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## IN VITRO ANTIMICROBIAL, ANTIBIOFILM AND CYTOTOXIC ACTIVITIES OF THE EXTRACTS OF *ARUM ITALICUM* MILLER LEAVES

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### Abstract

In this study, the antimicrobial, antibiofilm and cytotoxic activities of four different extracts which were prepared from the leaves of *Arum italicum* Miller, were investigated against various pathogenic microorganisms. The minimum inhibitory concentration (MIC) values of the extracts were determined by a microdilution method according to CLSI criteria. Inhibition of biofilm adhesion and biofilm formation tests were performed for extracts showing antimicrobial activity. In addition, the cytotoxic activities and anti-proliferative effects of the extracts were examined by the MTT viability test on the human umbilical vein endothelial cell line (HUVECs) and human breast cancer line (MDA-MB-231). The obtained results suggest that *A. italicum* extracts showed antimicrobial activity and were effective for inhibiting the pre-formed biofilms. In addition, the extracts significantly reduced the cell viability in the breast cancer cell line. According to our results, *A. italicum* extracts can be used as antimicrobial, antibiofilm and anticancer agents.

### Rezumat

Studiul prezintă activitatea antimicrobiană, antibiofilm și citotoxică a patru extracte preparate din frunze de *Arum italicum* Miller, studiate asupra mai multor specii microbiene. Concentrația minimă inhibitoare a fost determinată prin metoda microdiluției. Inhibiția adeziunii biofilmului și teste de formare a biofilmului au fost determinate pentru extractele care au prezentat activitate antimicrobiană. Activitatea citotoxică și efectele anti-proliferative ale extractelor au fost evaluate folosind testul MTT pe două tipuri de linii celulare: HUVEC și MDA-MB-231. Rezultatele arată că extractele prezintă activitate antimicrobiană și de inhibare a biofilmului, alături de reducerea viabilității celulelor MDA-MB-231. Astfel, extractele de *Arum italicum* Miller pot fi considerate potențiale substanțe anticancerigene.

**Keywords:** antimicrobial activities, antibiofilm activities, cytotoxic activities, *Arum italicum*

### Introduction

Plants have been used worldwide for centuries, in folk medicine; in herbal tea preparations, spices and for therapeutic purposes [23]. In particular, medicinal plants with antimicrobial effect have gained importance as concerns have been expressed about the rising prevalence of pathogenic microorganisms, which are resistant to modern antibiotics [19]. In this context, studies have been conducted which reported that plant extracts and natural substances obtained from various parts of the plants have antimicrobial properties against bacteria and fungi [2, 24, 34]. The *Araceae* family is represented in Turkey by 32 taxa, consisting of 5 genera (*Arisarum* Mill., *Arum* L., *Biarum* Schott, *Dracunculus* Schott, *Eminium* (Blume) Schott), 22 species, 5 subspecies and 12 varieties [9, 20, 27, 28]. *Arum* is the largest genus among the other aroids of Turkey. Representatives of the genus are distributed in

Central Asia, Europe, Macronesia, the Mediterranean and the Middle East regions. *Arum* taxa have been familiar to Eastern Mediterranean people for centuries. Arums have many vernacular names; “Yılan Bıçağı”, “Yılan Burçağı”, “Yılan Kaması” and “Yılan Yastığı” (“Serpent knife”; “Serpent dagger”; “Serpent vetch”; “Serpent pillow” respectively, in translation from Turkish) are most common among them [5, 6]. *Arum italicum* belongs to the *Arum* class of the *Araceae* family which is an angiosperm, a monocotyledonous perennial plant. The species in the *Arum* genus most discussed in the literature are *Arum palaestinum* and *Arum maculatum*. A review of the literature determined that, there are a limited number of studies of antimicrobial effects of *A. italicum* extracts [3, 9]. Tubers, leaves and fruits of *A. italicum* are mainly used as a traditional medicine and food industry in Turkey. While the leaves are boiled in water and consumed as wraps and salads, is common for the

whole plant to be cooked with rice and bulgur and used to reduce the labour pain.

Biofilms are communities of microbial cells adherent to biotic or abiotic surfaces within an extracellular matrix [11]. According to the National Institutes of Health, biofilms are estimated to account for over 80% of all nosocomial infections and are particularly common with device implants, such as contact lenses, ventricular assist devices, vascular and urinary catheters and endotracheal tubes [7, 12]. Because the use of conventional antimicrobial compounds in many cases cannot eradicate biofilms, there is an urgent need to develop alternative compounds and approaches to combat biofilm-based infections [4, 21]. For this purpose, in this study, the effects of four different extracts prepared from leaves of *A. italicum*, on the inhibition of biofilm adhesion and biofilm formation were investigated against various pathogenic microorganisms. In addition, *A. italicum* extracts were tested on the human umbilical vein endothelial cell line (HUVECs) and the human breast cancer cell line (MDA-MB-231) by MTT assay, to assess their anticarcinogenic effects.

## Materials and Methods

### *Preparation of plant materials and extracts*

The plant materials were collected from Istanbul and deposited as herbarium samples at the Biruni University, Turkey, Faculty of Pharmacy. The leaves of the plant were air-dried in shade at room temperature, then, they were ground into fine powder using an electric blender. The powdered plant materials (30 g), were extracted with 300 mL of four solvents (acetone, ethanol, methanol, distilled water) (plant material to solvent ratio was 1:10, m/v) for 72 hours at room temperature. Extracts were filtered through Whatman No.1 filter paper. All filtrates were concentrated and evaporated at 40°C until dry and were maintained at +4°C until further experiments [25, 31].

### *Antimicrobial activity*

The *in vitro* antimicrobial activities of the extracts were determined using the microbroth dilution technique described by the Clinical and Laboratory Standards Institute (CLSI) [13, 14]. The minimum inhibitory concentrations (MICs) of the extracts were investigated against *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750. Serial twofold dilutions ranging from 5000 to 4.8 µg/mL were prepared in Mueller-Hinton broth (MHB) (Difco, Detroit, MI, USA) for bacteria and RPMI-1640 medium (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS for yeasts,

dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA) was used as a solvent for the extracts. Each well was inoculated with 50 µL of a 4 - 6 h broth culture to give a final concentration of  $5 \times 10^5$  CFU/mL for bacteria and  $0.5 \times 10^3$  to  $2.5 \times 10^3$  CFU/mL for yeast in the test trays. The trays were covered and placed in plastic bags to prevent evaporation. The trays containing MHB were incubated at 37°C for 24 h, while those containing the RPMI-1640 medium were incubated at 30°C for 48 h. The MIC of each extract was defined as the lowest concentration of compound required for complete inhibition of visible growth. Ciprofloxacin and fluconazole were used as reference antimicrobials for bacteria and yeast, respectively. Also as a control, the antimicrobial effects of the DMSO were investigated using microorganisms. The results were evaluated according to the control values. The MIC values of ciprofloxacin and fluconazole were within the accuracy range in CLSI throughout the study.

### *Biofilm attachment assay*

After MIC assay, inhibition of biofilm attachment and biofilm formation assays were performed on those compounds which showed antimicrobial effects. Biofilm attachment assay was performed using the previously described method, with some modifications [10, 17]. The overnight cultures of isolates were prepared to a cellular density equivalent to  $1 \times 10^7$  cells/mL for bacteria and  $1 \times 10^6$  cells/mL for yeasts. Microorganisms were added to each well of 96-well tissue culture microtiter plates with 1/10 x MIC (MIC = 625 - 312.5 µg/mL) of plant extracts. The positive control without plant extracts and negative control without cells were also added. The plates were incubated for 1, 2 and 4 h at 37°C for bacteria and 2, 4 and 6 h at 37°C for yeast. After incubation, the wells were washed twice with PBS (phosphate buffered saline) solution and were measured spectrophotometrically at 450 nm, on a microplate reader (BioRad Novapath).

### *Inhibition of biofilm formation*

Bacteria ( $1 \times 10^5$  cells/mL) and yeast ( $1 \times 10^6$  cells/mL) were added to each well of a 96-well tissue culture microtiter plate with 1 x MIC, 1/10 x MIC and 1/100 x MIC (MIC = 625 - 312.5 µg/mL) of plant extracts. The positive control without plant extracts and negative control without cells were also added. The plates were incubated for 24 h at 37°C. After incubation, the wells were washed twice with PBS and were measured spectrophotometrically at 450 nm, on a microplate reader (BioRad Novapath) [8, 17].

### *Cell culture and plant extracts treatment*

For revealing of anticarcinogenic effects of *Arum* extracts on breast cancer, HUVECs and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (Virginia, USA). The cells were cultured at 37°C in a humidified incubator containing

5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium-F12 (DMEM-F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% Penicillin Streptomycin (Sigma, USA). The cells were maintained in the logarithmic phase of growth and sub-cultured every 3 - 4 days. Before treatment, 3 x 10<sup>3</sup> cells in each group were plated in a 96-well plate. After 24 h of cell plating, the cells were incubated with ethanol and acetone extract from *A. italicum* in increasing concentrations (5, 12.5, 25 and 50 µg/mL) for 48 h [29].

#### Cell Viability Assay

After treating the cells with extracts of *A. italicum*, cell viability was determined using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay that measures the activity of mitochondrial dehydrogenase. An MTT solution (5 mg/mL) was prepared by dissolving MTT powder in PBS, and the solution was filter-sterilized (0.22 µm pore size filter). Thereafter, MTT was transferred to each well containing cells and incubated in the dark for 3 h at 37°C. After incubation, the supernatant was decanted and DMSO was added to the cells. The optical density of the supernatant was measured at 570 nm with a microplate reader (Epoch Microplate Spectrophotometer, Biotek) [26]. Every concentration was repeated in three wells and then the half-maximal inhibitory concentration (IC<sub>50</sub>) values were calculated with a GraphPad Prism. Data were presented as mean ± SEM of at least triplicate determinations.

## Results and Discussion

Nowadays, although there are studies in the literature about the biological effects of many species in the *Arum* genus, there are limited studies on *A. italicum* activities *in vitro* [1, 3, 16, 18, 30]. The current investigations clearly indicate that antibacterial and anticandidal activity vary with the species of plant. In this study, it was determined that ethanol and acetone extracts prepared using the leaves of *A. italicum* plant were effective against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *C. albicans* and *C. tropicalis*, while the distilled water and methanol extracts had no effect against microorganisms. It was seen that both polar and apolar substances pass into ethanol used as a solvent in our study, whereas only polar substances passed into methanol. Therefore, the activity difference between ethanol and methanol extracts may be occurred due to the difference in the content of the extracts. The ethanol extracts were found to be active against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *C. albicans* and *C. tropicalis*, with MIC values which were identified as 625 µg/mL for bacteria and *C. albicans*, and 312.5 µg/mL for *C. tropicalis*. Furthermore, acetone extract was effective against *S. epidermidis* and *C. tropicalis* with MIC values which were determined as 625 µg/mL and 312.5 µg/mL, respectively (Table I). These results showed that *A. italicum* leaf extracts may have the potential to be used as an antibacterial agent in the research of new drugs.

**Table I**  
MIC values of extracts against microorganisms

Extracts	MIC values (µg/mL)								
	S.a	S.e	E.c	K.p	P.a	P.m	C.a	C.t	C.p
1/10 Ethanol	625	625	-	-	625	-	625	312.5	-
1/10 Acetone	-	625	-	-	-	-	-	312.5	-
1/10 Distilled water	-	-	-	-	-	-	-	-	-
1/10 Methanol	-	-	-	-	-	-	-	-	-
Ciprofloxacin	0.25	0.25	0.007	0.125	0.125	0.007	-	-	-
Amphotericin B	-	-	-	-	-	-	0.5	1	1

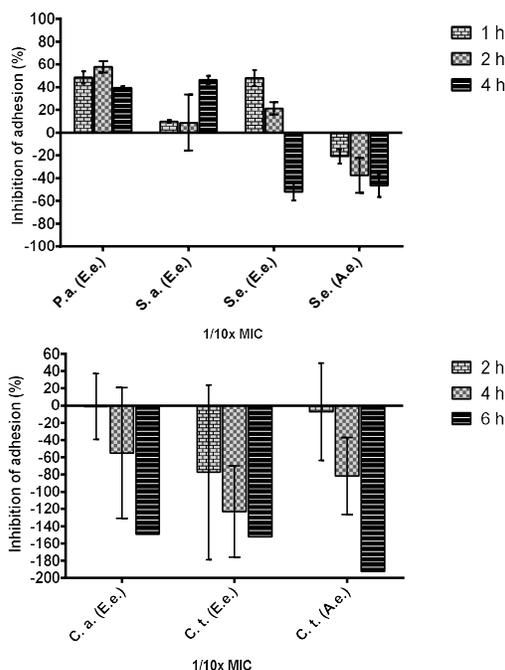
S.a: *S. aureus* ATCC 29213; S.e: *S. epidermidis* ATCC 12228; E.c: *E. coli* ATCC 25922; K.p: *K. pneumoniae* ATCC 4352; P.a: *P. aeruginosa* ATCC 27853; P.m: *P. mirabilis* ATCC 14153; C.a: *C. albicans* ATCC 10231; C.t: *C. tropicalis* ATCC 750; C.p: *C. parapsilosis* ATCC 22019.

Since biofilm-associated microorganisms are not affected by the therapeutically achievable concentrations of antimicrobial agents, current anti-biofilm therapies are generally focused on the inhibition of biofilm formation [22]. For this purpose, in addition to antimicrobial activities, we also investigated the *in vitro* activities of *A. italicum* extracts against the biofilm adhesion as well as the inhibition of the biofilm production of microorganisms by the MIC or subMIC values of the extracts. The 1/10 concentrations of *A. italicum* Miller extracts were incubated with microorganisms in the wells of tissue culture microtiter

plates, for 1, 2 and 4 h at 37°C for bacteria and 2, 4 and 6 h at 37°C for yeast to assess the adherence of microorganisms to the surface. According to our results *A. italicum* Miller ethanol extract inhibited the biofilm attachment of bacteria in a time dependent manner (Figure 1).

The highest adhesion inhibition rates (percentages) of ethanol extract varied over time for different bacteria; 57.91% (2 h) for *P. aeruginosa*, 46.29% (4 h) for *S. aureus* and 48.06% (1 h) for *S. epidermidis*. However, it was found that acetone extracts had no effect on the adhesion inhibition experiments. Despite all, the

effects of extracts on *Candida* biofilm adhesion were not determined (Figure 1).

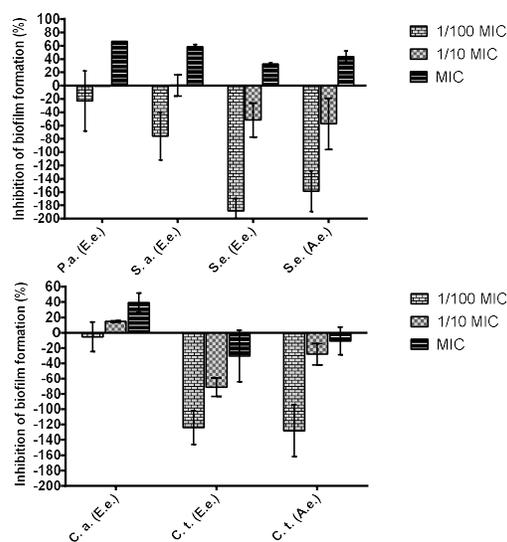


**Figure 1.**  
Inhibition of bacteria (a) and yeast (b) biofilm attachment by ethanol extract (E.e) and acetone extract (A.e)

Then the effects of the MIC and subMICs of ethanol and acetone extracts on the inhibition of biofilm formation at 24 h were investigated and concentration dependent inhibition was detected (Figure 2). It was observed that the ethanol extract significantly inhibited the biofilm formation at the  $1 \times$  MIC values. The inhibition rates of ethanol extract (at MIC values) against biofilm formation of *P. aeruginosa*, *S. aureus* and *S. epidermidis* were 66.94%, 58.35% and 32.53%, respectively. The inhibition rate of acetone extract on MIC values against *S. epidermidis* was determined as 43.72%. Inhibition of biofilm formation against *Candida sp.* was determined only for *C. albicans* (39.4% at MIC). Our results suggest that *A. italicum* extracts are effective for inhibiting pre-formed biofilms. Also, inhibition of biofilm formation in early critical stages seems to be more applicable.

Investigation of new anticancer compounds in bioactive plants is thought to be a promising strategy for the treatment and prevention of diseases [15, 32]. In our study, the cytotoxic and anti-proliferative effects of ethanol and acetone extracts from *A. italicum* were evaluated. For this purpose, HUVEC and MDA-MB-231 cell lines were treated with ethanol and acetone extracts of *A. italicum*. Cell proliferation of breast cancer cells and endothelial cells were investigated in the presence of increasing amounts of *A. italicum*

ethanol and acetone extracts. According to this study, viability of cells decreased to about 30% with 25  $\mu\text{g/mL}$  treatment with acetone extract in both cell lines. Furthermore, it was observed that the acetone extract was less toxic than the ethanol extract in HUVECs. Although ethanol extract did not have a remarkable effect on cell proliferation at lower doses (5 and 12.5  $\mu\text{g/mL}$ ) in HUVECs, ethanol extract induced cell death in breast cancer cells at these low concentrations.



**Figure 2.**  
Inhibition of bacteria (a) and yeast (b) biofilm formation by ethanol extract (E.e) and acetone extract (A.e)

In this study, ethanol extracts at concentrations of 5  $\mu\text{g/mL}$  and 12.5  $\mu\text{g/mL}$  were less toxic in HUVECs however, these doses effectively reduced cell viability to approximately 32% and 66% respectively, on the breast cancer cell line showing the cytotoxic and anti-proliferative properties of *A. italicum* ethanol and acetone extracts, as the first time.

The cytotoxic activities of the *A. italicum* extracts are shown in Table II. While all extracts induced strong cytotoxicity in cell lines ( $< 20 \text{ mg/mL}$ ), breast cancer cells showed striking sensitivity to treatment with *A. italicum* ethanol extract ( $7.023 \pm 0.2601$ ; Table II). Measuring the degree of cytotoxicity has established three groups of extracts from natural sources according their activity levels: inactive ( $\text{IC}_{50} > 100 \text{ mg/mL}$ ), moderately active  $\text{IC}_{50} 20 - 100 \text{ mg/mL}$  and active ( $\text{IC}_{50} < 20 \text{ mg/mL}$ ) were accepted by The U.S. National Cancer Institute (NCI) [33]. At 20  $\text{mg/mL}$ , the extract can be accepted as active, but also ethanol extract is less active in healthy cells in comparison with breast cancer cells. HUVECs, showed a slight differential effect between tumour and normal cells with ethanol extract treatment.

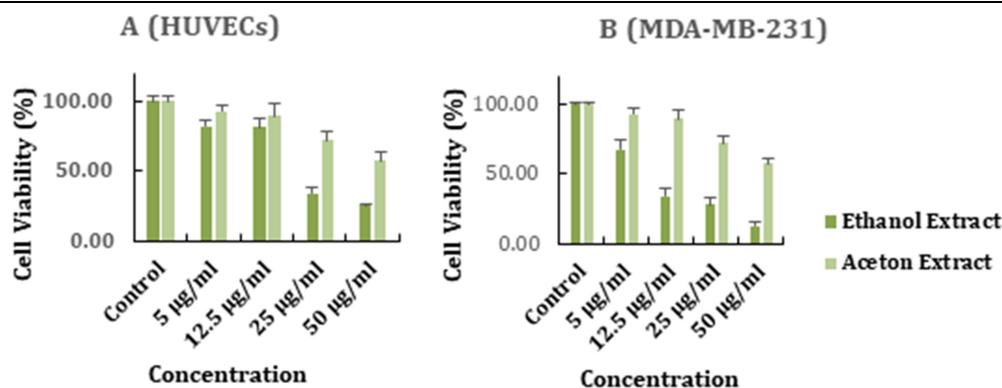


Figure 3.

(A) HUVEC and (B) MDA-MB-231 cell lines were treated with ethanol and acetone extract of *Arum italicicum* Mill. Cytotoxicity was measured by MTT assay. Cell viability was assessed at 570 nm by microplate reader after staining with MTT for 3 h. Mean  $\pm$  SEM of at least triplicate determinations.

Table II

IC<sub>50</sub> values ( $\mu\text{g/mL}$ ) of *Arum italicicum* ethanol and acetone extract on HUVECs and the breast cancer cell line. Human cancer cell lines were treated with different concentrations of *A. italicicum* methanol and acetone extracts in 96-well microculture plates for 24 h. IC<sub>50</sub> values are expressed as mean  $\pm$  standard error of mean (S.E.M) of triplicate determinations.

	Cell line and treatment IC <sub>50</sub> ( $\mu\text{g/mL}$ ) $\pm$ SEM
HUVECs + <i>A. italicicum</i> ethanol extract	19.66 $\pm$ 1.545
HUVECs + <i>A. italicicum</i> acetone extract	19.08 $\pm$ 0.1030
MDA-MB-231 + <i>A. italicicum</i> ethanol extract	7.023 $\pm$ 0.2601
MDA-MB-231 + <i>A. italicicum</i> acetone extract	18.12 $\pm$ 0.5381

Furthermore, our results show that ethanol extract may have significant effects on the proliferation of cancer cells (Figure 3). Also in our results, ethanol extract of *A. italicicum* on breast cancer cells yielded acceptable preliminary data. However, extracts should be studied more with respect to breast cancer and should be also evaluated for other cancer types in future studies.

### Conclusions

In the present study, four different extracts of *A. italicicum*, which are mainly used as medicine and food by East Mediterranean people, were studied. We found that two extracts of this plant (ethanol and acetone) displayed antimicrobial activities. Interestingly, the two extracts of *A. italicicum* also showed antibiofilm activity when used at subinhibitory concentrations. Additionally, our results suggest that the ethanol extract may have significant effects on the proliferation of cancer cells. Nevertheless, further studies are needed in order for making *A. italicicum* to be considered a viable candidate for the treatment of infectious diseases; biofilms as well as cancer.

### Conflict of interest

The authors declare no conflict of interest.

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