

Evaluate the effect of Sclareol on endoplasmic reticulum stress in KATO III human gastric carcinoma cancer cell line

Running title: Sclareol induces ER stress in KATO III gastric cancer cells

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Abstract

Background: The main goals of chemotherapy are to increase apoptosis (Cell death) and to halt the cell cycle in cancer cells. One important factor that induces apoptosis in cancer cells is to increase stress in the endoplasmic reticulum. In this study, the effect of Sclareol on the ATF6 pathway is investigated, as well as the effects of two other sub-genes, VEGF and E2F1, associated with the UPR pathways.

Methods: The target population in this research was KATO III human gastric carcinoma cancer cells, which were purchased from the Pasteur Institute of Iran, and they were cultured in the cell culture laboratory until their number reached at least 100,000 cells/well. The cells were treated with concentrations of 0, 20, 40, 60, 80 and 100 μ M of Sclareol for 5 hours. Analyzing the expression of the genes- expressed in the research objectives- derived from gastric cancer cells before and after treatment with Sclareol was performed using Quantitative Real Time-PCR from the spheres of cell lines cultured in suspension. SPSS Software was used for statistical analysis.

Results: *ATF-6* gene expression significantly increased in genes with doses of 20, 40 and 60 μ M of Sclareol ($P < 0.0001$). On the other hand, it was shown that the level of *VEGF* gene expression was significantly reduced in genes with doses of 20, 40 and 60 μ M of Sclareol ($P < 0.001$). Also, there was a significant increase in *E2F1* gene expression in doses of 20, 40 and 60 μ M of Sclareol ($P < 0.003$).

Conclusion: From the results obtained from the present study, it can be concluded that the administration of Sclareol may be useful for reducing the expression of *VEGF* gene and for increasing the expression of *ATF-6* and *E2F1* genes, which are responsible for increasing the stress of the endoplasmic reticulum system, in KATO III human gastric cancer cells. The doses of 40 and 60 μ M of Sclareol were considered to be effective doses.

Key words: Gastric Cancer, Endoplasmic Reticulum System Stress, Sclareol, KATO III Gastric Cancer Cells

Introduction

Stomach cancer is caused by the uncontrolled growth of malignant cells in the stomach, which grow slowly over years, but unfortunately, it does not display any symptoms in the early stages. Perhaps that is why it is difficult to diagnose (1).

Stomach cancer progresses slowly over several years. Before the development of actual cancer, precancerous changes often occur in the inner lining (mucosa) of the stomach. These initial changes strongly cause symptoms (1,2).

It is said that 90 to 95 percent of stomach cancers are of Adenocarcinoma type. This type of stomach cancer develops from the innermost layer of the stomach (mucosa). (Other types of stomach cancer are called lymphoma, stromal, carcinoid, etc. (3,4). *Helicobacter pylori* infection, smoking, high salt diet and insufficient intake of antioxidants have been reported to be the causes of this disease (5,6).

One of the main causes of stomach cancer is *Helicobacter* infection, which causes stomach ulcers and gastritis; although not every person with *Helicobacter* infection gets stomach cancer. If it were so, more than 55% of the world's people would have died from stomach cancer, because more than half of the world's population is infected with *Helicobacter* infection, while only 3% of these people may get stomach cancer (1).

This type of cancer may spread from the stomach to other body parts, especially to liver, lungs, bone, abdominal wall, and lymph nodes (1,2).

The onset of cancer in different parts of the stomach may cause different symptoms and also the location of the cancer can affect the treatment options. Various factors such as ethnicity, living environment, nutrition, gene mutations, pernicious anemia, stomach polyps, low social and economic status, obesity, etc. can play a role in stomach cancer development (1).

In the 20th century, stomach cancer was the most common cause of death from cancer in the world, but recently, lung cancer has taken the first place in death from cancer and death from stomach cancer has been reduced to the second place. In Iran, cancer is said to be the third cause of death. And every year between 30 and 40 thousand Iranians die of it (7).

To diagnose this disease, endoscopy and medical imaging should be resorted to, and the most common methods of treating this disease are considered to be surgery, chemotherapy, and radiation therapy (8).

Chemotherapy is one of the most effective treatments for metastatic tumors. Simultaneous resistance of cancer cells to different drugs, without structural and functional connection with each other, and resistance to multiple drugs are still big obstacles in the way of successful chemotherapy. Three decades of research on drug resistance have revealed various ways to overcome this major obstacle. According to the reported studies, drug resistance is also developed to any new effective treatments. Therefore, the ability to predict and overcome drug resistance will probably be very effective in improving chemotherapy (9).

Factors involved in cell resistance to anticancer drugs are divided into two general categories: 1- Factors that affect the access of tumor cells to anti-cancer drugs: In this case, the interference in reaching the drug to the cell can be the result of weak absorption of the used drugs, increased metabolism of drugs or increased excretion of them, and finally reduced level of the drug in the blood as well as its lowered diffusion to the tumor mass from the blood (6-8).

2- Factors that influence sensitivity to drug in cancer cells due to genetic and epigenetic changes.

Also, environmental factors such as the extracellular matrix or the location of the tumor play a role in drug resistance (6,9).

The main goals of chemotherapy are to increase apoptosis or cell death and to stop the cell cycle in cancer cells. One of the very important factors that induce apoptosis in cancer cells is to increase the stress of the Reticuloendoplasmic system.

Endoplasmic reticulum (ER) is one of the cell organelles and is considered the largest intracellular organelle facilitating intracellular communication. The endoplasmic reticulum consists of many folds that are covered by a membrane. (The space inside the ER is called lumen). Apart from this, the endoplasmic reticulum is responsible for transporting proteins and other carbohydrates to the Golgi apparatus, cell membrane, lysosome and anywhere else needed (10).

With the help of chaperones (Chaperones are in organic acids group and are three-dimensional molecules that help folding of proteins made in the cells) in the lumen, the endoplasmic reticulum system causes secretory proteins to be folded (Protein folding factory) and then to be transferred to the Golgi apparatus (10,11).

In various physiological conditions and when there is an excessive increase in metabolic factors such as glucose, lipids, unsaturated fatty acids, cholesterol and neurotransmitters, the need for protein synthesis increases in the cell. If the chaperones do not work properly, the unfolded proteins accumulate in the endoplasmic reticulum network and create stress in it, which stimulates apoptosis or repair (11 and 12). Disturbance in endoplasmic reticulum (ER) homeostasis causes stress conditions. Any disturbance caused by unfolded or misfolded proteins is reacted by a cascade of reactions called Unfolded Protein Response (UPR*) (11,12). Therefore, induction of endoplasmic reticulum stress condition leads to lack of nutrients, hypoxia and calcium depletion, etc., which will initiate the response stages through three main pathways and other secondary pathways.

Three integral proteins of endoplasmic reticulum are connected to BiP/GRP78 chaperone in normal conditions, but they are separated from them in stress conditions, leading to their activation (13).

With the goals of weakening the translation process, inducing the transcription of proteins located in the endoplasmic reticulum, inducing the breakdown system related to the endoplasmic reticulum and developing the endoplasmic reticulum in response to the pressure related to unfolded proteins, the above-mentioned three pathways try to improve the conditions of the endoplasmic reticulum and to return to balanced conditions, both of which lead to apoptosis if the conditions are not compensated (11,12).

The concept of using natural food chemicals such as Sclareol as cancer chemo preventive and therapeutic agents is very attractive because these chemicals are usually very inexpensive and readily available, can be readily metabolized in the human body, and usually do not cause significant harm to normal cells. Therefore, at the interface of cell biology and biochemistry, the use of food chemicals to activate the endoplasmic reticulum stress signaling pathway and apoptosis in cancer cells is a target for combating cancer.

In this study, the authors intended to investigate the effect of Sclareol on the *ATF6* pathway and on two other sub-genes related to the UPR pathways, which are called *VEGF* and *E2F1*. It has been reported that Sclareol in the sage plant with the scientific name of "*Salvia officinalis*" induces apoptosis in many cancer cells. Sclareol is an aromatic chemical compound found in and obtained from "*Salvia Sclarea*". It is classified as a bicyclic diterpene alcohol. It is an orange-colored solid with a sweet, safflower-like odor.

Sclareol is used as a fragrance in cosmetics and as a flavoring in food.

The results of studies conducted in recent years demonstrate that intratumoral injection of Sclareol is seen to have anti-tumor effects and modifies immune responses by reducing Treg cells and inhibits tumor growth in vivo (13,14).

It has been reported that Sclareol is a non-toxic compound and possesses the ability to induce apoptosis in leukemia cell line (In a time- and concentration-dependent manner). Scientists' findings show that chemical diterpenes or diterpenes in plants present in sage plant (Sclareol) own anti-cancer properties in vitro (15,16).

The results of previous studies emphasize that Sclareol is with the potential to be used as a chemotherapy agent and cytostatic activity in cancer cells. Therefore, it may potentially be used for drug development. To emphasize more on the molecular mechanisms of the compound, it is necessary to investigate more molecular pathways (17, 18).

Methods

This study is an experimental study that was analyzed based on the laboratory results obtained, and its target population was human gastric cancer cells of the cell line.

The target population in this research was KATO III human gastric cancer cells, which were purchased from the Pasteur Institute of Iran. The cells were cultured in the cell culture laboratory until their number reached at least 100,000 cells/well. For this reason, the sample volume for this study was considered to be at least 100,000 cells in each flask, which were divided into six groups being treated with six different concentrations of Sclareol.

KATO III cell line was cultured as a single adherent layer in RPMI-1640 (Roswell Park Memorial Institute) complete culture medium with fetal bovine serum (Inactivated by heat) at 100 ml/l, penicillin and streptomycin each at 100 mg/l and glutamine at 2 mmol/l liter, at a temperature of 37 degrees in a humidity level in the atmosphere and CO₂ gas with a relative pressure of 50 ml/liter. Reaching a suitable density of at least 100,000 cells per plate, it was separated by trypsin from the bottom of the flask and then passaged.

The cells were treated with concentrations of 0, 20, 40, 60, 80, and 100 μ M of Sclareol for 5 hours. The analysis of gastric cancer cell genes in line with the research objectives before and after treatment with Sclareol was performed using quantitative real-time PCR from spheres of cell lines cultured in suspension. The total RNA of cultured cells was extracted using Trizol solution according to the kit protocol and was quantitatively and qualitatively analyzed by agarose gel electrophoresis and spectrophotometry at 260 nm.

cDNA synthesis was done using RNA as a template with the enzyme reverse transcriptase. This enzyme, like other DNA-producing enzymes, needed a primer. Therefore, two types of primers, random hexamers and oligo dT, were used to make cDNA. The difference between these two types of primers is that oligo dT binds to the polyadenine tail of mRNA, while random hexamers bind to different parts of mRNA randomly. After binding to the primers, the enzyme reverse transcriptase makes DNA (cDNA) from the RNA template. The Synthesis Kit PrimeScript™ 1st Strand cDNA product (Takara) was used to synthesize cDNA.

Specific primers and probes for the target genes were designed using Primer-3 and Primer Express® software, based on sequences retrieved from the GenBank database. The final selected oligonucleotide sequences are presented in Table 1.

Table 1. Information on the sequence of primers designed for E2F1, VEGF, ATF-6 and β -Actin genes

Gene name	Primer type	Primer sequence (5' → 3')	Accession No.
<i>E2F1</i>	Forward	TACCTTTTCCTGGATGGCGG	NC_000020.11
	Reverse	TCCTGGTTACTGGGCATCCT	
<i>VEGF</i>	Forward	TGCAGATTATGCGGATCAAACC	NM_001287044.1
	Reverse	TGCATTACATTGTGTGTGCTGTAG	
<i>ATF-6</i>	Forward	GATTGGGGTGAGTGGGATCTG	NC_000001.11
	Reverse	GCCGAACGATACTCCCAGAC	
<i>β-Actin</i>	Forward	GGTGAGCTGCGAGAATAGCC	NC_000007.14
	Reverse	CTCCGACCAGTGTTCCTT	

In this study, real-time PCR was used to examine gene expression using a **Corbett Rotor-Gene 6000**. All samples were repeated three times. After the reaction was completed and the threshold line was determined, the threshold cycle (CT) of each sample was obtained. Relative

gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with the ratio of the threshold cycle of the gene of interest to the gene β -Actin as the housekeeping gene.

Data analysis in the descriptive statistics section was presented as mean, median, standard deviation, minimum, and maximum. Then, the normality of the data was examined using the Kolmogorov-Smirnov test. Finally, in the inferential statistics section, the research hypotheses were examined using one-way analysis of variance and SPSS 22 statistics.

Results

To check the quality of the extracted RNA, 18S rRNA and 28S rRNA bands were checked on a 2% agarose gel. For this purpose, 1.5 microliters of four RNA samples treated with 4 microliters of loading buffer were randomly electrophoresed in 2% agarose gel for 20 minutes and the relevant bands were observed as shown in Figure 1.

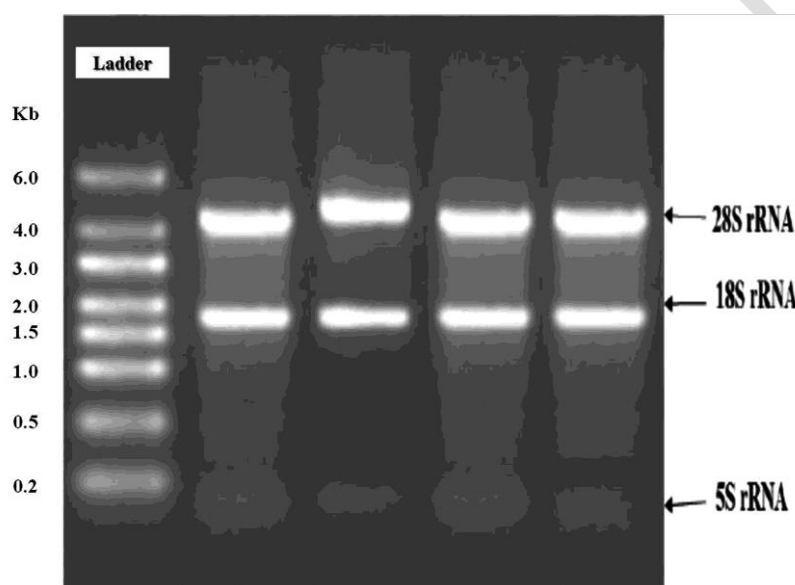


Figure 1. rRNA 18s and rRNA 28s electrophoresis bands of RNA samples extracted from culture media

Analysis of the quality of primers through performing PCR gradient reaction

To ensure the quality of the primers, a PCR gradient reaction was performed for the β -Actin gene primer.

PCR products were checked using electrophoresis with 2% agarose gel and the results were shown single band sharp at 57.8 degrees. Their detection was observed and recorded under UV light, which is shown in Figure (2).

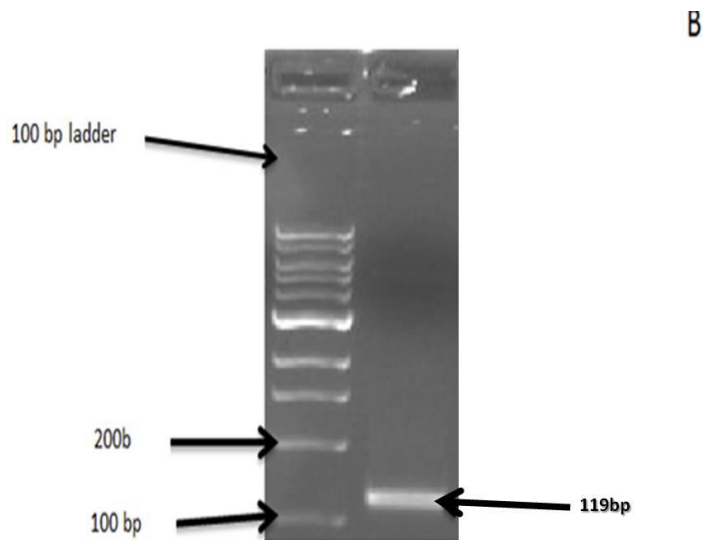


Figure 2. The image of β -Actin gene in electrophoresis gel, whose 119bp product gave a band at 57.8°C.

Analysis of the quality of synthesized cDNA through Positive PCR control

To ensure the quality of the synthesized cDNA, a PCR was performed for the housekeeping gene, β -Actin. In this reaction, cDNA was made as a template and Smar Taq DNA polymerase was used.

PCR products were checked using electrophoresis with 2% agarose gel and the results was single band sharp at 58.5 degrees. PCR products were observed and recorded by staining with ethidium bromide. Then they were revealed under UV light, as shown in Figure 3.

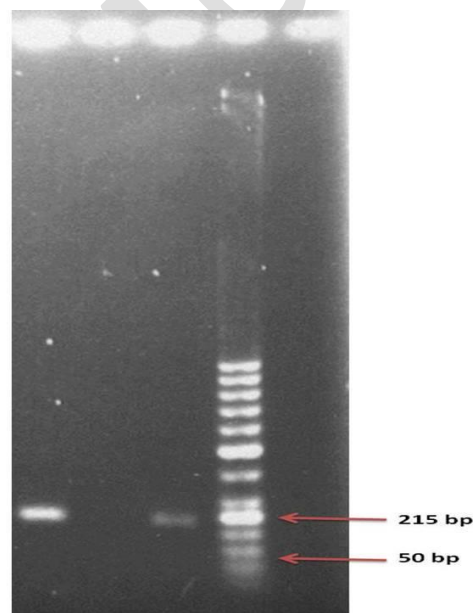


Figure 3. The image of the β -Actin gene on an electrophoresis gel, whose 218 bp products gave a band at 58.5°C.

Analysis of the melting curve obtained from Real-time PCR technique to show specific and non-specific products:

Figures 4 and 5 show melting curve diagrams after the end of the PCR reaction for β -Actin and ATF6 genes. As seen in these figures, non-specific products were minimized in this research.

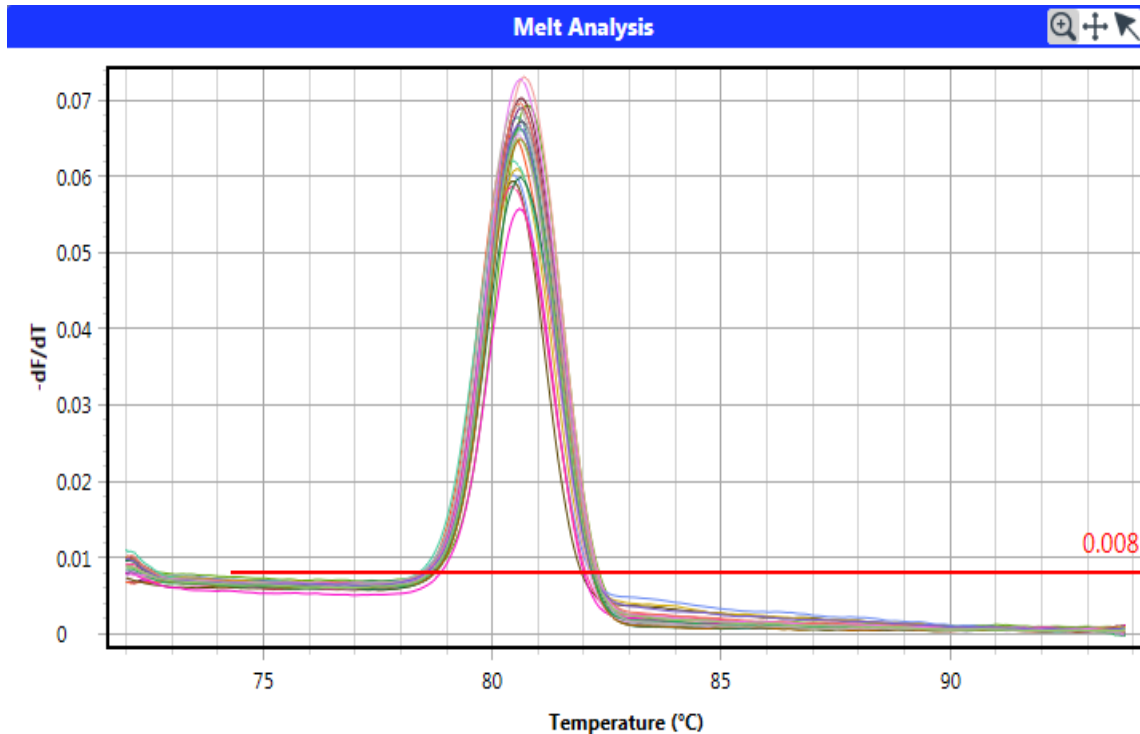


Figure 4. Melting curve diagram resulting from Real-time PCR technique for β -Actin gene

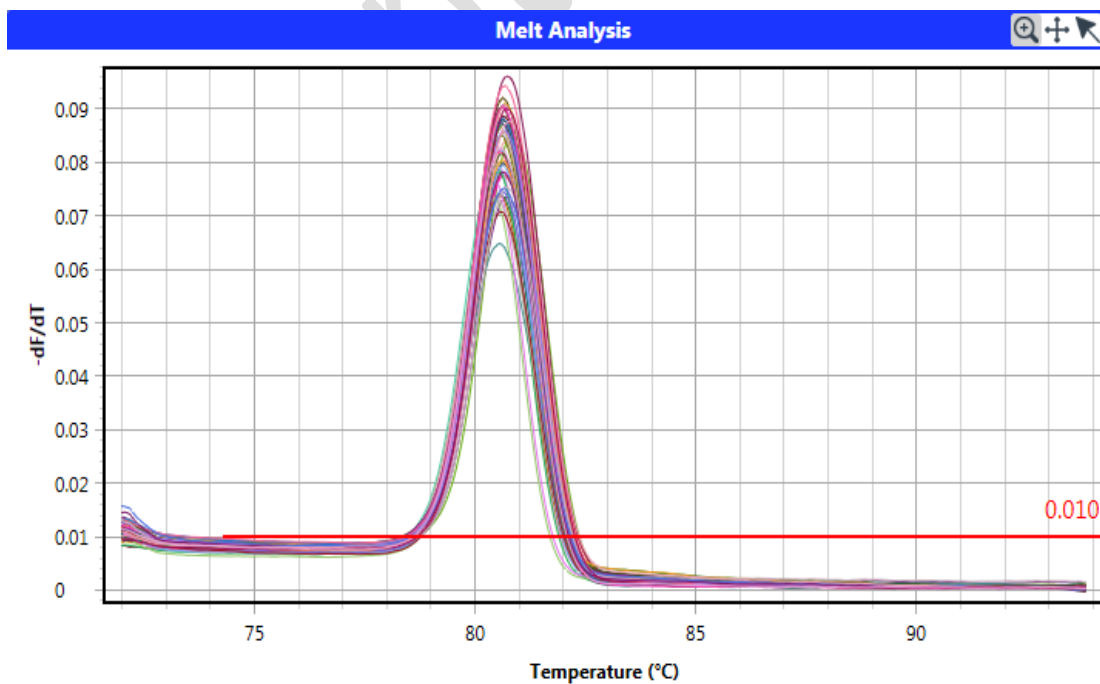


Figure 5. Melting curve diagram resulting from Real-time PCR technique for ATF6 gene

The results reported that the level of *ATF-6* gene expression in doses of 20, 40 and 60 μM of Sclareol had a significant increase, so that the level of expression of *ATF-6* gene in dose of 20 μM of Sclareol was equal to 0.21 ($2^{-\Delta\Delta\text{CT}}$). In dose of 40 μM of Sclareol, it was equal to 0.45 ($2^{-\Delta\Delta\text{CT}}$) and in dose of 60 μM of Sclareol it was 0.98 ($2^{-\Delta\Delta\text{CT}}$). This was while it resulted a decrease in doses of 80 and 100 μM . So that the level of *ATF-6* gene expression in the dose of 80 μM of Sclareol was equal to 0.56 ($2^{-\Delta\Delta\text{CT}}$) and in dose of 100 μM of Sclareol was equal to 0.23 ($2^{-\Delta\Delta\text{CT}}$) ($p < 0.0001$). The effect of different concentrations of Sclareol on *ATF-6* gene expression in cancer cells is shown in Figure 6.

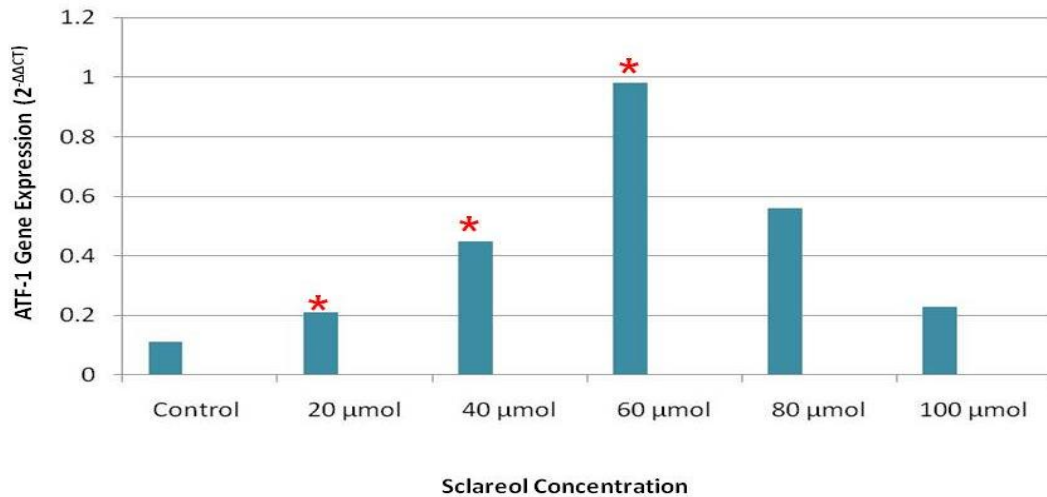


Figure 6. The graph showing the effect of Sclareol concentrations on *ATF-6* gene expression in cancer cells

On the other hand, in the present research it was shown that the expression level of *VEGF* gene in doses of 20, 40 and 60 μM of Sclareol had a significant rise, so that the expression level of this gene in a dose of 20 μM of Sclareol was equal to 0.59 ($2^{-\Delta\Delta\text{CT}}$). In dose of 40 μM of Sclareol, it equalled 0.39 ($2^{-\Delta\Delta\text{CT}}$) and it was 0.21 ($2^{-\Delta\Delta\text{CT}}$) in dose of 60 μM of Sclareol. However, it went up again in doses of 80 and 100 μM . Thus, the level of *VEGF* gene expression in dose of 80 μM of Sclareol was equal to 0.61 ($2^{-\Delta\Delta\text{CT}}$) and in dose of 100 μM of Sclareol it was equal to 0.77 ($2^{-\Delta\Delta\text{CT}}$) ($p < 0.001$). The effect of Sclareol concentrations on *VEGF* gene expression in cancer cells as shown in Figure 7.

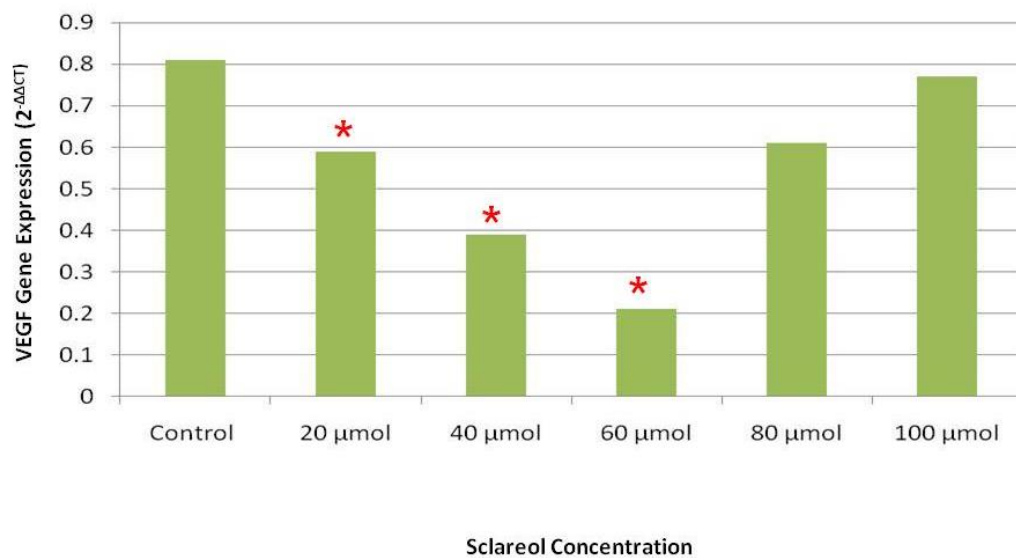


Figure 7. The graph showing the effect of Sclareol concentrations on *VEGF* gene expression in cancer cells

On the other hand, it was depicted that the expression level of *E2F1* gene increased significantly in doses of 20, 40 and 60 μmol of Sclareol, so that the expression level of this gene in dose of 20 μM of Sclareol was equal to 0.20 ($2^{-\Delta\Delta CT}$), in dose of 40 μM of Sclareol it was equal to 0.31 ($2^{-\Delta\Delta CT}$) and in dose of 60 μM of Sclareol it was 0.41 ($2^{-\Delta\Delta CT}$). However, it presented a reduction in doses of 80 and 100 μM . So that the level of *E2F1* gene expression in dose of 80 μM of Sclareol was equal to 0.51 ($2^{-\Delta\Delta CT}$) and in dose of 100 μM of Sclareol was equal to 0.26 ($2^{-\Delta\Delta CT}$) ($p < 0.003$). The effect of Sclareol concentrations on *E2F1* gene expression in cancer cells as shown in Figure 8.

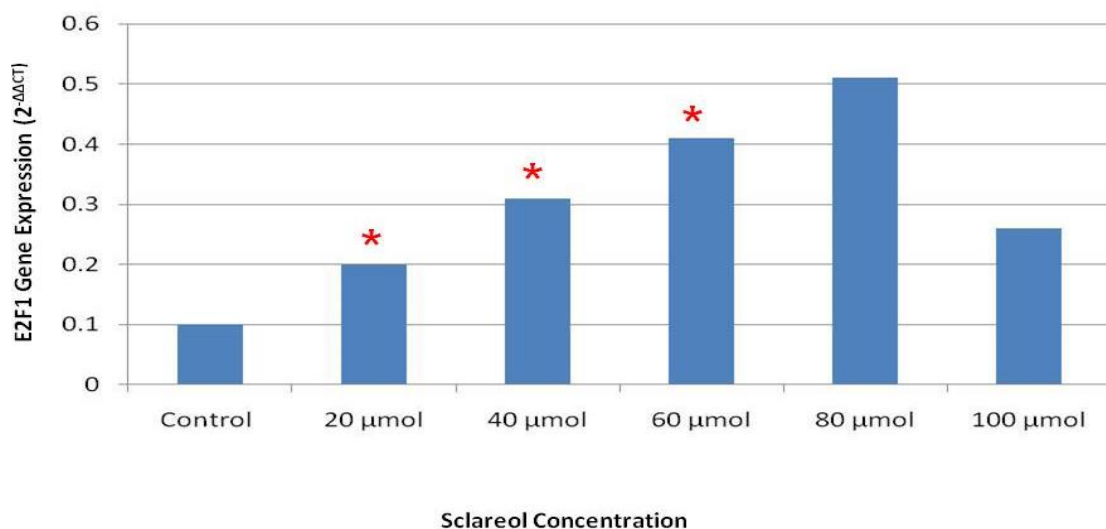


Figure 8. The graph showing the effect of Sclareol concentrations on *E2F1* gene expression in cancer cells

Discussion

As mentioned in the introduction, the main goal of the current research was to investigate the effect of Sclareol on the expression level of *VEGF*, *E2F1* and *ATF-6* genes- key genes of the Reticuloendoplasmic stress pathway in human gastric cancerous cells.

Nowadays, a lot of information is available about mechanisms of drug resistance in cancer cells. Contrary to the progress of cancer treatment with chemotherapy drugs, the protective mechanisms of cells against cytotoxic compounds are considered a big obstacle on the way to successful cancer treatments. Expanding information about the mechanisms of drug resistance can be effective in designing a strategy to overcome drug resistance through designing new chemotherapy methods with less resistance (19).

During the recent decades, the focus of studies on Sclareol has shifted from its function as a plant substance to the investigation of its function to prevent and reduce the risk of diseases if the substance is used adequately. Studies have shown the positive effects of Sclareol on all types of cancers. Reports emerged the plant extract inhibition of cancerous cells growth and the toxic effect increases on cancerous cells chemotherapy. Yet, the exact mechanism of how Sclareol affects cancer cells is not yet known. However, in different studies, different mechanisms have been mentioned for the anticancer effects of Sclareol (20).

Hassan and colleagues in 2003 (21) suggested that Sclareol should be an interesting alternative to voluntary routes in intratumoral injection. In the study conducted by Noori et al. (2010) (14), Sclareol was extracted from *S. Sclarea* plant and its effectiveness was evaluated: Sclareol was used through direct injection into the tumor. In his study, Sclareol had effective results in low doses (Between 20-60 mmol), which was consistent with the results of the present study. Although gastric cancer is a common solid tumor, there has been no report on the effect of Sclareol in doses above 80% on gastric tumor cells. This is definitely not the case, because one of the problems of the study was the fact that it failed to analyze Sclareol impact on healthy stomach cells. There is hope it will definitely be taken into consideration in future studies. However, since similar studies have not been reported to be conducted on human stomach cancer samples so far, it is not possible to reach a general conclusion with the results of this study.

Cancer cells induce UPR to relieve ER stress as a survival strategy (22). It has also been reported that UPR plays a significant role in resistance to chemotherapy or radiation in the field of cancer (23). Therefore, a therapeutic rationale for cancer suppression is to facilitate the accumulation of unfolded proteins by inhibiting UPR components involved in the survival response, which ultimately leads to apoptosis (24).

On the other hand, Karayiannakis et al. (25) pointed out that the serum levels of *VEGF* in patients with gastric cancer were higher than those in the control group. There was a significant relationship between *VEGF* serum levels and the stage of the disease, as well as the depth of tumor invasion and the presence of distant metastases. The serum level of *VEGF* decreases significantly after radical resection of the primary tumor and increases in patients with unrespectable tumors. Multivariate regression analysis showed that serum *VEGF* level was an independent prognostic factor for survival. The results of the present study depicted that the use of Sclareol can reduce the expression of *VEGF* gene which are said to be consistent with the results of Amirchaghmaghi et al.'s studies in 2015 (26).

Morishima et al. in 2011 (27) concluded that ER caused by oxidized derivatives of various substances may promote apoptosis. The results obtained from the study showed that the *ATF6* gene significantly increased in the study group. In other words, the use of Sclareol had been able to escalate the genes of the endoplasmic reticulum stress pathway.

As mentioned earlier, after induction of endoplasmic reticulum stress, a cAMP response element-binding protein dissociates *ATF6* and BiP/GRP78, and enters the Golgi apparatus, where it is broken down and then as a transcription factor, it enters the nucleus and causes the

activation of genes involved in the synthesis of chaperones. And the increase in chaperones causes linear proteins to be integrated and sent to the cytosol or ubiquitinated and directed to the proteasome for destruction. And thus, they can reduce the inflammation of the ER system and probably when it is seen that Sclareol increases the expression of the *ATF6* gene, therefore it must be able to affect the factors that facilitate the synthesis of *ATF6*. For example, it can increase the synthesis of cAMP or perform other roles for this task (28,29).

Regarding the *E2F1* gene, not many studies have been conducted in the past years, and it is considered as one of the genes for which there is not much information about the effect of Sclareol on both its function and its changes in gene expression (30). Montecillo-Aguado et al. (In 2023) (31) conducted a study on it, which showed that consuming high levels of polyunsaturated fatty acids (PUFAs) reduces the risk of cancer and increases the sensitivity of chemotherapy, stopping the G2 cell cycle and reducing *E2F1* transcription in cancer cells, but the results of the present study showed an increase in the level of *E2F1*, which could be due to the effect of Sclareol- not present in the previous study- having probably played a role in the continuation of the cell cycle process, or having created an effect on the pathway of apoptotic proteins, leading to their dysfunction. It is suggested that the causes of the discrepancy be clarified with further studies.

Conclusion

From the results obtained in this study, it can be concluded that the prescription of Sclareol reduces the expression of *VEGF* gene, and increases the expression of *ATF-6* and *E2F1*-genes responsible for increasing the stress of the Reticuloendoplasmic system in cancer cells- in KATO III human gastric cancerous cells. The doses between 40 and 60 μ M of Sclareol were considered effective doses, but doses above 60 μ M did not show to have such an effect. The results obtained in the study can be a promising point for reducing drug resistance in patients with stomach cancer.

Acknowledgement

The authors wish to thank Shahin Rashedi for their support in maintaining the cell lines and technical assistance with the laboratory assays.

Funding sources

This research received no external funding.

Ethical statement

This study was approved by the Ethics Committee of The Islamic Azad University, Tabriz Branch (Approval Code: IR.IAU.TABRIZ.REC.1403.399).

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

SAR and CA contributed to the study design and manuscript writing. MGG and RS contributed to data collection and analysis. All authors read and approved the final version of the manuscript.

Data Availability Statement

All data relevant to the study are included in the article.

AI Statement

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) solely for language editing and improving the clarity and readability of the manuscript. The authors reviewed and revised all AI-assisted content and take full responsibility for the accuracy, originality, and integrity of the final manuscript. AI was not used for study design, data collection, data analysis, interpretation of results, or generation of scientific findings.

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