INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2006/08–5–609–614
http://www.fspublishers.org

In Vitro Antiproliferative Effect of Fresh Red Garlic on Human Transitional Cell Carcinoma (TCC-5637 Cell Line)

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ABSTRACT

The present study was carried out to compare the antiproliferative effect of aqueous extract of fresh red garlic on the cells obtained from human transitional cell carcinoma (TCC) and normal cells (L929). TCC and L929 cells were cultured in the presence of various concentrations of the plant extract. Then cells were observed for morphological changes (by light microscopy) and cytotoxicity (by MTT assay). After 24 hours, a dose dependent cytotoxicity effect was observed that was augmented after 48 and 72 hours. MTT assay results confirmed the morphologic observations statistically. IC_{50} for TCC cells was 300 μg mL^{-1} and for L929 cells was 379 μg mL^{-1}. Results confirmed the anti-proliferative effect of fresh garlic extract on neoplastic TCC cells and also revealed that at concentrations less than 300 μg mL^{-1}, it has no significant cytotoxicity effect on non-neoplastic L929 cells.

Key words: Red garlic; Aqueous extract; Cytotoxicity; Bladder Cancer

INTRODUCTION

Bladder cancer is the second most common cancer of the genitourinary tract and the average age at diagnosis is 65 years (Tanagho & McAninch, 2000). Intravesical chemotherapy has been used since 1950s to treat existing transitional cell carcinoma and to reduce tumor recurrence and progression (Lamm & Riggs, 2000).

Garlic (*Allium sativum* L.) has been considered as a valuable healing agent by many different cultures for thousands of years, particularly for treating heart diseases (Koch & Lawson, 1996). Garlic has been demonstrated in epidemiologic studies to be associated with a reduced risk of stomach cancer and, in animal models, to have anti-tumor activity in sarcoma, mammary carcinoma, hepatoma, colon cancer, and squamous cell carcinoma of the skin and esophagus (Lamm & Riggs, 2001).

Garlic contains a large number of compounds, but only the thiosulfinates (allicin) have been found to have significant activity at levels representing normal garlic consumption (3–5 g/day) (Koch & Lawson, 1996). Allicin potentially becomes the main precursor of various other transformation compounds, such as allyl sulfides, ajoenes, and vinyldithiins, depending on the method of processing.

Purple-skinned garlic (red garlic) is a variety which is considered stronger than ordinary white-skinned one. Hence, we aimed to evaluate the possible anti-proliferative effect of aqueous extract of fresh red garlic (AEFRG) on human transitional carcinoma cells (TCC5637) by qualitative and quantitative methods, in comparison with non-neoplastic fibroblast cells (L929).

MATERIALS AND METHODS

Preparation of fresh garlic extract. Fresh red garlic bilbs were harvested from Veirani's garlic farm (Mashhad, Iran), peeled and separated into cloves. Cloves were mixed with distilled water in a 1/9 (w/v) ratio and crushed using a porcelain mortar and pestle (Ku *et al*., 2002). The resulting homogenates were allowed to stand at room temperature for 10-20 min, centrifuged at 2000 g for 15 minutes and filtered through a 0.2 μm filter. This solution was stored at 4-8°C. Various concentrations of garlic extract (0, 50, 100, 200, 250, 400, 800 and 1600 μg mL^{-1}) were prepared immediately before experiments and refrigerated.

Culture and growth of TCC5637 and L929 cell lines. Human transitional carcinoma cells (TCC5637) and L929 were obtained from National Cell Bank of Iran (NCBI). TCC5637 is an epithelial-like adherent cell line originally established from the primary bladder carcinoma of a 68-years-old man in 1974. L929 (mouse, C3H/An, areolar and adipose) cell morphology is like fibroblast that is derived from mouse C3H/An connective tissue. Cells were grown in DMEM with 10% fetal calf serum (FCS) (Gibco Company, Scottland). The media were supplemented with 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Jaberebne-Hayan laboratories , Tehran, Iran). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 2-3 days before experimental use. Viability of cells throughout the experiment was always >95% as determined by trypan blue.

MTT assay for cell proliferation. *In vitro* cytotoxicity was determined by using 3-(4,5-dimethyl

thiazol-2yl)-2,5-diphenyl tetrazolium Bromide (MTT) colorimetric assay originally described by Mossman (1983) and modified by Alley *et al*. (1988). About 2×10^6 cells/mL/well were incubated with increasing concentrations of garlic extract (0, 50, 100, 200, 250, 400, 800 and 1600 μg mL^{-1}) in 96 well plates for 24, 48 and 96 hours. 5 mg mL^{-1} MTT dye (Sigma, St. Louis, MO) in PBS was prepared freshly before each test and filtered through a 0.2 μm filter to sterilize and remove un-dissolved particles. For each 250 μL growth medium, 25 μL MTT solutions was added and incubated for 4 hours. After removing the growth medium, resulted crystals were dissolved in 200 μL DMSO (Dimethyl sulfoxide) and 25 μL glycine buffer by shaking microplates for 2-3 minutes. The absorbance of formazan dye was recorded at 570 nm using ELISA plate reader (Carmichael, 1987). All the tests were done two or three times. Since the formation of formazan as a product of MTT has been found to correlate with the number of living cells (Mossmann, 1983), and MTT assay was performed before density limitation of cell growth was occurred in control wells, the optical density read from the drug-treated wells was converted to a percentage of living cells against the control using the following formula:

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

The dose response curves were calculated for WEFRG at the above-mentioned concentrations and expressed as the mean percent fraction of control ± standard error of means (SEM). IC_{50} was determined by plotting the extract concentration against the mean percentage of living cells at each dose of WEFRG, using a software program (Durmaz *et al*., 1999).

Morphological alterations: Various concentrations of garlic extract (0, 50, 100, 400 and 1600 μg mL^{-1}) were prepared and co-cultured with TCC and L929 cells. Cells were observed under light inverted microscope for morphological alterations; for example, proliferation and distribution, granulation of cytoplasm and anchorage dependency from 12 to 72 hours.

Statistical analysis. Significance level was ascertained by one way analysis of variance, followed by Tukey's multiple comparison tests. Results were expressed as the mean ± SEM. A p-value of < 0.05 was considered significant. All statistical procedures were performed with SPSS software version 13 (SPSS, Inc., Chicago, IL).

RESULTS

Morphological Alterations.

TCC5637. We assessed effect of four different concentrations (50, 100, 400 and 1600 μg mL^{-1}) over four consequent days, when control flask became confluent by cell proliferation. After 24 hours of co-culture with 100 μg mL^{-1} of extract, cell population was decreased compared to control. Also, mild to moderate cytoplasmic granulations and decreased intercellular connections was apparent (Fig. 1A and 1B). At the concentrations 400 and 1600 μg mL^{-1}, cytotoxic effects were more prominent. Nearly all cells were granulated, cell proliferation was arrested and a large number of cells became round. Intercellular connections were also disrupted. There was no significant difference between 400 and 1600 μg mL^{-1} concentrations for TCC. After 48 hours, the difference between control and other flasks were more prominent. Cell proliferation in 50 and 100 μg mL^{-1} flasks was continued but it was completely arrested at 400 and 1600 μg mL^{-1} concentrations (Fig. 1C and 1D). On day 3, nearly 80% of surface in the control flask became confluent by cells but in 50 and 100 μg mL^{-1} flasks, only 50% of surface was contributed by TCC cells. Number of dead cells was increased at 400 and 1600 μg mL^{-1} concentrations. On day 4, the control flask was completely confluent. Nearly all cells became detached from surface at 1600 μg mL^{-1} concentration.

L929 cells. The morphologic assay of L929 cells was done two times during this study. The first was in our pilot study for determination of appropriate extract concentration and the second was done during the main study. During the study, the cells in flasks containing 50, 100 and 200 μg mL^{-1} of extract (Fig. 2B) had better condition compared to the control flasks (Fig. 2A). At 400 μg mL^{-1} concentration, the number of cells was decreased slightly (Fig. 2C) and in 1600 μg mL^{-1}, the changes in morphology and number was apparently significant (Fig.2D).

Quantitative assessment by MTT assay. The linear regression analysis showed a good correlation between extract concentrations and cell viability for TCC cells. This correlation was more prominent in the first day and improved when we omitted the concentrations above 400 μg mL^{-1} ($R = 0.892$, $R^2 = 0.796$, $p < 0.001$). Data analysis also showed that there was no difference in cell viability between 400, 800 and 1600 μg mL^{-1} concentrations for TCC cells ($p > 0.05$). But the difference between 250 and 400 μg mL^{-1} was significant ($p < 0.05$).

We performed the first MTT assay after 24 hours. As is shown in Table I, for TCC cells, the cell viability has an inverse correlation with extract concentration. In contrast to TCC cells, viability of L929 cells increased by increasing the extract concentration but decreased to less than control at concentrations more than 200 μg mL^{-1} (Fig. 4 and Table II).

There was no significant difference in percent of surviving cells between consecutive days (day 1, 2 and 4) for both L929 and TCC cells ($p > 0.05$). Fig. 3 and Fig. 4 show the estimated curves and their equations by regression analysis for viability data of TCC and L929 cells.

Fig 1. Morphologic effect of different WEFRG concentrations on TCC cells

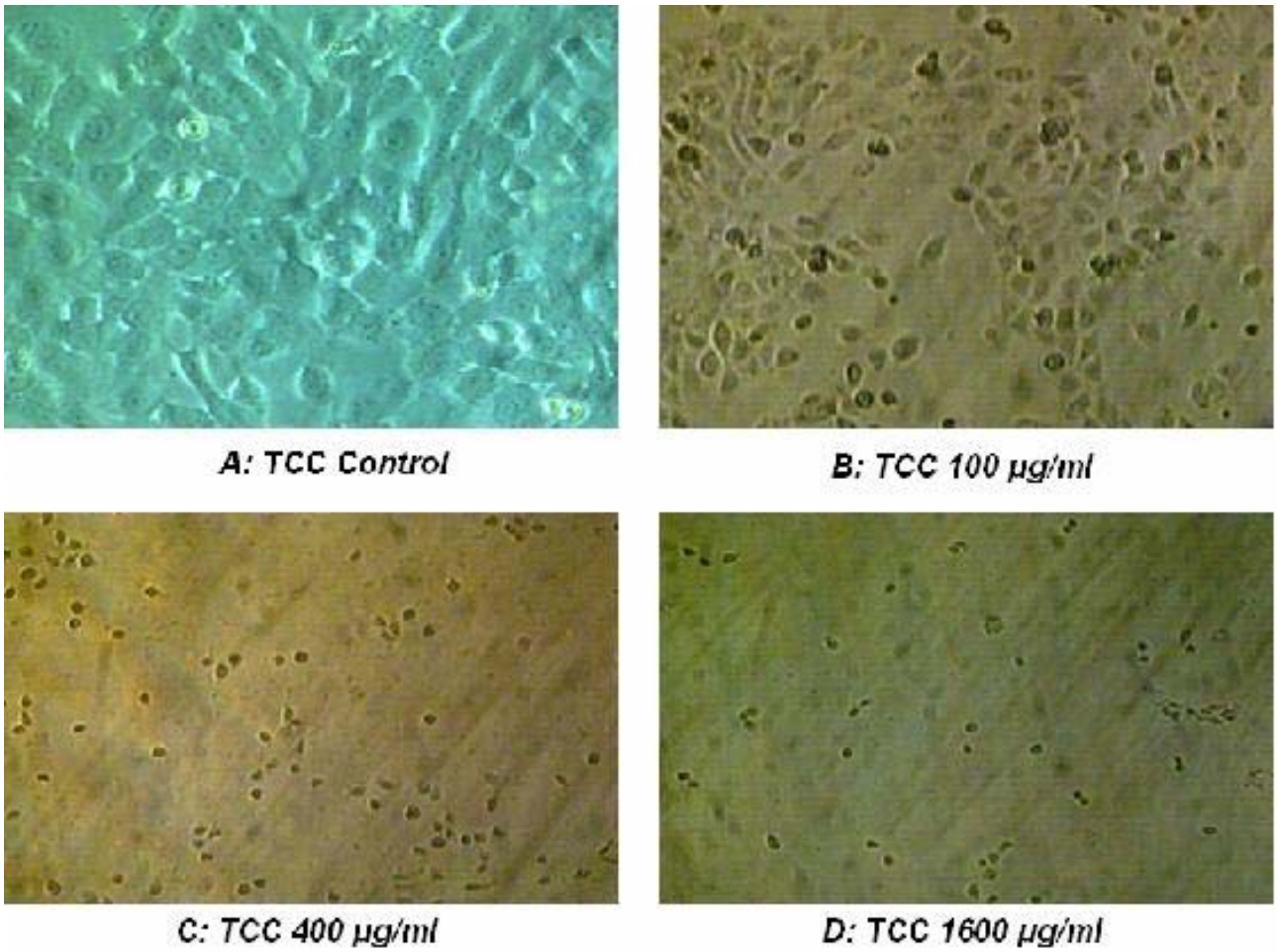


Table I. Percent of surviving TCC cells (mean±SD) in different garlic extract concentrations by MTT assay

TCC cell viability	Percent of surviving cells in different Garlic extract concentrations (µg mL^{-1})						
	0	**50**	**200**	**250**	**400**	**800**	**1600**
Mean±SD of the first and last day	100%	77±11%	58±13%	67±5%	22±2%	27±11%	29±6%
1st day: Mean±SD	100%	85±10%	67±18%	71±3.1%	24±7%	35±3.6%	33±9.4%
4th day: Mean±SD	100%	70±14%	49±17%	64±26%	21±6%	20±3%	25±4.6%

Table II. Percent of surviving L929 cells (mean±SD) in different garlic extract concentrations by MTT assay

L929 cell viability	Percent of surviving cells in different Garlic extract concentrations (µg mL^{-1})						
	0	**50**	**100**	**200**	**400**	**800**	**1600**
Mean±SD of the first and last day	100%	129±2%	117±55%	116±12%	36±30%	10.5±8%	10±9.9%
1st day: Mean±SD	100%	131±5%	78±4.5%	125±3.5%	58±3.5%	6.6±9.4%	3.7±1.7%
4th day: Mean±SD	100%	128±.33%	156±2.5%	108±3%	26%	16±7%	17±7%

In comparison to the controls, WEFRG reduced TCC cell proliferation in a dose dependent manner. Statistical analysis showed no significant difference between 0 and 50 µg mL^{-1} ($p > 0.05$). However, there was a significant difference between 0 and 100 µg mL^{-1} in reduction of cell survival ($p < 0.05$). The IC50 value of WEFRG for TCC cells was 300 µg mL^{-1}.

Concentrations equal or less than 200 µg mL^{-1} had no effect on L929 cell viability in comparison to the control ($p > 0.05$). But there was significant difference between the control and concentrations above 200 µg mL^{-1} ($p < 0.05$). IC50 for the L929 cells was 379 µg mL^{-1}.

Fig. 2. Morphologic effect of different extract concentrations on L929 growth

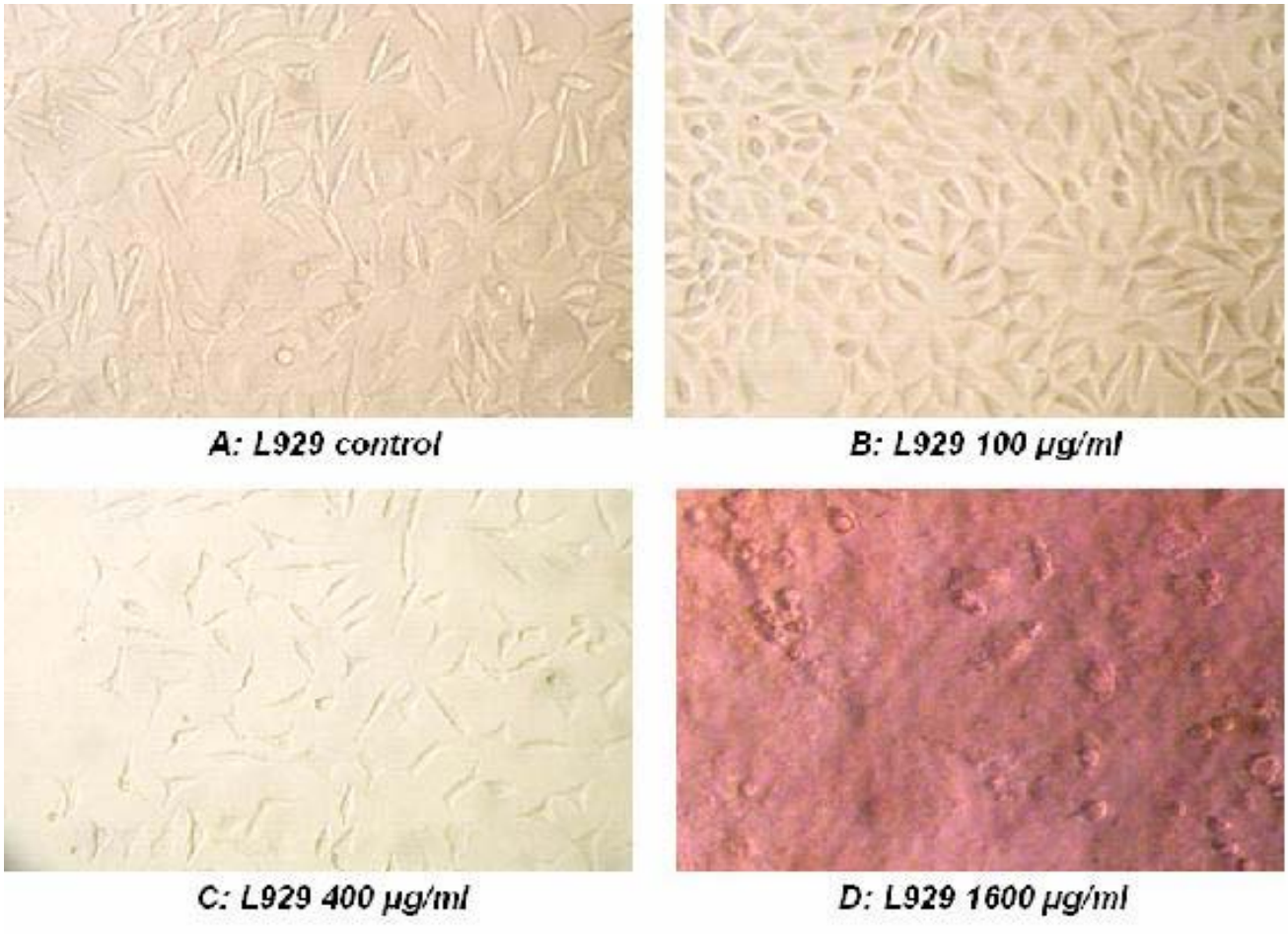


Fig 3. Curve estimation for TCC cells by regression analysis for the first day viability data

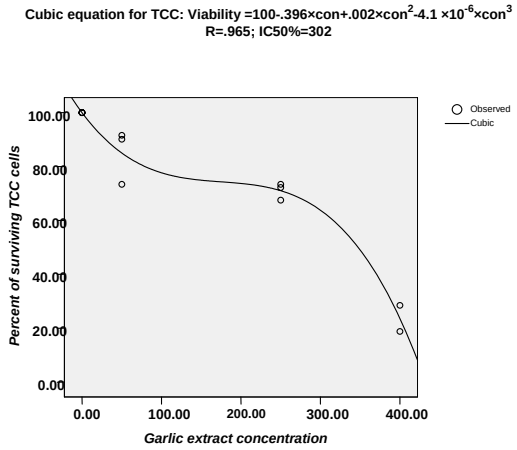
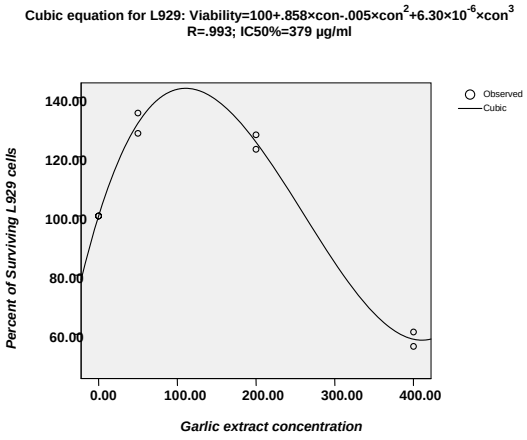


Fig 4. Curve estimation for L929 cells by regression analysis for the first day viability data



DISCUSSION

It was previously demonstrated that intralesional and even oral administration of aged garlic extract (AGE) can significantly reduce tumor growth and animal mortality in the MBT2 murine bladder cancer model (Riggs et al., 1997). In MBT2 murine bladder cancer model, it was shown that AGE concentrations of 31.3 mg mL^{-1} or higher have a moderate direct cytotoxicity (29.9% cell death by MTT assay) (Lamm & Riggs, 2000). It is mentioned also that the remarkable efficacy of oral, intravesical and intralesional AGE is not related to direct cytotoxicity alone and only immune stimulation can logically explain the observed inhibition of growth of transplanted cancer (Lamm & Riggs, 2001). These results are in contrast with our observations that WEFRG causes 50% cell death in extract concentration of 300 µg mL^{-1}, which is about 100 times more potent than the mentioned study. The proliferation of TCC5637 cells was significantly inhibited at concentrations more than 100 µg mL^{-1}. This study suggests that unlike previous studies on AGE, WEFRG has a more potent cytotoxicity in vitro. The most probable explanation for this observation is different constituents present in these extracts.

The anticancer effects appear to be shared equally between allicin and other unidentified compounds (Lawson, 1998). Non-sulfuric compounds in garlic include a variety of steroid saponins, Gamma-glutamylpeptides, scordinins, steroids, triterpenoids, flavonoids, and fructans also have anticancer effects (Tyler, 2000).

In our study, we used fresh garlic extract that is composed of sulfuric and non-sulfuric compounds. Research has revealed that garlic and its lipid- or water-soluble components have many pharmacologic properties (Song & Milner, 2001). According to some chemical reviews, aging garlic to produce the odorless AGE, reduces the content of all sulfur compounds. AGE contains only 3% of the alliin and 6% of the γ-glutamylcysteines found in fresh garlic (Lawson, 1998). So the higher potency of WEFRG may be due to the synergistic effect of multiple anti-carcinogenic agents in fresh garlic extract, specially the fresh sulfur compounds. Garlic can detoxify carcinogens by stimulation of cytochrome P-450 enzymes, antioxidant activity or sulfur compound binding. Studies demonstrate a direct toxic effect of garlic to sarcoma and gastric, colon, bladder and prostate cancer cells in tissue culture (Lamm & Riggs, 2001).

The most significant changes in morphology and number of cells occurred in the first 24 hours of our study when the proliferation of cells was at its highest rate. Evidence exists that cell number influences the anti-proliferative effects of allyl sulfur compounds (Sigounas *et al.,* 1997). Both concentration and duration of exposure can increase the anti-proliferative effects of lipid- and water-soluble allyl sulfides. Part of their anti-proliferative effects may relate to an increase in membrane fluidity and a suppression of integrin glycoprotein IIb-IIIa mediated adhesion. Allyl sulfides are also recognized for their ability to suppress cellular proliferation by blocking cells in the G2/M phase and by the induction of apoptosis. This increase in the G2/M and apoptotic cell populations correlates with elevated cellular peroxide production (Knowles & Milner, 2000).

According to the higher IC50 for L929 (379 µg mL^{-1}), it is suggested that in concentrations between these two IC50 (300 µg mL^{-1} for TCC and 379 µg mL^{-1} for L929), the extract has significant cytotoxicity on cancer cells without any significant cytotoxic effect on non-cancerous cells. This is ideal for chemotherapy in cancer.

Our research indeed was an in vitro study using an aqueous extract. In this extract, water-soluble and low concentrations of lipid-soluble compounds present. It is interesting to mention that lipid soluble compounds such as allicin are more stable in aqueous extracts. Pure allicin has a half-life of 2-16 hours at room temperature and 2.4 days in garlic juice or crushed garlic. In garlic juice diluted 1:10 in water, has a half life of 22 days. Refrigeration increases the life of allicin by a factor of twenty (Lawson, 1998).

According to the above discussion we can conclude that fresh red garlic aqueous extract has the following effects:

1. Significantly inhibits TCC5637 cell proliferation particularly at concentrations > 300 µg mL^{-1}.
2. Does not inhibit significantly L929 cell proliferation at concentrations < 379 µg mL^{-1}.
3. These concentrations are significantly lower than AGE concentrations for the same effects (> 31.3 mg mL^{-1}). This effect is probably due to the synergistic effect of anti-carcinogenic compounds especially fresh sulfur compounds.
4. As previous studies showed that AGE garlic has immunostimulatory effects, due to significant more potent cytotoxicity of WEFRG in comparison to AGE, it seems that in vivo administration of fresh garlic may cause superior effects. But further studies must be done for evaluation of its *in vivo* effects.

Acknowledgements. We thank Mr. Veirani, Dr. Sankian, Mr. Mezerji, Dr. Sazegar-nia, Dr. Rastin, Mr. Reza Feyzi and Ms. Azam Brouk for excellent support and technical help. Without their enthusiasm, this project would not have been possible.

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(Received 18 January 2006; Accepted 04 April 2006)