

Inhibition of silibinin on migration and adhesion capacity of human highly metastatic breast cancer cell line, MDA-MB-231, by evaluation of β 1-integrin and downstream molecules, Cdc42, Raf-1 and D4GDI

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Abstract Metastasis is a property of malignant cancer cells that requires integrins which with their downstream molecules participate in a number of signaling events in cells with pivotal roles in malignancy, migration and invasion of tumor cells. Silibinin, a flavonoid antioxidant from milk thistle (*Silybum marianum* L.), has attracted attention in the last decades for chemoprevention and chemotherapy of tumor cells. In the present study, the effect of silibinin on migration and adhesion capacity of MDA-MB-231 cells, a highly metastatic human breast cancer cell line, was investigated by evaluation of β 1-integrin and its important downstream molecules. MTT, migration and adhesion assays were performed to evaluate the silibinin effects on proliferation, migration and adhesion of MDA-MB-231 cells. In addition, the influence of the silibinin on the expression of β 1-integrin, Raf-1, Cdc42 and D4-GDI mRNAs was assessed by RT-PCR. Results showed significant dose-dependent inhibitory effect of silibinin on proliferation, migration and adhesion of MDA-MB-231

cells. It significantly inhibited the expression of Cdc42 and D4-GDI mRNAs but had no statistically significant effect on the expression of β 1-integrin and Raf-1 mRNAs although it indirectly but effectively modulated β 1-integrin signaling pathway and RAF1 function. In conclusion, the results showed the silibinin effect on reducing the rate of metastasis, migration and adhesion of MDA-MB-231 to distant organs.

Keywords β 1-integrin signaling pathway · Cdc42 · D4GDI · Raf1 · MDA-MB-231 breast carcinoma · Metastasis · Silibinin

Introduction

Since prevention of breast cancer cells metastasis is yet unattained, search for finding new agents to prevent and inhibit breast cancer cells invasion and metastasis seems to be essential [1]. There have been widespread studies on cancer treatment by polyphenols in the last few decades, indicating that these agents could be used in cancer chemotherapy, either alone or in combination [2]. Silibinin, a polyphenolic flavonolignan isolated from milk thistle, is one of the chemopreventive and anticancer agents that has attracted attention for the prevention of or treatment for cancer [3]. There are many studies on different aspects of the silibinin effects on the metastasis of various tumor cell types [4–8].

Invasion and metastasis, two properties of malignant cancer cells, are multistep processes involving numerous cell–cell and cell–extracellular matrix factors such as integrins [9, 10].

Integrins are essential for cell migration and invasion. Regulation of the expression of some genes, adhesion, cell

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survival and cytoskeletal organization are the results of integrin activation that enables cancer cells to become more invasive, migratory and better able to survive different microenvironments [9].

Following integrin–ligand binding and depending on the composition of ECM, clustered integrins activate focal adhesion kinase (FAK) which binds and activates multiple downstream signaling proteins [11]. A group of very important downstream signaling proteins of FAK are Ras, Rac and Cdc42 belonging to small G-proteins family. Cdc42 regulates formation of filopodia [12]. Disregulation or upregulation of these small G-proteins results in malignancy and cancer promotion [13]. A group of important regulatory factors of the small G-proteins family are Guanine dissociation inhibitors (GDIs), which by inhibiting Guanine dissociation factors, stabilize either the GDP or GTP-bound state of G-proteins [14]. D4-GDI, which belongs to this family, is reported to induce cell invasion by controlling β 1-integrin expression [15].

Another important downstream molecule of integrin signaling pathway is Raf-1 which is a downstream target of Ras, Cdc42 and Rac. Activation of the Ras-Raf-MEK-ERK (ERK) pathway is involved in promoting cell proliferation, survival, migration, invasion and cytoskeletal remodeling [16]. Thus, Raf-1 could be a logical target for novel therapeutic anticancer strategies [17].

The aim of the present study was to investigate the inhibitory effects of silibinin on proliferative potential and metastatic features of human breast carcinoma cell line, MDA-MB-231, by evaluation of the integrins and their important downstream molecules. Therefore, the effect of silibinin on cell proliferation was evaluated by MTT assay, and cell migration and adhesion were also assessed. The expression of β 1-integrin, Cdc42, D4-GDI and Raf-1 mRNAs were determined via qRT-PCR after the treatment of the cells with silibinin.

Materials and methods

Cell lines and reagents

MDA-MB-231, a highly metastatic human breast cancer cell line [18], was obtained from National Cell Bank of Iran (Tehran, Iran). The MDA-MB-231 cells were grown in RPMI1640 (Gibco, Scotland) containing 15 mM HEPES, supplemented with 10% fetal bovine serum (FBS) (Gibco, Scotland) and 1% penicillin–streptomycin (Gibco, Scotland) at 37°C and 5% CO₂ in an incubator [19].

Different amounts of the silibinin stock solution, 0.1 M in DMSO (Sigma, USA), were added to the culture medium as is mentioned for each assay. The final concentrations of DMSO did not exceed 0.1% [v/v] in any test.

Cell proliferation assay

Methyl thiazolyl tetrazolium bromide (MTT, Sigma, USA) colorimetric assay was performed to determine the inhibitory effect of silibinin on growth and proliferation of the MDA-MB-231 cells. Cells were cultured in 96-well plates (Orange Scientific, Brussels, Belgium) at a density of 7,500 cells per well, in quadruplicates for 24 h. Silibinin was added at different concentrations (0, 50, 100, 150, 200 and 400 μ M), and the cells were incubated at 37°C and 5% CO₂ for 48 h. Culture media was then removed, and the cells were incubated with 50 μ l MTT (0.5 mg/ml) for 4 h, after which, the supernatant was aspirated and formazan crystals were solubilized with 100 μ l DMSO for 15 min at 37°C. Absorbance was measured at 570 nm (ELISA reader, Organon Teknika, Netherlands) and the following equation was used to evaluate the inhibition rate (IR) of silibinin on MDA-MB-231 cells:

$$\text{IR (\%)} = 1 - \text{OD}_{\text{exp}}/\text{OD}_{\text{con}} \times 100,$$

where OD_{exp} and OD_{con} are the optical densitometries of treated and untreated cells, respectively [5].

Cell migration assay

CytoSelect cell migration assay kit (Cell Biolabs, USA), containing polycarbonate membrane (8- μ m pore size) in fluorometric format, was used to evaluate the effect of silibinin on migration of the MDA-MB-231 cells in vitro [4].

The assay was performed according to the manufacturer's protocol. Briefly, following pretreatment of the cells with 50, 70 and 100 μ M of silibinin for 48 h, control or treated cells were suspended in 100 μ l of RPMI without FBS and added to the upper chamber at a density of 4×10^5 cells/well, and 150 μ l RPMI containing 10% FBS (chemoattractant) was added to the bottom chamber and incubated at 37°C for 16 h.

Migratory cells passed through polycarbonate membrane pores in response to chemoattractant and clinged to the bottom side of membrane, which were dissociated from the membrane by cell detachment buffer. Cells were then lysed and analyzed by CyQuant[®] GR Dye, using fluorescent plate reader (Synergy HT, USA) at an excitation of 480 nm and an emission of 520 nm. In this test, the amount of the migrated control cells was considered as full migration (100%) and used as baseline for migration of the treated cells.

Cell–matrix adhesion assay

MDA-MB-231 cell line is highly metastatic to bone marrow [20]. To measure the relative attachment of the cells to immobilized type I collagen, 96-well plates were coated with 100 μ l of 100 mg/ml type I collagen (Sigma, USA)

and incubated overnight at 4°C. Then, the plates blocked with 100 µl PBS containing 3% (w/v) BSA for 30 min at 37°C. To measure baseline nonspecific binding, other wells were coated with 1 mg/ml BSA.

Following pretreatment of cells with 50, 70 and 100 µM silibinin for 48 h, the cells were resuspended in serum-free medium RPMI1640 (SFM) and BSA (1:1) and incubated at 37°C for 90 min to allow recovery of cell surface receptors and alleviating the effect of trypsin on the cells [21]. Approximately 5×10^4 cells in 100 µl of SFM-BSA were seeded in quadruplicates into each collagen-coated well and incubated at 37°C for 90 min. Nonadherent cells were removed by washing with PBS twice, and the adherent cells were fixed in ethanol for 10 min. After 5 min of crystal violet (0.1% (w/v) in 25% (v/v) methanol) staining at room temperature, the cells were gently rinsed five times with water to remove unbound stain and allowed to air-dry at room temperature. Fixed cells were lysed by 0.2% Triton X-100, and absorbance was measured at 550 nm [8, 19].

Quantitative real-time RT-PCR

Following pretreatment of cells with 50, 70 and 100 µM silibinin for 48 h, total RNA was extracted from cell pellets by FastPure™ RNA Kit (Takara, Japan) according to the manufacturer's instruction. The quantity and quality of the isolated RNA were determined by picodrop (Picodrop co, UK) and formaldehyde–agarose gel electrophoresis (1.2% agarose; Gibco/BRL), respectively. The amount of the 260/280 OD ratio of all samples was between 1.8 and 2.2, indicating their high purity [22].

Up to 500 ng of total RNA was reverse transcribed into cDNA by PrimeScript™ RT reagent Kit (Takara, Japan) according to the manufacturer's protocol. The reverse-transcribed RNA was amplified with SYBR® Premix Ex Taq™ (Takara, Japan) Master Mix containing gene-specific primers (Table 1), through 40 cycles on a Rotor-Gene 6000

Real-Time Thermal Cycler (Corbett Research, Australia). As the β 1-integrin and Cdc42 have some variants, the primers were designed so they flanked these variant regions. Also, to avoid the amplification of contaminating genomic DNA, the primers were designed so that they include exon junctions [22]. The PCR condition included an initial denaturation of 30 s at 95°C followed by a denaturation step of 5 s at 95°C and a combined annealing and extension step of 30 s at 60°C. The specificity of the PCR products was assessed by verifying a single peak in melting curve analysis. For complementary length verification, PCR products were visualized on 2.5% agarose gel stained with ethidium bromide.

Standard curves were generated using pooled cDNA of the samples [23]. No template controls were also included in each run. All samples and controls were normalized against reference gene, hypoxanthine phosphoribosyl transferase (HPRT) [24]. It has been reported that silibinin does not affect the expression of the HPRT [5].

REST 2008 Relative Expression software tool V2.0.7 (Corbett research Pty. Ltd. USA) that combines gene quantification and normalization was used to analyze data and determine the statistical significance [25]. This software is based on pairwise fixed reallocation randomization test to calculate the significance of results. $P < 0.05$ was considered statistically significant. Data are represented as fold of differences between the means of normalized expression values \pm standard error (S.E.M.) (<http://www.gene-quantification.info>) [25, 26].

Results

Effect of silibinin on proliferation of the MDA-MB-231 cells

In this study, we first determined the cytotoxicity of silibinin by treating the MDA-MB-231 cells with various

Table 1 Nucleotide sequences of the primers used for real-time RT-PCR

Gene	Accession number	Forward primer (5'–3')	Reverse primer (5'–3')	Size (bp)
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111
ITGB1 ^b	NM_002211 NM_133376 NM_033668	GCTCACCTGTCCAGAAAC	^a CTGAGGAAAGGGAATTGTATG	184
ARHGDI ^c	NM_001175	^a TGGGGTGAAAGTGGATAAAG	GTCATCGTCGGTGAAGAAG	151
RAF-1	NM_002880	^a AAGACGCAGCATCAGTATTC	AAGACGCAGCATCAGTATTC	167
CDC42	NM_044472 NM_001039802 NM_001791	CAGGGCAAGAGGATTATGAC	AGTCCCAACAAGCAAGAAAG	166

^a The primer is located on exon junction

^b The gene name of β 1-integrin

^c The gene name of D4-GDI

concentrations of silibinin (0–400 μM) for 48 h followed by MTT assay. Compared with controls, the cell viability was not significantly altered by silibinin, except at the concentration of 400 μM (Fig. 1). Therefore, it was confirmed that a 48-h treatment of silibinin with a concentration ranging from 0 to 100 μM , has no cytotoxic effect on the highly metastatic MDA-MB-231 cells. Thus, this concentration range was used in the subsequent experiments.

Silibinin inhibits migration of MDA-MB-231 cells

Following pretreatment of the cells with different concentrations of silibinin (50, 75 and 100 μM) for 48 h, the inhibitory effect of silibinin on migration of the treated cells was 81.5% ($P < 0.01$), 53.21% ($P < 0.001$) and 40.78% ($P < 0.001$) compared to controls, respectively (Fig. 2). This indicates that silibinin significantly inhibited the migration of the MDA-MB-231 cells dose dependently ($P < 0.001$).

The migration percent is expressed as the ratio of the mean cell number of the migrated sample cells to the mean cell number of the migrated control cells \pm standard deviation, from at least three independent experiments.

Silibinin inhibits adhesion of the MDA-MB-231 cells

Based on the fact that bone is the first site of breast cancer invasion [20], we decided to determine the effect of silibinin on the adhesion of MDA-MB-231 cells to type I collagen. Different silibinin concentrations of 50, 75 and 100 μM significantly reduced the adhesive capacity of the MDA-MB-231 cells to 75.9% ($P < 0.01$), 49.87%

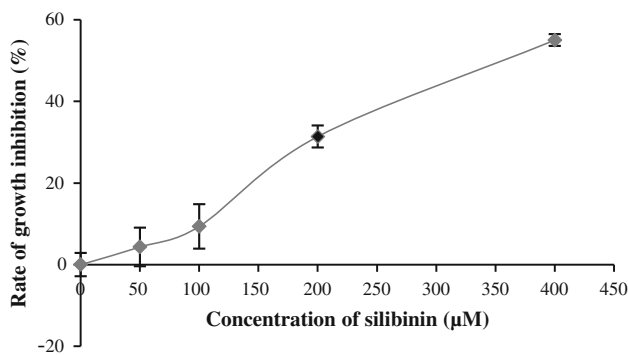


Fig. 1 Effect of silibinin on proliferation of the MDA-MB-231 cells by MTT assay. The MDA-MB-231 cells were treated with up to 400 μM silibinin concentration for 48 h. The inhibition rate of silibinin was measured as described in materials and methods, and the values are given as mean \pm SD, from three independent experiments repeated in quadruplicate. Data were analyzed by one-way ANOVA. Significance was set at $P < 0.05$. Statistically different values of $P = 0.553$ (50 μM), $P = 0.773$ (100 μM), $P < 0.001$ (200 μM) and $P < 0.001$ (400 μM) for 48 h after plating were determined compared with the control

($P < 0.001$) and 43.62% ($P < 0.001$), respectively, compared with the controls (Fig. 2).

These results indicate that silibinin could significantly reduce cell adhesion to type I collagen dose dependently ($P < 0.001$). This implies that silibinin can prevent the adhesion of migrated cells to the bone.

Silibinin decreases the mRNA levels of D4-GDI and Cdc42

Comparing with the controls, silibinin concentrations of 50, 75 and 100 μM caused a significant reduction in mRNA level of D4-GDI by a factor of 0.265, 0.560 and 0.531, respectively (Fig. 3a). It also significantly reduced the mRNA level of Cdc42, by a factor of 0.209, 0.403 and 0.588, at the concentrations of 50, 75 and 100 μM , respectively (Fig. 3b).

On the other hand, irrespective of the concentration, no statistically significant effect of silibinin was detected on Raf-1 and $\beta 1$ -integrin expression. The effect of silibinin on the expression of $\beta 1$ -integrin at the concentrations of 50, 75 and 100 μM was 1.55 ($P = 0.164$)-, 1.35 ($P = 0.332$)- and 1.12 ($P = 0.161$)-fold compared with the controls (Fig. 3c).

The silibinin effect on Raf-1 expression at the concentrations of 50, 75 and 100 μM was 0.962 ($P = 0.164$), 0.844 ($P = 0.681$)- and 1.059 ($P = 0.332$)-fold, respectively, compared to the controls, (Fig. 3d). The expression ratio and P values related to the investigated genes were computed by the Relative Expression Software Tool (REST), and the studied genes were normalized against HPRT.

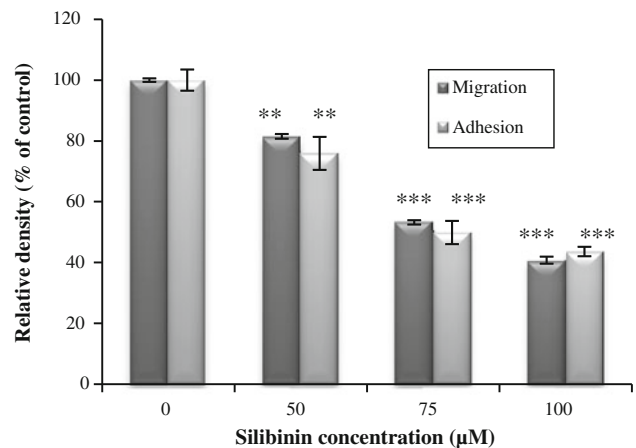


Fig. 2 Comparison of dose-dependent effects of silibinin on migration and adhesion of the MDA-MB-231 cells. After pretreatment of the cells with silibinin concentrations of 0, 50, 75 and 100 μM for 48 h, cells were harvested and used for adhesion and migration assays. Data are expressed as mean \pm standard deviation (S.D). Data were analyzed by one-way ANOVA, followed by LSD post hoc. Significance was set at $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$

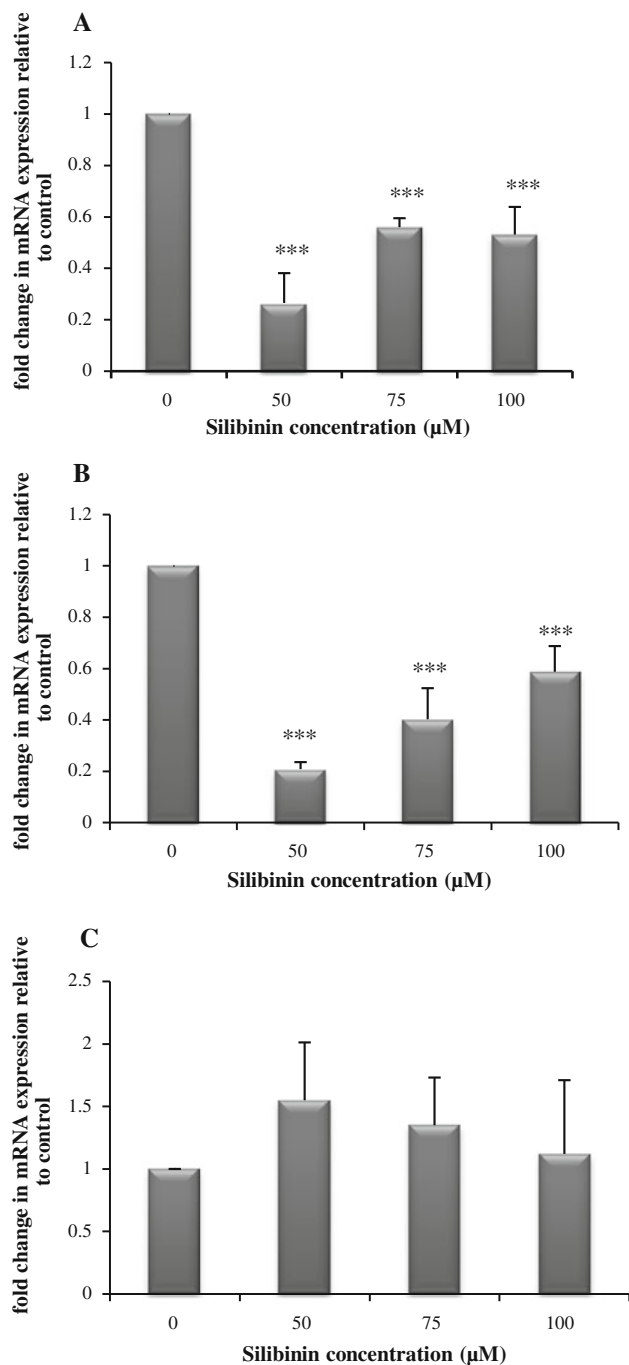


Fig. 3 Inhibitory effects of silibinin on expression of target genes mRNA. All data of qRT-PCR were normalized against HPRT, by REST software. Bars represent fold differences of the mean of normalized expression values \pm S.E.M. *** $P < 0.001$. **a** Silibinin effect on D4-GDI, **b** Cdc42, **c** $\beta 1$ -integrin and **d** Raf-1 mRNA expression

Discussion

There have been numerous studies on natural chemopreventive and chemotherapeutic agents during the last decades, indicating their importance in cancer treatment due

to their lower cytotoxicity and side effects for normal cells [2].

Silibinin is a natural flavonoid extracted from *silybum marianum* L. Silibinin anti-metastatic potential on malignant tumor cells has been reported in several studies [3, 4, 6, 27].

Here, we studied the restrictive effects of silibinin on migration and adhesion capacity of the MDA-MB-231 breast cancer cells by investigating $\beta 1$ -integrin signaling pathway and its important downstream molecules. One of the important reasons of cancer treatment failures is the metastasis of malignant tumor cells to healthy organs. $\beta 1$ -integrin signaling has been shown to critically mediate breast cancer progression in culture models and to be essential for tumorigenesis in vivo [28]. Alteration in $\beta 1$ -integrin signaling, as well as changes in its downstream molecules like Rac1 and Cdc42, has important effect on cancer cells survival and their metastatic properties [13, 28]. Therefore, identification of novel molecular targets and biomarkers among adhesion molecules or their downstream pathways seems to be promising for developing new strategies in cancer therapy [10].

Any factor capable of inhibiting or modulating the $\beta 1$ -integrin signaling pathway may be beneficial for targeted therapy of cancer. Thus, the Cdc42 and Raf-1, two important downstream molecules of $\beta 1$ -integrin signaling pathway, were investigated in this regard. Furthermore, D4-GDI, a regulator factor of small G-proteins, which is reported to induce cell invasion by controlling $\beta 1$ -integrin expression [15], was also studied.

Data obtained from the migration assay revealed that silibinin has significant inhibitory effect on migration of the MDA-MB-231 cells; therefore, inhibition of cell motility might be a useful strategy in the management of very early stages of tumor progression [1].

The main target for breast cancer cells metastasis is bone tissue [20]. In the current study, silibinin was found to inhibit cell adhesion to type I collagen, the major component of bone indicating that silibinin could inhibit the attachment of migrated cells to bones [29].

Moreover, silibinin significantly inhibits the expression of Cdc42 and D4-GDI mRNAs without any statistically significant effect on the $\beta 1$ -integrin and Raf-1 mRNA expression.

Cdc42 is a member of Rho family whose over expression occurs in breast cancer [13]. Activation of Cdc42 promotes actin polymerization to form filopodium, which is required for movement [12]. Thus, the inhibitory effect of silibinin on Cdc42 expression prevents filopodium formation, which subsequently inhibits the movement and migration capability of the cells. On the other hand, activation of the Cdc42 stimulates integrin-mediated migration and invasion across collagen matrix [30]. The reduction in

adhesion of the MDA-MB-231 cells to collagen could be a consequence of the inhibitory effect of silibinin on Cdc42 expression. In addition, it has been reported that the activation of PI3 K by Cdc42 alters actin organization and increases motility and invasiveness [30]. Silibinin has inhibitory effects on both Cdc42 (this study) and PI3 K [8]. This augments the inhibitory effect of silibinin on motility and invasion of malignant cells.

The reduction in D4-GDI expression by silibinin modulates small G-proteins family activation which are over expressed in malignant tumor cells [15]. Although Zhang Y and colleagues reported that the inhibition of D4-GDI expression could inhibit the expression of β 1-integrin and decrease the adhesion to laminin [15], but in the present study, no significant alteration in the expression of β 1-integrin was observed in spite of decrease in D4-GDI expression caused by silibinin. Furthermore, we showed that the inhibitory effect of silibinin on D4-GDI expression effectively prevents cell attachment to type I collagen. This implies that silibinin decreases adhesion and migration capability of malignant cells by affecting D4-GDI expression but via a different mechanism.

Moreover, in this study, no significant effect of silibinin on β 1-integrin expression was observed, but there are some evidence showing that silibinin can indirectly modulate β 1-integrin signaling pathway. There is a potential synergy between EGFR signaling and integrin function in breast cancer [31]. Inhibitory effects of silibinin on both PI3 K [8] and EGFR signaling [2, 8] are two important modulators of β 1-integrin signaling pathway. Downregulation of focal adhesion kinase (FAK) by silibinin is another critical factor in modulating β 1-integrin signaling pathway in promoting malignancy and metastasis [11, 32]. Silibinin has affect on other cell adhesion molecules such CD44 [33], CD82 [34], intercellular adhesion molecule1 [35], E-cadherin [36] and KAI1 [37].

Here, also no significant effect of silibinin on Raf-1 expression was detected. It has been previously reported that silibinin inhibits Raf-1 activity via upregulation of Raf kinase inhibitor protein (RKIP) and other regulators like Spred1 and Spred2 [5]. On the other hand, Cdc42 activates p21-activated kinase (PAK) which phosphorylates and activates RAF kinase [38]; therefore, the reduction observed in Cdc42 expression in the present study suggests another mechanism of modulating Raf-1 activity in malignant cells.

In conclusion, the significant inhibitory effects of silibinin on migration and adhesion of the MDA-MB-231 cells can be due to the inhibition of D4-GDI mRNA expression, a key regulator of G-protein family, and Cdc42, an important member of G-protein family involved in cell movement. Although silibinin does not have any significant effect on β 1-integrin and RAF1 expressions, but it can

indirectly modulate β 1-integrin signaling pathway and RAF1 function.

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