Comparison of the Effect of S14161 Small Molecule and Glaucium Flavum Extract on A549 Cancer Cells

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Abstract

Background: Lung cancer is the fifth most common cancer in Iran. Due to the side effects of common cancer treatments, everyone has turned to herbal remedies and new treatments. This study aimed to compare the effect of S14161 small molecule and Glaucium flavum extract on the induction of apoptosis in A549 cancer cells. Materials and Methods: In this study, the A549 cell line was treated with different concentrations of S14161 small molecule and G. flavum extract compounds. MTT assay was performed to determine the half-maximal inhibitory concentration (IC50) and compare the viability of treated cells on days 1, 3, and 5. Also, the real-time polymerase chain reaction assay was used to investigate the expressions of pro- and anti-apoptotic genes. Results: MTT results showed that both the combination of S14161 and G. flavum extract resulted in cell death and reduced cancer cell viability. However, the viability rate was greater by S14161, and this small molecule significantly increased the expression of Bax, P53, and Bad apoptotic genes and decreased the expression of the Bcl2 gene, which shows the induced apoptotic effect of S14161 in comparison with G. flavum. Conclusion: The results of this study showed that S14161 had fewer IC50 and caused cell death by inhibiting the PI3K/AKT pathway, and Glaucium flavum caused cancer cell death due to its alkaloid compounds. Therefore, both compounds are recommended as drug candidates for the treatment of lung cancer.

Keywords: Lung Cancer; Glaucium Flavum; S14161 Small Molecule; Apoptosis; Gene Expression
Introduction

Cancer is a major health problem worldwide, and the number of patients is increasing every year. To date, more than 100 different types of cancer have been reported in mammalian tissues [1]. Lung cancer is the third most common cancer after breast and colorectal cancers in Europe [2] and the fifth most common cancer in Iran. The incidence of lung cancer is higher in men and is about 73%. The highest incidence is in people aged 55 to 60 years [3].

Carcinogenic factors are lifestyle factors such as environment, reproductive duration, diet, smoking, and air pollution. In addition to the above, there are other risk factors in the body. The metabolic byproducts and breakdowns that occur during DNA replication as well as the byproducts of aerobic metabolism that produce oxygen radicals are mutagenic and are involved in carcinogenesis [4].

The overall goal in the effective treatment of cancers is to inhibit DNA synthesis, control the production of free radicals, regulate the cell cycle, and induce apoptotic cell death [5]. In apoptosis, the regulatory molecules include the anti-apoptotic Bcl2, pro-apoptotic genes (e.g., Bax, p53, and Bad) [6]. It has been found that the consumption of some plant products with high antioxidant properties can help inhibit some types of cancers. Many of these plants are less likely to have adverse effects and have rich sources of antioxidants that can reduce the effects of oxidants or some diseases [7].

Glaucium flavum is known from the Papaveraceae family, the yellow horned poppy or the Kelatin (among the people of southern Iran) and is commonly found in farms, shady places, plain and mountainous areas, and its usage date goes back to ancient times because this plant has been transferred to different parts of the world due to its presence in fields, along with cereals, and due to this dispersion in vast expanses, it has been the therapeutic use of different nations [8].

In the phytochemical analysis of this plant, alkaloids such as aporphine, protopine, protoberberine, and glucine have been observed, and glucine is the most important alkaloid compound of the aporphine subfamily [9].

G. flavum alkaloids have been recognized in the pharmaceutical industry as an analgesic, anticonvulsant, and palliative in breast disease, refractory cough, and asthma [10]. Alkaloids are naturally occurring chemical compounds with the strongest pharmacological activity and toxic properties among plant-derived substances [11]. Recent studies have added antioxidant, antiproliferative, antimicrobial, and anti-inflammatory properties to the potential applications of G. flavum [12, 13].

Coumarins are also a group of natural polyphenolic compounds found mainly in food such as vegetables, fruits, and tea [14]. In recent years, special attention has been paid to the production of coumarin and its derivatives to develop new anticancer drugs. One of these compounds is S14161 or 8-Ethoxy-2-(4-fluorophenyl) -3-nitro-2H-1-benzopyran, 8-Ethoxy-2-(4-fluorophenyl) -3-nitro-2H-chromene [15].

Coumarin-derived S14161 has been identified as a high-throughput PIK3 inhibitor that directly inhibits PI3K phosphoinositide 3-kinase in the PI3K / AKT signaling pathway. PI3K levels are significantly increased in most cancers, increasing the proliferation of cancer cells [16].

Coumarins have a wide range of biological activities, including antioxidant, anti-inflammatory, anticancer, antiviral, and have antiproliferative and apoptotic stimulants in cancer cells [17]. The present study aimed to compare the effect of apoptotic induction and expression of apoptotic and anti-apoptotic genes by the extract of G. flavum and S14161 small molecule on the A549 cancer cells.

Materials and Methods

Collecting G. flavum

After collecting the G. flavum plant from the southern parts of Iran, the aerial parts and flowers of the plant were dried after exposure to light in a power grinder and transferred to the laboratory for extraction. Dissolve the resulting powder in a 50/50 ratio with 96% ethanol and water for 72 hours and then
smooth and placed in an oven at 40°C until water and alcohol evaporate, and a coffee syrup-like thick solution remains. From 1000 g dry weight, 100 g pure extract was obtained.

**Preparation of G. flavum Extract**
To prepare 1, 10, 100, and 1000 µg/mL *G. flavum* concentrations, first, the powder prepared for each concentration was dissolved in the culture medium and then filtered and kept at refrigerator temperature (4°C) until use.

**S14161 Small Molecule Preparation**
To prepare this small molecule, concentrations of 1, 2, 5, and 10 μM were prepared from S14161 (Sigma, Germany) and then filtered using a 0.22μm syringe filter at 4°C until use.

**Cell Culture and Passage**
The lung carcinoma cell line (A549) was obtained from the Pasteur Institute of Cell Bank (Tehran, Iran). The cells were counted, and their viability percentage was determined. Then, they were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA). They were then stored in an incubator (Sina Co., Iran) with 5% CO2 and 95% humidity at 37°C. When the density of cells in the flask reached 80%, cell passage was performed, and cells were cultured with approximately 1×104 cells/cm2 in a 96-well plate containing a normal medium. After 24 hours, cells were treated with different concentrations of the hydroalcoholic extract of *G. flavum* and S14116. Cell viability was evaluated on days 1, 3, and 5.

**Cell Viability Assessment**
The 3-(4, 5 Dimethylthiazol-2-yl)-2,5-Diphenyltertrazolium bromide (MTT; Sigma, USA) assay was used to evaluate cell viability, and cancer cells had cultured with approximately 1×104 cells/cm2 per each pit containing DMEM medium with 10% FBS. After 24 hours, cells were treated with 1, 10, 100, and 1000 µg/ml concentrations of *G. flavum* extract and 0, 5, 10, and 20 μM concentrations of S14116 for 1, 3, and 5 days. A control group was also assigned to each untreated cell.

Then the MTT test was performed as follows. At certain times, the culture medium was removed from the pits containing cells, and 100 μl of fresh medium containing 10 μl MTT solution (5 mg/ml concentration) was added to each pit. The cells were incubated for 3 hours at 37°C.

Then, MTT solution was extracted, and 100 µl of dimethylsulfoxide (DMSO; Merck, USA) was added, and optical absorption of the samples was measured at 570 nm using a microplate reader (Biotek, Germany).

**Imaging**
For the morphological study of A549, cells from control and treatment groups were photographed on days 1, 3, and 5 on 96-well plates using a digital camera connected to an inverted microscope (sigma, USA) with a lens of 20 and 40, respectively. Cells were examined and compared in control and treatment groups.

**RNA Extraction**
RNA extraction was performed according to the extraction kit protocol (CinnaGen, Iran), and finally, a NanoDrop was used to measure RNA and measure the properties and size of RNAs at wavelengths of 260-280 nm. The quality and quantity of extracted RNA were confirmed by electrophoresis and Nanodrop (Thermo Fisher's scientific instruments, USA).

**cDNA Synthesis**
HyperScript (GeneAll, Portugal) kit was used for cDNA synthesis according to the manufacturer's instructions. Then for each sample, 2µm of cDNA was synthesized and 0.5µm forward primer, 0.5µm reverse primer, 0.5µm SYBR Green, and 5µl of distilled water were performed using power SYBER Green master mix (Qiagen, Japan) in a final volume of 10µl by real-time polymerase chain reaction (RT-PCR).

**RT-PCR**
A thermal cycler was used to measure gene expression. The time-heat program was carried out.
out in three stages. Briefly, the denaturation reaction was performed at 95°C for 30 seconds, then melted at 95°C for 1 second, and finally, the third application stage was at 60°C for 33 seconds for 40 cycles. Data analysis of each sample was done using StepOne software (Australia), and normalization was performed using the GAPDH gene in order to estimate the fold change percentage of Bax, Bad, Bcl2, and P53 were compared to control samples in comparison to the small molecule and G. flavum treated samples, and each experiment was repeated three times. Gene sequences were extracted from the NCBI site, and direct and reverse primers were designed using Gen runner (USA) and primer express software (USA). The results of the genes expression were evaluated by the LIVAC method (Table-1).

Ethical Statement
This study was approved by the Institutional Review Board of the Tehran Medical Sciences, Islamic Azad University, Tehran, Iran (approved code: IR. IAU. PS.REC.1399.071).

Results

Evaluation of Cell Viability in Cells Treated with G. Flavum
Comparison of cell viability was performed using MTT assay and viability rate of A549 cancer cells treated with different concentrations of G. flavum on tested days compared to control. Based on the MTT assay, 10 μg/mL concentration of G. flavum was determined as half-maximal inhibitory concentration (IC50) in A549 cells within 24 hours (P<0.05). The lethal effects of this compound on A549 cell viability were dose-dependent and decreased with increasing concentration of cell viability. The percentage of cell viability showed based on the resuscitation test in cells treated with G. flavum concentrations (Figure-1). Maximum cytotoxicity (decrease in cell viability) and minimum cytotoxicity were seen at 1000 and 1 µg /mL concentrations of A549, respectively.

Evaluation of Cell Viability in Cells Treated with S14161 Small Molecule
In the cell viability assay using MTT assay, the IC50 of S14161 small molecule was determined as 5mM on A549 cells treated for 24 hours. The results showed that the cytotoxic effects of this compound on A549 cell viability were dose-dependent and decreased with increasing concentration of cell viability (P<0.05). Cell viability was

<table>
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<th>Genes</th>
<th>Primer Sequence (5’→3’)</th>
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| Bax   | F: GCTGGACATTGGACTTCCTC  
      | R: ACCACTGTCACCTGCTCCA  |
|       | F: CGGAGGATGAGTGACGAGTT  
      | R: CCACCAGGACTGGAAGACTC  |
| Bad   | F: GATGGGATCGTTGCCTTATGC  
      | R: CCTTGGCATGAGATGCAGGA  |
| Bcl2  | F: GGAGGGCGATAAATACC    
      | R: AACTGTAACTCCTCAGGCAGGC  |
| P53   | F: GCAAGAGCACAAGAGGAAGA  
      | R: ACTGTGAGGAGGGAGATTC  |
| GAPDH | F: GCAAGAGCACAAGAGGAAGA  
      | R: ACTGTGAGGAGGGAGATTC  |
measured by MTT assay in cells treated with concentrations of 1, 2, 5, and 10 mM S14161 (Figure-2). Maximum and minimum cytotoxicity were observed at concentrations of 10 and 1 mM, respectively.

**Comparison of the Viability of A549 Cells Treated with S14161 Small Molecule and G. Flavum on Days 1, 3, and 5**

Cell viability by MTT assay showed that A549 cancer cells showed a significant decrease on the first day after treatment with IC50 concentration of S14161 small molecule and *G. flavum* (P<0.05). Cell viability was significantly decreased after exposure to the same concentrations on the third day (P<0.01). The percentage of cell viability in the experimental groups decreased significantly on the fifth day compared to the control and the other days, which showed a significant decrease compared to the control and the first and third days’ samples (P<0.001, Figure-3).

**Effect of S14161 and G. Flavum on Morphological Changes of A549 Cancer Cells**

The cells treated with IC50 concentrations of *G. flavum* and S14161 showed significant and dose-dependent differences compared to control cells.

One of the major changes was that the nuclei of the cells contracted, and their membranes were damaged, while intact cells were observed with intact membranes. Our observations were in agreement with MTT results that the cells apoptotic in the S14161-treated group were higher than in the *G. flavum* group (Figure-4).

**Evaluation of Gene Expression Changes in A549 Cells Treated with IC50 Concentration of S14161 Small Molecule and G. Flavum**

Treatment of A549 cells with IC50 concentration of S14161 small molecule and *G. flavum* significantly increased apoptotic *Bax*, *P53*, and *Bad* genes expression and decreased *Bcl2* expression. However, this increase was more pronounced in 24-hour-treated cells with an IC50 concentration of S14161 small molecule than in the *G. flavum*, especially in the expression of *P53* (P<0.01) and *Bad* (P<0.05) gene expression, this increase was significant between S14161 and *G. flavum* (Figure-5).

Also, the expression ratio of *Bax/Bcl2* genes and *Bad/Bcl2* ratios were higher in A549 cells treated with IC50 concentration of S14161 compared to cells treated with IC50 concentration of *G. flavum* (P<0.001 and P<0.01, respectively).

This significant difference in gene expression ratio indicates that *Bax* and *Bad* genes were more expressed in S14161-treated cells than in *G. flavum* and *Bcl2* gene compared to cells treated with S14161. *G. flavum* is less frequently expressed as a result of the induction of apoptotic death by the S14161 small molecule more strongly than *G. flavum* (Figure-6).
Figure 3. Effects of IC50 concentration of G. flavum (G3) and S14161 (G1) on A549 cell viability on days 1, 3, and 5 after treatment. Comparison of significant differences between different days in each individual group, number of different signals indicating differences significance at the level (P<0.05).

Figure 4. Morphology of A549 cells by invert microscope. S14161- and G. flavum-treated cells with IC50 concentration compared to control cells (original magnification: 20×)

Figure 5. Gene expression changes in 24-hour A549 cells treated with IC50 concentration of S14161 small molecule (G1) and G. flavum (G3). Increased expression of Bax, Bad, and P53 genes and decreased expression of Bcl2 in A549 cells treated with IC50 concentration of S14161 small molecule and G. flavum in cell line compared to the control group showed induction of apoptosis in cancer cells. *P<0.05, **P<0.01.

Discussion

Cancer is one of the deadly and worrisome diseases in developed countries today. These include cancer, surgery, chemotherapy, and radiotherapy. Due to the side effects of chemotherapy and conventional cancer radiotherapy, everyone is turning to herbal remedies and new treatments. Extensive research has been carried out to identify natural compounds with anticancer properties, which necessitate the identification of the anticancer molecular mechanism of many compounds, especially plant-derived compounds [18]. Recently, both in Iran and in other countries, much attention has been paid to the cytotoxic effects of plant extracts, including Meimandi and Yaghoobi have tested the effect of aqueous and ethanolic extracts of Rosa damascena flowers on human gastric cancer cells and observed the induction of programmed death in these cells [19]. Haji et al. (2013) also evaluated and confirmed the cytotoxicity of G. flavum extract on cancer cell lines such as HT-29, Caco-2, T47D, and NIH/3T3 by MTT assay. Properties were attributed to the alkaloids of this plant [20]. In another study on the properties of the same extract, it has been reported that protoberberine-derived berberine from G. flavum alkaloids can counteract the tumor [21]. Also, the cytotoxic effect of Papaver rhoeas plant extract alkaloids on breast cancer cells, a human clone, has been studied by researchers and has shown that the berberine combination of other extract compounds has the highest cytotoxicity on cancer cell viability [22].
Several other species of the family and genera of the Papaveraceae, such as Chelidonium Mojus and Sanguinaria Canadensis L. have been studied and shown to be used today for the treatment of human tumors due to the presence of alkaloids [23, 24]. Consequently, due to the importance of the alkaloids of the Papaveraceae family, these compounds have been the subject of many drug studies by researchers as anticancer drugs. It should be noted that there have been no studies on the cytotoxic effects of the G. flavum extract on the studied cancer cell lines (A549).

Therefore, the present study investigated the anticancer activity of this extract on the A549 cell line. MTT assay confirmed that 100 μg/ml concentrations of G. flavum, as the IC50 of A549 cells within 24 hours, were able to induce apoptosis in the A549 cell line. Also, the cells that were treated with this extract on the fifth and third day had more cell death than the first day and the control group.

The expression of pro-apoptotic genes such as Bax, P53, and Bad by G. flavum increased, and the anti-apoptotic Bcl2 gene expression decreased. Generally, this extract induced cell death on A549 cells.

Concerning the mechanism of the effect of plant extracts on cancer cells, alkaloids have been shown to have various pathways for anticancer activity, including induction and activation of apoptotic inducer proteins, the progression of apoptosis by induction of DNA damage, and activators of caspases or inhibitors of cell growth [25].

The major constituent of the G. flavum root is protopine alkaloid, which was shown in a study that protopine exerts its antiproliferative effect by stopping mitosis in human prostate cancer cells [26].

Similar research was done on other cell lines called MDA-MB-435, MDA-MB-231, and HS578T as human tumor cells and MCF10A, HUVEC, and skin fibroblast cells as healthy controls. It has led to apoptosis in cancer cells but has also been shown to be less toxic to healthy cells [27]. Plant antioxidants have also been shown to prevent cancer and selective killing of cancer cells, such as inducing apoptosis, preventing angiogenesis, and metastatic cancer growth [28].

Therefore, in the present study, the mechanism of action of G. flavum in anticancer activity on cancer cells of A549 cell line has its antioxidant activity and neutralizes harmful free radicals as well as inducing apoptosis. An increase in the expression of Bax, Bad, and P53 genes and a decrease in Bcl2 expression and cell cycle arrest were attributed to the presence of alkaloids.

Nevertheless, in addition to investigating the antiproliferative effect of this extract in the present study, the anticancer activity of the S14161 small molecule has also been studied and compared.

Today, new compounds called small molecules are used to treat diseases. The S14161 small molecule with the ring structure of gasoline and Pyron is abundantly found in nature. S14161 has been implicated as a potent inhibitor of PIK3 in the PI3K/AKT signaling pathway [14].

The PI3K pathway is crucial in regulating cyclin D expression and transcription, and PI3K has several effects, including stimulating cell proliferation and inhibiting apoptosis [29]. As cyclin D levels increase in cancers, small molecules that can inhibit cyclin D expression can play an effective role in cancer treatment [29]. S14161 exerts its anticancer properties against cyclin D inhibition (PI3K). The positive results of this compound have been proven in inherently malignant cancers such as leukemia and melanoma [30].

In the present results, the percentage of cell
viability in A549 cancer cells treated with IC50 concentration of S14161 was dose and time-dependent, and 5 μM was determined as IC50. In MTT evaluation on day 5, the viability rate was significantly decreased compared to days 1 and 3 as well as the control group. Morphological studies also confirm the cell death caused by this small molecule. In gene expression assays, the expression of pro-apoptotic genes such as \textit{Bax}, \textit{P53}, and \textit{Bad} was increased by S14161 and decreased expression of the anti-apoptotic \textit{Bcl2} gene. These data suggest that this small molecule induces cell death in A549 cancer cells. In recent years, the combination of coumarin and its derivatives has been the focus of many researchers' studies as anticancer drugs [15]. The S14161 small molecule is also one of these derivatives [15]. The mechanism of cell death induction by S14161 is associated with activation of caspases-3 and -9, a decrease in the levels of anti-apoptotic proteins, including \textit{Bcl2}, and an increase in the levels of pro-apoptotic proteins (e.g., \textit{Bim}). It blocks the translocation of AKT to the plasma membrane in cells stimulated with insulin-like growth factor 1 [16]. AKT is an important factor in regulating cell cycle and proliferation, protecting cells from programmed cell death or apoptosis, and forming and growing blood vessels. This pathway is increased in cancer cells [31]. Han et al. (2014) identified an analog of S14161 (BENC-511) as an effective combination in the treatment of myeloma and leukemia [32]. It has also been shown that BENC-511 decreased the cell viability of human lung adenocarcinoma cancer cells (A549) by blocking the cell cycle in the S phase and decreasing cyclin D expression by reducing the expression of \textit{Bcl2} and caspase-9 [33]. The effect of caspase-dependent apoptosis and autophagy induction by a coumarin hybrid compound on lung cancer cells has also been investigated, which inhibits the expression of the \textit{Bcl2} gene effective in the apoptotic pathway and activates \textit{Bax} gene expression and, like most chemotherapeutic drugs, this compound has been able to induce cell death in lung cancer cells [34], which is consistent with the results of this study. The inhibitory effect of S14161 small molecule on PI3K/AKT pathway and expression of downstream cell cycle genes such as cyclin D1 has been studied in a wide range of cells [35]. S14161 has been shown to express cyclin D at the transcriptional level. The vector inhibits far beyond translation levels and post-translational modifications [35]. Harris (2003) stated that S14161 decreases cyclin D expression and that cells arrest at the G0/G1 stage of the cell cycle, a finding that was consistent with myeloma cell lines [36]. Also, it is consistent with our results because of the specific effect of S14161 on the PI3K pathway and the lack of toxic effects at very high concentrations. So, this inhibitor has been proposed as a major strategy in the treatment of cancers [36]. However, in the present study, the comparison between the two compounds showed that S14161 performed better than the \textit{G. flavum} and had a significant difference in its lethal effect on the first, third, and fifth days compared to the \textit{G. flavum}. Also, in the study of the expression of genes involved in the apoptosis process, although both compounds significantly increased apoptotic \textit{Bax}, \textit{P53}, and \textit{Bad} expression and decreased \textit{Bcl2} expression, which indicated better effects of S14161. Also, the expression ratios of \textit{Bax/Bcl2} and \textit{Bad/Bcl2} were lower in S14161-treated A549 cells compared to cells treated with \textit{G. flavum}. Hence, it is suggested that the small molecule acts better than the \textit{G. flavum}. As mentioned, S14161 is specifically involved in the PI3K/AKT signaling pathway, a potent PI3K and cyclin D expression inhibitor, and can subsequently inhibit \textit{Bcl2} anti-apoptotic genes. Since in this study the IC50 concentration of S14161 small molecule was lower than the IC50 concentration of \textit{G. flavum}, and its effect on cancer cell death was greater and better.
Conclusion

Generally, this study showed that S14161 had fewer IC50 and caused cell death by inhibiting the PI3K/AKT pathway, and G. flavum caused cancer cell death possibly due to its alkaloid compounds.

Therefore, both compounds are recommended as drug candidates for lung cancer treatment.

Conflict of Interest

The authors have no conflicts of interest to declare.

References