Original Article
http://mjiri.iums.ac.ir
Medical Journal of the Islamic Republic of Iran (MJIRI)

Med J Islam Repub Iran. 2024 (9 Jul);38.78. https://doi.org/10.47176/mjiri.38.78

Effect of Silymarin on Expression of micro-RNA-21 and Matrix Metalloproteinase (MMP) 2 and 9 and Tissue Inhibitors of Matrix Metalloproteinase (TIMP) 1 and 2 in Hepatocellular Carcinoma Cell Line (HepG2)

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Received: 7 Nov 2023 Published: 9 Jul 2024

Abstract

Background: Silymarin is a flavonolignan that has various medicinal properties such as liver protection, antioxidant, anti-inflammatory, anti-cancer and heart protection activities. The aim of this study was to investigate the effect of silymarin on the expression level of *mir-21*, matrix metalloproteinase (*MMP*), and their tissue inhibitors (*TIMPs*) in liver cancer HepG2 cell line.

Methods: An *in-vitro* experimental study was conducted on the human HepG2 cells prepared from Pasteur Institute, Tehran, Iran. Four concentrations of 0 (control), 50, 100, and 150 µM of silymarin were considered as the study groups according to the MTT assay. Gene expression study was performed using real-time PCR. The studied genes were *mir-21*, *MMP-2*, *MMP-9*, *TIMP-1* and *TIMP-2*. In addition, some apoptosis-related genes including *BAX*, *BCL2* and *Caspase3* (*CAS3*) were investigated. *GAPDH* was used as an internal control. Relative expression was calculated by REST program using t-test on the logarithm of expression considering a significance level of 0.05.

Results: The significant up-regulations consisted of *TIMP* genes for doses 100 µM and 150 µM, and the apoptosis activating genes *CAS3* and *BAX* ($P < 0.05$). The significant down-regulations consisted of *MMP-9* in all concentrations, *MMP*-2 in concentration 100 µM, and the apoptosis inhibitory gene *BCL2* in concentrations 50 µM and 100 µM ($P < 0.05$). In addition, *mir-21* as an oncogenic micro-RNA showed significant down-regulation for all doses ($P < 0.05$). All the comparisons were with the control group.

Conclusion: The present study showed that silymarin could affect the HepG2 cell line at the gene expression level *via* increasing apoptosis and changing the expression of *MMP-2*, *MMP-9*, *TIMP-1*, *TIMP-2* and *mir-21*. These findings were in line with each other and in favor of suppression of tumoral activity in this cell line.

Keywords: Gene Expression, Apoptosis, Cancer, Hepatocellular Carcinoma

Conflicts of Interest: *None declared*
Funding: *Lorestan University of Medical Sciences with grant number #2622*

Cite this article as: Hormozi M, Moulaee M, Alaee M, Beigi Boroujeni N, Beigi Boroujeni M. Effect of Silymarin on Expression of micro-RNA-21 and Matrix Metalloproteinase (MMP) 2 and 9 and Tissue Inhibitors of Matrix Metalloproteinase (TIMP) 1 and 2 in Hepatocellular Carcinoma Cell Line (HepG2). *Med J Islam Repub Iran.* 2024 (9 Jul);38:78. https://doi.org/10.47176/mjiri.38.78

Introduction

According to the studies conducted in recent years, it was found that micro-RNAs (miRNA) play an essential role in cellular processes; hence, these factors are related to many diseases, including many types of malignancies. In cancers,

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↑What is "already known" in this topic:
Micro-RNA-21 (*mir-21*) triggers epithelial to mesenchymal transition (EMT) and, consequently, metastasis through increasing matrix metalloproteinase (MMP). This increasing effect on MMP-2 and MMP-9 is through inhibition of Phosphatase and tensin homolog (PTEN) signaling. In addition, *mir-21* as an oncogenic micro-RNA, may inhibit apoptosis.

→What this article adds:
Silymarin, as an antioxidant, could successfully inhibit oncogenic activities of the HepG2 cell line by moderating the gene expression of the mentioned pathways.

phenomena like cell invasion and metastasis are very important (1). The effect of a miRNA in different cancers is not the same and depends on factors such as the type of tissue or the type of cancer. In other words, a miRNA can target different genes in different cancers, and as a result, different effects in different disorders are observed. Therefore, miRNAs are able to appear both in the role of tumorigenesis and in the role of tumor suppressors in various cancers (2-4). Disruption of the regulation of miRNA expression is closely related to many cancers and can perform an important function in the process and progression of cancers as an inhibitor or inducer (5). Micro-RNA-21 (mir-21) is one of the famous miRNAs, and it has been determined through various research that its expression increases in many different cancers, including stomach cancer, pancreas, hepatocellular carcinoma (HCC), and colon cancer, and is capable of regulating growth (6). Evidence shows that mir-21 increases cell division and on the other hand, reduces the apoptosis process in pancreatic cancer. This miRNA, with its effect on the expression of extracellular matrix-degrading enzymes, stimulates the migration and invasion of cancer cells in HCC (7). How cancer cells migrate and invade their surrounding tissues is of particular importance. By increasing the expression of certain enzymes called matrix metalloproteinase, cancer cells can destroy the extracellular matrix and the basement membrane and in this way, enter the bloodstream and other tissues of the body (8).

Liver cancers have a high mortality and burden worldwide (9). The main causes of liver cancer are cirrhosis caused by hepatitis B and C, as well as alcohol consumption (10). In 2012, 770,000 cases of liver cancer were reported worldwide (11). The very poor prognosis in some of the most common liver cancers is due to the phenomenon of metastasis, and today, the molecular process and how it starts remains unclear (12). Targeting metastasis for treatment is much more difficult than various other factors such as cell division, because this process, like angiogenesis, includes many complex interactions between tumor and tissue (13). The most important regulators of matrix metalloproteinase are tissue inhibitors of these enzymes (TIMP) (14). These inhibitors are able to prevent the growth, division and migration of cancer cells by inhibiting matrix metalloproteinase enzymes (15). Four types of matrix metalloproteinase tissue inhibitors have been discovered in vertebrates and from this, the four types of TIMP -1, -2, -4 are part of proteins secreted by cells, but TIMP-3 is anchored in the extracellular matrix (16). When the balance between matrix metalloproteinase and their inhibitors (TIMPs) is broken, direct inhibition of these enzymes as well as increasing the expression of their inhibitors, can be attractive and effective therapeutic targets. In order to prevent the invasion of tumor cells, they should be considered (17). In some cancers, the increased expression of MMP-9, and also the decreased expression of TIMP-2, indicates a poor prognosis of the disease and the probability of cancer cell migration is high. Therefore, during the conducted studies, it was determined that TIMP-2 could prevent the invasion of tumor cells by inhibiting MMP-9 (15). On the other hand, in other research, it has been found that by increasing the expression of MMP-2 and MMP-9 and reduction in the expression of TIMP-1, the invasiveness of cancer cells increases (18).

Although environmental and genetic factors play the main role in cancer, many studies have shown that the nutrients in a person's diet can be a stimulating or inhibiting factor in the development of cancer (19). Silymarin is a flavonolignan extracted from the plant *Silybum marianum (L.) gaernt*. It has been shown that silymarin has various medicinal properties such as liver protection, antioxidant, anti-inflammatory, anti-cancer, and heart protection activities (20). Although some studies show the anti-cancer activity of silymarin, there are some gaps in knowledge. The mechanisms of this anti-cancer activity are currently known consisting of affecting MMPs and TIMPs. There was no study regarding human HCC. The only evidence reported by the review article of Koltai and Fliegel (2022) regarding the effects of silymarin on human HCC was the induction of apoptosis (21).

The rationale for investigating *mir-21* along with MMPs is that *mir-21* triggers epithelial-to-mesenchymal transition (EMT) and consequently metastasis through increasing MMPs. This increasing effect on MMP-2 and MMP-9 is through inhibition of Phosphatase and tensin homolog (PTEN) signaling. In addition, *mir-21* as an oncogenic miRNA (onco-mir), may inhibit apoptosis (22). Therefore, investigation of apoptosis-related genes may also be helpful.

There are studies on the effects of antioxidant compounds such as silymarin in liver cancer, and also, there are studies on the effects of these compounds on the expression of *mir-21* and factors whose expression is influenced by this miRNA, such as *MMP-2* and *-9* genes, the aim of this study is to investigate the effect of silymarin on the expression level of *mir-21*, *MMPs*, and their tissue inhibitors in liver cancer HepG2 cell line.

Methods

Study design

The present experimental study was conducted on human HepG2 cells with an *in-vitro* design. Gene expression study was performed using real-time polymerase chain reaction (real-time PCR). The protocol of this study was approved by the ethics committee of Lorestan University of Medical Sciences with registration number IR.LUMS.REC.1402.122.

Outcomes

The studied genes were *mir-21*, *MMP-2*, *MMP-9*, *TIMP-1* and *TIMP-2*. In addition, some apoptosis-related genes including *BAX*, *BCL2* and *CASPASE 3* (*CAS3*), were investigated. *GAPDH* was used as an internal control. The primers are shown which were collected from the literature (23-27) (Table 1).

Groups and treatments

The study was carried out on the liver cancer cell line (HepG2) prepared by the Pasteur Institute (Tehran, Iran). The experiments were repeated for each factor and the number of investigated groups was four groups (including

Table 1. Primers of the studied genes

Gene	Forward/Reverse	Primer sequence (5′→ 3′)	Product size (bp)	Reference
MMP-2	Forward	CTCATCGCAGATGCCTGGAA	104	(23)
	Reverse	TTCAGGTAATAGGCACCCTTGAAGA		
MMP-9	Forward	ACGCACGACGTCTTCCAGTA	94	(24)
	Reverse	CCACCTGGTTCAACTCACTCC		
TIMP-1	Forward	AAGGCTCTGAAAAGGGCTTC	105	(25)
	Reverse	GCAGGATTCAGGCTATCTGG		
TIMP-2	Forward	GAAGCATTTGACCCAGAGTG	165	(26)
	Reverse	CCTTTCAGACCGAACCTACT		
GAPDH	Forward	CTCTCTGCTCCTCCTGTTCG	114	(27)
	Reverse	ACGACCAAATCCGTTGACTC		

three different concentrations of treatment and one control group). After the preparation and cultivation of liver cells, the MTT method was used to confirm the effective time and dose through investigation of metabolic activity. For this purpose, after transferring the cell suspension to each well of a 96-well plate, the cells would be exposed to different concentrations of the treatment after different times have passed (24, 48 and 72 hours). From the start of the treatment, MTT solution was added to each well and after incubation, the supernatant liquid was removed, and dimethyl sulfoxide (DMSO) was added and the optical absorbance of each well was read with an ELISA reader. After confirming the doses and incubation time with the treatment, the cells were treated with three appropriate doses of silymarin (50, 100 and 150 µM) which were the three highest safe doses and a carrier as a control with the time of 24h (Figure 1).

Gene expression study

After the treatment of the cells, the cells were collected, and total RNA was extracted using the RNA extraction kit (Yekta-Tajhiz Azma, Iran). Then, cDNA was synthesized using a cDNA synthesis kit (Sinaclon, Iran) for mRNAs. For *mir-21*, cDNA was synthesized based on the stem-loop method using a cDNA synthesis kit (RNAbiotech, Iran). Finally, real-time PCR was run using the SYBR green master mix. All the processes were performed according to the manufacturers' guides.

Statistical analysis

The cyclic thresholds (CT) were reported and statistically compared by the REST program based on PCR efficacies. Microsoft Excel 2013 was used to draw the graphs of fold changes (FC). Further statistical analyses were performed, including t-test on the logarithm of expression using SPSS 24 (IBM Corp., NY, US). P <0.05 was considered as the significance level.

Results

Metabolic activity

According to the MTT results, metabolic activity was 100% in the control group (concentration zero) with standard error of the mean (SEM) 2.22, 5.45 and 6.07, 24h, 48h and 72h after treatment, respectively. The final metabolic activity of more than 50% was related to a concentration of 150 µM at 24h after treatment which was 64% (SEM =3.63, P < 0.05, H0: 50%). Considering these results, concentrations 50, 100 and 150 µM at 24h after treatment were studied (Figure 1).

Quality assessment

The concentration of the extracted RNA was approved

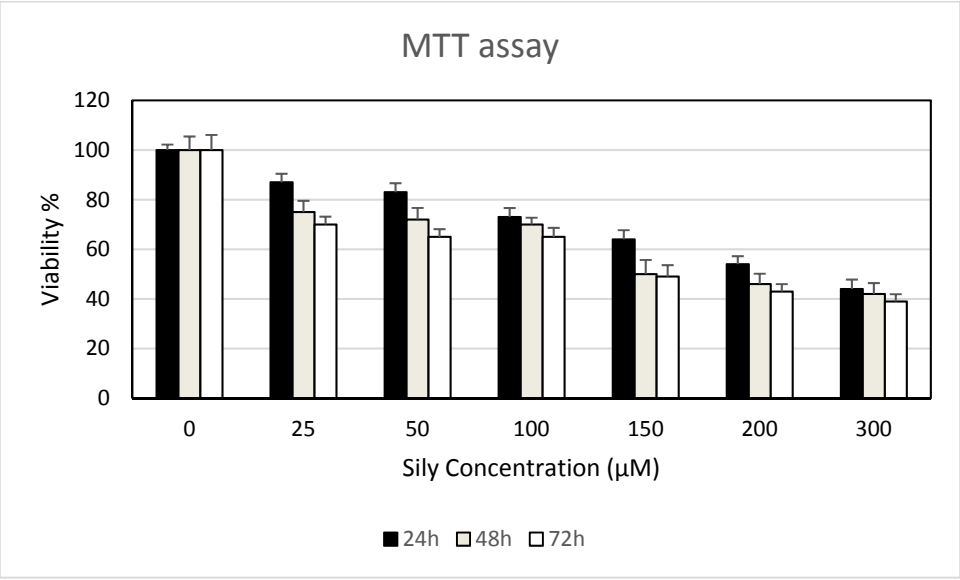

Figure 1. Results of MTT assay. The error bars indicate the standard error of the mean

according to the results of nanodrop spectrophotometry. In addition, one 18s and one 28s bound was observed, showing the quality of the extracted RNA.

Gene expression study

FCs of the target genes were calculated considering concentration zero µM as the calibrator. For *TIMP* genes, non-significant up-regulations were observed for concentration 50 µM ($0.05 < P < 0.2$), while significant up-regulations were observed for doses 100 µM and 150 µM ($P < 0.05$). For *MMP* genes, significant down-regulations were observed for *MMP-9* in all concentrations ($P < 0.05$), while for *MMP-2*, non-significant down-regulations were observed for concentrations 50 µM and 150 µM ($0.05 < P < 0.2$) and a significant down-regulation was observed for concentration 100 µM ($P < 0.05$) (Figure 2). For *mir-21*, significant down-regulations were observed for all concentrations ($P < 0.05$) (Figure 3). Regarding apoptosis-related

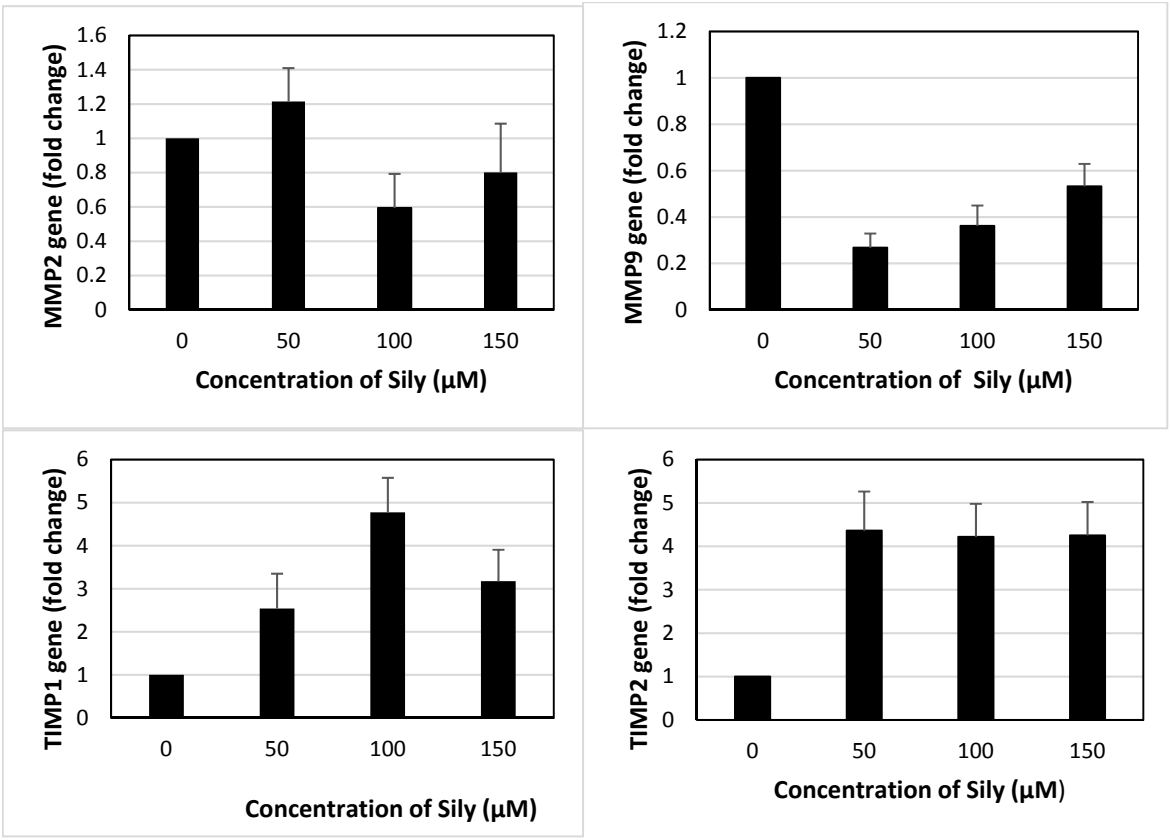

Figure 2. Expression of MMP and TIMP genes. The error bars indicate the standard error of the mean

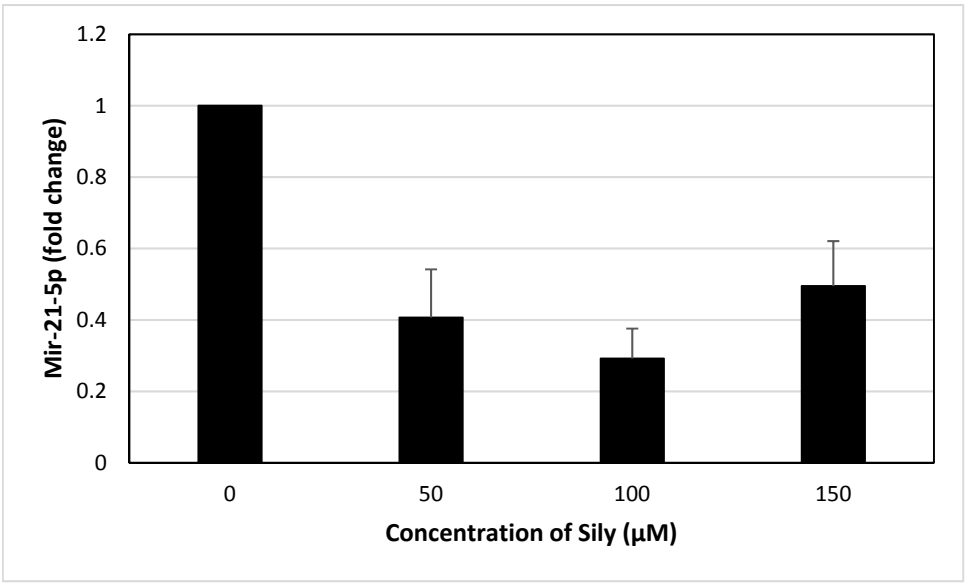

Figure 3. Expression of mir-21. The error bars indicate the standard error of the mean

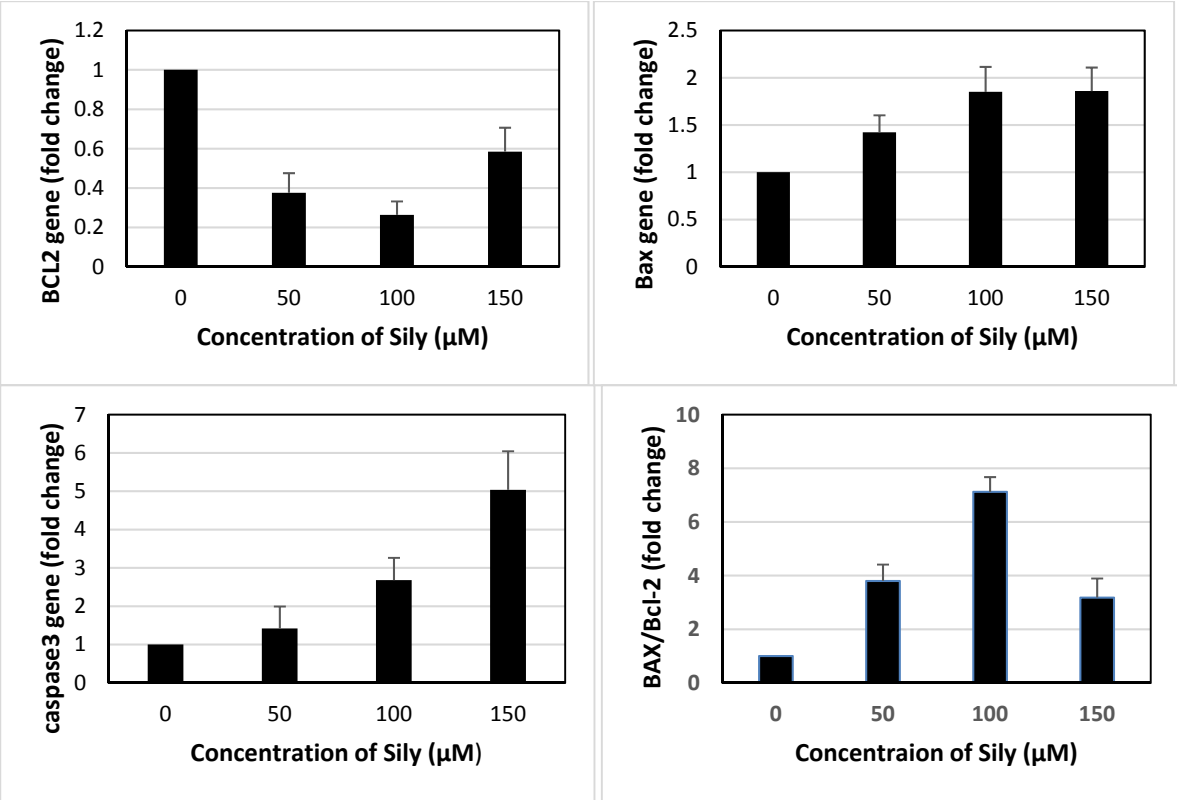

Figure 4. Expression of apoptosis-related genes. The error bars indicate the standard error of the mean

genes, significant up-regulations were observed for *CAS3* and *BAX* ($P < 0.05$), while for *BCL2*, significant down-regulations were observed for concentrations 50 µM and 100 µM ($P < 0.05$) and no significant change was observed for concentration 150 µM ($P > 0.2$) (Figure 4).

Discussion

Considering the importance of the invasion of tumor cells from the molecular and cellular aspects and considering the importance of miRNAs, including *mir-21* as well as MMP enzymes and their inhibitors in this condition, the purpose of this study was to investigate the effect of silymarin on the gene expression level of *mir-21* and MMP enzymes and the gene expression level of their tissue inhibitors in liver cancer cells. In addition, some apoptosis-related genes were studied. Briefly, gene expression changes were observed for all the studied genes, and the changes were in favor of tumor suppression.

According to the review article of Koltai and Fliegel, silymarin has a wide spectrum of anti-cancer effects, including induction of apoptosis, inhibition of angiogenesis, suppression of EMT and so on. Regarding MMP-2 and MMP-9, silymarin could inhibit the rat model of liver carcinogenesis (21). The present study was performed on human HCC cell lines and similar results were observed for *MMP-2* and *MMP-9* genes.

Extensive studies were done on the effect of *mir-21* in various cancers, including the study conducted by Wang *et al.* on breast cancer. This research was performed with the aim of the effect of *mir-21* on the invasion and metastasis of breast cancer cells. During the investigations carried out on the patient group, it was found that the plasma level of *mir-21* in the serum of the patients was much higher than that of the healthy group. Also, in this study, the scientists concluded that one of the direct targets of *mir-21* is the *LZTFL1* gene, which, by affecting it, increases the division and invasion of tumor cells (28). In the present study, *mir-21* was down-regulated showing the anti-tumoral activity of silymarin. Since *mir-21* is known as a famous onco-mir (29), this result of our study was in line with the study of Wang *et al.* (28). In order to identify the metastasis of tumor cells in HCC by measuring the amount of *mir-21* in the serum of different patients, Guo *et al.* conducted a study with this aim. These scientists concluded that the plasma level of *mir-21* was increased. There was a direct relationship between the disease and the invasion of cancer cells. Therefore, the measurement of this miRNA helped in the diagnosis and progression of HCC (30). As it was mentioned, the down-regulation of *mir-21* in the present study was in line with the results of the Guo *et al.* study (30).

The transformation of epithelial characteristics into mesenchymal characteristics of cells is one of the main causes of migration and invasion of cancer cells in HCC, which is called the EMT process. During this process, by acquiring mesenchymal characteristics, cancer cells are separated from the basement membrane and move to places far away

from their original location. A study was performed to investigate the effect of the puerarin compound on the migration rate of cancer cells. In this research, Zhou *et al.* studied the effect of this combination on *mir-21* and *PTEN*, as well as the relationship of these factors with the EMT process. Finally, these researchers came to the conclusion that the combination of puerarin reduced cell division, tumor formation, migration, invasion and metastasis of cancer cells. They showed that this combination reduced the oncogenic activity of *mir-21*. The EMT process was stopped and the invasion rate of cancer cells decreased in HCC (31). All the mentioned evidence supported the oncogenic role of *mir-21* which was down-regulated in the present study.

Shen *et al.* conducted a study with the aim of investigating the effect of solasodine on the expression of *MMP-2* and *MMP-9* genes, as well as tissue inhibitors of these enzymes in the A549 lung cancer cell line. They also investigated the expression level of *mir-21* and its relationship with the expression of the mentioned genes. During this study, they found that the expression level of *MMP* and *mir-21* genes decreased and the expression level of tissue inhibitors of these enzymes increased (32). These results were in line with the results of our study. Another study on the effect of *mir-21* on the migration and invasion of cancer cells in renal cell carcinoma was carried out by Fan *et al.* In this study, which analyzed a large number of previous studies, they finally concluded that the use of *mir-21* inhibitors and its suppression increased PDCD4 protein expression, and it caused a decrease in the expression of MMP enzymes, and on the other hand, the expression of tissue inhibitors of TIMP-1 and TIMP-2 increased. It was also found that inhibiting *mir-21* increased apoptosis. Therefore, *mir-21* was able to increase the migration and invasion of cancer cells by directly targeting the PDCD4 protein and reducing its expression level (33). Similarly, in the present study, down-regulation of *mir-21* and increasing apoptosis was observed.

One of the targets of *mir-21* in HCC is the PDCD4 protein. This protein is considered a tumor inhibitor, and it is able to prevent the expression of many MMPs including MMP-2 and MMP-9. Many studies have shown that this miRNA reduces the expression of PDCD4. Thus, it is assumed that *mir-21* indirectly plays an important role in the development of HCC by reducing the inhibition of genes expressing these two enzymes participating in the invasion of cancer cells. On the other hand, some studies show that *mir-21*, by targeting the regulators or inhibitors of MMPs, increases the migration and invasion of cancer cells (34).

In general, the gene expression changes observed in the present study were in line with the results of the previous studies that were in favor of tumor suppression. However, there were some limitations, including a lack of study at the protein expression level and a lack of other groups such as an oxidative stress-induced group. Another limitation was the lack of morphological study and survival analysis. Although these were important limitations, choosing a microRNA helped us to study gene expression regulation. According to the literature, gene expression regulation might be a proxy of morphological changes.

Conclusion

The present study showed that silymarin could affect the HepG2 cell line at gene expression level. It increased apoptosis *via* up-regulation of *CAS3* and *BAX* and down-regulation of *BCL2*. In addition, it could up-regulate *TIMP-1* and *TIMP-2* and down-regulate *MMP-1* and *MMP-2*. Also, *mir-21* was down-regulated. These findings were in favor of suppression of tumoral activity in this cell line and the results were consistent with the literature. Animal and human studies should be conducted in the future considering ethical issues. Future studies may be regarding the preventive or therapeutic roles of silymarin. However, clinical trials are needed before using such herbal medicines and natural antioxidants.

Authors' Contributions

MH: critical revision, design and conceptualization, MM: drafting, data collection, MA: drafting, data collection, NBB: critical revision and interpretation, MBB: critical revision, design and conceptualization. All the authors approved the final version of the manuscript.

Ethical Considerations

The protocol of this study was approved by the ethics committee of Lorestan University of Medical Sciences with registration number IR.LUMS.REC.1402.122.

Acknowledgment

The present study was supported by Lorestan University of Medical Sciences with grant number #2622.

Conflict of Interests

The authors declare that they have no competing interests.

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