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Antimicrobial and Antibiofilm Activity of Methanol and Ethyl Acetate Extract of *Ferula* sp. Growing in Erzurum

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Abstract

This study investigated the antimicrobial and antibiofilm activities of Ferula species (sp.) obtained from Erzurum province. Antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Candida albicans, and Candida tropicalis were determined by agar well diffusion assay. Ethyl acetate extract showed antimicrobial activity against S. aureus, and methanol extract showed antimicrobial activity against E. coli and C. albicans with a zone diameter of 21, 18, and 15 mm, respectively. The extract's minimum inhibitory concentrations (MIC) were 16, 32, and 32 µg/mL ml for S. aureus, E. coli, and C. albicans, respectively. Furthermore, it was determined for the first time that Ferula sp. extracts were effective against biofilm formations of S. aureus and C. albicans. In the light of these results, we think that extracts of Ferula sp. contain potential candidate molecules against biofilm-associated infections, and further characterization studies are needed.

Keywords: Antimicrobial, Antibiofilm, Extraction, *Ferula* sp.

Introduction

Nowadays, antibiotic resistance has become one of the biggest and most significant problems to human health. One of the most important reasons for antibiotic resistance is the formation of biofilms by microorganisms. Biofilms are cell communities composed of an extracellular matrix of polysaccharides, proteins, and DNA (1). Biofilms are highly resistant to antimicrobial molecules. There is a need for new antimicrobial and antibiofilm compounds in the fight against infections caused by biofilm structure and multi-drug resistant bacteria, which are

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a severe problem worldwide. Therefore, the discovery of antimicrobial sources becomes a necessity.

Plants are potential candidates for natural products to help combat such problems. There are plants with antimicrobial, antibacterial, antifungal, and antioxidant activity (2,3,4). So far, more than 30,000 compounds have been isolated from these plants (5). In addition, plant extracts with antimicrobial activity are used in many biotechnological applications. Compounds from plants that are effective on pathogens and have low toxicity for humans are essential in developing new antimicrobial drugs (6,7).

Ferula sp. belonging to the Apiaceae family have been identified as a rich source of antimicrobial compounds. It contains more than 150 species, mainly in the Mediterranean and Central Asia. For hundreds of years, products from Ferula sp. have been used in traditional medicine for skin infections, diarrhea, killing intestinal parasites, malaria, and microbial diseases (8,9,10,11). Ferula sp. in Turkey are called çakşir, çakşir grass or çaşir. Ferula L., the third largest genus of the Apiaceae family, is 18 in the flora of Turkey, only 9 of which are endemic (12,13). These species contain rich secondary metabolites and antimicrobial phytochemicals such as tannins, alkaloids, terpenoids, flavonoids, and coumarins (13). Studies show that it protects against bacteria and fungi that cause food contamination.

The current study evaluated Ferula sp. obtained from Erzurum province and Tortum town for antimicrobial and antibiofilm activity against pathogenic bacterial and fungal microorganisms.

Materials and Methods

Plant, Microbial Strains and Growth Conditions: *Ferula* species were obtained from Erzurum province and Tortum town in this study. *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *E. faecalis* (ATCC 29212) bacteria, and *C. albicans* (ATCC 10231) and *C. tropicalis* (KUEN 1025) yeasts were used in this study. In a shaking incubator, bacterial cultures were incubated in Mueller Hinton broth (MHB) for 24 hours at 37°C and 120 rpm. In a shaking incubator, yeast cultures were incubated in Potato dextrose broth (PDB) for 24 hours at 30°C and 120 rpm.

Extraction of *Ferula* Sp.: The plants were washed in a 0.04% bleach and then rinsed with distilled water. The plants were divided into sections by weighing 10 g as stems and leaves. These sections were homogenized separately with ethyl acetate and methanol. The shredded samples were taken and incubated in a magnetic shaker at 500 rpm for 2 hours. At the end of the period, the solid particles were separated by filtration and evaporated in the rotary evaporator. The resulting substantial extracts were used for further studies and dissolved with 1% dimethyl sulfoxide (DMSO) before use (14).

Agar Well Diffusion Assay: The agar well diffusion test was used to qualitatively assess the efficacy of antimicrobial activity of *Ferula* sp. Three wells were drilled in each petri dish with a cork borer. 150 µL ml *Ferula* sp. extracts were placed in the wells of agar plates inoculated with the test microorganisms. Petri dishes were incubated at 37°C for 24 hours in a flat position. At the end of the period, the zone diameters were measured. DMSO (1%) was used as a negative control (15).

Microdilution Assay: The microdilution assay was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (16). Briefly, 96-well plates were used to determine the minimum inhibitory concentrations (MIC) of the extracts. 100 µl of bacteria and yeast cells, the concentration of which was adjusted to OD_{600} =0.08-0.1, was added to each well, and the extract was added to the wells in the range of 1-128 µg/mL ml. The total volume was brought to 200 µl. The group with no cells

was added as a negative control, and the group with no extract was added as a positive control. Microorganism growth in the wells was evaluated after 24 hours of incubation at 37 °C. The MIC value was determined as the lowest concentration without visual growth.

Crystal Violet Assay: To evaluate the antibiofilm activities of *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 10231), 96-well plates were cultivated similarly to the microdilution assay. These plates were incubated at 37 °C for 48 hours. After that, all the contents in the wells were discarded and washed three times with phosphate buffer. 200 µl of 0.1% crystal violet dye was added and the plates were incubated in the dark for 20 minutes. Then, the wells were washed and were fixed with 30% acetic acid. An absorbance measurement was taken at 590 nm. The evaluation was made according to negative and positive control (17).

Results

Ethyl acetate and methanol extracts of *Ferula* sp. showed antimicrobial activity against *S. aureus, C. albicans,* and *E. coli*. However, the best results were determined as ethyl acetate extract against *S. aureus* shown in Figure 1 and methanol extract against *E. coli* and *C. albicans*. The zone diameters measured are represented in Table 1. Neither extract showed antimicrobial activity against other pathogenic bacteria.

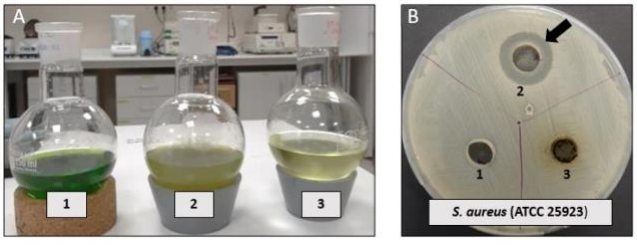


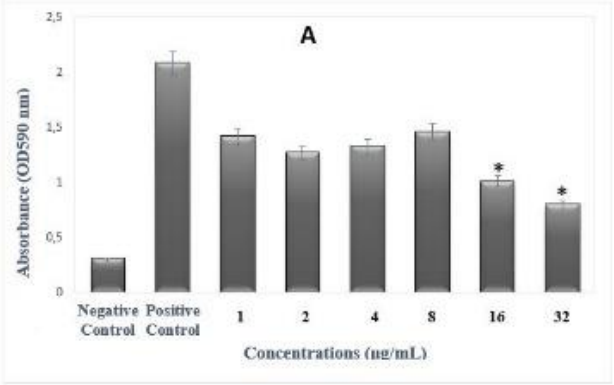
Figure 1. A. Extraction flask of *Ferula* sp. (1) Methanol extract of *Ferula* sp. leaf. (2) Ethyl acetate extract of *Ferula* sp. root. (3) Methanol extract of *Ferula* sp. root. B. Antimicrobial activity of ethyl acetate extract against *S. aureus*.

MIC values of *Ferula* sp. extract were 16, 32 and 32 µg/mL ml against *S. aureus*, *E. coli* and *C. albicans*, respectively (Table 1).

S. aureus and *C. albicans* were selected for antibiofilm activity studies, where we had the best results. The ethyl acetate extract of the collected *Ferula* sp. showed antibiofilm activity against *S. aureus* at increasing concentrations. In addition, the methanol extract showed antibiofilm activity against *C. albicans* at increasing concentrations. Antibiofilm activity graphs are given in Figure 2. While 8 µg/mL ml extract application inhibited biofilm formation in *C. albicans*, 32 µg/mL ml extract application reduced biofilm formation in *S. aureus* by half. Consequently, *Ferula* sp. extract inhibits *C. albicans* biofilm formation at a value four times lower than the MIC value. This shows that it contains molecules with strong antibiofilm effect.

Table 1. Zone diameter (mm) and MIC values (µg/mL) for pathogens

Patogens	Zone diameter (mm)	MIC value (µg/mL)
S. aureus (ATCC 25923)	21	16
E. coli (ATCC 25922)	18	32
C. albicans (ATCC 10231)	15	32



Discussion

Infectious diseases are a significant problem all over the world. In particular, biofilm-related infections cause serious problems and great economic loss. The effect of antibiotics on biofilm-induced infections are

limited (18). Therefore, there is a need for more reliable therapeutic approaches. Ferula sp. extracts have great potential as antimicrobial and antioxidant compounds (8,9). Therefore, it is an essential plant that can be used in therapeutic agents. The aim of this study was to determine whether or not the Ferula species obtained from Erzurum possessed antimicrobial and antibiofilm activity.

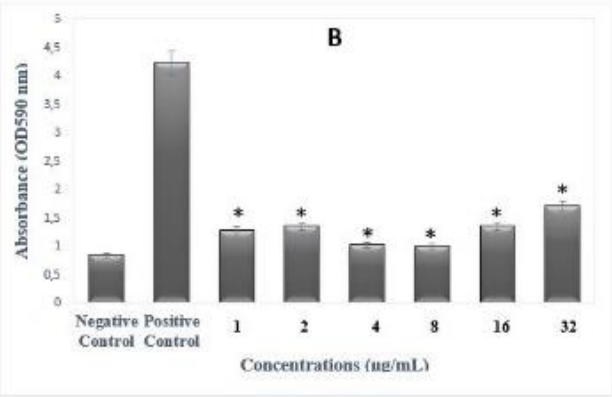


Figure 2. Antibiofilm activity of *Ferula* sp. extracts A. It showed antibiofilm activity at increasing concentrations against *S. aureus*. B. Antibiofilm activity at increasing concentrations against *C. albicans*.

First of all, we performed methanol and ethyl acetate extract of Ferula sp. obtained from the Erzurum province in the current study. Then, we examined the antimicrobial and antibiofilm activities of the obtained extract against the test strains. Many researchers have studied the antibacterial, antifungal and antioxidant properties of Ferula sp. These studies were reviewed in Daneshniya et al, 2021 (18).

Baldemir et al, in 2006, investigated the antimicrobial activity of the methanol extract of the Ferula halophila against various pathogens by disc diffusion method. Chloroform extract of F. halophila did not show activity against E. faecalis, E. coli, P. aeruginosa, and C. albicans. However, its methanol extracts showed a weak effect against Bacillus subtilis, Bacillus cereus and S. aureus (19). We obtained similar results in our study. One reason for the lack of antimicrobial activity of Ferula sp. against P. aeruginosa, E. faecalis, and C. tropicalis maybe that Ferula sp. contain volatile compounds with antimicrobial activity (20). Another reason may be the degradation of a molecule effective against these pathogens during extraction.

Alipour et al. (2014) showed that essential oils of Ferula cupularis have high antimicrobial activity against S. aureus, S. epidermidis, B. subtilis, E. coli, K. pneumoniae, and P. aeruginosa. Also, In addition, the most increased activity was obtained from the roots of F. cupularis (21).

Bhatnagar et al. (2015) found that polar extracts showed more antimicrobial activity than nonpolar extracts in their study with Ferula asafoetida (14). Daneshkazemi et al. (2019) showed that F. assa-foetida extracts are antimicrobial against oral pathogens such as Streptococcus mutans and Streptococcus sanguinis (22).

Karakaya et al. in 2019 showed that essential oils from the aerial parts of F. orientalis obtained from Erzurum were effective against S. aureus and C. albicans. Similar results were obtained in our study. However, in our study, unlike in the article, antimicrobial effects were also observed on E. coli. One reason for this is that we may have worked with different subspecies of Ferula (24).

One of the most important causes of antibiotic resistance is biofilm formation. Therefore, molecules that inhibit biofilm formation need to be discovered. Plant extracts are thought to be rich in molecules with antibiofilm activity. To our knowledge, there is only one study of antibiofilm activity against Candida species. Zomorodian et al, (25) showed that essential oils of F. assafoetida have antibacterial and antifungal activity. They also showed antibiofilm activity on C. albicans, C. tropicalis, and C. krusei. They stated that 4 µL/mL ml essential oil completely inhibited biofilm formation (23). Similarly, we observed antibiofilm activity against C. albicans in our study. Our best result was obtained with 8 µg/mL ml extract. This result indicates that the

concentration required for biofilm inhibition is lower than the MIC value. This shows that Ferula sp. are effective candidate molecules for infections caused by C. albicans biofilm.

Furthermore, the antibiofilm activity of Ferula extract against S. aureus was demonstrated for the first time in this study. Ferula extract inhibited half of the biofilm formation at a value of 32 µg/mL ml. Therefore, Ferula sp. are therapeutic candidate molecules in biofilm-induced infections of S. aureus. However, synergism with different antimicrobials or antibiotics needs to be examined.

In this study, antimicrobial and antibiofilm activities of *Ferula* sp. collected from the Erzurum region were investigated. Antimicrobial activity of *Ferula* sp. against *S. aureus*, *E. coli* and *C. albicans* was observed. It was also found to have antibiofilm activity by increasing concentrations against *S. aureus* and *C. albicans*. This is the first study to show the antibiofilm activity of *Ferula* sp. against *S. aureus*. Further characterization of *Ferula* sp. and our understanding of the nature of their antibiofilm properties are needed.

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