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Silibinin Inhibits TGF-β-induced MMP-2 and MMP-9 Through Smad Signaling Pathway in Colorectal Cancer HT-29 Cells

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ABSTRACT

Background: Metastasis of cancer cells is the primary responsible for death in patients with colorectal cancer (CRC). Transforming growth factor-β (TGF-β)-induced matrix metalloproteinases (MMPs) are essential for the metastasis process. Silibinin is a natural compound extracted from the Silybum marianum that exhibits anti-neoplastic activity in cancer cell lines. In this study, we evaluated the effects of silibinin on MMP-2 and MMP-9 induced by TGF-β in human HT-29 CRC cell line and the potential mechanism underlying the effects.

Methods: The present in vitro study was done on the HT-29 cell line. The HT-29 cell line was cultured in RPMI1640 and exposed to TGF- β (5 ng/ml) in the absence and presence of different concentrations of silibinin (10, 25, 50, and 100 µM). The effect of silibinin on HT-29 cell viability was measured with the MTT assay. A real-time polymerase chain reaction (Real-Time PCR) determined the relative mRNA expression of MMP-2 and MMP-9. Western blotting was employed to examine MMP-2 and MMP 9 protein expression and Smad2 phosphorylation.

Results: Silibinin inhibits cell viability of HT-29 cell line at 24 hours in a dose-dependent manner. TGF-β increased the mRNA and protein expression of MMP-2, MMP-9, and phosphorylated Smad2 compared to controls. Pharmacological inhibition with silibinin markedly blocked TGF-β–induced MMP-2 and MMP-9 mRNA and protein expression and Smad2 phosphorylation.

Conclusion: Silibinin decreased the cell viability of HT-29 cancer cells in a dose-dependent manner. Silibinin also inhibited TGF-β-stimulated MMP-2 and MMP-9 expression in HT-29 cells, possibly mediated with the Smad2 signaling pathway.

Keywords: Silibinin, Colorectal Neoplasms, TGF-β, MMP-2, MMP-9

INTRODUCTION:

Colorectal cancer (CRC) is the third primary common cancer around the world. The high mortality rate in the CRC patients is attributed to tumor metastasis to another organs, usually the liver (1). CRC cells interact with the extracellular matrix (ECM), causing remodeling and degradation of the ECM, a process essential for the migration of cancer cells from the primary tumor site (2). Matrix metalloproteinases (MMPs) are one specific group of proteolytic enzymes, which were studied widely as key mediators of ECM degradation and angiogenesis during tumor progression (3, 4). Increased expression and activation of MMP-9 and MMP-2, as the major subtypes of the MMPs family, generally promote ECM degradation by proteolytic activity against ECM molecules, including type IV collagen (5). Researches have shown that multiple signaling pathways can play a role in the activation of MMP2 and MMP9. Among them, transforming growth factor beta (TGF-β) is the well identified and studied (6, 7). The cellular effects of TGF-β signaling are mediated through serine/threonine kinase cell surface receptors, namely type 1 TGF-β receptor (TβRI) and type 2 TGF-β receptor (TβRII), and Smad transcription factors (8, 9). The activated TβRI phosphorylates specific serine residues within the carboxy region of a subclass of Smad transcription factors, namely receptor-activated Smads (R-Smads) (8, 10, 11). Subsequently, the phosphorylated R-Smads (Smad2 and Smad3) hetero-oligomerize with the Smad4 (co-Smad4), and then this heteromeric complex imports into the nucleus and regulate the expression of specific target genes, including MMP-2 and MMP-9, which are important responsible genes for CRC cells migration and invasion (6). It has been suggested that the inhibition of MMP-2 and MMP-9 expression may hinder the progression and metastasis of tumor cells (12-14). Previous studies show that medicinal plants have potential to inhibit the growth of solid tumors, including CRC, in both in vitro and in vivo models (15, 16). Silibinin, the major component of silymarin, was originally isolated from the seeds and dried fruits of the medicinal plant Silybum marianum (also known as milk thistle) (17). This flavonoid compound has also been shown to exhibit significant anti-cancer activity in various tumor types (in vitro and in vivo), including breast, colon, skin, prostate, and bladder cancers (17-22). Evidence indicates that the anti-cancer effects of silibinin are mainly through targeting cell growth, inflammation, proliferation, angiogenesis, apoptosis, metastasis, and the metabolism of tumor cells (21). Numerous experimental studies have reported the anti-metastatic activity of silibinin against CRC cells (23, 24). However, the mechanism of the anti-metastatic of silibinin in colon cancer is not entirely understood. In this study, we selected human HT-29 CRC cell line as a cell model to investigate the potential role of TGF/Smad2 signaling cascade in CRC invasion and possible inhibitory effect of silibinin on this process. We investigated the anti-neoplastic activities of silibinin in CRC cells using this cell model by evaluation of several key molecules related to cancer invasion and metastasis, such as MMP-2, MMP-9, and Smad2.

METHODS:

All experiments were performed in the Clinical Biochemistry laboratory at Lorestan University of Medical Sciences (Khorramabad, Iran) and the Farhangian University of Tehran in a multi-centric manner.

Materials

Silibinin, SB431542, and secondary antibody Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Sigma Aldrich (St. Louis, MO, USA). Primary anti-MMP-2 and anti-MMP-9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS), RPMI 1640, trypsin-EDTA, and Penicillin-Streptomycin were obtained from Gibco (Invitrogen, Carlsbad, CA, USA).

All primers (forward and reverse) were obtained from Takapouzist (Tehran, Iran). Anti-phospho-Smad2C (Ser465/467) and human recombinant TGF-β were obtained from Cell Signaling Technology (Beverly, MA, USA).

Human HT-29 culture and treatment

The present in vitro study was performed on human colon cancer cells, HT-29 cell line. Human HT-29 cells were purchased from the Pasteur Institute (Iran, Tehran) and then cultured and grown in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% penicillin (100 units/ml)/ streptomycin (100 µg/ml), at 37°C in an incubator containing 5% CO2.

Assessment of cell viability by MTT assay

The MTT assay was used for 24 h after silibinin treatment to evaluate the effect of silibinin on the HT-29 cell viability. Briefly, cells were seeded in a 96-well plate at 104 cells/well, and then HT-29 cells were incubated with different concentrations of silibinin (0 to 100 µM) for 24 hours. Next, the culture medium was eliminated, and 20 µL MTT solution (5 mg/mL dissolved in PBS) and 180 µL of serum-free RPMI1640 were added to each well incubated for 4 hours at 37 0C. The optical density (OD) of each experimental well was measured using a microplate reader at 540 nm (BioTek® ELx800, USA). The percentage of cell viability was calculated using the following formula:

Viability % = 100 - Cytotoxicity %

$$cytotoxicity\ \% = 1 - \frac{\text{mean OD of treatment group}}{\text{mean OD of control group}} \times 100$$

Quantitative real-time polymerase chain reaction (q-RT-PCR)

For measuring the mRNA levels of MMP-2 and MMP-9, total RNA was extracted from cultured HT-29 using Trizol reagent (Invitrogen) according to the standard producers. One µg of total RNA was applied for cDNA synthesis using reverse transcriptase kit (Takara, Japan). The q-RT-PCR was performed using SYBR Green PCR Master Mix (Takara, Japan) to amplify the mRNA expression. GAPDH was selected as an internal control. The primer pairs to detect human MMP-2, MMP-9, and GAPDH were organized in **Table 1**. All experiments were carried out in triplicate.

Western blotting

HT-9 Cells in all experimental groups were collected, and total proteins of each experimental group were extracted. The concentration of proteins of each experimental group was measured with the Bradford assay. 50 µg of protein samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins

Table 1. Sequences of primers

Genes	Sequences of primers	
MMP-2	Forward	5′- AGCTCCCGGAAAAGATTGATG -3′
	Reverse	5′- CAGGGTGCTGGCTGAGTAGAT -3′
MMP-9	Forward	5′-CACGCACGACGTCTTCCA-3′
	Reverse	5′- AAGCGGTCCTGGCAGAAAT -3′
GAPDH	Forward	5′-ACCCACTCCTCCACCTTTGA-3′
	Reverse	5′- CTGTTGCTGTAGCCAAATTCGT -3′

were transferred onto a polyvinylidene difluoride (PVDF) membrane. Next, non-specific binding sites were blocked with 5% non-fat milk for about 1 hour at room temperature. Membranes were incubated with specific primary antibodies against phospho-Smad2, MMP-2, MMP-9, and GAPDH at 4°C overnight and then were incubated with an anti-rabbit horseradish peroxidase-conjugated antibody at room temperature for one hour, and enhanced chemiluminescence (ECL) detection system was used. GAPDH was used as an internal control to normalize protein expression levels.

Ethical Considerations

The Study was approved following the Ethical recommendations of Lorestan University of Medical Sciences. The authors have observed ethical issues.

Statistical analysis

Data normalization was determined by Kolmogorov-Smirnov (K–S) test. All results are presented as means ± standard error of the mean (SEM). Statistical significance between groups was evaluated using a one-way analysis of variance (one-way ANOVA) followed by Least Significant Difference (LSD) post-hoc test. Statistical significance difference was indicated as $P < 0.05$ and $P < 0.01$. The IC50 (50% inhibition concentration) values of silibinin on HT-29 cells at 24 hours were determined by Microsoft Excel (Office 2016) and GraphPad prism software (version 8.0.2) using non-linear regression curve fitting with the normalized response.

RESULTS:

The effect of silibinin on cell viability of HT-29 cells

In this experimental study, we first examined the effects of silibinin on cell viability of the HT-29 cell line by treating cancer cells with various concentrations of silibinin (0, 10, 25, 50, and 100 µM) at 24 hours followed by a MTT assay. As shown in **Figure 1A**, the growth of the HT-29 cell line was inhibited by silibinin in a dose- dependent manner. Compared to the control, treatment of the HT-29 cells with silibinin at 10, 25, 50, and 100 µM for 24 hours decreased HT-29 cell viability by 94.82%, 79.16%, 58.22%, and 20.33%, respectively (Figure 1A). Data analysis of MTT assay showed that IC50 value of silibinin on HT-29 cell line was 54.41 µM during the 24-hours (**Figure 1B**).

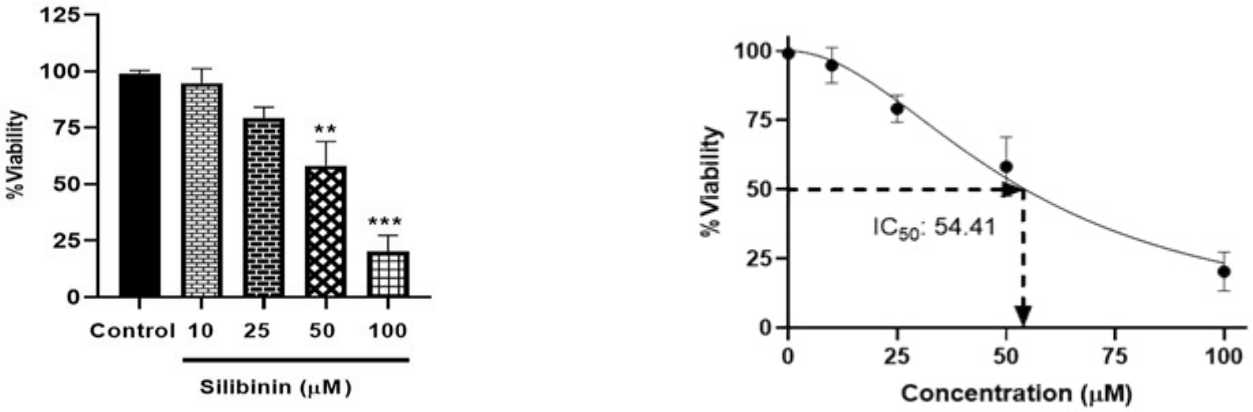

Figure 1. A) Effect of silibinin on cell viability of HT-29 cell line. Cells were exposed to different concentrations of silibinin (0-100 µM) for 24 hours, and cell viability was measured by the MTT assay method. All data are presented as a percentage of cell viability from three separate experiments.
B) IC50 value of silibinin on HT-29 cell line during 24 hours.

Silibinin suppresses TGF-β1-induced MMP-2 and MMP-9 mRNA expression in HT-29 cells

Previous studies have reported that TGF-β increases MMP-2 and MMP-9 mRNA levels in different cancer cells (25, 26). In the present study, we investigated whether silibinin interfered with the TGF-β-induced MMP-2 and MMP-9 mRNA expression in the HT-29 cancer cell line. We pre-treated HT-29 cells with 25 and 50 μM silibinin and 10 μM SB431542 (specific TGF-β receptor antagonist) for 1 hour, and then treated with TGF-β (5 ng/ml) for 24 hours and finally the mRNA levels of MMP2 and MMP-9 were analyzed by q-RT-PCR. Our results showed that TGF-β (5 ng/ml) increased MMP-2 and MMP-9 mRNA expression increased to 2.2-fold and 2.88-fold of the control group, respectively (P<0.01) (**Figure 2A** and **Figure 2B**). Our data also showed that TGF-β-induced MMP-2 and MMP-9 mRNA expression was decreased after pre-treatment with 10μM of SB431542 . Furthermore, TGF-β-induced MMP-2 and MMP-9 mRNA expression was significantly blocked by 25 and 50 μM silibinin pre-treatment in a dose-dependent manner (**Figure 2A and Figure 2B**) (P < 0.01).

Silibinin suppresses TGF-β1-induced MMP-2 and MMP-9 protein expression in HT-29 cells

Here, we determined whether silibinin blocked the TGF-β-induced MMP-2 and MMP-9 protein expression in HT-29 cancer cell line. After pre-incubation with different concentrations of 25 and 50 μM of silibinin for 1 hour, the cells were treated with TGF-β (5 ng/ml) for 24 hours and finally the protein expression of MMP-2 and MM-9 was analyzed by western blotting method. Our data showed that TGF-β (5 ng/ml) induced the protein levels of MMP-2 and MMP-9 to 2-fold and 2.1-fold of the control group (P<0.01) (**Figure 3A** and **Figure 3B**). Silibinin (50 μM) significantly decreased TGF-β-induced MMP-2 and MMP-9 protein expression (**Figure 3A** and **Figure 3B**) (P<0.01).

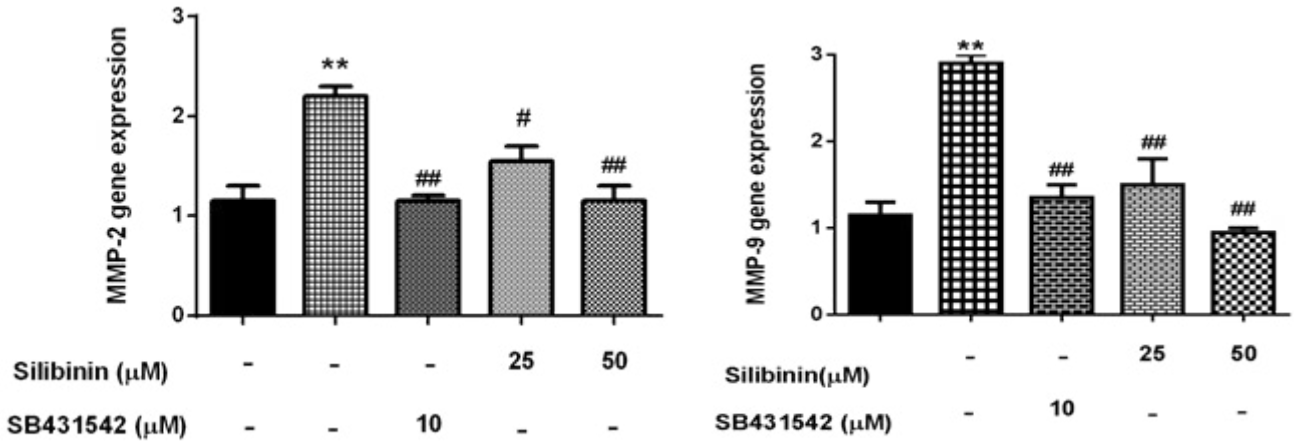

Figure 2. Effect of silibinin on TGF-β-mediated **A)** MMP-2 and **B)** MMP-9 mRNA expression in the HT-29 cell line. Cells were exposed to TGF-β (5 ng/ml) for 24 hours in the presence and absence of the silibinin (25 and 50 μM), and then the mRNA levels of MMP-2 and MMP-9 were analyzed by q-RT-PCR. Data are presented as mean ± SEM of at least three separate experiments. **P values < 0.01 compared with the control group, and #P < 0.05 and ##P < 0.01 compared with TGF-β group.

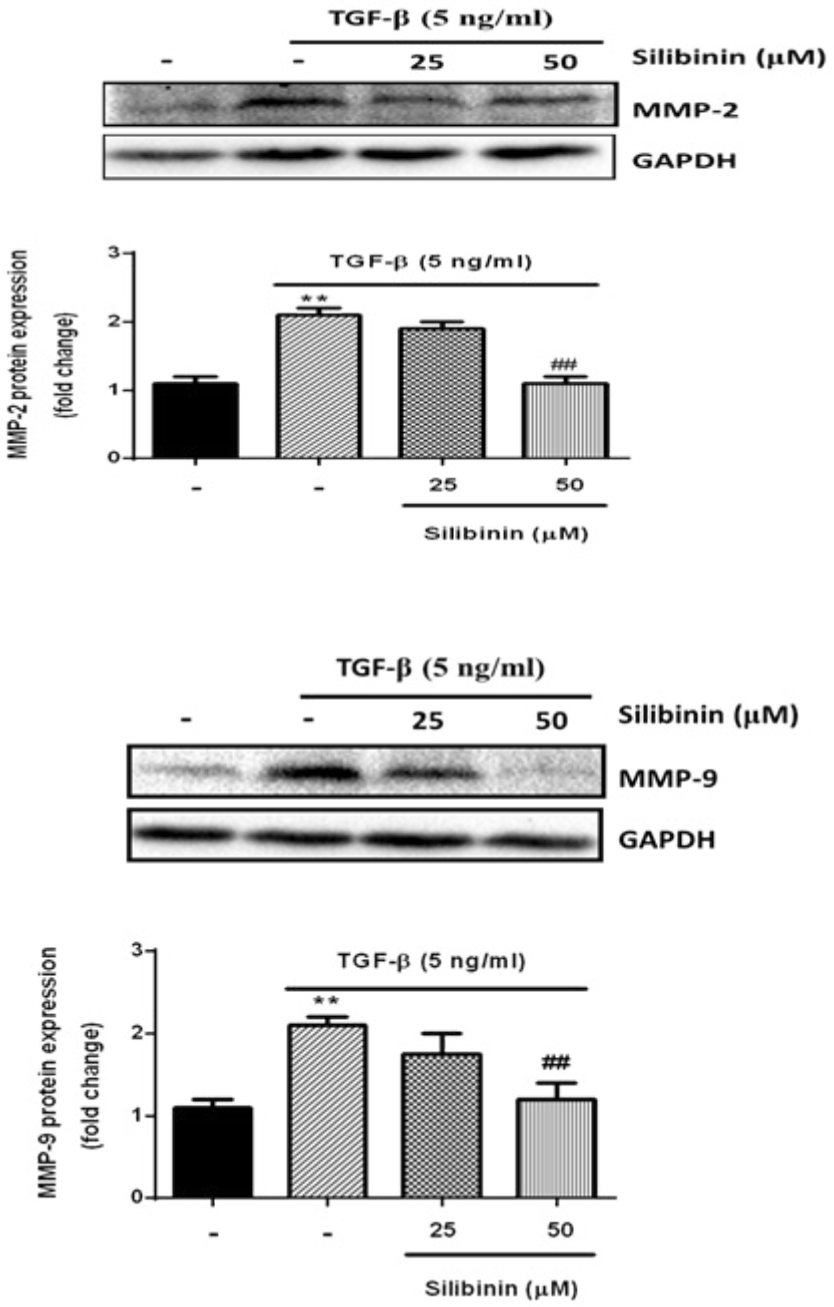

Figure 3. Silibinin reduces TGF-β-mediated A) MMP-2 and B) MMP-9 protein expression in the HT-29 cell line. Cells were exposed to TGF-β (5 ng/ml) for 24 hours in the presence and absence of the silibinin (50 μM) and SB431542 (10 μM), and then the protein levels of MMP-2 and MMP-9 were analyzed by western blotting method. Data are presented as mean ±SEM of at least three separate experiments. **P < 0.01 compared with the control group, and ##P < 0.01 compared with the TGF-β group.

Silibinin blocks TGF-β-mediated Smad2 phosphorylation in HT-29 cell line

Finally, we evaluated whether silibinin blocks TGF-β-mediated Smad2 phosphorylation in HT-29 cell line. Cells were pre-treated with 10 μM SB431542 and 50 μM silibinin for 1 hour, and then treated with TGF-β (5 ng/ml) for 24 hours and finally the phosphorylation of Smad2 was analyzed by western blotting method. As shown in Figure 4, the result of western blot revealed that TGF-β markedly increased Smad2 phosphorylation at 24 hours (P<0.01). Silibinin (50 μM) and SB431542 (10 μM) significantly inhibited TGF-β-induced Smad2 phosphorylation in HT-29 cells (P<0.01) (**Figure 4**).

DISCUSSION

In this study, we demonstrated the effects of silibinin on MMP-2 and MMP-9 expression induced by TGF-β in the HT-29 colorectal cancer cell line. Employing the human HT-29 cell line, we indicated that silibinin, as a natural flavonoid with anti-cancer effects, could block TGF-β-induced MMP-2 and MMP-9 mRNA and protein expression via suppressing Smad2 signaling.

Evidence now illustrates that ECM degradation is the essential process in tumor cell invasion to neighboring tissues, blood vessels, and then invasion of other distinct tissues (27, 28). Active MMP-2 and MMP-9 enzymes play a pivotal role in colon cancer migration and invasion via the degradation and destruction of

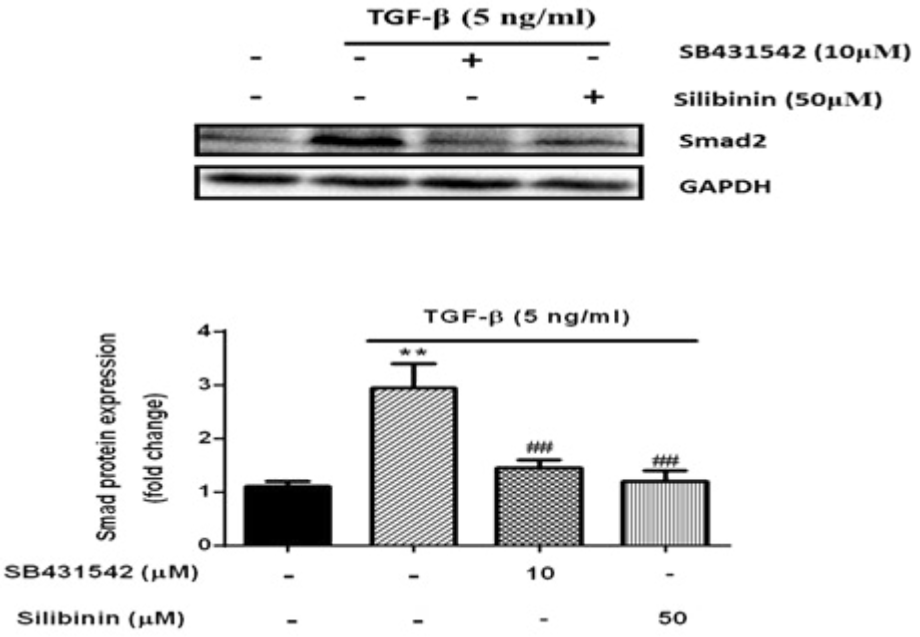

Figure 4. Silibinin reduces TGF-β-mediated Smad2 phosphorylation in the HT-29 cell line. Cells were exposed to TGF-β (5 ng/ml) for 24 hours in the presence and absence of the silibinin (50 μM) and SB431542 (10 μM), and then the phosphorylation levels of Smad2 were analyzed by western blotting method. Data are presented as mean ±SEM of at least three separate experiments. **P < 0.01 compared with the control, and ##P < 0.01 compared with the TGF-β group.

ECM components, such as type IV collagen (29, 30). These enzymes are produced by various growth factors which secreted by cancer cells and/or other cells in the tumor microenvironment, including TGF-β, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), Nerve growth factor (NGF), and fibroblast growth factor (FGF). Among these growth factors, TGF-β is high associated to tumor cell migration and invasion (31). A previous study reported that inducing MMP-2 and MMP-9 expression by TGF-β plays a vital role in invasion of breast cancer cells. Cancer cell invasion decreased following LY2109761 treatment, the TGF-β receptor type I and type II inhibitor, in triple negative breast cancer (TNBC) cells (32). Based on different reports, many natural agents can also repress TGF-β-induced MMP-2 and MMP-9, and employed for the prevention of invasion of tumor cells (31, 33). Previous reports have revealed that silibinin exerts anti-neoplastic effects in various in vivo and in vitro models, without any side-effect on the normal cells (33, 34). In human gastric cancer SGC7901 cells, silibinin suppressed the expression of MMP-2 and MMP-9 that indicated the anti-invasive impact of this compound (33). In human breast cancer cells, silibinin attenuated TGF-β-induced MMP-9 and invasion through extracellular-regulated kinase (ERK) and Smad signaling pathway (32). However, whether silibinin can suppress TGF-β-stimulated MMP-2/9 expression in colon cancer cells is not clear. This research focuses on the anti-invasive and anti-neoplastic effects of silibinin on colon cancer HT-29 cells via inhibiting TGF-β-induced MMP-2 and MMP-9 expression. Our data indicated that TGF-β significantly elevated the MMP-2 and MMP-9 mRNA and protein expression in the HT-29 colon cancer cells, and this action of TGF-β can be blocked by silibinin. In the classical TGF-β signaling pathway, TGF-β transduces it's signals through serine/threonine kinase receptors and Smad transcription factors. Smad complexes translocate into the nucleus and control the expression of target genes (35). So far, several studies have reported that TGF-β regulates MMP-2 and MMP-9 expression through rapidly intracellular Smad phosphorylation and activation (6). Na Mo et al. have found that TGF-β-induced MMP-9 and invasion are associated with the Smad signaling pathway in human breast cancer cells (6). Our results in the present study indicated that silibinin can markedly block Smad2 phosphorylation induced by TGF-β. Furthermore, SB431542 (the specific TGF-β receptor inhibitor) and silibinin had similar inhibitory effects on TGF-β-induced Smad2 phosphorylation. Taken together, the data obtained from this research demonstrate that TGF-β is an important regulator of MMPs (MMP-2 and MMP-9) in CRC cells model. We also demonstrate a novel mechanism by which silibinin acts as an anti-metastatic agent via inhibiting invasion, and migration by down-regulating the MMP-2 and MMP-9 expression. However, one of the limitations of our study is that these experiments were not investigated in animal models. So, animal studies should be performed to confirm the in vivo anti-metastatic effects of silibinin against CRC cells and explore additional molecular mechanisms of silibinin in the migration and invasion of CRC cells.

CONCLUSION

In conclusion, our findings indicated that silibinin down-regulated TGF-β-induced MMP-2 and MMP-9 expression via a mechanism involving Smad2 signaling in human HT-29 cells. These results provide a further understanding of silibinin's anti-tumor effect on the signaling pathways controlling metastasis, which leads to the inhibition of invasion and the development of colon cancer.

AUTHORS' CONTRIBUTION

Contribution of each author made to the manuscript in-

cluded:

(1) Participation in study design: Zahra Zare, Parisa Khanicheragh, Fahimeh Hosseinabadi

(2) Participation in the implementation of methods: Zahra Zare, Parisa Khanicheragh, Zakieh Sadat Sheikhalishahi, Amirhooman Asadi, Tina Nayerpour dizaj

(3) Participation in data analysis: Mana Zakeri

(4) Contribution in the writing of the manuscript: Zahra Zare, Parisa Khanicheragh, Armaghan Lohrasbi, Amirhooman Asadi, Mojtaba Abbasi

(5) Edit of the manuscript: Omid Abazari

(6) Photograph Processing: Tina Nayerpour dizaj,

(7) Responsible for overall supervision of authors: Parisa Khanicheragh

(8) Administrative support: Mojtaba Abbasi

(9) All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript, and approved the final draft.

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CONFLICT OF INTERESTS:

The authors declare that there is no conflict of interests associated with this work.

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