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ORIGINAL ARTICLE

Screening of Antimicrobial, Antibiofilm, and Cytotoxic Activities of Some Medicinal Plants from Balıkesir Province, Türkiye: Potential Effects of *Allium paniculatum* Flower

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ABSTRACT

Objectives: Plant extracts are important natural resources that may have antimicrobial and antibiofilm effects against pathogens. This study was conducted to investigate the *in vitro* antimicrobial activities of methanol extracts of some medicinal plants (*Achillea nobilis* subspecies *neilreichii* (A. Kern.) Velen., *Aetheorhiza bulbosa* (L.) Cass, *Allium paniculatum* L, *Asphodelus aestivus* Brot., *Ballota nigra* L., *Cistus laurifolius* L., *Cistus salviifolius* L., *Dioscorea communis* (L.) Caddick and Wilkin, *Galium verum* L., *Hypericum triquetrifolium* Turra, *Paliurus spina-christi* Mill., *Primula vulgaris* Huds. subspecies *rubra* (Sm.) Arcang., *Ranunculus arvensis* L. and *Teucrium polium* L.) from Balıkesir province in Türkiye.
Materials and Methods: Preliminary antimicrobial activity screening was conducted for all extracts. Antibiofilm activity studies were conducted on mature *Candida albicans* biofilms. Moreover, the cytotoxicities of *A. paniculatum* flower extract on A549 and Vero cell lines were determined using a colorimetric tetrazolium-based assay.
Results: *A. paniculatum* flower, *P. vulgaris* root, *C. laurifolius, C. salviifolius,* and *A. nobilis* displayed good activity [minimum inhibitory concentrations (MIC): 9.75, 156, 312, 312 and 312 µg/mL, respectively] against *C. albicans* American Type Culture Collection 10231. Biofilm studies were conducted on these plant extracts. The methanol extract of *A. paniculatum* flower decreased the number of *C. albicans* [colony-forming unit (CFU)/mL] in mature biofilm statistically at 32 x MIC and higher concentrations ($p < 0.01$). *A. paniculatum* flower extract had a cytotoxic effect (killing more than 50% of cells) at high concentrations, and its effect on Vero cells was similar to that on A549 cells.
Conclusion: This study demonstrated the importance of the methanol extract of *A. paniculatum* flower as a natural alternative against *C. albicans* infections, including biofilms.
Keywords: *Allium paniculatum*, antibiofilm activity, antimicrobial activity, cytotoxic activity

INTRODUCTION

Researchers around the world have been exploring the benefits of medicinal herbs for treating various diseases for many years.[1] An important part of these studies is investigating their effects against human pathogens. The effectiveness of antibiotics against pathogens is gradually disappearing, and plants are an important resource for researchers.

The treatment of bacterial and fungal infections has become a significant health concern in recent years because of the rise of multidrug resistance. Apart from the challenge of antibiotic

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resistance, the formation of biofilm by bacteria and fungi on medical devices inserted into the body, such as urinary catheters, central venous catheters, and contact lenses, further complicates the management of these infections.[2,3] Biofilm is a community of microorganisms that irreversibly bind to a specific surface or living tissue and are embedded in a self-secreted extracellular matrix. Biofilms show more resistance to antibiotics and host defense systems than planktonic cells. Therefore, high doses of antibiotics must be used for treatment, resulting in unwanted side effects.[4,5] Because biofilm formation on catheters and medical devices is a major challenge for treatment, biofilm removal is difficult except for device removal and/or replacement, which is an undesirable or high-risk procedure. Therefore, it is important to investigate natural antimicrobial agents for biofilm treatment.[6,7]

Studies have shown that some plant extracts can inhibit quorum sensing, thus preventing the formation of biofilms and being effective on mature biofilms. Therefore, plant extracts can be an effective source for antibiofilm therapy, because of the active molecules found in their structure.[8]

Balıkesir province in Türkiye is located in western Anatolian, on the border between the Marmara and Aegean regions with a surface area of 14.299 km^2. It is adjacent to Bursa in the northeast, Kütahya and Manisa in the southeast, İzmir in the southwest, the Aegean Sea and Çanakkale in the west. Because of its climatic characteristics, geological structure, and geographic location, the region features diverse flora. For this purpose, in this study, *in vitro* studies were conducted with 14 plants (17 different extracts) belonging to the Balıkesir province. The following plants were used in the study; *Achillea nobilis* L. subspecies *neilreichii* (A. Kern.) Velen., *Aetheorhiza bulbosa* (L.) Cass., *Allium paniculatum* L, *Asphodelus aestivus* Brot., *Ballota nigra* L., *Cistus laurifolius* L., *Cistus salviifolius* L., *Dioscorea communis* (L.) Caddick and Wilkin, *Galium verum* L., *Hypericum triquetrifolium* Turra, *Paliurus spina-christi* Mill., *Primula vulgaris Huds.* subspecies *rubra* (Sm.) Arcang., *Ranunculus arvensis* L. and *Teucrium polium* L. These plants have a very important ethnobotanical value, and their importance for treating different diseases has been recorded in the literature. The selected plants are commonly used for wound healing, diarrhea, urinary tract infections, stomach pain, fungal infections, and cough by local people in Balıkesir.[9-13]

A. paniculatum belongs to the *Amaryllidaceae* family and the genus *Allium* L. The genus *Allium* contains many species that are frequently used as food and natural remedies.[14] The local name of *A. paniculatum* in Balıkesir is "yoğurtçuk". The aerial part is cooked as a meal with eggs and is freshly eaten.[15] In this study, the antimicrobial and antibiofilm properties of medicinal plant extracts from the Balıkesir province were investigated. Further studies were conducted with *A. paniculatum*, which showed promising antibiofilm activity against *Candida* spp. Moreover, the cytotoxic activity of *A. paniculatum* was screened on the A549 and Vero cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MATERIALS AND METHODS

Collection and identification of plants

The plant species were collected from Savaştepe and Kepsut (Balıkesir). Studied species, herbarium numbers, and localities are given in Table 1. The plants were identified and the voucher specimens were deposited in the ISTE (Herbarium of the Faculty of Pharmacy of İstanbul University).

Plant extracts

Plant materials were air-dried and extracted by percolation at room temperature with 95% methanol. Then, the obtained

Table 1. Studied species information

Species	Locality	Herbarium number
Achillea nobilis subspecies *neilreichii*	Balıkesir, Kepsut; Kayacıklar village, 440 m.	ISTE 109654
Aetheorhiza bulbosa	Balıkesir, Savaştepe forest, 300 m.	ISTE 109632
Allium paniculatum	Balıkesir, Savaştepe; Karaçam village, 285 m.	ISTE 109758
Asphodelus aestivus	Balıkesir, Kepsut; Örenli village, 550 m.	ISTE 109969
Ballota nigra	Balıkesir, Kepsut; Bükdere village, 630 m.	ISTE 109820
Cistus laurifolius	Balıkesir, Kepsut; Bükdere village, 660 m.	ISTE 109587
Cistus salviifolius	Balıkesir, Kepsut; Serçeören village, 720 m.	ISTE 109591
Dioscorea communis	Balıkesir, Kepsut; Bükdere village, 600 m.	ISTE 109674
Galium verum	Balıkesir, Savaştepe; Soğucak village, 430 m.	ISTE 109947
Hypericum triquetrifolium	Balıkesir, Kepsut; Örenli village, 550 m.	ISTE 109716
Paliurus spina-christi	Balıkesir, Savaştepe; Kocaören village, 522 m.	ISTE 109894
Primula vulgaris subspecies *rubra*	Balıkesir, Kepsut; Örencik village, 730 m.	ISTE 109887
Ranunculus arvensis	Balıkesir, Savaştepe; Madenmezar village, 450 m.	ISTE 109890
Teucrium polium	Balıkesir, Kepsut; Örenharman village, 535 m.	ISTE 109781

methanolic solvents were then concentrated in a rotavapor at a low temperature. The resulting dense extract was dried in a lyophilizer. Dried extracts were stored at -180 ˚C.

Broth microdilution assay

The broth microdilution assay was performed to determine the *in vitro* antimicrobial activities of the plant extracts according to the Clinical and Laboratory Standards Institute guidelines against *Staphylococcus aureus* American Type Culture Collection (ATCC) 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 8739, *Proteus mirabilis* ATCC 43071, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352, and *Candida albicans* ATCC 10231.[16,17] The plant extracts were weighed in the desired amount, and dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, USA) was used to prepare the concentrations. Two-fold serial dilutions were performed on the extracts, ranging from 1250-0.6 µg/mL, in the microplate and treated with 5 x 10^5 colony-forming unit (CFU)/mL for the bacteria and 0.5 x 10^3 to 2.5 x 10^3 CFU/mL for the yeast final inoculum and incubated at 37 ˚C. The lowest concentration at which no growth was observed the next day was determined as the minimum inhibitory concentration (MIC). In addition, DMSO was tested against the test microorganisms. The studies were repeated at least three times.

Inhibitory effects of plant extracts on C. albicans mature biofilms

Considering the results of the study, *the in vitro* effects of the methanol extracts of *A. paniculatum* flower, *C. laurifolius, C. salviifolius, P. vulgaris* root, and *A. nobilis* on mature *C. albicans* biofilms were investigated because they were determined to have inhibitory effects on *C. albicans* planktonic cells. The overnight culture of *C. albicans* ATCC 10231 was diluted in Brain Heart Infusion Broth at 1 x 10^6 CFU/mL, and the prepared suspensions were transferred to 96-well polystyrene flat bottom microplates and incubated at 37 ˚C for 24 hours. After biofilm formation, the medium was carefully aspirated, and the wells were washed twice using sterile phosphate-buffered saline (PBS). The desired concentrations of plant extracts were added to the appropriate wells and incubated for an additional 24 hours. After incubation, the wells were washed twice with PBS. Finally, PBS was placed in the wells and sonicated for 5 minutes in an ultrasonic cleaner, and the biofilm was disintegrated. Microplates were then vortexed at 900 rpm. This process was repeated twice, and the collected supernatants were diluted and planted onto Tryptic Soy Agar. The CFU values were determined by counting the colonies. The logarithms of the CFU values were implemented in the GraphPad Prism program, and the results were expressed graphically.

Cell culture and effects of A. paniculatum flower extract on cells

The effects of *A. paniculatum* flower`s methanol extract, which was found to be significantly effective on both planktonic and biofilm cells of *C. albicans*, on the human lung cancer cell line (A549, ATCC®-185™) and African green monkey kidney cell line (Vero, ATCC® continuous cell line-81™) were determined. Culture media included Dulbecco's modified Eagle's medium (DMEM; Gibco; USA) supplemented with 10% fetal bovine serum (FBS; Gibco; USA) and 1% Penicillin-Streptomycin (Sigma, USA). 10^4 cells were cultured in wells of the 96-well flat-bottom microplate. Then, they were kept for 24 hours at 37 ˚C in a humidified incubator containing 5% CO_2. After incubation, increasing concentrations of the extract (9.75-1250 µg/mL) were placed into the corresponding wells and incubated for an additional 24 hours. To determine the viability of cells, MTT assay was performed. Following exposure, MTT stock solution (5 mg/mL) was prepared in PBS. After discarding the medium from the wells, 10 µL of MTT solution and 90 µL DMEM without phenol red were added to all wells and kept in the incubator for 3 hours. In addition, positive and negative controls were added, and upon incubation, the supernatants were removed. To dissolve the formazan crystals formed in the wells, 100 µL DMSO was added to the wells, and the plate was left in a shaker for 10 minutes. Optical density$_{570}$ was measured using a microplate reader (EON-BioTek Instruments, Winooski, VT, USA).

Statistical analysis

To evaluate the results statistically, GraphPad Prism 8 was used. Results are represented as mean value ± standard deviation. The data was subjected to One-Way analysis of variance (ANOVA), and subsequently, Tukey's post hoc test was employed. A significance level of $p < 0.05$ was used to determine statistical significance.

RESULTS

Antimicrobial activities of the extracts

The antimicrobial activity results are shown in Table 2. All studied extracts displayed better antimicrobial activity against *C. albicans* than against bacteria. According to the antibacterial activity results, *C. salviifolius* methanol extract showed the highest activity against *S. aureus*. The antimicrobial efficacy of any extract against *E. coli* and *K. pneumoniae*, which are important Gram-negative pathogens, could not be determined. However, significant activities were observed against *C. albicans,* especially for the methanol extract of *A. paniculatum* flower (9.75 µg/mL). Methanol extracts of *P. vulgaris* root, *C. laurifolius*, *C. salviifolius*, and *A. nobilis* subspecies *neilreichii* above ground showed 156, 312, 312 and 312 µg/mL MIC values against *C. albicans*, respectively.

Antibiofilm activity

Because the effects of antimicrobial agents against the biofilms of microorganisms are much lower than planktonic forms, and therefore high doses are needed for treatment, biofilm studies have been conducted with at least four times the MIC values of the extracts. According to the MIC (µg/mL) values of plant extracts; 4 x MIC and 8 x MIC of *C. laurifolius, C. salviifolius,* and *A. nobilis*; 4 x MIC, 8 x MIC and 16 x MIC of *P. vulgaris*; 8 x MIC, 16 x MIC, 32 x MIC, 64 x MIC, and 128 x MIC of *A. paniculatum* were prepared. The inhibitory properties of the studied concentrations on *C. albicans* ATCC 10231 mature biofilms were investigated.

Table 2. Antibacterial and antifungal activities of the plant extracts (µg/mL)

	Microorganisms							
Plant extracts	***S. aureus* ATCC 29213**	***S. epidermidis* ATCC 12228**	***E. faecalis* ATCC 29212**	***E. coli* ATCC 25922**	***K. pneumoniae* ATCC 4352**	***P. aeruginosa* ATCC 27853**	***P. mirabilis* ATCC 14153**	***C. albicans* ATCC 10231**
A. bulbosa tuber	1250	› 1250	625	› 1250	›1250	› 1250	› 1250	› 1250
A. paniculatum bulbous	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
A. paniculatum flower	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	9.75
A. aestivus root	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
B. nigra leaf	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
C. laurifolius leaf	625	› 1250	› 1250	› 1250	› 1250	625	› 1250	312
C. salviifolius leaf	312	› 1250	1250	› 1250	› 1250	625	625	312
D. communis root	1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
D. communis leaf	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
G. verum aerial part	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
H. triquetrifolium aerial part	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
P. spina-christi root	625	› 1250	› 1250	› 1250	› 1250	625	625	› 1250
P. vulgaris subspecies *rubra* root	› 1250	› 1250	1250	› 1250	› 1250	› 1250	› 1250	156
P. vulgaris subspecies *rubra* leaf	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
R. arvensis aerial part	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
T. polium aerial part	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250
A. nobilis subspecies *neilreichii* aerial part	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	› 1250	312

According to the results, 32 x MIC, 64 x MIC, and 128 x MIC concentrations of *A. paniculatum* flower methanol extract significantly inhibited *C. albicans* biofilm, whereas no effect was detected in other extracts (Figure 1).

Cytotoxicity

After determining the significant effects of the methanol extract of *A. paniculatum* flower on *C. albicans*, its cytotoxicity to A549 and Vero cell lines was also determined. When the results were analyzed statistically, it was revealed that all studied concentrations caused a statistically significant reduction ($p < 0.05$) in the percentage of viable cells (Figure 2). Based on these findings, 312.5 µg/mL and higher concentrations of the extract inhibited cell viability by more than 50% in both cell lines. Cytotoxicity was higher in Vero cells when lower concentrations were compared.

DISCUSSION

Because of the increasing antibiotic resistance to pathogens, the search for new antimicrobial agents is a high priority. There is a growing trend toward natural products as an alternative drug source, and the antimicrobial properties of plants are being widely studied as a solution against multidrug-resistant pathogens.[18] This study was undertaken to understand the *in vitro* properties of 17 plant extracts belonging to the Balıkesir province of Türkiye. In addition to investigating the antimicrobial activities of the herbal extracts used in our study, the possible

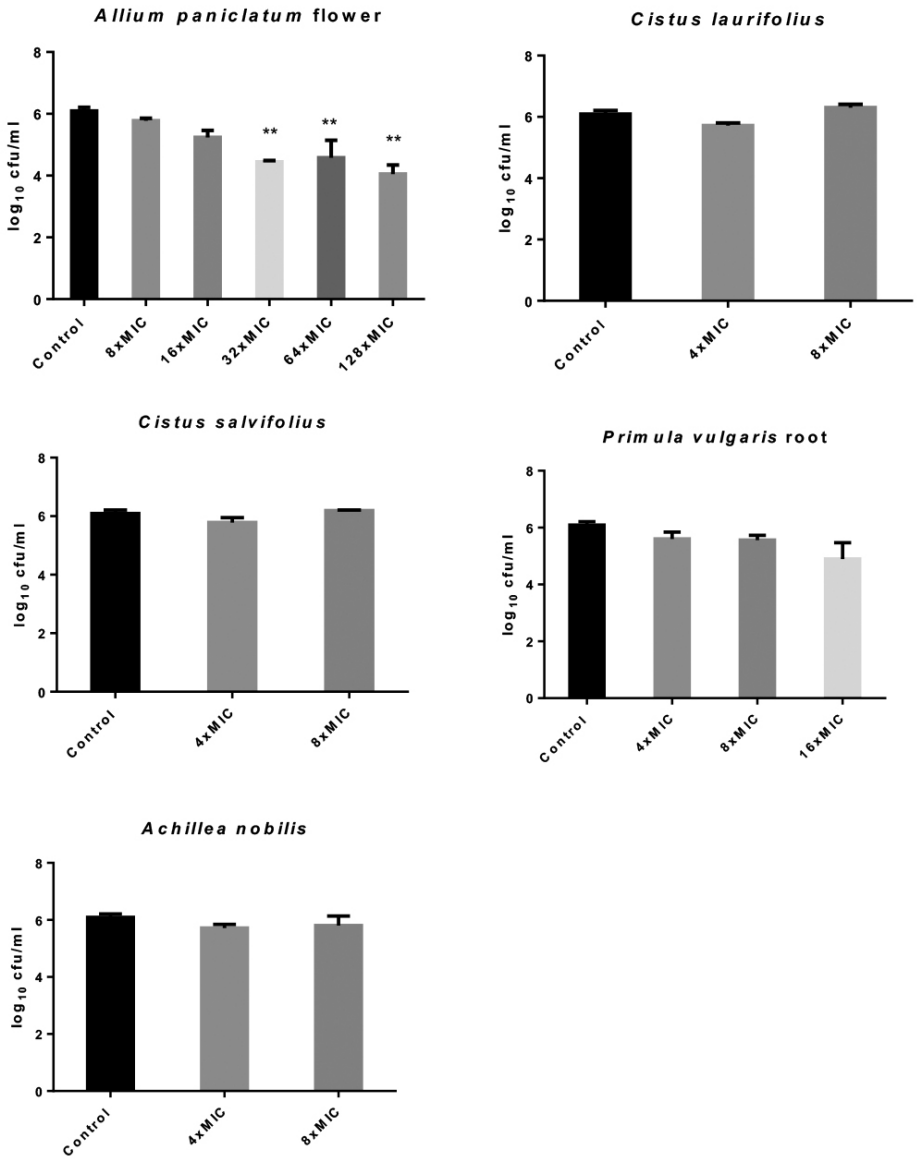

Figure 1. Antibiofilm activities of extracts against *Candida albicans*, $^{**}p < 0.01$

cytotoxicities of the extracts, which had promising antimicrobial effects, were also investigated.

The traditional uses of medicinal plants provide an idea for activity studies. In this study, traditionally utilized plants for their beneficial effects on wound healing in the Balıkesir province of Türkiye were selected for the activity studies. Wound infection is widely prevalent and is a significant clinical obstacle to wound healing. Consequently, exploring the potential antimicrobial properties of plants traditionally employed in wound healing practices is promising. The tuber part of *A. bulbosa* was taken with water for hemorrhoids, intestine problems, constipation, heel cracked, and allergy. The roots of *A. aestivus* are grated and cooked with tarhana (Turkish soup mixture) and applied to the skin for abscesses. The infusion of *B. nigra* leaves is used for colds and stomachaches. The roots of *D. communis*

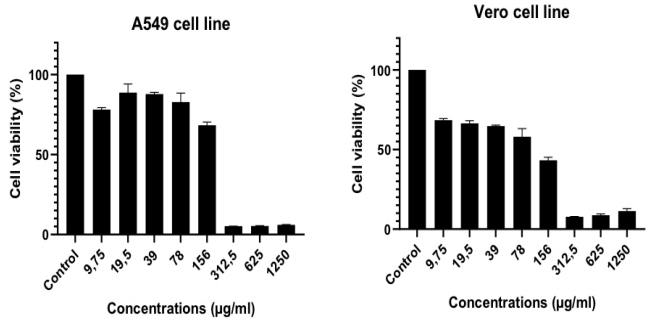

Figure 2. Effects of *Allium paniculatum* flower extract on A549 and Vero cell viability (%)

are used as a decoction for hemorrhoids. The aerial part of *G. verum* is crushed and applied to the skin for wound healing. The infusion prepared from the aerial part of *H. triquetrifolium* is used externally for wound treatment. The roots of *P. spina-christi* are used for allergy and itching. The aerial part of *R. arvensis* is applied on the skin for eczema, abscess, joint pain, and allergy. The aerial part of *A. nobilis* subspecies *neilreichii* is used to treat acne, wound healing, abdominal pain, cough, pain relief, and gynecological diseases. The aerial part of *A. paniculatum* is eaten for health.[11-13,19]

In the Balıkesir region, the roots of *P. vulgaris* are collected and sold by the local people for rheumatism treatment. According to Kahraman et al.,[20] the butanol fraction of *P. vulgaris* roots exhibited the strongest wound-healing efficacy. Primulasaponin I (1) and primulasaponin I methylester (2) were identified as the main active molecules using activity-guided fractionation and isolation procedures.[20] In our study, although the methanol extract derived from *P. vulgaris* root demonstrated efficacy against *C. albicans* (MIC: 156 µg/mL), it did not exhibit noteworthy activity against other tested bacteria.

Ethnomedicinal applications of *Cistus* species are prevalent. The aerial part of *C. laurifolius* is used for diarrhea, urinary tract infections, stomach pain, fungal infection between the fingers, cough, and kidney stones. The aerial part of *C. salviifolius* is used for snake bites, burns, wound healing, diarrhea, urinary tract infections, and prostate.[11-13] Based on the findings of this study, *C. salviifolius* methanol extract showed the highest antibacterial activity (MIC: 312 µg/mL) against *S. aureus* compared with all other extracts. The efficacy of *C. salviifolius* against *S. aureus* has also been confirmed by a previous study conducted by Álvarez-Martínez et al.[21] tested *C. salviifolius* extracts against 100 *S. aureus* clinical isolates and MIC_{50} values were found as 50-80 µg/mL. In addition, it was shown that higher antibacterial activity against methicillin-resistant *S. aureus* isolates than sensitive ones was observed because it contains hydrolyzable tannins and flavonoids such as myricetin and quercetin derivatives.[21] High-performance liquid chromatography revealed the presence of (+)-catechin, (-)-epigallocatechingallate, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, and luteolin in hydroethanolic extracts of five *Cistus* species, including *C. laurifolius* and *C. salviifolius*.[22] Three flavonoids were identified as the primary active components from the *C. laurifolius* ethanol extract: 3,7-O-dimethylquercetin, 3,7-O-methylkaempferol, and 3-O-methylquercetin, which are responsible for strong antinociceptive and anti-inflammatory activities.[23] Therefore, further studies should be conducted to understand its antimicrobial activity better. However, because the highest antimicrobial activity was determined against *C. albicans* in our study, biofilm studies were continued with *C. albicans*.

Although *C. albicans* is a harmless commensal fungus found in the oral cavity or gastrointestinal tract, it is also an opportunistic pathogen that can cause infections. Antimicrobial resistance threatens the treatment of *C. albicans*. In this study, the efficacy of some plant extracts against *C. albicans* was promising. *A. paniculatum* flower extract showed the highest activity (MIC: 9.75 µg/mL), followed by *P. vulgaris* root extract (MIC: 156 µg/mL), *C. laurifolius* (MIC: 312 µg/mL), *C. salviifolius* (MIC: 312 µg/mL), and *A. nobilis* extracts (MIC: 312 µg/mL). According to the MIC results, the methanol extract of the bulb of *A. paniculatum* was found to be ineffective against all microorganisms, including *C. albicans*, whereas the methanol extract of the flower displayed high activity against *C. albicans*. Different parts of *A. paniculatum* have different total flavonoid and phenolic contents; therefore, their antioxidant and enzyme inhibitory properties may vary.[14] The reason for the different antimicrobial activities may be the different contents of the extracts.

Antimicrobials may be up to 1000 times less effective on biofilms than planktonic cells; therefore, biofilms are challenging to eradicate.[24] In this study, it was shown that at least 32 times the MIC value (312 µg/mL) of the methanol extract of *A. paniculatum* flower significantly inhibited *C. albicans* biofilms. The leaves and bulbs of *Allium* plants are known for their antimicrobial properties because of their high thiosulfinate content, especially allicin, polyphenols, and flavonoids. In a recent study, Barbu et al.[25] investigated the antimicrobial activity of hydroalcoholic extracts of six *Allium* species, including *Allium sativum* L. and *Allium ursinum* L. According to their results, both extracts have shown antimicrobial activity against *Candida* species and *S. aureus*. Different studies on the *Allium* genus but different species have also determined the efficacy against *Candida* and *Candida* biofilms.[26,27] However, to date, there have been very few studies on *A. paniculatum* in the literature, but no data showing anti-*Candida* activity.

Organosulfur compounds (such as allicin, ajoenes, dialkenyl and dialkyl sulfides) and saponins found in the structure of *Allium* species have antimicrobial and cytotoxic properties.[28] Although these studies are generally carried out with the bulbs of *Allium* species, it has been shown that the methanol extract obtained from the flowers also contains saponins.[29] Mskhiladze et al.[29] demonstrated that the methanolic extract of *A. leucanthum* flowers inhibited the growth of A549 cells (IC_{50} of 15 ± 3 µg/mL). In our study, the methanol extract of *A. paniculatum* flowers, another *Allium* species, at concentrations of 9.75 µg/mL and higher, statistically inhibited the growth of A549 cells, but more than 50% inhibition occurred after 312.5 µg/mL. Furthermore, it was determined that the cytotoxic effect on cancer cells was similar to that on normal, non-cancerous Vero cells.

CONCLUSIONS

Consequently, the incidence, diagnosis, and clinical severity of *Candida* infections have dramatically increased in recent years. According to our results, *A. paniculatum* was found to be effective on both planktonic and biofilm cells of *C. albicans*, making this plant extract a potent source of antifungal drugs or adjuvant treatment for *Candida* infections. Nevertheless, further analysis studies should be conducted to determine which active compound of *A. paniculatum* has antimicrobial and cytotoxic effects to understand its anticandidal activity better. To determine the suitability of these plant extracts for clinical use, further in-depth investigations are needed.

Ethics

Ethics Committee Approval: There is no requirement for ethical approval.

Informed Consent: Not required.

Authorship Contributions

Concept: Ö.O., M.H., E.Ö., Ş.K., Ç.B.G., Design: Ö.O., M.H., M.Ş.E., Data Collection or Processing: Ö.O., M.H., E.Ö., Ş.K., Ç.B.G., Analysis or Interpretation: Ö.O., E.Ö., M.Ş.E., Ş.K., Ç.B.G., Literature Search: Ö.O., M.H., M.Ş.E., Writing: Ö.O., M.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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