

Cytotoxic effects of hydro-alcoholic extracts of cress (*Lepidium Sativum*) - made from different stages of the plant - on k562 Leukemia cell line

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Original Article

Abstract

Introduction: Chronic myeloid leukemia (CML) is a malignant clonal disorder of hematopoietic stem cells resulting in the increase of myeloid cells, erythroid cells and platelets in the peripheral blood and hyperplasia in bone marrow. The research evaluated the cytotoxic effects of hydro-alcoholic extracts of *Lepidium Sativum* (Cress plant) shoots before and after flowering on K562 cell line as a model of CML.

Methods: In this laboratory experimental study, the *Lepidium Sativum* shoots including stems and leaves of the plant before flowering and its shoots after flowering including stems, leaves and flowers were collected from Afoos city (Iran). They were extracted using maceration (50% Ethanol 96% and 50% water) method. K562 cells were cultured. Then the cells were treated with different concentrations of the extract (12.5-100 µg/ml) at different time intervals (24, 48 and 72 hour). The *Lepidium Sativum* cytotoxicity was evaluated by the MTT test method before and after flowering against K562 leukemia cells. The absorption was measured using an ELISA plate reader at 540 nm wave length. Data were analyzed using SPSS15 software and one-way ANOVA test analysis as well as Tukey test; where $P < 0.05$ was considered significant.

Results: Hydro-alcoholic extracts of *Lepidium Sativum* showed the most optimum cytotoxicity both before and after flowering with a dose of $IC_{50} = 25$ µg/ml and 72 hour after treatment on K562 cell line. In other words, hydro-alcoholic extracts of *Lepidium Sativum* prepared before and after flowering exhibited a dose and time dependent cytotoxic effect on K562 cell line.

Conclusion: Considering the cytotoxic effect of hydro-alcoholic extracts of *Lepidium Sativum* shoots before and after flowering on K562 cells, the plant can be considered as a potential candidate for further studies on CML treatment.

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Introduction:

Chronic Myelogenous Leukemia (CML) is the most common proliferative clonal disease resulted from genetic variations in the hematopoietic pluripotent stem cells (1). Fusion of Break Point Cluster Region (BCR) from chromosome 22 and Abelson murine leukemia viral oncogene homolog 1 (ABL1) on chromosome 9 encodes a hyperactive tyrosine kinase which results in further proliferative effect of the cells. It is one of the main characteristics of this leukemia. Based on clinical characteristics, CML is divided into three phases: Chronic, Accelerated and Blastic (1-4). The goal of CML treatment is to keep the patients in chronic phase and prevention of disease progression to the next phases, and also to reduce the toxicity of the common medication. Today, Imatinib Mesylate is considered as the first line treatment of the disease. However, the decisive treatment is bone marrow transplantation (5).

The protective effect of plants against cancers is attributed to the antioxidant ingredients of the plants (6) among which cress plant is one of the plants from Brassicaceae family and scientific name of *Lepidium Sativum*. The plant is indigenous to the Eastern Mediterranean and contains glucotropaeolin. Cress has a glycoside called tropaeolide. The plant is rich in antioxidants including vitamins A, B, C, E and isothiocyanate (7). Formerly, there has been such assumption that components found in vegetables had anti-cancer effects which were attributed to the fiber, vitamins E and C, and the products resulted from hydrolysis of glucosinolates. The suggested mechanisms include banding with carcinogens, antioxidant effects, and inhibition of promogens and carcinogens activation. Yet, no scientific study has been reported based on the cytotoxicity of this plant in CML (8). Antioxidants stop oxidation process by neutralization of free radicals. For the process to be carried out, the antioxidants are oxidized. That is why resources of antioxidants are required for the body (9). Hence, the protective effect against cancers caused by the plants is attributed to the antioxidant components (10). Components such as vitamins E and C, and phytochemicals eliminate the effect of active and deleterious molecules on vital macromolecules like DNA and proteins by

trapping properties for free radicals. Since free radicals have a short half-life and exert their effect quickly, the antioxidants needs to be adjacent to vital molecules to neutralize them immediately in order to eliminate the free radicals (11).

Phytochemicals include flavonoids as coerstine, glucosinolates of Brassicaceae vegetables and isothiocyanates of Brassicaceae vegetables. A lot of studies have been carried out on the antioxidant activities of herbal drugs and Brassicaceae family (12). In 2005, the cytotoxic effect of methanolic extract of cress seeds was reported on human bladder cell line with endothelial properties (13).

In another study in 2013, a comparison of the cytotoxic effect of aqueous seeds of cress on breast cancer cell line and control group was carried out. It reported that the cytotoxicity effect was dependent on concentration and time (14).

Warin et al (2010) showed that benzyl isothiocyanate in Brassicaceae family such as cress inhibited breast cancer cell line (15). In a comprehensive study, the vegetables in Brassicaceae family were introduced as the main source of glucosinolates in diets (16).

Since 1) there is not a comprehensive study on the anticancer effects of cress plant shoots in different growth stages on leukemia models, 2) researchers usually ignore the life cycle of plants (growing and flowering) on one hand and lack of definitive treatment for CML on the other, this study aims to determine the cytotoxicity effect of hydro-alcoholic cress extracts before and after flowering on K562 cell line as a model of CML. The anticancer effect of this plant for the treatment of CML is preliminarily evaluated for the first time.

To do this, it seems important to assess the survival and proliferation rate of cells to determine the effectiveness of the anticancer drugs for which several methods have been standardized methods (17). Nowadays, colorimetric methods are often employed because they are easy to handle and provide precise results (18). MTT colorimetric method is quite fast with high precision which can measure the proliferation of all cell lines. The principle of this method for the measurement is the power of mitochondrial dehydrogenase enzyme (19). One of the effective factors in MTT is the number of viable cells. To ensure the

percentage of viable cells, the viability of cells is calculated by Trypan blue. It should be noted that evaluation of drugs or medicinal plants cytotoxicity is based on the observation of morphologic changes in cells (20). The present research studied, the cytotoxicity of cress medicinal plant before and after flowering with concentrations of 12.5, 25, 50 and 100 $\mu\text{g/ml}$ on CML cell line (K562). The study answered whether cress medicinal plant in different stages of growth with pre-scheduled concentrations had anticancer effects on CML cell line.

Methods:

Collection of plants and Extraction

To carry out the present laboratory experimental study, cress was collected before and after flowering in May-June, 2012 from the farms in a city called Afoos near Isfahan (IRAN). The code of the plant was verified by botanical herbarium affiliated to Falavarjan Azad University. After collection, the plants (leaves and stalks separated from those which were collected before flowering; the leaves, stalks and flowers of the plants which were collected after flowering) dried in a cool environment away from light. Maceration method for extraction was preferred to other methods because it could better preserve the vitamins and antioxidants in the plant. Dried plants were comminuted. Fifty gram of dried powder was weighed. It was done separately for both of the collected plants (before and after flowering). The weighed powder bags were placed in two Erlenmeyers. 1500 ml solvent (50% Ethanol 96% and 50% water) was added to each Erlenmeyer to fully cover the powder. After covering the Erlenmeyers with aluminum sheets, they were positioned on a shaker (90 rpm) for 48 hours. After homogenization, the solutions were filtered through filter paper. To separate the solvent from the extracts, the Erlenmeyers were placed on a rotary device. The pure extracts were transferred to a refrigerator in sterile dishes for future experiments.

Cell line

K562 cell line was prepared from Pasteur Institute of Iran in October 2012. It was

transferred to research laboratory at Flavarjan Azad University. Culture medium used for the growth of the cell line in this research was: RPMI 1640 (Bia Idea, Iran) enriched with Fetal Bovine Serum-FBS 10% (Bia Idea, Iran), Streptomycin (100 $\mu\text{g/ml}$, Sinagen, Tehran, Iran) and Penicillin (100 $\mu\text{g/ml}$, Sinagen, Tehran, Iran). The cells were in cell cultures at an incubator (holding British Standard) with 5% CO_2 , 95% humidity and 37°C.

Assessment of cytotoxicity based on MTT

To evaluate the effects of cress before and after flowering on the morphology of K562 cell line, 10^5 cells were positioned in 96-well plates (Surface, Denmark). After 24 hours, the cells were treated with different concentrations of cress (12.5, 25, 50, 100 $\mu\text{g/ml}$) prepared separately from both cress before and after flowering for 24, 48 and 72 hours. The morphologic changes of the cells treated by cress before and after flowering were evaluated with inverted light microscope (Hm-Lux, Germany), and then were compared with control sample (untreated cells). Moreover, Trypan blue exclusion test and hemocytometer were used to evaluate the effect of cress on the growth and cell viability. To do those 2×10^4 cells were positioned in the wells of 96-well plates.

The researchers added 180 μl of culture medium to the wells in the first row of the 96-well plate, and added 180 μl of cell suspension to the wells in the remaining rows. After 24 hours of incubation, 20 μl of culture medium was added to the two first rows. Therefore, the first row was considered as blank, and the second as negative control. 20 μl standard Doxorubicin (200 mg/ml) was added to the third row. This row was considered as positive control. 20 μl of different concentrations of cress extracts were added to the wells in 4th to 12th rows of the plate for those 24 to 72 hours. In time output, the number of the cells were counted in each well using hemocytometer lamella and Trypan blue (Merck, Germany). The tests were carried out three times separately. Then to evaluate the cytotoxicity of cress on K562 cell line, Methyl Thiazol Tetrazolium (MTT) reduction test was employed. Tetrazolium salt is reduced to Formazan crystals (with a different absorption) through the mitochondrial activity of

live cells. To do this, 10^4 cells were loaded on each well. Different concentrations of cress extracts (before and after flowering) were added after 24 hours. Next, after either of time intervals, 20 μ l Tetrastazolium salt (Sigma, Malaysia) was added. After 24 hours, absorption rates of treated and untreated were measured using of ELISA (Statfix-2100, America).

Statistical analysis

All the experiments of present study were repeated at least three times. The results were shown as the mean of the three time repetition \pm Standard Deviation. One-way Variance analysis of variance (ANOVA) and T-test were applied on the collected data using SPSS (Version 15). $P < 0.05$ was considered statistically significant.

Results:

MTT test results

It is noteworthy that the time interval and the cress extract concentrations in the different growth stages of the plant were based on its anticancer effects in different studies and the preliminary investigations in laboratory. Table 1 indicates the percentage of cell survival (K562) in the presence of control groups. As shown in Table 2, the maximum percentage of cell survival (K562) is in concentration of 12.5 μ g/ml in 24 hours for both growing and flowering of Cress. Another noteworthy result in this table is $IC_{50} = 25$ μ g/ml (a concentration of the extract in which 50% of the cell in the culture medium are killed) and 72 hour time after the treatment of both growing and flowering stages. The results show that there is a significant difference between the survival rate of cells treated by Cress before and after flowering in

comparison with control cells after 3 days, especially in 25 μ g/ml concentrations.

The results show that the survival rate of K562 cells reduced in different concentration within three days. Moreover, statistical analysis related to the results from all the data show P value less than 0.05 which is considered significant. In other words, Cress has cytotoxic effects (anticancer) both before and after flowering on K562 cell. The best toxicity property is observed in 25 μ g/ml after 72 hours of treatment. In 24 hours survival rate started to reduce. The minimum percentage of K562 cell survival rate has been recorded in 72 hours. Survival has reduced in all concentrations, but 100 μ g/ml has had the minimum percentage of cell survival rate (Table 2).

Morphologic results

Investigation of morphologic changes of the cells treated by Cress in different growing stages show those certain morphologic changes in the treated cells in comparison with the control group. Figure 1 indicates the fragmentation of chromatin and transformation of the spherical shape of cells in $IC_{50} = 25$ μ g/ml. In this concentration, the cells either individually or collectively are dwindled and vacuolated. They show the reduction of cytoplasm and pigmentation in comparison with the control group. The results indicate the cytotoxicity of Cress medicinal plant in both before and after flowering on K562 cell line.

Table 1. The viability percentage of K562 cells in negative control (suspension cell) and in presence of positive control (Doxorubicin)

Positive control (Doxorubicin)	Negative control
14.1 \pm 1.5*	100 \pm 4.6

* $P < 0.05$

Table 2. The viability percentage of K562 cells in presence of different concentrations of cress plant before and after flowering

Plant	Concentration	Mean of survival percentage			P-value	Mean of the total survival percentage Mean±SD
		1 st day Mean±SD	2 nd day Mean±SD	3 rd day Mean±SD		
Cress Before Flowering	12.50	85.58±4.36	63.5±1.72	53.02±1.15	0.0001***	67.37±14.59
	25	83.88±5.81	61.96±1.15	50.77±0.24	0.0001***	65.54±14.89
	50	82.7±6.33	60.07±0.92	47.60±1.71	0.0001**	63.49±15.79
	100	81.27±7.16	58.43±0.54	45.71±2.57	0.0001***	61.80±16.06
Cress After Flowering	12.50	76.19±1.68	61.63±1.64	53.39±0.87	0.0001***	63.74±10.07
	25	74.74±1.79	59.59±1.52	50.61±0.38	0.0001***	61.64±10.63
	50	73.28±1.40	58.11±1.72	47.37±1.05	0.0001***	59.59±11.34
	100	72.08±2.10	57.00±2.09	45.17±0.99	0.0001***	58.08±11.76

* P-value < 0.05, ** P-value < 0.01, ***P-value < 0.001

The effects of cress – before and after flowering – on the survival percentage of K562 cells. Cells were treated with different concentrations of hydro-ethanolic extracts at time intervals of 24, 48 and 72 hours. Cell survival rate was evaluated by the use of cell count and Trypan blue exclusion test. The results are shown by the mean of three independent repetition of the tests ± Standard Deviation.

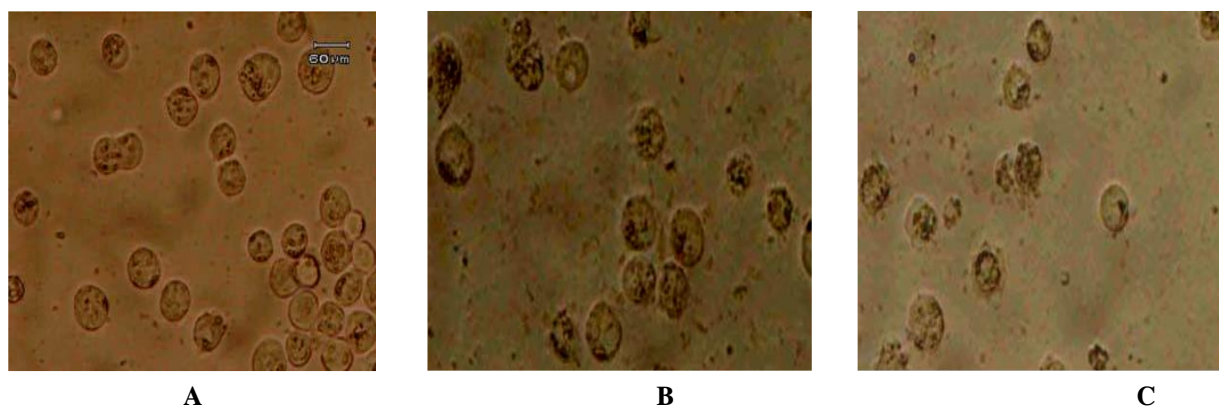


Figure 1. Morphologic changes: A) K562 cells before treatment, B) Cells treated with Cress before flowering, C) Cells treated with Cress after flowering. Cells treated with 25 μg/ml Cress before and after flowering after 72 hours, and morphologic changes were studied by the use of inverted HM-LUX. Note the fragmentation of chromatin and changes in the shape of the cell (magnification: 400X)

Conclusion:

Using culture methods creates comprehensive understanding of the effects of drugs and pharmaceutical plants on normal and cancerous cells.

The effects and changes created by different combinations such as *lepidium sativum* extract before and after flowering (which were analyzed in this research) on cells in studied and controlled culture lead to recognizing precisely the mechanism and biological effects as well as various intracellular factors. These facilities help to recognize intracellular processes and functions

during cancer treatment by pharmaceutical plants which can promote treatment methods (21).

Therefore, pharmaceutical plants with toxic effect (specially cytotoxic effect) are one of the most important candidates to synthesis anticancer drugs which the toxicity can be measured by cellular culture.

In other hand, due to frequency, lower side effects and pharmaceutical intervention, combinations with vegetative source attract pharmacists attention to synthesis new drugs in order to treat refractory disease such as cancers (22).

In consideration to the tables and pictures can be understood that hydroalcoholic extract in pre-post flowering has had cytotoxic on cell line of human chronic myeloid Leukemia (K562).

In the recent research, the highest rate of cytotoxicity in low concentration of *Lepidium sativum* extract compared to control group shows that there are strong cytotoxic combinations such as vitamins, glucosinolate and other antioxidant combination in that plant.

In the recent investigation, *Lepidium sativum* is from cruciform vegetables and according to kotasic, phytochemicals act better next to other materials in vegetables (23) among which glucosinolate and FITC in *lepidium sativum* next to vitamins and other antioxidant combinations has presented good cytotoxic effect on K562 in low concentration which is consistent with kotasic's results.

According to 15 human studies, 3738 patients under chemotherapy which consume complements containing antioxidants has had longer life.

In this studies, nutrients such as vitamins A, C, E and vitamins of group B a selenium were used (24-26). Hence there is a hope in using *Lepidium sativum* in pre-post flowering for complication decline and longer life in treatable patients with chemotherapy.

Neozil et al (2001) showed that the concentrations 4,6,9,12,15,18,20 and 25 μ l/ml of alpha-tocopherol - D (vitamin E) are dependent to dose and time and effective on cellular proliferation so that in concentration 20 μ l/ml after 48 hours and in concentration 12 μ l/ml after 72 hours, the survival of human promyelocytic (acute promyelocytic leukemia which is a type of leukemia) decreased to 50 percent in HL-60 cellular line. After 24 hours, no alive cells found in high concentration 3 μ l/ml of vitamin D.

Also in recent study, vitamin E is a part of main combination in *lepidium sativum* which according to surveys, cytotoxic effect of K562 can affect chronic myeloid leukemia due to vitamin E in combinations.

In recent study, *lepidium sativum* has reached IC50 in concentration 25 μ l/ml after 72 hours while in higher concentrations, alive cells percent has been below 50%.

Of course, Kozin has proven that vitamin E induces cellular mortality in blood and breast cancerous cells (27), vitamin is another vitamin in *lepidium sativum*. Ascorbic acid -L (vitamin C) can contrast to cancer by neutralizing reactive species and also prevents from tumor to other tissues by improving collagen synthesis (28).

The studies showed that ascorbic acid -L has the effectiveness of control on growth and proliferation in promyelocytic acute Leukemia.

In 2003, Kong surveyed vitamin C effect on HL-60 growth based on apoptosis induction and revealed that vitamin C in concentration above 10⁻⁴ M possesses the highest effect on proliferative control (29).

Therefore vitamin C existing in *Lepidium sativum* is probably one of the reasons for cytotoxic.

Fatimi et al reported cytotoxic effect of methanol extract present in *lepidium sativum* seed on ECV-304 (cellular line of human bladder cancer) in a concentration above 50 μ l/ml (13). In a similar research (2013) about cytotoxic effects of blue extract of that plant on cellular line of breast cancer (MCF-7), it is characterized that increasing concentration and time cause increasing cytotoxic effect in which cytotoxic property is attributed to glucosinolate (14). Respecting to antioxidants in aerial organs of this plant in comparison to Fatimi et al study, it is clear that because cytotoxic effect of aerial has been in a concentration lower than plant seed and extraction has been of hydroalcoholic type and also cytotoxic effect on growth of human chronic myeloid Leukemia in various growth stages in low concentration (25 μ g/ml), it is recommended that investigation about clinical usage of the plant in obtained dose to prevent and to treat next to chemotherapy drugs for this current in vivo be a introduction to next researchs.

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بررسی اثر سایتوتوکسیک عصاره هیدروالکلی گیاه شاهی در مراحل مختلف رویشی بر لاین سلولی سرطان خون (k562)

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چکیده

مقدمه: لوسمی میلوئیدی مزمن (CML) یک اختلال کلونال بدخیم سلول‌های بنیادی خون ساز است که منجر به افزایش سلول‌های میلوئید، سلول‌های اریتروئیدی و پلاکت‌ها در خون محیطی و هیپرپلازی در مغز استخوان می‌شود. این تحقیق به منظور بررسی اثر ضد سرطانی عصاره هیدروالکلی اندام‌های هوایی گیاه شاهی در مرحله قبل و بعد از گل‌دهی بر روی لاین سلولی K562 به عنوان مدل لوسمی میلوئید مزمن صورت گرفته است.

روش کار: در این مطالعه تجربی آزمایشگاهی، اندام‌های هوایی (برگ و ساقه) شاهی قبل از گل‌دهی و (برگ و ساقه و گل) شاهی بعد از گل‌دهی از شهر افسس جمع‌آوری و با استفاده از روش خیساندن (۵۰ درصد اتانول ۹۶ درصد: ۵۰ درصد آب) عصاره‌گیری شد. سلول‌های K562 کشت داده شد و با غلظت‌های عصاره (۱۲/۵-۱۰۰ میکروگرم بر میلی‌لیتر) و در فاصله‌های زمانی مختلف (۲۴، ۴۸، ۷۲ ساعت) تحت درمان قرار گرفتند. سمیت سلولی عصاره شاهی قبل و بعد از گل‌دهی علیه سلول‌های K562 لوسمی با استفاده از روش MTT برآورد شد. جذب با استفاده از دستگاه الیزا در طول موج ۵۴۰ نانومتر اندازه‌گیری شد. بررسی داده‌ها با استفاده از نرم‌افزار SPSS 15 و آنالیز یک طرفه ANOVA و آزمون توکی صورت گرفت و سطح معنی‌داری کمتر از ۰/۰۵ در نظر گرفته شد.

نتایج: عصاره هیدروالکلی گیاه شاهی هم در مرحله قبل از گل‌دهی و هم بعد از گل‌دهی، بهترین اثر سمیت سلولی را در $IC_{50} = 25$ میکروگرم بر میلی‌لیتر و ۷۲ ساعت پس از تیمار، از خود نشان داد. به عبارت دیگر، عصاره هیدروالکلی گیاه شاهی در مراحل مختلف رویشی اثر سمیت سلولی وابسته به دوز و زمان را بر رده سلولی K562 از خود بروز داده است.

نتیجه‌گیری: با توجه به اثر سمیت سلولی اندام‌های هوایی (برگ، ساقه و گل) گیاه شاهی در مراحل مختلف رویشی بر لاین سلولی K562 گیاه می‌تواند به عنوان یک کاندید بالقوه برای مطالعات بیشتر در مورد درمان سرطان لوسمی میلوئیدی مزمن در نظر گرفته شود.

کلیدواژه‌ها: سمیت سلولی - لوسمی - شاهی

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