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ORIGINAL ARTICLE

In vitro Cytotoxic Activity of Aqueous Root Extract of *Althea kurdica* against Endothelial Human Bone Marrow Cells (line k562) and Human Lymphocytes

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ABSTRACT
Althea kurdica is, a well- known plant drug, widely used in Iran as well as other countries. Althea flowers extract have been used extensively for urinary and respiratory disorders. The cytotoxic effects of A. kurdica flowers extracts were evaluated using Human Leukemia Cell Lines (line k562) and human lymphocytes. The cytotoxic activity was determined by anti-proliferative effects on the human lymphocytes and human bone marrow cell line were cultured as a model of normal and cancerous cells respectively under different concentrations. After 24 h treatment with the extracts, cell proliferation was measured using a colorimetric method based on the ability of metabolic active cells to cleave the yellow tetrazolium salt MTT to an insoluble purple formazan crystal. The soluble formazan dye was directly quantified using a scanning multiwall spectrophotometer (ELISA plate reader). A dose response assay with the extracts was carried out to determine the maximum effective dilution. The results showed that the extract exhibited a potent cytotoxic activity against the k562 cell lines and human lymphocytes in specific manner with an IC_{50} value of 7.5 mg and 5 mg respectively. The traditional extract of A. kurdica roots showed in vitro cytotoxic effects on two different cell lines. Interestingly the IC50 value was higher in the normal human lymphocytes comparing to the Human Bone Marrow Cells. Thus, the concentrations between 5 mg and 7.5 mg could be used for cancer treatment.
Keywords: *Althea kurdica*, aqueous extract, cytotoxic, MTT assay

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INTRODUCTION

Plants based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. On the other hand, plants have been a source of medicine for thousands of years, and phytochemicals continue to play an essential role in medicine [1]. The use of medicinal plant extracts for the treatment of human diseases is an ancient practice; this has greatly increased in recent years. For a long time, plants are being used in the treatment of cancer [2]. Natural compounds have provided many effective anticancer agents in current use. Currently, over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them [3].

Simply, in recent times, scientific study of their effects has flourished. Despite, the availability of rich synthetic drugs, plants remains– even today – a fundamental ingredient of health care. In developing countries, the practice of medicine still relies heavily on plant and herbal extracts for the treatment of human ailments. Dietary agents consist of a wide variety of biologically active compounds that are ubiquitous in plants, and many of them have been used as traditional medicines [4] [5]. According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products [6].

On a whole, cancer is a disease in which there is uncontrolled multiplication and spread within the body of abnormal forms of the body's own cell, is the second leading cause of more than six million deaths each year in the world. A large portion of Iranian still uses traditional medicine as an alternative for the treatment of this condition [7].

In the Iranian traditional medicine, the use of plants in the form of infusions or decoctions is a common practice among people of rural communities and their use is increasing in urban populations. Iranian medicinal plants were already studied for their use in different human diseases [8]. Some epidemiological studies have suggested that high consumption of fruits and vegetables

could help prevent several chronic diseases including cancer [9]. Some phytochemicals derived in spices and herbs as well as other plants possess substantial cancer preventive properties [10].
These indigenous plants have been used for various therapeutic purposes. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body [2].
Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers [11] [12]. More than 60% of currently used anticancer agents are derived in one way or another from natural sources [13]. But many of their potential effects especially anti-cancer effect have not been well studied. To date, approximately 100 species of plants have been examined, and some active constituents isolated and identified, for instance several of the current chemotherapeutic drugs like vinblastine, methotrexate, taxol, and so forth, were first identified in plants [14] [15] [16]. There are two main strategies for the selection of plants species in anticancer drug discovery: random screening and ethnomedical knowledge. The second approach includes plants used in organize traditional medical systems like herbalism and folklore [17]. The utility of cell lines acquired from tumors allows the investigation of tumor cells in a simplified and controlled environment; uncontrolled proliferation is a universal property of tumor cells [18]. In the cancer drug discovery program, a paradigm based on ethnobotanical and ethnopharmacological data would be more economical and beneficial for identifying potential anticancer molecules than mass screening of plant species [19]. Investigation of the cellular growth control mechanisms has contributed to the understanding of carcinogenesis and identification of compounds with specific antitumoral activities. Thus, cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antitumoral properties for future studies [20]. Among several medicinal plants used in Iran, plants of Malvaceae family have been frequently and widely used in traditional systems of medicine practiced in many Asian countries, and their medicinal functions have been broadly discussed and accepted in many traditional recipes. The phytochemical study of this family demonstrated the presence of quercitrin, a glicosidic flavonoid, and also tanines, saponines, resins and essential oils in the tips, as well as inulin and rutine diterpines, quinine acid, rhamnoides and caffeic acid, chlorogenic and hydrocynamic and their derivates in the roots [21] which has reported antitumour activity [22].
As plants of Malvaceae family are considered safe for human consumption, It is a medicinal plant used in traditional medicinal practice and has been reportedly useful in the treatment of many diseases. The great demulcent and emollient properties of Marsh Mallow make it useful in inflammation and irritation of the alimentary canal, and of the urinary and respiratory organs. It is excellent in painful complaints of the urinary organs, exerting a relaxing effect upon the passages, as well as acting curatively. This decoction is also effective in curing bruises, sprains or any ache in the muscles or sinews and for promoting menstruation in traditional medicine in Asia [23]. Anticomplement activity Yamada [24], Anti-inflammatory activity [25], Antimycobacterial activity effect [26], Antitussive activity [27] [28], Antiviral activity [29], Antiyeast activity, Antibacterial activity [30], Radical scavenging effect of this plant rhizome have been reported [31].
Aqueous extracts of *Althea* exhibit antifungal activity *in vitro* and it has been demonstrated that some of its organic solvent extracts are antibacterial [30] [22]. Babu et al. provided evidence for the antibacterial activity in plant extracts made with organic solvents, including methanol, ethanol, ethyl acetate, acetone, chloroform, and n-hexane petroleum ether, benzene, chloroform, methanol and ethanol [32]. They have shown that the antibacterial activity is more significant in solvent extracts as compared to aqueous extract of Althea indicating that the active principle responsible for antibacterial activity is more soluble in organic solvents. These species are excellent candidates for development of novel chemotherapeutics. *A. kurdica*; common name, "marsh mallow" is an annual weed that is native to Europe, a medicinal plant belonging to this family, is a close relative of *Althaea officinalis* from which the mucilage is isolated.
In Iran, these herbal plants are commonly used in the treatment of various ailments and are also consumed regularly as a part of the daily diet. Despite their widespread use, however, no scientific assessment for anticancer effect has been conducted in most cases. Considering their increasing recognition and consumption, the present study was undertaken to evaluate the anticancer potential of these plant extracts in the inhibition of cell proliferation. On a whole, the purpose of this study is to investigate the cytotoxic potential of various extracts from flowers of *A. kurdica* on normal and cancer cell lines using MTT assay.

MATERIALS AND METHODS

Collection of *Althea. kurdica*.

The flowers of *A. kurdica* were collected from the campus of Agriculture Department, Bu-Ali Sina University.

Preparation of the aqueous extracts of *A. kurdica* flowers.

Based on an analysis of published literature the following plant parts were considered for the assessment of anticancer properties: Plant material (flowers) were dried at room temperature in the dark and ground finely using blender. Exactly 20 g of each powdered sample was extracted in a flask by adding distilled hot boiling water. The flask was then placed on a shaker (200 rpm) for 4 hours, and the temperature was maintained at 37°C, after which the flask was brought to room temperature and then the suspension was filtered through a series of Whatman filters. The filtered aqueous extract was pooled and evaporated to dryness under reduced pressure at 40°C and a stock solution was then prepared by dissolving the extract powder in distilled water and the experimental concentrations were diluted in basal medium. Extracts were sterilized by filtration using sterile 0.22 µm pore size filters.

In vitro cytotoxic activity assay

Cell Lines and Culture Conditions. Endothelial human bone marrow (line k562) cell lines were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 Ìg/ml streptomycin (Invitrogen,Carlsbad, CA, USA). Cells were maintained in a humidified atmosphere of 5% CO2 at 37°C in an incubator. Prior to use in the assay the cells were grown to 80 - 90% confluence and synchronized by incubation in the assay media for 4 hrs. Cell suspensions ($5*10^3$ cells/100 µl/well) were then incubated with various concentrations (0.1, 0.4, 3, 7.5, 10 mg) of aqueous plant extract solutions for 24 hrs.

Isolation of lymphocytes from whole blood. Three ml of blood were taken from normal healthy individuals and collected in heparinised test tube. Five ml of Phosphate Buffered Saline (PBS) were added and mixed well. Two ml of ficoll hypaque solution were taken and carefully layered blood PBS mixture on to the ficoll hypaque solution. It was centrifuged at 2000 rpm for 30 minutes. The opaque interface containing mononuclear cells was collected, mixed with PBS, and centrifuged at 1500 rpm for 10 minutes, and supernatant was discarded. The centrifugation was repeated thrice, and normal lymphocytes were resuspended in RPMI medium with 10% fetal bovine serum. Then the cells were plated in 96-well plates at 10^4 cells/100 µl/well for the normal lymphocytes and used for cytotoxicity analysis.

In vitro cytotoxicity studies

Viability staining by trypan blue dye exclusion method. The aqueous extract was studied for short term *in vitro* cytotoxicity using human bone marrow cells (k562 cell line) and lymphocyte cells as normal cells. The cells were seeded in 96-well plates. Four wells for each concentration were seeded and triplicate plates were used the cell line. Then, the cells were incubated at 37°C. After 24 h the medium was replaced by fresh medium containing different concentrations of the plants extract. After incubation 0.1 ml trypan blue was added and number of dead cells determined by using haemocytometer. The percent viability was calculated by using formula:

% viability = (live cell count/total cell count)*100

Cytotoxicity assay. Cytotoxicity of sample on tumor cells was measured by microculture tetrazolium (MTT) assay [33]. For the assays, 96-well microplates were seeded with 100 µl medium containing 5, 000 cells. After 24 h incubation and attachment, the cells were treated with 6 fourfold dilution of crude extracts. Exactly from the stock solution (40 mg/ml), each extract sample was applied in a series of 6 dilutions (final concentrations ranging from 15.6 to 500 µg/ml) with a final DMSO concentration of 0.1% and was tested in quadruplicate. After 48 h incubation, cell viability was determined by adding (Sigma) tetrazolim salt as cytotoxicity indicator, so after 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. Tetrazolium salts are cleaved to formazan dye by cellular enzymes (only in the viable cells). The % cell inhibition was determined using following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

The Inhibitory concentration required for 50% cytotoxicity (IC50) value was analysed using sigmaplot software.

Light microscopy

Human bone marrow cell cancer and normal cells were grown to 70% confluence and treated with 5% aqueous plant extracts for 24h and the photographs were taken at 40X magnification using a phase-contrast inverse microscope (Olympus, Japan).

Statistical Analysis

All experiments were conducted with 4 replicates and the results expressed as mean ± standard deviation. Differences between the control and the individual dosage group of each extract were analyzed by means of the Duncan's test of significance at the $P < 0.05$ level. The concentration that results in 50% inhibition (IC50) value of the extracts were calculated from the equation of the logarithmic line determined by fitting the best line to the curve formed from the data using Microsoft Excel.

RESULTS

Using the ethnomedical data approach, one Iranian plant that are used in the Iranian traditional medicine for various diseases, including cancer, was collected and evaluated for their cytotoxic activities. The search for new anti-cancer drugs is one of the most prominent research areas of natural products. To investigate the cytotoxic potential of extracts were prepared according to the traditional use in Iran from Iranian plants used in traditional medicine for the treatment of various diseases such as cancer, inflammation or infectious diseases. We collected the flowers of *A. kurdica* in order to screen them for possible cytotoxic activity against human bone marrow cancer cell lines and lymphocytes. The cytotoxic activity was evaluated on human blood cancer cell line and lymphocytes cells respectively.

Evaluation of effects on cell viability using MTT test

Metabolic activity can be evaluated by measuring the activity of a mitochondrial enzyme succinate dehydrogenase using MTT test. MTT is designed to be used for the quantification of both cell proliferation and cell viability in cell population using 96-well plate format. This test is widely used in the *in vitro* evaluation of the biosafety of plant extracts. In the present study, we applied the MTT test to evaluate the biosafety of cytotoxic effect of aqueous plant extract on k562 cell line and lymphocyte cells. Therefore, cancer and normal cells were exposed to increasing concentrations (0.1-10 mg ml^{-1} of culture medium) of the aqueous of *A. kurdica* extracts for 24 h. Following removal of the plant extracts from each well, cells were washed in phosphate-buffered saline, and the MTT assay was carried out as described. The MTT assays data are presented respectively in Figures 1(A and B) and the corresponding IC50 are summarized in Table 2.

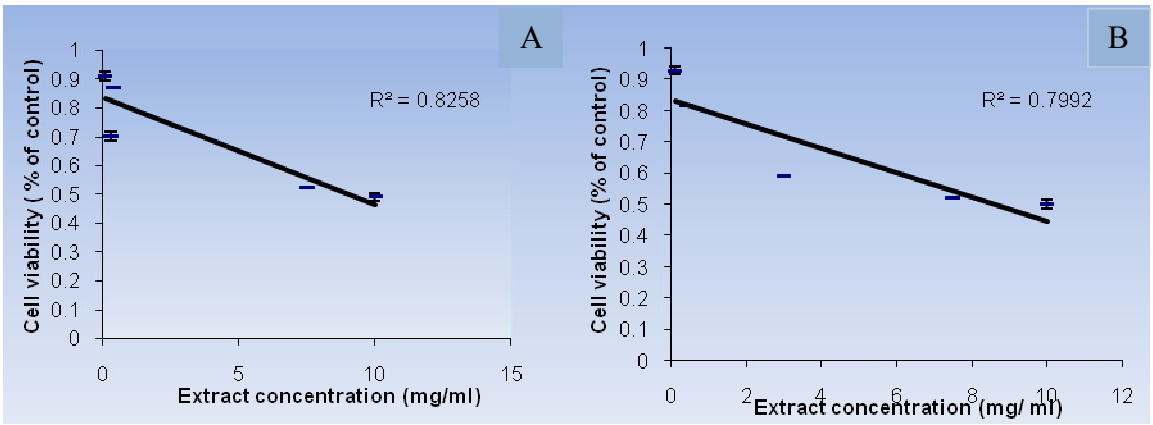

Figure 1. MTT assay in k562 cells (A) and in lymphocytes cells (B) after an overnight incubation with various concentrations of extracts from A. kurdica. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells. Values given represent the mean ± standard deviations of three independent experiments carried out in triplicate.

The extract produced a reduction in cell proliferation of lymphocytes cells at 24-hrs incubation (Figure 1B) with *A. kurdica* resulting in the best growth inhibition at lower concentrations. At the highest concentration tested (10 mg) with a 24-hrs incubation period, *A*. kurdica resulted in 95% inhibition of cell proliferation, while this extract provided 85% inhibitions of cancer cell line (k562).

Percentage cell viability of both of cells was carried out by using Trypan blue dye Exclusion technique (Figure 2). The results show dose dependent response. The extract showed different anti-proliferative profiles regarding extract concentrations. There were different inhibitions produced by different concentrations of at 24-hours incubation. On the other hand, there was no difference in the level of inhibition produced by concentrations lower than 0.4 mg. The inhibition level of each concentration (7.5 and 10 mg) was significantly different from those in lower concentrations ($P < 0.05$ and $P < 0.001$ respectively) (Table1). There was however no significant difference between the inhibition produced by 7.5 and 10 mg. The cytotoxicity activity was carried out by using MTT assay. The inhibition percentage with regard to cytotoxicity was found to be 99.66 % at 10 mg with IC50 value of 266.8µg/ml (Table 2)

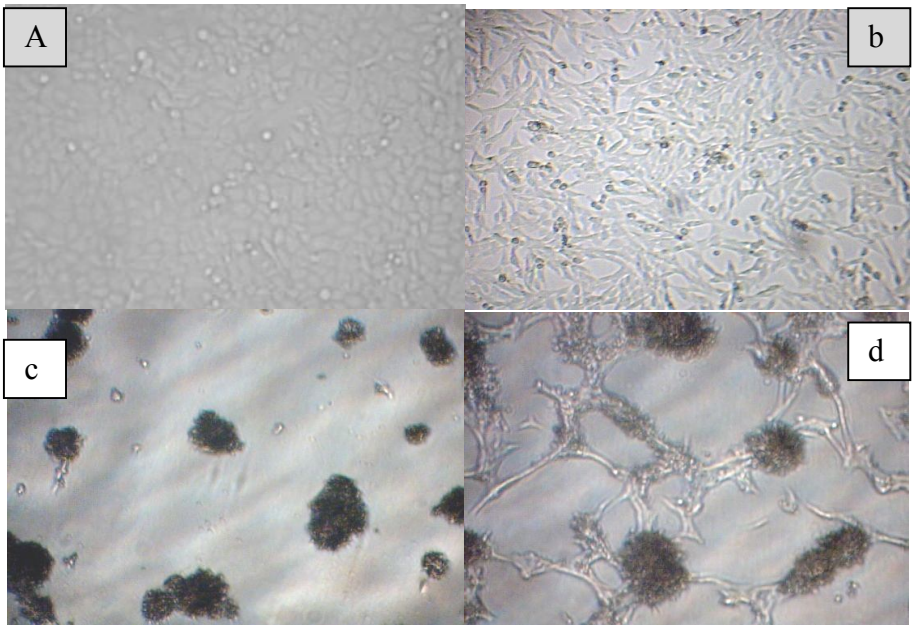

Figure 2: Photomicrograph (Pictoral view) of k562 cell lines by using Trypan blue dye exclusion technique. K562 cell lines (a) control (b) *A.kurdica* 0.4 mg/ml (c) *A.kurdica* 7.5 mg/ml (d) *A.kurdica* 10 mg/ml. The *in vitro* screening of the aqueous extract showed that Althea had cytotoxic effect on k562 cell lines.

Table 1. The result of analysis of variance of *Althea kurdica* extract on cancer cell line (k562) and lymphocyte cells.

Source of variation	Degree of freedom	Cytotoxic concentration
Cells	1	0.20**
Concentration	13	0.104**
Concentration* cells	13	0.004**
Experimental error	28	0.001
Coefficient of variation	-	% 1.81

Table 2: Determination of cytotoxicity by MTT Assay

Plant extract	Concentration mg/ml	Absorbance	% inhibition	IC 50 mg/ml
Althea kurdica	Control	0.498		
	0.1	0.427	14.25	
	0.4	0.380	23.69	
	3	0.268	46.05	
	7.5	0.165	66.73	
	10	0.00166	99.66	266.8

DISCUSSION

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around the world [34] [35]. Approximately, 60% of the anticancer drugs currently used have been isolated from natural products from the plants. At this time, more than 3000 plants worldwide have been reported to possess anticancer properties. The parts of *A. kurdica* have been

commonly used in traditional Iranian medicine for the treatment of various human ailments for many years [36]. Extracts of this medicinal plant are believed to contain a wide array of polyphenolic compounds which might possess cancer preventive and/or therapeutic properties [22]. On a whole, our goal was to determine whether the extracts of these plants exerted an inhibitory effect on cancer cell proliferation and caused cell death. The results of our studies suggest that aqueous extract of *A. kurdica* possess the strongest cytotoxic effects on both human cancer cell and normal cell. The initial screening of plant for its anticancer properties use cell-based assays and established cell lines, in which the cytotoxic effects of plants extracts or isolated compounds could be measured. In the present study, we observed that the extract of *A. kurdica* caused marked cell growth inhibition in the human blood cancer k562 cell line and lymphocytes cells in a dose –dependent, but this effect is caused in the lower concentration. In fact, *A.kurdica* extract had the greatest activity with lowest IC50 values. Studies have shown differential sensitivities to several natural compounds between tumor and normal cells *in vitro*, and the results obtained from the present study show that the aqueous extract from *A.kurdica* is cytotoxic to k562 cell lines and lymphocytes. To be a good drug candidate, the IC50 value of such agent should be sufficiently low to avoid any possible unspecific effects. The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product for future bioguided studies if it exerts an IC50 value < 30 μg/ml [37]. In this preliminary study, we have focused our interest on crude plant extracts, the cytotoxic activity could be due to the presence in the aqueous extract of active products that could probably have highly anti-growth effects. On the other hand, our phytochemical screening revealed the presence of terpenoid, flavonoids and alkaloids in the extract of *A. kurdica*, which could be responsible for this activity [28] [23]. Flavonoids have been found to possess antimutagenic and antimalignant effects [33] [38]. Moreover it has protective effect against cancer by their effect on signal transduction in cell proliferation and angiogenesis. Several studies have been reported on the phytochemical and other biological properties of *A. kurdica* (L.) [28]. In addition, this plant is a source of a number of bioactives compounds as well as flavonoids and phenoloic derivatives [39].

This study provides an important basis for further investigation into the isolation, characterization and mechanism of cytotoxic compounds from the screened medicinal plants. We also plan to carry more biological activities, including the *in vivo* studies and the statute of inhibition of blood cancer. Thus, these plants could be as a source for new lead structures in drug design to combat cancer. It also justifies the folklore medicinal uses and claims about the therapeutic values of this plant as curative agent against cancer and we therefore, suggest further, the purification and characterization of the phytochemicals and isolatation the active constituent. In addition, it can be subjected to pharmacological screening of this plant along with investigations that are needed to provide some additional insight into the *in vivo* cytotoxic activity of the plants with a view to obtaining useful chemotherapeutic agent. In addition, further studies are required to elucidate the precise molecular mechanisms and targets for cell growth inhibition which will allow the rationale design for more effective molecules for the eventual use as cancer chemopreventive and/or therapeutic agents.

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