Cytotoxic Effect of Freeze-Dried Extract of Ecballium elaterium *Fruit on Gastric Adenocarcinoma (AGS) and Esophageal Squamous Cell Carcinoma (KYSE30) Cell Lines*

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ORIGINAL RESEARCH

Cytotoxic Effect of Freeze-Dried Extract of *Ecballium elaterium* Fruit on Gastric Adenocarcinoma (AGS) and Esophageal Squamous Cell Carcinoma (KYSE30) Cell Lines

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Abstract
Purpose *Ecballium elaterium* (L.) A. Rich (Cucurbitaceae), also known as the "squirting cucumber," is a wild medicinal plant found abundantly in Moghan, Ardabil province, Iran. This study was undertaken to examine possible cytotoxic effect of freeze-dried aqueous extract of *E. elaterium* fruit on cell lines of gastric and esophageal origin namely called AGS (human gastric carcinoma) and KYSE30 (human esophageal squamous cell carcinoma).
Methods The aqueous extract of the fruits of *E. elaterium* was prepared and freeze-dried. AGS and KYSE30 cancer cell lines were treated by the extract and incubated for 24, 48, and 72 h. Cytotoxicity was examined by MTT assay. Ethidium bromide/acridine orange (EB/AO) staining was used for apoptotic cell detection. A DAPI staining method was used to analyze cell cycle by flow cytometry.
Results The IC_{50} values were 2.5, 0.7, and 0.7 μg/ml for AGS cell line after 24, 48, and 72 h, respectively. IC_{50} values for KYSE30 cell line were 500, 150, and 125 μg/ml after 24, 48, and 72 h, respectively. The EB/AO staining showed an increase in apoptotic cells. Cell cycle analysis showed a significant increase in cell density at G2/M phase.
Conclusions The results of the current study showed that the freeze-dried aqueous extract of *E. elaterium* fruit has a cytotoxic effect on gastric and esophageal cancer cell lines by means of apoptosis. The gastric cancer cells (AGS) showed a remarkably higher sensitivity. It seems that several compounds are possibly responsible for the cytotoxic effect of the extract.

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Keywords *Ecballium elaterium* · Moghan · Ardabil · Gastric cancer · Esophageal cancer · Cytotoxic · Apoptosis

Introduction

Ecballium elaterium (L.) A. Rich (Cucurbitaceae), also known as the "squirting cucumber," is a wild medicinal plant found abundantly in Moghan, Ardabil province, Iran. The fruits contain black seeds and a liquid known as "juice of *E. elaterium*." The Natives of Moghan usually use the plant's fruit juice for the treatment of sinusitis. Extracts of *E. elaterium* consist of Triterpenoids (cucurbitacins), carbohydrates, gum, leucoanthocyanins, tannins, and peptides (*E. elaterium* protease inhibitors) [1].

Beneficial effects of the fresh juice have been reported including analgesic, antipyretic, and anti-inflammatory effects. *E. elaterium* and its constituent cucurbitacin B have preventive and curative effects against CCl4-induced hepatotoxicity [2]. The cytotoxic effect of *E. elaterium* extract on HepG2 cell lines was previously reported to be at 1 mg/ml [3].

Cancers of the gastrointestinal tract account for more than a third of total cancer incidence and nearly half of the cancer-related deaths in the world [4]. It appears that the cancers of the esophageal and stomach are the most common form of cancer mortality in northwest of Iran. The esophageal and gastric cancers ranked in the first and second place in the prevalence in this area, respectively [5].

The current study was undertaken to evaluate cytotoxic effect of aqueous extract of *E. elaterium* fruit on cell lines of gastric and esophageal cancers namely called AGS (human gastric carcinoma) and KYSE30 (human esophageal squamous cell carcinoma). This study has been done based on

the high prevalence of esophageal and gastric cancers in Ardabil province located in northwest of Iran as a part of a series of screening programs to explore cytotoxic effects of native medicinal plants of the area to find out a potentially putative therapeutic solution.

Methods

The human cancer cell lines including AGS and KYSE30 were provided from Pasteur Institute of Iran's Cell Bank (Tehran, Iran). All reagents and medium were prepared just before use.

Preparation of the Extract

At the end of summer, the fruits of *E. elaterium* were collected from suburb area of Moghan, Ardabil province, Iran. Fruits were washed and cut into small pieces and homogenized by SilentCrusher M (Heidolph, Germany) in distilled water. The subsequent mixture was further treated by an ultrasonic homogenizer (UP200H, Hielscher, Germany) with maximum amplitude and complete cycle for 15 min. Then, the milky mixture was centrifuged at 4,000 rpm for 15 min. the supernatant was filtered through gauze into a 100-ml glass bottle and allowed to freeze at −80 °C. The next day, the frozen supernatant was lyophilized using Freeze dryer ALPHA 2-plus (Martin Christ, Germany) and dried residue kept at −20 °C.

Cell Culture and Treatment

All cell types were cultured in RPMI-1640 (Cat. No. 51800-035, GIBCO, UK) medium supplemented with 10 % FBS (Cat. No. 10270-106, GIBCO, UK), penicillin 100 units/ml, and streptomycin 100 μg/ml. The cells were incubated at 37 °C in a water-saturated atmosphere of 5 % CO_2 and 95 % air until confluence. Cell types were trypsinized and plated in 96 microtiter plates at a density of 1×10^4 cells per well in a 200-μl medium and incubated overnight. After attachment, the medium was removed, and cells were incubated with a serum-free medium containing 10 mg/ml of the extract by 1/4 serial dilutions for 24, 48, and 72 h. The cytotoxicity of the extract was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MTT Assay

Four hours before completion of the incubation times, the medium was removed, and 180 μl of serum-free medium and 20 μl of 2.5 mg/ml MTT (Merck, Germany) were added to each well. Plates were incubated again for additional 4 h for completing the incubation time. The medium was removed, and 200 μl of DMSO was added to each well. Plates were shaken for 10 min. The absorbance at 540 nm was measured by a plate reader (Synergy HT, BioTek), using wells without cells as blank. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells that served as control [3].

EB/AO Staining for Apoptotic Cells

Cells were seeded in 96-well plates at a density of 1×10^3 cells per well and incubated overnight. After attachment, they were treated with 0.03–2×10^3 μg/ml of the extract. To stain apoptotic cells, the plates were centrifuged for 5 min (129×*g*, 1,000 rpm) at 4 °C. The ethidium bromide/acridine orange (EB/AO) dye mix (100 μg/ml EO and 100 μg/ml AO was prepared in phosphate-buffered saline (PBS), and 10 μl was added to each well. Cells were viewed and counted under an inverted fluorescence microscope (IX 71, Olympus, Japan). Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of the apoptotic bodies. Necrotic cells were identified by uniform labeling of the cells with ethidium bromide [6]. Images were taken with a microscope equipped with a digital camera (DP 71, Olympus). Tests were done in triplicate, counting a minimum of 100 cells each time.

Cell Cycle Analysis by Flow Cytometry

Cells were seeded in 25-cm^2 flasks at a density of 1×10^6 cells/flask. Cells were treated with the extract for 24 h at a range of concentrations for each cell line (AGS cells with 2.5, 5 and KYSE30 cells 62.5, 125, and 250 μg/ml). After treatment, the DNA content and cell cycle distribution were determined by flow cytometry. Briefly, cells were trypsinized and harvested and fixed with 70 % ice ethanol at 4 °C for 2 h. Fixed cells were centrifuged (300×*g*, 4 °C, 5 min) and washed with cold PBS, and then stained with diamidino-2-phenylindole (10 μg/ml; Triton X-100 0.1 % *v*/*v* in PBS). The stained cells were then transferred to flow tubes by passing through a nylon mesh with a pore size of 30 μm. Flow cytometric analysis was performed using a flow cytometer (Partec CyFlow space, Germany). The distribution of cells in the different cell cycle phases was analyzed using Partec FloMax software.

Statistical Analysis

Concentration for 50 % inhibition of cell growth (IC_{50}) value was calculated using Sigma Plot 11 software. Data

values for the growth inhibition study are presented as mean ±SD, except in figures where error bars represent standard error of mean. The statistical significance of the results was evaluated by Student's *t* test or one-way analysis of variance followed by the Newman–Keuls test for multiple comparison. $P<0.05$ was considered significant.

Results

Growth Inhibition Study

Viability assays showed the crude extract of *E. elaterium* fruit exerted a cytotoxic effect on cancer cell lines after 24, 48, and 72 h of incubation (Fig. 1). The extract exerted its greatest cytotoxic effect on the AGS cell line. The extract induced a decrease in the viability of cells when assessed by the MTT. IC_{50} values were 2.5, 0.7, and 0.7 μg/ml for AGS cell line after 24, 48, and 72 h, respectively. IC_{50} values for KYSE30 cell line were 500, 150, and 125 μg/ml after 24, 48, and 72 h, respectively.

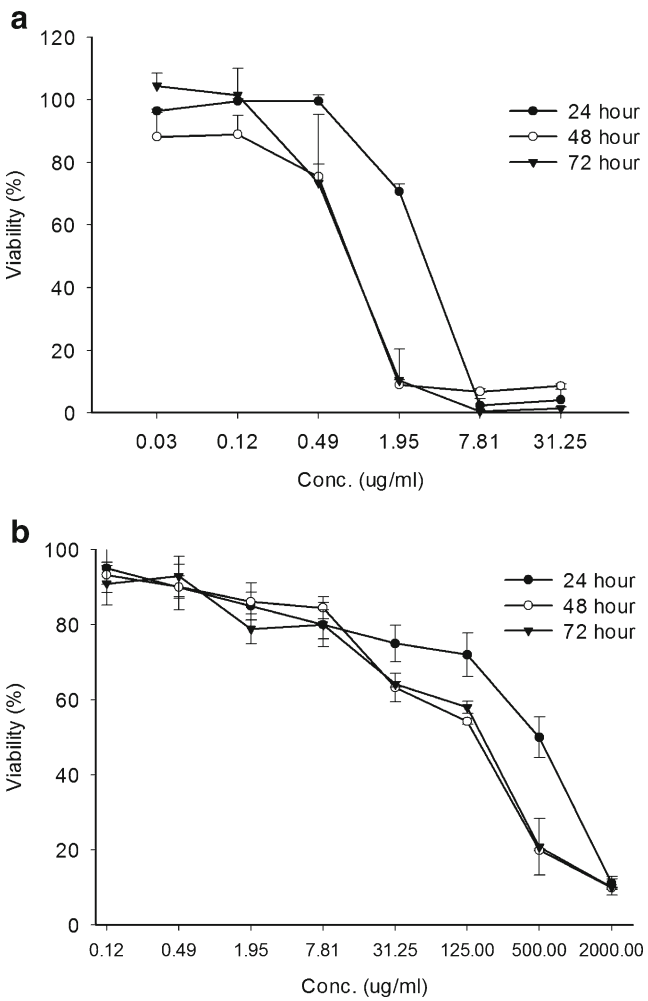

Fig. 1 MTT assay of cytotoxic activity of the freeze-dried aqueous extract of *E. elaterium* fruit on AGS and KYSE30 cell lines after 24, 48, and 72 h compared to normal (control)

EB/AO Staining for Apoptotic Cells

Morphological characteristics of the extract-induced cancer cells were determined by EB/AO staining and analyzed under a fluorescent microscope (Fig. 2). The results showed that after incubation at 2–250 μg/ml of the extract for 24 h (Fig. 2), the cells displayed a series of morphological changes including condensation and fragmentation of chromatin and nucleus, and formation of apoptotic bodies (Fig. 2b), which were designated as typical evidence of apoptotic bodies. In contrast, control cells exhibited a normal appearance (Fig. 2a). As shown in Table 1, there was a significant increase of apoptotic cells in cancer cells after treating with the extract ($P<0.001$).

Cell Cycle Analysis by Flow Cytometry

In this study, the DNA content of extract-treated cancer cells during cell cycle phases was measured in order to obtain information about the cell cycle progression. The effect of extract of *E. elaterium* fruit on cell cycle was evaluated using flow cytometric analysis (Fig. 3). The percentage of cells in G1, S, and G2/M phase was calculated using Partec FloMax software and is shown in Table 2. A dose-dependent effect of the extract on the cell cycle was observed. After 24 h of extract treatment at different concentrations, population of cells in the G2/M was increased significantly in respect to controls ($P<0.05$ for 62.5 μg/ml and $P<0.001$ for 125 and 250 μg/ml of the extract for KYSE30 cell line and $P<0.001$ at all concentrations of the extract for AGS cell line). The increase of cell population at the G2/M phase was accompanied by a decrease of cell population in the G1 phase of cell cycle (Table 2).

Discussion

The results of this study showed that crude extract of *E. elaterium* fruit exerts cytotoxic effect on gastric and

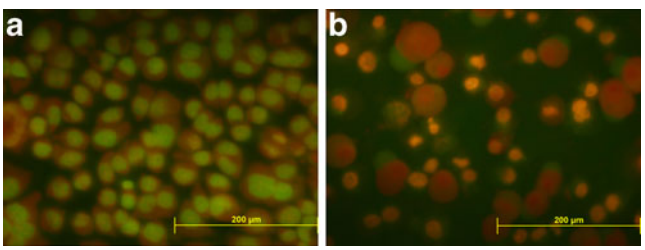

Fig. 2 Representative photomicrographs showing apoptosis induction of the freeze-dried aqueous extract of *E. elaterium* fruit on AGS cell line. **a** Normal untreated cells, **b** cells treated with the extract

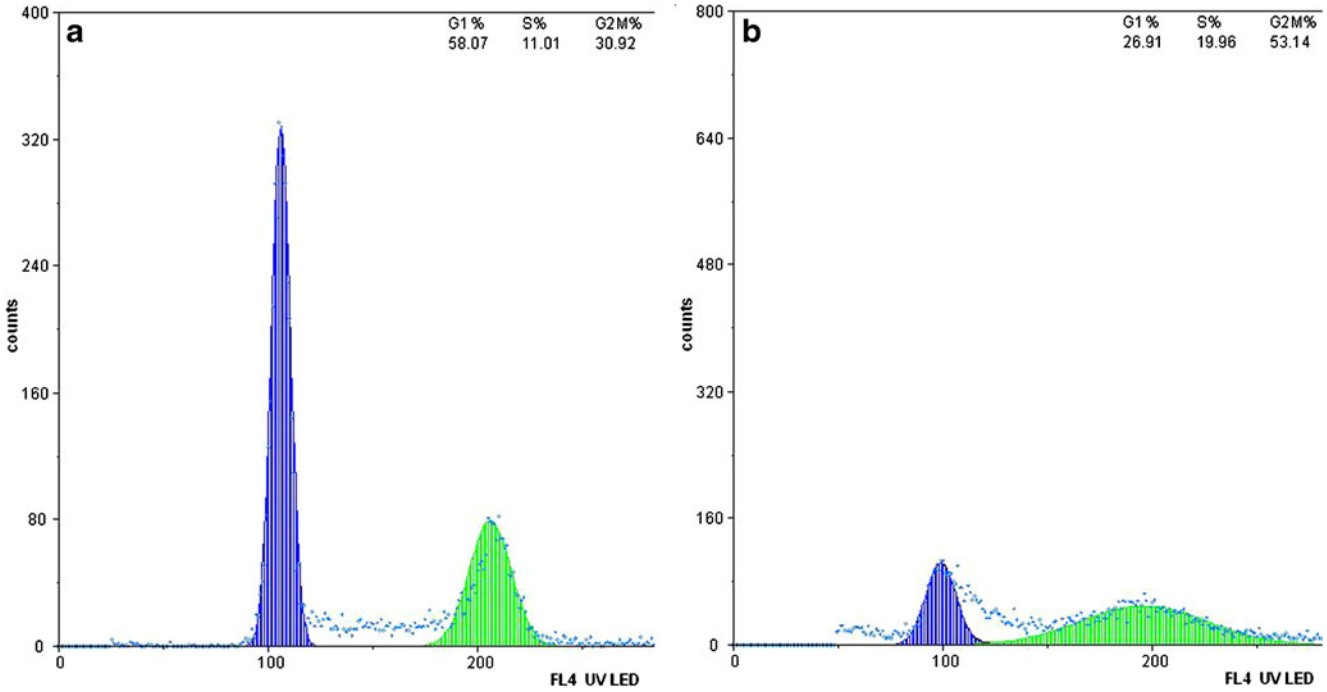

Fig. 3 Effect of the freeze-dried aqueous extract of *E. elaterium* fruit on cell cycle distribution in AGS cell line **a** control cells and **b** cells treated with the extract. Results represent similar results from three independent experiments

esophageal cancer cell lines of AGS and KYSE30 types. The gastric cancer cells (AGS) expressed remarkably higher sensitivity to cytotoxic effect of the extract. The result of this study also showed that the extract applies its cytotoxic effect via apoptotic pathway and cell cycle arrest at G2/M phase.

Ljubuncic et al. have previously reported the cytotoxic effect of aqueous extract of *E. elaterium* on HepG2 cell line with IC_{50} value of 1,000 μg/ml at 24-h incubation [3]. This concentration is close to IC_{50} value of the extract on KYSE30 cell line in our study (500 μg/ml at 24-h incubation), but it is much higher than the IC_{50} value of the extract on AGS cell line (2.5 μg/ml at 24-h incubation).

The freeze-dried extract of *E. elaterium* fruit may induce cytotoxicity by means of several compounds found in the fruit's juice and the seeds. *E. elaterium* fruit contains cucurbitacins, particularly cucurbitacin E [7]. Cucurbitacin E can bind to actin filament [8] and disrupt cell actin which leads to inhibition of proliferation by destroying cellular skeleton [9]. It was also reported that cucurbitacin E may induce G2/M cell cycle arrest and apoptosis [10]. In line with such studies, our results demonstrated that apoptotic cell death and cell cycle arrest at G2/M phase which may be introduced by cucurbitacins could be accounted, at least in part, for cytotoxic effect of the extract on AGS and KYSE30 cell lines.

Table 1 Apoptosis induction of freeze-dried extract of *E. elaterium* fruit; data are expressed as percentage of cells

Cell line	Live cells	Apoptotic cells	Necrotic cells
AGS control	95.2±2	3.8±0.2	1±0.1
AGS treated with the extract	47.8±2.5*	49.5±2.8*	2.54±0.9
KYSE30 control	96.5±4	4.2±0.4	0.5±0.1
KYSE30 treated with the extract	52±5.7*	45.6±4.2*	2.1±1

Data are presented as mean±SD (n=3)

*P<0.001 vs. control

Table 2 Effect of *E. elaterium* on cell cycle distribution in AGS and KYSE30 cells after 24-h incubation

Cell line (the extract, μg/ml)	G1%	S%	G2/M
KYSE30 control	25±4.5	35±5.2	40.4±2.5
KYSE30 (62.5 μg/ml)	22.65±3.2	31±3.1	47.6±3.5**
KYSE30 (125 μg/ml)	22.5 ±7.4	26.5±6.5	51.5±4*
KYSE30 (250 μg/ml)	16±4.7	15.3±7	69.3±3*
AGS control	50.8±6.5	17.3±7.1	33±4.7
AGS (2.5 μg/ml)	37.2±3.6**	14.4±4.3	47.6±5.3*
AGS (5 μg/ml)	25.6±4*	22±7.3	51.54±2.2*
AGS (10 μg/ml)	25.4±5.2*	12.8±6	63.6±3.5*

Data are presented as mean±SD (n=3)

*P<0.001 vs. control; **P<0.05 vs. control

Cucurbitacins show their cytotoxic effect at concentrations below 0.5 μg/ml [11]. Nevertheless, at the best conditions, only 2.2 % (*w*/*w*%) of *E. elaterium* fruit's dry matter is composed of cucurbitacins [7]. In our study, the IC_{50} value of the fruit extract for AGS cell line was 0.7 μg/ml after 48-h incubation. This finding shows that cucurbitacins are likely one of the compounds responsible for cytotoxic effect of the extract, and other ingredients of fruit such as peptides (trypsin inhibitors) and polyphenolic compounds (leucoanthocyanins) [1] may also play a role in part. Although plant-derived trypsin inhibitors may cause cytotoxic effect on tumors [12], such activity for trypsin inhibitors found in *E. elaterium* has not been clearly established and needs to be addressed.

Conclusion

The results of the current study showed that the freeze-dried aqueous extract of *E. elaterium* fruit has cytotoxic effect on gastric and esophageal cancer cell lines by means of apoptosis. The gastric cancer cells (AGS) showed remarkably higher sensitivity. It seems that several compounds are possibly responsible for the cytotoxic effect of the extract.

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