

Anticancer and anti-inflammatory activities of shallot (*Allium ascalonicum*) extract

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Abstract

Introduction: *Allium* plants are an important part of the diet of many populations and there is a long-held belief in their health-enhancing properties such as cancer prevention. In this study, the anticancer and anti-inflammatory activities of the aqueous extract of the *Allium ascalonicum* bulbs have been studied.

Material and methods: The antiproliferative and anti-growth activity of the aqueous extract of *A. ascalonicum* was examined *in vitro* on different tumor cell lines. Furthermore, the acetic acid-induced vascular permeability as an *in vivo* assay was used for studying anti-inflammatory activity of the extract.

Results: The aqueous extract of *A. ascalonicum* had the most anti-growth activity on the cancer cell lines; Jurkat and K562 against Wehi 164 with lower cytotoxic preference. The extract also showed much less cytotoxicity against the normal cell (HUVEC) line and significant anti-inflammatory activity *in vivo*.

Conclusions: It is of interest that the extract of this plant has shown much less cytotoxicity against the normal cell line, and, if this also occurs *in vivo*, the use of this plant clinically for the treatment of cancer patients would have some scientific support. The results of these assays indicated that *A. ascalonicum* can be a candidate for prevention and treatment of many diseases related to inflammation and malignancy.

Key words: *Allium ascalonicum*, anti-inflammatory, anticancer, LDH assay.

Introduction

Cancer is one of the most prevalent diseases worldwide, which is a major public health issue in both developed and developing countries. Because of the high demand for cancer drugs, the need to less side effects products from natural sources and the constraints of present production technologies for pharmaceuticals, attention has recently been focused on investigation and screening of pharmaceutical anticancer compounds in plants. Indeed, plants have a long history of use in the treatment of cancer [1]. However, the undeniable role of diet containing plants should not be disregarded in the prevention of many diseases such as cancer.

Until recently, a number of biological active phytochemicals have been identified in plant foods [2]. Plant-derived compounds have been also an important source of several clinically useful anticancer agents, which include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel (taxol). Moreover, several promising new agents, including flavopiridol and combretastatin A4 phosphate, are in clinical development based on

selective activity against cancer-related molecular targets while some agents which failed in earlier clinical studies have been noticed again [3].

Shallot (*Allium ascalonicum*) is a member of the *Liliaceae* family, which has been used mainly as a spice traditionally from the ancient times. It has many different benefits including antibacterial and anti-fungal properties [4, 5], beneficial hematological influences [6], antioxidant properties [7], anti-*Helicobacter pylori* potential effect [8] and peroxynitrite-scavenging capacity [9] which have been studied until now. Besides, the chemical composition of *A. ascalonicum* was analyzed by other researchers, and some effective components such as a mannose-specific lectin [10], an anti-fungal peptide [11], new furostanol saponins [12], selenium and sulfur species [13], and various flavonol glucosides [14], inhibit proliferation and growth of tumor cell lines as HeLa and MCF-7 cell lines extract [15]. Cytotoxic effect of selenized odorless garlic and shallot against human leukemia cells (HL-60) [16], arresting cell cycle progression and inducing apoptosis in human cervical carcinoma HeLa cells [17] have been identified and isolated from it.

The aim of this study was to investigate anticancer and anti-inflammatory activities of the aqueous extract of *A. ascalonicum* which were performed using in vitro three cancer cell lines and an in vivo acetic acid-induced vascular permeability assay in mice.

Material and methods

Reagents

DMEM (Dulbeccos modified minimum essential medium), RPMI 1640 (Life Technologies, Grand Island, NY), fetal bovine serum (FBS), Trypan blue 0.4% (Gibco, New York, USA), LDH (Lactate dehydrogenase) cytotoxicity assay kit (Roch Chemical Co.), Penicillin/streptomycin, trypsin /EDTA (Sigma Chemical Co.).

Plant material

The bulbs of *A. ascalonicum* were prepared from local vegetable markets at Kermanshah (West of Iran), and authenticated by one of the authors (Dr. Ali Mostafaie).

Preparation of plant extract

Preparation of the aqueous extract of *A. ascalonicum* was performed as previously described [18]. In brief, the bulbs (1 kg) were grounded in a mortar with one liter of distilled water and stirred overnight to complete extraction. Then, it was filtrated through a cheese cloth and centrifuged at 16000 xg and 4°C for 30 min. After

complete extracting by distilled water and drying the extract by freeze dryer, it's the yield of which was about 27.4% as compared to original bulbs weight. Before use, it was weighed and dissolved in sterile phosphate buffered saline (PBS).

Cell culture

The cancer cell lines including Wehi164 (mouse fibrosarcoma cells), Jurkat (human-acute T-cell leukemia) and K562 (human erythroleukemia), and human umbilical vein endothelial cells (HUVEC) as a normal cell line were purchased from the National Cell Bank, Pasteur Institute of Iran.

Cell lines were seeded in 75-cm² tissue culture flasks and maintained in RPMI 1640 and Dulbecco's MEM supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. The medium was renewed every two days and the cell cultures were incubated at 37°C in a humidified atmosphere (95% air and 5% CO₂).

Cell viability inhibition assays

Cytotoxicity effects on cancer cell lines by *A. ascalonicum* were evaluated by Trypan blue exclusion assay and activity of lactate dehydrogenase (LDH) method.

Trypan blue assay

Cancer cell lines at a density of 5×10^4 per well were seeded in 24-well plates overnight. The day after, the medium was changed by fresh complete medium which contained increasing concentrations of *A. ascalonicum* (0, 25, 50, 100, 200 400, 600, 800, 1000, 1500, and 2000 µg/ml). At 24, 48 and 72 h of treatments, the cells were washed with PBS and harvested. Trypan blue dye was further added to the cell suspensions. The Trypan blue-staining cells were examined as damaged or dead cells.

LDH cytotoxicity assay

The cytotoxic effect of the aqueous extract of *A. ascalonicum* was also examined using LDH assay as described by Linford [19] with some modifications. Briefly, the cell lines were separately plated at a density of 5×10^3 per well in 96-well microplates with RPMI 1640-DMEM medium containing 10% FBS, and allowed to incubate overnight. After 24 h of early cell culture, the fresh medium with extract at concentrations of (0, 25, 50, 100, 200 400, 600, 800, 1000, 1500, and 2000 µg/ml) was renewed. At 72 h of treatments, the plates were centrifuged at 200 xg, and 100 µl of the media from each well was then transferred to new 96-well plates. Thereafter, 100 µl of LDH assay mixture was added to each well and plates were incubated at 37°C for 30 min. A group of wells

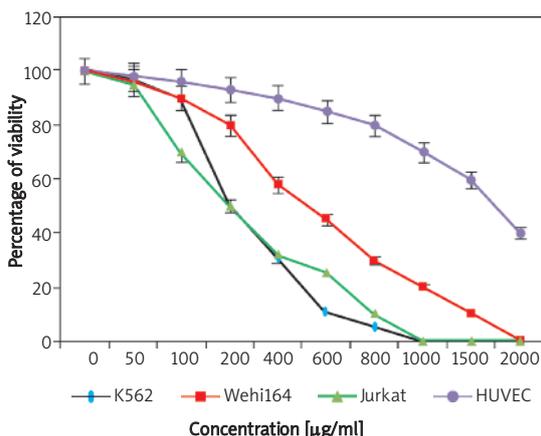


Figure 1. The cytotoxic effects of different concentrations of the aqueous extract of *A. ascalonicum* on K562, Jurkat and Wehi164, and HUVEC cell lines. The viability percentage of cell lines was assayed using Trypan blue 4% exclusion and LDH assays. Each data shown are one representative example of three independent experiments, expressed as a percentage of control and standard deviations were within 5% of all experimental values

was treated with 1% Triton X-100 solution for 45 min to maximum LDH release. The LDH release was estimated using a microplatereader at 495 nm according to the manufacturer's instructions. Tests were performed in triplicate. The mean cell viability was expressed as a percentage of the control.

Proliferation assay

The cell lines were plated in 24-well plates at a density of 5×10^4 cells/well in complete medium and incubated at 37°C under a humidified atmosphere containing 5% CO₂ for 24 h. The day after, the cells were treated with fresh medium containing 10% FBS and the determined concentrations of the extract (0, 25, 50, 100, 200, 400, 600, 800, 1000, 1500, and 2000 µg/ml). After 72 h, the treated and untreated cells were removed and counted against control wells using a Coulter counter (KX-21 Sysmex Co).

Acetic acid-induced vascular permeability in mice

For acetic acid-induced vascular permeability test, the Whittle's method [20] was used with somewhat modifications. Briefly, one hour after oral administration of the aqueous extract of *A. ascalonicum* (50-200 mg/kg), and Indomethacin (INN) (5 mg/ml); 0.1 ml/10 g body weight of 1% Evans blue solution was injected intravenously in each mouse. Thirty minutes later, 0.1 ml/10 g body weight of 0.7% acetic acid in saline was intra-

peritoneally injected for 30 min. Following the administration of acetic acid, the mice were killed by cervical dislocation and after extravasation of 10 ml of saline into the peritoneal cavity then, the washing solutions were collected in separated test tubes. Concentrations of Evans blue leaked into the peritoneal cavities were measured by the absorbance at 590 nm of the collected washing solutions. The vascular permeability was represented in terms of the absorbance (A590).

Statistical analysis

All measurements were evaluated statically expressed as mean \pm standard deviation. Results were analyzed by one way ANOVA. Values of $p < 0.05$ were considered significant. All experiments were performed in triplicate unless otherwise noted.

Results

Cytotoxicity effects of the aqueous extract of *Allium ascalonicum* on cancer cell lines and normal cell line

In this study, we evaluated the effects of the aqueous extract of *A. ascalonicum* on viability of the three cancer cell lines including K562, Wehi164 and Jurkat, and normal cell line (HUVEC) using Trypan blue and LDH assays evaluation. On the basis of obtained results, after 24, 48 and 72 h of incubation, the extract significantly reduced the viability of three cell lines at different concentrations compared with the control group, and these effects were stronger as time increased. On the other hand, the studied cytotoxicity treatments resulted in a dose and time-dependent manner. Furthermore, the observed results from effective concentrations of the extract were accompanied with differences for various cancer cell lines. In fact, and based on data of Figure 1, the cytotoxic effects of *A. ascalonicum* for Wehi164 cell line were considerably lower than those on K562 and Jurkat cell lines. As shown in Figure 1, the extract inhibited viability of K562 and Jurkat cell lines at 100 µg/ml significantly, while this effect for Wehi164 cell line occurred at higher concentrations (400 µg/ml). The IC₅₀ values of the *A. ascalonicum* extract (the concentration required for a 50% viability inhibition) for each cancer cell line have been shown in Table I. Furthermore, treatment of HUVECs as normal cell lines with the aqueous extract of the *A. ascalonicum* bulbs up to 1000 µg/ml or even higher concentrations for 72 h showed no considerable cytotoxic effect on the HUVECs. In fact, under the concentration of 1000 µg/ml, there was no significant difference in viability between the test and control wells. The IC₅₀ value of the extract for growth inhibition of the cells was determined above 1 mg/ml (Table I). The IC₅₀ values of the

A. ascalonicum extract (the concentration required for a 50% viability inhibition) for each cancer cell line have also been shown in Table I.

Aqueous extract of *Allium ascalonicum* induced suppression of cancer cell lines proliferation

The antiproliferative activity of the *A. ascalonicum* extract on the cancer cell lines was determined by the Coulter counter, the results of which are shown in Figure 2. After treatment with 25-200 µg/ml and higher concentrations of the extract for 72 h, the proliferation ratio of K562 and Jurkat cell lines decreased gradually as compared with controls. While, the extract could not inhibit the proliferation of Wehi164 cell line significantly at similar concentrations, unless they are higher than 400 µg/ml. These results showed that *A. ascalonicum* inhibited the proliferation of all cell lines in a dose-dependent manner. The GI₅₀ values of the *A. ascalonicum* extract (50% proliferation or growth inhibition) tested in these cancer cells were also different after 72 h treatment (Table I). Indeed, K562 and Jurkat cells showed the highest susceptibility (GI₅₀: 100 µg/ml), and Wehi164 cells displayed the lowest susceptibility to *A. ascalonicum* with GI₅₀ at 400 µg/ml.

We also examined the effects of the aqueous extract of *A. ascalonicum* on HUVECs proliferation. As the results showed (Figure 2), the extract inhibits proliferation of HUVECs in a dose-dependent manner. At concentrations up to 50 µg/ml the extract did not significantly decrease the number of cells, but at 100 µg/ml and at higher concentrations, it potently inhibited proliferation of HUVECs. Finally, the 50% proliferation inhibition (IC₅₀) of the aqueous extract of *A. ascalonicum* on HUVECs was estimated at 400 µg/ml.

Anti-inflammatory effect of the *Allium ascalonicum* extract

In order to assess an anti-inflammatory activity of the aqueous extract of *A. ascalonicum*, the acetic

Table I. IC₅₀ and GI₅₀ value data of the aqueous extract of *A. ascalonicum*, for K562, Jurkat and Wehi164, and HUVEC after 72 h of treatment

Cell line	GI ₅₀ [µg/ml]	IC ₅₀ [µg/ml]
K562	100 ±10	100 ±8
Jurkat	100 ±12	100 ±10
Wehi164	400 ±8	400 ±6
HUVEC	400 ±5	1600 ±8

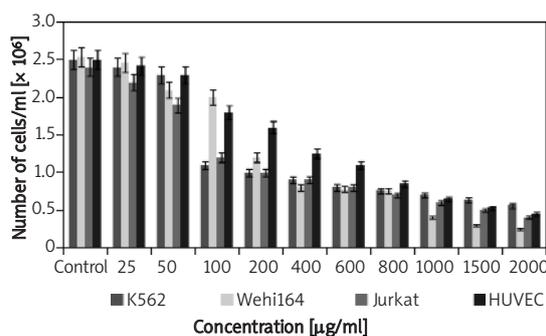


Figure 2. Inhibition of cancer and normal cell lines by the aqueous extract of *A. ascalonicum* after 72 h. The cells were counted by the Coulter Counter (Sysmex KX21). Each column represents mean ± S.E. of three independent experiments
**p* < 0.04, compared with the control group

acid-induced vascular permeability model was used. In this regard, mediators of inflammation released following stimulation, leads to dilation of arterioles and venules and increased vascular permeability [21]. The extract at the oral doses of 25, 50, 100 and 200 mg/kg showed an inhibition in vascular permeability and this activity was increasing in the dose-dependent manner (Table II). On the other hand, the *A. ascalonicum* extract at the oral doses of 25, 50, 100 and 200 mg/kg showed an inhibition of 10.2%, 21.4%, 38.3% and 80.1% in vascular permeability assay, respectively. In turn, the Indomethacin (INN) could cause 35.4% inhibition of vascular permeability (Table II). From this

Table II. Effect of the *A. ascalonicum* extract on acetic acid-induced vascular permeability in mice. The results are expressed as mean ± S.E. of *n* = 5. Inhibition percentages were calculated regarding the control treated with saline only. Indomethacin (INN) was chosen as a positive control. *P* < 0.05 compared with the control group

Group	Dose [mg/kg, p.o.]	A590	Inhibition (%)
<i>A. ascalonicum</i>	50	2.56 ±0.14	10.2
	100	2.24 ±0.18	21.4
	200	1.76 ±0.14	38.3
	400	0.57 ±0.11	80.1
INN	5	1.84 ±0.04	35.4
Control	–	2.85 ±0.08	–

observation, it is assumed that an anti-inflammatory activity of the *A. ascalonicum* extract results from its protection on the release of inflammatory mediators at the first stage.

Discussion

Diet plays a major role in cancer etiology and prevention. Although inconsistencies exist across studies that have investigated the relationship between diet and cancer, dietary factors undoubtedly influence cancer risk [22]. There is increasing evidence that fruits and vegetables have chemopreventive properties because of the supplement and synergistic effects of various phytochemicals present in these nourishments [23-26].

From the other point of view, herbs and spices are generally considered safe and proved to be effective against various human ailments and their medicinal uses have increased gradually in many countries. In this regard, to assess the anticancer properties of plants which have been widely consumed in a human diet, the use of vegetable extracts provide an interesting approach, because these extracts contain several bioactive molecules [27-29].

Allium ascalonicum is one of the important *Allium* species commonly used in many Asian diets and in traditional medicine since ancient times. A lot of evidence also suggested that *Allium* genus possesses anticancer properties as shown by their ability to suppress tumor proliferation *in vivo* and *in vitro* [30-32]. In spite of wide consumption of *A. ascalonicum*, reports regarding its biological effects are rarely compared to other *Allium* species such as garlic and onion. *A. ascalonicum* is usually known for hypocholesterolemic, antimicrobial, antidermatophytic, and anti-angiogenic properties [5, 8, 33-36].

Allium ascalonicum has been also reported to exhibit antioxidative and free radical scavenging capacities. These properties appear to be related to the high contents of flavone, sulfur-containing compounds, and polyphenolic derivatives in the bulb of *A. ascalonicum*. Furthermore, it is shown that the antioxidant potential of *A. ascalonicum* comparing several onion varieties and some garlic preparations is more noticeable [7, 37].

Furthermore, researchers demonstrated that it can be useful to protect against oxidant-induced various illness conditions, including as an adjunct therapy in patients suffering nephrotoxicity from consuming CsA [38]. In previous studies, we could show that *A. ascalonicum* exhibits significant anti-angiogenic effects on *in vitro*, *ex vivo* and also *in vivo* assays without the toxic effect on the endothelial cells. Finally, we have demonstrated that heat and acid conditions had no effect on its anti-angiogenic activity [18, 39].

The present study was performed to examine the possible potential of the *A. ascalonicum* extract on anticancer and anti-inflammatory activities. For this purpose, we examined *in vitro* antiproliferative and cytotoxic effects of the *A. ascalonicum* extract against three well known cell lines including Wehi164, K562 and Jurkat and HUVEC as a normal cell line. The findings showed that *A. ascalonicum*, which contains many benefit phytochemicals; significantly affects the viability of all of the cancer cell lines in different IC_{50} values as shown in Table I.

To examine the influence of the aqueous extract of *A. ascalonicum* on cancer cell proliferation further; the cells were treated with increasing extract concentrations for 72 h. The obtained data of counting by a Coulter counter showed that *A. ascalonicum* induced inhibition of the cell proliferation, resulting in a considerable reduction of cell growth with dose increasing against the control group. The 50% inhibitory concentrations of *A. ascalonicum* for the above-mentioned cell lines, which significantly resulted in restraint of their growth, were estimated (Table I). In agreement with 50% inhibition of cell proliferation (the concentration of the extract that is required for half-maximal inhibition), the order of sensitivity of the cell lines to extract was K562, Jurkat > Wehi164 with GI_{50} of 100 and 400 $\mu\text{g/ml}$, respectively (Table I). Meanwhile, the results showed that the proliferation of HUVEC cells was inhibited by the aqueous extract of *A. ascalonicum* with GI_{50} about 400 $\mu\text{g/ml}$. In general, K562, Jurkat and Wehi164 cell lines exhibited both decreased viability and proliferation in a dose- and time-dependent manner under experimental conditions. These results further support previous observations concerning inhibition of cell proliferation by shallot on tumor cell lines, which showed that treatment of HeLa and MCF-7 cell lines with different concentrations of the chloroformic extract of shallot resulted in decreased growth in a dose-dependent manner [15]. Finally and on the basis of this investigation, we found that *A. ascalonicum* had significant antiproliferative effects on Wehi164 representing solid, and K562 and Jurkat cell lines as soft tumor cell lines in comparison with HUVECs as a normal cell.

Inflammation is a healthful response to offense or injury and an important part of innate immunity. Although inflammation acts as an adaptive host defense against infection or injury and is primarily a self-limiting process, inadequate resolution of inflammatory responses often leads to various chronic ailments including cancer [40, 41]. On the other side, chronic or recurrent acute inflammation, as a result of infectious agents or other sources, has potential promotional functions in each of tumor initiation, progression, invasion, and metastasis phases [42, 43]. Besides, most solid

tumors are known to exhibit highly enhanced vascular permeability, similar to or more than the inflammatory tissues. Cancer and inflammatory tissues have various common vascular mediators, such as NO, BK and PGs, and their most predominant physiological effect is vascular permeability enhancement. Enhanced vascular permeability of tumor and inflammatory tissue is the key to be considered for future drug development for more selective targeting for the desired tumors or inflammatory lesions, based on enhanced permeability and retention (EPR) effect using macromolecular drugs [44-46].

Acetic acid-induced vascular permeability causes an immediate reaction which is lasting a long period of time over 24 h and arrives to infiltration of fluid rich in plasma proteins [20, 47, 48]. It is therefore used as a well-characterized mouse model of acute inflammation. Thus, inhibition of vascular permeability is considered a major feature for the suppression of the exudative phase of acute inflammation [49]. In the present study, in the well-known in vivo acetic acid-induced vascular permeability model, we observed that the *A. ascalonicum* extract shows significant anti-inflammatory activity at a dose-response correlation with extract increasing concentrations. However, the aqueous extract of *A. ascalonicum* at low doses had no considerable effects on prevention of vascular permeability of acetic acid; it showed meaningful differences comparing the inhibitory effect of INN as a common drug in treating the inflammation status. These findings suggest that the significant anti-inflammatory activity of *A. ascalonicum* may be due to affecting membrane-stabilization to reduce vascular permeability or through inhibition of various inflammatory mediators.

Regarding our and previous findings of beneficial properties of *A. ascalonicum*, particularly for prevention and treating of cancer and inflammation situations, further studies are needed to clarify the responsible compounds and their possible mechanisms in this regard.

In conclusion, the aqueous extract of *A. ascalonicum* bulbs had the most cytotoxic activity on cancer cell lines Jurkat and K562 against Wehi164 with lower cytotoxic preference. Treatment of HUVECs with the aqueous extract of the *A. ascalonicum* bulbs up to 1000 µg/ml or even higher concentrations for 72 h showed no considerable cytotoxic effect on the HUVECs but the extract inhibits proliferation of HUVECs in a dose-dependent manner. It is of interest that the extract of this plant has showed much less cytotoxicity against the normal cell line, and, if this also occurs in vivo, the use of this plant clinically for the treatment of cancer patients would have

some scientific support. Moreover, because of anti-inflammatory activity in vivo, it would be regarded as an excellent candidate for the development of an anti-inflammatory drug with fewer side effects, which needs to be more investigated.

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