Exploring the Antiproliferative Mechanisms of *Urtica dioica L*. extract in Human Promyelocytic Leukemia Cell Line

İnsan Promiyelositik Lösemi Hücre Hattında Isırgan Ekstraktının Antiproliferatif Mekanizmalarının Araştırılması

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Background: Nature is a source of therapeutic compounds which have fewer side effects compared to synthetic drugs. Stinging *Urtica dioica L*. extract, widely-used in Anatolia, has a strong antiproliferative effect on many types of cancer. However, the underlying molecular mechanisms of this effect is still not known.
Materials and Methods: In this study, the interaction of *Urtica dioica L*. extract at different concentrations with apoptosis and autophagy pathways in the human promyelocytic cell line (HL-60) was studied to elucidate how it triggers the antiproliferative effects. In this context, firstly, the plant leaves were extracted in water with the Soxhlet extraction method. HL-60 cells were incubated with the extract at different concentrations for 24 hours, and the activated antitumoral effect pathway was investigated with advanced following molecular tests: MTT staining, Nitric oxide (NO) level, Annexin-V in flow cytometry, cell cycle, mitochondrial membrane potential measurement (MMP) and qPCR for evaluation of apoptosis and autophagy mediator genes.
Results: It was determined that cell proliferation was suppressed at a concentration of 100 µg/mL and cells were kept in G0/G1 phase, MMP was impaired in cells and the rate of apoptotic cells increased. These apoptotic markers were confirmed by statistically increased expression of apoptotic and autophagy genes and NO level.
Conclusions: Taken together, it is predicted that *Urtica dioica L*. water extract initiates apoptosis in HL-60 cells and could be promising compound candidate for cancer treatment.
Keywords: HL-60*, Urtica dioica L*., Cancer, Apoptosis, APL

ÖZ.

Amaç: Doğa, sentetik ilaçlara kıyasla daha az yan etkiye sahip terapötik bileşiklerin kaynağıdır. Anadolu'da yaygın olarak kullanılan ısırgan otu özütü, birçok kanser türü üzerinde güçlü bir antiproliferatif etkiye sahiptir. Bununla birlikte, bu etkinin altında yatan moleküler mekanizmalar hala bilinmemektedir.
Materyal ve Metod: Bu çalışmada, insan promiyelositik hücre hattında (HL-60) farklı konsantrasyonlarda hazırlanan ısırgan ekstraktının apoptozis ve otofaji yolakları ile etkileşimi ve antiproliferatif etkileri incelenmiştir. Bu kapsamda öncelikle bitki yaprakları Soxhlet ekstraksiyon yöntemi ile suda ekstrakte edilmiştir. HL-60 hücreleri, 24 saat boyunca farklı konsantrasyonlarda ekstrakt ile inkübe edildi ve aktif anti-tümöral etki yolu, ileri moleküler testler ile araştırıldı: MTT boyama, Nitrik oksit (NO) seviyesi, flow sitometride Annexin-V, hücre döngüsü, mitokondriyal zar potansiyeli ölçümü (MMP) ile apoptozis ve otofaji yolak genlerinin değerlendirilmesi için qPCR.
Bulgular: 100 µg/mL konsantrasyonda hücre proliferasyonunun baskılandığı ve hücrelerin G0/G1 fazında tutulduğu, hücrelerde MMP'nin bozulduğu ve apoptotik hücre yüzdesinin arttığı belirlendi. Bu apoptotik belirteçler, apoptotik ve otofaji genlerinin istatistiksel olarak artan ekspresyonu ve NO seviyesi ile doğrulandı.
Sonuç: Tüm sonuçlar birlikte ele alındığında, ısırgan su ekstraktının HL-60 hücrelerinde apoptozisi başlattığı ve kanser tedavisi için umut verici bitki adayı olabileceği tahmin edilmektedir.

Anahtar Kelimeler: HL-60, *Urtica dioica L*., Kanser, Apoptozis, APL

Introduction

Acute Promyelocytic Leukemia (APL) is a bone marrow malignancy characterized by an excess of immature cells called promyelocytes. APL, was developed as a result of the disruption of the gene encoding for retinoic acid alpha receptor on the 17. chromosome, and this chromosome exhibits unique clinical features which are totally different from the other types of cancer (1).

Although there are many types of leukemia, some of them are more common in children. Other types are generally seen in adults. APL constitutes 5-8% of all acute myeloid leukemias (AML). Among the most recent treatment options, all-trans retinoic acid (ATRA) therapy and chemotherapy combinations are considered to be the most preferred approach, meanwhile a complete remission can be achieved in almost 90% of newly diagnosed APL patients (2).

APL is one type of cancer whose genetic and molecular activities have been well studied (3), however, most of the molecular and genetic mechanisms involved in the pathogenesis of APL are still unknown. Therefore, detailed molecular analyses of both the functional signaling pathways and the interaction of natural/synthetic agents with these signaling pathways can also play a substantial role in understanding the molecular etiology of the disease and developing new sophisticated therapeutic strategies. The main problems facing cancer therapies are that cancer cells can develop resistance to chemotherapy and other existing therapies which cause serious side effects. Therefore, the number of studies for investigating supportive and alternative drugs with less side effects, low cost and high cytotoxicity on cancer cells has been increased in recent years (4). The most popular agents in supportive and alternative drug development studies are obtained from medicinal plant extracts. Many studies have shown that herbs can reactivate blocked apoptotic pathways in cancer cells. It needs to be pointed that highly studied herbs are *Urtica dioica L*. plants (5, 6).

This plant has 40 species and about 500 subspecies, is endemic to Malaysia, India, America and tropical countries. However, it spreads less frequently in Europe and Africa. Although there are differences between the parts of the *Urtica dioica L*. plant but the main compounds that make up this extract are tannin, formic acid, phytosterin, iron and mucilage. It can be used use in the treatment of various diseases such as diabetes, atherosclerosis, cardiovascular disease, prostate cancer (7-10). There are three types of cell death pathways: apoptosis, necrosis, and autophagy. They play a large substantial role in eliminating abnormal cells in the tissue, keeping the cell number constant, regulating growth and eliciting the immune response. The reduction of apoptotic flow or the development of resistance plays an initiating role in cancer formation. therefore, new anti-tumor compounds must be specifically formed to target mediators that are located in the apoptotic pathway (11, 12). As we know, there are no studies showing the anti-tumors potential of the extract obtained from *Urtica dioica L*. plants in the HL-60 cell line. Therefore, in this study, it was aimed to elucidate the antiproliferative effects of *Urtica dioica L*. plants and it was supposed to have anti-proliferative effects in many types of cancer especially in the HL-60 cell line, this study was done to reveal their interaction with cell death pathway mediators.

Materials and Methods

Materials

The cell-culture medium (DMEM: F-12), fetal bovine serum (FBS), streptomycin, dimethyl sulfoxide (DMSO), MTT and penicillin were purchased from Sigma (Sigma-Aldrich, USA). The culture plates were purchased from Nunc (Brand products, Denmark). ELISA kits were purchased from Nitric oxide (NO) (My BioSource, USA).

Cell Culture

The following cell lines purchased from ATCC and kept in liquid nitrogen were used for the study: HL-60 (CCL-240-human leukemia cell line). The cell was cultured in DMEM: F-12 media supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/mL streptomycin/100 IU/mL penicillin in incubators at 37 ℃ under humid conditions containing 5% CO_2 and 95% air.

Plant Extraction

The stems and leaves of the plants to be used in the study were dried in a 40°C incubator. After the dried plant samples were pulverized, 100 gr was taken and extracted in polar solvent (water) using the Soxhlet extraction method. It was dried and lyophilized with the help of a rotary evaporator. 10 mg/mL stock was prepared from lyophilized plant extracts and used by dilution (DMSO was used> 1/1000 as solvent).

Cell Viability Assay

The cytotoxicity of the extracts obtained from plant were assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma-Aldrich, USA) assay. The cells were incubated in 24-well sterile plates for 24 hours with 1×10^5 cells per well. The media was removed and the *Urtica dioica L*. extracts were added at doses of 0, 50 and 100 µg/ mL subsequently, the media and the control (DMSO) were incubated for 24 hours and stained with MTT. Measurements in the luminescence mode were performed in a plate-reader (Thermo Scientific Multiskan GO, USA).

Annexin V/PI Double Staining

FITC Annexin V Apoptosis Detection Kit I (Cat No./ID:556547, BD, New Jersey, ABD) was used to detect the apoptosis of the HL-60 cells by flow cytometry pursuant to the manufacturer's instruction. According to the kit protocol, 5×10^6 cells were seeded in 6 well plates and incubated by applying DMSO and plant extract at dose of 100 µg/ml. It was transferred as 1×10^6 cells in 1x binding buffer. 5 µL of fluorochrome-conjugated Annexin V and 5

µL Propidium Iodide Staining dyes were added and incubated for 15 minutes at room temperature, 100 µL of 1x binding buffer was added and analyzed by flow cytometry (BD Via, New Jersey, USA).

Cell Cycle Assay

Cell cycle analysis was performed using the BD Cycletest ™ Plus DNA Reagent kit (BD Biosciences, New Jersey, USA) in accordance with the manufacturer's instructions. According to the kit protocol, $1x10^6$ cells were seeded in the 6-well plate for 24 hours and the cells were incubated with the plant extract at dose of 100 µg/ml. After incubation, 250 µL solution A was added and incubated in a light-free environment for 10 min. 200 µL of solution B is added for 10 minutes, and 200 µL of solution C is added for 10 minutes in a protected environment at +4 ◦C. All these analyzes were performed by flow cytometry (BD Via, NJ, USA).

JC-1 staining for mitochondrial membrane potential (MMP, Δψm)

To detect changes in mitochondrial function induced by MMP. The mitochondrial membrane potential was measured by JC-1 dye (5.5',6.6'-tetrachloro-1.1',3,3'-tetra ethyl imida carbo cyanine iodide) (BD ™ Mito Screen, New Jersey, USA). $1x10^6$ cells were seeded in the 6-well plate for 24 hours and the cells were incubated with the plant extract at dose of 100 µg/ml. After incubation, JC-1 (1 µg/mL) was added and incubated for 15 min at 37 °C in a dark environment. After incubation, cells were washed twice with 1x wash solution. All these analyzes were performed by flow cytometry (BD Via, NJ, USA).

Gene Expression

All gene expression analyses were performed after treating plant extract with a dose of 100 µg/ mL for 24 hr. Total RNA isolation was performed with miRNeasy mini-kit (Qiagen Hilden, Germany), and complementary DNA synthesis was done using the RT² First strand kit (Qiagen Hilden, Germany) according to the kit procedure. RT-qPCR, Rotor-Gene Q was performed on a Real Time PCR System (Qiagen Hilden, Germany) with QuantiTect SYBR Green PCR kit (Qiagen Hilden, Germany). qPCR protocol can be done at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, at 60 °C for 60 s and at 65 °C for 1 min. Each sample was studied in triplicate by using the primary series and GAPDH housekeeping gene. The gene expression was calculated using the 2^-ΔΔCt (fold change) method in comparison with the controls, and the p values were analyzed with GraphPad Prism 8 software. Primers were designed by using Primer blast on the National Center for Biotechnology Information website. All primers were determined to be 95-100% efficient and all showed only one dissociation peak. Sequences are listed in Table 1.

Determination of Nitric oxide (NO) level with ELISA

A total of $1x10^6$ HL-60 cells were incubated in a 6-well plate with plant extract (100 µg/ mL) for 24 hrs. We harvested the incubated cells and used cold PBS for washing. Then, the cells were decomposed at 0°C and centrifuged at 4°C, 12.000xg for 10 min. The absorbance was detected using a microplate reader (Thermo Scientific Multiskan GO, USA). A standard curve provided by the kit was used to calculate the activity.

Table 1. Primer sequences used in gene expression.

Primer	Forward(5'-3')	Reverse(3'-5')
CASPASE-3	TGTCGGCATACTGTTTCAGCA	TGGCGAAATTCAAAGGATGGC
CASPASE-8	TGGGAGAAGGAAAGTTGGACA	CGTTGATTATCTTCAGCAGGCTC
CASPASE-9	GCCCTGGCCTTATGATGTTTT	GCTGTTTCGGTGAAAGGGATTT
BAX	GCTGACATGTTTTCTGACGGC	TTGAGCACCAGTTTGCTGGC
BCL-2	TGACCCCACCGAACTCAAAG	GACTTCGCCGAGATGTCCA
P53	TAACAGTTCCTGCATGGGCG	ACCTCAAAGCTGTTCCGTCC
P27	ACCTGCAACCGACGATTCTT	GTCCATTCCATGAAGTCACGCG
NFKB	ATCCATATTTGGGAAGGCCTGA	GGAAACGAAATCCTCTCTGTTTAGG
PTEN	GCGGAACTTGCAATCCTCAG	GGTAACGGCTGAGGGAACTC
LC3	ATCATCGAGCGCTACAAGGG	AGAAGCCGAAGGTTTCCTGG
BECLIN-1	CGACTGGAGCAGGAAGAAG	TCTGAGCATAACGCATCTGG
GAPDH	CCTGACCTGCCGTCTAGAAA	TGGGTGTCGCTGTTGAAGTC

Statistical Analysis

The distribution of data was controlled by the Shapiro-Wilk test. While Student- t-test was used for the groups with a normal distribution (p>0.05), the Mann Whitney U test was used to compare two groups without a normal distribution (p<0.05). One-way ANOVA test was used to compare three or more groups with a normal distribution. SPSS 25 (SPSS, Inc, Chicago, IL) and GraphPad prism 8 (GraphPad Software, Inc, San Diego, USA) were used for statistical analysis (*p<0.05; **p<0.01).

Results

Anti-cancer activity of *Urtica dioica L*. extract on HL-60 cells

HL-60 cells that was treated with *Urtica dioica L*. extract showed a remarkable suppression in cell proliferation at a dose of 100 µg/mL according to the results of MTT staining after 24 hours, and this decrease was statistically significant (Figure 1A). When the cell morphology between the groups was evaluated, it was observed that the cells were lysed and the number of cells decreased at a dose of 100 µg/mL compared to the cells treated with negative and DMSO (X20) (Figure 1B). According to the results of Annexin V/PI staining that we had conducted to determine its effect on cell death, it was found that cells died in a plant extract at a dose of 100 µg/mL (live: 5.69%; early apoptotic: 5.8%; late apoptotic: 42.76%; necrotic: 45.75%) (Figure 1C). According to the result of Propidium Iodide (PI) staining that we had performed to determine the effect of *Urtica dioica L*. extract on the cell cycle, it was observed that the cells were arrested in the G0/G1 phase (70.2%) at a dose of 100 µg/mL and the cell division was slowed down (Figure 1D). According to the results of JC-1 staining that we had done to determine the effect of HL-60 cells on mitochondrial membrane potential, the mortality rate was found to be 18.9% and that was due to organelle loss at a dose of 100 µg/mL (Figure 1E).
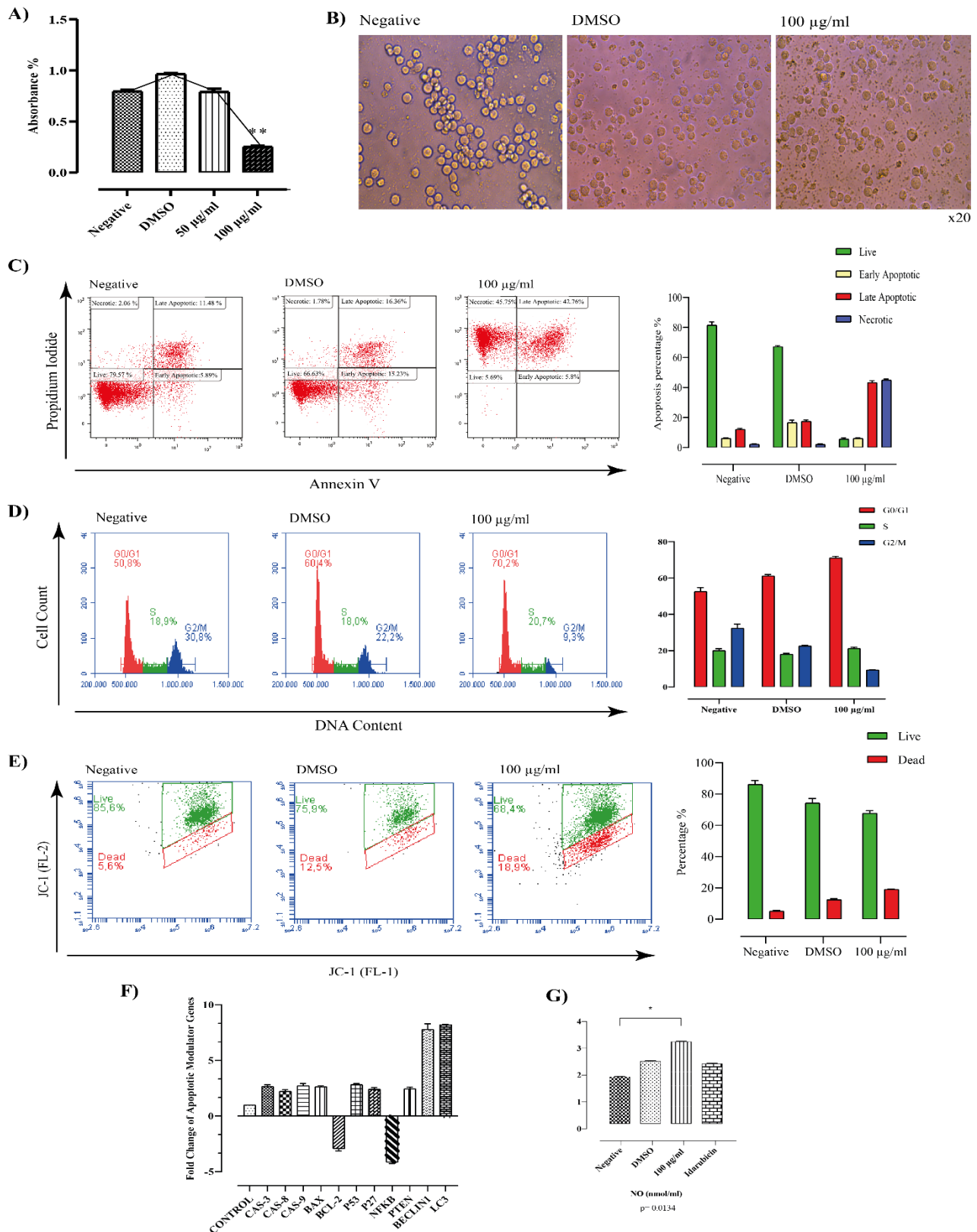
Figure 1. Effect of *Urtica dioica L.* extract on HL-60 cell line**.**
A. Graph of changing cell viability as a result of Urtica dioica L. extract application to HL-60 cells (Negative; DMSO (used during dissolution of the extract and its ratio in the extract was adjusted as 1/1000); 50 and 100µg/mL Urtica dioica L. extract). B. Changing cell morphological image after substance administration (X20). C. Annexin V/PI staining result after treatment of Urtica dioica L. extract. D. PI staining result showing the effect of Urtica dioica L. extract on cell division. E. JC-1 staining result showing mitochondrial membrane potential change. F. Graph showing the expression levels of genes involved in the apoptotic pathway and cell division check points. G. Measurement plots of NO levels after treatment of Urtica dioica L. extract.

It has to be noted that P53 expression which is considered as a DNA damage marker and cell cycle checkpoint, has been found to be increased. In addition, the increase in pro-apoptotic BAX gene expression and the decrease in anti-apoptotic BCL-2 gene expression were determined as an improvement that apoptosis was triggered in the cell. It was also found that the expression of the apoptotic cascade regulator of CAS-3, 8 and 9 genes was increased. The increase in P27 gene expression working with P53 at the cell division and control point indicates that apoptosis is triggered by halting cell division in the presence of DNA damage. NF-κB prevents cell death which is caused by tumor necrosis factor (TNF) and the other genotoxic attacks by directly inducing anti-apoptotic target genes. According to the results of our study, the decrease in BCL-2 and NF-κB gene expressions leads to make cell death to be markedly observed moreover the increase in PTEN expression confirms these results.

BECLIN1 and BCL-2 expression levels play a role in controlling the mechanism that regulates the change in cell death pathway type I and II. The BECLIN1 level was responsible for the decrease that occurred in cells as cancerous and the poor prognosis of the cancer. It was also noted that BECLIN1 and LC3 expressions were increased in HL-60 cells treated with *Urtica dioica L*. extract and BCL-2 expression was decreased (Figure 1F). After the treatment of *Urtica dioica L*. extract, Nitric oxide (NO) level was found to be increased significantly at the effective dose (Figure 1G).

Discussion

Cancer has been described as the disease of the 21st century with a few effective treatment options. The most commonly used treatment methods, chemotherapy; radiotherapy, hormone therapy, surgical treatments and most recently, nanotechnology-based applications and gene silencing methods are found to be used (13). The inadequacy and side effects of these used treatment methods have paved the way for the development of alternative treatment strategies. In particular, there is a need for a specific anti-cancer treatment tool that will effectively halt the initiation and progression of cancer, with few side effects and high cytotoxicity (14). As a traditional treatment method, herbal compounds are used in the production of modern anti-cancer drugs as well as being a valuable source producing agents that inhibit the proliferation of cancer cells (15, 16). The used herbal compounds (phytochemicals) in treatment of cancer consist of biologically active substances such as carotenoids, flavonoids, anthocyanins or terpenoids. These active substances are used today to extract chemical agents with important pharmacological effects. Some of the compounds that were obtained from plants and were used as neoplastic agents; irinotecan, vincristine, etoposide and paclitaxel (17). In general, the mechanism of action of these plant-based chemicals works by controlling cell division, differentiation and regulating the apoptotic mechanism in cells whose structure is disrupted. In some of the recent carried out studies, the combined treatment method has been tried to obtain simultaneous treatment; It is aimed to increase the effectiveness of the treatment by using a traditional chemotherapeutic drug and a natural compound or two/more natural agents. Another objective for this method is to increase the sensitivity of cancer cells to chemotherapeutic drugs by targeting more than one metabolic pathway and reducing the development of resistance to anti-cancer drugs. A study was conducted by Mohammad et al. (2016) (18) they emphasized the importance of a combined approach and found that by combining *Urtica dioica L.* extract with paclitaxel reduces paclitaxel resistance in breast cancer cells, they sensitized the cells to the drug and increased the effectiveness of the neoplastic agent. In addition to the combined treatment proposition, only plant extract-based treatment strategies occupy a wide place in the literature. As a common result of the studies conducted in this field, it has been determined that herbal therapy can help preventing the development of cancer as well as positively affects the healing process by increasing the survival rate of patients (19).

In this study, we evaluated the effects of the water extract that was prepared using the leaves and stems of *Urtica dioica* plant targeting HL-60 cell proliferation and apoptotic mechanism. It has been determined that *Urtica dioica* plant has anticancer activity by studies conducted in different solvents and different cell lines. The first study conducted in this context was conducted by Nahata et al. in 2012. Anticancer activity experiments were carried out on 7 different cancer lines (PC-3, DU-145, A-549, MCF-7, IGR-OV-1, CoLo-205 and IMR-3) of the fraction prepared in 3 different solvents (petroleum ether, ethanolic, and aqueous) using *Urtica dioica* plant. As a result of the study, the anti-cancer activity of the *Urtica dioica L.* plant was not detected (20). In 2016, Mohammedi et al. in the study conducted by, MDA-MB-468 breast cancer cells were treated with *Urtica dioica* extract and paclitaxel formed in the cells, these two agents made the cells sensitive to the drug by reducing the drug resistance. Also it can lead to death of the cells by reducing cell proliferation (18). In 2017, Turk et al. In the study conducted by *Urtica dioica* root extract, the cytotoxic effect of the hemostatic agent (Ankaferd hemostat) that was prepared using five plants, it was conducted on melanoma cell line and it was shown that the drug had a negative effect on cell viability with increasing concentration and incubation time (21). In this study, we found that *Urtica dioica* water extract reduced HL-60 cell proliferation (100µg/mL) and altered morphologically (Figure 1A-B). According to Annexin V/PI staining results, we determined the rate of cell death as 48.56% compared to the negative group (Figure 1C).

These obtained results were compatible with the literature. According to a recent study, *Urtica dioica L.* methanol extract was found to induce apoptosis by exerting anti-proliferative effect in HCT-116 and HEPG-2 cells (22). As stated by the results of PI staining that were done to evaluate the effects on the cell cycle, we found that cell division was arrested in the G0/G1 phase (70.2%) (Figure 1D). We tested the effect on mitochondrial membrane potential using the JC-1 staining method and found that 18.9% of the mitochondrial membrane structure was disrupted in the group to which we applied the extract (Figure 1E). We tested the effects of these obtained data by flow cytometry method on apoptotic mediator genes with RT-PCR method. It was found that the expression of the BAX gene was increased. BAX gene is one of the apoptotic pathway mediator genes, a member of the impaired mitochondrial membrane marker (23) and pro-apoptotic gene family. Additionally, the expression of the anti-apoptotic gene BCL-2 was decreased significantly (rate of increase and decrease was evaluated on the basis of the two multiples and expressed fold change). The markedly increased P53 expression indicates the presence of DNA damage, meanwhile the increase in CAS-3, 8 and 9 expressions indicate that the apoptosis pathway of the cells is triggered. The increase in both the expression of PTEN (24) and a tumor suppressor gene (that regulates cellular metabolism, growth and survival mechanisms), indicates a decrease in cell proliferation and this result is correlated with cell cycle analyses. NF-κB prevents cell death caused by tumor necrosis factor (TNF) and other genotoxic attacks by directly inducing anti-apoptotic target genes (25). According to the results of our study, it was found that the decrease in BCL-2 and NF-κB gene expressions caused cell death and we emphasized the accuracy of these results by increasing PTEN gene expression. The cyclin-dependent kinase inhibitor P27 (KIP1) is a factor that inhibits cell cycle progression using specific molecular mechanisms. It also works in combination with P53 in the regulation of cell differentiation and apoptosis (26). In our study, we found that by increasing (P27) in correlation with P53, they have an inhibitory effect on cell division in the presence of DNA damage. BECLIN1 and BCL-2 expression levels play a role in the mechanism regulating autophagic-apoptotic change (27, 28). In addition, the decrease in BECLIN1 level has been associated with cancer pathogenesis and poor prognosis (29, 30). It was found that BECLIN1 and LC3 expressions were increased in HL-60 cells treated with *Urtica dioica L.* extract and BCL-2 expression, was decreased (Figure 1F). Nitric oxide (NO) is a free radical gas with complex and pleiotropic biological activity that plays a key role in various physiological and pathological processes (31, 32). It has been stated that unregulated NO synthesis (Figure 1G) contributes to the pathophysiology of many diseases, including cancer (33). The scarcity or abundance of NO in cancer cells is a complex process in which pro-apoptotic gene activation or inactivation occurs in cells (32, 34, 35).

Conclusions

In our study, the NO increases in HL-60 cells treated with *Urtica dioica L.* extract which caused a series of apoptotic gene activation. Our findings support this output. The increased apoptotic cell percentage, increase in cell involvement in the G0/G1 phase, increase in the expression of genes involved in the P53-CASPASE cascade, and decrease in NF-κB, BCL-2 gene expressions are considered be to perceivable evidence that triggers apoptosis by increasing NO levels.

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Concept: E.T., I.K., K.S.
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Design : E.T., I.K.
Data acquisition: E.T., O.Y., Y.A
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