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Screening of antimicrobial, antibiofilm and cytotoxic activities of some medicinal plants from Balıkesir province, Türkiye: pointing to the 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 the methanol extracts of some medicinal plants (*Achillea nobilis* L. subsp. *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 & Wilkin, *Galium verum* L., *Hypericum triquetrifolium* Turra, *Paliurus spina-christi* Mill., *Primula vulgaris* Huds. subsp. *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 performed against *Candida albicans* mature biofilms. Moreover, 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* were displayed good activity (MICs: 9.75, 156, 312, 312 and 312 µg/ml, respectively) against *Candida albicans* ATCC 10231. The biofilm studies were performed to these plant extracts. The methanol extract of *A. paniculatum* flower decreased the number of *C. albicans* (cfu/ml) in mature biofilm statistically at 32xMIC 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 cell was similar to that on A549 cell.
Conclusion: This study demonstrates the importance of the methanol extract of *A. paniculatum* flower as 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 in the treatment of various diseases for many years.[1] An important part of these studies is investigating their effects against human pathogens. Because 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 due to the rise of multi-drug resistance. Apart from the challenge of antibiotic 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 compared to planktonic cells. Therefore, high doses of antibiotics must be used for treatment and thus causes unwanted side

effects.[4,5] Since biofilm formation on catheters and medical devices is a major challenge for treatment, removal of biofilm 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 as well as being effective on mature biofilms. Therefore, plant extracts can be an effective source for the antibiofilm therapy, because of the active molecules found in their structure.[8]

Balıkesir province in Turkey located in western Anatolian the border between the Marmara and Aegean regions with a surface area of 14.299 km², 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 a 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. subsp. *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 & Wilkin, *Galium verum* L., *Hypericum triquetrifolium* Turra, *Paliurus spina-christi* Mill., *Primula vulgaris Huds*. subsp. *rubra* (Sm.) Arcang., *Ranunculus arvensis* L. and *Teucrium polium* L. These plants have very important ethnobotanical value and their importance in the treatment of different diseases has been recorded in the literature. The selected plants are commonly used for wound healing, diarrhea, urinary tract infections, stomach pain, fungal infection, 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 the *Allium paniculatum* in Balıkesir is "yoğurtçuk ". The aerial part is cooked as a meal with eggs and freshly eaten.[15] In this study, the antimicrobial and antibiofilm properties of some medicinal plant extracts from 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 by MTT assay.

Material and Methods

Collection and identification of plants

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

Plant extracts

Plant materials were air-dried and extracted by percolation at room temperature with 95% methanol. Then, the obtained 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 & Laboratory Standards Institute (CLSI) guidelines against *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus feacalis* 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 $5x10^5$ cfu/mL for the bacteria and $0.5x10^3$ to $2.5x10^3$ cfu/mL for the yeast final inoculum and incubated 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 test microorganisms. Studies were repeated at least three times.

Inhibitory effects of plant extracts on *C. albicans* mature biofilms

Considering the results of the study, *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 (BHIB) at $1x10^6$ 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 extra 24 hours. After incubation, the wells were washed twice with PBS, and finally, PBS was placed in the wells and sonicated for 5 minutes in an ultrasonic cleaner and the biofilm was disintegrated. Microplates then were vortexed at 900 rpm. This process was repeated twice and the collected supernatants were diluted and planted onto Tryptic Soy Agar (TSA). The cfu values were determined by

counting the colonies, and the logarithms of the cfu values were implemented in the GraphPad Prism program and the results were expressed graphically.

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

The effects of *A. paniculatum* flower`s methanol extract, which was found significantly effective on both planktonic and biofilm cells of *C. albicans*, on the human lung cancer cell line (A549, ATCC® CCL-185™) and African green monkey kidney cell line (Vero, ATCC® CCL-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 wells flat-bottom microplate. Then, they 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 additional 24 hours. To determine the viability of cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Following exposure, MTT stock solution (5 mg/mL) was prepared in PBS and after discarding the medium from wells, 10 µL of MTT solution and 90 µl DMEM without phenol red were added to all wells and kept into the incubator for 3 hours. In addition, positive and negative controls were also added and upon incubation, the supernatants were removed. To dissolve the formazan crystals formed into the wells, 100 µL DMSO was added to the wells and plate was left in a shaker for 10 minutes. OD_{570} was measured using the microplate reader (EON-BioTek Instruments, Winooski, VT, USA).

Statistical analysis

To evaluate the results statistically, GraphPad Prism 8 was used. Results were 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 were shown in Table 2. All studied extracts displayed better antimicrobial activity against *C. albicans* than 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* subsp. *neilreichii* above ground parts showed 156, 312, 312 and 312 µg/mL MIC values against *C. albicans*, respectively.

Antibiofilm activity

Since 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 studied with at least 4 times of MIC values of the extracts. According to the MIC (µg/mL) values of plant extracts; 4xMIC and 8xMIC of *C. laurifolius, C. salviifolius,* and *A. nobilis*; 4xMIC, 8xMIC and 16xMIC of *P. vulgaris*; 8xMIC, 16xMIC, 32xMIC, 64xMIC, and 128xMIC of *A. paniculatum* were prepared. The inhibitory properties of the studied concentrations on *C. albicans* ATCC 10231 mature biofilms were investigated.

According to the results, 32xMIC, 64xMIC and 128xMIC concentrations of *A. paniculatum* flower methanol extract significantly inhibited *C. albicans* biofilm, while 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 the 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 on Vero cells when lower concentrations were compared.

Discussion

Due to the increasing antibiotic resistance to pathogens, the search for new antimicrobial agents is a high priority. There is now a growing trend towards natural products as an alternative drug source and the antimicrobial properties of plants are widely studied as a solution against multidrug-resistant pathogens.[18] The present study has been undertaken to understand *in vitro* properties of 17 plant extracts, which were belonging to the Balıkesir province of Turkey. In addition to investigating the antimicrobial activities of the herbal extracts used in our study, the possible cytotoxicities of the extracts which had promising antimicrobial effects were also investigated.

The traditional uses of medicinal plants give us an idea for activity studies. In this study, the traditionally utilized plants for their beneficial effects on wound healing in Balıkesir province of Türkiye were selected for the activity studies. Wound infection is widely prevalent and a significant clinical obstacle to wound healing. Consequently, exploring the potential antimicrobial properties of plants traditionally employed in wound healing

practices can be promising. The tuber part of *A. bulbosa* taken with water for hemorrhoids, intestine problems, constipation, heel cracked, allergy. The roots of *A. aestivus* are grated and cooked with tarhana (Turkish soup mixture) and applied on skin for abscess. The infusion of *B. nigra*'s leaves of are used for colds and stomachaches. The roots of *D. communis* are used as a decoction for hemorrhoids. The aerial part of *G. verum* is crushed and applied on the skin for wound healing. The infusion prepared from the aerial part of *H. triquetrifolium* is used externally for the treatment of wounds. The roots of *P. spina-christi* are used for allergy, itching. The aerial part of *R. arvensis* is applied on skin for eczema, abscess, joint pain, allergy. The aerial part of *A. nobilis* subsp. *neilreichii* is used for acne, wound healing, abdominal pain, cough, pain relief, and gynecological diseases. The aerial part of *Allium paniculatum* is eaten for health.[11-13,19]

In Balıkesir region, the roots of *P. vulgaris* are collected and sold by the local people for rheumatism treatment. According to Kahraman et al (2022), the *P. vulgaris* roots' butanol fraction exhibited the strongest wound-healing efficacy. Primulasaponin I (1) and primulasaponin I methylester (2) were identified as the main active molecules by activity-guided fractionation and isolation procedures.[20] In our study, while 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 Citrus species are extensively 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 snakebites, burns, wound healing, diarrhea, urinary tract infection, 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 to 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. Álvarez - Martínez et al. tested *C. salviifolius* extracts against 100 *S. aureus* clinical isolates and MIC_{50} values were found as 50-80 µg/mL. Also, it was shown that higher antibacterial activity against methicillin-resistant *S. aureus* isolates than sensitive ones was observed since it contains hydrolysable tannins and flavonoids such as myricetin and quercetin derivatives.[21] HPLC study 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 is responsible for strong antinociceptive and anti-inflammatory activities.[23] For this reason, further studies should be carried out to better understand its antimicrobial activity. However, since 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 and 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 the *A. paniculatum* was found to be ineffective against all microorganisms including *C. albicans*, while the methanol extract of the flower part displayed high activity against *C. albicans*. Different parts of *A. paniculatum* have different total flavonoid and phenolic contents and therefore their antioxidant properties 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, so biofilms are difficult 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, due to their high thiosulfinate content, especially allicin, polyphenols or flavonoids. In a recent study, Barbu et al. (2023) 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*.[25] Different studies on the *Allium* genus but different species also have determined efficacy against *Candida* and *Candida* biofilms.[26,27] However, to date, there are 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 been shown by studies to 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. demonstrated that the methanolic extract of *A. leucanthum* flowers inhibited the growth of A549 cells (IC_{50} of 15 ± 3 µg / mL).[29] In our study, the methanol extract of *A. paniculatum* flowers, another *Allium* species, at concentrations of 9.75 µg/mL and higher, inhibited the growth of A549 cells statistically, but more than 50% inhibition occurred after 312.5

µg/mL. Furthermore, it was determined that the cytotoxic effect on a cancer cell was similar to the 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 effective on both planktonic and biofilm cells of *C. albicans* which can make this plant extract potent sources of antifungal drugs or adjuvant treatment for *Candida* infections. Nevertheless further analysis studies should be conducted to determine which active compound of *A. paniculatum* causes antimicrobial and cytotoxic effects to better understand its anticandidal activity. And, to ascertain the suitability of these plant extracts for clinical use, further in-depth investigations are needed.

Conflict of Interest: There is no conflict of interest to declare

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Table 1: Studied species information

Species	Locality	Herbarium number
Achillea nobilis subsp. *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 subsp. *rubra*	Balıkesir, Kepsut; Örencik village, 730 m.	ISTE 109887
Ranunculus arvensis	Balıkesir, Savaştepe; Madenmezarı village, 450 m.	ISTE 109890
Teucrium polium	Balıkesir, Kepsut; Örenharman village, 535 m.	ISTE 109781

Table 2: Antibacterial and antifungal activities of 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 subsp. *Rubra* Root	>1250	>1250	1250	>1250	>1250	>1250	>1250	**156**
P. vulgaris subsp. *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 subsp. *neilreichii* Aerialpart	>1250	>1250	>1250	>1250	>1250	>1250	>1250	**312**

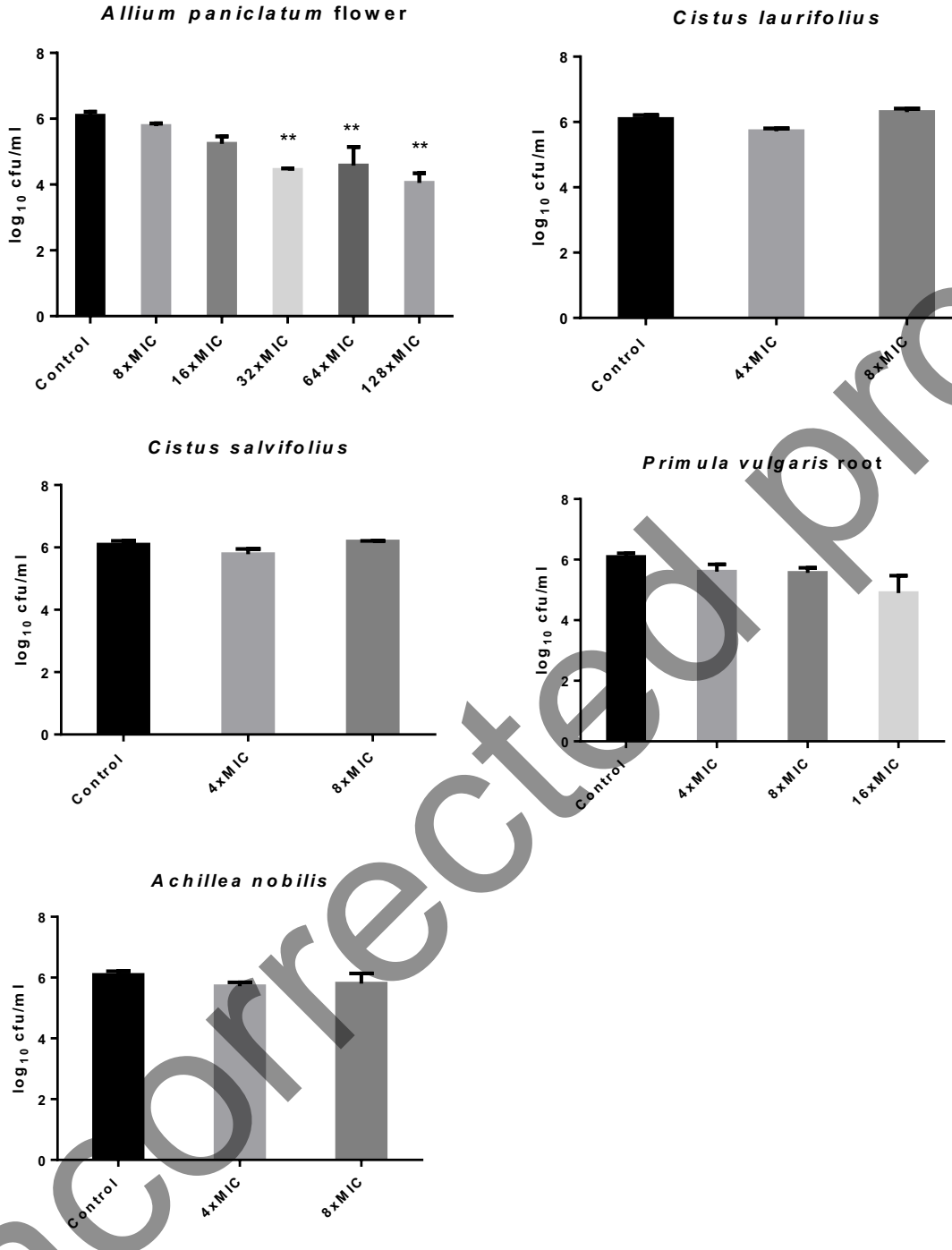

Figure 1: Antibiofilm activities of extracts against *C. albicans*. ** ; $p < 0.01$

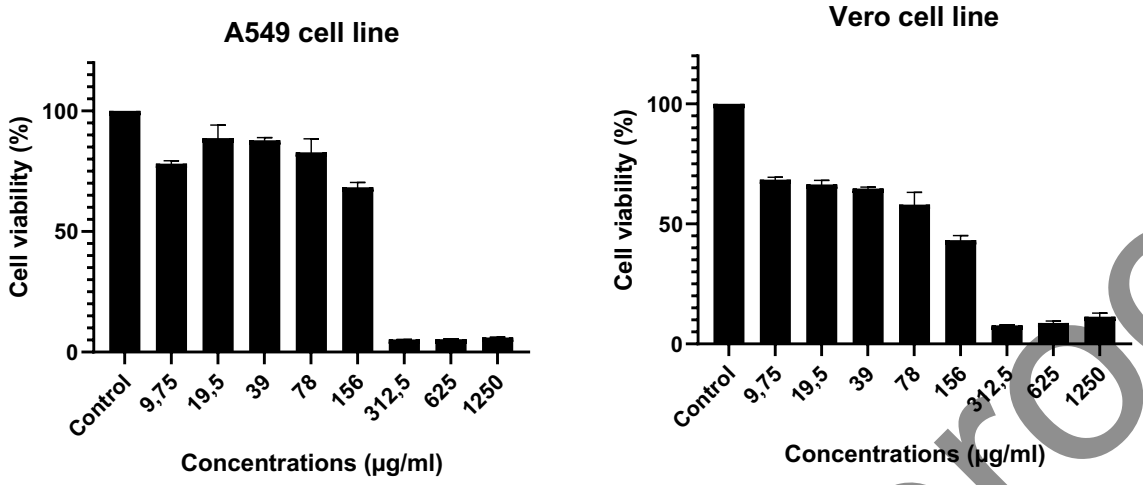

Figure 2: The effects of *A. paniculatum* flower extract on A549 and Vero cell viability (%)