sciences, Department of Biology Herbarium in Hatay (MKU1782).

Ethical approval was not necessary since no human or animal subject was used in this study.

Extract Preparation

The aerial parts, seeds and seed coats of *T. smyrnea* were dried and then macerated in ethanol at room temperature for three days. The mixture was later filtered via filter paper (Whatman Grade No.1) to remove impurities. Subsequently, the solvents were removed with a vacuum evaporator and the resulting extracts were kept in darkness at 4°C until further analysis.[11]

Biological Studies

Antimicrobial Activity Assay

Tests for antimicrobial activity were conducted with adjustments to methods that had been previously described.[12,13] The study included 5 reference bacterial strains *(Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 02026), additionaly *Candida albicans* ATCC 90028, *C. glabrata* ATCC 15126 and *C. parapsilosis* ATCC 90018 as reference yeast strains. The concentration of microbial cultures grown overnight before microdilution assay was adjusted to McFarland ($1x10^8$ CFU/mL) and stock solution of the extracts were prepared in dimethyl sulfoxide (DMSO). The extracts stock solutions were diluted in two-fold to produce concentrations covering the range of 500 to 3.90 µg/mL. Subsequently, the test microorganisms were introduced into 96-microplate wells. Additionally, a well for microbial growth control was set up with a suspension of bacteria or yeast alone, without any added extracts. Afterward, the microplates were incubated at 37°C for a period of 24 hours. Upon completion of the incubation, the minimal inhibitory concentration (MIC) was determined using a microplate reader (BioTek Inc., USA). Ampicillin (Sigma, USA) and fluconazole (Sigma, USA) served as reference drugs for comparison. Antimicrobial tests were carried out in duplicate to ensure accuracy. Additionally, the impact of DMSO on the growth of microorganisms included in this study was also assessed.

Determination of Biofilm Formation Capacity

The biofilm formation capability of *P. aeruginosa* strain was assessed using a modified crystal violet (CV) staining technique.[14] Mueller Hinton Broth (MHB) was added to 96-microplate wells, and microorganism cell suspensions were adjusted to McFarland ($1x10^6$ CFU/mL) before being added to each well. The microplate was then incubated at 37°C for 24 hours. Following the incubation, the wells were rinsed three times with sterile PBS to eliminate non-adherent cells. The wells were treated with pure methanol (150 µL) for 15 minutes, after which the methanol was removed and the microplates were dried. Subsequent to this, 150 µL of a solution containing 0.5% CV was introduced into the wells and left to incubate at a temperature of 25°C for 15 minutes. The CV solution in the wells was subsequently removed and the wells were washed with PBS. Following this, 150 µL of ethanol with a concentration of 95% was added to the microplate wells and incubated for an additional period of 15 minutes. Assessment of biofilm formation involved measuring absorbance at a wavelength of 550 nm using a microtiter plate spectrophotometer (BioTek Inc., USA). OD values from control wells without microbial inoculation served as negative controls. Biofilm production capacity was evaluated based on critical OD value (ODc); where biofilm formation is classified as negative (-) if OD ≤ ODc, weakly positive (+) if ODc < OD ≤ 2 x ODc, moderately positive (++) if 2 x ODc < OD ≤ 4 x ODc,and strongly positive (+++) if OD> 4 x ODc.[15] 2 sets of tests were conducted.

Biofilm-Prevention Assay

The impact of extracts on biofilm formation was determined with minor adaptations to the CV staining method.[16] 100 µl of MHB was added to each well in the microplate, followed by sequential dilutions of extracts at concentrations below the MIC (MIC/2 and MIC/4). A suspension of *P. aeruginosa* adjusted to McFarland standard $1x10^6$ CFU/mL was then added to each well except for the control, and incubated at 37°C for 24 hours. Then, the potential of the tested extracts to inhibit the formation of *P. aeruginosa* biofilm was determined by following the CV staining assay steps mentioned in the *"Determination of biofilm formation capacity"* section. The impact of extracts on the formation of biofilms was assessed by determining the optical density of the wells at 550 nm with a microplate reader. The minimum concentration of extracts that resulted in at least a 50% inhibition of biofilm formation was established as the minimum biofilm inhibition concentration. The MBIC50 value was calculated using the formula: Biofilm inhibition (%) = (A control – A sample / A control) × 100. The control indicates the absorbance of wells that only contain P. aeruginosa, while A sample represents the absorbance of wells containing extracts and *P. aeruginosa.*

Biofilm-Eradication Assay

The impact of extracts on the existing biofilm of *P. aeruginosa* was assessed using slight adjustments to the CV staining technique.[16] One hundred microliters of MHB and 5 µL of P. aeruginosa cell suspension (1x10^6 CFU/mL) were added in every well of a 96-well plate. The plate was then incubated at 37°C for 24 hours. Subsequently, the supernatants were carefully removed and replaced with a 100 µL extract diluted to concentrations equivalent to half the minimum inhibitory concentration (MIC/2), the MIC, and twice the MIC (2×MIC) in each well. Then, the plates were then incubated again (37°C, 24 h.). The ability of the extracts to prevent preformed biofilm by *P. aeruginosa* was subsequently assessed using the CV staining test protocol outlined in the "Determination of biofilm formation capacity" section. The pre-existing biofilm impacts of the extracts were assessed by determining the optical density at 550 nm using a microplate reader (BioTek Inc., USA). The minimum concentration of extracts needed to eliminate at least half of the preformed biofilm was established as the minimal biofilm reduction concentration (MBRC50). The MBRC50 was calculated using the subsequent equation: Inhibition of biofilm (%) = (A control – A sample / A control) × 100, where A control denotes the absorbance measurement of wells containing only *P. aeruginosa* and A sample represents the absorbance value of wells containing extracts and *P. aeruginosa.*

RESULTS AND DISCUSSION

The present research examined the antimicrobial and antibiofilm properties of ethanol extracts derived from various components (aerial parts, seeds and seed coats) of *T. smyrnea*. Boiss. The yields of seed, aerial part and seed coat extracts were 8.4%, 0.8%, and 10% (w/w), respectively.

Antimicrobial Activity Assay

In this study five reference bacterial strains and three reference yeast strains were included. Table 1 displays the antimicrobial effects of the extracts on bacteria and yeast strains. In comparison to standard antibacterial agents, the extracts exhibited modest to mild antimicrobial efficacy (MIC range: 250-62.5 µg/mL) against both bacteria and yeasts. When assessed for their antibacterial and antifungal properties, it was noted that there were minimal variations between the extracts. However, the seed extract demonstrated greater efficacy against *C. parapsilosis*, while the seed coat extract exhibited enhanced effectiveness against both *C. glabrata* and *C. parapsilosis* (Table 1). The varying antifungal activity may be attributed to the different contents of phenolic compounds, such as pyrogallol, gallic acid, 3-hydroxy tyrosol, and catechol, present in different parts of the plant species.[17]

T.foenum-graecum (fenugreek) is the most significant species in the Trigonella L. genus from both an economic and medical perspective, and most of the antimicrobial research on species within this genus focuses on fenugreek. A study involving fenugreek seeds revealed that a 50% aqueous ethanolic seed extract exhibited varying levels of antimicrobial activity against *E. coli, Klebsiella pneumoniae, B. cereus, S. aureus, P. aeruginosa, and Salmonella typhi.*[18] A another study[19] involving fenugreek demonstrated that the seeds' ethanol and water extracts displayed significant antimicrobial effects against a range of bacterial and fungal pathogens which is coherent to our study. Akbaş et al.,[20] found that the aqueous-ethanol extract from fenugreek seeds showed restricted antimicrobial effects against the tested bacterial and fungal pathogens. Al-Timimi et al.,[21] demonstrated that the ethanol-based fenugreek seed extract had a noteworthy effect on *P. aeruginosa* and *S. aureus*. Furthermore, a prior investigation by Singh et al.[22] on fenugreek, revealed that both the leaf and seed extracts of fenugreek exhibited notable antimicrobial properties against various microbial pathogens. Our research team investigated the *T. cylindracea* ethanol extracts derived from aerial parts, seeds and seed coats that the examined extracts displayed varying degrees of antimicrobial activity compared to standard antimicrobials.[23] In a prior study, ethanolic extracts of aerial part, seed and seed coat derived from *T. strangulata* exhibited moderate and limited antimicrobial efficacy in comparison to standard antimicrobials (fluconazole, ampicillin). Furthermore, upon assessment of their overall antimicrobial effectiveness, it was found that they displayed superior activity against fungal strains compared to bacteria.[24]

To the best of our knowledge, no studies about the antimicrobial effects of *T. smyrnea* were reported. Therefore, our findings regarding the antimicrobial activity of extracts from various parts of *T. symrne*a serve as valuable initial insights for the literature.

Biofilm Assays

P. aeruginosa strains were evaluated for their ability to form biofilms using the crystal violet method. The tested *P. aeruginosa* strain utilized formed a robust biofilm. *P. aeruginosa*, which was studied for its antibiofilm properties in this research, is a type of opportunistic pathogen capable of causing infections by effectively attaching to various surfaces such as medical equipment like implants, urinary catheters and contact lenses. It is associated with both acute and chronic infections.[25] *P. aeruginosa*, which has the ability to acquire resistance to antibiotics and is challenging to eliminate, can generate multiple virulence factors governed by the Quorum sensing (QS) regulatory mechanism, which plays a significant role in sustaining biofilm formation.[26] Hence, it is crucial to prevent or remove the development of biofilm triggered by this microorganism. This research investigated the impact of extracts on both preventing biofilm formation and disrupting pre-existing biofilms created by *P. aeruginosa.*

In the biofilm prevention test, it was examined that aerial parts, seed and seed coat extracts showed biofilm inhibition 36%, 28%, 40% and 30%, 25%, 33% at MIC/2 and MIC/4, respectively (Fig. 1A). The data showed that the extracts did not reduce biofilm formation by 50% at sub-MICs, so the extracts do not have MBIC50 values. In addition, the biofilm eradication test showed that aerial parts, seed and seed coat extracts inhibited biofilm formation 11%, 26%, 26%, and 15%, 30%, 31%, and 31%, 38%, 38% at MIC/2, 1X, and 2X MIC, respectively (Fig. 1B). In the study, none of the analyzed extracts could effectively reduce pre-existing biofilm by 50% at the concentrations that were tested. For this reason, extracts do not have an $MBRC_{50}$ value.

Table 1. MIC Values (in µg/mL) of the Examined Plant Extracts and Reference Antibiotics Against Different Species.

Used part/ Reference antimicrobials	*B. subtilis* ATCC 6633	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*A. baumannii* ATCC 02026	*C. albicans* ATCC 90028	*C. glabrata* ATCC 15126	*C. parapsilosis* ATCC 90018
Seeds	125	125	250	125	250	125	125	62.5
Aerial part	125	125	250	125	125	125	125	125
Seed Coat	125	125	250	250	125	125	62.5	62.5
Ampicillin	1.95	31.25	0.48	0.97	31.25	-	-	-
Fluconazole	-	-	-	-	-	0.12	8	0.24

- Not tested

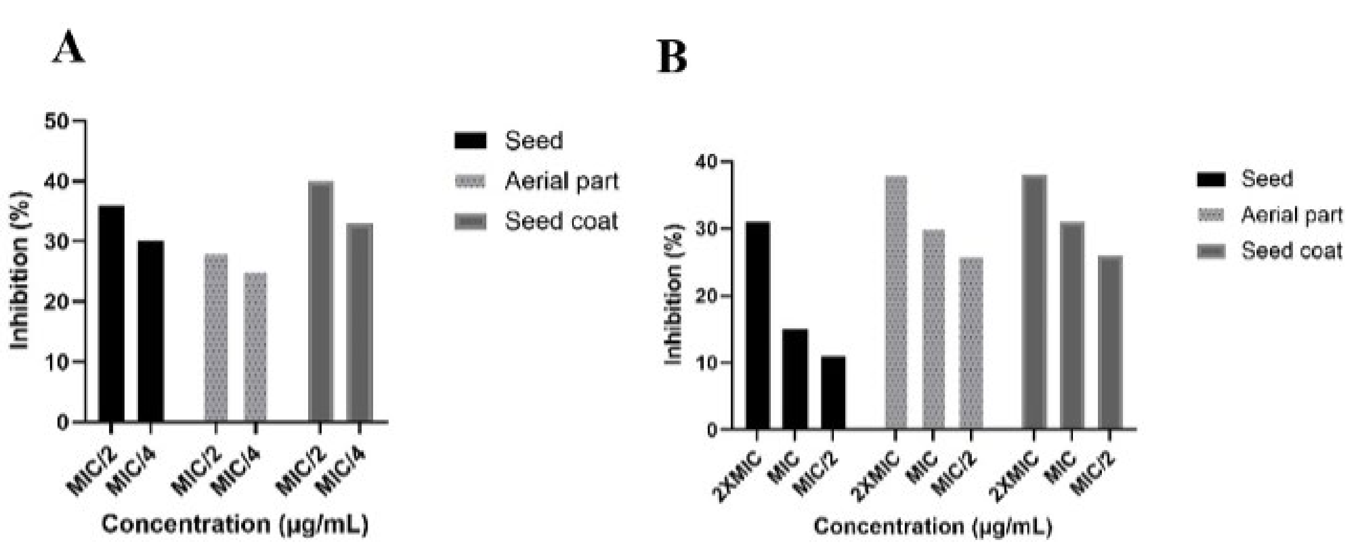

Figure 1. Biofilm inhibition assay (A) Prevention of biofilm formation. Inhibition (%) of biofilm formation by extracts at MIC/2 and MIC/4 concentrations. (B) Eradication of biofilm formation. Preformed biofilm inhibition (%) of extracts at MIC/2, MIC, and 2MIC concentrations.

When we evaluate the findings in general, the potential of the extracts in the study for biofilm prevention and inhibit previously formed biofilm is close to each other. However, the potential of seed coat extract to both prevent biofilm and inhibit pre-formed biofilm was found to be better than other extracts.

Upon reviewing the literature, we observed that there is limited research on the anti-biofilm characteristics of species belonging to the Trigonella L genus. According to Husain et al.[27] fenugreek seeds demonstrated inhibition of biofilm formation and QS in *Aeromonas hydrophila* and *P. aeruginosa*. In a previous investigation using ethanol extracts derived from aerial parts, seeds and seed coats and of *T. cylindracea*, our findings indicated potential antibiofilm effects of these extracts. Biofilm assays revealed that both the seed and seed coat extracts were capable of reducing preformed biofilms by 50% at double the minimum inhibitory concentration. Furthermore, it was established that the seed coat extract could decrease biofilm formation by 50% at sub-MIC levels.[23]

Our research results indicate that the extracts studied, including *T. smyrnea* aerial part, seed, and seed coat, did not exhibit biofilm formation for a 50% prevention. However, they did show inhibition of biofilm formation.

CONCLUSION

This study represented the initial effort to explore the antimicrobial/antibiofilm properties of *T. smyrnea* aerial parts, seeds and seed coats. Consequently, our investigation offers significant preliminary insights into this species in the field. Our findings indicated that the extracts exhibited limited to moderate antimicrobial effects but showed some potential for inhibiting biofilm formation. The growing utilization of natural substances as antimicrobials and food preservatives is driven by their cost-effectiveness and widespread availability. This study will stimulate further exploration into utilizing this species as an antibiofilm agent for treating infectious diseases, including subsequent animal studies and toxicity assessments. Additionally, it is anticipated that *T. smyrnea* could serve as a promising source for developing new therapeutic agents across various industries.

Ethics Committee Approval: Ethical approval was not necessary since no human or animal subject was used in this study.

Peer-review: Externally peer-reviewed.

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Declaration of Interests: The authors have no conflict of interest to declare.

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