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Antibacterial and Antifungal Activity of *Abies nordmanniana* subsp. *equi-trojani* (Aschers. & Sint. ex Boiss) Extracts

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Research Article

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Abstract

In this study, the antibacterial and antifungal activity of *Abies nordmanniana* subsp. *equi-trojani* cone spangles, which are endemic and endangered and grow in Çanakkale Ida Mountains, was investigated by agar well diffusion and microplate methods. The antibacterial and antifungal activity of distilled water, ethanol, methanol and dimethylsulfoxide (DMSO) extracts of cone spangles was evaluated against gram-positive and gram-negative bacteria and yeasts. As a result, it was determined that the DMSO extract had antibacterial and antifungal activity against the tested microorganisms, while the extract prepared with distilled water had no activity. The antimicrobial activity of the methanol and ethanol extracts varied depending on the microorganism type. When the results of this study were compared with the positive control (penicillin G and fluconazole), it was determined that the extracts were not as effective as the antibiotic and antifungal disc. As a result, it is important to use alternative products that are abundant in nature instead of products derived from endangered species.

Keywords: *A. equi-trojani*, Fir, Ida Mountains, antimicrobial activity

Abies nordmanniana subsp. *equi-trojani* (Aschers. & Sint. ex Boiss) Ekstraktlarının Antibakteriyel ve Antifungal Aktivitesi

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Öz

Bu çalışmada Çanakkale Kazdağlarında yetişen endemik ve yok olma tehlikesiyle karşı karşıya olan *Abies nordmanniana* subsp. *equi-trojani* kozalak pullarının antibakteriyel ve antifungal aktivitesi agar kuyu ve mikroplak yöntemiyle araştırılmıştır. Kozalak pullarının distile su, etanol, metanol ve dimetilsülfoksit (DMSO) ekstraktlarının antimikrobiyal aktivitesi gram pozitif ve gram negatif bakteriler ile mayalara karşı değerlendirilmiştir. Sonuç olarak özellikle DMSO ekstraktının test edilen mikroorganizmalara karşı antibakteriyel ve antifungal aktiviteye sahip olduğu tespit edilmiştir. Distile su ile hazırlanan ekstraktının ise herhangi bir aktiviteye sahip olmadığı belirlendi. Metanol ve etanol ekstraktlarının ise mikroorganizma türüne göre aktivite gösterdiği belirlendi. Bu çalışmanın sonuçları pozitif kontrol (penisilin G ve flukonazol) ile karşılaştırıldığında, ekstraktların antibiyotik ve antifungal disk kadar etkili olmadığı belirlendi. Sonuç olarak nesli yok olma tehlikesiyle karşı karşıya kalan bu türün ürünleri yerine doğada bol bulunan alternatif ürünlerin kullanılması önem arz etmektedir.

Anahtar Kelimeler: *A. equi-trojani*, Köknar, Kazdağı, antimikrobiyal aktivite

Introduction

The genus *Abies* includes more than 50 species worldwide and is widespread in Europe, North Africa, Asia and North America. *Abies* species grow at high altitudes, especially in mountainous areas [1]. Based on morphological and anatomical features such as hairy or hairless shoots, resinous or slightly resinous bud structures, seed structures, shape and size of cones and spangles, the classification of firs growing in Anatolia is well-known to botanists [2]. Four species are common throughout Turkey: *Abies bornmuelleriana* Mattf., *Abies cilicica* Carr., *Abies nordmanniana* Link. and *Abies nordmanniana* subsp. *equi-trojani* Aschers. & Sint. ex Boiss. The homeland of the Turkish Fir *A. bornmuelleriana* species is Central-Northern Anatolia. There are two subspecies of the *A. cilicica*. *A. cilicica* subsp. *cilicica* and *A. cilicica* subsp. *isaurica* are specific to the central-eastern Toros Mountains and are endemic. *A. nordmanniana*, Caucasian fir or Nordmann fir, which has a wide distribution area in the Caucasus extending to Georgia and Russia, is found in northeastern Turkey. *A. nordmanniana* subsp. *equi-trojani* species naturally grows only in the limited area from Southern Çanakkale to the ancient city of Troy and is endemic to that region [1, 2]. Different species of *Abies* are used for therapeutic purposes in folk medicine. *Abies numidica* has been used in folk medicine against lung, cold, stomachache, indigestion, vascular, and venereal diseases [3]. The species *A. nordmanniana* subsp. *equi-trojani* is used as an ointment for wounds and boils due to its microbiocidal effect, and the syrup created by boiling its leaves and fresh green cones is used for respiratory diseases. Additionally, it is also used in furniture production [4]. However, this species is classified as an endangered (in danger of extinction in the near future) tree species by the International Union for Conservation of Nature and has been included in the Red List due to its small population sizes, extinction risks and habitat degradation [1, 5]. It has been determined that extracts and essential oils from different parts of the *Abies* species contain compounds such as phenols, flavonoids, lignans, steroids, triterpenoids and fatty acids [3]. For this reason, there are different studies on the essential oils and extracts of *Abies* species growing in various geographical and ecological conditions [2, 3, 6, 7]. Additionally, studies have found that *Abies* species have different biological activities such as antibacterial, antifungal, antihypertensive, anti-inflammatory, anti-ulcerogenic, antitussive, antitumor, and activity on the central nervous system [3]. Therefore, in this study, the antibacterial and antifungal activity of *A. nordmanniana* subsp. *equi-trojani* cone spangle extracts were investigated.

Materials and Method

Plant Material

Dried cones of *Abies nordmanniana* subsp. *equi-trojani* were obtained from a commercial phytotherapy shop (Malatya Kuruyemiş) in Çanakkale Küçükkuyu, Türkiye, in May 2021.

Preparation of Extracts

The extraction process of the Ida Mountain *A. nordmanniana* subsp. *equi-trojani* cone was carried out using four different solvents: distilled water (DW), methanol (M) (Merck, Germany), ethanol (E) (Isolab, Türkiye), and dimethyl sulfoxide (DMSO) (Merck, Germany). For the extraction of the cone, a 40 g spangle and a 10 g high-speed ground spangle (Figure 1) were mixed, and a 1:10 (w:v) solvent was added. The mixture was then extracted with Soxhlet for 24 hours. After extraction, the extracts were filtered using sterile filter papers, placed in sterile dark-colored bottles, and stored at +4°C.

Figure 1. *Supplied Abies nordmanniana subsp equi-trojani cone spangles and ground powder*

Cultures

In the study, standard cultures and clinical isolates from the collections of Dr. Gülçin ÖZCAN ATEŞ and Dr. Nükhet ZORBA in Çanakkale Onsekiz Mart University Food Engineering Microbiology Laboratory were used.

The Inoculum Suspension Preparation

The stock cultures were inoculated on Tryptic Soy Agar (TSA) (Merck, Germany) and revived overnight at 37°C. After the incubation period, colonies selected from the petri dish were suspended in a physiological saline solution (0.85% NaCl w/v) until the turbidity matched the 0.5 McFarland standard.

Agar Well Diffusion Method

The extracts were tested for their antibacterial and antifungal properties by creating wells on the surface of the Mueller-Hinton Agar (MHA) (Merck, Germany) plates using a cork borer set, instead of using the disk specified in EUCAST [8]. The inoculum suspension was prepared and inoculated onto the dried surface of the MHA plate using a sterile swab, after which 20 µL of the extracted sample was added to the 6mm diameter wells. The Petri dishes were incubated at 37°C for 24 hours, and the zone diameters were measured using a digital calliper. Penicillin G (10 µg, Bioanalyse, Turkey) antibiotic and

fluconazole (25 mcg, Himedia, India) antifungal disc were used as positive controls, while solvents were used as negative control. The study was conducted in triplicate, and the results were presented as mean (M) ± standard deviation (sd).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC value of the DMSO extract, which was found to possess antibacterial and antifungal properties through agar well diffusion tests, was further determined using the microplate method with 96-well U-bottom microplates by CLSI guidelines [9]. First, the extract concentrations were prepared in Muller Hinton Broth (MHB) (Merck, Germany), containing double layers of medium. 100 µL of the prepared concentrations was added to the wells of rows B-G of the microplates, followed by the addition of 100 µL of cell suspension. The final extract concentrations were 40, 20, 10, 5, 2.5 and 1 µL/mL. Row A of the microplates was used as a negative (MHB containing 40 µL/mL extract) control, and row H was used as a positive (MHB+culture) control. After the microplates were incubated for 18-48 hours at 36±1°C, 10 µL of 1% (w/v) 2,3,5 tetrazolium chloride (Merck, Germany) solution was dropped into each well to observe the colour change. The first well with no colour change was determined as the MIC value. The study was carried out in three parallels.

Results

The current study includes the antibacterial and antifungal activity results of distilled water, ethanol, methanol and DMSO extracts of *A. nordmanniana* subsp. *equi-trojani* cone spangles against eight clinical isolates, 14 standard bacteria and six standard yeast cultures. As seen in Tables 1, 2 and 3 it was determined that DMSO extract exhibited antibacterial activity against the tested bacterial cultures. Although it was found that extracts did not have any antifungal activity against the five *Candida* cultures tested, it was recorded that ethanol, methanol, and DMSO extracts showed antifungal activity against the *C. neofermans* ATCC 90112 yeast (Table 3).

Table 1. *Agar well diffusion results of Abies nordmanniana subsp. equi-trojani cone spangles extract against Gram-negative bacteria (in mm)*

Microorganisms	**DW**	**M**	**E**	**DMSO**	**Penicillin G**
Acinetobacter baumannii (clinical isolate 1048)	6.00±0.01	6.00±0.01	9.77±0.63	11.17±0.86	15.48±0.17
Acinetobacter baumannii (clinical isolate 1132)	6.00±0.01	6.00±0.01	10.06±0.73	11.56±0.97	6.43±1.48
Acinetobacter baumannii (clinical isolate 2685)	6.00±0.01	6.00±0.01	9.68±0.92	15.27±1.80	6.00±0.01
Escherichia coli ATCC 8739	6.00±0.01	6.00±0.01	6.00±0.01	11.04±2.47	21.45±1.77
Escherichia coli ATCC 25922	6.00±0.01	6.00±0.01	6.00±0.01	8.55±0.21	20.63±1.07
Escherichia coli NRRL B-3704	6.00±0.01	6.00±0.01	6.00±0.01	10.28±0.38	10.11±1.11
Klebsiella quasipneumoniae ATCC 700603	6.00±0.01	7.89±0.69	7.71±2.57	9.91±1.57	6.55±1.90
Klebsiella pneumoniae (clinical isolate 1042)	6.00±0.01	6.00±0.01	6.00±0.01	6.00±0.01	7.26±0.21
Salmonella enterica subsp. *enterica* ATCC 13076	6.00±0.01	8.75±1.47	6.00±0.01	8.40±1.25	22.16±0.35
Pseudomonas aeruginosa ATCC 10145	6.00±0.01	6.00±0.01	6.00±0.01	8.61±0.16	11.43±0.28
Pseudomonas aeruginosa ATCC 27853	6.00±0.01	6.00±0.01	6.00±0.01	10.42±1.03	6.00±0.01
Proteus vulgaris ATCC 13315	6.00±0.01	6.00±0.01	6.00±0.01	7.00±0.01	19.47±0.88

Table 2. *Agar well diffusion results of Abies nordmanniana subsp. equi-trojani cone spangles extract against Gram-positive bacteria (in mm)*

Microorganisms	DW	M	E	DMSO	Penicillin G
Basillus subtilis	6.00±0.01	6.00±0.01	6.00±0.01	10.62±0.55	21.47±0.77
Basillus subtilis subsp. *spizizenii* ATCC 6633	6.00±0.01	9.72±2.51	10.44±1.89	15.79±4.30	6.00±0.01
Enterococcus faecalis ATCC 29212	6.00±0.01	6.00±0.01	6.00±0.01	10.37±1.23	6.00±0.01
Enterococcus faecalis (clinical isolate 1075)	6.00±0.01	6.00±0.01	6.00±0.01	9.71±0.52	8.98±0.40
Staphylococcus aureus ATCC 29213	6.00±0.01	8.52±1.24	6.00±0.01	11.00±1.14	23.42±2.20
Staphylococcus aureus ATCC 25923	6.00±0.01	6.00±0.01	6.00±0.01	10.06±1.59	11.52±0.59
Staphylococcus aureus ATCC 6538	6.00±0.01	6.00±0.01	6.00±0.01	9.31±0.31	22.55±0.36
Staphylococcus epidermidis (clinical isolate 2657)	6.00±0.01	12.03±1.20	6.00±0.01	13.12±1.83	6.00±0.01
Staphylococcus epidermidis (clinical isolate 2671)	6.00±0.01	6.00±0.01	12.55±1.35	11.88±2.46	6.00±0.01
Staphylococcus gallinarum (clinical isolate 1093)	6.00±0.01	12.88±3.16	11.99±1.45	13.69±1.34	28.63±1.91

Table 3. *Agar well diffusion results of Abies nordmanniana subsp. equi-trojani cone spangles extract against yeast (in mm)*

Microorganisms	DW	M	E	DMSO	Fluconazol
Candida albicans ATCC 10231	6.00±0.01	6.00±0.01	6.00±0.01	6.00±0.01	39.33±1.13
Candida albicans ATCC 24433	6.00±0.01	6.00±0.01	6.00±0.01	6.00±0.01	37.57±0.56
Candida albicans ATCC 90028	6.00±0.01	6.00±0.01	6.00±0.01	6.00±0.01	37.97±1.02
Candida tropicalis ATCC 1021	6.00±0.01	6.00±0.01	6.00±0.01	6.00±0.01	27.77±0.46
Candida parapsilosis ATCC 22019	6.00±0.01	6.00±0.01	6.00±0.01	6.00±0.01	36.25±0.94
Cryptococcus neofermans ATCC 90112	6.00±0.01	11.69±1.66	12.80±1.87	11.10±1.49	37.77±0.04

Agar well diffusion results of DMSO extract showed that the inhibition zone diameter ranged from 7.00±0.01 to 15.27±1.80 mm. It was determined that the lowest inhibition zone diameter was against *P. vulgaris* ATCC 13315, and the highest inhibition zone diameter was against the clinical isolate *A. baumannii* (2685). The antifungal activity of DMSO extract against yeasts was detected only against *C. neofermans* ATCC 90112, and the inhibition zone diameter was found to be 11.10±1.49 mm. The ethanol extract has been detected, giving an inhibition zone between 7.71±2.57 and 12.80±1.87 mm against three *A. baumanni* clinical isolates, *K. quasipneumoniae* ATCC 700603, *B. subtilis* subsp. *spizizenii* ATCC 6633 *S. epidermidis* (clinical isolate 2671), *S. gallinarum* (clinical isolate 1093), and *C. neofermans* ATCC 90112. It was determined that the methanol extract showed antimicrobial activity against *K. quasipneumoniae* ATCC 700603, *S. enterica* subsp. *enterica* ATCC 13076, *B. subtilis* subsp. *spizizenii* ATCC 6633, *S. aureus* ATCC 29213, *S. epidermidis* (clinical isolate 2657), *S. gallinarum* (clinical isolate 1093), and *C. neofermans* ATCC 90112 and gave an inhibition zone between 7.89±0.69 and 12.88±3.16 mm. It was found that the extract prepared with distilled water did not exhibit both antibacterial and antifungal activity. Additionally, it was found that DMSO extracts had a smaller inhibition zone against 12 bacteria (-178.14% to -14.51%) and a larger inhibition zone against 13 bacteria (1.65% to 62%). The MIC value of DMSO extract, which was found to have antibacterial and antifungal activity, was determined. Figure 1 shows that the MIC value for most of the microorganisms tested was 40 µL/mL. The lowest MIC value of the DMSO extract was determined to be 2.5 µL/mL against the *S. epidermidis* (clinical isolate 2657) isolate, and the second lowest MIC value was 5 µL/mL against the *A. baumannii* (clinical isolate 1048) isolates. However, the MIC value against the cultures of *E. coli* NRRL B-3704, *P. aeruginosa* ATCC 10145 and ATCC 27853, *E. faecalis* (clinical isolate 1075), *S. aureus* ATCC 25923 and 6538 was outside the tested concentrations, and the MIC value was >40 µL/mL.

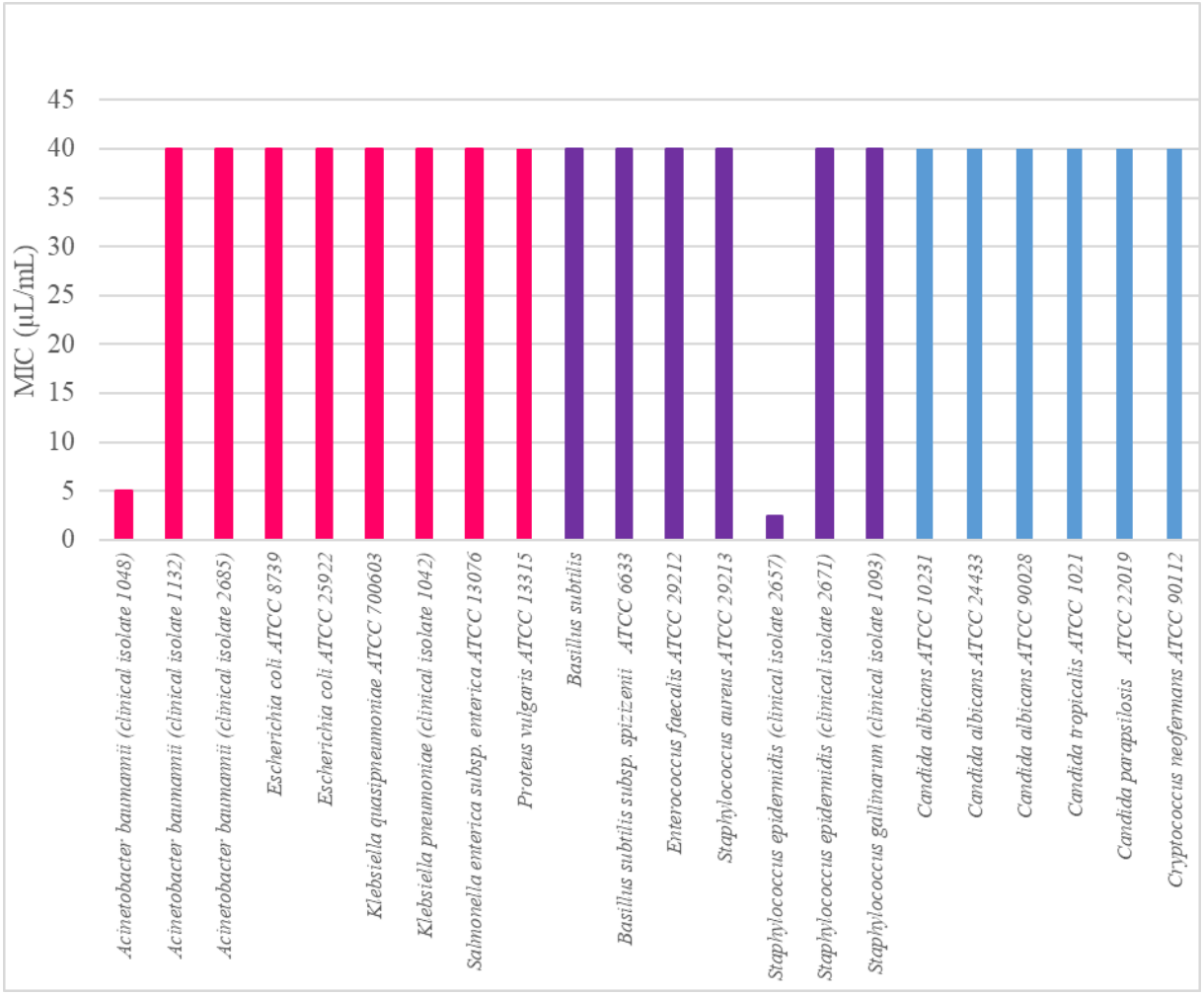

Figure 1. *MIC values of Abies nordmanniana subsp. equi-trojani cone spangles DMSO extract*

Discussion

It was determined that the *A. nordmanniana* subsp. *equi-trojani* cone spangle extracts used in this study were not as effective as the positive control antibiotic and antifungal disks. Bağcı and Dığrak [10] determined that the essential oil they extracted from the needle branches of *A. equi-trojani* by hydrodistillation for three hours did not have antimicrobial activity against *E. coli, S. aureus* and *S. cerevisiae*. Sakar et al. [11] reported that methanol, ethanol and chloroform extracts of *A. nordmanniana subsp. equi-trojani* cones collected from Ida Mountain Balıkesir in 1991 gave a 9 mm inhibition zone against *B. subtilis* ATCC 6633 at a 4000 µg/disc concentration. They found that chloroform and methanol extract gave 7- and 10-mm inhibition zones against *S. aureus*. They found that it did not give an inhibition zone against *S.* Typhimurium CMC 583, *E. coli* ATCC 11230 and *Candida utilis*. Eryılmaz et al. [12] reported that the ether extract of Balıkesir *A. nordmanniana subsp. equi-trojani* cones gave a 7 mm inhibition zone against *B. subtilis* ATCC 6633 and *P. aeruginosa* ATCC 27853. They noted that it did not show any inhibition against *S. aureus* ATCC 43300 (methicillin-resistant S. aureus), *E. coli* ATCC 25922, *K. pneumoniae* RSKK 574, and *C. albicans* ATCC 10231. Öztürk Pulatoğlu et al. [7] collected *A. nordmanniana subsp. equi-trojani* from Kastamonu in 2017. They determined that the

essential oil obtained from *A. nordmanniana subsp. equi-trojani* cones by Clevenger hydrodistillation had MIC values between 0.195 and 25 µg/ml against the gram-negative and gram-positive bacteria they tested. It has been determined that *A. nordmanniana subsp. equi-trojani* species collected at different times and places in our country have different effects on bacteria and yeasts, and the results of this study are parallel to the results in literature.

Conclusions

Over the last fifty years, the population of Fir trees has decreased significantly due to several factors, including environmental conditions and wildfires, like the 2023 Çanakkale forest fires. However, in Çanakkale districts, people still believe that the cones of the Kazdağı fir are good for respiratory diseases, so they consume the cones by brewing them as tea. These cones are also sold commercially. However, different studies found that Ida Mountain fir extracts were less effective than antibiotic substances. Therefore, it is recommended to use herbal products with higher antimicrobial activity that are abundant in our environment instead of this endemic species that is in danger of extinction. Additionally, it is essential to raise public awareness about this issue to ensure the continuity of the species.

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Conflicts of Interest The authors declared no conflict of interest.

Authors Contribution Gülçin ÖZCAN ATEŞ's contribution to the article is 50%; she planned the project, analysed and wrote the article. Tülay BİCAN SÜERDEM's contribution to the article was 50%; she analysed and wrote the article.

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