Original Article

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Effect of Valeriana Sysimberifolia Extract on VEGF Expression in A549 Cell Line

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ABSTRACT

BACKGROUND AND OBJECTIVE: Today, the use of medicinal plants in the treatment of cancer is more important due to its fewer side effects. Vascular endothelial growth factor (VEGF) is one of the most important factors in stimulating angiogenesis and promoting cancer. The aim of this study was to investigate the inhibitory effect of Valeriana Sysimberifolia root extract on proliferation of lung cancer cells (A549) and VEGF gene expression in treated cells.

METHODS: The hydro alcoholic extract of Valeriana Sysimberifolia root was prepared by percolation. After preparation of cell line A549 from the Iranian Biological Resource Center, cancer cells were treated in culture medium with different concentrations of plant root extract (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/ml) at 24, 48 and 72 hours and viability was performed by MTT assay at all three time. Real Time-PCR method was used to evaluate the expression of VEGF gene in cancer cells treated with IC50 concentration of plant extract.

FINDINGS: With increasing the concentration of the extract and the treatment time, a decrease in viability of cancer cells was observed. The highest inhibition of cell proliferation was observed at a concentration of 50µg/ml after 72 hours (94% growth inhibition) which was significant compared to the control group (p= 0.0001). Also, with increasing treatment time, decreased VEGF gene expression was seen compared to the control group. Thus, a two times decrease in VEGF gene expression was seen in cells after 72 hours (p= 0.0017).

CONCLUSION: Valeriana Sysimberifolia root extract has anti-proliferative and anti-angiogenesis activity. It seems that further studies can be used as a biological product in the treatment of cancer.

KEY WORDS: *Valeriana Sysimberifolia, VEGF, Lung Cancer*.

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Introduction

Lung cancer is still the most common cancer worldwide in terms of the number of new cases diagnosed (1.8 million cases, 12.9% of all cases) and mortality rate (1.6 million deaths, 19.4% of all cases). Lung cancer mortality rates vary widely in different parts of the world. Most lung cancer deaths occur in less developed countries. According to available statistics, the prevalence of lung cancer in Iranian men and women is constantly increasing. In 2012, 4361 deaths due to lung cancer were registered in Iran, which was 9.80% and 6.07% of the total deaths related to the ten most common cancers among Iranian men and women, respectively (1-4).

In a meta-analysis conducted by Hassanipour et al., the prevalence of lung cancer in Iran is slightly lower than in other geographical areas (5). The most common cause of lung cancer is smoking, which is the cause of 85% of lung cancers (6) and other causes are a combination of genetic factors, radon gas, asbestos, air pollution (7) and third-party cigarette smoke (8, 9). Common therapies include surgery, chemotherapy, radiation therapy, photodynamics, and clinical care, and the most important chemotherapy drugs are cisplatin and gemcitabine (10).

Adequate blood circulation and more blood vessels are essential for the rapid growth of tumor developing tissue, and tumor cells use at least two mechanisms: angiogenesis and vasculogenesis. The process of angiogenesis plays an important role not only in inducing tumor growth but also in the complex process of tumor spread and metastasis from the original site and migration along blood and lymph vessels to distant points (11). Angiogenesis is influenced by activating factors such as: bFGF, VEGF, MMPs, NO (12). VEGF is a heparin-binding hemodimer protein with a molecular weight of 45 KD that is capable of both pro-angiogenic activity in vivo and in vitro and has 7 isoforms: A, B, C, D, E, F and PIGF, which are obtained as a result of different splices of this gene (13).

VEGF is one of the most important stimulatory factors of angiogenesis that exerts its mitogenic and angiogenic activities through two tyrosine kinase receptors: VEGFR1 and VEGFR2 through autocrine or paracrine mechanisms (14). In lung cancer, studies of VEGF autocrine signaling to activate the ERK/MEK and PI3K / AKT signaling pathways for NSCLC cell proliferation and the effect of NRP1 on regulating of VEGF expression in the NSCLC cell line have been demonstrated (15, 16). For this purpose, by optimizing the treatment methods, anti-VEGF compounds are used, which can be effective not only in lung cancer but also in other cancers. Thus, by using plant polyphenolic compounds, in addition to increasing the body's immune system, by inhibiting free radicals as well as binding to growth factor receptors, it prevents the growth and proliferation of cancer cells, which in turn leads to a reduction in the use of chemical drugs in the treatment of lung cancer (17). The cytotoxic effects of various plants, including Phyllanthus (18), Momordica charantia (19), Justicia adhatoda (20), have been reported on A549 lung cancer cells. Ebenus boissieri root (21) has also been reported to inhibit cell proliferation and induce apoptosis in A549 cell line.

Valeriana sisymbriifolia is one of the six species of valerian in Iran, whose roots contain valuable active ingredients. According to scientists, 57 different compounds were identified by gas chromatography (GC/MS) of this plant (22), many of which Such as cario-phylene oxide (23), borneol (24), borneyl acetate (25), carvacrol (26), Guaiol (27), pinene (28), terpinene (29), elemene (30), Cadinene (31), etc have anti-cancer and growth inhibitory properties in different categories of cancer cells. However, no research was found on the effect of this plant in the A549 cell line. Therefore, in the present study, the effect of hydroalcoholic extract of this plant on inhibition of cell proliferation and expression of VEGF gene in A549 lung cancer cell line was studied.

Methods

This experimental study was conducted after approval by the Ethics Committee of the Islamic Azad University, Parand Branch, with the letter number of 08-18-6520.

Extraction: At first, hydroalcoholic extraction was performed by percolation method from the desired plant. Thus, the root of the plant was prepared from the Genetic and Biological Resource Center of Iran in dried form with herbarium number IBRC P1007034. After milling, 100 g of the resulting powder was added to Erlenmeyer flask containing 500 ml of 50° methanol and soaked in the laboratory for 48 h. To prevent evaporation of alcohol, the Erlenmeyer flask was closed with paraffin and kept in the dark to prevent unwanted reactions and changes in the effective factors of the plant. The clear supernatant was then separated with a strainer and the sediment was discarded. The filtered

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liquid was placed in a vacuum rotary machine at 42°C to separate the solvent. The extract was completely dried in an oven and prepared with the remaining powder at a concentration of 10 mg/ml of storage solution and stored in a dark container at 4°C until use.

Cell culture: Cell flask containing 60 ml of enriched cell culture medium was prepared from Iran Genetic and Biological Resources Center with registration number IBRC C10080 and stored at 37°C, 5% CO2 and 95% oxygen for 4 to 5 days until the density of cells in the medium reaches above 80%. The precipitated cells were washed with 1M PBS solution and pH 7.4, and 2 ml of DMEM culture medium enriched with L-glutamine and 10% FBS, combination of antibiotics including: neomycin 50 µg/ml, Penicillin 50 µg/ml, streptomycin 100 µg/ml were added. Then the number of cells and the percentage of cell viability were counted using a reverse microscope as well as the Trypan Blue and homocytometer Lam methods. After counting the cells and ensuring their health, this culture was used to perform the next steps of the experiment, including a test to evaluate the toxicity of the plant extract on cancer cells.

Evaluation of cytotoxicity: MTT colorimetric method was used to evaluate the effect of plant extract on the growth and proliferation of cancer cells. This method is a competitive mitochondrial metabolic test and is based on the breakdown of tetrazolium salt by the succinate dehydrogenase mitochondrial enzyme of living cells. For the experiment, a volume of 100 µl of culture medium containing 104 A549 cells in each 96-well plate was placed. Cancer cells were incubated with different concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/ ml of plant extract for 24, 48 and 72 hours. After these times, 100 µl tetrazolium with the chemical formula (3- [4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide) or MTT with a concentration of 0.5 mg/ml was added to each plate and incubated for 4 hours in incubator 37 ° C equipped with CO2. The MTT medium was then carefully removed and to each well plate 100 µl DMSO was added to dissolve purple crystals of color. After 30 minutes of incubation, the light absorption of each well was read at 570 nm using the BioTek it-ELX808 ELISA reader. The results were reported as cell survival with three replications.

Evaluation of VEGF gene expression: for this purpose first from cells treated with IC50 concentration of plant extract, RNA extraction and then cDNA synthesis was done and finally with a specific primer for VEGF gene, by Real Time PCR method VEGF gene expression was evaluated.

RNA extraction and cDNA synthesis: RNA extraction from A549 cells that had been exposed to IC50 dilution from plant extracts for 24, 48 and 72 hours was performed according to the instructions of Total RNA Purification Kit/RT01 (Gene Mark) by column method. The extracted RNA was transferred to a freezer at -70°C. The quality of RNA extracted was measured by gel method with 2% agarose and also RNA concentration was measured with Nabi Micro Digital nanodrop device and after confirming the desired concentration and quality in terms of non-contamination with DNA, protein and carbohydrates, it was used for cDNA synthesis. Complementary DNA molecules were synthesized with the Revert AidTM First strand cDNA Synthesis Kit (Fermentas, Lithuania) in which the reaction mixture contained 5 µl of x5 reaction buffer, 1 µg of RNA, 0.5 µl of random six nucleotide primer, 0.5 µl of Oligo dT primer, 2 µl of deoxynucleoside triphosphate mixture (10 mM), one microliter of RNase inhibitor (20 units per microliter), 1 µl of reverse transcriptase, and distilled water were twice distilled (to final volume of 20µl). Temperature-time program at 25°C for 5 minutes (for primer binding), 42°C for 60 minutes (cDNA synthesize), 70°C for 5 minutes (reverse transcript inactivation) and 4°C was performed for 5 minutes (32).

Real-Time-PCR: The real-time PCR reaction at the final volume of 20 µl was optimized as follows: 10 µl of SYBRTM (2X) Master Mix (Takara Company), 1 µl of forward-reverse primers with a concentration of 10 µM (Takapo Co. Bio) (33), 7 µl of deionized water and 1 µl of template cDNA. The b-actin gene was used as an internal control (34).

The thermal program was performed as follows: initial denaturation of the template DNA at 95°C for 10 minutes, the second phase alternately was performed over 40 cycles at 95°C for 20 seconds and 65° for 20 seconds and 72°C for 20 seconds. Fluorescence was measured by Heal Force X-960 and amplification and separation curves were plotted and analyzed using software.

Data analysis and statistical analysis: Raw data obtained from Real-Time-PCR were analyzed using software. After CT (cycle threshold) amplification, the samples were identified and PCR efficiency mean was determined. Based on RQ (Relative Quantification) method, and $2^{-\Delta\Delta CT}$ formulation was entered as a multiple of VEGF gene expression and compared with

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the internal b-actin gene. The test groups were compared with the control group and significance of data was analyzed using Graphpad Prims ver.6 software, T-test, one way ANOVA and HSD s'Tukey test hoc-post statistical method (for difference in expression of target genes between control and treated samples) and $p<0.05$ was considered significant.

Results

In this study, with increasing the concentration of extract and duration of treatment, an increase in inhibition of cell proliferation and a decrease in bioavailability were seen. At concentrations of 50 µg/ml, the highest inhibition of cell proliferation was observed, which was statistically significant compared to the control group ($p<0.001$). While slight cytotoxic effects were observed at concentrations of 0.78 and 1.56 µg/ml and the observed decrease in bioavailability compared to the control group did not show a significant difference. The IC50 value of the extract at 24 hours was 11.91±1.510 µg/ml and at 48 and 72 hours were 3.90±0.485 µg/ml and 2.30±0.419 µg/ml, respectively (Figure 1). Specific amplification of the desired gene fragments, no pairing of primers and no amplification of nonspecific fragments for each gene were determined using the melting curve. The melting curves of VEGF and β-Actin genes in A549 cell line are shown in Figures 2 and 3. Changes in gene expression were assessed in A549 cells treated with IC50 concentrations of the extract after 24, 48 and 72 hours. The expression ratio of VEGF gene in A549 cancer cell line treated with the extract decreased compared to the control group with increasing treatment duration. So that this decrease was significant after 48 hours ($p=0.00189$) and 72 hours ($p=0.0017$) compared to the control group. The decrease in expression observed at 24 hours after treatment was not statistically significant compared to the control group. With increasing the duration of treatment, a greater decrease in expression was observed so that a higher decrease in expression was observed at 72 hours compared to the other two times (Figure 4).

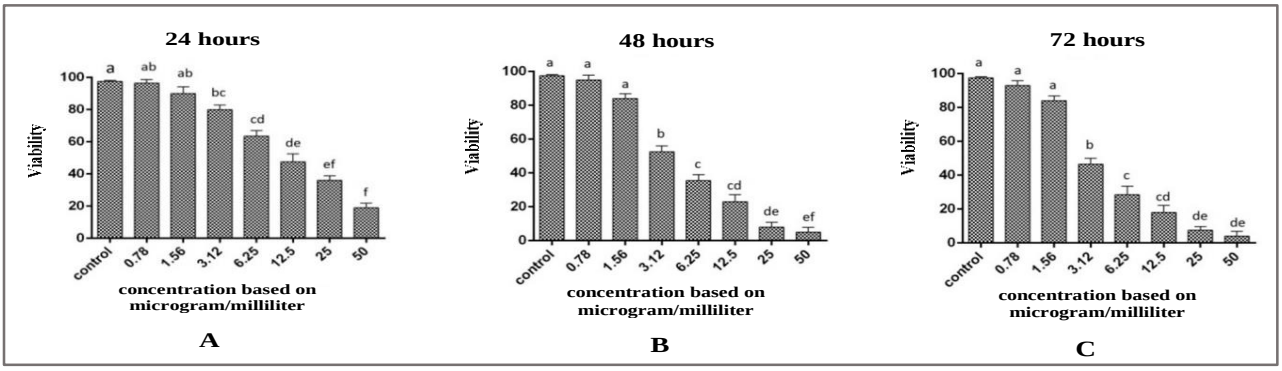


Figure 1. Comparison of cytotoxic effects of different concentrations of Valeriana sisymbriifolia extract on lung cancer cells (A549) after 24, 48 and 72 hours. The same Latin letters at the top of each column indicate no significant differences and different Latin letters indicate significant differences between the groups

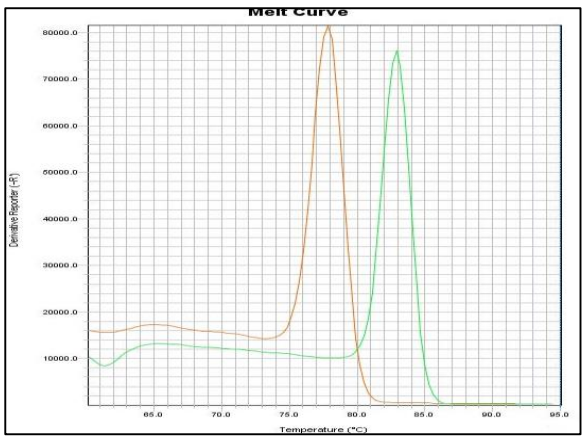


Figure 2. Pattern of melting curve of VEGF and β-Actin genes at 82.86°C and 77.89°C

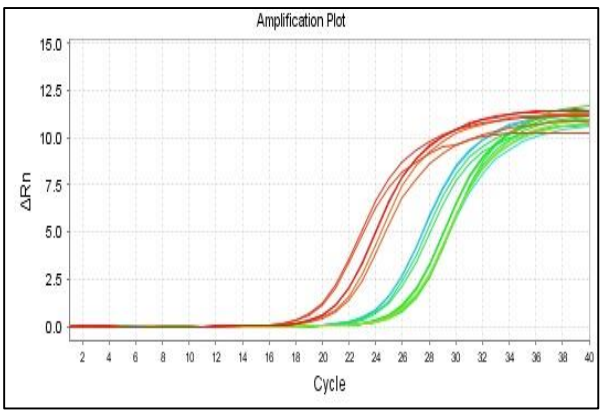


Figure 3. Diagram of amplification of VEGF and β-Actin genes in cells treated and untreated with plant extract

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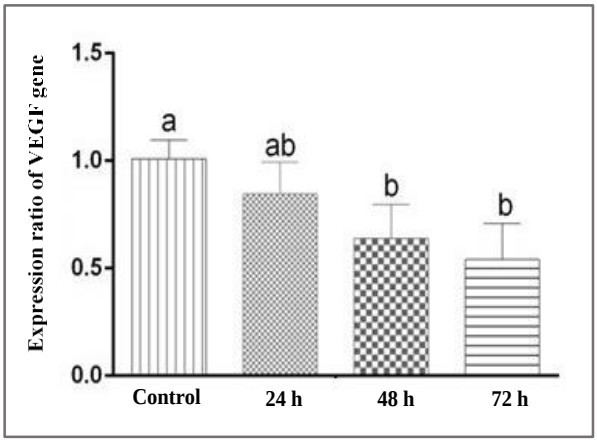


Figure 4. Evaluation of VEGF gene expression in A549 cells treated with plant extracts for 24, 48 and 72 hours. (The letter b at 48 and 72 hours indicates significance compared to the control group and no significance compared to 24 hours)

Discussion

The results of the present study showed that the plant extract in a time-dependent and dose-dependent pattern inhibits cell proliferation and reduces VEGF gene expression. Over time, gene expression decreased significantly compared to the control group and after 72 hours, the greatest reduction in gene expression was seen. So far, no study has been performed on the cytotoxic effect and inhibition of cell growth by the root extract of Valeriana Sysimberifolia (valerian) in lung cancer cell line A549. Cytotoxic and anti-proliferative effects of various plants including Phyllanthus (IC50: 61.3 µg/ml for 72 hours) (18), Momordica charantia (IC50: 32.5 µg/ml for 24 hours) (19), Justicia adhatoda (IC50: 80 µg/ml for first day) (20), Urtica dioica (IC50:5 µg/ml for 48 and 72 hours) (35), Haplophyllum tuberculatum (IC50:152.13 µg/ml diethyl ether root extract for 24 hours) (36), Sapindus mukorossi (IC50:30 µg/ml for 48 hours) (37), Lagenaria siceraria (IC50:6.5 µg/ml for 72 hours) (38), reported on A549 lung cancer cells. Comparison of the cytotoxic effect of Valeriana Sysimberifolia root extract with other plants on A549 cell line showed that Valeriana Sysimberifolia root extract has a much stronger ability to stop cell proliferation and inhibition, which is well determined by comparing IC50 of Valeriana Sysimberifolia root extract (IC50: 2, IC50: 3, IC50: 11.91 for 24, 48 and 72 hours). It has been shown that the roots of Ebenus boissieri (21) and ZINGIBER OFFICINALE (39) also have inhibitory activity of cell proliferation and induction of apoptosis. The inhibitory concentration of Ebenus boissieri root in 72 hours was 35.8 µg/ml and ZINGIBER OFFICINALE root after 24 hours was 79.57 µg/ml, which compared to our research, much lower concentrations of Valeriana Sysimberifolia root extract were able to reduce and It inhibit cell growth and has a stronger ability to induce cell death and inhibit cell proliferation. In a study, Chang et al. used curcuminoid extracts and nanoemulsions of Curcuma longa Linnaeus to evaluate cytotoxicity effect on the A549 cell line. In this study, the inhibitory concentration (IC50) for nanoemulsion was considered (3.9 µg/ml) and for curcuminoid was considered (3.75 µg/ml), which was similar to the present study (40). Sakamato et al. showed that Epigallacatechin 3 gallate extract was able to reduce VEGF gene expression between 0, 6, 12 and 24 hours. Contrary to our research, measuring the vital activity of cells at 24, 48 and 72 hours by trypan blue method did not show a significant difference (41). The results of the present study showed that the root extract of Valeriana Sysimberifolia has anti-proliferative and anti-angiogenic activity in A549 lung cancer cells. The root extract of this plant has strong anti-inflammatory and anti-angiogenic effects, which can prevent the proliferation and spread of lung cancer cells. It is hoped that by performing additional tests and identifying signaling pathways and its effect on cell apoptosis as well as clinical trials in vivo, this plant can be used as a biological product in the inhibition and control of lung cancer.

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