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Vitexin Induces Apoptosis in MCF-7 Breast Cancer Cells through the Regulation of Specific miRNAs Expression

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ABSTRACT

In this research, we investigated microRNAs (miRNAs) expression profile in MCF-7 breast cancer cell line which treated with 150 µM vitexin. Profiling of miRNAs expression was performed using TaqMan MiRNA Array. Apoptosis was analyzed by flow cytometry and the expression of some genes involved in "anti-proliferative" signaling pathways were evaluated by western blotting and real time PCR methods. Twenty microRNAs were differentially expressed in vitexin treated cells compared to the control. Among them, let-7- b, c were up regulated while miRNA-17-5p was down regulated with highest score. Also, we detected the expression changes of mentioned miRNAs target genes as well as genes involved in caspase apoptosis pathways. Our results provide the first evidence that vitexin can effect miRNA expression in MCF-7 cells. Also based on our finding, vitexin can be an attractive miRNA mediated chemo preventive and therapeutic agent in breast cancer.

Keywords: Vitexin, MicroRNA, breast cancer, apoptosis

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Introduction

Vitexin (apigenin-8-C-D-glucopyranoside), has shown "anti-tumor" efficacy against a wide variety of human cancers, including leukemia (1), hepatocellular carcinoma (2) and glioblastoma (3).This drug found in the traditional Chinese herb Crataegus pinnatifida (hawthorn) and naturally has flavonoid compound (4). Newly, plant derived compounds such as Vitexin, has been highly regarded for its "anti-tumor" properties.

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Anti-apoptotic effect of vitexin, has been shown in different cell lines such as breast, ovarian, prostate (5). Vitexin involved in the regulation of different process such as proliferation, differentiation, cell cycle regulation and apoptosis (6, 7).

Although many studies have been performed on the "anti-tumor" effect of vitexin, there are still many ambiguities regarding the molecular mechanisms and signaling pathways that this drug activates or suppresses. It has been shown that in MCF-7 cells, vitexin has a strong inhibitory effect on cell proliferation, by the decreased Bcl-2/Bax ratio and over expression of caspases (5).

Micro RNAs are small non-coding 21–25 nucleotide that involved in regulation of many physiological and pathological processes, like cell development, differentiation, infection, immunity, tumor suppression and carcinogenesis (8, 9). To date, as far as we know, there is no data on the role of vitexin on the expression profile of micro RNAs in breast cancer cell line. The aim of this research was to evaluation of the miRNAs expression profile in MCF-7 breast cancer cell line to understand molecular mechanism underlying the "anti-tumor" activity of vitexin.

Materials and methods

Cell culture

The human breast cancer cell line (MCF-7) was obtained from the Cell Bank of Pasteur Institute of Iran, Tehran, IRAN. Cells were cultured at 37 °C in a 5% CO2 humidified atmosphere and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1% l-glutamine. Sub-confluent cells were seeded in 6-well plates at the density of 1.5 × 105 cells/well to achieve 80% of confluence.

MCF-7 cells viability assay

Vitexin was purchased in powder form from Sigma Company (Sigma, USA) , dissolved in dimethyl sulfoxide (DMSO, Sigma) and diluted with pure water then was packed and stored at 4°C. 200 µL MCF-7 cells were plated in 96-well plates at a density of 5×10^3 cells/well. Upon 24-hour culture, the cells were treated with different concentrations of vitexin (0, 20, 50,100, 150µM) for 12,24,48 hours in a 5% CO_2 incubator at 37 °C and 4 replicate wells were used for each concentration with a final volume of 200 µL. Following incubation at $37^{\circ C}$ in a humidified atmosphere containing 5% CO_2, 20 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well, and the cells were cultured for another 4 h. The supernatant was then removed, 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and the plates were then shaken on a horizontal shaker for 15 min to allow for complete dissolution. The optical density (OD) was read at 492 nm on an enzyme-linked immunosorbent assay (ELISA) microplate reader. The inhibition of cell growth (IR) was calculated using the following formula: Inhibitory rate (%) = (1 - mean OD value in the experimental group/mean OD value in the control group) × 100%. The IC_{50} value was estimated from the OD value and was applied to evaluate the cytostatic efficacy of Vitexin on MCF-7 cells. Each experiment was repeated in triplicate.

CCK-8 assay

MCF-7 cells were harvested in a logarithmic growth phase. The cell density was adjusted to 1×10^3/ml and incubated in 96- well plates (100 µL of cell suspension per well). The 96 well plates were then placed in

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an incubator to continue the culture. Then, 10 µL of CCK-8 reagent (Dojindo, Japan) were added to the wells at 12, 24, 48, and 72 hours, followed by 2 hours of culture. Subsequently, the absorbance in each well examined at 450 nm using a microplate reader in a group of experiments was repeated three times.

miRNA profiling

In this regard total RNA was extracted from cultured treated cells or not, by using the miRVANA PARIS Kit (Thermo fisher, Germany), according to manufacturer instructions. To evaluate the concentration and purity of RNA, we used Nano Drop ND-1000 (Thermo, USA) and 260/280 ratios of >1.8 were accepted. In the next step cDNA was synthesized from total RNA by TaqMan MiRNA Reverse Transcription Kit and the Megaplex RT Primers (Thermo fisher, Germany). Taq Man PreAmp Master Mix and the Megaplex PreAmp Primers was used to amplify the quantity of desired cDNA. Taq Man Universal PCR Master Mix and sequence-specific primers and probes on the 384-well Taq Man miRNA Array CARD (Thermo fisher, Germany) was used for amplification of pre amplified cDNA.

qRT-PCR procedure

For validation of the miRNA expression real time PCR was performed using Rotor gene-Q real-time PCR system (Qiagene, Germany). Each real-time PCR (10 µL) master mix included 1 µL of reverse and forward primers (Exiqon, Denmarck), 5 µl of Ampliqon real Q plus 2x master mix green (Ampliqone, Denmarck), and 4 µL of diluted cDNA . The reactions were incubated in a 72 well optical strip at 95˚C for 15 min (enzyme activation), followed by 95 ˚C for 20 s and 60 ˚C for 60 s (40 cycles). For efficiency assessment we diluted one normal sample in 1/10 ratio, and 5 dilution was provided, then efficacy of primers (Target miRNAs and endogenous control miRNAs) was evaluated. Efficacy of primers were near the 100 and had not significant difference. All reactions were run in triplicate. After the reactions, the mean Ct was determined from the triplicate PCRs. We used Ct values to evaluate the expression levels of the three miRNAs. Note that hsa-miR30a-5p and hsa-miR100-5p are the endogenous control genes to normalize RNA contents among different samples. The expression value of miRNAs relative to internal controls was determined using the $2^{-\Delta\Delta Ct}$ method (10).

Analysis of apoptosis

Flow cytometry was used for evaluation of apoptosis. In this method two dye was used to distinguish apoptotic from necrotic cells (Annexin V-FITC and propidium iodide (PI)). Apoptotic cells were Annexin V-FITC-positive, PI positive and necrotic cells were Annexin V-FITC negative, PI positive. In order to do this process, 1.5×10^5 cells/well of MCF-7 cells were plated in 6-multiwell plates with or without 150 µM vitexin. After 48 h, cells were detached and analyzed by flow cytometry method. The experiment was carried out according to the Life Technologies Apoptosis Assay protocol and the samples were analyzed by flow cytometry within 1 hour using the FACS Calibur (Becton Dickinson, CA) in triplicate. Dot plot graphs were used to illustrate the viable cells (the lower left quadrant), early phase apoptotic cells (the lower right quadrant), late phase apoptotic or dead cells (the upper right quadrant) and the necrotic cells (the upper left quadrant).

Western blotting method

Cultured MCF-7 cells with 150 µM vitexin and without vitexin centrifuged. Plate was washed twice with ice cold PBS, and the pellet was lysed using 100 µL of RIPA buffer. Incubation time on ice was 30 min,

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then the cells were centrifuged at 18,000×g for 30 min at 4 °C, and the supernatant was recovered. Bradford method was used for protein concentration determination (10). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein separation, then proteins transferred to nitrocellulose membranes by Trans blot turbo (BIO-RAD, USA). Primary antibodies were used at a dilution of 1:1000, and secondary antibodies were used at a dilution of 1:5000. Primary anti-bodies were: Anti-AIB-1 (1:1,000 dilution; cat. no. ab *376-389*), Anti-TCF4 (1:1000 dilution; cat. no. ab 31179), Anti-Beta actin (1:1000 dilution; cat. no. ab 119716). Secondary horseradish peroxidase-conjugated was purchased from Sigma Aldrich, USA and employed for the chemo-luminescence detection. Blots were screened using enhanced chemo luminescence detection reagents ECL (Cyanagen, Bologna, IT) and exposed to X-ray film. All films were scanned by using Image J software (National Institutes of Health, USA).

Statistical analysis

The results were analyzed by the GraphPad software (GraphPad PRISM V 5.04analytical software). Data were based on three replications for each sample. Data are expressed as mean ± standard deviation (SD). The means were compared using analysis of variance (ANOVA) plus Bonferroni's t-test. A P-value of < 0.05 indicates a statistically significant result.

Results

Effect of vitexin treatment on MCF-7 cells viability

Vitexin solution of varying concentrations 0, 20, 50, 100, 150µM were used to treat MCF-7 cells and to determine the optimal therapeutic dose. Our results showed, compared with the control group cells, vitexin concentrations of under the 100 µM caused minimal toxicity to MCF-7 cells, while concentrations above 100 µM after 48h caused significant toxicity and decreased cell proliferation significantly ($P<0.0001$) Figure 1. 150 µM concentration were selected for the use of in subsequent experiments in this study.

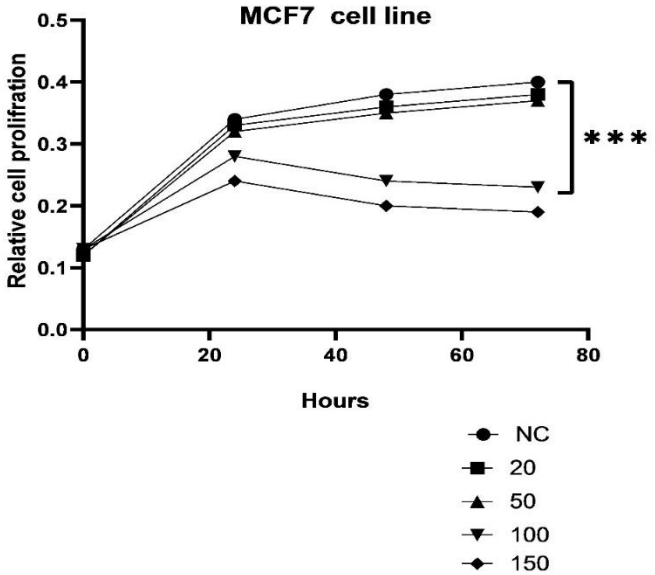

Fig.1. Cell proliferation assay to determine optimal dose of vitexin. As seen in figure in treatment with 150 µM vitexin after 48h the proliferation of cells decreased significantly.***p< 0.001.

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Table 1. Top up regulated miRNAs in MCF-7 cell line that treated with 150µM vitexin.

miRNA	Fold change	P-value
hsa-let7a-5p	5.0041	2.11E−01
hsa-let7b-5p	4. 1432	0.00321
hsa-miR-145-5p	4.027864	0.0223
hsa-miR-194-5p	3. 0019	0.00062
hsa-miR-106a-5p	3. 1234	0.02765
hsa-miR-23b-3p	2.011	0.000101
hsa -miR-24-3p	1.89	0.000032
hsa -miR-143-3p	1.578	0.0564
hsa -miR-99a-5p	1.443	0.0987

Table 2. Top down regulated miRNAs in MCF-7 cell line that treated with 150µM vitexin.

miRNA	Fold change	P-value
hsa-miR-17-5p	0.003241	1.10E−01
hsa-miR-195-5p	0.001432	0.005
hsa-miR-145-5p	0.002764	0.0123
hsa-miR-30b-5p	0.02419	0.00062
hsa-miR-21-5p	0.012334	0.02765
hsa-miR-103a-3p	0.011	0.0001
hsa -miR-19a-3p	0.23412	0. 0032
hsa -miR-181b-5p	0.2784323	0.0564
hsa -miR-99a-5p	0.543216	0.036

Profile of miRNA expression in treated MCF-7 cells

In this regard MCF-7 cells which treated with 150 µM vitexin after 48 h was evaluated for miRNAs expression profile using a 384-well TaqMan Array CARD. After expression analysis our results showed 20 miRNAs have differential expression in treated cells in comparison to control cells Tables 1, 2. Among the differentially expressed miRNAs, we evaluated those miRNAs that had highest score. Also real time PCR results confirm array results Tables 3. In this context miR let 7b, c were up regulated and miR 7-5p was down regulated.

Apoptotic effect of vitexin on the MCF-7 cell line

In order to investigate the effect of vitexin on the apoptosis of cancer cells, we used flow cytometry technique. Our results showed in the control cells %2.8 of cells were apoptotic but after treatment with 150 µM vitexin, apoptotic cells rate raised to 39.3% that was significant ($P<0.001$) Figure 2 (a,b). To confirmation apoptosis of cancer cells after exposure to vitexin, we evaluated expression of 3 genes involved in apoptosis process. Our results showed significant up regulation of caspase 3 after exposure to vitexin $2^{-\Delta\Delta ct} = 3.2$ ($P<0.001$). Also we showed significant over expression of caspase 6, 8 in treated cells in comparison to non-treated cells $2^{-\Delta\Delta ct} = 5.8$ ($P<0.001$), $2^{\Delta-\Delta ct} = 4.42$ ($P<0.0001$) respectively Figure 3. In conclusion, these data provided evidence for the apoptotic activity of vitexin, by a caspase dependent mechanism.

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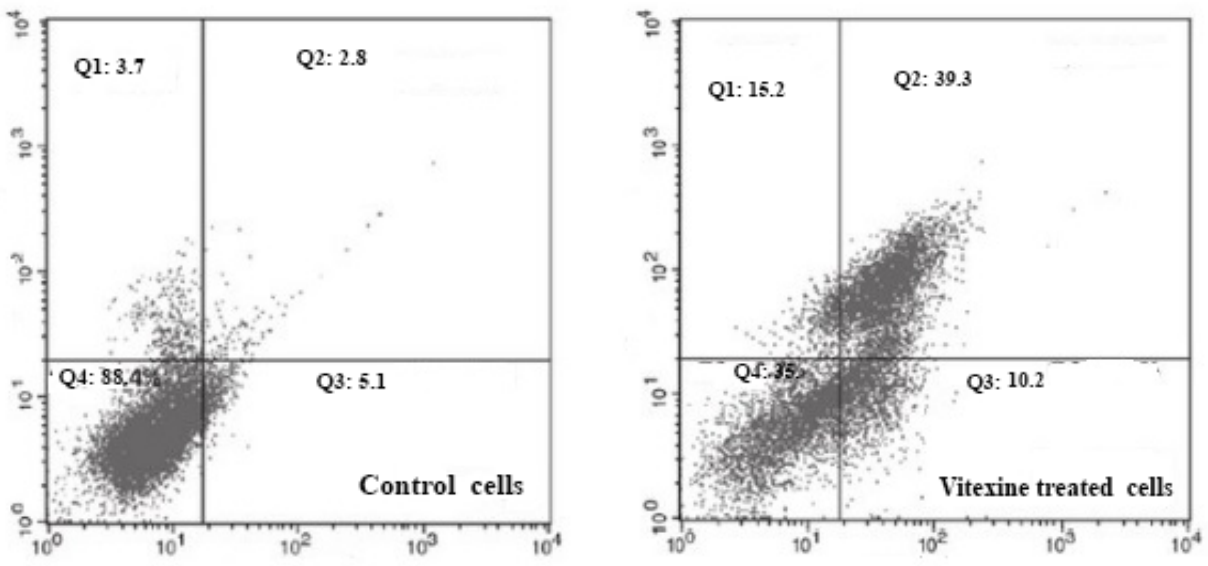
Table 3. Primer sequences of studied miRNAs.

miRNA	Primer sequences
hsa-let7a-5p	5'-GCAGTGAGGTAGTAGGTTG-3' 5'-GGTCCAGTTTTTTTTTTTTTTTAACTATAC-3'
hsa-let7b-5p	5′-AGCCAGGGACTTCCCAAGA-3′ 5′-AGTCTCATGACCTGGAACAG-3′
hsa-miR-17-5p	5'-GCAAAGTGCTTACAGTGCA-3' 5'-GGTCCAGTTTTTTTTTTTTTTTCTAC-3'
hsa-miR-195-5p	5'-UAGCAGCACAGAAAUAUUGGC-3' 5'-UAGCAGCACAGAAAUAUUGGC-3'
hsa-miR30a-5p	5'-CACACCCACTTTTCTGTATCAACT-3' 5'-GAATTCCACTCCCATTCTCTTATG-3'
hsa-miR100a-5p	F: 5'-GA ACCCGTAGATCCGAACT-3' R: 5'-CAGTGCGTG TCGTGGAGT-3'

Table 4. Comparison of fold changes in expression of miRNAs detected by microarrays qRT-PCR.

miR	Fold change	
	Array	Real time PCR
hsa-let7a-5p	5.0041	3.432
hsa-let7b-5p	4.1432	3.894
hsa-miR-17-5p	0.003241	-1.1
hsa-miR-195-5p	0.001432	0.0011



(A) **(B)**

Fig.2. Apoptosis was evaluated by FACS analysis. The different quadrants report the percentage of cells. Non-viable necrotic cells, upper left (Q1), late apoptotic cells, top right (Q2), early apoptotic cells, bottom right (Q3), viable cells lower left (Q4). The experiment was repeated three times and the results were always similar. a. Related to non-treated cells (control), b. related to treated cells.

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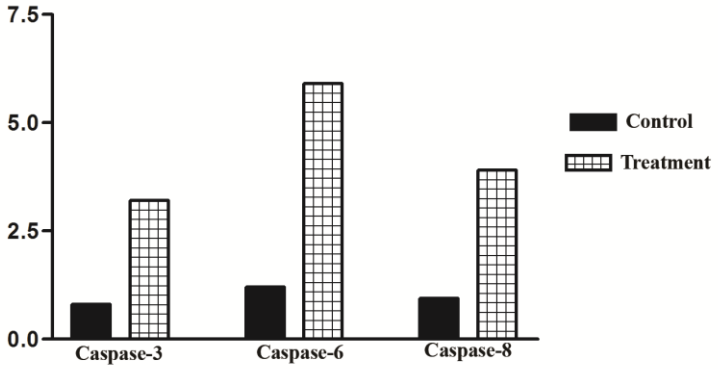
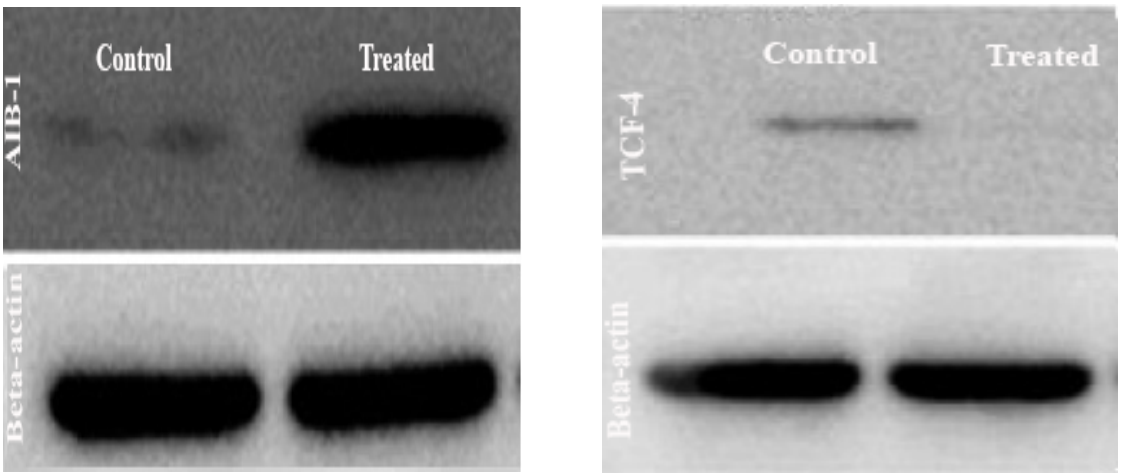


Fig.3. Real time PCR assay of MCF-7 cell extracts was evaluated for the expression of caspase-3, caspase-6 and caspase-8.

MiR-7b, c and miR-17-5p targeted TCF-4 and AIB1 genes

MiRNA-mRNA integration analysis was done by using the Target Scan miRNA target prediction software to identify potential mRNAs targets the miR-let7b, c and miR-17-5p. Our analysis results showed TCF-4 mRNA is potential target gene of miR-let7b, c and AIB1 is target mRNA of miR-17-5p. The transcription factor 4(TCF-4) gene plays an important role in malignant transformation and it forms an important component of the Wnt signaling pathway (5). Amplified in breast cancer 1 (AIB1) is widely implicated in nuclear receptor-mediated diseases, particularly malignant diseases (5). To confirm the interaction between miR-let7b, c and miR-17-5p with TCF-4 and AIB1 mRNAs western blotting analysis was performed to detect the expression of the both proteins in MCF-7 cells after vitexin treatment. Our results showed significant up regulation of AIB1 protein and down regulation of TCF-4 protein Figure 4 a-b.



A **B**

Fig.4. Treated and non-treated cells incubated with antibodies against the indicated proteins and analyzed by Western blotting. The housekeeping protein α-tubulin was used as loading control. The intensities of signals were expressed as arbitrary units. The images are representative of three immune blotting analyses obtained from at least three independent experiments.

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Discussion

Although breast cancer can be diagnosed and treated quickly, it is one of the leading causes of death in women (11-15). Due to the fact that in breast cancer there are significant cases of resistance to chemotherapy drugs and cell death, today studies have led to the use of drugs with less toxicity and greater effectiveness(14).Studies have shown the "anti-proliferative" effect of vitexin (5), but its exact molecular mechanism is not yet known. In recent years, a new class of small non coding RNA molecules, known as miRNAs have received much attention and can be used as prognostic, diagnostic biomarkers and even in the treatment of cancers, including breast cancer (16-17). Studies have shown that miRNAs can act as potential oncogenes or as tumor suppressor genes in the pathogenesis of cancers, and regulate oncogene and tumor suppressor genes expression through binding to the 3′UTR regions and influencing the expression of targets (17).To date, a direct correlation between the "anti-proliferative" effect of vitexin and the variation of miRNAs expression in breast cancer has never been shown. In this study, we demonstrated for the first time vitexin was able to effect on the miRNAs expression profile in MCF-7 breast cancer cell line. Treatment of cell line with vitexin significantly modulated 20 miRNAs, among them let-7b and let-7c were up regulated and miR-175p was down regulated with high score. In human, 10 members of the let-7 family have been identified, in normal physiological conditions these genes family involved in gene regulation, cell adhesion and muscle formation. Accumulating evidences suggest that let-7 is down regulated in numerous types of cancers including breast cancer (18). According to the results of this study, let-7b, c had the highest score in the up regulated miRNAs after treatment with vitexin. This miRNA expression is reduced in breast cancer tissues and is inversely associate with the tumor lymph node metastasis, patient overall and relapse free survival. Also it has been shown that induced let-7b expression effectively inhibit the growth of breast cancer cells in *vitro* (19). It has shown that Let-7c, inhibits cancer cells survival by regulating cell proliferation and apoptosis (19). Increased expression of Let-7 under the influence of the quercetin (as flavonoid) has been shown in breast cancer (20, 21), but no report has been done for vitexin. Based on the results of the target scan, TCF-4 gene had common binding sequence for let-7b, c and our western blot results showed significant down regulation of this oncon gene after 150 µM vitexin treatment. The TCF-4 protein is part of a group of proteins known as E-proteins. E-proteins bind to each other and then bind to a specific sequence of DNA known as an E-box. Abnormal up regulation of TCF-4 and stimulating downstream target genes is a common early event in tumori genesis (20). About the interaction of let-7 miRNAs and TCF-4 gene has been shown *Let-7* down regulates WNT signaling activity by targeting estrogen receptors in breast cancer and TCF-4. WNT pathway is major regulator of cell proliferation, differentiation, and migration (22-25). High score down regulated miRNA after treatment of vitexin was miR-17-5p, in all cancer types studied so far, miR-17-5p has been found at elevated levels in the circulation, and normally expressed in all tissues (26). It was found that patients suffering from several different types of cancers have high circulating miR-17-5p levels in their serum (24). In addition, miR-17-5p among other miRNAs is as potential molecular marker to evaluate grade, receptor status and molecular type in breast cancer (27). Expression of AIB1 as a target gene of miR-17-5p was studied after vitexin treatment and result showed significant up reregulation of AIB1 gene (28).The protein encoded by this gene is a nuclear receptor coactivator that interacts with nuclear hormone receptors to enhance their transcriptional activator functions. The encoded protein has histone acetyl transferase

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activity and recruits p300/CBP-associated factor and CREB binding protein as part of a multi subunit coactivation complex. This protein is initially found in the cytoplasm but is translocated into the nucleus upon phosphorylation. Several transcript variants encoding different isoforms have been found for this gene. In addition, a polymorphic repeat region is found in the C-terminus of the encoded protein (29).Overexpression of AIB1 in breast cancer has been described by several groups thus contributing to the development of cancer (30). The expression of this gene, regulated by exogenous substances such as tamoxifen and endogenous microRNAs such as miR-17-5p; and proteasomal degradation (30). Due to the increase in apoptosis of MCF-7 cells after treatment with vitexin, the expression of three genes (Caspase 3, 6 and 8) involved in apoptosis before and after treatment was compared and a significant increase in expression was observed. In conclusion we showed the first evidence that vitexin as flavonoid has more toxicity effect on MCF-7 cells in 150 µM after 48h treatment. Also we showed in this dose vitexin has regulatory effect on miRNA expression in MCF-7 cells, and causes up regulation and down regulation of some onco and suppressor miRNA$_S$ and is an attractive miRNA-mediated chemo preventive and therapeutic agent in breast cancer.

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