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Ethanolic extract of *Allium sativum* has antiproliferative effect on Hep2 and L929 cell lines

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ABSTRACT: Cancer of the larynx represents worldwide approximately 1-2% of all cancers and generally occurs predominantly in males. Experimentally, *Allium sativum* and its associated sulfur components are reported to suppress tumor incidence in breast, colon, skin, uterine, esophagus and lung. In this study, we investigated *in vitro* effect of ethanolic extract of *A. sativum* on human larynx tumor cell (Hep2) and non-tumor cell (L929) lines. The cells were cultivated and incubated with different concentrations (0 (control), 0.5, 1, 2, 4, 8 and 12 mg/ml) of the whole extract. After 24h, morphological changes in both cells showed growth inhibitory effects at concentrations of 4, 8 and 12 mg/ml of the extract. These changes became more prominent on the second and third day. MTT assay results indicated a significant difference between Hep2 and L929 cells incubated in all concentrations and the cells incubated in the absence of the extract in reduction of percentage of surviving cells. The results of this study demonstrate that this type of *A. sativum* extract exert anti-proliferative effect on both Hep2 and L929 cell lines.

KEYWORDS: *Allium sativum*, Ethanoli extract, Antiproliferative, Hep2, L929, MTT

INTRODUCTION

Cancer of the larynx represents worldwide approximately 1-2% of all cancers and generally occurs predominantly in males (1). Almost all laryngeal cancers (90-95%) are squamous cell carcinoma (SCC) (2). It has been clearly demonstrated that this disease is strongly associated with alcohol and tobacco consumption (3).

Garlic (*Allium sativum*) is used for medicinal purposes for more than 3000 years (4). Experimentally, *A. sativum* and its associated sulfur components are reported to suppress tumor incidence in breast, colon, skin, uterine, esophagus and lung (5). The antitumor effect of garlic components is a consequence of their direct cytostatic or cytotoxic properties as shown using different tumor models *in vivo* or on tumor cell line *in vitro* (6). When *A. sativum* is cut, chopped or crushed, the cloves membrane is disrupted and S-allylcysteinesulfoxide (alliine) is transformed enzymatically into allicin by alliinase (7). Allicine inhibit the proliferation of several human malignant cells. Ajoene is *A. ativum* derived compound produced most efficiently from pure allicin and has the advantage of greater chemical stability than allicin. Recently, ajoene was shown to inhibit proliferation and induce apoptosis of human leukemia CD34-negative cells (8). No reports are available on the effects of ethanolic extract of *A. sativum* on SCC. Our present study was therefore designed to examine the effect of this extract on the *in vitro* proliferation of Hep2 cells (SCC, type2), originated from laryngeal carcinoma. In addition, we chose L929 cell line as non-tumor cell, for comparison of the effect of extract on both cell lines.

MATERIAL AND METHODS

Preparation of ethanolic extract

Fresh whole plants of *A. sativum* were collected from a farm in Hamedan, Iran. The cloves of plant were crushed by knife and ethanolic extract was prepared by 50 g crushed cloves and 400 ml 70° ethanol in Soxhelet set for 17h. The extract was dried using a

Buchi evaporator and water bath. The solid extract was saluted in 200 ml distilled water and sterilized using 0.2-µm filter. 10 ml of sterile extract was transferred to tarred dishes and dried on a water bath and finally in a vacuum over at 40ºC. The solid sterile extract was weighed. Concentration of sterile extract was 52 mg/ml.

Morphologic examinations

Human larynx carcinoma cell line (Hep2) and mouse fibroblast cell line (L929) were provided from National Cell Bank of Iran (NCBI). Cells were cultivated in a 1:1 mixture of Dulbeccos Modified Eagles Medium (DMEM), supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution (9). The cultured were humidified at 37ºC and 5% CO_2 atmosphere and fed 2-3 times a week until they approached confluence (9,10). Then, cell aggregates were treated with 0.25% trypsin-EDTA solutions and mechanically dispersed using a 10 ml pipette. Trypsin activity was inhibited by adding growth medium and cells were centrifuged at 1000 RPM for 5 min. Supernatant was removed and pellet of each cells was seeded into seven 50 cm^2-flasks, each flask containing 5×10^5 cells. Under these conditions cell viability was found to be higher than 95% by trypan blue exclusion testing. After 24h, both cells were incubated by different concentrations (0 (control), 0.5, 1, 2, 4, 8 and 12 mg/ml) of the extract into the flasks. Cells were observed under light inverted microscope for shape, granulation and suspension (anchorage independency) from 24 to 72h. Morphologic examination was repeated 3 times for insure (10,11).

MTT assay

The effect of ethanolic extract of *A. sativum* on Hep2 and L929 cells proliferation was determined using 3-(4, 5- dimethyl thiazol -2-yl) -2, 5- diphenyl tetrazolium bromide (MTT) colorimetric assay, originally described by Mosman (1983) and modified by Alley et al (1988). For this examination, 96-well plates were prepared in this way: 21 (3 × 7) wells of each plate were selected and Hep2 cells were seeded in the plates (3×10^3 cells/well) while L929s cells were cultivated in another 21 (3 × 7) wells. The cultures were humidified at 37ºC and 5% CO_2 atmosphere for 24h. At the end of this procedure, culture medium of each plate was changed by fresh culture medium. Then the extract was added to growth medium at concentrations of 0 (control), 0.5, 1, 2, 4, 8 and 12 mg/ml in the way that each 3 well were incubated with one of these concentrations. After 24h, one plate was selected and cell proliferation was determined using MTT assay. For each 200 µl growth medium, 20 µl MTT (Sigma, Missouri, USA) solution was added and incubated for 4h (9). After all growth medium had been removed, crystal particles of MTT were dissolved by adding 200 µl Dimethylsulfoxide (DMSO) and 25 µl glycin and shaking plates for 2-3 min. The absorbance of formazan dye was read at 570 nm using ELISA plate reader (4,10). The whole procedure was repeated in the same manner for second and third plates after 48 and 72h.

Statistical analysis

Data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests for significant differences (HSD). The values were considered statistically significant if $P<0.05$.

RESULTS

Morphologic changes

After 24h, Hep2 and also L929 cells with low concentrations (0.5 and 1 mg/ml) of ethanolic extract of the *A. sativum* in the sense of morphology were very similar to the control (cells incubated in the absence of the extract). Morphological effects of the extract on both cells started at concentration of 2 mg/ml. A complete response from the extract was seen on both cells at concentrations of 4, 8 and 12 mg/ml, so that cells appeared as round and suspended with increase granulation of cytoplasm when compared to the control. These changes became more prominent on second and third day. The effect of ethanolic extract of *A. sativum* on cultured cells is shown in figure 1.

MTT assay results

Optical density (OD) of Hep2 and L929 cells incubated in the presence of the different concentrations of *A. sativum* extract was compared to the cells incubated in the absence of the extract and percentage of surviving cells was determined by the following formula:

$$\textit{Surviving cells (\%) in compared to the controls} = \frac{\textit{OD of cells in each well} \times 100}{\textit{Mean OD of controls cells}}$$

After 24h, percentage of surviving Hep2 and L929 cells in all concentrations of the extract showed significant decrease as compared to the control cells ($P<0.05$) and there was not a correlation between increase in the extract concentration and decrease of surviving Hep2 ($r=-0.003$) and L929 ($r=0.126$) cells. After 48h, decrease of surviving Hep2 cells was more than L929 cells. Also percentage of surviving Hep2 and L929 cells in all concentrations of the extract showed significant

decrease when compared with the control cells (P<0.001). These changes became more prominent in third day, so that percentage of surviving Hep2 and L929 cells in all concentrations of the extract showed significant decrease when compared with control cells (P<0.001). But there was not a correlation between increase in the extract concentration and decrease of surviving Hep2 (r=-0.459) and L929 (r=0.383) cells. Effect of the *Allium sativum* extract on percent of surviving Hep2 and L929 cells after 72h is shown in figure 2.

DISCUSSION

No previous literature was found showing the effectiveness of ethanolic extract of *A. sativum* on Hep2 and L929 cells. Our data demonstrated that Hep2 and L929 cell proliferation is influenced by this extract. Effect of the extract started from 24h and became more prominent in 48h and 72h, so that maximum morphologic changes and antiproliferative effect appeared after 72h. In MTT assay, statistical analysis indicated that the extract significantly inhibited the proliferation of both cells in all concentrations. In microscopic examinations, morphological changes in both cells started from 2 mg/ml and there was a correlation between increase of the extract concentration and morphologic changes, therefore, maximum morphologic changes appeared at 12 mg/ml for both cells. These results demonstrated that ethanolic extract of *A. sativum* can inhibit growth of human larynx tumor cell lines *in vitro*. But this inhibition can also occur in non-tumor L929 cells. These properties may arise from several mechanisms including inhibition of gene mutation, suppressing the formation of DNA-adducts, change of enzyme activity or induction of apoptosis (7). It has been reported that *A. sativum* induces apoptosis during 7, 12-dimethylbenz (a)anthracene-induced hamster buccal pouch carcinogenesis (12) and garlic lectin-induced apoptosis in human tumor cells (13). Also, research of the past two decades has consistently shown that start of cancer is related to genetic damage rate in somatic cells. Administration of *A. sativum* can prevent carcinogen-induced genetic damage (14).

Fig 1: Effects of ethanolic extract of Allium sativum on Hep2 and L929 cells morphology after 72h. A: control Hep2 cells (×40), B: Hep2 cells in 4 mg/ml (×40), C: Hep2 cells in 12 mg/ml (×10), D: control L929 cells (×20), E: L929 cells in 4 mg/ml (×20), F: L929 cells in 12 mg/ml (×40).

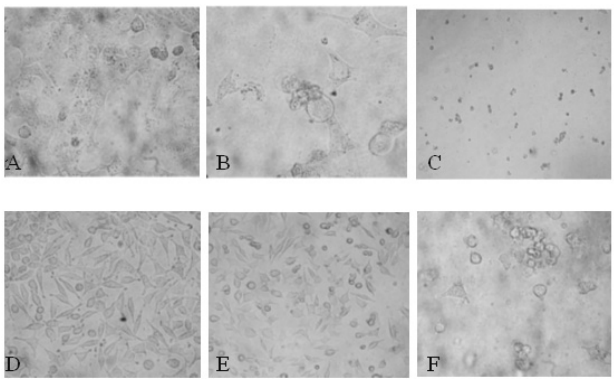

Figure 1

Fig 2: Effect of ethanolic extract of Allium sativum on percent of surviving Hep2 and L929 cells after 72h. Cells were cultivated into seven 50 cm^2–flasks, containing 5×10^5 cells in a 1:1 mixture of DMEM, supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution. Cell viability was found to be higher than 95% by trypan blue exclusion testing. Both cells were incubated with concentrations of 0 (control), 0.5,1,2,4,8 and 12 mg/ml of the extract. Percentage of surviving Hep2 and L929 cells in all concentrations of the extract was significantly decreased when compared with control cells (P<0.001). Each point represents the Mean ± SD.

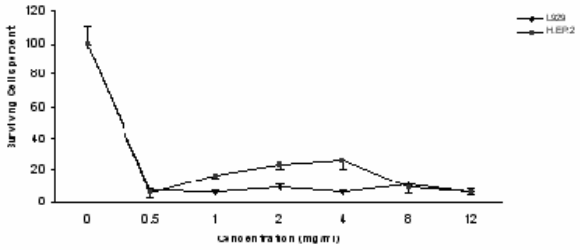

Figure 2.

According to these observations, the mechanism by which *A. sativum* prevents proliferation of human larynx tumor can be same, for example induction of apoptosis. As apoptosis also occurs in non-tumor cells, this mechanism can be due to the antiproliferative effect that was observed in L929 cell lines. Of course, more studies are required to ascertain the mechanism of action of this extract.

In conclusion, we have demonstrated, using cell culture model, antiproliferative effect of ethanolic extract of *A. sativum* on human larynx tumor (Hep2) and L929 cell lines.

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