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Electrochemotherapy enhances the efficacy of Asparagus officinalis, Arum elongatum and Urtica dioica extracts in breast cancer treatment

Elektrokemoterapi meme kanseri tedavisinde Asparagus officinalis, Arum elongatum ve Urtica dioica özütlerinin etkinliğini arttırır

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Abstract

The aim of the current study was to determine the phenolic substances in the stem of *Asparagus officinalis* L*,* leaves of *Arum elongatum S*teven and *Urtica dioica* L. plants collected from different regions and their cytotoxicities on MCF-7 human breast cancer cell line and to reveal the effects of electroporation (EP) on the antiproliferative activities of these plants. Antiproliferative activities of plant extracts were determined in MCF-7 human breast cancer cells, and their biocompatibility was determined in L-929 fibroblast cells by MTT analysis method. In electrochemotherapy (extract+EP) applications of MCF-7 human breast cancer cells, eight square wave electrical pulse sequences with an intensity of 800 V/cm were used with various doses of plants extracts. It was found that all three plant extracts were rich in phenolic compounds and only *A. elongatum* showed a relative cytotoxic effect on L-929 fibroblast cells. However, MCF-7 cancer cells showed very good sensitivity to the cytotoxic activities of *A. officinalis, A. elongatum* and *U. dioica* extracts, with IC50 values of 443.57, 361.88, and 448.55µg/mL, respectively. It was observed that with EP application, the cytotoxic activity of all three plant extracts on cancer cells increased significantly compared to extract application and cell viability percentages decreased significantly. As a result, our findings suggest that *A. officinalis, A. elongatum* and *U. dioica* extracts have anticancer potential and may be promising for breast cancer when used with EP.

Özet

Bu çalışmanın amacı, farklı bölgelerden toplanan *Asparagus officinalis* L. bitkisinin gövdesinde, *Arum elongatum* Steven ve *Urtica dioica* L. bitkilerinin yapraklarında bulunan fenolik madde içeriklerini ve bunların MCF-7 insan meme kanseri hücre hattı üzerindeki sitotoksisitelerini belirlemek ve bu bitkilerin antiproliferatif aktiviteleri üzerine elektroporasyonun (EP) etkilerini ortaya koymaktır. Bitki ekstraktlarının antiproliferatif aktiviteleri MCF-7 insan meme kanseri hücrelerinde tespit edildi ve biyouyumlulukları MTT analiz yöntemi ile L-929 fibroblast hücrelerinde belirlendi. MCF-7 insan meme kanseri hücrelerinin elektrokemoterapi (ekstrakt+EP) uygulamalarında, bitki ekstraktlarının çeşitli dozları ile 800 V/cm şiddetinde sekiz kare dalga elektriksel darbe dizisi kullanıldı. Her üç bitki ekstraktının da fenolik bileşikler açısından zengin olduğu ve sadece *A. elongatum*'un L-929 fibroblast hücreleri üzerinde göreceli sitotoksik etki gösterdiği bulundu. Ancak MCF-7 kanser hücreleri, sırasıyla 443.57, 361.88 ve 448.55µg/mL IC_{50} değerleriyle *A. officinalis*, *A. elongatum* ve *U. dioica* özütlerinin sitotoksik aktivitelerine karşı çok iyi duyarlılık gösterdi. EP uygulamasıyla, üç bitki özütünün de kanser hücreleri üzerindeki sitotoksik aktivitesinin özüt uygulamasına kıyasla önemli ölçüde arttığı ve hücre canlılık yüzdelerinin önemli ölçüde azaldığı gözlendi. Sonuç olarak, bulgularımız *A. officinalis, A. elongatum* ve *U. dioica* özütlerinin antikanser potansiyeline sahip olduğunu ve EP ile kullanıldığında meme kanseri tedavisi için umut verici olabileceğini düşündürmektedir.

INTRODUCTION

National and international organizations recommend the consumption of edible wild plants due to their different nutritional contents and therapeutic properties. This situation directs researchers to investigate the pharmacological properties of traditional and wild edible plants (Sommano et al. 2013, Tunçtürk and Özgökçe 2015, Cüce et al. 2018, Alam et al. 2020, Alaca et al. 2021, Cüce and Basançelebi 2021). As a result of these studies, it was noted that consuming plants in the diet has beneficial effects on health due to the pharmacological properties of the secondary metabolites that they synthesize as a result of complex metabolic reactions. It is also stated that plant phenolic compounds such as flavonoids,

stilbenes and lignans are powerful radical scavenging molecules that alleviate the adverse effects of oxidative damage in both *in vitro* and *in vivo* experiments (Pandey and Rizvi 2009, Cosme et al. 2020).

Asparagus officinalis, an edible plant from the Liliaceae family, is not only rich in many biologically active phytochemicals, including oligosaccharides, steroidal saponins, amino acid derivatives and essential minerals, but also used in the treatment of various cancers (Lei et al. 2017, Cheng et al. 2019, Sun at al. 2020, Zhang et al. 2021). Saponins, one of the most important secondary compounds of *Asparagus*, have proven antitumor, antihypertensive, antioxidant, immunomodulatory, hypoglycemic and antiepileptic effects (Guo et al. 2020).

Arum elongatum, which has traditionally been widely used in the treatment of abdominal pain, arterial hypertension, diabetes, rheumatism and hemorrhoids, shows promising biological activities depending on its phenolic compound composition under the influence of the ecological environment (Fawzi et al. 2023). Secondary metabolites such as saponin, phenols, tannin, flavonoids alkaloids and flavonoids are abundant in different extracts of *Arum* species (Jaradat and Abualhasan 2016). In different countries of the Middle East, different parts of *Arum* genus plants are used in the treatment of diseases such as abdominal pain, antibacterial activity, hypertension and diabetes. In addition, it has been determined that different extracts are effective against leukemia (k562) and colon cancer, as well as being consumed as hot drinks, spices, and herbal medicine against various types of cancer (Azab 2017, Al-Daghistani et al. 2021).

Urtica dioica, an herbaceous plant native to North America, India, Malaysia and tropical regions, is rare in Europe and Africa. It has pharmacological properties because it contains many secondary metabolites such as tannin, mucilage, wax-like substance, formic acid, phytoestrin, calcium, potassium nitrate, orticin, iron and a type of glycoside that has skin irritating effects (Hartmann et al. 1996, Ait-Mohamed et al. 2011). *U. dioica* extract was shown to have antitumor and antiproliferative properties in PC3 prostate cancer and MDA-MB-468 breast cancer cell lines (Mohammadi et al. 2016a, Mohammadi et al. 2016b).

Breast cancer remains one of the most common types of cancer in the world. In addition, its cutaneous and subcutaneous metastasis increase malignancy with high mortality (Di Prata et al. 2023). In recent years, there have been significant advancements in the treatment of individuals with breast cancer, but there is a necessity for the discover of new, efficient treatment methods that have low side effects (Pallerla et al. 2021).

Electrochemotherapy (ECT) is a local treatment technique that applies short and high-intensity electrical pulses to cancerous cells in order to increase the permeability of the cell membranes to poorly permeable or impermeable chemotherapeutics (Larkin et al. 2007). In ECT treatment, the efficiency will be higher if electroporation (electrical pulses) is applied when the substance is most concentrated around the cancer cells. It works well for treating skin metastases from different types of cancer, such as breast cancer (Schmidt et al. 2014) ECT is a safe and effective treatment technique to manage breast cancer; It can be done on areas that have been previously irradiated or treated differently and can be done repeatedly (Matthiessen et al. 2018). This therapy offers a more favorable option compared to chemotherapy due to its reduced side effects as a result of using a lower dosage of medication. As stated above, although there are many studies showings that *A. officinalis, A. elongatum,* and *U. dioica* plant extracts can be used in the treatment of various types of cancer, studies on the effectiveness of electroporation application of these plant extracts on cancerous cells are quite limited. To our knowledge, no study has been conducted on the effect of ECT technique on the cytotoxic activities of *A. officinalis, A. elongatum,* and *U. dioica* plants in MCF-7 cancer cells.

In the literature reviews, it is one of the pioneering studies conducted on breast cancer cells using the ECT method of *A. officinalis, A. elongatum* and *U. dioica* plant extracts.

The current study aims to determine the phenolic substance contents of *A. officinalis, A. elongatum* and *U. dioica* plant extracts by HPLC, as well as to elucidate

the cytotoxic effects of the extracts on MCF-7 human breast cancer cells and L-929 fibroblast cells using chemotherapy and ECT treatment techniques.

MATERIAL AND METHOD

Plant Materials

Fresh plants of *A. officinalis, A. elongatum,* and *U. dioica* was collected from rural area near the Muş City (Eastern Anatolia region, Türkiye) in April and May 2023. The plants used in our study were identified according to Davis (1970) Flora of Turkey. After the collected plant samples were taken to the laboratory, they were packaged in the freezer section of the refrigerator set at -20 °C to be used in the experimental stages.

Preparation of Extracts

After taking 1 g of the plant samples taken out of the freezer, they were transferred to 50 mL falcon tubes and 10 mL of extraction solution (consisting of methanol, 96% ethanol and distilled sterile water prepared in 1:1:1 ratio) was added. Then, the plant samples were completely fell aparted with a tissue lyser at 15 000 rpm (IKA, T18 digital Ultra Turrax, Germany). After the obtained extract was centrifuged at 500 rpm for 25 minutes at +4 ℃ degrees, the supernatant was transferred to 2 mL eppendorf tubes. The extract was stored in the freezer at -20 ℃ degrees to be used in later cell culture studies (Azmir et al. 2013).

In Vitro Anticancer Activity

Cell Lines and Culture

MCF-7 human breast cancer cell line and L-929 fibroblast cell line were employed in the study. The cell lines utilized in the research were acquired from Mus Alparslan University's Application and Research Center. Cell lines were cultured in DMEM media (Capricorn) mixed with pen-strept (100 IU/mL, 10 mg/mL) and 10% fetal bovine serum (FBS, Sigma) at 37 ℃ in a humidified environment with 5% CO_2. Once the culture had grown to 85–90% confluence, removed from flask bottom with 0.25% trypsin, 5 min centrifugated at 1300 rpm and used in experiments.

Cytotoxicity

10.000 cells from each cell line were seeded in 96-well plates and placed in a carbon dioxide incubator for 24 h. Following the incubation time, the DMEM inside the wells was removed and different doses of *A. officinalis, A. elongatum*, and *U. dioica* extracts (0, 10, 50, 100, 200, 400 and 800 µg/ml) and doxorubicin (0,1, 5, 10, 25, 50, 100 µg/ml) were added to the wells and incubated for another 24 h. Following period of incubation, the cells were examined to determine their viability through the utilization of MTT analysis, and the toxicity of the extracts on the cells was assessed.

Electrochemotherapy Protocol

In order to evaluate the effectiveness of electroporation with various *A. officinalis, A. elongatum,* and *U. dioica* extracts concentration (0, 10, 50, 100, 200 µg/ml) and reference drug doxorubicin (0, 1, 10, 50, 100 µg/ml) on MCF-7 cancer cells, cell solutions at a density of 1 × 106 were prepared and 400 µL of cell solution was placed in each electroporation cuvette (Bio rad, 0.4 cm). Cuvettes were subjected to 8 square wave electrical impulses with pulse widths of 100 µs and frequencies of 1 Hz at electric field strength of 800 V/cm employing a BTX Gemini X2 EP device (Harvard Apparatus, USA) electroporator. These ECT settings were already optimized for MCF-7 cancer cells in prior experiments (Bute and Alkis 2022). After the cells were incubated for 14-15 min at room temperature, they were seeded in 96-well plates (10.000 cells/each well) and left in a carbon dioxide incubator for 24 h. The viability of the cells was assessed using the MTT test following period of incubation.

MTT Analysis

The MTT assay was performed to assess the viability of cells and the extracts' cytotoxic activity (Ghasemi et al. 2016). After the incubation periods were completed, the growth medium was removed from the wells, MTT solution dissolved 1/9 ratio in PBS was applied to each well and left to incubate at 37 °C for 4 h. The solution with

MTT was thrown out after 4 h and 100 µl of Dimethylsulphoxide (DMSO) was administered to each well in order to dissolve the formazan crystals formed during the MTT process. Next, the absorbance was measured using an ELISA reader (Thermo Fisher Scientific, Finland) at a wavelength of 570 nanometers. This test was performed three times for each dose of extract. Since the MTT is affected by light, the experiments were carried out in a dark environment. The values of absorbance (AV) calculated from the wells containing *A. officinalis, A. elongatum* and *U. dioica* extracts and doxorubicin solutions were compared with the control absorbance value (AV), and the % viability ratio was determined using the formula below.

% Viability= (Treatment group AV/Control group AV) x100

The IC_{50} (50% inhibitory concentration) was calculated by plotting the percentage inhibition plotted against the concentration of a substance.

Measurement of Phenolics Content Composition

To determine the quantity of phenolic compounds using HPLC, the final concentrations of apigenin, abscisic acid, gallic acid, ascorbic acid, trans-p-coumaric acid, kaempferol, 3,4-dihydroxybenzoic acid, quercetin, 4-hydroxybenzoic acid, myricetin acid, catechol, rosmarinic acid, caffeic acid, cinnamic acid and vanillin standards were measured in order to prepare solutions with a 10 mg per mL concentration. Next, the standards were supplemented with a 1% mixture of acetic acid and acetonitrile (at the rate of 9/1), and an equal amount of methanol was included in order to create the stock standards. The calibration curve was established using diluted stock standards at 10, 25, 50, 75, and 100 µg/mL concentrations (Tapan 2016). The plant leaf extracts: concentration was set to 20 mg per mL using the same solutions as in the standard. The extracts samples were filtered (0.45 µm membrane filter) and then placed into an HPLC machine. Agilent Technologies 1260 Infinity II HPLC machine was utilized for the HPLC analysis (Agilent, USA). The HPLC machine had a 1260 DAD WR detector with wavelengths of 272 nm, 280 nm, and 310 nm. It also had a 1260 Quat Pump VL pump with a flow rate of 1.0 mL per min, a 1260 Vial sampler that injected 20 µL, and a G7130A column furnace set at 28°C. Analysis was conducted using the ACE 5 C18 column (4.6x250 mm).

Statistical Analysis

Each treatment was designed in randomized blocks with three biological replicates and included two pots (each containing four seedlings). The data were analyzed using SPSS (v.17, SPSS Inc., USA). Duncan's multiple range test was employed to establish statistical significance. In all the analyses, statistical significance was shown by $p < 0.05$ (Demiralay, 2022). The outcomes were calculated as the mean ± standard deviation of the average of three independent experimental results.

RESULTS

During the extraction of *A. officinalis, A. elongatum* and *U. dioica* plant leaves, it was observed that *A. officinalis* caused more foaming compared to *A. elongatum* plant. Additionally, it was determined that very little foaming occurred in the *U. dioica* plant.

Phenolic Compound Contents of Plant Extracts

In the phenolic compound analysis in our study, extracts of *A. officinalis, A. elongatum,* and *U. dioica* were found to contain ascorbic acid, 3,4-Dihydroxybnz acid, 4-Hydroxybenzoic acid, trans-p-coumaric acid, abcisic acid, quercetin, apigenin, kaempferol, myricetin, catechol, vanillin, caffeic acid, cinnamic acid, gallic acid, and rosmarinic acid contents are shown in Table 1.

Cytotoxicities of Plant Extracts

The breast cancer cell line MCF-7 and fibroblasts cell line L-929 were exposed for 24 hours to doxorubicin and an extracts of *A. officinalis, A. elongatum* and *U. dioica* at doses ranging from 0 to 800 µg/ml. L-929 fibroblasts were used as a reference cell line to evaluate the cytotoxicity of the extracts on healthy cells and chemotherapeutic drug doxorubicin was utilized as a positive control. To measure the inhibitory effects of the extracts and doxorubicin, we calculated IC_{50} (dose that reduces cell viability by half) values and presented them in Table 2.

Table 1. Phenolic substances content (µg/g). The outcomes were calculated as the mean ± standard deviation of the average of three independent experimental results (N/A: Non-available)

Phenolic substances	*A. officinalis*	*A. elongatum*	*U. dioica*
Ascorbic acid	67.4±0.13	664.3±0.71	68.1±0.14
3,4-Dihydroxybenzoic acid	N/A	N/A	0.0155±0.0000023
4-Hydroxybenzoic acid	13.3±0.0023	26.96±0.0018	21.1±0.053
Trans-p-coumaric acid	1367.79±0.517	1139.6±0.0532	453.88±0.0006
Abcisic acid	69.333±0.00226	26.47±0.01744	N/A
Quercetin	N/A	N/A	N/A
Apigenin	85.69±0.1302	182.37±0.0387	N/A
Kaempferol	13.9±0.01053	N/A	N/A
Myricetin	20.62±0.0059	N/A	19.96±0.0055
Catechol	6.45±0.006	842.3±0.41	1617.81±0.0007
Vanillin	N/A	494.7±0.0226	44.207±0.003
Caffeic acid	N/A	242.5±0.084	N/A
Cinnamic acid	10.09±0.014	11.95±0.044	14.2±0.306
Gallic acid	N/A	N/A	N/A
Rosmarinic acid	N/A	N/A	7.91± 0.002

Table 2. IC_{50} values of *A. officinalis, A. elongatum, U. dioica* extracts and doxorubicin in fibroblast and cancer cell lines

Plant extracts	L-929- IC_{50} (µg/ml) Extract	MCF-7-IC_{50} (µg/ml) Extract	MCF-7-IC_{50} (µg/ml) Extract+EP
Asparagus officinalis	1451.17	443.57	154.48
Arum elongatum	604.66	361.88	105.20
Urtica dioica	1101.61	448.55	235.88
Doxorubicin	92.09	22.30	6.04

Effects of Electroporation on The Cytotoxicity of Plant Extracts

The percentage of cell viability in the groups treated with extract alone and extracts combined with electroporation (EP) after 24 hours was measured using the MTT assay. Since ECT is an application specifically aimed at locally cancerous cells, it was applied only to MCF-7 cancer cells in this study.

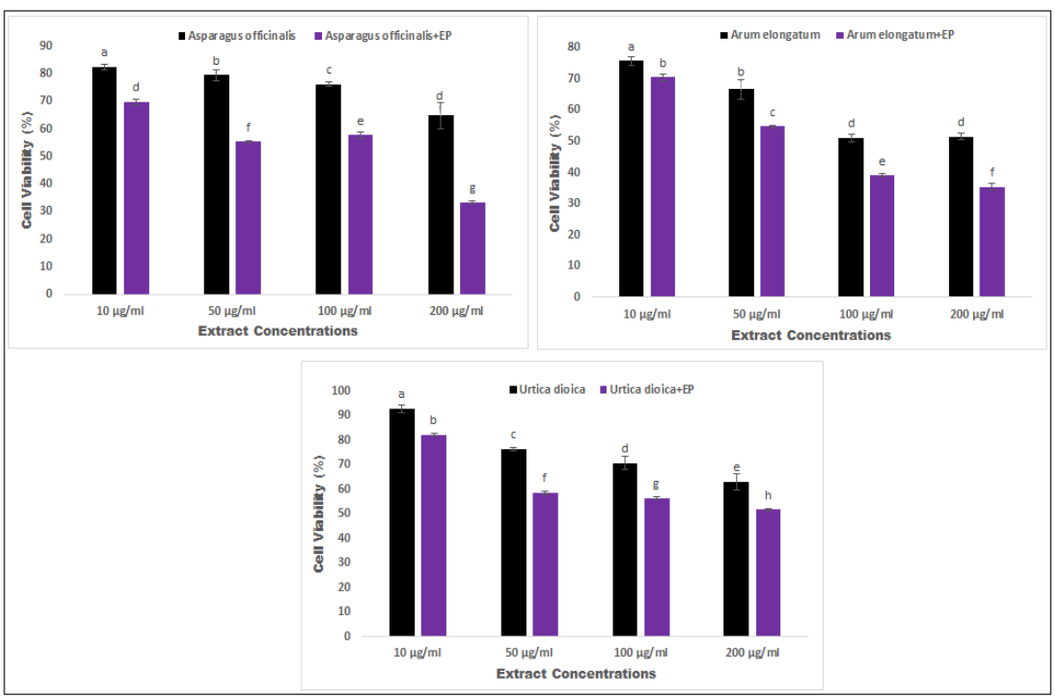

Figure 1. Effects of different doses of *Asparagus officinalis, Arum elongatum and Urtica dioica* extracts on MCF-7 cancer cells viability due to extracts alone, and extracts +EP after 24 hours of treatment ($p<0.05$)

The EP, when combined with the reference medication doxorubicin and extracts, significantly reduced their IC_{50} levels for MCF-7 breast cancer cells ($p< 0.05$). IC_{50} values of *A. officinalis, A. elongatum* and *U. dioica* extracts and doxorubicin fell from 443.57 to 154.48, 361.88 to 105.20, 448.55 to 235.88 and 22.30 to 6.04 µg/mL, respectively (Table 2).

DISCUSSION

Foaming may indicate that saponin contents, which are important secondary metabolites, are higher in *A. officinalis, A. elongatum* and *U. dioica* plants, respectively. Extracts containing saponins are used in the treatment of various types of cancer (Cheng et al. 2019). It is known that phenolic compounds, which are known to have antioxidative and anti-cancer properties on human health, are produced as a result of the secondary metabolism of plants and reinforce the antioxidant system (Cüce et al. 2017, Napoli et al. 2018, Cüce et al. 2019). Trans-p-coumaric acid, abcisic acid, kaempferol and myricetin phenolics were found to be higher in *A. officinalis* plant than other plant extracts. It was determined that the contents of ascorbic acid, 4-hydroxybenzoic acid, apigenin, vanillin and caffeic acid in *A. elongatum* were higher than the other two plants. It is observed that the phenolic compound contents of 3, 4-hydroxybenzoic acid, cinnamic acid and rosmarinic acid are higher in the *U. dioica* plant (Table 1). Additionally, during extraction, it was observed that *A. officinalis, A. elongatum* and *U. dioica* plants foamed, respectively, as an indicator of saponin content. This indicates that mixtures of phenolics and saponins may have strong cytotoxic effects on MCF-7 human breast cancer cells.

The strength of toxicity can be determined by the IC_{50} value; lower values indicate strong toxicity and higher values indicate weak toxicity (Alkış et al. 2021a). While *A. officinalis* and *U. dioica* extracts showed almost no cytotoxic effect (IC_{50}>1000 µg/mL) on L-929 fibroblast cells, *A. elongatum* showed low cytotoxic effect (IC_{50}=604.66 Table 2). The IC_{50} values in Table 2 show that the extracts exhibited good levels of cytotoxic effect on MCF-7 cancer cells. The IC_{50} values of the *A. officinalis, A. elongatum* and *U. dioica* extracts and doxorubicin were calculated as 443.57, 361.38, 448.55 and 22.30 µg/mL, respectively, for MCF-7 cancer cells. Although *A. elongatum* showed a better cytotoxic effect on MCF-7 cancer cells than *A. officinalis* and *U. dioica* extracts, it also adversely affected healthy cells, so it should be used with caution.

As seen in Figure 1, cells treated with extracts combined with EP show significantly reduced cell viability compared to cells treated with extract alone. It was observed that *A. officinalis* and *A. elongatum* showed better antiprolifreative activity than *U. dioica* extracts in ECT treatment. *A. officinalis* is thought to be the most efficient extract among the extracts used in the study, as it has no side effects on healthy cells and reduces MCF-7 cancer cell viability at a high level when used in combination with EP. The percentage of cell viability in groups treated with both extract alone and extracts plus EP decreased in a concentration-dependent manner. Combining electrical signals with plant extracts may have facilitated the entry of extracts into cells by increasing the permeability of the cell membrane (Mondal et al. 2023). In ECT treatment of our study, doxorubicin (reference drug) and extracts of *A. officinalis, A. elongatum* and *U. dioica* probably entered the cell more and increased their cytotoxic effects. Similar positive results were observed both in our previous studies examining the effectiveness of EP on different compounds (Alkış et al. 2022, Savcı et al. 2022a, Alkış et al. 2021b and Savcı et al. 2022b), and in many other studies (Bieżuńska-Kusiak et al. 2023). Poompavai et al. (2021) examined the effect of EP on the anti-cancer activity of neem extracts in MCF-7 cancer cells. At the end of their examination, they found that treatment of cancer cells with neem extracts + EP was much more effective than neem extracts alone. Wichtowski et al. (2019) also emphasized the effectiveness of ECT in the treatment of metastatic breast cancer. Overall, our findings support the combined use of *A. officinalis, A. elongatum,* and *U. dioica* extracts with EP as palliative therapy in MCF-7 breast cancer cells.

CONCLUSION

In light of the findings, it was determined that *A. officinalis, A. elongatum* and *U. dioica* extracts were

rich in phenolic compounds with antiproliferative properties. While all three plant extracts showed high cytotoxic activity on MCF-7 breast cancer cells, it was observed that only *A. elongatum* extract showed a relative cytotoxic effect on L-929 fibroblast cells. When the cytotoxicity of plant extracts on cancer cells was compared, it was observed that although *A. elongatum* extract showed the best cytotoxic effect on cancer cells, it also had a cytotoxic effect on L-929 fibroblast cells. However, although *A. officinalis* extract had less cytotoxic effect on cancerous cells than *A. elongatum* extract, it was observed that it did not show any cytotoxic effect on L-929 fibroblast cells. While a significant increase was recorded in the cytotoxic activity of all three plant extracts on cancerous cells with EP application, it was observed that cell viability percentages decreased significantly in extract+EP application compared to extract application. As a result, although *A. officinalis* is the best candidate, it can be said that all three plant species help in the treatment of MCF-7 breast cancer cells and electroporation application significantly increases the antiproliferative activities of the extracts.

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