Original Article

In vitro cytotoxic effect of urtica dioica extracts on acute myelogenous leukemia cell line (kg-1)

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Abstract

Background: Urtica dioica is one of the medicinal herbs with many uses in treating various diseases. In some studies, its antiproliferative and apoptotic effects on cancer cell lines have been shown. Therefore, the evaluation of U. dioica effect was performed on KG-1 cell line for acute myelogenous leukemia (AML) for the first time in this study.

Materials and Methods: KG-1 cell line was treated by various extracts (aqueous, hydroalcoholic, chloroform and ethyl acetate) of U. dioica aerial parts and roots in different concentrations. Metabolic activity of extracts on cell line was assessed by MTT assay. To evaluate the percentage of apoptotic cells, the flow cytometry was performed by FITC Annexin V-PI apoptosis detection kit in KG-1 cell line treated with root chloroform (UDC-R) and ethyl acetate (UDE-R) extracts. The results have been reported as percentage of cell viability and IC50.

Results: Based on MTT results, the strongest IC50 in KG-1 cell line (219.361µg/ml) was related to UDC-R. The flow cytometric analysis showed that UDC-R and UDE-R in IC50 concentration induced early (53.6% and 57.4%, respectively) and late (27% and 33.2%, respectively) apoptosis in KG-1 cells after 24 hrs. The inhibition of cell proliferation by various extracts of U. dioica was dependent on concentration (p=0.000).

Conclusion: Flow cytometric analysis confirmed that UDC-R and UDE-R extracts affect on proliferation reduction of KG-1 cells by activating the apoptotic pathway.

Keywords: Urtica dioica extract, acute myelogenous leukemia, KG-1, IC50, apoptosis

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Introduction

Hematologic malignancies appropriate 7% and 9% of all cancers in women and men, respectively (1). Current treatments for leukemias is usage of chemotherapy, radiation and immunotherapy. Unfortunately such treatments have considerable side effects. Hence, it is better to find more safe way to cure leukemias. Using the plants to treat the diseases has been increased recently. There are many medicinal plants. Their main advantages are less side effects and expenses (2-4). Urtica dioica (aka stinging nettle) is one of this plants that has a long

history in treating diseases such as allergies, inflammations, diabetes, high blood pressure and so on. It belongs to the family Urticaceae and is native to Africa and western Asia, but it can be found in all mild regions worldwide (5-7). It has been reported that U. dioica extracts can have antiproliferative, apoptotic and antioxidant activities on some human cancer cell lines. It believed that cytotoxic effects of U. dioica extracts can be due to its polyphenols (secondary metabolites of the plants) that have some features including antioxidants, antiproliferative activities and so on (8, 9). Therefore, we decided to evaluate the antiproliferative and apoptotic effects of various extracts (aqueous, hydroalcoholic, chloroform and ethyl acetate) of U. dioica on KG-1 cell line for acute myelogenous leukemia (AML) for the first time, in this study.

Methods

Plant extraction. Nettle was collected in May, 2015 from Lahijan, Guilan, Iran. The species was confirmed by herbarium of pharmacy faculty of Tehran University of Medical Sciences (Herbarium number: PMP-334). The plant was totally dried at room temperature and shadow within a week and then grounded in an electric mill. 100gr of aerial parts (leaves and stems) and roots were weighed. To prepare the aqueous extract, each part was boiled in 1000 ml water in water bath at 80°C for 30min. Then, they were filtered by filter paper and Büchner funnel and centrifuged in a megafuge centrifuge (Heraeus, German) at 4000rpm for 15min for further filtration. After that, the supernatants were transferred to an evaporator flask in rotary evaporator (Heidolph, Heizbad Hei-VAP, Germany) to evaporate the extracts and remove the solvents at 60°C. The extracts were freeze-dried (LTE science LTD, England) at -60°C and 10 µmHg for 24 hrs and then put in refrigerator (4°C) until used. To prepare the hydroalcoholic (ethanol 60°C), chloroform and ethyl acetate extracts, 100 gr of each parts subjected to 1000 ml related solvents were put in a separatory funnel for 48 hrs. After evaporating in rotary evaporator, extracts were put inside a vacuum oven (SHELLAB, USA) for 24 hrs until dried. Finally 8 extracts were provided including UDA-L (U. dioica aqueous extract-leaf), UDA-R (U. dioica aqueous extract-root), UDH-L (U. dioica hydroalcoholic extract-leaf), UDH-R (U. dioica hydroalcoholic extract-root), UDC-L (U. dioica chloroform extract-leaf), UDC-R (U. dioica chloroform extract-root), UDE-L (U. dioica ethyl acetate extract-leaf) and UDE-R (U. dioica ethyl acetate extract-root).

Cell culture. The KG-1 (human acute myelogenous leukemia) cell line was purchased from Pasteur Institute of Iran, Tehran, Iran. The cells were cultured in RPMI-1640 media contains Glutamax, NaHCO3 and HEPES (Biosera, England) supplemented with 10% FBS (Gibco, USA) and 1% antibiotics including penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Biosera, England). The cells were kept in 5% CO2 incubator at 37°C and 95% air. The number of viable cells was counted by trypan blue (Biosera, England) exclusion test to use in MTT assay and flow cytometry.

Preparation of extract and etoposide concentrations. 40 mg of each extract were weighed and dissolved in 1 ml of related solvents. Used solvents were water (for aqueous extracts), ethanol 60°C (for hydroalcoholic extracts) and DMSO + methanol (for chloroform and ethyl acetate extracts). The final concentration of the stock was 1 mg/ml. Other concentrations (62, 125, 250 and 500 µg/ml) were obtained from the stock.

For etoposide (EBEWE Pharma, Austria), a stock (4 mg/ml) was made at first and then the concentrations of 1, 10, 20 and 40 µg/ml were provided.

MTT assay. Antiproliferative effect of the U. dioica extracts on KG-1 cell line was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA). MTT assay is a colorimetric method to assess cell viability and proliferation. Alive cells reduce yellow water soluble MTT (methyl-thiazolyl-tetrazolium) to water insoluble dark blue formazan by mitochondrial dehydrogenase (10, 11). The cells (195µl) were seeded at a concentration of 5× 104 cells/well in 96-well plate. After 24 hr incubation, 5 µl of extracts at final concentrations of 62, 125, 250, 500 and 1000 µg/ml were added to each well. Each concentration was added as triplicate. The plates were incubated for 48 hrs in CO2 incubator. Then, 50 µl of MTT solution (12.5 mg MTT powder per 5 ml nonsterile PBS for

one 96-well plate) was added to each well and incubated for 4 hrs once more. After incubation time, supernatants were discarded. To dissolve dark blue MTT crystals, 100 µl DMSO (Sigma-Aldrich, USA) were added to each well. After 15 min, the OD absorbance was read in ELISA plate reader at 492 nm. Related solvents (water, ethanol 60° and DMSO + methanol) of extracts and etoposide were used as negative and positive control, respectively. The final concentration of DMSO and methanol in each related well was 1% and 1.5%, respectively. This experiment was repeated at least three times. IC50 (half maximal inhibitory concentration) values were calculated by logarithmic regression. The results have reported as percentage of cell viability and IC50.

Apoptosis evaluation by flow cytometry. Since most anti-cancer drugs induce apoptosis, to quantitatively evaluate of apoptotic cells within a population that was induced to undergo apoptosis, FITC Annexin V-PI double staining, using FITC Annexin V-PI apoptosis detection kit (BD biosciences, USA) was performed according to the protocol of the kit. The apoptosis is characterized by some morphologic features including cell shrinkage, chromatin condensation (pyknosis) and fragmentation (karyorrhexis) and membrane blebbing (12-14). In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner layer of the plasma membrane to the outer one. As a result, PS puts on the surface (15, 16). Annexin V is a Ca2+-dependent phospholipid-binding protein that has a high affinity for PS. Propidium Iodide (PI) is a nucleic acid dye and is used to distinguish viable from nonviable cells. The membranes of alive cells exclude PI, whereas the membranes of dead cells are permeable to PI. FITC Annexin V-positive and PI-negative cells are undergone early apoptosis. FITC Annexin V-positive and PI-positive cells are either in the end stage of apoptosis or undergone necrosis. FITC Annexin V-negative and PI-negative cells are alive and are not undergone measurable apoptosis. FITC Annexin V-negative and PI- positive cells are necrotic (17, 18). The KG-1 cell line was treated with UDC-R and UDE-R extracts (in their IC50 values) as representative in a 6-well plate. 2 wells were untreated as negative control. After 24 hrs, the cells were harvested and washed twice with cold PBS and then resuspended in 1X binding buffer (as a source of Ca2+) at a concentration of 1 x 106 cells/ml. 100 µl of the suspension (1 x 105 cells) transferred to a microtube. 5 µl FITC Annexin V and 5 µl PI were added. The cells were gently vortexed and incubated for 15 min at RT in the dark. Then, the cells were analyzed by flow cytometer (BDFACS Calibur, BD Biosciences, California, USA).

Results

Effects of extracts on cell viability. Based on MTT results, UDC-R extract caused the least percentage of

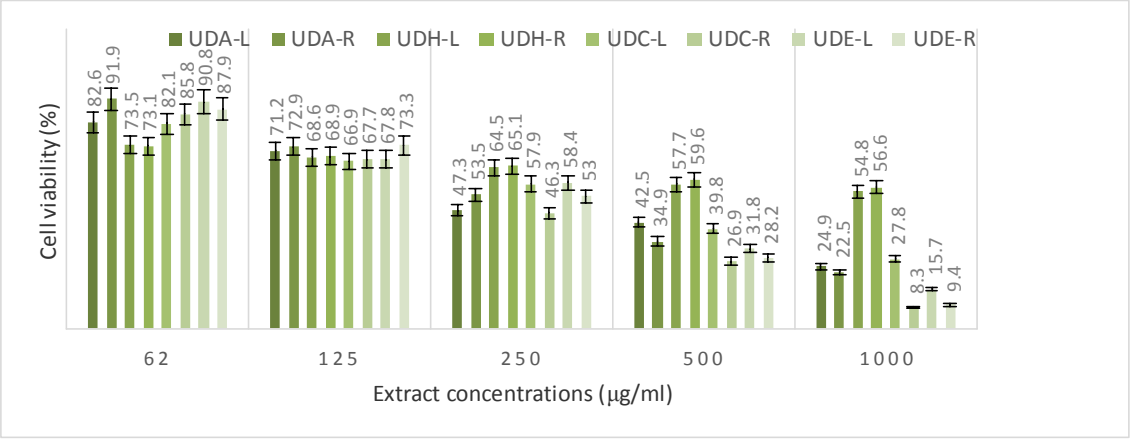

Figure 1. Viability percentage of KG-1 cell line after 48 hrs of treating extracts in various concentrations (62, 125, 250, 500 and 1000 µg/ml). Extract concentrations (µg/ml) in x axis and cell viability (%) in y.

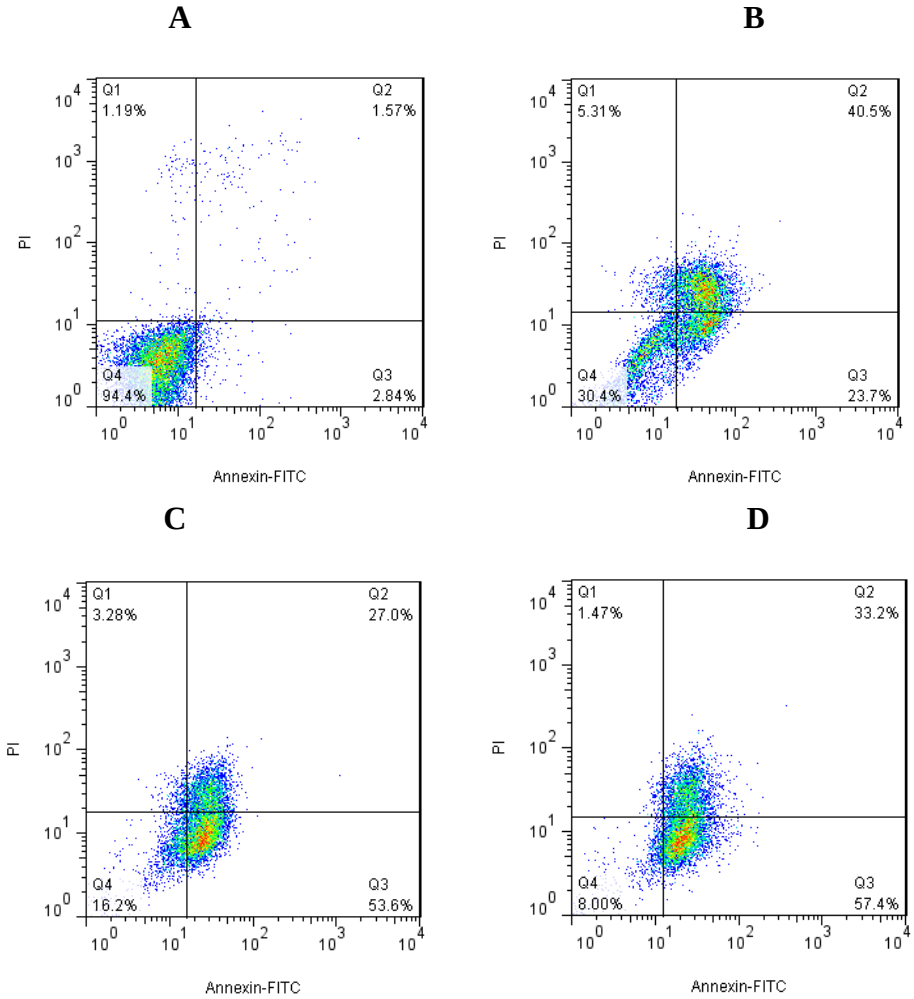

Figure 2. Flow cytometric analysis of apoptosis in KG-1 cell line after 24 hrs. Untreated cells as negative control (**A**), etoposide as positive control (**B**), UDC-R (**C**) and UDE-R (**D**) extracts. The lower left quadrant are alive cells (double negative), the lower right quadrant are early apoptotic cells (annexin V+: PI-), the upper right quadrant is either end stage apoptotic or necrotic cells (double positive) and the upper left quadrant are necrotic cells (annexin V-: PI+). UDC-R and UDE-R induced early (53.6% and 57.4%, respectively) and end stage (27% and 33.2%, respectively) apoptosis in KG-1 cells.

viability in KG-1 cell line (8.3%) at concentration of 1000 µg/ml. IC50 and R2 (regression coefficient) values for each extract were presented in table 1 and viability percentage graph of KG-1 cell line in figure 1. The least IC50 was belonged to UDC-R (219.361 µg/ml). IC50 of etoposide in KG-1 cell was 11.89 µM.

Effects of extracts on cell apoptosis. UDC-R and UDE-R extracts in their IC50 concentration 219.361 and 251.267 µg/ml and R2 values 0.9989 and 0.9863, respectively induced considerable percentage of early (53.6% and 57.4%, respectively) and late (27% and 33.2%, respectively) apoptotic cells after 24 hrs. The results are shown in figure 2.

The relationship between the doses of extracts and inhibition percentage. To evaluate the relationship between the doses of extracts and inhibition percentage in KG-1 cell line, Repeated Measure test was performed by SPSS 21. It showed that this relationship was significant (p: 0.000) at 95% confidence interval (Figure 3). As a result, the proliferation inhibition was dependent on the concentration.

Discussion

In recent years, using herbal products to cure the diseases specially cancers has been increased. It is due to various phytometabolic contents that have biologic activities. Nettle (Urtica dioica) has been used long time and widespread in folk medicine to treat different diseases (9). Hence, we evaluated antiproliferative and apoptotic effects of different extracts (aqueous, hydroalcoholic, chloroform and ethyl acetate) of U. dioica on KG-1 cell line. Antiproliferative of U. dioica was assessed by determining its effect on viability of cell line by MTT assay. Evaluating such effects of U. dioica was performed on KG-1 cell line for acute myelogenous leukemia for the first time, in this study. Since most drugs used in cancer treatment cause apoptosis to inhibit cell proliferation, we applied flow cytometry using FITC Annexin V-PI apoptosis detection kit (quantitative assessment of apoptosis) in KG-1 cell line treated with two extracts (root chloroform and ethyl acetate extracts). Apoptosis is a type of programmed cell death along with certain morphology (nucleus fragmentation, chromatin condensation, blebbing of plasma membrane and apoptotic bodies) (3, 12, 19). In the present study, the results of MTT assay showed that the minimum percentage of cell viability in KG-1 cell line (8.3%) was related to UDC-R extract in 1000 µg/ml. The strongest IC50 was belonged to UDC-R (219.361 µg/ml). UDA-L, UDA-R, UDC-L, UDC-R, UDE-L and UDE-R in KG-1 cell line inhibited 50% of cell proliferation (IC50) at concentrations less than 500 µg/ml. The association between the doses of extracts and inhibition percentage was significant (p: 0.000) which showed by Repeated Measure test in SPSS 21. UDC-R and UDE-R extracts of U. dioica caused early (53.6% and 57.4%, respectively) and late (27% and 33.2%, respectively) apoptosis in KG-1 cell line after 24 hrs. Therefore, the flow cytometry confirmed that the effects of these extracts were due to inducing apoptotic pathway in this cell line. In a previous study, hydroalcoholic (ethanol and double distilled water-4:1) extract of U. dioica leaves, roots, seeds and flowers had antioxidant activities, reduction ability and superoxide and free radicals and H2O2 destruction (20). A study demonstrated that methanolic extract (20%) of U. dioica roots caused considerable reduction in proliferation of prostate cancer cells (LNCaP) and was dependent on time and concentration (21). Methanolic extracts of U. dioica leaves collected from Alto Rio Grande Region, Brazil by Martins H. and coworkers, inhibited 56.2%, 72% and 38.2% of cell proliferation in HL-60 (acute promyelocytic leukemia), MCF-7 (breast cancer) and HCT-8 (colon cancer) cell lines, respectively (22). The results of a study showed that ethanolic, aqueous and petroleum ether extracts of U. dioica roots gathered from Nainital, India had not sensible inhibitory activity (<50%) on A-549 (human lung cancer), IGR-OV-1 (ovary cancer), UD-145 and PC-3 (prostate cancer), HL-60, MCF-7, CoLo-205 (colon cancer) and IMR-32

Table 1. IC50 and R^2 values of various extracts of *U. dioica* in KG-1 cell line

Extracts	IC50 (µg/ml)	R^2
UDA-L	293.298	0.9451
UDA-R	282.100	0.9909
UDH-L	1504.004	0.9894
UDH-R	2322.474	0.9937
UDC-L	315.093	0.9855
UDC-R	219.361	0.9989
UDE-L	270.610	0.9719
UDE-R	251.267	0.9863

Abbreviations: UDA-L, *U. dioica* aqueous extract-leaf; UDA-R, *U. dioica* aqueous extract-root; UDH-L, *U. dioica* hydroalcoholic extract-leaf; UDH-R, *U. dioica* hydroalcoholic extract-root; UDC-L, *U. dioica* chloroform extract-leaf; UDC-R, *U. dioica* chloroform extract-root; UDE-L, *U. dioica* ethyl acetate extract-leaf; UDE-R, *U. dioica* ethyl acetate extract-root.

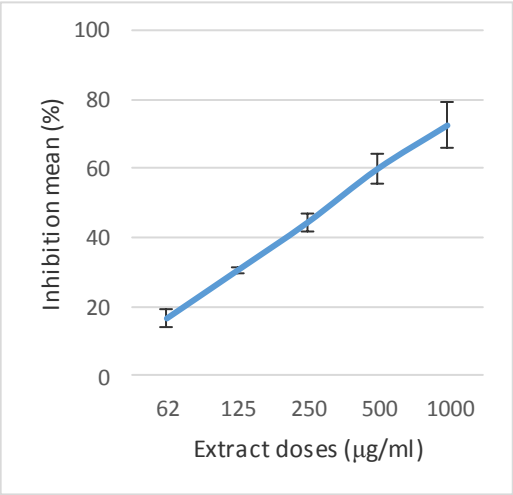

Figure 3. Relationship between the doses of extracts and inhibition percentage in KG-1 cell line was significant (p: 0.000) obtained by Repeated Measure test in SPSS 21.

(neuroblastoma) in 100 µg/ml after 48 hrs (23). In the present study, root hydroalcoholic extract inhibited 50% of KG-1 proliferation at concentrations more than 1000 µg/ml and root aqueous extract caused 50% inhibition in KG-1 cell line at concentration of 282.100 µg/ml after 48 hrs. The results of a study on aqueous extract of U. dioica leaves collected from Babol, Mazandaran, Iran revealed significant dose-dependent inhibition of MCF-7 cell proliferation. In this study, the aqueous extract of U. dioica leaves inhibited the proliferation of MCF-7 cells with IC50 2 mg/ml and flow cytometry using FITC Annexin V-PI apoptosis detection kit showed increased early and late apoptosis as concentration-dependent manner after 72 hrs (3). In the present study, leaf aqueous extract caused 50% inhibition at concentration of 293.298 µg/ml in KG-1 cells after 48 hrs. As mentioned, polyphenols of U. dioica can cause cytotoxic activities (antioxidant, antiproliferative and so on). In a related study, despite of high amounts of phenolic contents in aqueous extract of U. dioica, there was no direct association between the amounts of polyphenols and in vitro cytotoxicity against Hela (cervical cancer) and BT-4749 (ductal carcinoma) cell lines (9). Current in vitro study suggests that U. dioica has anticancer activity in AML. However, we suggest performing the isolation of effective herbal ingredients of U. dioica extracts and further evaluation of their effects on KG-1 cell line and also in normal cells and animal models as in vivo.

Conflicts of Interest

The authors declare that there is no conflict of interest in this study.

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