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The Pattern of Cell Death in the HCT–116 Cells after Exposure to an Extract of *Scrophularia Megalantha*

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Abstract

Background: Scrophularia species are used in traditional medicines to cure many diseases such as cancer. In this study, the anti-cancer and cytotoxic effects of a hydroalcoholic extract of Scrophularia megalantha on the colon cancer cell line, HCT–116 and on human fibroblasts were evaluated. Methods: The fibroblasts and HCT–116 cells were cultured with various concentrations of S. megalantha extract for 48 h. The MTT assay was used to determine cytotoxicity. The cell death pattern was determined by staining with Annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide). Results: The GI50 index of the S. megalantha extract was found to be 62.6µg/mL and 49.8µg/mL for the colon cancer cells and fibroblasts, respectively. Furthermore, the cytotoxicity of this extract was almost similar to the normal fibroblasts and colon cancer cells. Flow cytometry analysis showed that the dominant pattern of cell death induced by the S. megalanthaextract was necrosis, whereas apoptosis was also detected with higher doses of this extract. Conclusion: Taken together, our experiments indicated that the crude extract of S.megalantha induced necrosis and inhibited the proliferation of theses colon cancer cells. Hence, this extract might be a useful agent for improving the treatment of multidrug-resistant colon cancer tumors. However, further in vivo experiments are necessary.

Keywords: HCT–116, Scrophularia Megalantha, Cell Death Pattern, Extract.

Introduction

Cancer is a leading cause of death worldwide that potentially invades and spreads to various organs from its site of origin, destroys the normal cells, and is characterized by uncontrolled cell growth (Mohammadi and et al., 2016). There should be a fine balance between cell death and cell division because any disturbance in this cell death process can lead to cancer development (Baharara, 2016). In general, apoptosis or programmed cell death plays a key role in the development, clearance of injured, old or even premalignant cells, and regulation of cell growth. Cells can resist or reduce cell death through different mechanisms in a variety of diseases, including cancer or autoimmune disease (YongYanga and et al., 2017; Ravichandiran and et al., 2014; Safarzadeh, et al. 2014)

Surgery, radiotherapy, and chemotherapy are the commonly used treatment strategies in cancer patients and most of the chemotherapy drugs destroy the malignant cells through the activation of apoptotic pathways. However, these aggressive methods are not attractive due to their high cost and serious toxicity to both the normal and immune cells (Mohammadi and et al., 2016; Safarzadeh and et al., 2014). Therefore, in order to create innovative therapeutic strategies for cancer treatment, achieving newer, more effective, and safer anti-cancer drugs is an undeniable need (Mohammadi and et al., 2016). *Scrophularia* species are used in traditional medicines for treating different

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diseases such as eczema, ulcers, cancer, etc (Azadmehr, 2013). The effects of *S.megalantha* extract on cancer cell death are unclear. Thus, the objective of the present study was to investigate the cytotoxic effects and cell death pattern of *S.megalantha* on human colon cancer HCT-116 cells, and results indicated that this extract induced cell necrosis in vitro.

Materials and Methods

Preparation of the S. megalantha extract

S. megalantha plants were collected from the Kelardasht region (Mazandaran Province) located in the northern part of Iran in May 2011. A voucher specimen was deposited in the herbarium of the institute with the code number 1461. The plants collected were identified and authenticated by a botanist. The aerial parts of the plants were air-dried, powdered, and then an amount of 10 g of each plant was extracted by the percolation method, using 80% ethanol for three days at room temperature with three changes of the solution. The solvent was completely removed to dryness under reduced pressure at 40 °C in a rotary evaporator. The yield of *S. megalantha* was found to be 10%. The samples were then stored at 4 °C until further experiments were conducted.

Cell culture

In order to examine the effects of the extract on both the colon cancer and normal fibroblast cells, two kinds of cells were used in this study: the human colorectal carcinoma cell line, HCT–116, which was purchased from the Pasteur Institute of Iran and the normal human skin, which were isolated from the foreskin, following the method already described (Pandamooz and et al., 2012). Briefly, the HCT–116 and fibroblasts were cultivated in 75 cm^2 cell culture flasks, using the Roswell Park Memorial Institute(RPMI) 1640 medium and Dulbecco's Modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin. The cell culture flasks were then incubated in 5% CO_2 at 37 °C.A confluence of 80–90% was regarded as appropriate for passage (Fig1). The cell number and cell viability were determined using the Neubauer hemocytometer and the trypan blue exclusion test, respectively.

The MTT colorimetric assay

The cytotoxicity and viability of the fibroblasts and HCT–116 cells were measured by the MTT assay. In this assay, the HCT–116 (1.5×10^4 cells/well) and fibroblasts (8×10^3 cells/well) were seeded into 96-well plates, containing 200 µL of the cell culture media and were allowed to attach by incubating them for 48 h in 5% CO_2 at 37 °C. In the following step, the culture media were replaced by fresh media, containing different concentrations (0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000 µg/mL) of the *S. megalantha* extract and were added to the wells in triplicate. On the other hand, the control wells received no extract and such untreated cells were regarded as the control cells. The MTT assay was performed after 48 h, in which, the culture media were aspirated off, the cells were washed with phosphate buffered saline (PBS), and 50 µL of the MTT solution (5 mg/mL in PBS, Sigma) was added to each well, followed by incubation for an additional 4 h at 37 °C. Subsequently, 150 µL of the solubilization solution (isopropanol, 0.33% HCl, 0.1% Triton X–100) was added to each of the wells and mixed gently at room temperature, in order to dissolve the formazan crystals formed. The absorbance (OD) was measured at 570 nm. The following formula was used to calculate the percentage inhibition of cell growth:

$$\% \text{ Inhibition} = (\text{Test OD} - \text{Control OD}/\text{Control OD}) \times 100$$

All the above experiments were repeated three times separately and the results were shown as mean ±SD. The GI50 (50% of growth inhibition) index was determined on the basis of the percentage inhibition of cell growth versus the extract concentration curves.

Flow cytometry analysis

The necrotic and apoptotic cells were detected by the AnnexinV-FITC (fluorescein isothiocyanate) Apoptosis Detection Kit (eBioscience, USA).The HCT–116 cells were plated into 6-well plates (n=3) and then the cells were exposed to the *S.megalantha* extract at the concentrations of (922, 62.6, 10 µg/mL) as GI99, GI50, and GI1, respectively. A total of three wells were considered as the control wells and received no extracts. The plates were incubated at 37°C in 5% CO_2 for 48 h. Subsequently, the cells were harvested and resuspended in PBS and binding buffer. Ultimately, the stained cells were analyzed by flow cytometrywith Annexin-FITC and PI (propidium iodide) solution.

Statistical analysis

One-way ANOVA followed by Tukey's post-hoc test was performed to compare the differences between the group means. The SPSS version 16.0 software was used for statistical analysis. A value of P<0.05 was considered to be statistically significant.

Results

Inhibitory effect of S. megalantha on the growth of the HCT–116 and fibroblasts

For determining the cytotoxic effect of the herbal extract on the HCT–116 cancer cells and normal fibroblasts, the MTT assay was done after these cells were incubated with different doses of the *S. megalantha* extract (0.001–10000 µg/mL). The extract significantly inhibited the growth of both cells in a dose-dependent manner (Fig.2, 3). At a concentration of 1000 µg/mL, the *S. megalantha* extract inhibited the growth of both the cell lines by approximately 99% compared to the control groups. In both the cell lines, no significant reduction in cell growth inhibition was seen at lower concentrations of the extract (0–10 µg/mL). Our data showed that the sensitivity of both the normal fibroblasts and malignant cells, which were exposed to the various concentrations of the extract, was almost similar (the HCT–116 GI50/fibroblastGI50 index for *S.megalantha* was1.26).

The S. megalantha extract induces necrotic and apoptotic cell death pattern in the HCT–116 cells

In order to determine the effect of the extract on the kind of cell death in the HCT–116 cells, the Annexin V-FITC assay was performed. Data analysis revealed that the concentrations of the *S. megalantha* extract and cell death have a dose-dependent association (p<0.0001, r=0.9). Although increasing the concentrations of the *S. megalantha* extract leads to an increase in the necrosis rates compared to the control, however at high doses of this extract apoptotic cells rate also were increased (Fig3).

Discussion

In order to maintain tissue homeostasis, the immune system substitute's new cells with damaged, unwanted, and old cells, using the cell death processes such as apoptosis and necrosis (Gao and et al. 2011). One of the main characteristics of the cancer cells, which distinguish them from the normal cells, is their resistance to apoptosis (Mansoori and et al., 2014). On the other hand, chemotherapy is used to kill the cancer cells by the induction of apoptosis or necrosis (Nisa and et al., 2017)

Resistance to current chemotherapeutic agents in many colon cancer patients is known to be due to the over expression of the ATP-binding cassette transporters (ABC transporters) (Hu and et al., 2016). ABC transporters play a key role in warding off toxins and drugs from the cells just like pumps (Davidson and et al., 2008). Therefore, it is interesting to note that these transporters increase and help the efflux of anti-cancer drugs from the cancer cells in colon cancer patients. As a result, the therapeutic effects are decreased and the cancer cells evade apoptosis (Hu and et al., 2016)**.**

Hence, the medicinal plants have gained much attention in the treatment of cancer due to their fewer side effects compared to the chemical drugs (Gao and et al. 2011). In this study, the hydro alcoholic extract of *S. megalantha* was used to induce cell death and inhibit the growth of the colon carcinoma cell line, HCT–116and the human skin fibroblast in a dose-dependent manner. Fibroblasts were considered as the normal cells. The results of flowcytometry demonstrated that the dominant cell death pattern of the *S. megalantha* extract was necrosis when they were treated with 10µg, 62.6µg and 922µg of the extract as GI1, GI50 and GI 99 respectively, while apoptosis was observed mainly was observed after exposure to elevated extract concentration (922 µg/mL of the extract as GI99).

Azadmehr et al showed that the extract of S. megalantha inhibited tumor cell growth in vitro and could cause significant growth inhibition of Jurkat cell line in a dosedependent manner (Azadmehr and et al., 2011).

Similar results were reported in a study, using flow cytometry, KP018, an extract of *the Thai plant* Ellipeosis cherrevensis, induced necrosis in cancer cell lines, paclitaxel-resistant HepG2 (PR-HepG2)and colon-26 cells (Yumoto and et al., 2012).

Conclusion

To the best of our knowledge, this is the first report on the necrotic and apoptotic cell death caused by the plant *S. megalantha*. Although the underlying possible mechanisms on cell death are obscure, it is supposed that a change in the membrane permeability or a decrease in the ABC transporters leads to the death of the cancer cells.

The cytotoxicity of the crude extract on the normal fibroblast cells was also studied and it was concluded that the plant has the same effect on both the normal and cancer cells. The purification of the active components from these plants can perhaps eliminate the cytotoxicity of this extract on the normal fibroblast cells. Therefore, further *in vivo* experiments are needed to identify the potential anti-cancer properties of the *S. megalantha* extract and its components.

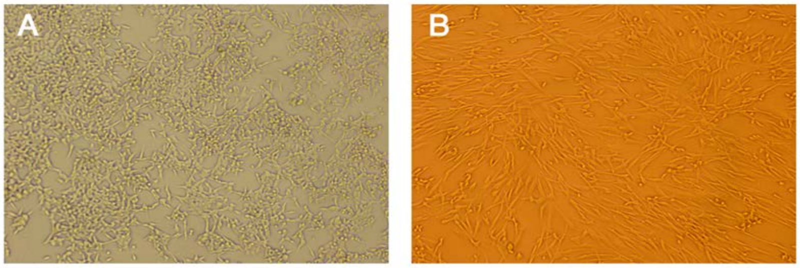

Fig. 1.A) HCT-116 cells, B) normal human skin fibroblasts in cell culture media. Magnification=×400

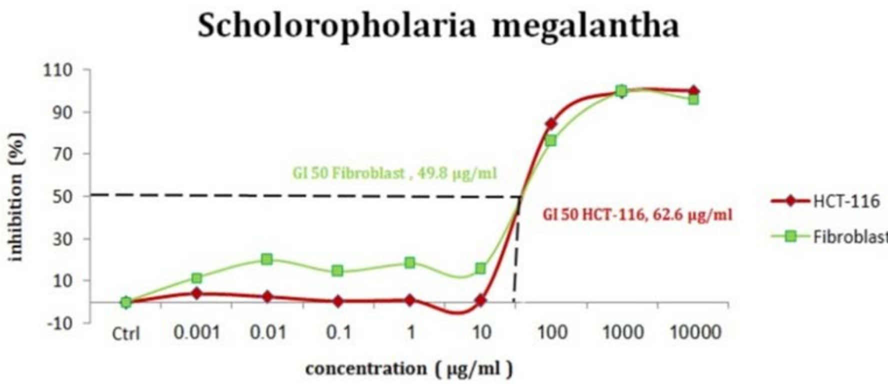

Fig 2. The Effect of Scrophularia megalantha extract on viability of HCT-116 cells and fibroblasts. Both cells were incubated with selected concentrations (0–10000 µg/ml) of extract for 48 h. Results indicated that the extract inhibited both cells population growth in compared to non-treated (control group) cells. Results shown are representative of three independent experiments.

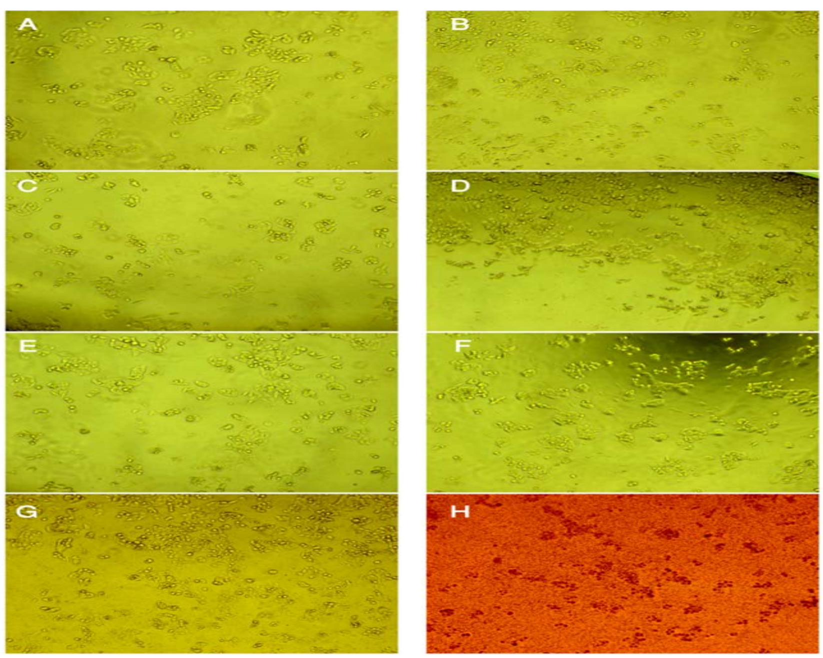

Fig 3. Microphotographs showing the inhibitory effect of Scrophularia megalantha on HCT-116 cell growth. (A,B, C, D, E, F, G, H) indicated accumulation of HCT-116 cells treated with 0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000 µg/ml of Scrophularia megalantha extract for 48 hr, respectively. The photographs were taken directly from culture plates using a phase microscope. Magnification=×400.

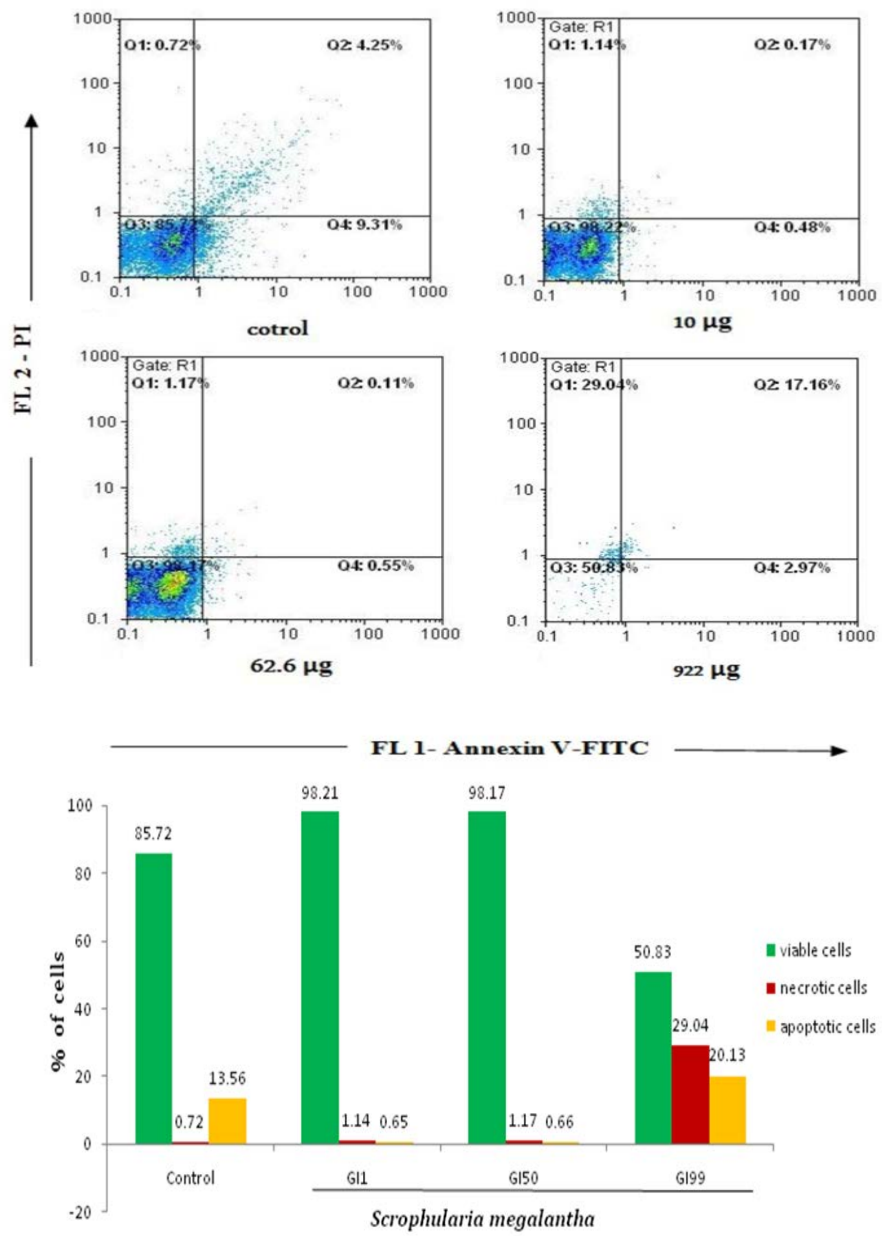

Fig 4. Flow Cytometry results of HCT-116 in the presence of ***Scrophularia megalantha*** extract (10 µg/mL, 62.6 µg/mL and 100µg/mL concentrations as GI1, GI50 and GI99, respectively) and a control group without ***Scrophularia megalantha*** extract, after 48 hours of incubation. X and Y axes are related to Annexin V and PI, Consecutively

Conflict of interest statement

The authors have declared no conflict of interest.

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