



The effect of Sclareol on the expression of MDR-1 gene and Glycoprotein-P Level in MKN-45 human gastric cancer cells

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Abstract

Background & Aims: In recent years, the emergence of multidrug resistance in gastric cancer has been a major challenge in treatment of gastric cancer. To deal with the problem, studies and researches were conducted on Sclareol and have turned up the anti-cancer effect of the compound and have also determined the molecular mechanism of it to some extent. Therefore, the main purpose of this study was to investigate the effect of the substance extracted from *Salvia Officinalis* called Sclareol on MDR-1 gene expression and consequently on the rate of P-glycoprotein in human gastric cancer cell line MKN-45.

Materials & Methods: Cell line MKN-45 was purchased from the Pasteur Institute of Iran and cultured in complete RPMI 1640 Medium with Fetal Bovine Serum, with 20, 40, 60, 80 and 100 μ M concentrations of Sclareol treatment for 5 hours. The rate of expression of MDR-1 gene was assessed by Real Time-PCR method and that of P-GP was assessed by Western blotting method.

Results: The expression of MDR-1 gene was significantly reduced at doses of 20, 40 and 60 μ mol of Sclareol, while at doses of 80 and 100 μ mol there was not seen much effect ($p < 0.0001$). Also, P-glycoprotein showed a very high decrease at doses of 40 and 60 μ mol of Sclareol, but no decrease was seen at doses of 80 and 100 μ mol ($p < 0.0001$).

Conclusion: From the results of this study, it seems that doses between 20 and 60 μ mol of Sclareol can be useful in reducing drug resistance, but doses above 60 mmol do not have such an effect.

Keywords: Gastric Cancer, Sclareol, Multi-drug resistance (MDR1) gene, Glycoprotein-P

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Introduction

The emergence of Multidrug Resistance (MDR) to chemotherapy is considered a serious obstacle in cancer treatment (1). Chemotherapy as an alternative treatment

option is used to treat the patients suffering from the final process of progressed and metastatic cancers, the point at which the main challenge is to resort to applying the drug with a dose that has the highest efficacy and the

least toxicity. Unfortunately, in many patients the tumor does not respond to therapeutic agents. This is due to the resistance to chemotherapy in clinical treatment. Whether hereditary or acquired, there is always resistance to any anticancer drug (2).

MDR is one of the most common causes of treatment failure in cancer patients. Although most of tumors show an initial response to chemotherapy, in a recurrence there are some tumors that no longer respond to the drugs being initially used or to any of the other anticancer drugs. Laboratory researches have determined that the cellular basis of this phenomenon is drug resistance (3). Resistance exists to any effective anticancer drug and develops with several mechanisms, including decreasing drug absorption, increasing drug excretion, activating the detoxification system, activating DNA repair mechanisms, escaping from induced apoptosis by drug and etc (3).

Several mechanisms have been determined for MDR. One of most important of them, which is associated with altered drug delivery of anticancer drugs, is mediated through the superfamily proteins of ABC (Adenosine triphosphate Binding Cassette) transporter. These transporters were able to reduce drug concentrations in vitro. One of the most well-known multidrug resistance genes is MDR1 gene which is located on chromosome 7 and codifies a P-glycoprotein (P-gp). Expressing P-gp occurs in tumors that are associated with their drug resistance. Since the P-gp is involved in both hereditary and acquired drug resistance in human cancers, it plays an important role in improving the effectiveness of chemotherapy (3).

Considering the above-mentioned information on the one hand and according to the results of studies published in recent years on the other hand, it was concluded that in general, the killers of cancer cells and antitumor cells act selectively, and also kill the natural proliferating cells. Identifying specific compounds in cancer cells is a significant point in screening and discovering drug mechanisms. It has been reported that Sclareol in the sage plant (the scientific name, *Salvia Officinalis*) induces apoptosis in several cancer cells (4).

The results of a study conducted in recent years show that intra-tumoral injection of Sclareol has antitumor effects, modulates immune responses by reducing Treg cells and inhibits tumor growth in vivo as well (5).

Sclareol has been reported to be a non-toxic compound, having the ability to induce apoptosis in the leukemia cell line (in a time- and concentration-dependent manner). Scientists have found that the herbal-chemical diterpene in *Salvia Officinalis* (Sclareol) possesses anti-cancer properties in vitro (6). The results of previous studies emphasize that Sclareol offers the potential of being used both as a chemotherapeutic agent and as a cytostatic agent active in cancer cells. Therefore, it may potentially be used for pharmaceutical development as well. To emphasize more on the molecular mechanisms of this compound, it is necessary to study more molecular pathways (7).

In studies and researches on Sclareol, the anti-cancer effects of this compound were discovered and its molecular mechanism was partially determined. Therefore, our hypothesis is that Sclareol in human gastric cancer cell lines MKN-45 may reduce the expression of MDR-1 gene and consequently reduce P-glycoprotein synthesis.

Materials & Methods

The present study was of experimental type which was analyzed on the basis of the obtained experimental results. The target population was human gastric cancer cell line MKN-45.

The target population in this study was human gastric cancer cell line MKN-45 which was purchased from Iran Pastor Institute. The cells were cultured in the laboratory so that the number of them reached up to at least 100,000 cells/well. For this reason, the sample size for this study was at least 100,000 cells per flask, were divided into six groups and each was treated by six different concentrations of Sclareol.

Cell culture method by Sclareol injection:

Cell line MKN-45 was purchased from Pasteur Institute of Iran and then cultured in RPMI 1640 complete medium with Fetal Bovine Serum (100 ml/L) (inactivated by heat), penicillin and streptomycin (100

mg/l each) and glutamine (2 mmol/L), at a temperature of 37°C in the presence of the moisture in the atmosphere and CO₂ with a relative pressure of 50 ml/l as a single adhesive layer. Having reached the appropriate density of about the minimum cell of 100,000 per plate, the cells were separated from the bottom of the flask by trypsin and labeled with passage number.

The cells were then treated with 0, 20, 40, 60, 80 and 100 µM concentrations of Sclareol for 5 hours. From spheres obtained from cell lines, cell suspension was prepared. The total RNA of the cultured cells was

extracted using Trizol solution with the kit protocol made by EUR X Polish manufacturer and evaluated quantitatively and qualitatively by Agarose gel electrophoresis and Nano Drop at 260 nm.

CDNA synthesis was performed from template RNA with reverse transcriptase enzyme. To make cDNA, the Synthesis Kit PrimeScripttm 1st Strand cDNA product of Takara commercial Company was applied.

Using Primer 3 and Primer Express[®] software, investigation of related sequences in Gene Bank primers and specific probes for MDR-1 gene was designed and chosen (Table 1).

Table 1: Information on the sequence of primers designed for MDR-1 and GAPDH genes

MDR-1 PRIMER	SEQUENCE (5'→3')	LENGTH	TM	GC%	SELF COMPLEMENTARITY	SELF 3' COMPLEMENTARITY
Forward primer	CAACGGGACTCAGGAGCACA	20	62.10	60.00	4.00	0.00
Reverse primer	CGCCATGGAGTAGCCAAAC	20	61.38	60.00	6.00	0.00
GAPDH Primer	Sequence (5'→3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAAGGTGAAGGTCGGAGTC	19	57.18	57.89	3.00	3.00
Reverse primer	GAAGATGGTGATGGGATTTC	20	53.72	45.00	3.00	2.00

In this study, Real Time PCR method was used to examine the expression of MDR-1 gene with the use of the Corbett Rotor-Gene 6000 instrument. All samples were repeated three times. After the end of the reaction and determination of the threshold line, the threshold cycle (CT) of each sample was obtained. The relative expression of MDR-1 gene was calculated by $2^{-\Delta\Delta CT}$ method by the ratio of the threshold cycle of the desired gene to MDR-1 gene and GAPDH as the housekeeping gene.

To confirm the expression of MDR-1 gene, the amount of its active protein -P-glycoprotein- was determined by Western Blotting.

Data analysis was presented in the descriptive statistics section as the mean, median, standard deviation, the minimum and maximum. Then the

normality of the data was checked using Kolmogorov-Smirnov test and finally in the inferential statistics section, the research hypothesis was investigated using One-Way ANOVA and SPSS Statistics 22.

Results

Assessment of the quality of RNA extracted from 2% agarose gel:

To assess the quality of the extracted RNA, 18s rRNA and 28s rRNA bands were observed in 2% agarose gel. To do this, randomly 1.5 µl of the four treated RNA samples were electrophoresed with 4 µl loading buffer in 2% agarose gel for 20 minutes and the corresponding bands were observed as shown in Figure 1.

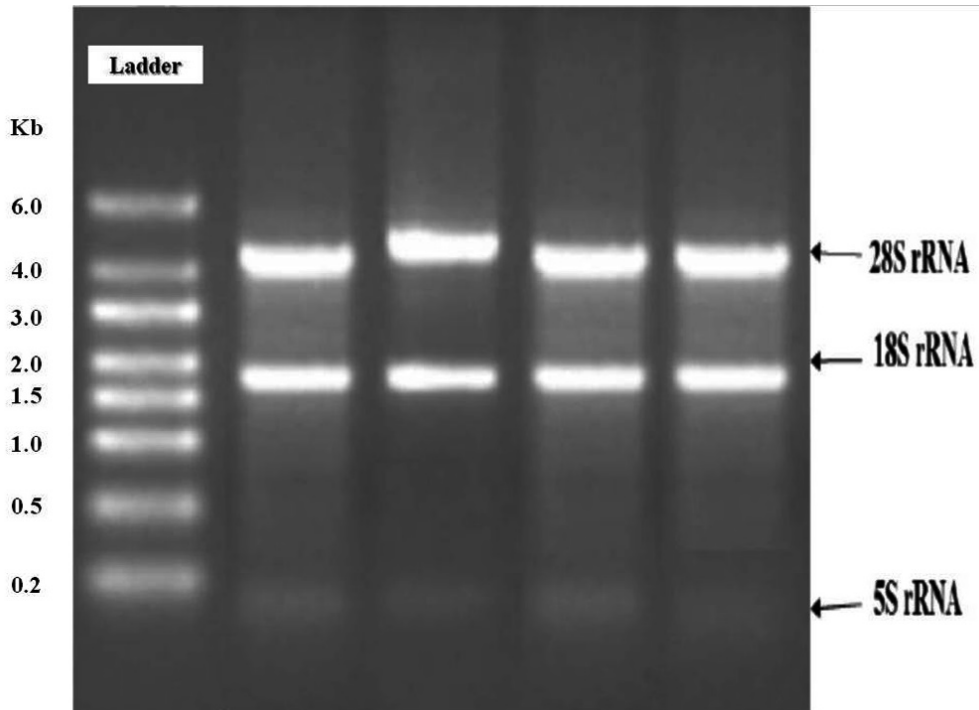


Fig 1: 18s rRNA and 28s rRNA electrophoresis bands corresponding to RNA samples extracted from culture media

Assessment of the quality of primers by performing reactions of gradient PCR:

In order to make sure of the quality of the primers, a gradient PCR reaction was run for the primer of GAPDH

gene. The PCR products were examined by electrophoresis with 2% agarose gel and the results were given as a single Sharp band at the temperature of 57.8°C, the detection of which was observed and recorded under UV light, as shown in Figure 2.

B

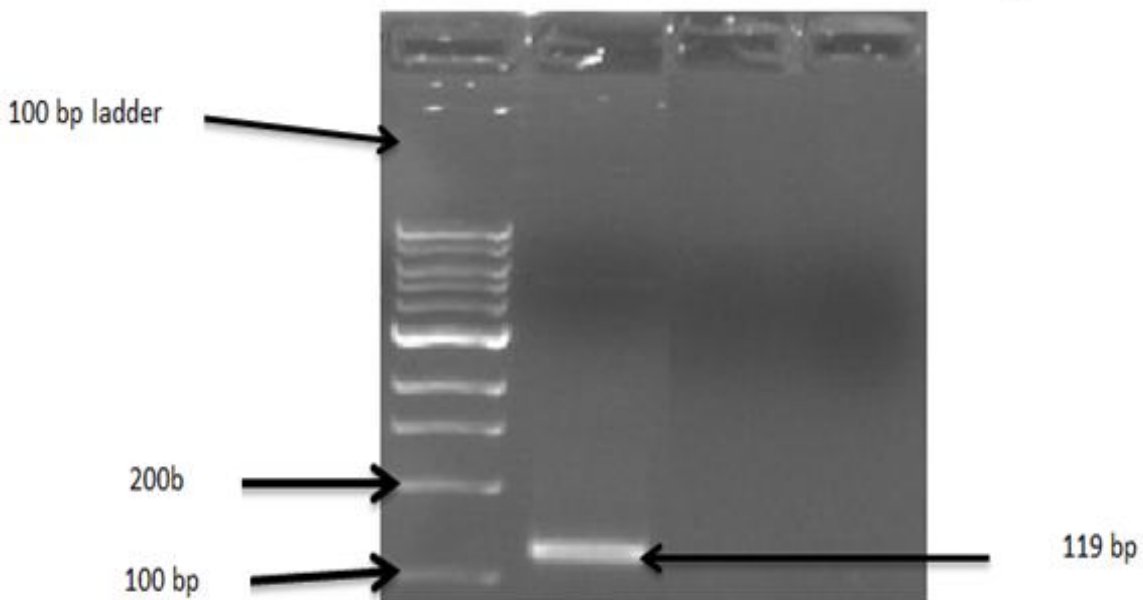


Fig 2: Image of GAPDH gene in electrophoresis gel with 119 bp product banded at 57.8 ° C.

Investigation of MDR-1 gene expression in the study groups:

The results showed that the expression of MDR-1 gene at doses of 20, 40 and 60 μM of Sclareol was significantly reduced: the rate of MDR-1 gene expression at dose of 20 μmol of Sclareol was equal to

0.87 ± 0.026 ($2^{-\Delta\Delta\text{CT}}$); at dose of 40 μmol of Sclareol, 0.61 ± 0.026 ($2^{-\Delta\Delta\text{CT}}$); and at dose of 60 μmol , 0.08 ± 0.010 ($2^{-\Delta\Delta\text{CT}}$). This was while at doses of 80 and 100 μmol there was an increase. And MDR-1 gene expression at dose of 80 μmol of Sclareol was equal to 0.51 ± 0.062 ($2^{-\Delta\Delta\text{CT}}$) and at dose of 100 μmol of Sclareol it was equal to 0.93 ± 0.052 ($2^{-\Delta\Delta\text{CT}}$) ($p < 0.0001$) (Figur 3).

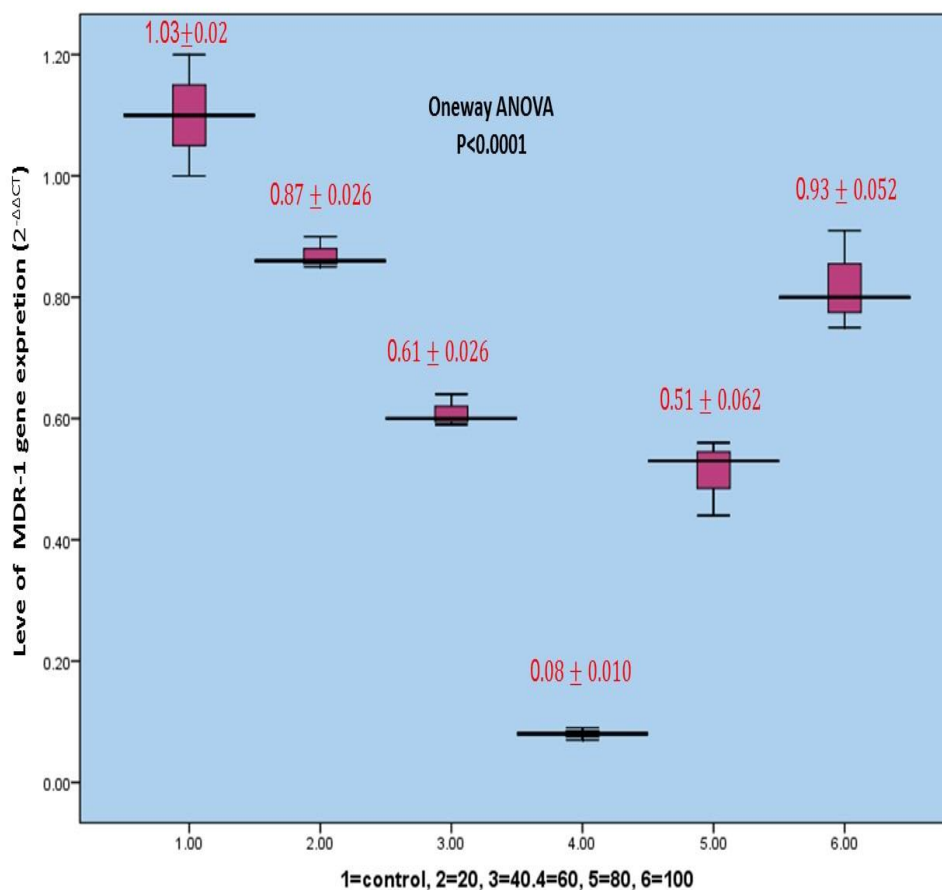


Fig 3: BoxPlot graph showing the effect of Sclareol concentrations on MDR-1 gene expression in cancer cells

Comparison of Western blotting technique in P-glycoprotein and GAPDH:

The cultured cells were treated with 0, 20, 40, 60, 80 and 100 μM concentrations of Sclareol for 5 hours. The rate of glycoprotein and GAPDH at doses of 0, 20, 40, 60, 80, and 100 μM concentrations of Sclareol were assessed by Western blotting as shown in Figure 4.

Comparison of P-glycoprotein protein rate in MKN-45 gastric cancer cells:

The results obtained from Western blotting technique showed that the rate of P-glycoprotein protein expression at doses of 40 and 60 μmol of Sclareol was significantly in decline: the rate of P-glycoprotein protein at dose of 40 μmol of Sclareol was equal to 0.596 and at dose of 60 μmol of Sclareol it was 0.236. However, the rate of P-glycoprotein at dose of 80 μmol of Sclareol was equal to 0.896 and at dose of 100 μmol of Sclareol it was equal to 0.985 which showed an increase in comparison to those of the doses of 40 and 60.

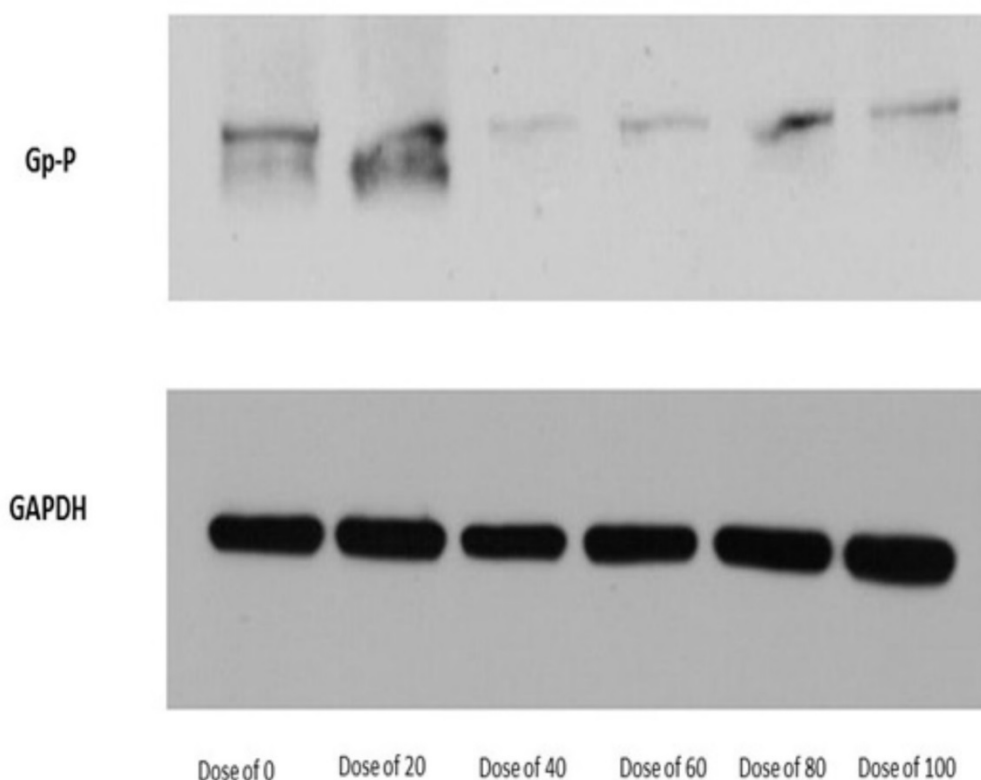


Fig 4: Western blotting technique- comparing P-glycoprotein and GAPDH

Discussion

Drugs used in cancer control as routine chemotherapy drugs for gastric cancer control, such as platinum, are among the newer anti-cancer drugs that bind to chlorine atoms in the form of Cis-configuration, creating anti-cancer properties. This drug reacts easily with the N7 site of purines and causes disruption and cessation of the cell cycle and eventually apoptosis by creating intersecting cross-links (8). Resistance to these drugs is created by mechanisms that reduce the binding of these drugs to DNA (such as changes in drug entry and exit into the cell and inactivation of the drug by metallothionein and glutathione) as well as by the mechanisms that repair DNA damage caused by these drugs such as Nucleotide excision repair (NER) (9). On the other hand, most of the herbal compounds used in chemotherapy such as taxol, vincristine, etc. not only induce programmed death in cancer cells, but also cause destructive damage to normal host cells (10).

Different types of ABC vectors appear to be involved in resistance to chemotherapy drugs in gastric cancer cells. In recent years, most clinical studies on drug resistance have been performed on P-glycoprotein. Various studies have shown that P-glycoprotein is highly expressed in many human cancers, especially in gastrointestinal cancers. It has been proved that high expression of P-glycoprotein in cancer cells affects the response of cancer cells to chemotherapy drugs (11, 12). Therefore, it was hypothesized that drug resistance in cancer cells could be inhibited using P-glycoprotein inhibitors.

The effect of Sclareol on the rate MDR-1 gene expression and on P-glycoprotein gene in MKN-45 gastric cancer cells in the present study:

In the present study, the effect of Sclareol on MDR-1 gene and P-glycoprotein in MKN-45 human gastric cancer cells was investigated. MDR-1 gene expression was significantly reduced at doses of 20, 40 and 60 μmol

of Sclareol ($p < 0.0001$), while this effect was not seen at doses of 80 and 100 μmol . Also, the amount of P-glycoprotein at doses of 40 and 60 μmol of Sclareol showed a significant decrease ($p < 0.0001$), but at doses of 80 and 100 μmol , this effect was very small. In this study, it seems that doses between 20 to 60 μmol of Sclareol can be great for reducing drug resistance and work in fact well as the effective doses of this extract on MKN-45 cancer cells line. However, doses above 60 μmol do not have this effect. Hassan et al. (2003) (13) suggested that Sclareol is an interesting alternative to voluntary routes in intra-tumor injections. And in a study by Noori et al. (2010) (14), Sclareol was extracted from *Salvia Sclarea* plant, its effectiveness was evaluated and Sclareol was used by direct injection into the tumor. In the same study, low-doses of Sclareol (between 20-60 μmol) yield effective results that are consistent with the results of the present study. Although gastric cancer is more common than solid tumors, there have been no reports of the effect of Sclareol at doses above 80 on gastric tumor cells. This is not certain, and one of the drawbacks of the present study was that the study should also have been performed on healthy stomach cells that can be taken into account in future studies. However, because similar studies performed on human gastric cancer samples have not been undertaken, it is not possible to reach a general conclusion merely on the basis of the results of the current study.

Studies on drug resistance associated with MDR-1 genes and P-glycoprotein:

The main reason for the failure of treatment in gastric cancer is accounted for the development of multidrug resistance in chemotherapy. Recently, there has been a report published on drug resistance of gastric cancer that ATP-binding transporters such as P-glycoprotein or the protein associated with the MDR-1 gene, and a number of other molecules in humans may cause drug resistance in gastric cancer. Newer researches have included the use of new chemotherapeutic agents such as taxanes and platinum which could be one of the best clinical and oncological treatments. All the facts show the inherent and complex nature of the multidrug resistance of gastric

cancer. In each of the two monotherapy and combination therapies for gastric cancer, which are used routinely, the response rate in patients is seen to be about 30-40%. Using cellular and molecular biology techniques, the altered genes that cause drug resistance in simple cells have been identified, and these cells have been known as drug resistance cell lines. Due to the specific nature and genetic background of gastric cancer cells, many of these altered genes are associated with the MDR1 gene. According to the results of these studies, the most common reason for drug resistance to a wide range of anticancer drugs lies in the high expression of ATP-dependent transporters that are responsible for removing the drug from the cell. The most famous of these ATP-dependent vectors is P-glycoprotein, which is regarded as a product of the MDR1 or ABCB1 gene (15).

In a review article, Ruby et al. (2008) (16) explained that some studies have reported high expression of ABCC1 or ABCB1 in human gastric cancer specimens. But the results are not generated in all studies. It was concluded that the presence of both protein and mRNA of P-glycoprotein are said to be essential for accurate assessment of changes in ABCC1 expression between tumor and normal tissue.

The major families of ABC transporters, such as P-glycoprotein and ABCC1, are highly expressed in many gastric tumors. Some studies have also made high expression of ABCB1 or ABCC1 clear in human gastric cancer samples (16).

Zhao et al. (2009) (17) announced that they witnessed significant rate of mRNA expression of P-glycoprotein in 41% of 22 cases suffering gastric cancer, the results of which are somewhat consistent with the findings of the present study, for in the cultured cells in this study, both MDR-1 gene expression and active P-glycoprotein were significantly elevated before cell treatment with Sclareol, too.

In an investigation by Gurel et al. (1999) (18) in Turkey, they found that MDR-1 gene expression increased in more than 87% of 55 gastric cancer patients.

A study by Choi et al. in 2002 (19) also revealed remarkable expression of ABCB1 (41%) in 103 cases of gastric cancer, but chemotherapy with 5-Fu and

doxorubicin in these patients failed to reduce the expression.

However, some studies have presented low ABCB1 expression in gastric cancer. For instance, Yabuki et al. (1996) (20), who worked on human gastric and intestinal cancer cell line, published a report, using reverse transcriptase PCR, highlighting that none of the five gastric cancer cell lines expressed ABCB1, and more than 10 signs of gastric cancer tissue of the lower levels of ABCB1 are expressed, which contradicts the results of the present study. The contradiction between the works of the two researchers refers to use of different cell lines in their research.

Studies on the anti-cancer effects of Sclareol:

Prior to this, studies such as Nouri et al. (2010) (14), Dimas et al. (2007) (21), and Zheng et al.'s (2017) (22) had indicated that Sclareol can inhibit tumor growth in vitro; it has low toxicity, reduces tumor growth in vivo and has anti-cancer properties against cancer tumor cells. They had also announced that Sclareol provides its own anti-cancer effects by regulating caveolin-1 level which is a scaffolding protein crucial in the regulation of several signaling pathways including apoptotic pathway. However, different studies have suggested different mechanisms for the anticancer influence of Sclareol which were discussed below.

Nouri et al. (2010) (14) shed light on Sclareol's inhibition of tumor growth in vitro, its low toxicity and tumor growth reduction in vivo. They pointed out it can also direct the immune response to Th1 by enhancing $INF\gamma$ level and reducing IL4 level. Additionally, Sclareol has cytotoxic activity and by inducing apoptosis, it can, in human leukemia cells, kill them. The results of their study are consistent with the present study results only in terms of the effectiveness of Sclareol substance. In other words, this study showed that Sclareol can achieve the desired effect in the treatment of gastric cancer by diminishing the expression of MDR-1 gene and consequently by producing P-glycoprotein can be used as a treatment option.

Similarly, Dimas et al. (2006) (23) clarified the influence of Sclareol on breast cancer cell line of MN1 and MDD2 derived from human MCF-7 stem cells, expressing that Sclareol induces apoptosis from a pathway independent of the P53 gene. In addition, it was discovered that Sclareol can enhance the effect of doxorubicin, etoposide and cisplatin on routine chemotherapy drugs used to control breast cancer in MDD2 breast cancer cell line.

The results of the researches mentioned above are the manifestation of Sclareol influential impact on treatment of the disease, which all are in line with the results obtained from this study. Of course, there are contradictory results which are outnumbered by the results of the studied revealed positive effects of Sclareol. This makes demands on more research on various cell lines and other cancers.

Conclusion

From the results of the study in hand, we can draw the conclusion that the administration of Sclareol can be beneficial to the decrease of the MDR-1 gene expression and its protein production -P-glycoprotein- in human gastric cancer cell line MNK-45 which increases drug resistance to cancer cells. The doses between 20 and 60 mmol of Sclareol were considered the effective doses, but doses above 60 mmol do not possess such an effect. The results obtained from this study can be a promising point for reducing drug resistance in patients with gastric cancer.

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