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ORIGINAL RESEARCH

Antiproliferative activity of chloroformic extract of Persian Shallot, *Allium hirtifolium*, on tumor cell lines

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Abstract *Allium hirtifolim* (Persian Shallot) belongs to *Allium* genus (*Alliaceae* family). We investigated the in vitro effects of chloroformic extract of *A. hirtifolium* and its Allicin on the proliferation of HeLa (cervical cancer), MCF7 (human, caucasion, breast, adenocarcinoma) and L929 (mouse, C3H/An, connective) cell lines. Our results showed that components of *A. hirtifolium* might inhibit proliferation of tumor cell lines. This inhibition in HeLa and MCF-7 cells was dose-dependent. The presence of Allicin was evaluated by TLC method in bulbs and the extract of *A. hirtifolium* was analyzed by HPLC. MTT test was performed 24, 48 and 72 h after cell culture. A significant decrease in cell lines was observed in HeLa and MCF-7 as compared to L929 cell lines. DNA fragmentation analysis revealed a large number of apoptotic cells in treated HeLa and MCF-7 cell groups, but no effects in L929 cells. Therefore *A. hirtifolium* might be a candidate for tumor suppression.

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Abbreviations

DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate-buffered saline

Introduction

Antibacterial, antifungal, antiviral, antiprotozoal, and antihelmintic properties of *Allium* genus have been reported recently (Cavallito and Bailey 1944; Ariga and Seki 2006; Ankri and Mirelman 1999; Yamada and Azuma 1977; Moore and Atkins 1977; Taran et al. 2006). It is believed Alliums can treat diabetes, arthritis, colds and flu, stress, fever, coughs, headache, hemorrhoids, asthma, arteriosclerosis, cancer, rheumatic and inflammatory disorders (Eidi et al. 2006; Abdou et al. 1972; Lawson 1996; Bordia et al. 1997; Kojuri and Vosoughi 2007; Hirsch et al. 2000). In the invading organisms and microorganisms, *Allium* genus extracts were shown to decrease the oxygen uptake, reduce the growth of the organism, inhibit the synthesis of lipids, proteins, and nucleic acids and damage the membranes (Borek 2001; Dirsch and Kiemer 1998).

Analysis of steam distillations of crushed garlic cloves performed over a century ago showed a variety

of allyl sulfides (Lee et al. 2003). Allicin (diallyl thiosulfinate) was isolated and identified. It is responsible for the remarkable antibacterial activity of crushed garlic cloves (Cavallito and Bailey 1944). Alliin was found to be the stable precursor that was converted to Allicin by the action of allinase which was more than 10% of the total protein content in garlic (Baghalian et al. 2005). Allicin was reported to possess diverse biological actions such as antimicrobial, antiparasite, and antifungal activities. It has been found that lipid peroxidation is inhibited and OH is scavenged (Borek 2001; Lawson and Hughes 1992).

Garlic consumption reduces the risk of cancer, as its extract blocks effectively induced tumors in skin, breast, uterine cervix, and colon (Oommen and Anto 2004; Vainio and Weiderpass 2006). Aqueous garlic extract might exert its chemopreventive effect by inducing apoptosis (Balasenthil and Rao 2002). Many organosulfur compounds, the major active principles in garlic, inhibit the proliferation of cancer cells, and some of them cause apoptosis in tumor cells of different tissue origin (Kwon and Moon 2005; Thatte and Bagadey 2000; Arditti and Rabinkov 2005; Jakubikova and Sedlak 2006). Hence, apoptosis could be a potential general mechanism providing a mechanistic basis for the anticarcinogenic activities of individual garlic components, although the actual mechanism is not known (Thatte and Bagadey 2000).

Allium hirtifolium with the common Persian name of 'Moosir', a native edible plant in Iran, has been widely used as medicine and condiment predates. It belongs to the same biological genus as *Allium sativum* (garlic) and other onions (Mozaffarian 1996). Therefore, the aim of this study was to investigate the anticancer effects of *Allium hirtifolium*.

Materials and methods

Reagents

Allicin (standard) was obtained from Nopex Company as Allisure™ (Nopex, England). Fetal calf serum (FCS) was obtained from Gibco BRL (Grand Island, NY, USA). RPMI 1640, Ampicillin, MTT (3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide), Trypsin, and other reagents were obtained from Sigma (St. Louis, MO, USA).

Samples of *A. hirtifolium*

The bulbs of *A. hirtifolium* were collected from Isfahan province, Iran in June 2006. The plant specimen was identified by The Iranian Research Institute of Forests and Rangelands, Tehran, Iran.

Preparation of chloroformic extract of *A. hirtifolium* for cell culture

The bulbs of *A. hirtifolium* were washed with tap water and cut into small slices. The slices were powdered after air-drying. Then 100 g of powder was added to 1 l chloroform and the mixture kept for 24 h at 10°C. It was filtered through cotton cloth and evaporated in a rotating evaporator under reduced pressure until completely dried. The dried extract was preserved at 4°C and added to RPMI medium up to a final concentration of 200 μg ml^{-1}. For preparation of Allicin, RPMI was used up to a final concentration of 18 μg ml^{-1}.

Analysis of Allicin by thin layer chromatography method (TLC)

The presence of Allicin was evaluated in bulbs by TLC. Chloroformic extract of *A. hirtifolium* was tentatively identified on silica gel GF254 pre-coated plate. Pure Allicin was used as a control for comparison. Briefly, the toluene–ethyl acetate (100:30) was used as mobile phase and UV detection was performed at 254 nm and Rf value was calculated by Vanilln–glacial acetic reagent (Sreevidya and Mehrotra 2003). Total thiosulfinates and Allicin contents of *A. hirtifolium* bulbs were determined using a spectrophotometric method (You et al. 1989; Miron et al. 1998; Miron et al. 2002; Ogra and Suzaki 2005).

Reversed-phase HPLC was a method of choice for detection and quantitation of Allicin in *A. hirtifolium* extract. The HPLC Reversed-phase HPLC/UV (methanol/water/formic acid (40:60:0.1), λ: 240 nm, flow rate: 0.9 $mlmin^{-1}$) was used (Lawson and Wood 1991).

Cell culture

The human cervical carcinoma cell line (HeLa, NCBI-115), estrogen receptor—positive human breast cancer cell line (MCF-7 NCBI C135) and

mouse fibroblast cells (L929 NCBI C161) were purchased from the National Cell Bank, Pasteur Institute of Iran. All the cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS serum, streptomycin (100 μg ml^{-1}) and penicillin (100 IU ml^{-1}) in culture flasks at 37°C in 5% humidified CO_2 incubator. The cells were fed until confluence (2×10^6) and expanded by trypsinization and subculture at lower numbers in new culture flasks.

The cultured cells were treated with Allicin and *A. hirtifolium* extract and examined their effects in different concentrations. The cells were evaluated after 24, 48 and 72 h of the treatment.

DNA fragmentation analysis

The DNA from cultivated cells was isolated (Herrmann and Lorenz 1994). In brief, cells (2×10^6) were treated with the extract and Allicin then collected by centrifugation (2,000 *g*, 10 min). The pellet was resuspended in 0.5 ml DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris–HCl, pH = 8.5). The lysate was incubated with 0.1 mg ml^{-1} proteinase k and then incubated for 4 h at 37°C. The DNA was precipitated with 70% ethanol after addition of isopropanol. The suspension was centrifuged and DNA treated with RNase A in 10 mM Tris–HCl (pH = 7.5) at 37°C for 18 h. The samples were analyzed by electrophoresis on a 1.5% agarose gel and assessed under UV illumination.

Morphological changes of cells after treatment

After treating of cells with chloroformic extract and Allicin, shrinkage, detachment, and colony forming of cells were evaluated under inverted microscope.

Evaluation of cultured cells by MTT colorimetric method

A colorimetric assay using MTT was performed (Mosmann 1983). Cells seeded in 96-well microplates (5,000 cells $well^{-1}$ 200 μl^{-1}) and routinely cultured in a humidified incubator for 24 h. The medium was aspirated off after a 24 h pre-culture and exchanged for medium containing *A. hirtifolium* extract and Allicin at various concentrations ranging from 0 to 44 μg ml^{-1} and 0 to 0.44 μg ml^{-1}, respectively. Cells were then re-incubated for 24, 48 and 72 h. A control group (RPMI without extract) and a blank group (without cells or medium) were also included. This assay was performed in triplicate. The medium replaced with 100 μl of medium containing MTT solution (0.5 $mgml^{-1}$ in RPMI). Cells were re-incubated for an additional 2 h. Then after addition of 0.15 ml DMSO, the plates were shook for 10 min to dissolve the formazan crystals. Then, optical density of 96-well culture plates was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% viability. The obtained optical densities from the treated wells were converted to a percentage of living cells (cell survival rate) against the control using the following formula:

$$\text{Absorbance of treated cells in the each well} \times 100/\text{Mean absorbance of control cells}$$

Statistical analysis

Computer program (Graph Pad Prism) was used to calculate the IC50 (50% inhibition of cell proliferation) values. Student's *t*-test was used for statistical analyses while *P*-values < 0.05 were considered to be statistically significant.

Results and discussion

Allicin was identified by observing the characteristic violet-brown zone. The Rf of Allicin was determined 0.6 based on TLC experiment. In *A. hirtifolium* chloroformic extract, Allicin was quantified in the amount of 3.4 ± 0.1 mg g^{-1}.

Shrinkage, granulation of cytoplasm and detachment were observed in treated cells with chloroformic extract of *A. hirtifolium* and Allicin. Particularly in higher concentrations of extract and Allicin and longer period of incubation the number of colony forming cells was decreased. Most sensitive cells were HeLa and MCF-7 cell lines, respectively (Fig. 1). The inhibitory effect of chloroformic extract of *A. hirtifolium* was stronger than Allicin. The growth of L929 cells was suppressed only in high doses of *A. hirtifolium* and Allicin (200 μg ml^{-1} and 18 μg ml^{-1}, respectively).

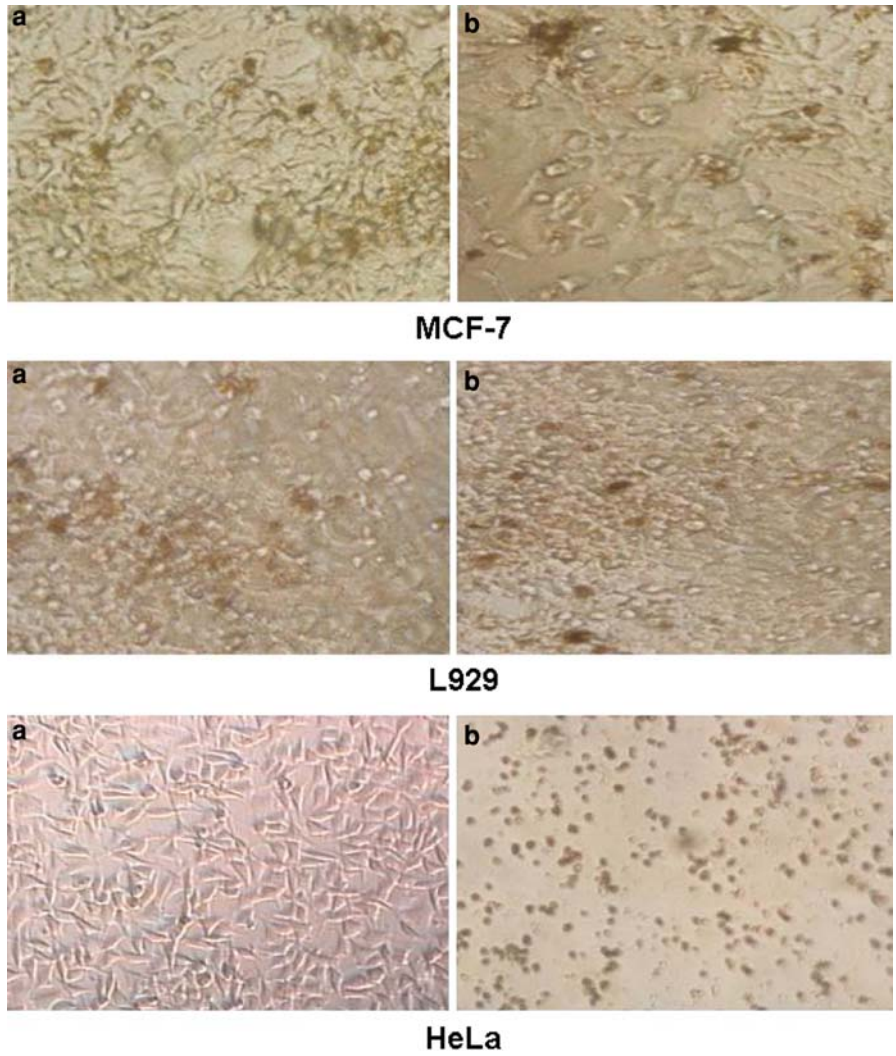

Fig. 1 Morphological changes of L929, MCF-7, and HeLa cells after treating with *A. hirtifulium* extract for 72 h. Shrinkage and detachment effects were visible in treated cells (**a**, untreated; **b**, treated cells)

Fifty percent inhibition of cells occurred with 20 μg ml^{-1} *A. hirtifolium* and 0.15 μg ml^{-1} Allicin in Hela cells, 24 μg ml^{-1} *A. hirtifolium* and 0.20 μg ml^{-1} Allicin in MCF-7 cells and 250 μg ml^{-1} *A. hirtifolium* and 1.25 μg ml^{-1} Allicin in L929 cells. The IC50 values of *A. hirtifolium* extract and Allicin for HeLa and MCF-7 and L929 cell lines are shown in Table 1. At the highest concentration, the inhibitory rate of *A. hirtifolium* on HeLa cell was 100% while that of Allicin was 94%.

The first MTT assay performed after 24 h. The cell viability had an inverse correlation with extract concentration. In contrast, viability of L929 cells increased in higher extract concentrations. There was significant difference in percent of surviving cells between consecutive days (day 1, 2, and 3) for both HeLa and MCF-7 cells ($P < 0.05$) as shown in Fig. 2.

HeLa and MCF-7 cell lines exhibited decreased growth in a dose-dependent manner under experimental condition used in the 72 h treatment, According to 50% inhibition of cell proliferation (IC50), the order of sensitivity of the cell lines to this extract was HeLa > MCF-7 > L929 cells.

Incubation of MCF-7 and HeLa cells with extract and Allicin showed typical DNA ladder by electrophoresis after 72 h. We did not find the DNA ladder shape in L929 cells (Fig. 3).

Several pieces of evidences suggested that *Allium* genus possess anticancer properties as shown by their ability to suppress tumor proliferation in vivo and in vitro (Ahmed et al. 2001; Miron et al. 2002;

Table 1 Growth-inhibitory effects of Allicin and *A. hirtifulium* extract on HeLa, MCF-7, and L929 cells

Cell line	IC50 Allicin (μg ml^{-1})	IC50 *A. hirtifulium* (μg ml^{-1})
HeLa	0.15	20
MCF-7	0.20	24
L929	1.25	250

The cells were incubated with various concentrations of Allicin and *A. hirtifulium* extract in RPMI medium for 72 h. Cell proliferation was measured by the MTT reduction assay. The IC50 value was the estimated concentration that results in 50% inhibition of cell proliferation under the specified experimental conditions

Zhang et al. 2007). It was shown that concentration and duration of the exposure to allyl sulfides increased the antiproliferative effects. In our study the amount of Allicin formed from *A. hirtifolium* was 3.4 ± 0.1 mg g^{-1} and the theoretical amount of Allicin formed from garlic is reported as 3.5 mg g^{-1} (Lawson and Wood 1991).

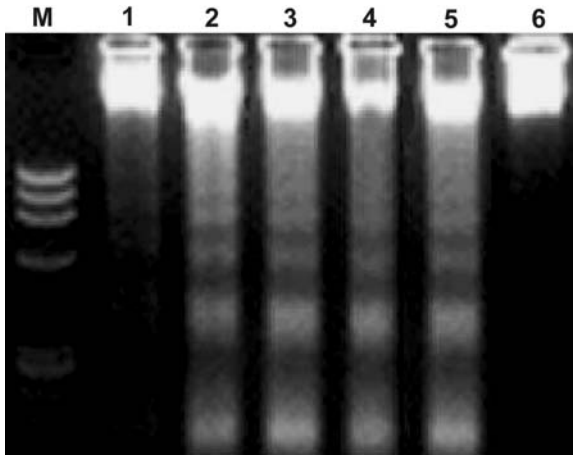

Fig. 3 Agarose gel electrophoresis of DNA extracted from cells treated with *A. hirtifolium* (44 μg ml^{-1}) and Allicin (0.44 μg ml^{-1}). Lane M: DNA marker; 100 bp, Fermentas. DNA laddering, typical for apoptotic cells, was visible for treated HeLa with *A. hirtifolium* (lane 2), HeLa with Allicin (lane 3), MCF−7 cells with *A. hirtifolium* (lane 4), and MCF-7 with Allicin (lane 5), but it was not found for L929 cells with *A. hirtifolium* and Allicin, respectively (lane 1, 6)

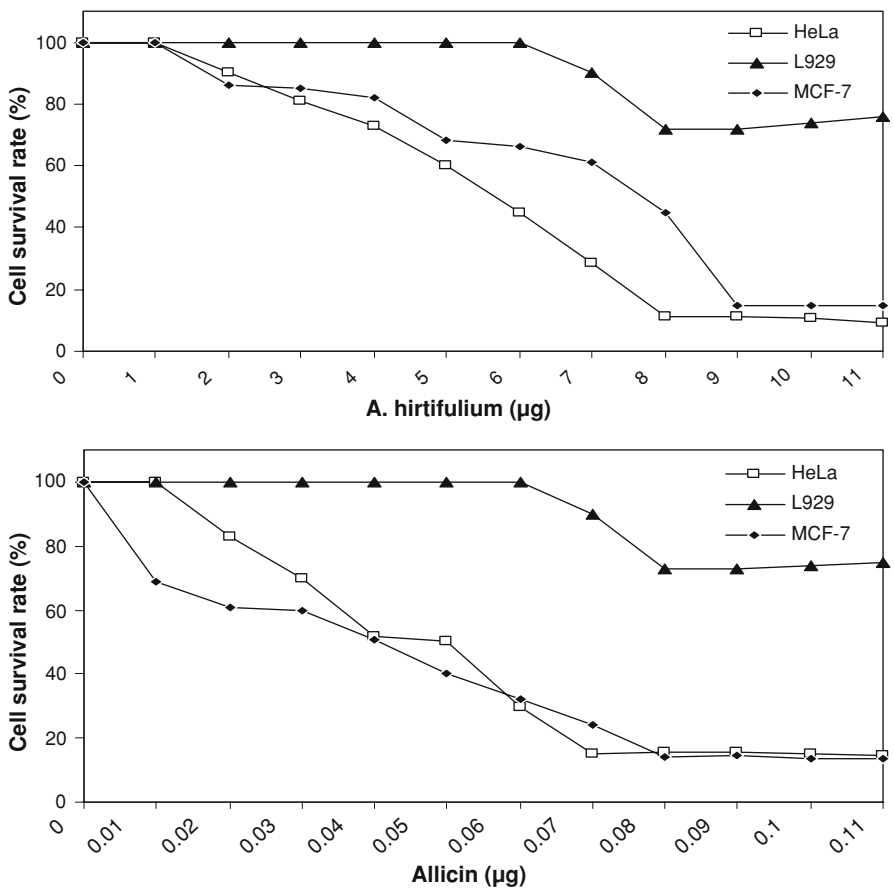

Fig. 2 The effect of the extent of confluence on cell survival after treatment with Allicin and *A. hirtifulium* extract. The cells were seeded on a 96-well plate. The cells were then treated with Allicin and *A. hirtifulium* for 72 h and cell survival was measured by MTT assay. Percentage of surviving HeLA and MCF-7 cells in all concentrations of Allicin and *A. hirtifulium* extract was significantly decreased when compared with L929 cells ($P < 0.001$). *A. hirtifolium* and Allicin were shown as μg per each well microplate. Each point represented the mean ± SD ($n = 3$)

This antineoplastic effect was greater for lipid-soluble than for water-soluble allyl sulfides (Rabinkov et al. 1998; Tattelman 2005). Diallyl disulfide suppresses the growth of human colon tumor cell xenografts in athymic nude mice (Miron et al. 2003; Zhang et al. 2007).

Allium hirtifolium (Moosir) belongs to the same biological genus as *Allium sativum* (garlic) and other onions. This species is a native plant in Iran that its antiproliferative effects have received little attention (Taran et al. 2006). No previous literature was found to show the effectiveness of chloroformic extract of *A. hirtifolium* on HeLa, MCF-7, and L929 cells.

In the present study we assessed the antiproliferative effect of *A. hirtifolium* extract on HeLa, MCF-7, and L929 cell lines by MTT method and the DNA fragmentation analysis. This investigation clearly showed a cell growth inhibition on cancer HeLa and MCF-7 cell lines at concentrations about 44 μg ml^{-1} of *A. hirtifolium* extract. With respect to the cell lines studied, IC_{50} values varied from 20 μg ml^{-1} in HeLa to 24 μg ml^{-1} in MCF-7. In contrast, *A. hirtifolium* extract had less effect on a normal cell (L929) (Table 1).

The effect of *A. hirtifolium* on these cells started from 24 h and became more prominent in 48 and 72 h. Therefore, maximum morphologic changes and antiproliferative effect appeared after 72 h. In MTT assay, the statistical analysis indicated that *A. hirtifolium* extract significantly inhibited the proliferation of HeLa and MCF-7 cells. But this inhibition could not occur in non-tumor L929 cells. Interestingly the *A. hirtifolium* extract affected the tumor cells much stronger than Allicin.

The results showed that *A. hirtifolium* could inhibit proliferation of tumor cell lines in a dose-dependent manner. The inhibitory effect on tumor cell lines was 25 times stronger than that in normal cells. Therefore, our results provided important insights into the use of *A. hirtifolium* as an additive to food or as a drug without any side effects.

The present study was the first report to provide evidence of *A. hirtifolium* activity on tumor cell lines. Further investigations were needed to elucidate subcellular mechanisms involved in the suppression of growth in tumor cell lines.

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