Original Research

Cytotoxicity Effect of *Arnebia Euchroma* against Human Gastric Adenocarcinoma Cell Line (AGS)

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Abstract

Introduction: Despite the reduction in the incidence of gastric cancer during the past 75 years, it remains the most common cause of death from cancer in Iran and now despite all the progress made, the 5-year survival of patients is generally 15%. Thus, improvement in current treatments seems necessary. Given the richness of Iranian medicine in ancient texts, a detailed study of the effects of medicinal plants native to Iran in particular is essential. In this research, the cytotoxicity effect of *Arnebia euchoroma* on AGS was reviewed.

Materials and Methods: In this study, cells were cultured in RPMI at different concentrations of *Arnebia euchoroma* extract (5,2,1,0.2,0.1,0.05 and 0.02 mg/ml) at intervals of 24,48 and 72 h and the number of living cells compared with the control group and cytotoxicity effect were measured by MTT assay.

Results: Within 24 hours, drug effects on reduction of cell growth of AGS in comparison to the control group at 5, 1, 0.1, 0.05 and 0.02 mg/ml were significant (P values are 0.0305, 0.0465, 0.0137, 0.0274 and 0.0003, respectively). Within 48 hours, concentrations of 0.1 and 0.02 mg/ml were significant (P values are 0.0398 and 0.0143).Within 72 hours, 5, 0.05 and 0.02 mg/ml reductions were significant (P values are 0.0402, 0.0073 and 0.0349, respectively).

Conclusion: According to the results, *Arnebia euchoroma* significantly has no killing effect on AGS. Only in the 0.02 mg/ml concentrations the reduction was significant at three times, with a fatality rate of about 20%, which is not enough to be considered cytotoxic.

Key words: Gastric adenocarcinoma, Cytotoxic, *Arnebia euchroma*, *In Vitro*

Introduction

Currently, cancer is the biggest health problem in most countries of the world (1). It was estimated that about 12.7 million people worldwide were affected in 2008. Of those, 6.6 million are men and 6 million women. It is expected the number of patients to rise to 21 million by 2030 (2). In a 5-year study (2003-2008) the decline in cancer mortality rate was 1.8% per year in men and 1.6% in females (1).

Gastric cancer is one of the most common cancers worldwide, with 989,600 people diagnosed each year and 73,800 patients dying. Gastric cancer incidence is approximately 8% per year (3). The incidence and mortality rate of stomach cancer during the last 75 years shows a significant decline, but the incidence of gastric cancer in Japan, China, Chile and Ireland remains

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high (4). Reduction of gastric cancer incidence perhaps is due to identification of some of the risk factors, such as *Helicobacter pylori* and other nutritional and environmental risk factors (5).

In Iran, cancer is the third leading cause of death, with an annual mortality of 30,000. The incidence of cancer is more than 70,000 people each year. Gastric cancer is the third most common cause of cancer in Iran (6).

Stomach cancer has a very poor prognosis. Some statistics demonstrate that the 5 year survival of patients is only 5-15% in Western countries, including the United States (7, 8). According to some sources, the 5-years survival is estimated in men at 18.6% and in women at 25.2%. Even after gastrectomy there is a less than 20% chance of survival and survival of patients with untreated metastatic gastric cancer is estimated at only 3-4 months (9).

From the low survival rates we can estimate that available treatments do not have much impact on the quality of life. On the other hand, these methods are associated with many side effects (10).

In recent years, many studies have focused on using herbal medicines. The main advantage of natural products is their milder side effects in comparison with chemical drugs. Therefore, discovery of novel anti-cancer herbal drugs is important (11, 12). Traditional medicine is one of the ways that can help to discover new drugs. Extracted plants' substances have a less cytotoxic property (13, 14). About 30 years ago, the WHO recognized the role of complementary medicine in the health care system (15,16).

Given the richness of several anti-cancer drugs in traditional Iranian medicine and ancient texts, a detailed study of the effects of Iran's native drugs and medicinal plants is essential.

In this research, *Arnebia euchoroma,* a plant of the Boraginaces family developed in the Kerman province, is reviewed. In Iranian traditional books it is noted that the root of this plant has healing effects on wounds and burns and destroys swelling and lumps (17). Recent studies have shown that *shikonin,* an active ingredient extracted from the chorion layer, inhibits the growth of cancer cells and also has antimicrobial effects (18). In the current study, the root's extract of this plant was examined for the detection of cytotoxic effect on gastric adenocarcinoma cells (AGS). We also determined the most appropriate time and dose for the cytotoxic effect of *Arnebia euchroma* on AGS cells.

Materials and methods
Reagents:
RPMI-1640 medium and fetal bovine serum (FBS) was obtained from Gibco. MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was obtained from Sigma. Powder and phosphate buffer saline (PBS) were obtained from Merck (Germany). Serum NaCl 10%. 10% trypsin solution obtained from Sigma.

Cell culture
Adenocarcinoma gastric cell line (AGS) was purchased from Pasteur Institute (Tehran, Iran) and maintained in RPMI with 10% FBS (both of them from Gibco) incubated at 37.8C and 5% CO_2. Exponentially growing AGS cells were digested by 2ml trypsin 0.25% for 1-2 min. RPMI 1640 medium containing 10% FBS was subsequently added. Final cell suspensions were placed in 96-well plates (1×10^4/ 200 ml/well) in an incubator containing 5% CO_2 and incubated at 37.8C for 24 h. Then, 200 ml RPMI 1640 medium containing different concentrations (5-0.02 mg/ml) of *Arnebia euchroma* herbal extract were added to each well of the plate. Cells were cultured for 24, 48 and 72 h.

Preparation of herbal extracts
In the present study, total aqueous herbal extracts were prepared by mixing with distilled and deionized water and centrifuged (5000 g/30 min), and the resultant supernatant was collected and stored in refrigerator until used. All extracts were used in different concentrations (5, 2, 1, 0.2. 0.1, 0.05 and 0.02 mg/ml) based on their weight (mg) per water volume (ml).

The powder of *Arnebia euchroma* rhizome was

dissolved in 80% alcohol. The supernatant was placed in a decanter funnel and percolator device, after 24 hours the alcohol products were drained. This step was performed 3 times. Finally, the solvent was dried by rotator-evaporator and remaining powders were stored in a vacuum oven for 24-48 hours.

Cell viability

We used MTT reduction assays for evaluating cells viability. Briefly, MTT powder (Merck, Germany) was dissolved in PBS (5 mg/ml). Cells were seeded at 20,000/well onto 96-well culture plates and allowed to grow. Then, the cells were treated with different concentrations of each herbal extract for 24, 48 and 72 hours. Four hours before reading of the absorbance, 20ml of MTT solution was added to each culture and the cells were incubated for 4 h with MTT solution. Subsequently, MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The supernatants were then gently removed, the formazan crystals were resolved in 100 ml acidic isopropanol (0.04M HCl in isopropanol), and absorbance was read at 290 nm with a plate reader.

Statistical analysis

The results are presented as means. Analysis of variation was done with SPSS v.16 software and comparisons between study groups were made using student's t- test. Differences were considered significant at p<0.05.

Results

Effect of Arnebia euchroma extract on cell viability of AGS cell line

This experiment prepared concentrations of 5 mg/ml, 2 mg ml, 1 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml and 0.02 mg/ml of Arnebia euchroma that were exposed to AGS cell line within 24, 48 and 72 hours. The results are illustrated in Table 1. Within 24 hours, medication effects on reduction of cell growth of AGS in comparison to the control group in doses 5 mg/ml, 1 mg/ml, 0.1 mg/ml, 0.05 mg/ml and 0.02 mg/ml at the 95%

confidence limits were significant (P values are 0.0305, 0.0465, 0.0137, 0.0274 and 0.0003, respectively). Within 48 hours, the reduction in the AGS cell in 0.1mg/ml and 0.02 mg/ml doses at 95% confidence limits were significant (P values obtained for each are 0.0398 and 0.0143, respectively).

AGS cells at 72 hours in 5 mg/ml, 0.05 mg/ml and 0.02 mg/ml doses at 95% confidence range compared to the control group were statistically significant (P values obtained for each are 0.0402, 0.0073 and 0.0349, respectively).

Table 1: Average of absorbance and percentage of viability relative to the control group after 24, 48 and 72 hours of exposed to Arnebia euchroma to AGS cell line

% of viability	Mean of Absorbance number(O.D)		Groups
100	49.92	24h	Control
100	65.92	48h	
100	55.34	72h	
84.65	42.41	24h	5 mg/ml
99.24	65.42	48h	
76.21	42.18	72h	
95.63	47.74	24h	2 mg/ml
92.87	61.22	48h	
97.18	53.78	72h	
89.06	44.46	24h	1 mg/ml
96.17	63.4	48h	
90.1	49.88	72h	
95.95	47.9	24h	0.2 mg/ml
90.23	59.48	48h	
95.62	52.92	72h	
82.71	41.29	24h	0.1 mg/ml
82.64	54.48	48h	
98.66	54.6	72h	
88.34	44.1	24h	0.05 mg/ml
94.78	62.48	48h	
62.52	34.6	72h	
76.92	38.4	24h	0.02 mg/ml
73.39	48.38	48h	
74.41	41.18	72h	

Survey of change in the number of epithelial AGS cells (control group) at different times:

The means of absorbance obtained from control group of the AGS cell line at intervals of 24, 48 and 72 hours are 49.92, 65.92 and 55.34. The result is illustrated in Figure 1.
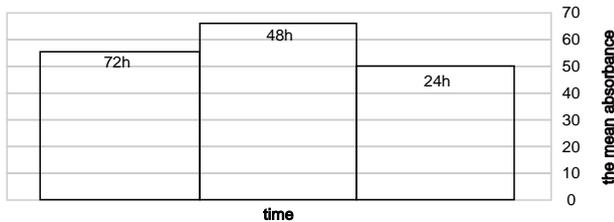
Figure 1: Average absorbance on epithelial cell of AGS cell line at different times.

The results illustrated in Figure 2 show that there are no significant differences between test and the control groups.
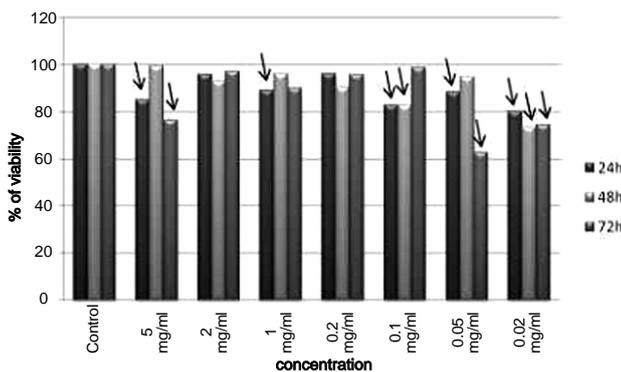

Figure 2. Effect of aqueous extract of Arnebia euchroma on cell viability of AGS cell line at 24, 48 and 72 h. denotes significant differences compared to control group. (p<0.05).

Discussion

The results obtained in this study are shown in Table 1 and Figures 1 and 2. Arnebia euchroma showed no significant cytotoxic activity on the AGS gastric cancer cell line. Comparing different doses over time, just the single dose of 0.02 mg/ml was cytotoxic after exposure to *Arnebia euchroma* at 24, 48 and 72 hours. The largest reduction of AGS cells was at 0.05 mg/ml and 0.02 mg/ml doses. The cytotoxic rate was 25-30%. This rate should be more than 50% to prove cytotoxicity. Therefore, this drug does not have significant cytotoxic effects on the AGS cell line (20).

In the majority exposure to Arnebia euchroma within 48 hours cytotoxic effects was unlikely and the number of living cells increased. The increase in the number of viable cells in comparison with 24 hours in the majority of the dose was significantly different. After 72 hours of contact with *Arnebia euchroma* there was a reduction in the number of viable cells, but the reduction compared with the first 24 hours was not significantly different.

The average absorbance of the control group of the AGS cell line at 24, 48 and 72 hours, as well as concentrations of living cells increased after 48 hours. The enhancement of the living cells was significantly different in comparison with the first 24 hours. After 72 hours the mean absorbance of living cells was reduced.

According to the results, *Arnebia euchroma* does not have significant cytotoxic effect on the AGS cell line. The results of the control group were similar to other groups.

The reason of the enhancement of living cells at 48 hours can be due to the presence of adequate FBS serum to grow in the plate. After 72 hours, the concentration of FBS was not sufficient for growth. Therefore, cells died and the absorbance decreased. Perhaps the cytotoxic effect requires more than 72 hours of contact with cells.

In the previous studies, the cytotoxic effect of *Arnebia euchroma* extract has been evaluated on various gastric cancer cell lines. Lia and Lu in a research project in China detected anticancer effects of naphthoquinone that was extracted from *Arnebia euchroma.* The extract could apparently inhibit the proliferation of stomach and esophagus cancer cell lines. At the effective concentration of 5 micrograms/ml, the mitotic index and growth curve declined without showing any damage to normal human cells. Anticancer effect of this compound might be related to its role in influencing the amount of RNA and the ultrastructure of cancer cells (19).

It should be noted in that study, *Arnebia euchroma* was grown in China where soil and climate may affect the properties of plant extracts. In that study the active ingredient of *Arnebia euchroma* was examined.

We suggest further review to assess the growth and cytotoxic effects of *Arnebia euchroma*, other than the MTT method. Other methods such as direct observation of cells and their nuclei, DNA assessment and evaluation calorimetrics (methylene blue, trypan blue, Janos Green, Natural red) should be used to increase accuracy. The active substance of *Arnebia euchroma* should be examined for detection of the lethal effect. We suggest further evaluation of the effect of *Arnebia euchroma* on normal cells compared with a normal gastric epithelial cell line.

Acknowledgements

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