KSÜ Tarım ve Doğa Derg 27 (2), 293-303, 2024
KSU J. Agric Nat 27 (2), 293-303, 2024
DOI:10.18016/ksutarimdoga.vi.1292478

Investigation of Cytotoxic, Antimetastatic and Apoptotic Activities of Jerusalem Artichoke (*Helianthus tuberosus* L.) Extracts: Comparison with MCF-7 and MCF-12A Cells

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ABSTRACT

Jerusalem artichoke (*Helianthus tuberosus* L.) is an important medicinal plant due to its composition of several bioactive substances, high antioxidant activity, rich inulin content and produces functional food ingredients. Complementary alternative medicine therapies become more popular for cancer treatments because of higher efficiency, lower cost and minimum side effects. This study reported anti-cancer efficiency of both shell and tuber extract of Jerusalem artichoke (JA) on different breast cell lines with the purpose of discovering new alternative medicine therapies. Cytotoxic effects of JA were evaluated by XTT method by using different concentrations on different incubation times (24h, 48h and 72h). Invasion, adhesion and apoptotic studies were performed with IC_{50} values for 48h and 72h. JA extracts do not show cytotoxic activity on healthy human breast cell, while they induce tumoral cell death with dose and time-dependent manner. The most effective doses of tuber and shell extracts were 108.8 µM and 134.2 µM at 48h and 20 µM ve 5 µM at 72h, respectively. It demonstrated robust anti-metastatic activity on MCF-7 cell lines because it inhibited adhesion, and invasion, significantly. TMRE and AnnexinV/7AAD staining were used for determine to mitochondrial membrane potential and nuclear morphology, respectively The results were found consistent with each other. Overall, this paper which first in literature demonstrates the anti-cancer efficacy of the JA both tuber and shell extract which can be proposed as a potent candidate for the treatment of breast cancer.

Moleculer Biology

Research Article

Article History
Received : 05.05.2023
Accepted : 28.09.2023

Keywords
Adhesion
Apoptosis
Cytotoxicity
Invasion
MCF-7 cells

Yer Elması (*Helianthus tuberosus* L.) Ekstraktlarının Sitotoksik, Antimetastatik ve Apoptotik Aktivitelerinin Araştırılması: MCF-7 ve MCF-12A Hücrelerinin Karşılaştırılması

ÖZET

Tamamlayıcı alternatif tıp tedavileri, daha yüksek verim, daha düşük maliyet ve yan etkilerinin minimum olması nedeniyle kanser tedavileri için daha popüler hale gelmektedir. Bu bağlamda çeşitli biyoaktif maddelerden oluşması, yüksek antioksidan aktivitesi, zengin inülin içeriği ve fonksiyonel gıda bileşenleri üretmesi nedeniyle yer elması (*Helianthus tuberosus* L.) önemli bir tıbbi bitkidir. Bu çalışma, yeni alternatif tıp tedavilerini keşfetmek amacıyla, yer elmasının hem kabuk hem de yumru özütünün farklı meme hücre hatları üzerinde kansere karşı etkinliğini araştırmak için yapılmıştır. Yer elmasının sitotoksik etkileri, farklı inkübasyon sürelerinde (24s,48s,72s) farklı konsantrasyonlar kullanılarak XTT yöntemi ile değerlendirilmiştir. İnvazyon, adezyon ve apoptotik çalışmalar 48 saat ve 72 saat uygulamalardan elde edilen IC_{50} değerlerine göre yürütülmüştür. Yer elması özütleri sağlıklı insan meme hücresi üzerinde sitotoksik aktivite göstermezken, doz ve zamana bağlı olarak tümöral hücre ölümünü indüklemiştir. Yumru ve kabuk özlerinin en etkili dozları 48 saatte sırasıyla 108.8 µM ve 134.2 µM ve 72 saatte 20 µM ve 5 µM olarak gözlenmiştir. Yer elması özleri MCF-7 hücre hatları üzerinde adezyon ve invazyonu önemli ölçüde engellemesi

Moleküler Biyoloji

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 05.05.2023
Kabul Tarihi : 28.09.2023

Anahtar Kelimeler
Adezyon
Apoptoz
İnvazyon
MCF-7 hücreleri
Sitotoksisite

nedeniyle güçlü bir anti-metastatik aktivite göstermiştir. Mitokondriyal membran potansiyelini ve nükleer morfolojiyi belirlemek için sırasıyla TMRE ve AnnexinV/ 7AAD boyaması kullanıldı. Sonuçlar birbiriyle uyumlu bulundu. Literatürde bir ilk olan bu çalışma, yer elmasının hem yumru hem de kabuk özütünün anti-kanser etkinliğini ortaya koyarak meme kanseri tedavisi için önerilebilir.

Atıf Şekli: Beyazyüz, F., Arslan, E., & Koygun, G (2024). Yer Elması (*Helianthus tuberosus* L.) Ekstraktlarının Sitotoksik, Antimetastatik ve Apoptotik Aktivitelerinin Araştırılması: MCF-7 ve MCF-12A Hücrelerinin Karşılaştırılması. *KSÜ Tarım ve Doğa Derg 27*(2), 293-303. DOI: 10.18016/ksutarimdoga.1292478.

To Cite : Beyazyüz, F., Arslan, E., & Koygun, G (2024). Investigation of Cytotoxic, Antimetastatic and Apoptotic Activities of Jerusalem Artichoke (*Helianthus tuberosus* L.) Extracts: Comparison with MCF-7 and MCF-12A Cells. *KSU J. Agric Nat 27*(2), 293-303. DOI: 10.18016/ksutarimdoga.1292478.

INTRODUCTION

Cancer is a worldwide disease which is significantly affects human health due to uncontrolled division, reproduction and accumulation of cells in organism. Approximately 18% of cancer deaths are of breast cancer ranked origin third after lung and colorectal cancers (Çakır et al. 2016; Çapanoğlu and Bakar 2018). Most breast cancer deaths are substantially depend on metastasis to different parts of organs such as the lymph nodes, bones, lungs, brain and liver (Wang et al. 2010). Decreasing of cell-cell adhesion amount and anchorage-independent growth are two main hallmarks of cancer formation. Tumor cells circulating in the bloodstream are found in clusters and these cells migrate massively (Janiszewska et al. 2020). Cluster formation of circulating tumor cells originates from the primary tumor, and this cluster formation is more effective in metastasizing than individual cancer cells. Surgery, chemotherapy and radiotherapy have been used for cancer treatments (Debela et al.,2021) but these have various side effects, such as destruction of healthy cells, reaching insufficient dosage to the tumor site and drug instability. Several complementary and alternative medicine threapies have become increasingly popular for cancer tratments (Akhtar et al. 2018). In these sense, cancer chemoprevention strategies using foods and herbs has been recognized as one of the most visible areas for new and safe anti-cancer products (Islam et al. 2022) and various studies revealed that natural products are associated with a reduction in the incidence of breast cancer remains to be resolved (Yap et al. 2021).

Various studies are about proposed a series of compounds from medicinal plants with potential anti-cancer activities (Roy,2021). So, in a study which is about metastatic breast cancer patients showed the five-year survival rate was found to be 25%, indicating the importance of targeted therapy (Hortobagyi et al. 2003; Majumder et al. 2019). Besides, several phytochemical researches demonstrated that JA which include coumarins, unsaturated fatty acids polyacetylenic derivatives could be a therapeutic agent for medicinal property (Pan et al.,2009; Gupta D & Chaturvedi, 2020).

Antioxidant, anti-inflammatory, antitumor and antibacterial capacity of plant extracts are related to phenolic, flavonoids, isoflavonoids and anthocyanins compunds which play a highly significant role in drug discovery and development process (Pan et al. 2009; Yuan et al. 2013; Wang et al. 2020). Therefore, high antioxidant capacities plants are commonly preffered to novel chemical compound for cancer research field (Iqbal et al. 2017; Gupta et al. 2020) and JA tuber has high antioxidant activity (Seljåsen et al. 2005; Saikaew et al. 2010; Amarowicz et al. 2020) and also it has biologically active substances constituents eg, caffeoylquinic acid isomers (Kapustaet al. 2013). JA is has all the essential amino acids for human (Mariadoss et al. 2021, includes flavonoids, terpenoids, and some protein complexes which are inhibited tumor cell proliferation and has toxic effects on tumor cells and also flavonoids and phenolic acids can exert antioxidant effects by removing various free radicals (Wang et al. 2020). JA tubers are rich in inulin, protein and other bioactive compounds which lead to it is traditionally used in folk medicine (Michalska-Ciechanowska et al. 2019; Sawicka et al. 2020). Inulin has been successfully applied in several anticancer therapy. It has been determined that inulin confers selective in vitro cytotoxicity to cancer cells both as a drug-loaded, self-forming nanocarrier and as self-organizing prodrugs (Giammona, et al. 2016). A study revealed that JA leaf and tuber extracts altered the cell proliferation (Nizioł-Łukaszewska et al. 2018). However, heliangin (source of sesquiterpene lactones) obtained from JA extracts showed significant activity against Ehrlich ascites carcinoma cells (Yang et al. 2015; Sawicka et al. 2020).

In this study it was aimed to determine the cytotoxicity effect of the methanol extracts of the peeled shell and tuber extracts of JA on MCF-7 breast cancer cell line and MCF-12A human breast ephitelial cell line with comparasions to each other and also aimed to examine the effect on adhesion and invasion abilities for antimetastatic activity depend on obtained cytotoxic

doses for JA shell and tuber extracts. Furthermore this study is the first in the literature in terms of determining the cytotoxic effect of JA using the XTT method, performing adhesion and invasion analysis to determine the antimetastatic effects of both tuber and shell extracts of JA on MCF-7 cells and determining its apoptotic effects.

MATERIAL and METHOD

Cell Proliferation Kit (XTT based) -(Biological Industries), Cell Adhesion Assay Kit (Thermo V13181), Cell Invasion Assay Kit (Collagen I)-(Biovision), Tetramethylrhodamine Ethyl Ester (TMRE) (Perchlorate) (Cayman), Annexın V, 7AAD Peridinin Chlorophyll Protein percp, (Becton Dickinson-Bd), Dimethyl sulfoxide (DMSO)(Biochrom), Tripan Blue (ABCAM), Trypsin-Edta (TE) (Biochrom -%0.05W/V, without Ca2+, Mg^{+2}), Fetal Bovine Serum (FBS)(Biochrom), %1 Penicillin- Steptromycin (Biochrom), Phosphate Buffer Saline (PBS) (Biochrom), Dulbecco's Modified Eagle Medium (DMEM).

Extraction of plant

JA materials were purchased from local market in Turkey. JA gently washed and then tuber and shell were dried separately on blotter paper at room temperature. The dried plant material was ground separately with grinder and turned into powder. The extraction process was carried out by Downey et al. (2007) method. JA tuber and shell were extracted separately at 60 °C with orbital shaker at room temperature in the dark. After the mixture was filtered with Whatman filter paper no1 and then the clear filtrate was removed from methanol at 40 °C using a rotary evaporator. The crude extracts obtained were weighed in order to calculate the extraction efficiency. These extracts were lyophilized by lyophilizer device. After that, the extracts were weighed in accordance with the doses and prepared by dissolving in sterile water and stored at + 4°C to be applied to the cells.

Cell culture

MCF-7 breast cancer cells (ATCC® HTB-22™) were obtained from American Type Culture Collection (Rockville, MD, USA). RPMI 1640 medium with broad applicability to support the proliferation of MCF-7 cell line and DMEM medium for human breast cell line, MCF-12A (ATCC® CRL-10782™) was used in this study (Figure1). RPMI 1640 medium was prepared by adding 10% FBS and 0.1% gentamicin and DMEM was prepared by adding 10% FBS, 0.1% penicilin strep, 20 ng/ml EGF, 500 ng/ml Hydrocortisone, 0.01 mg/ml Human insulin and %1 NEAA (nonessential amino acids).

Cells were proliferated at 37°C in a humidified atmosphere of 5% CO2 incubator by changing the medium for every 24 to 48 h. The cells were counted by staining with trypan blue in order to evaluate whether the cells had grown in sufficient number and cell viability.

Cell viability determination

The 2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium5-carboxanilide inner salt (XTT) assay was used to measure metabolic activity of viable cell lines. Cells were seeded (1×10^4 cells/mL), in a final volume of 200 µL, in a 96-well microplate and treated with different concentrations of JA extracts (5000, 2500,1250, 615, 312.5, 156.25, 78.125, 39.06,19.53, 9.765 µM) for 24h,48h,72h. 100 µL of XTT solution was added to each well and further incubated for 4 h at 37 °C. A medium supplemented with DMSO was used as a control. The absorbance was measured at 490 nm with ELISA reader (Biotek). Then IC_{50} values were calculated with linear regression plots as the sample concentration which resulted in 50% reduction of absorbance compared to controlled (untreated) cells. Cell viability was calculated by using the Graphpad Prism. Each concentration of JA extracts was independently assayed with three replicates.

Adhesion analysis

The cell-adhesion study was analyzed by using Vybrant Cell Adhesion Assay Kit (V-13181). The method was applied according to the manufacturer's instruction. MCF-7 cells were seeded at

2.5×10^5 cells/well in 100 µl solution and incubated for 48h and 72h. Cytotoxic doses of shell extracts and tuber extracts of JA were added into plate wells MCF-7 cells were washed with PBS and resuspended with serum-free RPMI-1640 medium. Then, Calcein AM stock solution which is a sensitive method for determining the effect on DNA polymerization activity and for cell viability testing 27 was added and incubated for 120 min. After the non-adherent cells were washed away, 200 µl of PBS was added. The incubated cells were scanned in a fluorescent microplate (Biotek) reader at Ex / Em = 504 nm / 523 nm wavelength and the results were analyzed.

Invasion analysis

The cell-invasion assay was analyzed with "EZCell Cell Invasion Assay Kit" (Biovision, K917-24). The kit procedure used a Boyden chamber lined with collagen-I in a 24-chamber plate, where cells invade the matrix and pass through a semipermeable membrane in the chamber in response to stimulants or inhibitory compounds. Cells were seeded at 1.5×10^5 cells /well in the upper chamber. Serum-free medium was added to the lower wells. 200µl of plant cell was added to the

upper chambers and incubated for 48h in accordance with the IC_{50} concentrations. The cells were counted and the suspension in the upper chambers of the plate was aspirated. The upper chambers were dried and centrifugated at 1000×g for 5 min. Bottom chambers were washed with 500 µl and then wash buffer aspirated. Cell dye was added to the separation solution and mixed thoroughly. 500 µl of mix was added to each well of the lower chambers and the upper chambers were placed on the lower chambers and incubated at 37 °C for 30 min. The suspension in the lower chambers was transferred to plate for fluorescence reading (BioTek) at Ex / Em = 485/530 nm wavelength.

Analysis of MMP and determining the rates of early apoptotic and late apoptotic cells

Mitochondrial membrane potential (MMP) of MCF-7 cells treated with JA extracts was visualized by TMRE staining and nuclear morphology was analyzed by AnnexinV-7AAD staining for determination of apoptotic stages. Annexin V is a Ca^{+2}-dependent protein that binds to phosphatidylserine (PS) molecules on the surface of the cell membrane in cells undergoing apoptosis (Demchenko,2013).7-Aminoactinomycin D (7-AAD) which is used to distinguish and count living cells from dead or damaged cells is a nucleic acid dye used for the detection of late apoptotic cells (Wadkins and Jovin, 1991). The combination of AnnexinV and 7AAD staining was analyzed with fluorescence microscope which was used to identification for early and late apoptotic cells. Early apoptotic cells express phosphatidylserines (PS) on the outer leaflet of the plasma membrane. PS can be stained by labeled annexin V. Late apoptotic cells and necrotic cells lose their cell membrane integrity and are permeable to vital dyes such as 7-AAD (Zimmermann and Meyer, 2011). Cells were seeded into the plate and treated with JA extracts with IC_{50} concentrations. Untreated cells were used as the control group. The cells were washed with PBS for 10 min at room temperature after 48h and 72h treatment. Next, MCF-7 cells were incubated with TMRE and Annexin V-7AAD solutions for 15 min, then washed twice with PBS solution and visualized with fluorescence microscope (ZEN 2.3 SP1).

Statistical analysis

The MCF-7 and MCF-12A cells were incubated 24h, 48h and 72 h for the cytotoxicity assay was performed in three replicates. Absorbance/fluorescence values of each repeat were calculated by the microplate reader as mean and ± SD values. Various concentrations of dose response curves of shell and tuber extracts of JA were performed by Graph-Pad Prism 8.2.0 software. The statistical difference among the experimental groups was measured by using one-way ANOVA followed by Duncan's analysis. p-value of <0.05 was considered statically significant.

RESULTS

Results of cell viability

The cytotoxicity effects of 10 different concentrations (5000,2500,1250,615,312.5,156.25, 78.125, 39.06, 19.53 and 9.765 µM) of both JA tuber and shell extracts were determined for 24h, 48h and 72h incubation time. Tuber and shell extracts of JA were obtained for cytotoxicity effect on MCF-7 cells. Table 1 shows the inducing cytotoxicity doses of MCF-7 cells. The cytotoxic doses at 48h (108.80µM for tuber extract and 134.23µM for shell extract) and 72h (20.08µM for tuber extract and 4.75µM for shell extract) were used for adhesion, invasion, and apoptosis assays and the of IC_{50} doses and lower concentration was used for the control group for these assays.

Tablo 1. Yer elmasının yumru ve kabuk özlerinin MCF-7 hücreleri üzerindeki IC*50* değerleri.
Table 1. *IC_{50} values of tuber and shell extracts of Jerusalem artichoke on MCF-7 cells.*

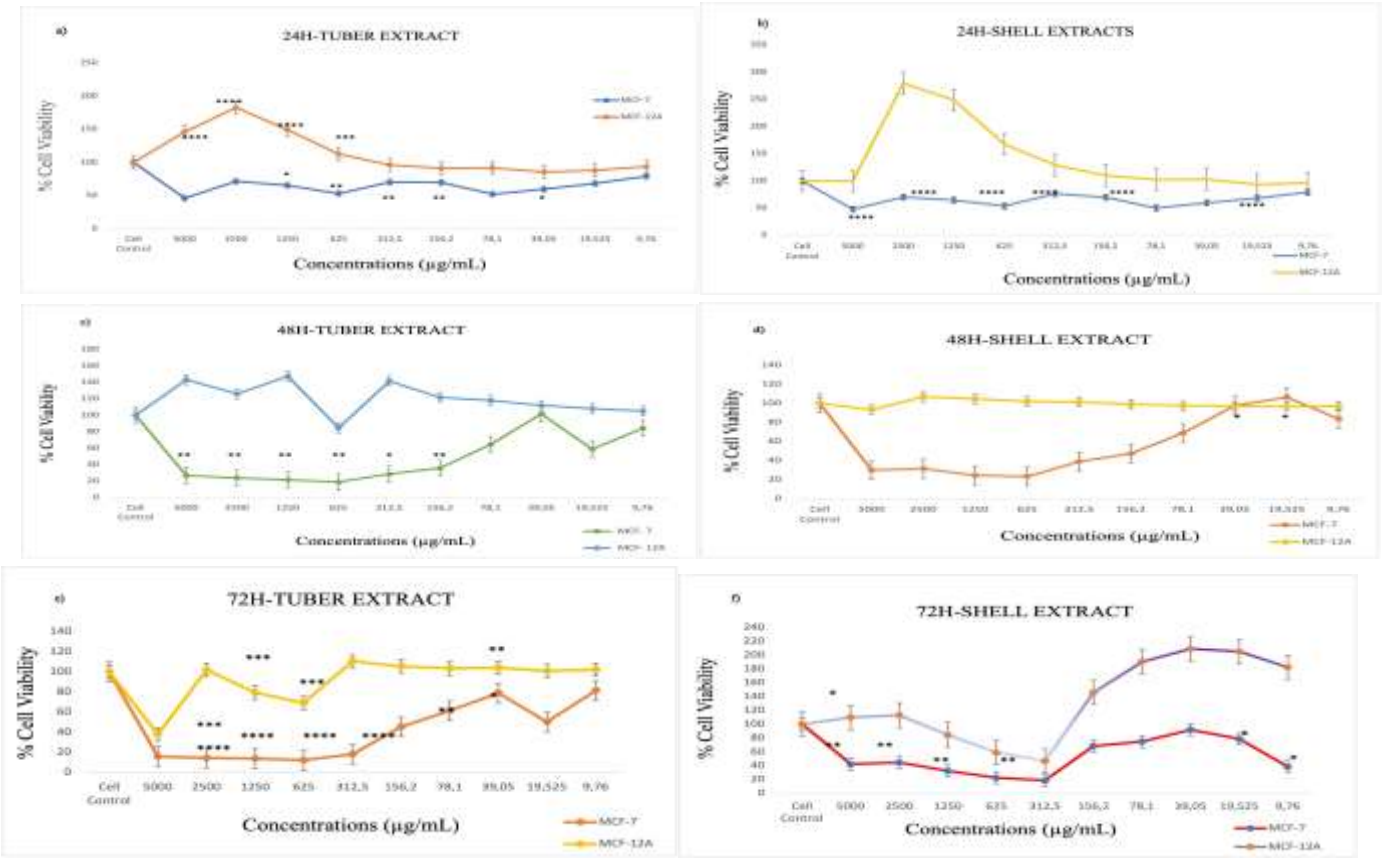
Incubation Time İnkübasyon süresi	Tuber extract Yumru özütü	Shell extract Kabuk özütü
24h	79.16µM	78.69µM
48h	108.80µM	134.23µM
72h	20.08µM	4.75µM

Tuber extracts of JA were 21.46µM on MCF-12A cells and it was determined that the shell extracts of JA did not fall below 80% at 24h (Figure 1a and 1b). The results showed that the tuber extracts did not have toxic effects on MCF-12A cells on 24h, 48h. A cytotoxic effect was observed only on 72h of both extracts' application (Figure 1e, 1f). The IC_{50} value of shell extract was 225.99µM.

Adhesion results

The ability of malignancy is mostly dependent on the degree of increasing number of adherent cells. The amount of adhesion of cancer cells treated with JA extracts is expected to decrease compared to untreated cancer cells. In this context, the JA tuber extracts which treated with MCF-7 cell line on 20, 50, 100µM concentrations was decreased compared to the control group by 19%, 29% and 50% at 48h, respectively (Figure 2a). It was determined that a significant result

at 100µM concentration which is the IC_{50} dose. However, it was observed that the shell extracts the treated 48h with MCF-7 cell line at 10, 50, 100 and 134µM decreased adhesion properties compared to the control group by 3%, 18%, 48% and 59%, respectively (Figure 2b). It was observed that applying JA tuber extract at concentrations of 10, 20 and 40µM on 72h for MCF-7 cells decreased the adhesion properties of the cells compared to the control group by 19%, 50% and 62%, respectively (Figure 2c).



Şekil 1. Yer elmasının MCF-7 ve MCF-12A hücre hattı üzerindeki hücre canlılığına etkileri a) Yumru özlerinin 24 saat inkübasyonu, b) Kabuk özütlerinin 24 saat inkübasyonu, c) Yumru özütlerinin 48 saat inkübasyonu d) Kabuk özütlerinin 48 saat inkübasyonu, e) Yumru özütlerinin 72 saat inkübasyonu, f) Kabuk özütlerinin 72 saat inkübasyonu (Grafiklerdeki veriler ± standart hata 'dir. **** p <0,0001, ** p <0,001, * p <0,005).

Figure 1. Cell viability effects of Jerusalem artichoke on MCF-7 and MCF-12A cell line a) 24h of tuber extracts, b) 24h shell extracts, c) 48h tuber extracts d) 48h extracts, e) 72h tuber extracts, f) 72h shell extracts (Data in graphs are mean ± SD **** p <0.0001*, ** p <0.001, * p <0.005).*

Furthermore, it was observed that applying JA shell extract at concentrations of 2.5, 5 and 20µM on 72h for MCF-7 cells decreased the adhesion properties of the cells compared to the control group by 67%, 47% and 17%, respectively (Figure 2d).

Invasion results

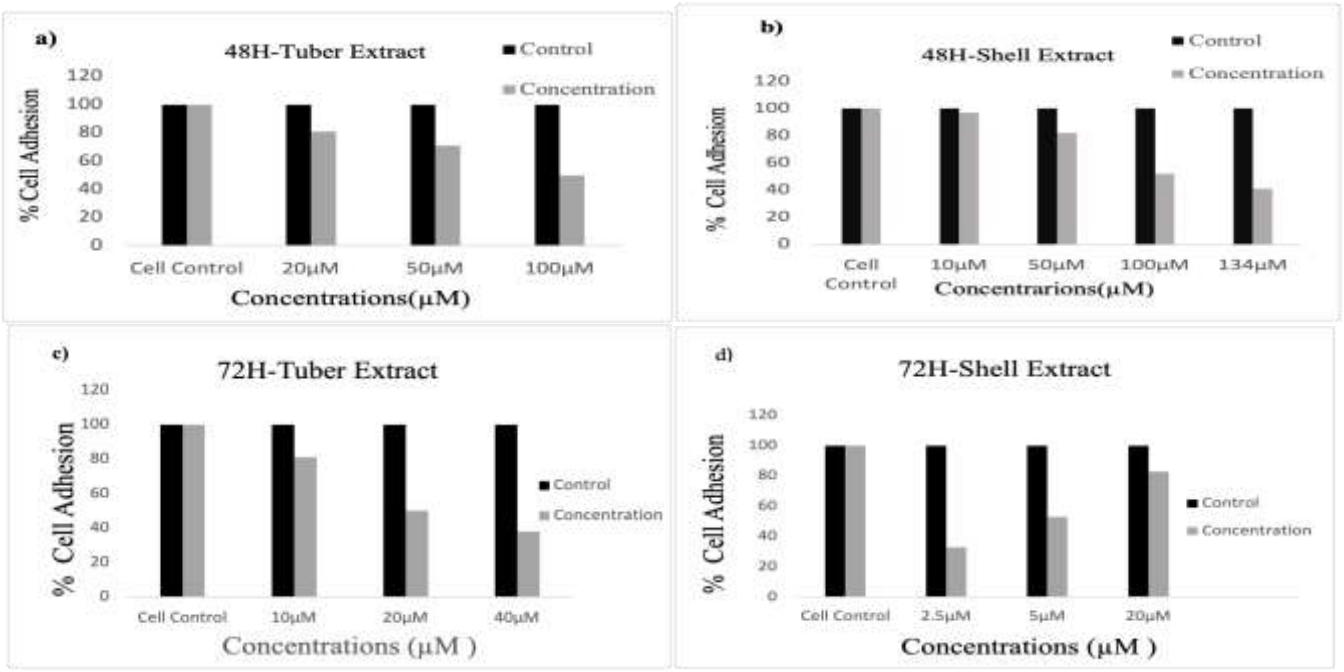
We analysed the invasive properties of MCF-7 cells treated with both tuber and shell extracts of JA with different concentrations depend on to IC_{50} doses. It was determined that 20µM, 50µM and 100µM concentrations of tuber extract decreased the invasion properties on 48h of the cells compared to the control group by 2%, 30% and 35% respectively (Figure 3a). Also, 10µM, 50µM, 100µM and 134µM concentrations of JA shell extract decrease the invasion properties on 48h of the cells compared to the control group by 2%, 8%, 9% and 11%, respectively (Figure 3b).

10µM concentration of tuber extract on 72h increased invasion properties by 1% while 20µM and 40µM concentrations of extracts were decreased invasion properties by 2% and 3%, respectively (Figure 3c). On the other hand, 2.5µM, 5µM and 20µM concentrations of shell extracts on 72h were decreased invasion properties compared to the control group by 1%, 2% and 50%, respectively (Figure 3d).

Analysis of MMP

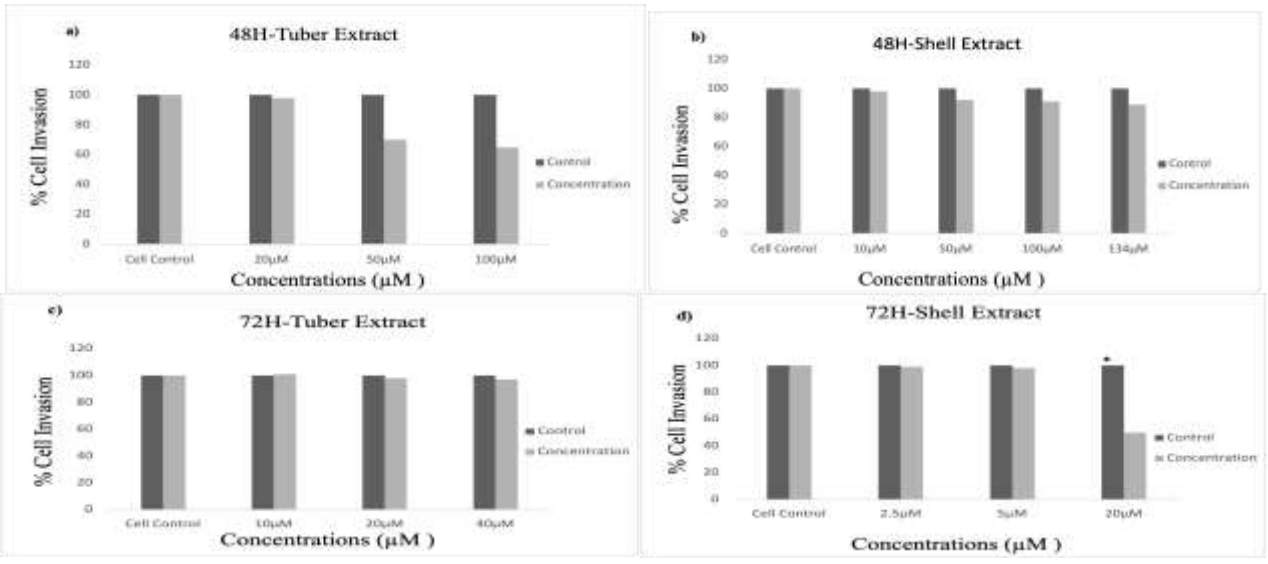
MMP was visualized by Tetrametilrodamin etil ester (TMRE) staining. According to the results, tuber extract application on MCF-7 cells 20µM, 50µM and 100µM concentrations decreased by MMP absorbance

12%, 18% and 20%, respectively at 48h while the shell extract application on MCF-7 cells 10µM, 50µM, and 134µM concentrations increased by MMP absorbance 0%, 5%, and 20% compared to the control group, respectively (Figure 4a). In this context significant



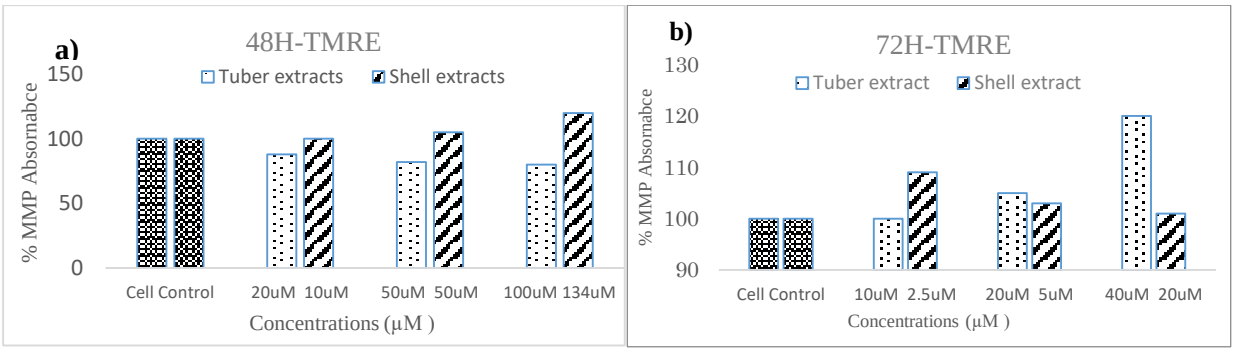
Şekil 2. Yer elmasıyla muamele edilmiş MCF-7 hücre hattının adezyon üzerindeki konsantrasyona bağlı etki grafiği a) 48 saatlik uygulamada yumru özütlerinin 20,50 ,100µM konsantrasyonları, b) 48 saatlik uygulamada kabuk özütlerinin 10, 50, 100,134 konsantrasyonları, c) 72 saatlik uygulama yumru özütlerinin 10,20,40µM konsantrasyonları, d) 72 saatlik uygulamada kabuk özütlerinin 2.5,5,20µM konsantrasyonları.

Figure 2. Concentration-dependent effect on adhesion of MCF-7 cell line treated with JA a) 20,50,100µM concentrations of tuber extracts in 48h application, b) 10, 50, 100,134 concentrations of shell extracts in 48h application, c) 10,20,40µM concentrations of tuber extracts in 72h application, d) 2.5,5,20µM concentrations of shell extracts in 72h application.



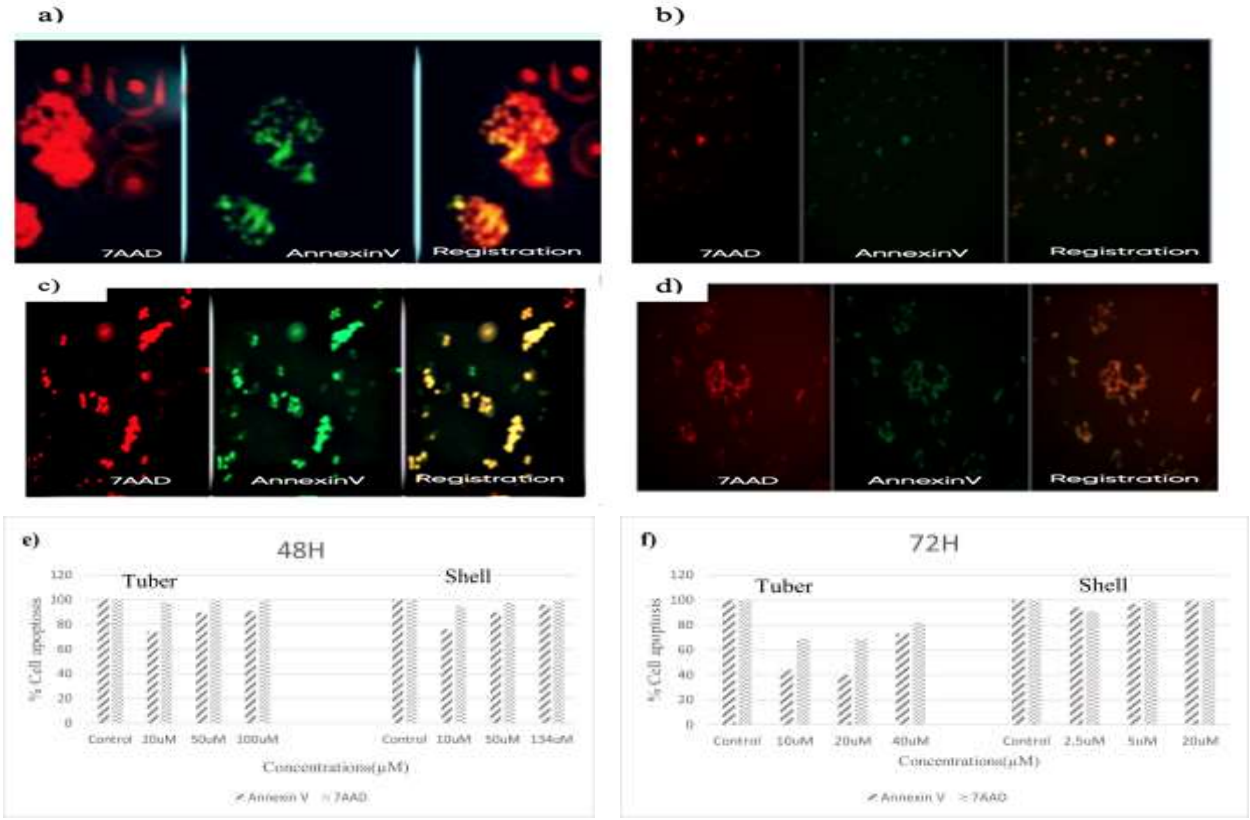
Şekil 3. Yer elmasıyla muamele edilmiş MCF-7 hücre hattının invazyon üzerindeki konsantrasyona bağlı etki grafiği a) 48 saatlik uygulamada yumru özütlerinin 20,50,100µM konsantrasyonları, b) 48 saatlik uygulamada kabuk özütlerinin10,50,100,134µM konsantrasyonları, c) 72 saatlik uygulamada yumru özütlerinin 10,20,40µM konsantrasyonları, d) 72 saatlik uygulamada kabuk özütlerinin 2.5, 5,20µM konsantrasyonları.

Figure 3. Concentration-dependent effect on invasion of JA-treated MCF-7 cell line a) 20,50,100µM concentrations of tuber extracts at 48h b) 10,50,100,134µM concentrations of shell extracts at 48h, c) 10,20,40µM concentrations of tuber extracts at 72h, d) 2.5, 5,20µM concentrations of shell extracts at 72h.



Şekil 4. TMRE boyamasının MCF-7 hücre hattında analizi a) 48 saatlik uygulamasının yumru özütlerinin 20,50,100µM konsantrasyonları; kabuk özütlerinin 10, 50, 134µM konsantrasyonları, b) 72 aatlik uygulamasının yumru özütlerinin 10,20,40µM konsantrasyonları;kabuk özütlerinin 2.5,5,20µM konsantrasyonları,

Figure 4. Analysis of TMRE staining in MCF-7 cell line a) 48h treatment with 20,50,100µM concentrations of tuber extracts; 10, 50, 134µM concentrations of shell extracts, b) 72h treatment with 10,20,40µM concentrations of tuber extracts; 2.5,5,20µM concentrations of shell extracts.



Şekil 5. MCF-7 hücreleri üzerinde Annexin V ve 7AAD boyaları ile boyanmış yer elması özütlerinin floresan mikroskop görüntüsü a) 48 saat 100µM konsantrasyonda işlenmiş yumru özütleri için b) 48 saat 134µM konsantrasyonda işlenmiş kabuk özütleri için. c) 72 saat 40µM konsantrasyonda işlenen yumru özütleri için d) 72 saat 5µM konsantrasyonda işlenen kabuk özütleri için. Veriler, MCF-7 hücre hattındaki yer elması karşılaştırmalı kabuk ve yumru özlerinin apoptotik hücrelerin yüzdesini floresan plaka ile doza bağımlı bir şekilde değiştirdiğini gösterdi. e) 48 saatlik uygulamada, yumru özütleri için 20µM 50µM 100µM konsantrasyonları ve kabuk özütleri için 10µM, 50µM ,34µM konsantrasyonları f) 72 saatlik uygulamada yumru özütleri için 10µM, 20µM ,40µM konsantrasyonları ve kabuk özütleri için 2.5µM, 5µM,20µM konsantrasyonları.

Figure 5. *Fluorescent microscope image of Jerusalem artichoke extracts stained with Annexin V and 7AAD dyes on MCF-7 cells a) for tuber extracts treated 48h 100µM concentration b) for shell extracts treated 48h 134µM concentration. c) for tuber extracts treated 72h 40µM concentration d) for shell extracts treated 72h 5µM concentration.The data showed that Jerusalem artichoke comparative shell and tuber extracts on MCF-7 cell line changes the percentage of apoptotic cells a dose-dependent manner by fluorescent plate reader e) 48h treatment, for tuber 20µM 50µM 100µM concentrations and for shell extracts 10µM, 50µM ,34µM concentrations f) 72 h treatment for tuber 10µM, 20µM ,40µM concentrations and for shell extracts 2.5µM, 5µM,20µM concentrations.*

MMP change was not observed in the tuber extract, while the shell extract significantly increases by MMP absorbance level at 48h applications. Furthermore, tuber extract application on MCF-7 cells 10µM, 20µM and 40µM concentrations increased by MMP absorbance 18%, 17% and 19%, respectively at 72h while the shell extract application on MCF-7 cells 2.5µM, 5µM, and 20µM concentrations increased by MMP absorbance 9 %, 3%, and 1% compared to the control group, respectively (Figure 4b). According to these results, MMP values were found to be significant in tuber extract for 48h, while significant in shell extract for 72h.

Determining the rates of early apoptotic and late apoptotic cells

When Annexin-V and 7-AAD are used together, early apoptotic cells are stained with annexin-V, while late apoptotic cells are stained with both Annexin-V and 7-AAD and living cells do not stain (Figure 5 a, b, c, d). The 20µM, 50 µM and100 µM concentration of tuber extract on 48h showed that 26%, 10% and 9 %undergone early apoptosis and 2%,1% and 0.9% gone to late apoptosis relative to the control, respectively and also the 10µM, 50µM, 134µM concentration of shell extract on 48h showed that 24%, 10%, and 4% undergone early apoptosis and 5%, 2% and 1% gone to late apoptosis relative to the control, respectively (Figure 5.e)

The 10µM, 20µM and 40µM concentration of tuber extracts showed that 56%, 60%, and 27% undergo early apoptosis at 72h and 31%, 31% and 18% went to late apoptosis, respectively and also the 2.5µM, 5 µM and 20 µM concentration of shell extracts showed that 6%, 3% and 0.9% undergo early apoptosis at 72h and 9%, 1% and 1% went to late apoptosis relative to the control, respectively (Figure 5.f). Furthermore 2.5µM, 5µM and 20µM concentration of shell extracts for 72 h results showed that 6%, 3%, and 0.9% undergo early apoptosis and 9%, 1% and 1% went to late apoptosis, respectively (Figure 5 f).

DISCUSSION

Various plants have been used to develop new cancer treatments (Roy,2021). JA is an alternative plant for cancer treatment because of "inulin" content which has protective properties against many diseases, and helps to eliminate toxin substances in the intestines (Wang et al.,2020). Although rare studies about the cytotoxicity of JA by MTT method (Pan et al. 2009; Yuan et al. 2013; Griffaut et al. 2007) no literarure could found about antimetastatic and apoptotoic effect of JA on breast cancer. In our study, XTT assay was used for JA tuber and shell extracts on MCF-7 cells. Researchers showed that stressed JA (non-dormant) tuber extracts decreased cytotoxicity on MCF-7 cell lines (Griffaut et al. 2007) and we obtained both tuber extracts and shell extracts decreased cytotoxicity on MCF-7 cells time and dose-dependent manner. In this study, the effective dose for shell extracts of JA on cancer cell line at 24h and 48h, the doses do not toxic on healthy cell lines (MCF-12A) and effective doses at 72h for shell extracts of JA has changed on healthy cells by dose-dependent manner. Shortly, the JA extracts were effective on MCF-7 cells while MCF-12A cells (control group) were not effective at 24h,48h and 72h application.

Pan et al. (2009) showed that methanol extract of JA contained several bioactive substances eg, amino acids, flavonoids, phenolic acids and they determined that JA methanol extracts have cytotoxicity on the MCF-7 cell line. Furthermore, Petkova et al.(2014), showed that ethanol extracts of JA tubers and leaves have higher antioxidant activity than water extracts and Niziol Lukaszewska et al. (2018), determined that the JA ethanol extracts of leaves and tubers changes cell proliferation and may alter the oxidative stress gene expression levels on HaCaT and BJ fibroblast cells. We used methanol extraction method for JA tuber and shell similar to the Pan et al. (2009) and our results showed that JA tuber and shell extracts changes cell proliferation on MCF-7 cells. Yuan et al.(2013) investigated the cytotoxic effect of the compounds (Hydroxy-8ß-tigloyloxy-dehydroaliglovine, a newly known sesquiterpene lactone and sesquiterpene lactones and two known flavones) obtained from JA leaves for MCF-7 cells on 48h incubation with MTT method and as a result, they reported that some of these compounds (sesquiterpene lactones) had a cytotoxic effect for the MCF-7 cell line. On the other hand we used XTT method for cell proliferation different from these researchers and our results supported to them that JA both extracts significantly inhibits the proliferation of MCF-7 cells at 48h incubation. According to the researchers' MTT results, the proliferation and vitality decreased depending on the dose increase. In this context, it can be said that both the tuber and the shell extracts have an antiproliferative effects on MCF-7 cells and XTT method could be used as an alternative to MTT method.

An antimetastatic formulation would be a potential biological weapon in breast cancer treatment cause there are no antimetastatic drugs are available (Majumder et al. 2019). Determining the migration, adhesion and invasion phenomen of cancer cells and molecular mechanism determinations are essential for new clinical strategies on cancer diagnosis, prognosis, drug development and treatment (Friedl and Wolf 2003)In addition, metastasis is the main cause of cancer death, 90% of deaths can be attributed to metastatic spread (Kramer et al. 2013) and determination adhesive and invasive properties of

progression is a very important and challenging step in cancer treatment (Anderson et al. 2019). Similarly to our study Beşli et al. (2019) showed that metformin caused a decrease in the expressions of some proteins involved in the breast cancer invasion pathway.

Various plant extracts that can be used for breast cancer treatment have been reported to have significant cytotoxic activity in a dose-time dependent manner and inhibit metastasis based on adhesion and invasion analysis (Majumder et al. 2019; Majumder et al. 2020; Tavares-Carreón et al.2020, Demir et al 2020). This study was the first to determine the antimestatic effects (adhesion and invasion) of both the tuber extracts and the shell extracts of the JA on MCF-7 breast cancer cells the discussion was rather limited. In this context, the results showed that the shell extracts IC_{50} dose (134uM) more effective to decreasing of adhesion ability for 48h while the tuber extracts IC_{50} dose (100uM) was more effective for invasion rate at 48h. The tuber extract IC_{50} dose (20uM) decreased 50% of adhesion rate while the shell extract IC_{50} dose (5uM)) decreased 47% of adhesion rate, so it can be said that both tuber and shell extracts are effective for decreasing adhesion rate on MCF-7 cell at 72h. The invasion results of tuber and shell extracts on MCF-7 cells at 72h the IC_{50} values of both extracts were not effective but the 20 µM concentrations of shell extracts decreased 50% of invasion rate at 72h. These results showed that the invasion and adhesion analysis are significantly related to the cytotoxicity results.

Annexin-V was used for analysis of the early stages of apoptotic cells and also it combined with 7AAD for analysis apoptotic and necrotic cells. Analysis of the early stage of the intrinsic pathway indicates that MOMP leads to a collapse of the mitochondrial membrane potential. The mitochondrial membrane potantial changes are another pointer of mid-phase apoptosis. TMRE is accumulated in the living cells of intact mitochondria when compared with cytosol (O'Reilly et al. 2003; Ricci et al. 2003). $\Delta\Psi m$ is measured by loss of TMRE fluorescent intensity which is a cationic, cell-permeant, and fluorescent dye (Matissek et al. 2003). After depolarization $\Delta\Psi m$ the TMRE intensity decreases in mitochondrial fluorescence intensity (Okal et al. 2013).

Afoakwah et al (2023)J showed that tuber extracts were able to inhibit cancer growth in HT-29 colon cancer cell line (HT-29 cc cell line) in a dose-dependent manner and they were also determined that these extracts inhibited HT-29 cc cell line growth resulting in programmed cell death. In this study, it was observed that JA tuber and shell variably reduced MMP in MCF-7 cells depending on IC_{50} doses. It was observed that tuber extract decreased MMP in MCF-7 cells in 48h application, while shell extract application increased MMP. In addition, it was observed that both extracts increased MMP in 72h application, but the increase in tuber extract was higher. In addition, in the study conducted to determine early and late apoptotic cells, it was determined that the effects of JA tuber and shell extract varied depending on the application hours and IC_{50} doses. In this context, tuber and shell extract application with 48h caused significant effects in the early apoptotic phase of MCF-7 cells, but no significant changes were detected in the late apoptotic phase. It was determined that tuber extract application caused significant effects in both early and late apoptotic stages of MCF-7 cells with 72h application, and shell extract application did not show any significant changes in both stages. In short, the results of early and late apoptosis and TMRE staining showed that 72h of application of JA tuber extract had a higher apoptotic effect on MCF-7 cells than the other extracts and hour applications.

CONCLUSION

Phytotherapy is one of the oldest methods of treatment for cancer. Because medicinal plants which are used in traditional medicines are cheaper than drugs from pharmaceutical companies medicinal plants derived from folk medicines are frequently used worldwide. In this sense, JA is one of the most popular plant widely cultivated in the Mediterranean area due to its nutritional and medicinal benefits. In this study, JA tuber and shell extracts showed high cytotoxic effect on the other hand MCF-12A breast epithelial cells have no cytotoxicity effect. The cytotoxic concentrations were used for metastatic and apoptotic analysis. This study is the first in the literature to use the XTT method to determine the cytotoxic effect of JA extracts in MCF-7 cells and also metastatic and apoptotic assays depend on cytotoxic doses of JA is also firstly analyzed. The results showed that JA extracts have antimetastatic properties and apoptosis-inducing ability. It is predicted that JA is a new therapeutic agent candidate for breast cancer treatment by determining for the first time the existence of antimetastatic and apoptotic effects on MCF-7 breast cancer cells. This further adds to the importance of validating using traditional medicinal plants and herbs in breast cancer therapy.

ACKNOWLEDGEMENT

This research was supported by Selçuk University Scientific Research Project (BAP). This study was presented as an oral presentation 1st International Plant Biology Congress in 2018 (10-12May) and published as an abstract in the congrees book. F. B and E.A. conceived the original idea and build E.A. supervised the project. The experiments were performed by F.B and G.K. and I would like to thank Mert SUDAĞIDAN for helping us with the devices for the experiments. This study was produced from the master thesis.

Conflict of Interest Statement

The authours report no conflict of interest.

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