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Apoptotic and antiproliferative effects of *Urtica dioica* L. extract on K562 chronic myeloid leukemia cell line

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Urtica dioica L. (stinging nettle) is an herb commonly used as a medical supplement for its anticarcinogenic, antiinflammatory, analgesic, antifungal, and antioxidant effects. In this study, we analyzed phytochemical composition of *Urtica dioica* ethanol extract and investigated its apoptotic and antiproliferative effects in K562 cell line, a model cell line for chronic myeloid leukemia (CML). K562 cells exposed to different concentrations of *U. dioica* extract (0, 25, 50, 75 and 100 µg/mL) were tested for cell proliferation and apoptosis using appropriate methods and different phases of the cell cycle were analyzed using flow cytometry. Phenolic content of the plant was analyzed by LC–MS/MS. We showed that *U. dioica* ethanol extract has four phenolic compounds: caffeic acid, chlorogenic acid (major compound), malic acid and rutin. *Urtica dioica* significantly repressed proliferation of K562 cells compared to control mononuclear cells (PBMCs) isolated from the peripheral blood of healthy donors. An increase in Bax/Bcl-2 and PARP cleavage ratio, decrease in mitochondrial membrane potential indicated that the extract inhibited cell proliferation through an apoptosis-mediated process. Furthermore, cell cycle arrest at G0/G1 phase increased in a concentration dependent manner. Our results explicitly demonstrate that ethanol extract of *Urtica dioica* leads to a decrease in cell viability and proliferation, initiates apoptosis, and induces G0/G1 cell cycle accumulation in this cell line. This is the first study to have demonstrated the antiproliferative and apoptotic effects of *Urtica dioica* on chronic myeloid leukemia (CML) cell lines K562 *in vitro*.

Keywords: Apoptosis, Caffeic acid, Cancer, Chlorogenic acid, Malic acid, Rutin, Stinging nettle

Urtica dioica L. (Stinging nettle) is a subsidiary herbal plant from Urticaceae family with a variety of health benefits. Its nutritional usage involves use as a vegetable, tea, or juice, sometimes directly as a dried, powdered seed, as well as used as a blood nutritive tonic and for allergic rhinitis. *U. dioica* shows different biological activities involving anti-inflammatory, anticancer, local analgesic, anti-allergenic, antioxidant, tonic, diuretic, nutritive and homeostatic effects[1-4]. The shoots of *U. dioica* are especially rich in dietary fibers (6-7%) and proteins (3-4%), but also contain essential minerals like calcium, sodium or iron, as well as fatty acids (as lineoleic and alfa-lineoleic acid), together with vitamins A and C, presenting a beneficial nutritional source with total calorie ingredient of 45-50 kcal/100 g[5]. In addition, it also has phenolic components (involving flavonoids and phenolic acids), sterols and poly-saccharides[6].

The pharmacological effects of different components were investigated through various physiological responses and diseases. Its anti-haemorrhagic property was shown to reduce intensive menstrual flow and nose bleedings, while its fresh freeze-dried leaves were shown to suppress allergic reactions. The roots are already in use for benign prostate hyperplasia and the herbs are utilized for treatment of urinary tract infections or rheumatic cases[7,8].

Similarly, different extracts of *U. dioica* were studied and found to have medical benefits such as aqueous extract with antiasthmatic activity[9,10] or leaf extract with antiinflammatory activities on inflammatory joint diseases[10-13], and its different activities were reported such as antioxidant[13-18],

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antimicrobial[19,20], antidiabetic[21-24], antimutagenic[25] or immunomodulatory activities[26,27].

The cytotoxic properties and anticancer activities of *U. dioica* have been demonstrated in colon and gastric cell lines[28-31], non-small cell lung cancer cell model[32], several prostate cancer[33-36] and breast cancer cell lines[15,37-41]. Until now, there have been reports only on local solid tumors. Here, we investigated a blood malignancy, chronic myeloid leukemia (CML), originated from abnormalities in bone-marrow myeloid cells. It is projected that in 2023, there will be 59,610 new cases of leukemia identified in the US, and 23,710 deaths from the illness[42,43]. Since in these types of cancers, malignant cells easily spread to any part of the body through the bloodstream, chemoradiotherapy applications remain inefficient due to serious systemic side effects, weakened immune response, drug resistance and relapses.

As a known nutritional supplement with multiple benefits, study of the mechanisms of action of *U. dioica* may provide a promising chance for the use of this herb in anticancer therapies. Therefore, studies to specifically characterize and ascertain the effects of distinct *U. dioica* extracts, determine their dose-response relationships, potential supplementary or therapeutic roles in different types of cancers would be a significant asset.

In the present study, we explored the anticancer activity of the stinging nettle, *Urtica dioica* ethanol extract by examining cellular regulations in cell-cycle and apoptotic processes, using highly undifferentiated leukemia cell line K562 as a cell model.

Material and Methods

Plant material and extraction

Leaves of *Urtica dioica*, identified by Dr. Ahmet Doğan, a botanist from Marmara University, Faculty of Pharmacy, were gathered at the flowering stage from Adapazarı region, Sakarya. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE No: 22297). Dried and powdered material (90 g) was extracted using 96% of ethanol in a Soxhlet apparatus. The extraction procedure was carried out until the solvent becomes colorless in the Soxhlet loop. The filtrate was evaporated and concentrated at 40°C. After extraction, the yield was determined as 14.64%. The obtained ethanol extract was stored at 4°C until analysis.

Cell culture

K562 leukemia cells were grown in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% glutamine and penicillin/ streptomycin (pen/strep) in a humidified atmosphere with 5% CO_2 at 37°C.

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood (5-10 mL) was collected from healthy volunteer and mononuclear cells were isolated from fresh whole blood by centrifugation on Ficoll-Hypaque density gradients. Briefly, whole blood 1:1 diluted with PBS was layered onto the same volume of Ficoll-Hypaque medium, centrifuged at 500 ×g for 20 min and mid-layer was removed. Cells were washed with PBS and cultured in RPMI-1640 medium, supplemented with 10% FBS, 1% glutamine and pen/strep as described above.

MTT assay

The antiproliferative activity of the extract was assessed by MTT assay **(**Cell Proliferation Kit I, Roche**)**. For this purpose, K562 cell lines were planted at a concentration of 5×10^3 cells/well, in 100 µL of culture medium, onto 96-well plates. Different concentrations (0, 25, 50, 75 and 100 µg/mL) of the extract were added into cultures and left to incubate for 24 h. After incubation time, 10 µL of the MTT labeling reagent (0.5 mg/mL) was added to each well and the plates were further incubated for 4 h, at 37°C in 5% CO_2 humidified atmosphere**.** At the end of incubation, formazan crystals were dissolved in solubilization solution and absorbance was evaluated in a microplate reader, at 570 nm **(**Synergy H1, Biotech Instruments Inc., Vermont, USA**).**

Tali apoptosis assay

Following 24 h incubation with *U. dioica* ethanol extract, cells were stained using Tali® Apoptosis Kit, containing Annexin V Alexa Fluor® 488 and Propidium Iodide, according to the manufacturer's protocol (Molecular Probes A10788). The fluorescence was analyzed using Tali Imaged- Based Cytometer.

Mitochondrial membrane potential assay

Apoptosis was also evaluated by detecting mitochondrial membrane potential changes using a cationic dye JC-1. JC-1 acts as an indicator for mitochondrial function and has been used to assess the ratio of healthy and apoptotic cells. K562 cells

were treated with JC-1 probe, according to manufacturer's protocols (JC-1 mitochondrial membrane potential assay kit, Abnova KA1324). Healthy cells with high polarization ($\Delta\psi_M$) produce red fluorescence and apoptotic cells with low polarization ($\Delta\psi_M$) exhibit green fluorescence. Green to red fluorescence absorbances were measured and the ratio was determined using multi-mode microplate reader with appropriate filter sets (Synergy H1, BioTek Instruments Inc., Vermont, USA).

Western blot

The cells were cultured in 6-well plate and were incubated with *Urtica dioica* ethanol extract for 24 h. Following 24 h incubation, cells were transferred to the 1.5 ml microtubes and proteins were isolated using 1×RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Complete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche). Total protein levels were quantified using Take3™ Multi-Volume Plate (Synergy H1, BioTek Instruments Inc., Vermont, USA). The extracted proteins were analyzed through gel electrophoresis and immunoblot was performed. Primary antibodies used in the study were β-actin (Novus Biologicals, NB600-501), Bax (SPM336, NBP2-32809), Bcl-2 (100/D5, NBP2-15200) and cleaved PARP (194C1439, NBP2-27335) monoclonal antibodies produced in mouse (Novus Biologicals). The bands were visualized with HRP chemiluminescent substrates (Western Bright ECL-Advansta, K-12045-050). Quantification was evaluated using built-in software of Chemiluminescence Imaging System (Celvin S, BioStep).

Cell cycle assay

Quantification of cellular DNA content is a commonly used process for monitoring cell cycle progression. For cell cycle analysis, cells were cultured in 6-well plate and treated with *Urtica dioica* extract for 24 h. Cells were rinsed with Dulbecco's phosphate buffered saline (DPBS), fixed in 70% ice-cold ethanol and placed at –20°C overnight. Staining was carried out in propidium iodide solution, which contains RNase A (2 mg/mL) and Triton X-100, for 30 min at room temperature (25°C). The fluorescent intensity represents the amount of DNA on each phase of the cell cycle. The DNA content of the cells was determined using flow cytometer (FACS Calibur, BD, Franklin Lakes, NI) and Sub G1, G0/G1, S and G2+M cell phases were analyzed by Cell Quest software (Becton Dickinson Japan, Tokyo, Japan).

LC-MS/MS analysis of U. dioica ethanol extract

The LC-MS/MS method was performed according to our previous study[44].

Statistical analysis

Statistical analysis of the experimental results was performed with the t-test using GraphPad Prisim 6. Statistical significance was defined as $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results and Discussion

Effects of U. dioica extract on cell proliferation and viability

Firstly, for determination of cytotoxic effects of *Urtica dioica* extract, MTT assay (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed on K562 cells. The cells were treated with *U. dioica* ethanol extract at different concentrations as described in methods. Cell counts were determined by TALI image-based cytometer and direct counts after trypan blue staining. All data indicated that treatment with *U. dioica* didn't show up a dose-dependent cytotoxic effect on K562 cell line. However, according to repeated MTT test data and cell counts, there was a non-specific suppression of cell proliferation and viability in leukemia cells, when compared to peripheral blood mononuclear cells (PBMCs) isolated from healthy people (Fig. 1 A and B). Compared to control group (no agent, +DMSO), healthy cells showed approximately 40% increase in 24 h, while leukemia cells maintained their initial levels through 24 h incubation period, at all concentrations. *U. dioica* induced a small drop at 25 μg/mL with respect to control, and displayed a cytostatic effect through 50-100 μg/mL concentrations. This effect was not observed in healthy lymphocytes, a potential indicator of promising selective activity for tumorogenic cell line.

U. dioica dose dependently induced apoptosis

In order to assess anticancer activity further, we investigated apoptosis in K562 cell line. For this purpose, we investigated phosphatidyl serine (PS) exposure, together with reduction in mitochondrial membrane potential ($\Delta\psi_m$) and Bcl-2 family protein expressions. Interestingly, unlike MTT findings, *U. dioica* treatment induced apoptosis in a dose-dependent manner as confirmed by all three methods.

PS exposure was determined using annexin-V stain that has a strong affinity for PS. After the cells were incubated with Annexin V/PI fluorescent dyes, the ratio of apoptotic cells was found to be 3% of the total cell population at 25 μg/mL and rised to 12% at

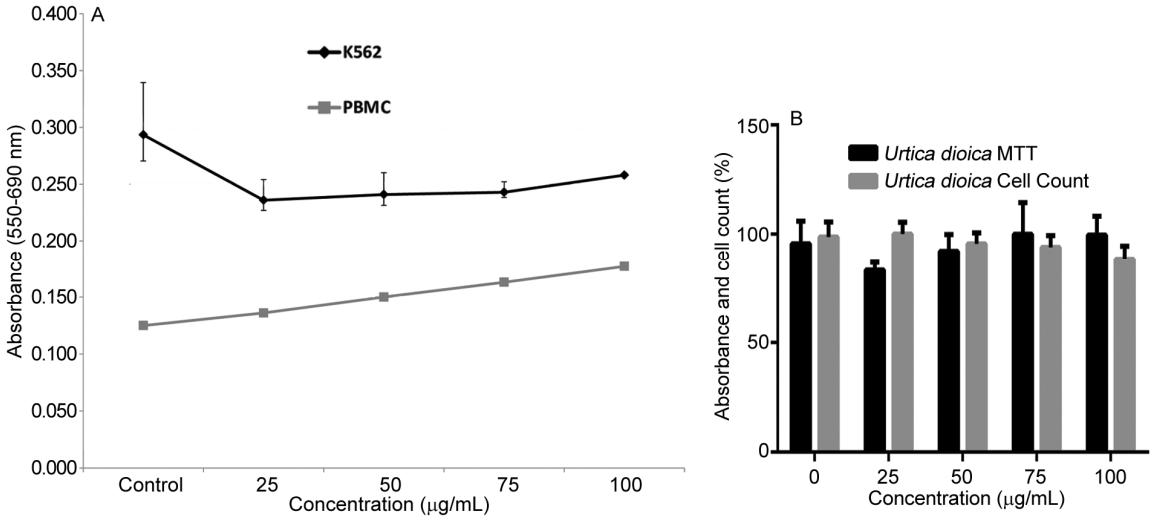

Fig. 1 — Cytotoxic and antiproliferative effects of *Urtica dioica* extract for different concentrations (0-100 µg/mL) on K562 and PBMCs at the end of 24 h incubation, determined by MTT assay and trypan blue staining. (A) Determination of cytotoxic effects by MTT assay; and (B) Comparison of MTT viability test results and direct cell counts on K562 cells.

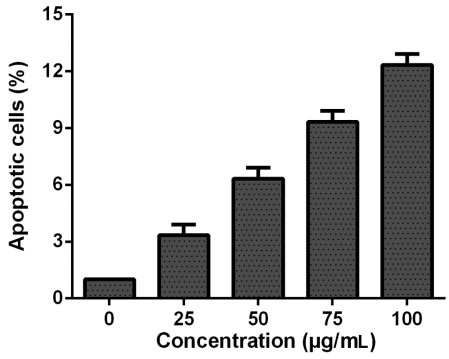

Fig. 2 — Apoptotic cells were evaluated using Annexin V/PI staining in K562 cells.

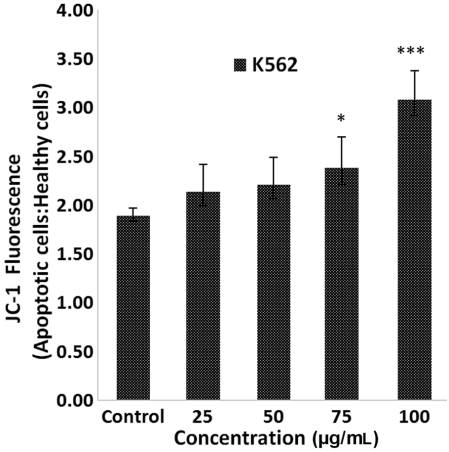

Fig. 3 — Mitochondrial membrane potential was detected by JC-1 dye (n=3). [**P* <0.05, ****P* <0.001]

100 µg/mL (Fig. 2). This change was consistent with the cytostatic effects observed in proliferation assays.

Mitochondrial function, a sign of cell health, can be evaluated by observing changes in mitochondrial membrane potential (MMP). After the apoptotic signal is received by the cell, a change in the permeability of the mitochondrial membrane results in depolarization. The pro-apoptotic and anti-apoptotic mitochondrial proteins, which are members of the Bcl-2 family, regulate cell death by checking mitochondrial activity during apoptosis.

According to the signals from JC-1 stain that cause to a red-to-green shift in fluorescence as the permeabilisation of membrane changes, *U. dioica* extract yielded to an increase in apoptosis 13% and 41% for 25 and 100 µg/mL, respectively, in comparis onto untreated control cells (Fig. 3).

Roles of apoptotic proteins in *U. dioica* induced response

The apoptotic process regulated by coordinated action of pro- and anti-apoptotic Bcl-2 family proteins were analyzed using immunoblot. The Bax/Bcl-2 ratio is a significant marker of sensitivity against apoptotic stimulus[45,46]. Bax and Bcl-2 protein expressions were measured by immunoblot analysis as band intensities were quantified relative to housekeeping protein beta-actin (Fig. 4). The increase in Bax/Bcl-2 ratio was observed only after the addition of 75 and 100 µg/mL *U. dioica*.

PARP-1 is another marker with significant roles in various cellular mechanisms such as DNA damage response, cell viability, cell death and apoptosis under

different stressful conditions. Cleavage of PARP-1 is another indicator of apoptotic cell death that occurs through caspase 3 or 7. At the end of cleavage, two specific fragments could be specified: an 89-kD catalytic fragment and a 24-kD death binding domain. In our study, the primary antibody specific for 89-kD fragment explicitly showed that cleaved fraction markedly increased at 100 µg/mL concentration (Fig. 5).

Urtica dioica induces G0/G1 cell cycle arrest on leukemia cancer cells

To observe a possible delay in the cell-cycle process due to *U. dioica* extract, cell cycle analysis was performed using flow cytometry. Cells were treated with various concentrations of *U. dioica* extract for 24 h and distributions of phases were monitored. Flow cytometric analysis indicated that treatment with the extract initiated accumulation of cells in G0/G1 phase and caused by a reduction in G2/M phase population (Fig. 6). This indicates that *U. dioica* treatment may slow down cell growth through inducing cell cycle arrest at G0/G1 phase, in a dose-dependent manner. Accumulation at G0/G1 phase increased 9.6, 10 and 14% in 50, 75 and 100 µg/mL concentrations, respectively, compared to the control group.

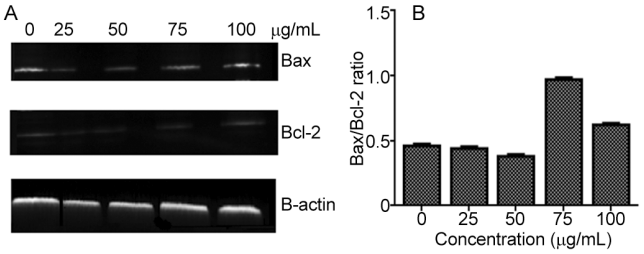

Fig. 4 — (A) Representative immunoblot image of Bax and Bcl-2 protein expression with β-actin in K562 cells treated with different concentrations of *U. dioica* ethanol extract at the end of 24 h incubation; and (B) The expression levels of each band were quantified using scanning densitometry and normalized to beta-actin standart.

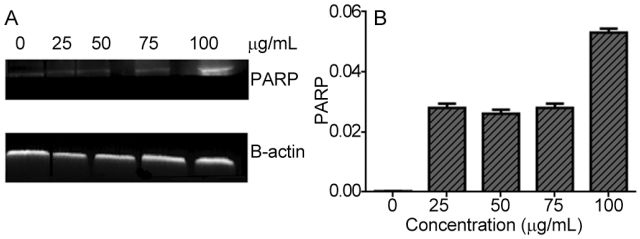

Fig. 5 — (A) Representative immunoblot image; and (B) densitometric analysis showing cleaved PARP protein expression in K562 cells. The Western Blot analyse was carried out using primary antibody for (89 kD)-cleaved-PARP fragment after treatment with *Urtica dioica* extract at the end of 24 h incubation. β-actin protein was used as the loading control.

LC-MS/MS characterization of phenolic compounds in Urtica dioica ethanol extract

Three phenolic acids, caffeic acid, chlorogenic acid, malic acid and one flavonoid rutin were identified in the ethanol extract obtained from *Urtica dioica* leaves. Also, chlorogenic acid was found as a major compound in the extract (Table 1 and Fig. 7).

After cardiovascular diseases, cancer is the second most common cause of death in modern societies, and studies indicate increased incidence in the near future.

Table 1 — Characterization of phenolic compounds in the ethanol extract of *Urtica dioica*[47-49]

Rt min	[M-H]$^-$ *m/z*	MS^2	Identified as
3,4	179	135	Caffeic acid[45]
6,5	353	191, 179, 135	5-Caffeoylquinic acid (chlorogenic acid). (Main compound)[46]
9,3	133	115	Malic acid[45]
9,5	609	300, 271, 179	Rutin[47]

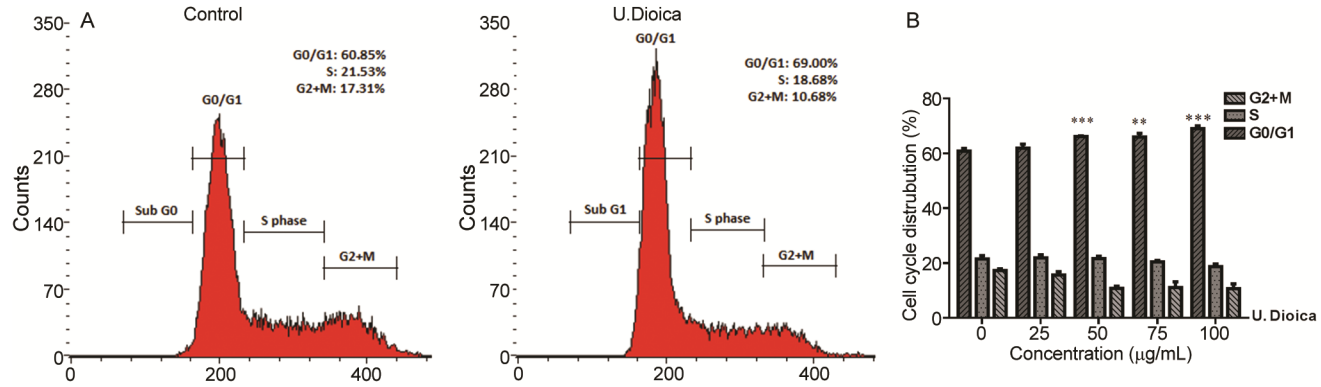

Fig. 6 — (A) Cell cycle progression was measured by flow cytometry. Representative distribution of Sub G1, G0/G1, S and G2/M phases for control and 100 µg/mL *U. dioica*; and (B) The distribution of cell cycle (%) at different phases and doses in K562 cells. ***P* <0.01, ****P* <0.001.

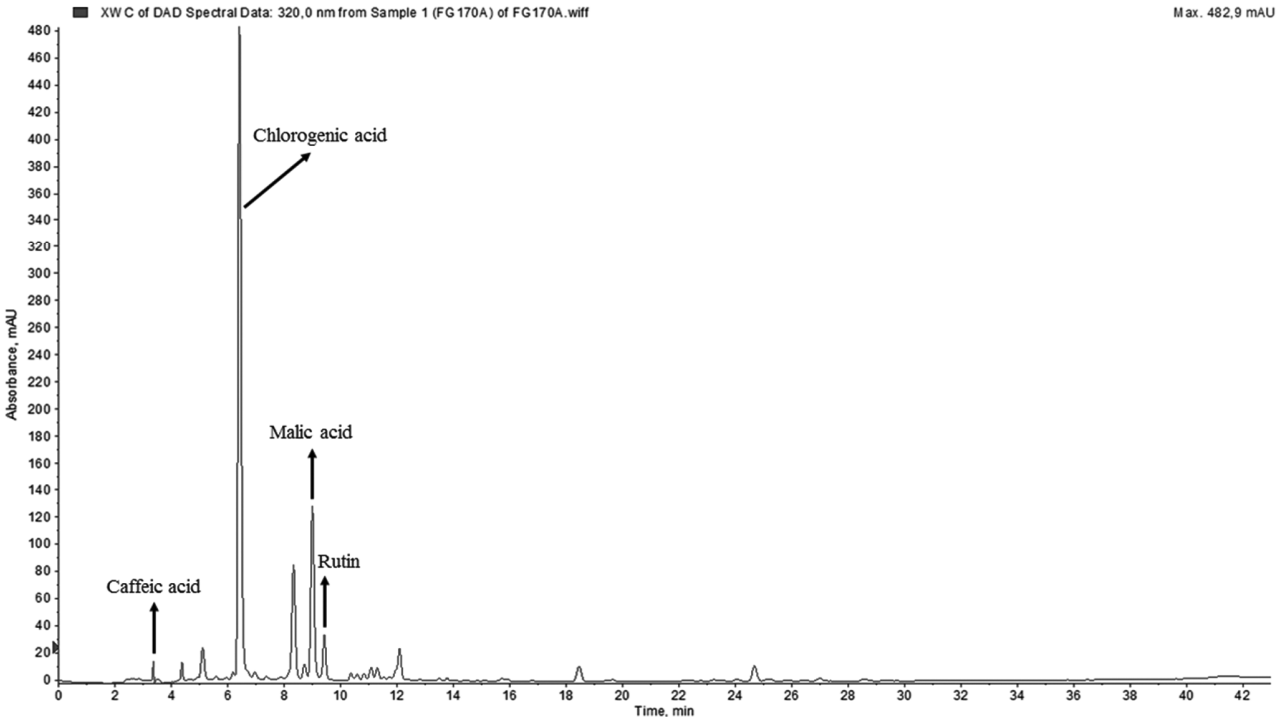

Fig. 7 — LC-MS/MS chromatogram of ethanol extract of *Urtica dioica*

In recent years, alongside searches for the new approaches in anticancer therapies, substantial toxicity and drug resistance studies based on medicinal plants have emerged, to diminish severe side effects of classical chemo-radio therapies and increase their efficacy. Most of drugs used in cancer therapy have cytotoxic and/or cytostatic effects[50]. For an effective cancer therapy, these effects would be through induction of apoptosis in cancer cells without damaging healthy cells[51]. In addition to apoptosis, another important feature of cytotoxic factors is their ability to induce cell cycle arrest.

Within this framework, phytotherapeutic approaches are gaining more interest to find new alternatives and numbers of ongoing researches for possible candidates are piling up. *Urtica dioica* is one of plants with medicinal value and its benefits for human health have been known since ancient times. The plant is commonly used as supplemental to known therapies or nutritional dietary[52].

In this research, we attempted to investigate anticancer potential of *U. dioica* ethanol extract by examining its antiproliferative and apoptotic effects on highly undifferentiated leukemia cell line K562 *in vitro*. Cellular and molecular effects of *U. dioica* have been reported on different types of cancers; however, there are very small number of studies inspecting its role in hematopoietic cells, and to our knowledge, a research involving various aspects of *U. dioica* on chronic myeloid leukemia cells has not been conducted except a very recent report on acute myeloid leukemia cell lines U-937, KG-1,and HL-60[53,54]. Since the genetic history of the cells will be a leading factor for observed responses, such as cell survival or apoptosis, the response of CML K562 cells were study of interest. The cytotoxic and anticancer activities of *U. dioica* have been demonstrated in various cancer types. The concentration of *U. dioica* with a growth inhibition of 50% (IC_{50} value) for MCF-7 cells was previously reported as 18 µg/mL for 48 h[40].

A study demonstrated that chlorogenic acid (CA) is the main compound in *U. dioica* leaves[55]. Here, it has been reported that CA possessed antitumor activity via the induction of apoptosis in acute promyelocytic leukemia cell line HL-60. According to the same study, CA induced apoptosis and inhibited proliferation, together with G0/G1 phase arrest in this cell line in a dose- and time-dependent manner, especially after 48 h[56]. An earlier study had reported its effects in K562 cells as well, emphasizing its differential role between Bcr-Abl–positive and negative cell lines[57]. Another interesting point

reported in this study was the fact that the sodium salt of CA was twice effective in its anticancer properties. There are also reports on the role of other two phenolic components, caffeic acid and rutin, investigating their anti-leukemic effects in K562 leukemia cells[57,58]. Therefore, CA along with other phenolic compounds may be responsible for the antileukemic effects of this plant. Furthermore, combined effects of these components could have a higher therapeutic potential than the individual constituents.

Based on this rationale, we used the total ethanol extract of the plant to determine whether the combined action of components is more efficacious or not. Our results from the proliferation and viability assays showed a cytostatic effect of the *U. dioica* on K562 cells, repressing their growth after 25 µg/mL. After a marked drop in this concentration with respect to control cells, growth of cells stayed in a stationary phase and no further inhibitory effect was observed, possibly due to the low toxicity of *U. dioica* and high proliferation rate of these cells. On the other hand, this cytostatic effect was not observed in PBMCs isolated from healthy donor.

Unlike viability results, apoptosis was remarkably induced in cells with the application of *U. dioica.* We used several approaches to investigate apoptotic effects. Annexin V/PI staining data clearly showed that *U. dioica* extract could suppress the growth of K562 cells through induction of apoptosis in all doses. Cleavage of PARP-1 is another apoptotic sign implying that apoptotic cell death occurs through activation of caspase 3 or 7. At the end of the cleavage, two unique fragments could be detected: catalytic fragment with a MW (molecular weight) of 89 kD and death-binding domain with a MW of 24 kD. In this study, we used a primary antibody that is unique to the 89-kD fragment. Similar to annexin V/PI staining data, immunoblot analysis of PARP cleavage showed that, as the concentration of extract increased to 25 µg/mL or higher, the cleaved fraction increased dramatically.

Another approach for determination of apoptosis induction is the evaluation of mitochondrial Bcl-2 family protein expressions. Bcl-2 protein family members are well known as the regulators of apoptosis, and coordinated actions of these proteins, which involve anti-apoptotic (Bcl-2, Bcl-xL and Bcl-W) and pro-apoptotic (Bax, Bak and Bid) proteins, define the resultant outcome. The Bax/Bcl-2 ratio can be used to assess apoptosis induction following treatment with an apoptotic drug[59]. To show apoptotic progress, we first examined Bcl-2 and Bax protein expressions using appropriate antibodies. These proteins play important roles both in the extrinsic and intrinsic mitochondrial cell death pathways. Induction of apoptosis is normally associated with a significantly increased Bax/Bcl-2 ratio. In our study, an increase in Bax/Bcl-2 ratio was observed only after high concentrations *U. dioica* (75 and 100 µg/mL). This may be because Bax is partially dependent on p53 and K562 cells are known as p53-deficient cells[60]. K562 cell line is known to have a frameshift mutation in exon 5 of p53 gene. p53 acts as an upstream regulator for the Bax gene and deficiency of this protein could suppress the expected observable changes in Bax/Bcl-2 ratio, even though other compensatory mechanisms also exist for the Bax regulation[61,62].

Similarly, changes in mitochondrial membrane permeabilities were also evident after this concentration, clearly demonstrating initiation of an apoptotic process. Overall, our results show that *U. dioica* concentrations, especially after ≥50 µg/mL, induced apoptosis effectively, in a dose-dependent manner.

Cell cycle deregulations are another aspect of abnormal growth and proliferation of cancer cells; thus, monitoring cell cycle phase shifts are a plausible strategy to detect anticancer effects of a potential drug agent. An earlier study by Mohammadi *et al*.[38] reported that *U. dioica* inhibited cell proliferation by modifying cell cycle transition especially through G2/M phase arrest in MDA-MB-468 breast cancer cell lines. A recent study conducted with lectin component of *U. dioica*, on the other hand, recorded a substantial accumulation in the number of sub G1 cells[54]. Here, we observed an accumulation in the G0/G1 phase, where differences were significant after 50 µg/mL compared to control group. Our findings show, in agreement with reported observations and data, that the *Urtica dioica* extract induces cell cycle arrest at G0/G1 phase and this effect proceeds in a dose-dependent manner.

Conclusion

Thus far, there are only few studies reposting anticancer activity of *Urtica dioica* extract on human chronic myeloid leukemia (CML) cell line K562. The

present study has clearly demonstrated the antiproliferative and apoptotic effects of *U. dioica* extract on the leukemia cell line *in vitro*. Our data reveal that ethanol extract of *U. dioica* suppress proliferation, and induces apoptosis and cell cycle arrest at G0/G1 phase in the human chronic myeloid leukemia (CML) cell line K562. Induction of apoptosis has been shown at reasonably low concentrations. Our study is possibly the first one to show antiproliferative and apoptotic effects of *U. dioica* ethanol extract on leukemia cell lines *in vitro.*

Conflict of Interest

The authors declare no competing interests.

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