Original Article

Antiproliferative and Pro-Apoptotic Effects of Glaucium Flavum Extract on A549 Lung Cancer Cells

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Abstract

Introduction: Glaucium Flavum has recently been studied by researchers and pharmacists and has been attributed to its antioxidant, antiproliferative properties. The alkaloid compounds of this plant are also widely used in the pharmaceutical industry as decongestants and antitussives.

Materials and Methods: In this experiment, first, the cell class (A549) was cultured in DMEM culture medium containing 10% FBS and then treated with different concentrations of Glaucium Flavum. MTT assay was performed to determine IC50 and compare the viability percentage of treated cells with different concentrations of Glaucium Flavum on days 1, 3, and 5. The qRT-PCR test was used to investigate the effects of Glaucium Flavum with IC50 concentration on the induction of apoptosis, and expression of genes including P53, Bax, Bad, and Bcl2. Obtained results were analyzed by SPSS software using ANOVA test.

Results: MTT results showed that Glaucium Flavum causes cell death and reduces the viability of cancer cells, which was observed in the form of cell shrinkage, nucleus shrinkage, and chromatin density and determination of 10 µg/ml concentration as IC50 of A549 cells. An increase in the expression of Bax, P53 and bad apoptotic genes, and a decrease in the expression of the Bcl2 gene also indicate the induction of apoptotic death and the lethal effect of Glaucium Flavum.

Conclusion: Finally, it can be said that Glaucium Flavum, due to its rich content of alkaloid and antioxidant compounds, can be a good option to replace it with chemical drugs in the treatment of lung cancer.

Keywords: Glaucium Flavum, lung cancer, apoptosis

1. Introduction

Lung cancer or bronchogenic carcinoma is the second-best known cancer all over the world. This is the second deadliest and most sumptuous cancer in the world. The origin of this cancer is epithelial cell lining of air ways that is known as lung cancer [1]. Today, lung cancer is the leading cause of cancer mortality in women (26%) and men (29%) and the second most common cancer, accounting for 15% of all cancers after breast cancer in women and prostate cancer in men. The main cause of lung cancer is long-term exposure to carcinogens, especially in tobacco smoke and generally tobacco use. A large proportion of lung cancer is related to smoking [2]. Other factors influencing the carcinogenicity of the body's by-products of metabolism and aerobic metabolism and error that occur during DNA replication include oxygen-free radicals from aerobic metabolism and involvement in carcinogenesis [3]. Preventing DNA synthesis, controlling the production of free radicals and regulating the cell cycle, and inducing cell death in apoptosis are among the goals of cancer treatment [4]. The process of apoptosis, or

the programmed cell death, is a way of protecting and controlling genes that are used in order to remove abnormal and unwanted cells in living organisms and prevent the tissues from becoming cancerous [5]. Another major damaging factor in cancer is oxidative stress caused by free radicals, and since many plants contain antioxidant compounds, they also have anti-cancer effects [6]. From ancient times to the present, traditional medicine has been used for treating a variety of diseases, including cancer. Herbal medicines are among the medicines used in traditional medicine, and have attracted the attention of researchers due to their minor side effects compared to chemical drugs [7]. The species of Glaucium Flavum, known as Yellow Horned Puppy, belongs to the genus Papaveraceae family of the order Ranunculales, one of which is a herbal remedy that has been used for a long time [8]. It is a saltwater plant and grows widely in salt marshes and swamps such as Europe, North Africa, West Asia, and the United States. The name Glaucium is derived from the Greek word glaucos and comes in greenish-blue leaves and the word Flavum refers to its yellow flowers [9]. In the phytochemical analysis of this plant, the presence of alkaloid compounds such as Aporphine, Protopine and Protoberberine and Glaucine has been shown to be among the most important alkaloid compounds under the Aporphine family [10]. Alkaloids are a group of natural and chemical compounds that have the strongest pharmacological activity and toxic properties among plant-synthesized substances [11]. Glaucium Flavum has also been studied by researchers and pharmacists due to its alkaloid composition and has been attributed to its antioxidant, antiproliferative, antimicrobial and anti-inflammatory properties [12]. Also, the alkaloid compounds of this plant are widely used in the pharmaceutical industry as pain relievers, decongestant, and antitussives [13]. There are several other plant species of the genus Glaucium, such as Allium Jesdianum and Chelidonium majus, which are used today for the treatment of human tumors due to the presence of alkaloid compounds [14, 15]. Therefore, due to the importance of alkaloids in the pupae family and their widespread use in the pharmaceutical industry, they have been the subject of many researchers' studies on anti-cancer drugs [16]. The cell and its mechanism of action in the treatment of cancer. Therefore, in the previous study, we tried to investigate its effect on cellular toxicity and expression of Bax, Bad, P53 and BCl-2 genes involved in the apoptosis process on lung cancer cells or A549 category.

2. Materials and Methods

2.1 Collecting & Preparation of Glaucium Flavum Extract

In order to collect Glaucium plants from the southern regions of Iran, the aerial parts and flowers of the plant were dried in the presence of light in the electric mill after drying in the vicinity of the light and were transferred to the laboratory for preparing the extract. The resulting powder was soaked in a ratio of 50/50 with 96% ethanol-water and alcohol for 72 hours and was then strained. Finally, it was placed in an oven at 40°C until the water and alcohol evaporated and thick coffee-milk-like liquid remained. From 1000 grams of the dry weight of the plant, 100 grams of the pure extract was obtained.

2.2 Cell Culture

For cell culture and passage, the cellular class of lung carcinoma (A549) was first prepared from the cellular bank of Pasteur Institute of Tehran. The cells were then counted and the viability percentage was determined and cultured in DMEM (Dulbecco's modified Eagle's medium Gibco, USA) containing 10% FBS (Fetal bovine serum, Gibco, USA). Then in the incubator (Sina Company, Iran) with 5% CO2 and 95% humidity was kept at 37°C.

When the density of the cells in the flask reached 80%, a cellular passage was performed and the cells were cultured with an approximate number of cells of 1×10^4 cell/cm in a 96-well plate containing the usual culture medium. Different concentrations of Glaucium Flavum hydroalcoholic extract, including 1, 10 100 and 1000 µg/ml, which were dissolved in a suitable solvent (water with final DMSO concentration of 0.2%) were added to the culture medium containing the flasks containing these cells and incubated for 24 hours, and on days 1, 3, and 5, cell viability was assessed.

2.3 MTT Test

The MTT test (3- (4, 5 Dimethylthiazol-2-yl) -2.5-Diphenyltertrazolium Bromide) was used to assess the viability and determine IC50. The basis of MTT is the use of formazione color reduction test in the presence of dehydrogenase enzymes. When cancer cells numbered about 1×10^4 cell/cm^2 in each well, plates containing DMEM culture medium with 10% FBS were cultured from 96-well plates. Twenty-four hours later, the cells were treated with concentrations of 1, 10, 100, and 1000 µg/ml of Glaucium Flavum extract for 1, 3 and 5 days. For each cell, a control group was considered that was not treated. The MTT test was then performed as follows. At the desired time, the culture medium was removed from the wells containing the cell and about 100µl fresh medium containing 10µl MTT solution (with a concentration of 5 mg/ml) was added to each cell. The cells were stored for 3 hours at 37°C. They were incubated and then the MTT solution was removed and added to each 100 ml DMSO well (Dimethyl sulfoxide Merck, USA, 100%).

To morphologically study A549 cells from control groups treated with IC50 concentration on days 1, 3, and 5, 96-well plates are photographed with a digital camera connected to a reverse microscope with a 20 and 40 object lens, and microscopic images for each cell in control and assess groups were evaluated and compared.

2.4 Real-Time Quantitative PCR

Extraction of RNA was performed according to the kit protocol of Cinnagen and Iran's manufacturing company and all the steps were done according to the protocol, using kit solutions. Finally, Nanodrop device was used for measuring RNA and the quality and size of RNAs at 260/280 nanometers. After ensuring the purity of RNA, cDNA synthesis was performed using the HyperScript kit (GeneAll, Portugal) and the steps were performed according to the instructions. For 2µl of synthesized cDNA and 0.5µl of Forward Primer, 0.5 µl of Reverse Primer, 2µl of SYBR Green and 5µl of distilled water using Power SYBER Green master mix (Qiagen, Japan) in the final volume of 10 and by qRT-PCR was performed.

The qRT-PCR test is performed in 3 steps in the thermal cycler. The first stage of the denaturation reaction was performed at 95℃ for 30 seconds, then the melting operation at 95℃ for 1 second, and finally the third application at 60℃ for 33 seconds at 40 cycles. Data analysis (CT) of each sample was performed using StepOne software and normalization was performed using GAPDH (Housing Control Gene) gene, so that to estimate the percentage of changes (Fold Change), the gene expression of Bax, Bad, P53, and Bcl2 were determined in comparison with the difference between the GAPDH gene threshold cycle among the control sample (without drug treatment) compared with the sample treated with Glaucium Flavum and each test was repeated three times. From the NCBI site, the sequence of primary primer gene sequences was obtained, and direct and reverse primers were designed using Gen runner software and primer express. The sequence of related primers is presented in the table below. Obtained results were

analyzed by SPSS software, using ANOVA test.

Table 1. Used promoter sequences

Name	Primer Sequence (5'→3')	Tm (C)
BAX (F)	GCTGGACATTGGACTTCCTC	58.5
BAX (R)	ACCACTGTGACCTGCTCCA	
GADPH (F)	GCAAGAGCACAAGAGGAAGA	57
GADPH (R)	ACTGTGAGGAGGGGAGATTC	
BCL-2 (F)	GATGGGATCGTTGCCTTATGC	58.8
BCL-2 (R)	CCTTGGCATGAGATGCAGGA	
BAD (F)	CGGAGGATGAGTGACGAGTT	58.3
BAD (R)	CCACCAGGACTGGAAGACTC	
P53 (F)	GGAGGGGCGATAAATACC	57.25
P53 (R)	AACTGTAACTCCTCAGGCAGGC	

3. Results

MTT Analysis in Cells Treated with Glaucium Flavum

The viability percentage of cancer cells class A549 treated with different concentrations of Glaucium Flavum in the tested days were compared to the control sample in the image below. Based on the results of the MTT test, the concentration of 10 µg/ml of Glaucium Flavum was determined as IC50 of A549 cells within 24 hours (P <0.05). The rate of cellular toxicity of Glaucium Flavum depends on the dose and decreases significantly with increasing cellular viability concentration. According to the diagram, the maximum cell toxicity (decrease in cell viability) and the minimum cellular toxicity were observed at a concentration of 1000 and 1 µg/ml of Glaucium Flavum, respectively, for the A549 (P <0.05) (Figure 1). Also, the comparison of cell viability with IC50 concentration of Glaucium Flavum on the first, third and fifth days in Figure 2 shows the viability of cells after exposure to IC50 concentration on the third day compared to the control group and also compared to the first day of treatment. The percentage of living cells in the tested groups decreased sharply on the fifth day compared to the control group and on other days, it showed a significant decrease compared to the control sample and the first and third-day samples (P <0.001) (Figure 2).

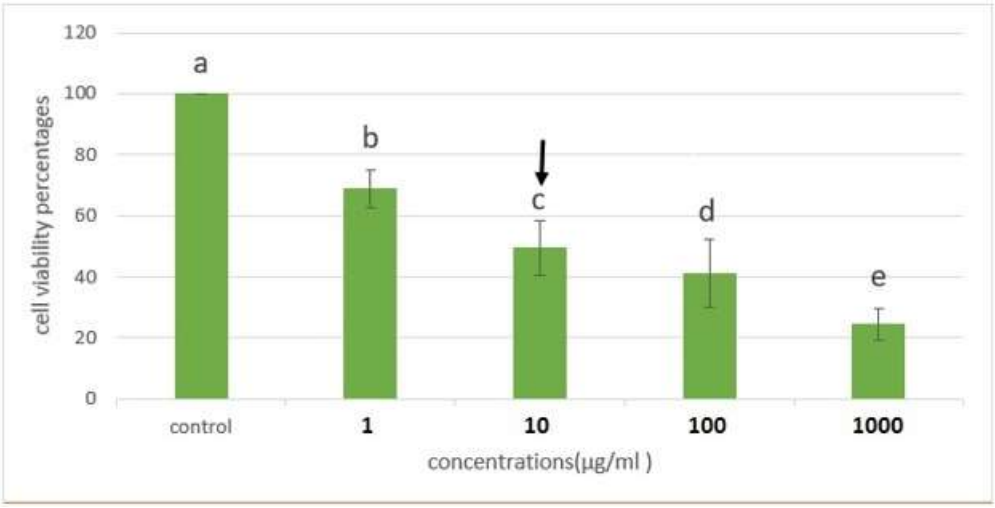

Figure 1. Effects of different concentrations of Glaucium Flavum on A549 cell viability using MTT test and determination of 10 µg/ml concentration as IC50 of A549 cells (different letters indicate significant differences between groups (P <0.05). The arrow indicates the IC50 concentration).

Figure 2. Effects of IC50 Glaucium Flavum concentration on the viability of A549 cells on days 1, 3 and 5 after treatment - Comparison of significant differences between different days in each separate group, different number of symptoms indicates a significant difference in level (P <0.05).(* P <0.05, ** P <0.01, *** P <0.001)

Morphological Changes of A549 Cancer Cell,s Treated with Glaucium Flavum

The morphological examination was performed using conventional invert microscope imaging, as shown in the images below. The nuclei of the cells under treatment became smaller and their membranes were damaged, while healthy cells with intact membranes were observed (Figure 3).

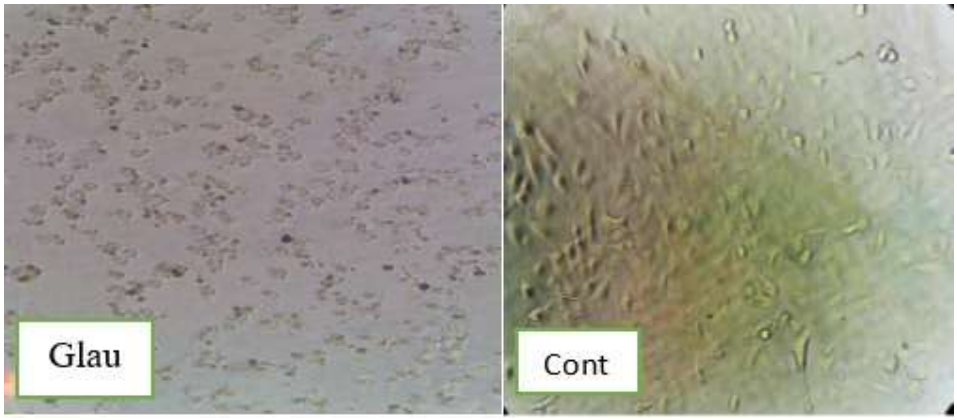

Figure 3. Examination of A549 cell morphology by conventional invert microscopy: Glau- cells treated with an IC50 concentration of Glaucium Flavum; Cont- cells of the control group - small and shrunken nuclei and damaged membranes are signs of cell death.

Evaluation of Expression of Apoptotic Index Genes (Bax, Bad, P53, and BCL-2)

Treatment of A549 cancer cells with IC50 Glaucium Flavum concentration significantly increased the expression of Bax, P53 and Bad apoptotic genes and reduced the expression of the Bcl2 antiapoptotic gene relative to the control group. Glaucium Flavum causes cell death in cancer cells (Figure 4).

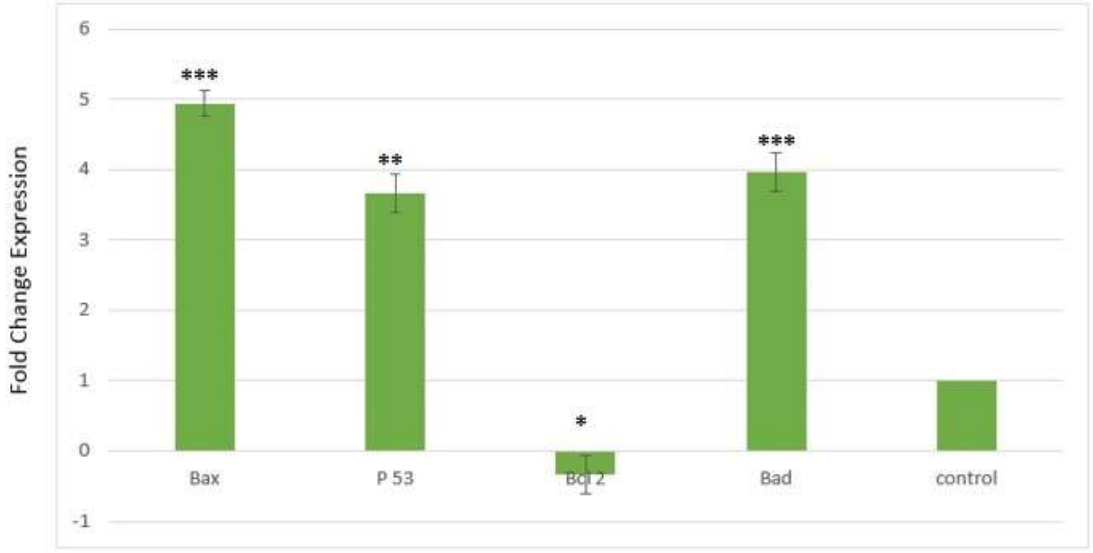

Figure 4. Changes in gene expression in A549 cells under 24-hour treatment with IC50 concentration of Glaucium Flavum- increased expression of Bax, Bad and P53 genes and decreased expression of Bcl2 genes; The control group indicates the induction of apoptotic death in cancer cells by these substances. (*** (P <0.001); ** (P <0.01); * (P <0.05))).

4. Discussion

Cancer is a major public health problem worldwide, and the number of infected patients is increasing every year. In recent decades, radiation therapy, chemotherapy, and surgery have been used to remove cancerous tissues, each with its own weaknesses. Due to the side effects such as not getting the right concentration of the drug to the target tissue, the damaging effects of radiation therapy on other healthy tissues, as well as the problem of both drug resistance, which can be due to the tumor's intrinsic resistance to the drug or acquired during chemotherapy, new anticancer drugs are required. Also, side effects of anticancer chemical drugs are another limiting factor for their use in cancer treatment [17, 18]. Most of the people still use herbal medicine as an alternative for the treatment of this disease [19]. The use of herbal medicine has a long history behind it and medics have used many herbs to manufacture drugs [20]. Glaucium Flavum is known as an analgesic, decongestant, and analgesic in chest diseases, resistant coughs, and asthma due to its richness in alkaloid compounds in the pharmaceutical industry [13]. Therefore, the aim of this study was to investigate the cytotoxic effects of Glaucium Flavum on cancer cells and to the inducing of apoptosis in them, and to suggest a non-chemical, natural drug without side effects. The results of the biological viability analysis of the present study showed that Glaucium Flavum extract was able to cause cell death and reduce the biological viability of A549 cancer cells, and this cytotoxic effect was dose- and time-dependent so that on the fifth day compared to the first day and third, Glaucium Flavum had the highest cellular toxicity. Cell treatment with this extract also led to morphological changes such as the nucleus and cell shrinkage and their condensation, which confirms the occurrence of apoptosis. PCR findings also showed that the Bax, Bad and P53 genes were increased, but the Bcl-2 gene was reduced. In fact, with increased expression of apoptotic genes, apoptosis is induced in cells. Recently, many studies have been conducted on the anti-cancer properties of plant extracts, including Glaucium Flavum. L. In their study, Bournine et al. (2013) found that Glaucium Flavum extract reduced the growth of glioma tumors with signs of tumor shrinkage and extensive necrosis in the treated cells, indicating cell

death of the treated cancer cells. [21]. In a study similar to that of L. Bournine, some authors reported that Glaucium Flavum extract, due to the presence of various alkaloids such as protopine, glaucine, aporphine, and antioxidants, could have anti-proliferative and apoptotic effects on glioblastoma cancer cells, and quantitative MTT results showed that the IC50 concentration for glioblastoma cells is 500 µg/ml [22].

Alkaloids are compounds found in abundance in all plants, especially those used in medicine [23], and most herbal medicines used to treat cancer are alkaloid compounds [24]. Aporphine and glaucine are among the most important alkaloid compounds found in Glaucium Flavum extract, and researchers have found that these compounds have antioxidant and antiviral activity [25]. The main constituent of the Glaucium Flavum root is protopine alkaloid. Protopine reduces the growth of human prostate cancer cells by stopping the division of mitosis [26]. The roots of the Algerian Glaucium Flavum also inhibit the growth of breast cancer cells and the induction of apoptosis into the body due to the presence of protopine alkaloids. The root monopoly of this plant has been able to reduce the biological viability of breast cancer cells (IC50 = 15 µg / ml) and lead to morphological changes such as chromatin shrinkage, DNA shrinkage and shrinkage of treated cells [21]. Of course, Mondher. B et al. (2019) in a study on the properties of compounds found in mountain climates reported that its phenolic compounds are also able to reduce the proliferation of MCf-7 cancer cells, and this effect is dose-dependent. In addition to the antiproliferative properties, the antioxidant properties of Glaucium Flavum extract were also presented [27]. Glaucium Flavum decongestant effect may be due to the apoptotic effect of alkaloid compounds by activating extracellular MAP kinase on MCF-7 cells [28] or by activating the P53 tumor suppressor protein in colon cancer cells (HCT 116) [29 .]. Hippeastrum Vittatum extract is also rich in alkaloid compounds that have the potential to reduce the viability of 5 categories of human cancer cells such as breast cancer (MCF-7), colon cancer (HT29), and non-small cell lung cancer (H460), kidney cancer (RXF393) and ovarian cancer (OVCAR3) [30]. Various pathways for anticancer activity of alkaloids have been reported, including cell death induction by activating BAX, BAD, and P53 genes, and apoptosis progression by induction of DNA damage and caspase activators or cell growth inhibitors. [31]. Plant antioxidants are also involved in the prevention of cancer and the death of cancer cells in various ways, such as induction of apoptosis, prevention of angiogenesis and metastatic growth of cancer [32]. Therefore, Glaucium Flavum has compounds such as glaucine and aporphine and the mechanism of action of Glaucium Flavum in having antiproliferative power can be attributed to the presence of alkaloid compounds with its antioxidant properties and by increasing the expression of BAX, BAD and P53 genes and reducing the expression of BCl-2 gene causes cell death.

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Conflict of interest

The authors declare no conflict of interest.

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