Investigating the effect of Sclareol on IRE-1 and PERK genes The pathway of reticulandaplasmic system stress in gastric cancer cells MKN-45

Elmira Aboutalebi[1], Vand Beilanokhi[1], Ebrahim Sakhinia[2], Homayoun Dolatkhah*[3]

[1] MSc Student in Genetics, Dept. of Biology, Allameh Amini Faculty of Basic Sciences, Azad Islamic University, Tabriz Branch, Tabriz, East Azarbaijan, Iran
[2] Associate Professor in Human Genetics, Dept. of Medical Genetics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, East Azarbaijan, Iran
[3] PhD in Clinical Biochemistry, Dept. of Clinical Biochemistry and Laboratories Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, East, Azarbaijan, Iran

*Corresponding authors: Homayoun Dolatkhah, Address: Dept. of Clinical Biochemistry and Laboratories Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, East, Azarbaijan, Iran. Email: dolatkhahh@gmail.com,

Tel:+989143117197

Abstract

Background & Aims: Despite the decline in the prevalence of gastric cancer in recent years, it remains the fourth leading cause of death from cancer in the world. Common cancer treatments may reduce the size of the tumor, but it is transient and does not have a positive effect on the patient's survival and there is a possibility of recurrence of the disease. Strong induction of reticulom endoplasmic has been shown to increase the susceptibility to anti-cancer therapy. Regarding the importance of medicinal herbs in recent years and its low side effects after administration, compared with synthetic drugs, this study investigated the effects of salvia Sclareol purification from sage on the induction of reticulum endoplasmic system stress.

Materials and Methods: The MKN-45 cell line from the Pasteur Institute of Iran was purchased and cultured in a complete culture medium of RPMI-1640 with cetacean embryos. Cells cultured with 0, 20, 40, 60, 80 and 100 μm concentrations of Sclareol treatment for 5 hours. The rate of expression of IRE-1 and PERK genes by quantitative real time -PCR and the level of proteins IRE-1 and PERK by western blotting method was investigated.

Results: The rate of expression of IRE-1 in doses of 20, 40 and 60 μM Sclareol was significantly increased while decreasing in doses of 80 and 100 μM (p <0.0001). Also, the expression of PERK gene expression at doses of 20, 40 and 60 μM Sclareol was significantly increased, but no increase was observed in doses of 80 and 100 μM (p <0.0001). Also, the levels of IRE-1 and PERK proteins in doses of 20, 40 and 60 micromoles of Sclareol showed a high increase in doses of 80 and 100 μM.

Conclusion: From the results of this study, it seems that doses between 20 and 60 μmol of can be Sclareol helpful in increasing the amount of reticuloendoplasmic stress, but doses higher than 60 milimoles do not have like this effect.

Keywords: gastric Cancer, Sclareol, reticuloendoplasmic system stress

Introduction

Gastric cancer is the fourth most common cancer in the world, with the world's worst mortalities the second rate in the world. The main causes of gastric cancer are helicobacter pylori and nutritional factors, while gastric reflux and obesity also play a significant role in the development of this cancer (1). According to global estimates, 93,000 cases of cancer are detected every year, with 700,000 deaths occurring in areas at risk for the disease in Iran, northern and north-western parts of the country. Adenocarcinoma is the most common form of gastric cancer, and infection with Helicobacter pylori, tobacco use, high salt intake and inadequate antioxidant intake have been reported in Iran (2). In most cases, diagnosis takes place at advanced stages of the disease. Common therapeutic approaches are based on the assumption that tumor mass is homogeneous (3). And target rapidly differentiated and proliferating cells (4). But since more than a century ago, cancer has been morphologically expressed as a heterogeneous population of cells (5). And in the last decade, they have also made some functional differences (6).

In fact, cancers tissue contains a limited subset of cells with special properties, which are responsible for the appearance of tumors, metastases (7). Relapse, and resistance to common treatments (8). Although cancer is considered as a serious disease, but today it is believed that many cancers can be prevented. Statistics show that the incidence of cancer is reduced by one-third in the absence of smoking, weight control, proper diet and exercise (at least 3-5 hours per week). On the other hand, the endoplasmic reticulum (ER) is the largest intracellular organ and is known as a folding protein plant (9).

Many of the newly synthesized proteins go to the lumens of the reticulo-neoplastic network, and they are folded by the chaperones (10). In various physiological conditions and excessive increase in metabolic factors such as glucose, lipid, unsaturated fatty acids, cholesterol and neurotransmitters, the need for protein synthesis in the cell increases, as well as during disturbance in the protein folding pathway (For example, with poor functioning of the chaperones),

tinned or poorly folded proteins accumulate in the reticuloondoplasmic network. As a result of these conditions, the stress of the reticuloondoplasmic network occurs (11,12).

Eukaryotic cells are responsive to the stress of the reticulo-neoplastic network, with a cascade of reactions called Unfolded Protein Response (UPR) (13). The process of UPR through three signal paths, by weakening the translation process, increasing the amount of folding chapron protein in the reticuloondoplasmic network and inducing the system of decomposition with the reticuloondoplasmic network, attempts to restore the condition of the reticuloondoplasmic network back to balance, and in the absence of Compensation induces the process of apoptosis (14).

With regard to studies in the field of understanding the mechanisms of UPR and inflammation, it is possible to target these pathways with a new therapeutic approach to the disease (15). In the early stages of the growth of solid tumors, the proliferation of uncontrolled cells leads to the formation of a cell nucleus, which is characterized by a lack of hypoxia and glucose. Two, Stress stimulation immediately stimulates the stress of the reticuloondoplasmic reticulum and the next reaction program of the UPR protein, which maintains the survival of the tumor cells. In addition, the UPR program can also be stimulated as a result of chemotherapy treatment to inhibit cell death, thereby maintaining tumor growth (16).

The collected evidence suggests that the three branches of UPR can specifically interact with different tumor types, and it is also interesting at different stages of the tumor, such as development, progression, and resistance to treatment (17). In fact, several examples of features that support this concept are that the IRE1 signal is very important at the onset of cancer and cancer cells (18). While PERK activation is required when the tumor is created (19). Considering the importance of medicinal plants in recent years, as well as the low post-consumption side effects compared with synthetic drugs, the effect of the cleaved sclareol extract from the herb on the expression of IRE-1 and PERK genes The

pathway of stress to the reticulandaplasmic system has been studied. The results of previous studies emphasize that sclareol has the potential to be used as a chemotherapy agent and cytostatic activity in cancer cells.

Therefore, it may also be potentially used for drug development. To emphasize more on the molecular mechanisms of this compound, more molecular pathways are needed. In studies and studies on Sclareol, the anti-cancer effects of this compound were discovered and its molecular mechanism was somewhat determined.

Materials and Methods

This is a case - control study that was analyzed based on the results of the laboratory tests. The target population of this study was human-type gastric cancer cells of the MKN-45 class, purchased from the Pasteur Institute of Iran, and these cells were cultured in a cell culture medium complete culture medium of RPMI-1640 with cow's fetal serum (by heat Inactivated (100 ml / liter), penicillin and streptomycin (100 mg / l) and glutamine (2 mmol / L) at 37 ° C at a moisture content of atmospheric pressure and CO2 gas at a pressure The relative volume of 50 ml per liter was grown as a single-layer sintered crop to reach at least 100000 Cell / Well.

After reaching the appropriate density, at least 100,000 cells per plate were dissected from the flask floor by trypsin and passaged. Treatment of slaughtered cells with 0, 20, 40, 60, 80 and 100 μm for 5 hours was treated. . The total RNA of the cells cultured using Trizol solution was extracted and quantitatively and qualitatively evaluated by electrophoresis of Agarose gel and Nanodrop. A review of the expression of IRE-1 and PERK genes of gastric cancer cells before and after treatment with Sclareol was performed by Quantitative Real Time – PCR.

Table 1. Information on the sequence of primers designed for the studied genes

IRE-1Primer	Sequence (5'->3')	NCBI Ref. Sequence
Forward primer	GCTCCAGAACGGGTGTT	NM_138711.3
Reverse primer	CTGTGGCTTTGGACTGGTAGT	
PERK primer	Sequence (5'->3')	NCBI Ref. Sequence
Forward primer	GAAAGTCCACCTTCCCCAACA	NM_001289746.1
Reverse primer	GCGCGTTTGCTCTCTCATTG	
β-Actin Primer	Sequence (5'->3')	NCBI Ref. Sequence
Forward primer	GGTGAGCTGCGAGAATAGCC	NC_000007.14
Reverse primer	CTCCGACCAGTGTTTGCCTT	

To confirm the expression of the genes in this study, the active protein levels were also measured using Western Blotting.

Data analysis method:

First, the CTs calculated for each gene were calculated by the formula ($2^{-\Delta\Delta CT}$) the semi-quantitative amounts of proteins obtained by Western blotting were converted into quantitative values by Image-J software and then compared with each other.

Results

**Evaluation of RNA quality extracted with agarose gel 2%:**

To evaluate the quality of RNA extracted, r18 and rRNA s18 bands were observed in agarose gel 2%. To do this, 1.5 μl of the four RNA treated with 4 μL buffer ligation in 2% agarose gel were electrophoresed for 20 minutes, and the corresponding bands were observed as shown in Fig. 1.
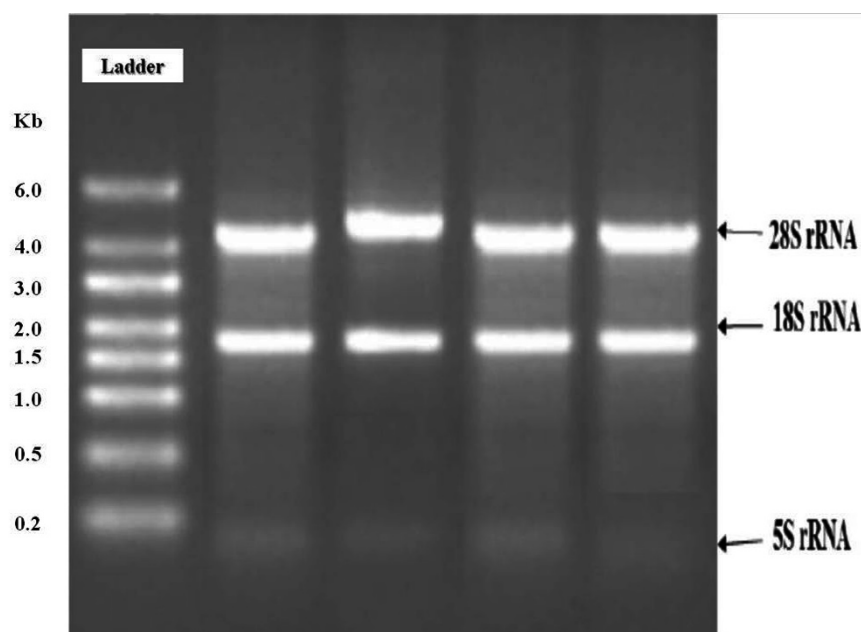
Fig.1: 18S rRNA and 28S rRNA electrophoresis bands for RNA samples extracted from samples

Melting curve graphs obtained from the Real-Time PCR technique to show specific and non-specific products:
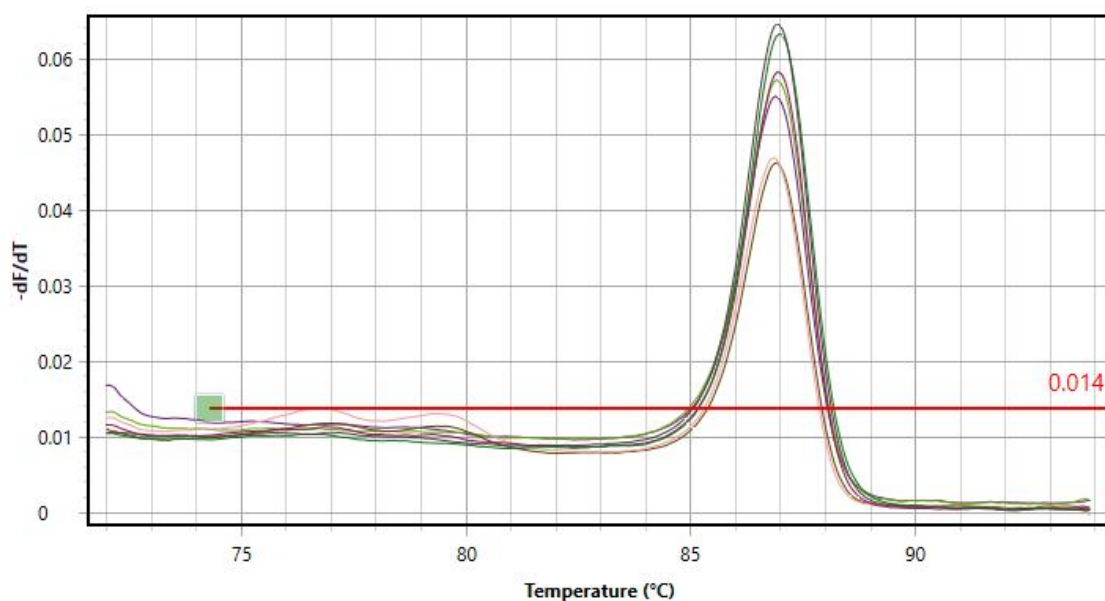
In Figures 2 to 4, the melting curve graphs after the end of the PCR reaction are presented for PERK, IRE-1, β-Actin genes, and as in this figes, in this research, nonspecific products have reached the minimum.
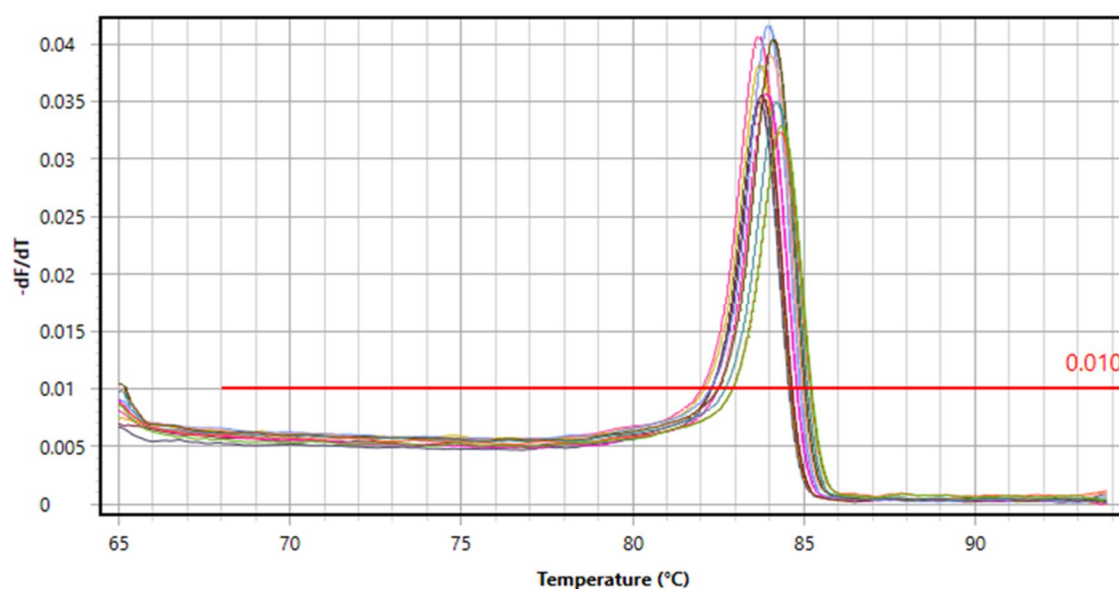
Fig 2: Real-time PCR melting curve for the β-Actin gene
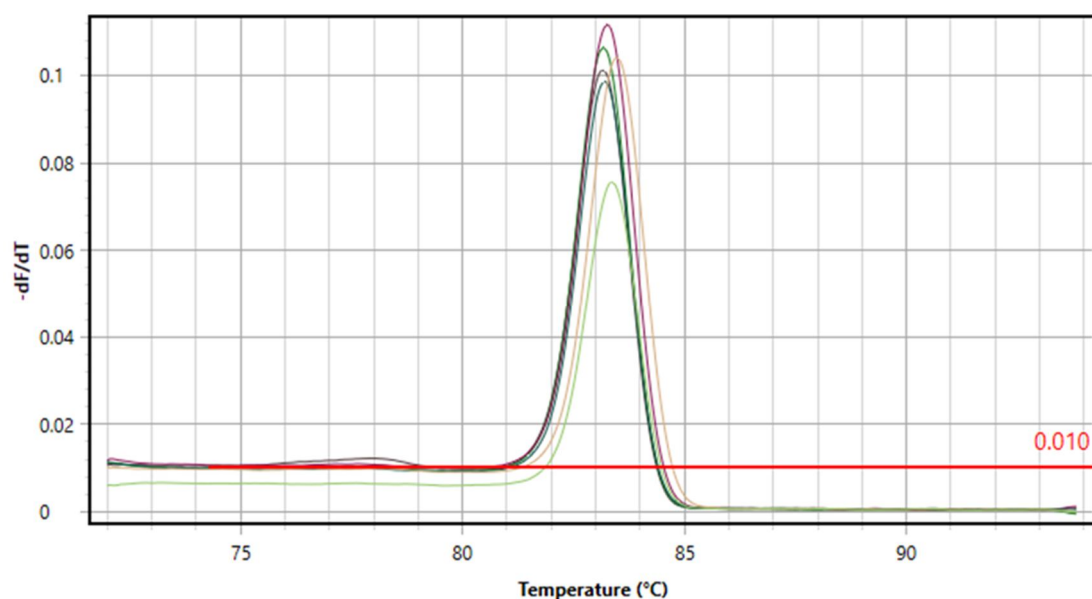
**Fig 3:** Real-time PCR melting curve for the IRE-1 gene

Fig 4: Melting curve graph of the Real-Time PCR technique for the PERK gene

Evaluation of the Effect of Scalreol on the Level of Expression of IRE-1 Gene in Human Stomach Cancer Cells MKN-45:

As shown in Fig. 1, the expression level of IRE-1 in doses of 20, 40 and 60 µM of Sclareol increased significantly, so that the expression of IRE-1 gene in a 20 micromolar Sclareol dose was equal to 1.77 ($2^{-\Delta\Delta CT}$), 40 µm Sclareol was equal to 01.35 ($2^{-\Delta\Delta CT}$)and at 60 µM Sclareol dose was 07.53($2^{-\Delta\Delta CT}$). If decreased in doses of 80 and 100 micromoles. As the amount of IRE-1 expression at 80 µM Sclareol dose was 62.14($2^{-\Delta\Delta CT}$) and at 100 µM Sclareol dose, it was 60.2 ($2^{-\Delta\Delta CT}$).
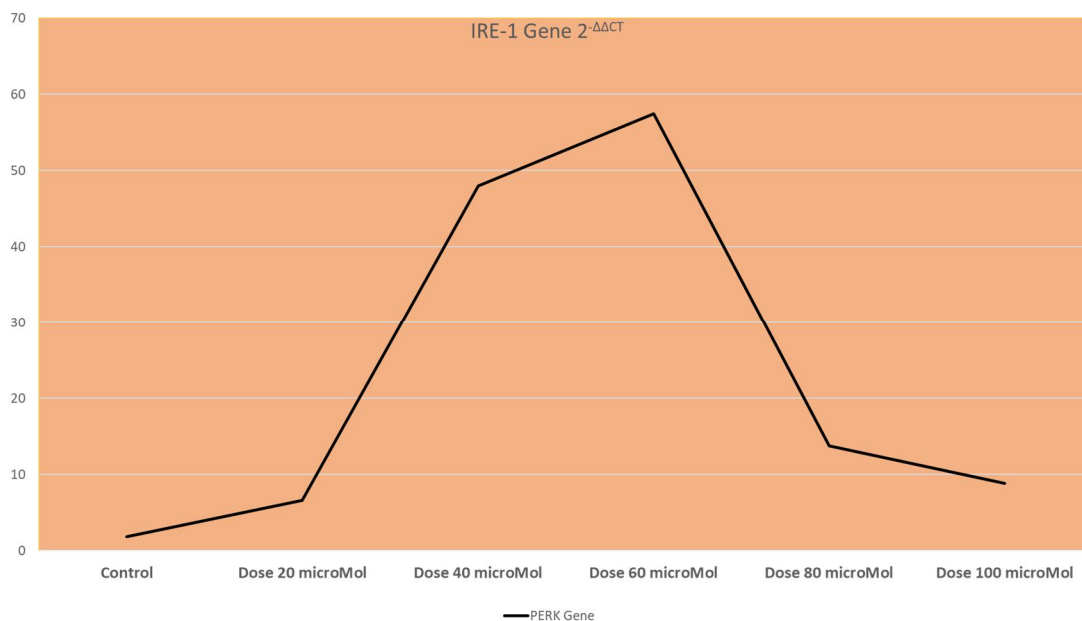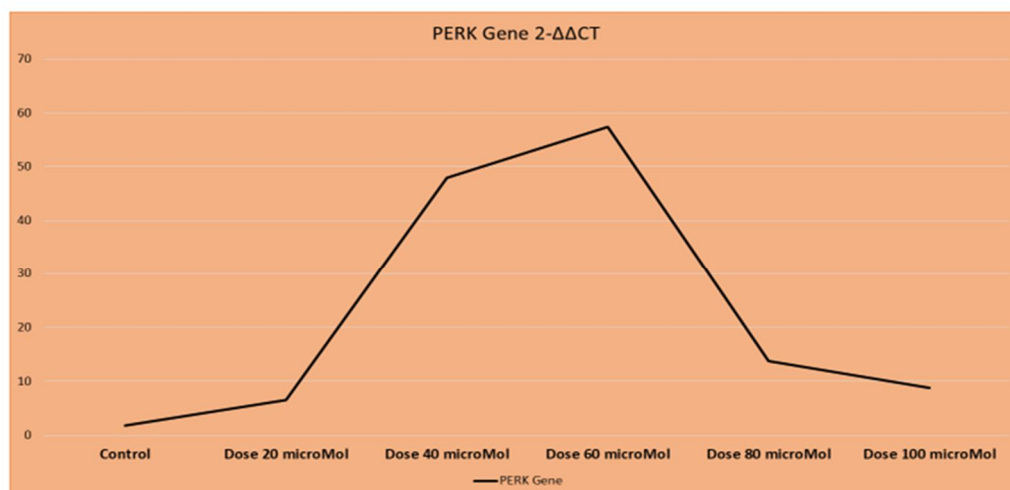
Diagram 1: Effect of Sclareol Concentration on Intracellular IRE-1 Receptor Intracellular Cancer In Cancer Cell

Evaluation of the Effect of Sclareol on PERK Gene Expression in Human MKN-45 Gastric Cancer Cells:

As shown in Diagram 2, the PERK expression rate in doses of 20, 40 and 60 μM Sclareol was increased, so that the expression of PERK gene in a 20 micromole Sclareol dose is equal to 58.6 $(2^{-\Delta\Delta CT})$In a 40-micromole Sclareol dose, it was 16.47975 $(2^{-\Delta\Delta CT})$at 60 μM Sclareol 23 / 574,49 (ΔΔCT-2). If there was no increase in doses of 80 and 100 μM, in that the PERK expression in the 80 μM d Sclareol ose was 3777.25($2^{-\Delta\Delta CT}$) and at 1009 μM Sclareol dose equal to 879.7 ($2^{-\Delta\Delta CT}$).

**Diagram 2:** Effect of Sclareol Concentration on the Percutaneous Transcription of PERK Intracellular Receptor in Cancer Cells

Comparison of Western blotting technique in expression of IRE-1 and-Actin β proteins:

As shown in Fig. 5, the levels of IRE-1 and Actin-β in the doses of 0, 20, 40, 60, 80 and 100 micromoles of Sclareol concentration were measured by western blotting method.
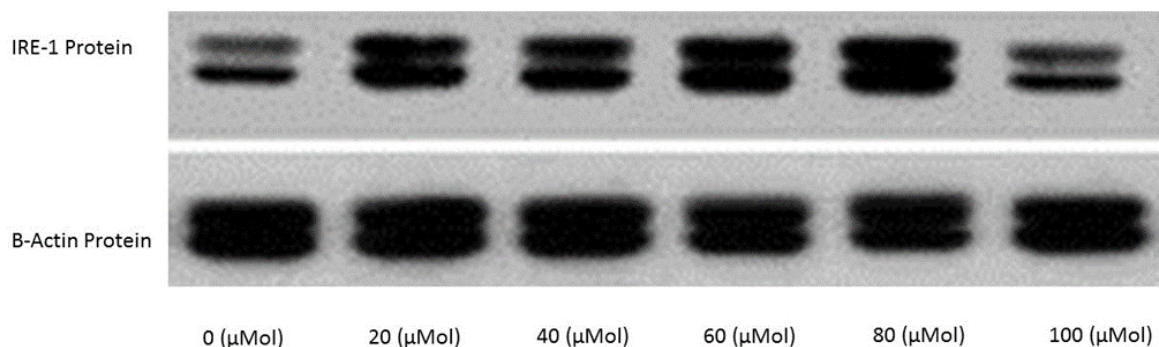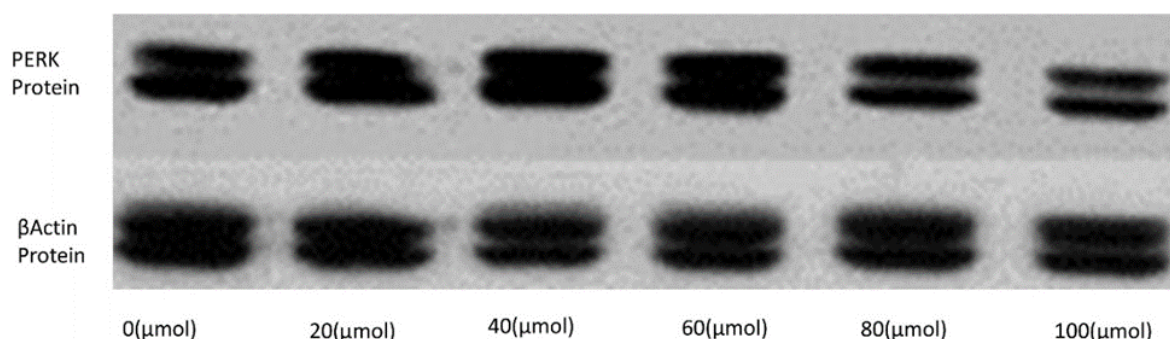

Fig 5: Western Blotting Technique Comparison of IRE-1 and Actin -β Proteins

Comparison of Western Blatting Technique in Expressing PERK Proteins and Actin β: As shown in Fig. 6, PERK and Actin -β proteins were measured in 0, 20, 40, 60, 80 and 100 micromoles of Sclareol concentration by western blotting method.

Fig 6: Western blotting technique Comparison of PERK and Actin -β proteins

Comparison of IRE-1 Protein in Gastric Cancer:

As shown in Figure 4, IRE-1 proteins in doses of 20, 40 and 60 micromoles of Sclareol increased significantly, with IRE-1 proteins at 20 micromolar Sclareol dose equal to 1.5 at a dose 40 micromoles of Sclareol was 5.1 and at a dose of 60 μm Sclareol 7.1. If it did not show an increase in doses of 80 and 100 micromoles. So that the expression of IRE-1 proteins in the 80 micromolar Sclareol dose was 0.2 and in the 100 micromole Sclareol dose was 2.1

Studied groups
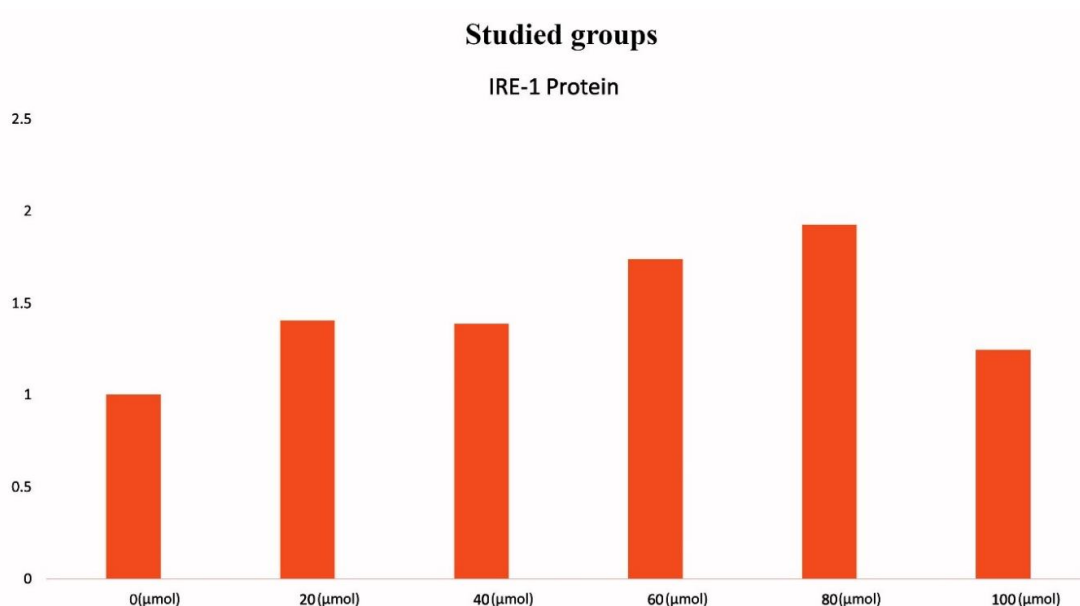
IRE-1 Protein

Diagram 3: The rate of IRE-1 protein in the 6 groups studied. As shown in the diagram, the x-axis of the dose used is Scalareol and in the y-axis, the amount of protein IRE-1

Comparison of PERK Protein in gastric Cancer:

As shown in Fig. 5, the PERK expression levels expressed in doses of 20, 40 and 60 µM were Sclareol significantly increased, so that the expression of PERK proteins in a 20 micromole Sclareol dose was 2.1 in a dose of 40 Sclareol micromole was equal to 4.1 and at a dose of 60 µM Sclareol was 2.1 If it did not show an increase in doses of 80 and 100 micromoles. As the expression of PERK expression in the 80 micromolar Sclareol dose was 9.0 and in the 100 micromole Sclareol dose, it was equal to 0.1

Studied groups
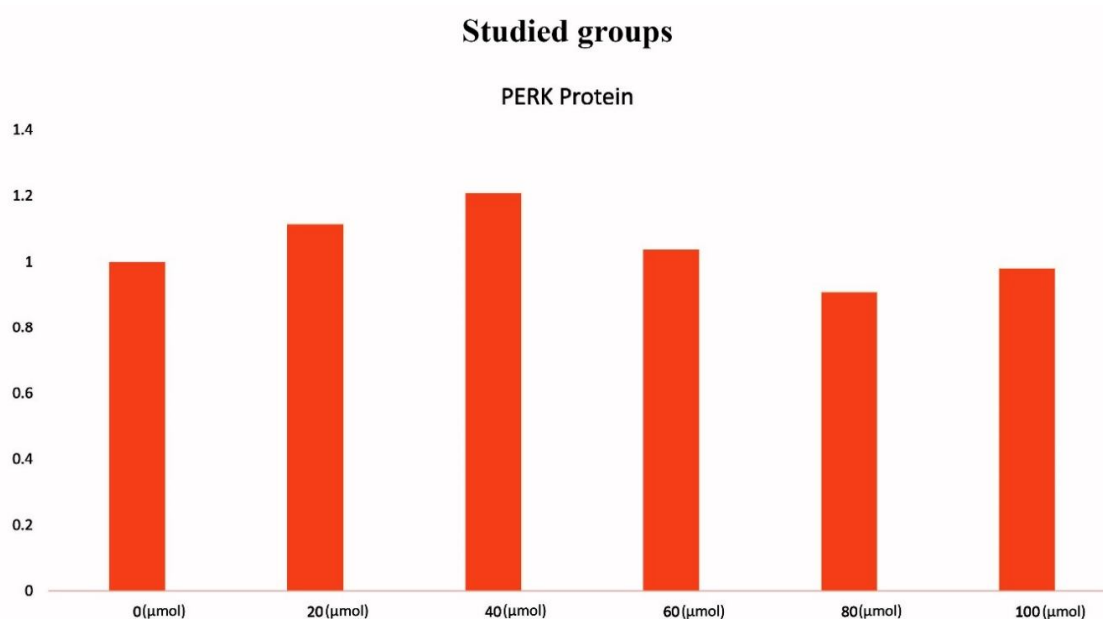
PERK Protein

Diagram 4: PERK protein content graph in the 6 groups studied. As shown in the diagram, the skeletal dose is given in the x-axis and in the y-axis, the PERK protein level

Discussin

The purpose of this study was to investigate the effect of Sclareol on IRE-1 and PERK genes of the reticulandaplasmic reticulum system in human MKN-45 gastric cancer cells. Earlier in various studies such as the study Nuri et al In 2010 (20) , Dimas et al In the year 2007 (21) and Zhang et al In 2017 (22), reported that Sclareol can inhibit tumor growth in vitro and have a low toxicity and tumor growth in vivo Reduces And had anticancer activity against cancer tumor cells, and also reported that Sclareol provides its anticancer effects by adjusting the amounts of caveolin-1, which is a very important scaffold protein in regulating several signaling pathways, including the pathway for apoptosis. However, in various studies, various mechanisms have been proposed for the anticancer effects of Sclareol. Here's a discussion below.

Noury et al in 2010 (20) reported that Sclareol can inhibit tumor growth in vitro. Has a low toxicity and reduces tumor growth in vivo. It can also lead to immune response by increasing the level of INFγ and reducing the level of IL4 to Th1. It also has cytotoxic activity and can be destroyed by induction of apoptosis in human leukemia cells. Dimas et al in 2007(21) Found that this compound has anti-tumor activity against colon cancer tumors by studying Sclareol. The addition of this compound in vitro to the culture medium of HCT116 colon cancer inhibits DNA synthesis and disrupts cellular cycle and keeps HCT116 cells in the G1 phase

 Also Huang et al In 2012 (23) Reported in 2012 that Sclareol can exhibit anti-inflammatory properties in the vicinity of LPS-stimulated macrophage cells and, in vivo, can also reduce the edema in the tumor of the heel of the mouse.

Zhang et al In 2017 (22), Studying the effects of Sclareol on cervical cancer cells, reported that Sclareol provides its anticancer effects by adjusting the amounts of caveolin-1, which provides a very important scaffold protein in the regulation of several signaling pathways, including the pathway for apoptosis.

Signoretto et al in 2016 (24) reported that Sclareol causes red blood cell lysis by eliminating the membrane phospholipids of these cells, which is due to the activation of the P38 kinase and casein kinase -1α enzymes.

Wang et al In 2015 (25) reported that Sclareol inhibited the growth of osteosarcoma cancer cells by inducing apoptosis by studying the effects of Sclareol - dependent on osteosarcoma cancer. This induction, along with the stop of the cell cycle in the G1 phase, and the loss of the mitochondrial membrane potential in these cancer cells.

Also Dimas et al In 2006 (26) reported on the effects of Sclareol in breast cancer cells MN1 and MDD2 derived from human MCF-7 maternal cells, which reported that Sclareol induces apoptosis from the nonbody pathway to the P53 gene . In addition, they reported that can Sclareol increase the effect of duxurubicin, autopsy and cisplatin drugs on routine chemotherapy used to control breast cancer in MDD2 cell line.

Effect of Sclareol in gastric cancer cells:

n this study, the effect of Sclareol on IRE-1 and PERK genes of retinal doplasmic system was investigated in human gastric cancer cells of MNK-45. MKN-45 was purchased from the Pasteur Institute of Iran and in a complete culture medium, RPMI-1640 was cultured with cow's serum. Cells cultured with 0, 20, 40, 60, 80 and 100 μm concentrations of Sclareol for 5 hours. The expression levels of IRE-1 and PERK genes were measured by Quantitative Real Time-PCR. The expression level of IRE-1 in doses of 20, 40 and 60 μM Sclareol was significantly increased (p <0.0001), However, there was no significant effect on doses of 80 and 100 μM. Also, the PERK expression in doses of 20, 40 and 60 μM Sclareol was significantly increased (p <0.0001), but no significant increase was observed in doses of 80 and 100 μM. In this study it seems to be that doses between 20 and 60 micromoles of Sclareol can be helpful in increasing the amount of reticuloendoplasmic stress, and the effective dose of this extract is on the cancer cells of the MNK-45 group. But doses above 60 milimoles do not,

Hassani et al reported in 2002 and 2003 (27, 28) that Sclareol was an interesting alternative to voluntary

pathways in intrauterine infusion, and in the 2010 report by Noury et al (20) , In 2010 Which extracted Sclareol from S. Sclareol, and its study of the effectiveness of immunity Treated with Sclareol injected directly into the tumor and in this study, it seems that Sclareol can be effective in low doses (we make between 20-60 milimoles).

Although gastric cancer is commonly found in solid tumors, no reports have been reported on the effects of Sclareol in doses greater than 80 in gastric tumor cells. This is not an end in itself, and one of the drawbacks of our study is that this study should be done on healthy stomach cells, which will certainly be considered in later studies. However, similar studies on the sample Gastric cancer in humans has not been reported so far, so the results of this study can not be achieved with a general result.

Noury and Wang et al (20,29) First reported on the isolation and cleansing of Sclareol and extracted 1000 grams of S Sclareol from northeast. Dry plant was mixed with n-hexane / ethyl acetate: acetate / methanol (1: 1: 1) and in the room temperature was maintained for 24 hours. Filtration was then evaporated by evaporation of a rotary vacuum tank at 45 ° C. A concentrated filtration was performed by chromatography of a silica gel column in a solution of various poles starting from a nonpolar solvent to a polar medium (n-hexane / ethyl acetate) and finished with acetate ethyl.

Then mixing different concentrations of n-hexane / ethyl acetate / methanol with increasing methanol concentration compared to higher polarity was used for 2 hours in a freezer. The extract was then passed through the Whitman filter paper and tested in adult mice the inbreds were aged 6 to 8 weeks. Due to free access to food and water, the rats were placed for one week and kept in special places. The standard conditions before the test and all the tests according to Protocol, animal care and their use were carried out at Tarbiat Modares University, Tehran, Iran.

Isolated molecules using 1 H NMR spectroscopy of S. Sclareol were collected using previous processes and the purity of the components in each section was evaluated by thin layer and Sclareol chromatography.

Spectrometry of 1H NMR 500 MHz was shown using CDCl3 as a solvent. The effect of Sclareol in the DTH response to assess the effect of Sclareol on immune response, a group of 10 mice were divided into two groups and the results showed that animals treated with Sclareol The effect of intra Sclareol tumor injection on tumor volume in order to evaluate tumor volume in animals containing tumor, Sclareol was injected into the tumor daily measurement of tumor size. The injected dose from Sclareol decreased significantly compared to the control group (p <0.05). The effect of intra-tumor scralareol injection on lymphocytes Sclareol index. The proliferation of intra-tumor injection using duplicate Muscle tumor cells are injected 6 days after the onset of internal tumors and again stimulate with injected with lysis antigen, Proliferative and responses of skilled rats treated with Sclareol. As well as in treated animals, the effect of Sclareol has increased. The effect of intra-tumor Sclareol injection on IFN-c and IL-4 changes from intramuscular injection was studied in mice for producing IFN-c and IL-4. The effect of intra-tumor Sclareol injection on CD4 + CD25 + Foxp3 + T-monitored T cells The effect of intra Sclareol tumor on CD4 + CD25 + Foxp3 + T lymphocytes was measured by flow cytometry (P <0.05) Reduction in CD4 + CD25 + Foxp3 + T tumor In lymphocytes.

Norie et al In 2010 (20) Also reported that direct injection of Sclareol into the tumor increases and antitumor activity develops in the immune system and prevents tumor growth, and, accordingly, stimulates the more potent anti-cancer cell T response One of the crucial stages of success. In addition, Au et a al In 2001 (30) Reported that topical injection of Sclareol can be safely.

And may require a higher dose of Sclareol to produce a more potent effect and may develop an anti-tumor system and, therefore, direct injection into the Sclareol tumor may be a desirable candidate for cancer treatment.

The Effect of IRE-1 and PERK Genes on the Stress of the Reticulandoplasminic System in Human Gastric Cancer Cells MKN-45:

Lin et al In (2007) (31) , by studying the complexity of the metazoan system, showed that there are two sensitive protein-responsive protein reticulo-adenoplastic systems that include transcription factor kinase PERK and ATF6. There are conflicting studies on how these genes are present. It is related to the stress of the reticulo-adenoplastic system of mammals. And in the recent study found that IRE1 can account for cellular survival as a result of it, and causes the stressor of the reticuloendoplasmic system on thapsigargin and tunicamycin.

Wu and Yamamoto and colleagues in 2007 (32) , did not find any result in studying the activity of the IRE1 gene that cells could survive or the remarkable genes of reticuloendoplasmic stress stress, instead, IRE1 was identified as a regulator of cell proliferation In all cases where the activity of the IRE1 base was impaired in the absence of the stressors of the reticulo-anaphylactoid system, cell proliferation slowed down, while the promotion of the basic activity of IRE1 was able to stimulate cell proliferation in all three cell lines, and the method Electrophoresis of polyacrylamide gel was used in this study, Wu and Yamamoto showed that the I642G IRE1 mutation had an increased Xbp-1, even in the absence of 1NM-PP1, the spelling activity, while the mutation K599A IRE1 blocked the Xbp-1 beam. Thus, the activity of endrobinoclase IRE1 is absent. ER stimulus and can promote cell proliferation additionally, the use of target siRNA represents the sum of Xbp-1s a downward downward mechanical link. The basic activity of IRE1, as those cells with reduced The Xbp-1 levels were weakened in their ability to reproduce. These data are consistent with a model in which IRE1 controls an arm's response to the stress of the reticulo-anaplastic system and highlights the biological function of IRE1 in controlling cell proliferation. The activity of IRE1 in the expression of the stress gene of the known reticulo-adenoplastic system in human cells has been confirmed in this study. Recently, it has been shown that the transcription of Grp78 and Grp94 induction was significant and was a defect in MEFs and a shortage of ATF6α gene.

Lacroix and Leclercq, and Shuda et al In 2003 and 2004 (33,34) , studying in the stress pathways of the reticulandoplasmic system and tumor growth, became a region of intense interest and the pathways that have been modulated in human cancer in IRE1 / XBP-1. Connected to Xbp-1 and IRE1 / XBP-1, both in the intravenous carcinoma and in breast cancer.

In a study by Kulshreshtha R et al (35), although stomach cancer is common in solid tumors and hypoxia is a hallmark of its features, there are no reports of the effect of hypoxia on the expression of miR-107 in gastric tumor cells. Is. In this study, the relationship between hypoxia and increased expression of miR-107 in gastric cancer cells was 45-MKN and AGS.

Conclusion

From the results obtained in this study, it seems that the effect of Sclareol administration on the IRE-1 and PERK genes in the pathway of the reticulandaplasmic system's stress in gastric cancer cells of the human grade MNK-45 used to control gastric cancer can be be useful. If doses ranging from 20 to 60 μmol of Sclareol can be helpful in increasing the amount of reticuloendoplasmic stress, doses higher than 60 mM can not be. And the effective doses in this study range from 20 to 60 Mil mol. This study showed that it could be a hopeful tip for reducing drug resistance in patients with gastric cancer.

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