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Activity Guided Fractionation of *Arum italicum* Miller Tubers and the LC/MS-MS Profiles

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Abstract: In this study, the polar extract of *Arum italicum* Miller tubers was fractionated by 'biological activity guided fractionation' method. MCF-7 (human breast adenocarcinoma) and A549 (non-small lung cancer) cancer cell lines were used to screen cytotoxic activity. Possible antiproliferative and apoptotic effects of each subfraction were evaluated by *in vitro*. As a result, E2 were cytotoxic against A549 cell lines while E3 and E6 showed good cytotoxic activities against both cancer cell lines. These three subfractions showed apoptotic and antiproliferative effects comparable with positive control. The chemical composition of each subfraction was determined by LC/MS-MS. Hydroxycinnamic acid derivatives, lignans and glycosides, hydroxycinnamic acid-spermidine conjugates and oxylipins identified in subfractions were major secondary metabolites.

Keywords: *Arum italicum*; Araceae; cytotoxic effect; apoptosis; LC/MS-MS.

1. Introduction

Nowadays, cancer is among the most difficult diseases to cure. Since formation mechanisms of this disease is complex and not fully understood, but also since cancer cells bear so much resemblance to healthy cells, medication can not achieve complete success. In this respect, researches on natural resources that can be used effectively in cancer treatment are still popular.

In studies conducted in recent years, articles published in the field of ethnobotany have gained importance. With these studies, information is recorded about what purpose medicinal plants are used in a specific region, which parts of these plants are prepared in what way and how much time they are used [1,2].

A. italicum, a member of Araceae, is distributed in South and West Europe, Balkans, North Africa, Caucasia, Iraq and Turkey [3]. *A. italicum* is known to be grown in gardens due to beautiful posture of their orange fruits. It is recorded that their tubers and leaves are used as soap in Europe. Tubers contain high amounts of sugar and starch (especially, 2-year-old *A. italicum* tubers contain up to 20% of starch) [4]. It is poisonous when fresh, effects of its toxic substances are lost when dried and boiled up. Eating fresh plant is characterized with nausea, vomiting, diarrhea and heart rhythm

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disorders, and it may cause poisoning that leads to death. Tubers, leaves and fruits of *A. italicum* are used in folk medicine in Turkey. It is suggested that dried tubers be swallowed in 1-2 g per day for laxative and expectorant effects. Mixture of powdered tubers with honey is used for stomach ulcer. Fresh tubers are consumed for in the treatment of eczema, foot skin cracks and nipple cracks. Fresh tubers are used for wound and abscess [5]. It was reported that fresh/with honey/decoction of tubers were used for treatment of disease such as cancer, eczema, peptic ulcer and gynaecological diseases [6].

The fact that use of *Arum* species is not very common suggests that this species has attracted attention of researchers less. In literature review, it has been determined that, in limited number of studies on *A. italicum*, antioxidant and antimicrobial effects of extracts were studied as *in vitro* [7, 8]. Species with most literature information with *Arum* genus are *A. palaestinum* and *A. maculatum*, these studies can be summarized as follows; it is quite noticeable that cytotoxic effects of two substances that are alkaloid derivatives and isolated from *A. palaestinum* leaves: it has been reported that "piperazirum" showed significant cytotoxic effect on lung, ovary, melanoma and column cancer [9] and "3-hydroxypiperazine-2,5-dion" showed significant cytotoxic effect on uterine carcinoma [10]. In *A. maculatum* samples, positive effects on the immune system [11], antimicrobial [12] and antioxidant [13] were observed; in *A. palaestinum* samples, antioxidant [14,15], antifungal effect [15] and cytotoxic effect were observed against breast [15,17], lymphoblastic leukemia [16], larynx, cervix, liver [17] cancer cells. There are just a few pharmacological studies conducted on *A. italicum* that constitutes the research material. In ethanol extracts prepared from root, fruit, petiole and leaves of *Arum italicum* used in skin and soft tissue infections in Southern Italy, no effects against methicillin-resistant *Staphylococcus aureus* (ATCC 33593) were observed [7]. Methanol extracts were prepared with 2% infusion of aerial parts of the plant that was collected from Ordu/Fatsa region, Turkey. DPPH, reduction power capability, hydrogen peroxide cleaning capability, metal chelating activity were analysed to determine antioxidant effects of prepared extracts. It was determined that methanol extract with 0.5 g/L concentration had 56% DPPH scavenging effect and hydrogen peroxide cleaning ability, also had low reduction ability. In addition, it showed 82% metal chelating capacity in 0.1 g/L concentration [8].

In tubers, lignans were defined such as (+)-lariciresinol 4'-O-β-D-glucopyranoside, (+)-lariciresinol 9-O-β-D-glucopyranoside, ferulic acid, coniferyl alcohol 4-O-β-D-glucopyranoside, *p*-coumaryl alcohol-4-O-β-D-glucopyranoside, (+)-pinoresinol-O-β-D-glucopyranoside, (+)-syringaresinol-O-β-D-glucopyranoside, (+)-lariciresinol and (+)-lariciresinol-4-O-β-D-glucopyranoside [18,19], in another work, 8-O-3'neolignan and 8-O-4'neolignan were defined. Besides, 4-O-β-D-glucopyranoside and 9-O-β-D-glucopyranoside derivatives of dehydroconiferyl alcohol were found [20]. Gellerstedt *et al*. [21] identified a neolignan derivative called treo-guayaquilglycerol-β-coniferyl ether-4-β-D-glucopyranoside, also 8-O-3' neolignan structure. Also, steroidal compounds and oxygenated stigmasterol derivatives were obtained [18, 22].

In species belonging to *Arum* genus, determination of such effects is remarkable and important in terms of detailed analysis of these species. It is considered that analysis of *A. italicum* tubers *in vitro* biologic activities and having an idea on compounds that may be responsible for such an effect will serve as a resource for future studies.

Starting from these information, fractions of *Arum italicum* tuber extract were evaluated for their cytotoxic, antiproliferative and apoptotic effect by *in vitro* models. Phytochemical profiles of fractions were determined by LC/ESI-MS-MS system.

2. Materials and Methods

2.1. Plant Material

Arum italicum was collected from Armutlu Cemetery, Bursa, Turkey in 4th July 2013. The material was identified by Prof. Dr. Hulusi Malyer (Uludag University, Faculty of Arts & Science,

Department of Biology, 16059-Görükle, Bursa-Turkey). The tubers separated from the aerial parts were sliced and dried in the well-ventilated room. The voucher specimens are kept in Anadolu University, the Herbarium of Pharmacy Faculty with ESSE number 14620.

2.2. Cell Cultures, Chemicals and Solvents

A549 (lung carcinoma, human, ATCC®CCL-185TM), MCF-7 (breast adenocarcinoma, human, ATCC®HTB-22 TM) and L929 (healthy fibroblast cell, mouse, ATCC® CCL-1) cell lines were obtained from American Type Culture Collection (ATCC, USA). Annexin V-FITC early and late apoptosis measurement kit (BD Pharmingen), BRdU colorimetric kit (Roche) and all remaining reagents were of the highest purity available: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT salt) (Sigma), mitoxantrone (Sigma), Silica gel 60 for column chromatography (Merck), C18 column material (Macherel Nagel), 100 IU/mL penicillin-streptomycin solution (Gibco), 90% RPMI supplemented with 10% fetal bovine serum (Sigma-Aldrich), PBS solution (Sigma), dimethyl sulphoxide extra pure (Sial), formic acid (Sigma-Aldrich), *n*-hexane (Sigma-Aldrich), acetone chromasolv (Riedel), dichloromethane (Sigma-Aldrich), methanol chromasolv (Sigma-Aldrich). Ultra-pure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., MA, USA)

2.3. Extraction Procedures

The preparations of the extract and fractions were based on the biological activity guided fractionation method (see Supporting Data). Therefore, the cytotoxic activity results against MCF-7 and A549 cell lines and the yields of the samples were used for the fractionation of the crude extract.

2.3.1. The Preparation of Crude Extract

The grounded and air-dried tubers (793 g) were subjected to continuous extraction by Soxhlet apparatus with acetone:water (1:1, *v/v*) for 8 h. The extract was freeze-dried after removal of acetone.

2.3.2. The Preparation of Fractions

The fractionation of the crude extract (50 g) based on the polarity of the solvent by using non-polar (*n*-hexane), semi polar (dichloromethane), more polar (dichloromethane:methanol (1:1, *v/v*) and polar (methanol) solvents was evaluated with Silica gel 60 (0.063-0.2 μm particle size) by flash chromatography. Each of these fractions were concentrated under reduced pressure using a rotary evaporator. The yield of each fraction was calculated.

2.3.3. The Preparation of Subfractions of Methanol Fraction

Elution of the methanol fraction with C18 apolar flash chromatography were employed with 500 mL of water, methanol:water (2:8, *v/v*), methanol:water (4:6, *v/v*), methanol:water (6:4, *v/v*), methanol:water (8:2, *v/v*) and methanol, respectively. The subfractions were freeze-dried or concentrated by a rotary evaporator. The yield of each subfraction was calculated.

2.4. LC/ESI-MS-MS Analyses of Subfractions

Separation and detection of phytochemicals of subfractions of *A. italicum* tuber were performed on a Shimadzu UPLC system consisting of a vacuum degasser, an autosampler (SIL20A Shimadzu Autosampler), a binary pump (LC20AD Shimadzu), an oven (CTO20A Shimadzu Column Oven) and UV dedector (SPD20A Shimadzu UV Detector). The instrument was equipped with an

Intersil ODS column (4.6 x 250 mm, 5 µm particle size). The mobile phase was methanol:water:formic acid (10:89:1, *v/v/v*) (solvent A) and methanol:water:formic acid (89:10:1, *v/v/v*) (solvent B) at a flow rate of 0.7 mL/min. The gradient elution started with 10% solvent B at 0 min, 100% solvent B reached at 40 min. The column temperature was kept at 40ºC with an injection volume of 10 µL. The chromatograms were recorded at 280 nm.

The UPLC system was connected directly to a 3200 Q TRAP (AB Sciex, Toronto, Canada), supported with an electrospray ionization interface (ESI). For enhanced mass scan (EMS), the mass system was operated in negative polarity at a scan rate of 1000 Da/s within the mass range of 100-1200 amu. Mass scan (MS) and daughter (MS-MS) spectra were measured from *m/z* 100 up to *m/z* 1200. Collision-induced fragmentation experiments were performed in the ion trap using nitrogen as the collision gas, with the collision energy set at 50. The parameters were as follow: Collision Energy Spread (CES), 0; Declostiring Potential (DP), 40; Enterance Potential (EP), 10; Curtain Gas (CUR), 20; Gas Source 1 (GS1), 50; Gas Source 2 (GS2), 50; CAD, high; Ihe, on; Temperature (TEM), 500. The software used for data acquisition and analysis is Analyst 1.5. The chromatographic conditions were used as described above.

Each peak in the chromatograms was identified by their UV spectra and by total ion chromatograms, their molecular weights and their fragmentation patterns was screened by using in-house library which was obtained with standard compounds and published data.

2.5. The Evaluation of Cytotoxic Activity

The methods for cell culture preparation, sample treatment, cytotoxicity, DNA synthesis inhibition activity and apoptotic effects by flow cytometric analyses were measured as our previous study [41].

2.5.1. Cell Culture, Sample Treatment

A549, human lung adenocarcima cells and MCF-7, breast carcinoma cells were incubated in 90% RPMI supplemented with 10% fetal bovine serum. All media were supplemented with 100 IU/mL penicillin-streptomycin and cells were incubated at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were plated at $2x10^4$ cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h before the addition of the samples (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). Stock solutions of samples were prepared in dimethyl sulphoxide (DMSO) and further dilutions were made with fresh culture medium (the concentration of DMSO in the final culture medium was <0.1% which had no effect on the cell viability).

2.5.2. Cytotoxic Activity (MTT Assay)

A tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), was used as a colorimetric substrate for measuring cytotoxicity (MTT assay) as previously described in the literature with small modifications [23]. The tested samples and mitoxantrone (positive control) were incubated with cells to give final concentration in the range 3.9-1000 µg/mL. The plates were incubated for 24 h at 37ºC in 5% CO_2 humidified incubator together with untreated control sample. After the incubation period, 20 µL (0.5 mg/mL) MTT dye was added. After the cells were incubated for further 4 h at 37ºC, the plates were measured with an ELx808-IUBio-Tek apparatus at 540 nm. Every concentration was repeated in three wells and IC_{50} values were defined as the sample concentrations that reduced absorbance to 50% of control values.

2.5.3. Antiproliferative Activity (Analysis of DNA Synthesis Inhibition)

This method was performed in the 96 well flat-bottomed microtiter plates by using a Roche BrdU colorimetric kit [24]. Hence, this method provides a colorimetric measurement for DNA synthesis inhibition ratio of the carcinogenic cells. Suspensions of cells were seeded into 96 well flat-bottomed microtiter plates at a density of $2x10^3$. The tumor cell lines were cultured in the presence of various doses of the test samples or mitoxantrone. Microtiter plates were incubated at 37ºC in a 5% CO_2 / 95% air humidified atmosphere for 24 h. The cells were labelled with 10 µL BrdU solution for 2 h and then fixed. Anti-BrdU-POD (100 µL) was added and incubated for 90 min. Finally, microtiter plates were washed with phosphate buffer saline (PBS) three times and the cells were incubated with substrate solution until the colour was sufficient for photometric detection. Absorbance of the samples were measured with an ELX808-IU Bio-Tek apparatus at 492 nm. All experiments were repeated two times. For each sample dose, dublicate wells were used.

2.5.4. Flow Cytometric Analyses of Apoptosis

After A549 and MCF-7 cells were incubated with each sample at IC_{50} concentrations for 24 h, Annexin V staining protocol was applied according to manufacturer's instructions (BD, Pharmingen). After cells were washed with cold PBS, suspended in binding buffer at a concentration of ~$1x10^6$ cells/mL. Then 100 µL of this solution containing ~$1x10^5$ cells, were transferred to a 5 mL test tube. After 5 µL of Annexin-V and PI was added, the cells were incubated for 15 minutes at room temparature in the dark. Then 400 µL of 1xBinding Buffer was added to each tube and cells were processed for data acquisition, and analyzed on a flow cytometer using FACSDIVA Software Version 6.1.1.

2.5.5. Toxicity on L929 Healthy Mouse Cell Lines

To determine possible toxicity of subfractions against normal healthy cell, L929 mouse normal skin fibroblast cell lines were used. The cell culture and MTT assay procedures were used as mentioned at above.

2.6. Statistical Analyses

Statistical Package for the Social Science (SPSS) for Windows 15.0 was used for statistical analysis. Data was expressed as Mean ± SD. Comparisons were performed by one way ANOVA test for normally distributed continuous variables and post hoc analyses of group differences were expressed by the Tukey test.

3. Results and Discussion

3.1. Cytotoxic Activity Studies

3.1.1. Cytotoxic Activity

IC_{50} values of fractions of the crude extract are given in Table 1. Since yield of B fraction was very low, its cytotoxic effect was not considered. As given in Table 1, the higher the polarities of fractions are, the more their effects become. The cytotoxic effect of the dichloromethane fraction was quite poor. The effect comparable to mitoxantrone was observed in D and E. While D showed weak cytotoxic effect against MCF-7 (IC_{50}-296.7 µg/mL) cancer cells, it showed noticeable cytotoxic effect against A549 (IC_{50}-44.0 µg/mL) cells. On the other hand, it was determined that E showed significant cytotoxic effect against both MCF-7 (IC_{50}-58.3 µg/mL) and A549 (IC_{50}-32.3 µg/mL) cells comparable to mitoxantrone.

Cytotoxic effects of the subfractions against both cells were studied under the same conditions. In Table 1, obtained findings are given. When the results were compared, it was found that A549 cells were more sensitive against subfractions. The highest cytotoxic effect against A549 cells was observed in E2 (IC_{50}-13.67 µg/mL) and IC_{50} value was found close to mitoxantrone (IC_{50}-10.33 µg/mL). In terms of the cytotoxic effect against A549, E3 (IC_{50}-26.67 µg/mL) and E6 (IC_{50}-26.33 µg/mL) yielded similar results. While the effect in E1 (IC_{50}-31 µg/mL) were lower, it showed better effect than E5 (IC_{50}-56.67 µg/mL) and E4 (IC_{50}-63.33 µg/mL). IC_{50} value of mitoxantrone against MCF-7 cells was calculated as 22.33 µg/mL. The nearest effect to mitoxantrone was obtained in E6 and E2 as 28 µg/mL and 33.67 µg/mL, respectively.

Table 1. The cytotoxic activity results of fractions and subfractions.

Code	Fractions	Yields*	Cytotoxic activity (IC_{50}- µg/mL)	
			MCF-7	A549
Fractions				
B	*n*-Hexane	0.005	-	-
C	Dichloromethane	0.40	416.67±28.87	286.67±77.67
D	Dichloromethane:methanol (1:1, *v/v*)	11.84	296.67±90.77	44.00±5.29
E	Methanol	45.11	58.33±7.64	32.33±2.52
Mito	Mitoxantrone		22.00±6.56	15.33±6.11
Subfractions				
E1	Water	83.9	65.00±15.00	31.00±3.60
E2	Methanol:water (2:8, *v/v*)	0.51	33.67±3.20	13.67±1.50
E3	Methanol:water (4:6, *v/v*)	0.25	51.67±12.60	26.67±7.40
E4	Methanol:water (6:4, *v/v*)	0.16	90.00±10.00	63.33±18.90
E5	Methanol:water (8:2, *v/v*)	0.22	63.33±12.60	56.67±10.4
E6	Methanol	0.36	28.00±2.00	26.33±1.50
Mito	Mitoxantrone		22.33±2.10	10.33±1.50

*Yields were calculated as *w/w* of the sample.

3.1.2. Antiproliferative Activity (DNA Synthesis Inhibition)

While mitoxantrone showed approximately 41% of DNA synthesis inhibition in MCF-7 breast cancer cells at 22.33 µg/mL concentration, this rate was found to be 63% in 33.5 µg/mL concentration. In general in subfractions, an inhibition increase was observed based on dose. Findings regarding DNA synthesis inhibitions of subfractions in MCF-7 cancer cells are given in Figure 1. E2 and E6 among subfractions showed high DNA synthesis inhibition in MCF-7 cells. E2 provided approximately 37% inhibition in 33.67 µg/mL and high inhibition at the rate of 72.3% in 50.51 µg/mL concentration. It was observed that E6 provided around 70% inhibition in concentration with IC_{50} value and higher, and this effect was found to be higher than mitoxantrone. In other subfractions, no significant findings were found.

When inhibitions of subfractions on DNA synthesis of A549 cells, an effect higher than 10% was found at IC_{50} value and higher concentrations, and concentration-dependent increase was observed. Mitoxantrone made 56.7% of DNA synthesis inhibition at IC_{50} dose (10.33 µg/mL) and 66.2% of DNA synthesis inhibition at 15.5 µg/mL. DNA synthesis inhibition results of E2 was at 54.9% rate in IC_{50}-13.67 µg/mL dose and 68.3% rate in 20.51 µg/mL concentration. It was observed that E2 inhibited DNA synthesis in A549 cells at a level comparable to mitoxantrone. In E6, respectively 65.4% and 72.1% DNA synthesis inhibition were determined at 26.33 µg/mL (IC_{50} dose) and 34.5 µg/mL concentration. E3 showed 40.4% effect in 26.67 µg/mL dose and 55.7% effect in 40.01 µg/mL concentration (Figure 2). When these findings are analyzed, it was determined that E2 and E6 inhibited DNA synthesis in MCF-7 cells and E2, E3 and E6 inhibited DNA synthesis in A549 cells at IC_{50} dose and higher concentrations, and therefore, they showed antiproliferative effect. It is possible to say that these subfractions showed cytotoxic effect by inhibiting DNA synthesis.

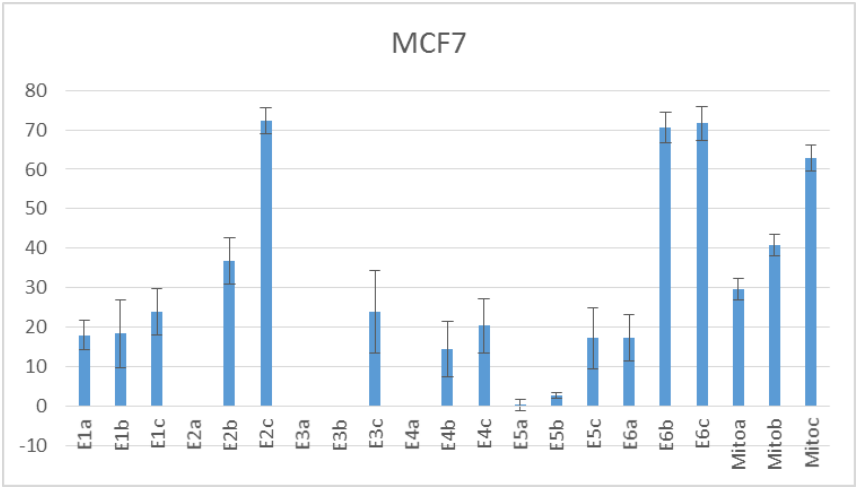

Figure 1. DNA synthesis inhibitory activitiy of the subfractions and mitoxantrone on MCF cancer cells (Mean percent absorbance of untreated control cells were assumed 0%; (E1), water subfraction; (E2), methanol:water (2:8, *v/v*) subfraction; (E3), methanol:water (4:6, *v/v*) subfraction; (E4), methanol:water (6:4, *v/v*) subfraction; (E5), methanol:water (8:2, *v/v*) subfraction; (E6), methanol subfraction; (Mito), Mitoxantrone; E1a 32.5 µg/mL; E1b 65 µg/mL; E1c 97.5 µg/mL; E2a 16.84 µg/mL; E2b 33.67 µg/mL; E2c 50.51 µg/mL; E3a 25.84 µg/mL; E3b 51.67 µg/mL; E3c 77.51 µg/mL; E4a 45 µg/mL; E4b 90 µg/mL; E4c 135 µg/mL; E5a 31.67 µg/mL; E5b 63.33 µg/mL; E5c 95 µg/mL; E6a 14 µg/mL; E6b 28 µg/mL; E6c 42 µg/mL; Mitoa 11.17 µg/mL; Mitob 22.33 µg/mL; Mitoc 33.5 µg/mL. $p < 0.05$).

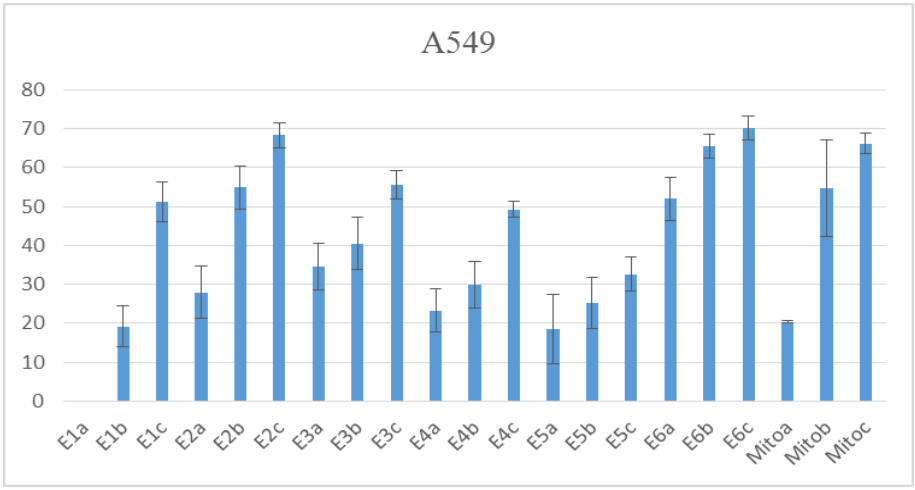

Figure 2. DNA synthesis inhibitory activitiy of the subfractions and mitoxantrone on A549 cancer cells (Mean percent absorbance of untreated control cells were assumed 0%; (E1), water subfraction; (E2), methanol:water (2:8, *v/v*) subfraction; (E3), methanol:water (4:6, *v/v*) subfraction; (E4), methanol:water (6:4, *v/v*) subfraction; (E5), methanol:water (8:2, *v/v*) subfraction; (E6), methanol subfraction; (Mito), Mitoxantrone; E1a 15.5 µg/mL; E1b 31 µg/mL; E1c 46.5 µg/mL; E2a 6.84 µg/mL; E2b 13.67 µg/mL; E2c 20.51 µg/mL; E3a 13.34 µg/mL; E3b 26.67 µg/mL; E3c 40.01 µg/mL; E4a 31.67 µg/mL; E4b 63.33 µg/mL; E4c 95 µg/mL; E5a 28.34 µg/mL; E5b 56.67 µg/mL; E5c 85.01 µg/mL; E6a 13.17 µg/mL; E6b 26.33 µg/mL; E6c 34.5 µg/mL; Mitoa 5.17 µg/mL; Mitob 10.33 µg/mL; Mitoc 15.5 µg/mL. $p< 0.05$)

3.1.3. Apoptotic Activity

For apoptosis, the results are given in Table 2. When apoptotic effects of subfractions in MCF-7 cells, it was observed that E6 drove cells to early apoptosis at 19.3% rate as a result of incubation

with MCF-7 cells, and this effect was comparable to mitoxantrone. Mitoxantrone drove cells to early apoptosis at the rate of 10.5% in 22.33 µg/mL. It was determined that other subfractions drove MCF-7 cells into early apoptosis more than 10% at IC_{50} doses that were studied. When apoptotic effects of subfractions on A549 cells were analyzed, early apoptotic effect in E2, E3 and E6 was higher than 5% in IC_{50} doses studied. While mitoxantrone showed 5.4% effect in 10.33 µg/mL dose, E2 drove cells to apoptosis at 9.7% rate in 13.67 µg/mL dose. E6 showed early apoptotic effect at 11.6% rate in IC_{50} dose (For the quadrant analysis, see Supporting data).

Table 2. Flow cytometric analysis of apoptosis on MCF-7 and A549 cell lines.

Group	**MCF-7**			**A549**		
	% EAC	**% LAC**	**% VC**	**% EAC**	**% LAC**	**% VC**
Control	1.4	1.2	91.0	0.1	1.6	91.0
E1	13.1	6.8	71.6	4.2	5.1	82.9
E2	11.0	9.3	68.6	9.7	14.8	71.9
E3	12.4	7.5	70.6	5.9	7.6	83.0
E4	17.2	6.1	70.6	2.7	5.2	83.7
E5	11.4	12.2	65.0	2.1	7.0	85.3
E6	19.3	10.1	63.3	11.6	43.9	40.4
Mito	10.5	6.7	74.1	5.4	39.9	46.3

(EAC), early apoptotic cells; (LAC), late apoptotic cells; (VC), viable cells; Control, untreated cells; (E1), water subfraction; (E2), methanol:water (2:8, *v/v*) subfraction; (E3), methanol:water (4:6, *v/v*) subfraction; (E4), methanol:water (6:4, *v/v*) subfraction; (E5), methanol:water (8:2, *v/v*) subfraction; (E6), methanol subfraction; (Mito), Mitoxantrone.

3.1.4. Toxicity on Healthy Cells

As a result of toxicity tests, it was found that E1 and E4 were toxic effective against L929 normal mouse fibroblast cells respectively in 106.7 and 68.3 µg/mL IC_{50} doses. IC_{50} values were not reached at doses where the highest level of concentration was 80 µg/mL and that were prepared from other subfractions, therefore, IC_{50} values were considered to be higher than 80 µg/mL.

When these findings were analyzed, it was found remarkable that E2, E3 and E6 with their cytotoxic effects found considerable against MCF-7 and A549 cell lines showed cytotoxic effect in cancer cell lines at IC_{50} doses but did not show any toxic effect against normal cells.

3.2. LC/MS-MS Results

As a result of this assessment, it was observed that hydroxycinnamic acid derivatives, lignans and glycosides of compounds identified in subfractions were included in secondary metabolites such as hydroxycinnamic acid-spermidine conjugates and oxylipins (For detail information, see Supporting Data).

In subfractions of *Arum italicum*, ferulic acid, caffeic acid and its derivatives and *p*-coumaric acid were found. In previous studies on Araceae plants, ferulic acid was identified in *A. italicum* tubers, *Pinellia ternata* and *Acorus calamus* rhizomes; caffeic acid and its derivatives were identified in *Arum palaestinum* leaves; *p*-coumaric acid was identified in *A. dioscoridis* seeds within Araceae family. There is an increasing number of studies indicating that hydroxycinnamic acids prevent invasion and metastasis of cancer cells. As shown in various sources, caffeic acid and their esters, *p*-coumaric acid, ferulic acid showed anticancer effects against cancer cells such as column, lung, breast, liver, prostate, cervix and gastric [25-29]. It has been determined that *p*-coumaric acid, ferulic acid and caffeic acid significantly reduce survival rate of MDA-MB 468 and HBL 100 breast cancer cells, also colony formations of MDA-MB 468 cells. Caffeic acid and ferulic acid were found as directly antiproliferative effective against MCF-7 and T47D human breast cancer cells [30]. *p*-Coumaric acid's antitumor effects against MCF-7, NCI-H460 and HCT15 cancers cells have been recorded. The

importance of antioxidant-rich diet is emphasized in the prevention of lung cancer. In this regard, it is known that ferulic acid inhibited related enzymes such as nitric oxide synthase, caspase and COX-2 with cytotoxic effect. It is indicated that caffeic acid esters prevent metastasis of tumor cells into lung. In addition, caffeic acid-rich *Prunella* (Apiaceae) extracts are effective in prevention and treatment of lung cancer [25]. Another group found in subfractions is benzoic acid derivatives. Benzoic acid derivatives are known to be effective due to their radical scavenging effects, antimutagenic, anticarcinogenic, anti-inflammatory effects have been identified from natural sources [31].

Flavonoid derivatives are known to have antiproliferative effect in various cancer cell lines and to prevent tumor growth with some animal models [31]. In subfractions, puerarin, vitexin and apigenin-6,8-C-pentoside-hexoside and a flavonoid derivative have been identified by interpreting spectra information. Vitexin has been identified in *Arum dioscoridis* and *A. palaestinum* extracts in previous studies [32, 33]. Vitexin drags colon cancer cells into apoptosis and prevents cell growth; and drags leucocythemia cells (U937) into apoptosis due to its effects on mitochondrial signaling pathways [34].

Lignans were found in subfractions. Lignan and its derivatives are used in cancer treatment. In subfractions, it was considered that pinoresinol, hidroxypinoresinol and lariciresinol glycosides were found. Pinoresinol-rich olive oil showed apoptotic effect in colon cancer cells [35].

Fatty acids are key components for cell membranes and storage lipids in all living organisms. Oxygenated unsaturated fatty acids are generally called oxylipins. Oxylipins synthetized from plants are known to have important roles against pathogens, and regulating growth and development in the plant [36]. Hayashi *et al*. [37] found out cytotoxic effects of 9-hydroxy-10,12-octadecanoic acid, 13-hydroxy-9,11-octadecanoic acid isolated from water extract of rice bran against P388 mouse leukemia cells. Bioactivity studies were not found in the literature on oxylipins defined in this study.

Another group found in subfractions is hydroxycinnamic acid-spermidine conjugations. Ponchet *et al*. [39] mentioned about the existence of hydroxycinnamic acid-amides in flowery sections of plants belong to Araceae. In species belonging to *Arum* genus, presence of alkaloids is mentioned but there are no detailed studies on this subject. Bienz *et al*. [38] mentioned about the presence of N^1,N^6-dicoumaroyl-putrescine, N^1,N^5-dicoumaroyl-spermidine and N^1,N^{10}-dicoumaroyl-spermidine in *A. maculatum*. Putrescine, spermidine and spermine derivatives that are taken as both endogenously and dietary play a role in wound healing and tumor growth. The excessive increase of polyamine levels in cancer cells is remarkable. Studying possible effects of a polyamine analog on K562 chronic myeloit leukemia cells, Wang *et al*. [40] found its effects on key enzyme in catabolic pathway in K562 cells, proliferation prevention and apoptosis inducing effects based on dose and time.

4. Conclusion

Present study represents the first compherensive phytochemical analysis of *A. italicum* tuber growing wild in Turkey. Totally forty compounds were tentatively identified in tuber samples providing sensitive and rapid analyses by using the correct and acceptable data of MS and MS/MS together with the information previously reported in the literature and introduced data in the in-house library.

With this study, the results of cytotoxic, antiproliferative and apoptotic effects *A. italicum* tuber samples were firstly reported. According to our results, E2 showed powerful cytotoxic effect comparable to mitoxantrone in A549 lung cancer cells. It is suggested that, in future studies, compound(s) of E2 responsible for this effect be elucidated. Thus, it will be possible to assess this effect on compound(s) basis or mention synergistic effect. Besides, it is necessary to emphasize the necessity to study cytotoxic effect in *in vivo*.

When cytotoxic effects were studied, E2 and E6 showed appreciable effect against MCF-7 cells and E2, E3 and E6 showed appreciable effect against A549 cells. Especially, the cytotoxic effect of E2 against A549 is at a comparable level to mitoxantrone used in the treatment. In the formation of cytotoxic effect, it can be said that E2 and E6 inhibit DNA synthesis in MCF-7 cells; E2, E3 and E6 both inhibit DNA synthesis and drag cells into early apoptosis in A549 cells. That subfractions with their effects defined were not toxic on healthy cells at studied doses was shown on mouse origin

normal fibroblast cells. This finding may provide preliminary insight on selectivity of these subfractions.

In the light of all findings obtained in this study, comments and assessments, it is considered that especially combinations of E2, E3 and E6 may be clarified in more detailed studies to assist in the discovery of new natural compounds and/or combinations that will encourage hope in terms of cancer treatment.

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Supporting Information

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