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Research Article

Antimicrobial and antibiofilm studies on three endemic species of *Verbascum* L. (Scrophulariaceae) in Türkiye

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Abstract: The consumption of traditional medicinal herbs has gained popularity as a viable alternative approach for addressing microbial infections or infectious structures. In this study, *Verbascum detersile, Verbascum eskisehirensis,* and *Verbascum gypsicola* endemic species belonging to the Scrophulariaceae family, spreading in Eskişehir and its surroundings, were collected from natural habitats and evaluated in terms of antimicrobial and antibiofilm activities. In biological activity studies, different concentrations of three plant extracts showed various antimicrobial and antibiofilm activities on selected standard microorganism cultures (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, and *Candida krusei* ATCC 6258). It was observed that the selected three endemic *Verbascum* extracts had a minimum inhibitory and minimum biofilm eradication concentration value of 1250 µg/mL.

1. INTRODUCTION

Antimicrobial and antibiofilm agents are the compounds employed to inhibit the growth of bacteria or induce their elimination, while concurrently destroying the biofilm structure they form on various surfaces. Nevertheless, the efficacy of pharmaceuticals in terms of their antimicrobial or antibiofilm capabilities is constrained by the presence of resistant microorganisms. There is an urgent need for the development of novel, biologically active substances that are both environmentally friendly and devoid of toxicity to effectively combat diseases and minimize their negative consequences. The structures in question are known as microbial biofilms, which provide a conducive environment for microorganisms to thrive within a sophisticated matrix (Sánchez *et al*., 2016). Biofilm formation is considered a significant pathogenicity component in microorganisms. Plants have been widely recognized as highly promising bioactive agents. In contemporary times, there has been a notable increase in the focus on the bioactivities exhibited by plants, which have the potential to provide protection against infections or act as preventive measures.

The genus *Verbascum* L. (mullein) is one of the medicinal plants belonging to the family of Scrophulariaceae commonly known as “Sığırkuyruğu”. The family of Scrophulariaceae is one of the largest plant families in dicotyledonous angiosperms with 200 genera (Gökmen, 2021).

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Verbascum genus exists predominantly in Europe, North America, and West and Central Asia (especially Anatolia). The *Verbascum* genus is represented in the world with nearly 360 species (Hunt, 2009). In Türkiye, the *Verbascum* genus consists of approximately 255 species, 200 of which are endemic (about 80% endemism rate), and it has 130 additional hybrid species. The genus exhibits a notably high endemism ratio of 80%, as seen by the presence of 196 species that are native to certain regions (Duman *et al.*, 2020). *Verbascum* species are a popular herb with medicinal uses. In traditional Turkish folk medicine, these medicinal plants are used in the treatment of expectorant, stomachache, stomach ulcer, diabetes, hemorrhoid, rheumatism, and urinary tract infection (Kargıoğlu *et al*., 2008; Mükemre *et al*., 2015). It has been reported that they are used for respiratory disorders, expectorants, stomach tonic, dyspepsia, diarrhea, diuretics, snake bites, blood clotting of women after childbirth, wound disinfection, and sedative in Iran (Ghorbani, 2005; Mohamadi *et al*., 2015). In addition, the Herbal Medicinal Products Committee (HMPC) reported that Mullein flowers (*Verbascum phlomoides*, *Verbascum thapsus*, and *Verbascum densiflorum*) can be used to soothe the throat in colds and dry coughs (EMA, 2021). In some studies, *Verbascum* has been found to have an antiviral (Escobar *et al*., 2012), antimicrobial (Senatore *et al*., 2007), enzyme inhibitory activities (Georgiev *et al*., 2011), and wound healing properties (Süntar *et al*., 2010). The medicinal benefits of *Verbascum* species are attributed to their biologically active components, including phenylethanoids, flavonoids, glycosides, neolignan, monoterpene glycosides, and saponins. (Küçük *et al*., 2016).

In Türkiye, *Verbascum detersile* Boisse & Heldr., *Verbascum gypsicola* Vural & Aydoğdu and *Verbascum eskisehirensis* Karavel., Ocak & Ekici are known as "Zinemit", "Mermer Sığırkuyruğu" and "Eski Sığırkuyruğu" respectively (Karavelioğulları *et al*., 2012). *Verbascum detersile* is in the VU category and has been reported to have a high risk of extinction. *Verbascum gypsicola* and *Verbascum eskisehirensis* species are in the CR category according to IUCN criteria and are reported as highly endangered. In this research, these three endemic species were evaluated for their antimicrobial and antibiofilm properties. These species are endemic to the Anatolia region. *V. detersile* is a biennial herb with yellow flowers. This plant prefers *Pinus* forests and rocks from sea level up to 300 m altitude and generally grows in Antalya (Saltan *et al*., 2011). *V. gypsicola* has been documented in three specific locations inside Ankara, where it thrives in very soil conditions (Vural *et al*., 1993). Additionally, it has been observed in a single locality in Eskişehir (Öztürk *et al*., 2018). *V. eskisehirensis* is a biennial herb that exclusively thrives in specific regions, including Eskişehir, Sivrihisar, Karacaören, and the Mountain of Arayit. According to Karavelioğulları *et al.* (2009), this specific plant exhibits a preference for limestone rocks and scree places. This study, therefore, aims to investigate the potential antimicrobial and antibiofilm activities of these three distinct endemic species of *Verbascum.*

2. MATERIAL and METHODS

2.1. Preparation of Plant Extracts

The aerial parts (above-ground parts) of *Verbascum* species were gathered and subsequently brought to the laboratory. Table 1 presents the geographical data about three distinct species. The collected plants were identified by Prof. Dr. Sevim Küçük (Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 26470, Tepebaşı, Eskişehir). The plant materials, which had been dried and ground into a powder, were accurately weighed and subjected to extraction using a 70% ethanol solution. This process was carried out in appropriate glass flasks. The extracts were incubated in a water bath set to room temperature with continuous agitation, and the resulting filtrates were collected by replacing the solvent every 24 hours. The procedure was sustained for three consecutive days. The extracts obtained were concentrated using a rotary evaporator and subsequently subjected to lyophilization. The desiccated extracts were stored at a temperature of +4°C following the processes of evaporation and lyophilization (Öztürk *et al*., 2019).

Table 1. Locations of the collected *Verbascum* species

Verbascum Species	Locations
V. detersile Boiss. & Heldr.	B3 Eskişehir: Alpu-Gölalan yolu, 768 m., 39°45'39.09- 30°58'22.00, 05.07.2019 (ESSE 15614)
V. gypsicola Vural & Aydoğdu	B4 Ankara: Beypazarı: Çayırhan-Beypazarı, 2 km, jipsli step, 503 m., 40°11'89.23- 31°63'89.53, 2.07.2019 (ESSE 15615)
V. eskisehirensis Karavel., Ocak & Ekici	B3 Eskişehir: Sivrihisar: Sivrihisar-Kaymaz, 1100 m., 39°26' 57.73- 31°31' 57.18, 01.06.2019 (ESSE 15616)

2.2. Determination of Antimicrobial Activity

2.2.1. *Well diffusion test*

In the test standard pathogenic microorganisms were used; *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028 and *Candida. krusei* ATCC 6258. The microorganism strains were prepared on appropriate media Mueller Hinton Agar (MHA) for bacteria and Saboroud Dextrose Agar (SDA) for yeasts. The number of microorganisms per milliliter was adjusted to $1x10^8$ cfu/mL according to the McFarland 0.5 standard. 100 µL of microorganism cultures were inoculated into suitable solid media with a Drigalski spatula. 6 mm wells were made on the medium and 25 µL of V1 (*V. detersile*), V2 (*V. eskisehirensis*), and V3 (*V. gypsicola*) was added to the wells. The plates were then incubated at 37 °C for 24 hours (Ferraro, 2000). The experiment was repeated 3 times, and the results were averaged.

2.2.2. *Broth microdilution method*

The broth microdilution method, according to the guidelines set by the Clinical and Laboratory Standards Institute (CLSI), was employed to determine the minimum inhibitory concentration (MIC) of three plant extracts (CLSI, 2002; CLSI, 2012). MIC values were determined in 96-well plates for concentrations of 5000-39.0625 µg/mL of plant extracts V1 (*V. detersile*), V2 (*V. eskisehirensis*), V3 (*V. gypsicola*). Fresh cultures of microorganisms were prepared overnight on suitable media, such as MHA for bacteria and SDA for yeasts. The pathogenic microorganisms used in this study were the same as in well diffusion method. These microorganisms were prepared following the McFarland 0.5 standard, and the concentration of microorganism colonies per milliliter was adjusted to $1x10^8$. Plant extracts were diluted in a 96-well plate and 100 µL of pathogenic microorganisms were added to them. The plates were incubated at 37 °C for 24 hours. The standard antibiotics (Ketoconazole, Chloramphenicol) 0.4-1000 µg/mL were used as a positive control group. As the negative control group, MHB and SDB medium, as well as growth control groups for microorganisms, were transferred to a 96-well plate in 2 parallels. After incubation, 20 µL of resazurin dye was added to the wells and incubated for 4 hours. After 4 hours, the results were analyzed based on the observed alterations in the wells' coloration, which spanned from a blue-green hue to a pink shade. The experiment was replicated three times, and the results were subsequently averaged.

2.3. Determination of Antibiofilm Activity

The antibiofilm activity of microorganisms was determined by the Minimum Eradication Concentration (MBEC) method (Cruz, Shah & Tammela, 2018). Biofilm-forming pathogenic microorganisms (*S. aureus* ATCC 29213, *S. epidermidis* ATCC 14990, *P. aeruginosa* ATCC 27853) were prepared according to McFarland 0.5 standard and the number of microorganisms per milliliter was adjusted to $1x10^8$ cfu/mL. 200 µL of microorganism cultures were transferred to wells of 96-well plates and incubated at 37 °C for 48 hours to the formation of biofilms. After incubation, the suspensions (200 µL) in the wells were withdrawn and the wells were washed 2 times with 0.9% NaCl solution. The concentrations (5000, 2500, 1250, 625, 312.5 µg/mL) of the plant (V1 (*V.detersile*), V2 *(V. eskisehirensis*), V3 (*V. gypsicola*)) extracts were obtained by macrodilution in MHB medium for bacteria and SDB for yeasts. Subsequently, 100 µL of plant

extracts were individually introduced into the wells, followed by incubation of the plate at a temperature of 37 °C for 24 hours. Following the incubation period, a volume of 20 µL of resazurin dye was introduced into the wells and subsequently incubated at a temperature of 37 °C for 3 hours. Following the designated waiting period, the outcomes were assessed based on the observed alterations in the wells' coloration, which might vary from a blue (or green) hue to a pink shade. MBEC values (without living cells in blue (green) color) were determined at different concentrations according to the microorganisms. The experiment was repeated three times, and the results were subsequently averaged.

3. FINDINGS

3.1. Well Diffusion Test

In the good diffusion test, it was observed that 156.25 µg/mL concentrations of the three *Verbascum* extracts did not show any antimicrobial effect against *C. albicans* and *C.krusei* yeast cells. All the *Verbascum* extracts showed antimicrobial activity against *S. aureus*, *S. epidermis*, and *P.aeruginosa* bacteria strains. *V. eskisehirensis* and *V. detersile* extracts demonstrated moderate antimicrobial activity when compared with amoxicillin, especially against *S. aureus* and *P.aeruginosa*. In addition to these results, *V. gypsicola* extracts showed strong antimicrobial activity with a 10 mm zone inhibition diameter as amoxicillin. The values of the zone diameters in (mm) of the extracts against the standard pathogenic microorganisms are given in Table 2.

Table 2. Values of the zone diameters (mm) formed in the well Diffusion results (V1: *V. detersile*, V2: *V. eskisehirensis*, V3: *V. gypsicola*)

Plant Extract Codes – Standard Tests Microorganisms	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*C. albicans*	*C. krusei*
V1 (156.25 µg/mL)	4±1	4±1	6±1	-*	-*
V2 (156.25 µg/mL)	10±1	6±1	6±1	-*	-*
V3 (156.25 µg/mL)	2±1	2±1	10±1	-*	-*
Amoxicillin (30 µg/mL)	4±1	32±1	10±1	-**	-**
Ketokonazol (1 mg/mL)	-**	-**	-**	14±1	30±1

* Insufficient-Low Concentration, ** Ineffective

3.2. Broth Microdilution Method

MIC values of plant extracts are given in Table 3. According to the test result, three plant extracts showed antimicrobial activity with different concentration values. The MIC values were in the range of 312.5 to 1250 µg/ml. The most prominent effect was achieved for *V. gypsicola* extract against *S. aureus* and *C. krusei*. All extracts demonstrated strong antifungal activity on *C. krusei* when compared with ketoconazole.

Table 3. MIC Values (µg/mL)

Plant Extract Codes – Standard Tests Microorganisms	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*C. albicans*	*C. krusei*
V1	1250	1250	625	1250	312.5
V2	1250	1250	625	1250	156.25
V3	625	1250	625	1250	312.5
Chloramphenicol	31.25	31.25	62.5	-**	-**
Ketoconazole	-**	-**	-**	500	500

** Ineffective

3.3. Determination of Antibiofilm Activity

Antibiofilm activity was determined by MBEC, and the results are given in Table 4. The MBEC values were in the range of 625 to 2500 µg/ml. As a result of the antibiofilm test, the biofilm structure of *S. aureus* was more sensitive to *V. eskisehirensis* and *V. detersile* extracts at the 625 µg/mL concentration. *S. epidermidis* is a prevalent pathogen with the primary pathogenic factor of creating cohesive biofilms (Knobloch *et al*., 2001). Because of this property, all *Verbascum* extracts demonstrated weak antibiofilm activity against *S. epidermidis* at the concentration of 2500 µg/mL.

Table 4. MBEC Values (µg/mL).

Plant Extract Codes – Standard Tests Microorganisms	*S. aureus*	*S. epidermidis*	*P. aeruginosa*
V1	625	2500	1250
V2	625	2500	1250
V3	1250	2500	1250

4. DISCUSSION and CONCLUSION

Certain plants belonging to the genus *Verbascum* have been employed for numerous millennia in the treatment of both internal and exterior illnesses. In the relevant literature, it has been reported that *Verbascum* species have various antimicrobial activities. In some studies, it was observed that *Verbascum* spp. was especially effective against Gram-positive and Gram-negative bacteria but did not show antifungal activity against *Candida* yeasts (Khafagi, 2002). In another study, it was commented that some *Verbascum* species showed antibacterial activity only against Gram-positive bacteria and were ineffective against Gram-negative bacteria and yeasts (Dülger *et al*., 2002). Amirnia *et al.* (2011) studied the possible antimicrobial activity of alcoholic and aqueous extracts of flowers of *V. speciosum*. Antimicrobial tests were performed by using the disc diffusion method, which included the *B. subtilis, B. cereus,* and *E. coli* bacteria strains. The extracts exhibited concentration-dependent inhibitory effects on bacterial strains. Additionally, results showed that ethanolic extract exhibited more potent activity on bacterial strains. Amirnia *et al.* (2011) reported that the highest level of activity was detected against *B. cereus*, followed by *B. subtilis*. Additionally, it was found that *E. coli* exhibited the highest level of resistance among the strains tested. Unlike these studies, both antibacterial and antifungal activities were observed in *Verbascum* species used within the scope of the research. According to the data reported by Saltan *et al.* (2011), chloroform and methanol extracts of *Verbascum detersile* showed antibacterial activity at MIC concentrations in the range of 7.5-150 mg/mL. The species on which *V. detersile* was most effective were *E. coli* ATCC 25922, *P. aeroginosa* ATCC 15442, *Streptococcus salivarius* RSHE 606, and these results were reported as 7.5 mg/mL for chloroform extract and 9.3 mg/mL for methanol extract, respectively. Öztürk *et al.* (2019) studied *V. eskisehirensis* methanol extract and stated that it has moderate antibacterial activity thanks to its flavonoid content. This is due to the verbascoside and luteolin substances in the flavonoid content. When the data obtained as a result of antimicrobial tests in this study were evaluated, antifungal activity was observed only as a result of the microdilution method. The reason for this is that the microdilution method is more sensitive than the well diffusion method and plant extracts were tested at different concentrations in the microdilution method. In the article published by Ocak *et al.* (2020), contrary to the result obtained in this study, it was reported that the methanol extract of *V. gysicola* applied by the good diffusion method had antifungal activity on *Candida albicans*. When the MIC results were evaluated, it was observed that the three plant species showed both antibacterial and antifungal activity at concentrations close to each other. In the publication of Grigorov *et al.* (2023), the antibacterial activity results obtained from the ethanol extract obtained from the flowers of *V. niveum* species are similar to the results obtained in this study. In addition to antibacterial results, antifungal values obtained

against yeasts are also similar. In the study of Göse and Hacıoğlu Doğru (2019), it was reported that ethanol extracts obtained from *V. pinnatifidum* have antibiofilm activities, especially against the biofilm structure formed by Gram-positive bacteria. This activity was proven for the first time in this study at concentrations of 2.5-10 µg/ml of the plant. In another study, Göse and Hacıoğlu Doğru (2021) reported that *V. pinnatifidum* and *V. antinori* extracts showed an inhibition activity on the biofilm structure of *B. subtilis* ATCC 6633 by 92.18% and 91.19%, respectively. Bacterial biofilms possess significant pathogenicity attributes owing to their notable resistance capabilities against chemotherapeutic agents (Grant & Hung, 2013). Bacterial biofilm formation could be controlled by a communication mechanism named quorum sensing. Hence, our study hypothesized that *Verbascum* extracts can suppress the quorum-sensing mechanism. In this study, different concentrations of the three *Verbascum* extracts showed various antimicrobial and antibiofilm activities on selected standard cultures of microorganisms. The diversity of the results of biological activity studies with *Verbascum* species is directly related to the locality where the plants were collected, ecological conditions, seasonal changes, and differences in extraction processes.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Pervin Soyer: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing-original draft. **Sevim Küçük**: Methodology, Supervision, and Validation. **Yağmur Tunalı**: Supervision, and Validation.

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