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Silibinin, Synergistically Enhances Vinblastine-Mediated Apoptosis in Triple Negative Breast Cancer Cell Line: Involvement of Bcl2/Bax and Caspase-3 Pathway

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) with a poor prognosis and survival is the most invasive subtype of breast cancer. Usually, TNBC requires a chemotherapy regimen at all stages, but chemotherapy drugs have shown many side effects. We assumed that combination therapy of vinblastine and silibinin might reduce the vinblastine toxicity and dose of vinblastine.

Materials and Methods: The MDA-MB-231 were cells subjected to MTT assay for IC50 determination and combination effects, which were measured based on Chou-Talalay's method. The type of cell death was determined by using a Flow-cytometric assay. Cell death pathway markers, including Bcl-2, Bax, and caspase-3 were analyzed by western blot and Real-Time PCR.

Results: The treatment of MDA-MB-231 cells exhibited IC50 and synergism at the combination of 30 µM of silibinin and 4 µm of vinblastine in cell viability assay (CI=0.69). YO-PRO-1/PI double staining results showed a significant induction of apoptosis when MDA-MB-231 cells were treated with a silibinin and vinblastine combination ($p<0.01$). Protein levels of Bax and cleaved caspase-3 were significantly upregulated, and Bcl-2 downregulated significantly. Significant upregulation of Bax (2.96-fold) and caspase-3 (3.46-fold) while Bcl-2 was downregulated by 2-fold.

Conclusion: Findings established a preclinical rationale for the combination of silibinin and vinblastine. This combination produces synergistic effects in MDA-MB-231 cells by altering pro- and anti-apoptotic genes, which may reduce the toxicity and side effects of vinblastine.

Keywords: Vinblastine; Silibinin; Drug combination; Triple negative breast cancer (TNBC); Apoptosis

INTRODUCTION

Lack of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2) characterize a heterogeneous subtype of breast cancer known as Triple-negative breast cancer (TNBC). Although TNBC comprises only 15-20% of all diagnosed breast cancer patients, it is associated with a poor prognosis, metastasis, and a high recurrence rate[1,2]. Due to lack of receptors expression, TNBC is unresponsive to targeted therapies, and management of this type has been mainly limited to chemotherapy and surgery[3].

Microtubules possess highly dynamic structures and play a crucial role in cellular physiology and cell division. This dynamic structure makes them appropriate candidates for anticancer therapeutic development such as vinca alkaloids, epothilones, taxanes and eribulin[4]. Vinblastine is a natural alkaloid derived from Catharanthus roseus that disrupts tubulin polymerization and induces cell death. Additionally, vinblastine interferes with spindle formation, resulting in chromosome segregation. It is used to treat some solid tumors such as breast cancer, bladder cancer, brain cancer, melanoma, and testicular tumors. Vinblastine, when combined with other chemotherapy agents, has been shown to be effective in patients with metastatic breast cancer or those who have relapsed after adjuvant therapy[5]. In addition to the benefits of vinblastine, side effects including white blood cells and platelet loss, anemia, hair loss, gastrointestinal problems, sweating, hypertension, muscle cramps, depression, headache, dizziness, and cardiac problems limit its use in the realm of clinical application [6,7].

Silibinin, a natural polyphenolic flavonoid, is the foremost bioactive flavanone found in milk thistle seeds. Silibinin has a wide range of phytotherapeutic applications. It has been reported that this compound has antioxidant, anti-inflammatory, and anti-cancer properties[8]. On the other hand, it has been reported that consumption of silibinin can be safe and non-toxic in animals and humans. By reviewing recent publications on different cancers, it is clear evident that silibinin has shown its efficacy mainly through targeting proliferation, inflammation, angiogenesis, apoptosis, and tumor cell metabolism. It has been described that silibinin targets a range of cellular signaling cascades and molecules, including NF-κB (nuclear factor-kappaB) transcription factor, MAPK (mitogen-activated protein kinase), epidermal growth factor receptor, HIF-1α (hypoxia-inducible factor 1-alpha), transcription factor AP-1, cyclooxygenase, phosphoinositide 3-kinase/Akt, and β-catenin. Accordingly, silibinin's biological effects occur through these molecular alterations[9,10].

Nowadays, researchers are focusing on the investigation of dietary supplements and other phytotherapeutic agents for their synergistic efficacy in combination with anticancer drugs to minimize the systemic toxicity of chemotherapeutic agents[11]. Research has shown that combining dietary phytochemicals with chemotherapeutics can sensitize cancer cells to apoptosis and reduce the side effects of chemotherapy[12]. For illustration, silibinin in combination with paclitaxel, cisplatin, carboplatin, and doxorubicin in human breast carcinoma models including MCF-7, MDA-MB-435, and MDA-MB-468 cells was studied, and results indicated that this concomitant use made more substantial apoptotic death compared to either agent alone in the cell lines [11, 13].

However, there are no reports on the synergistic effect of vinblastine and silibinin in TNBC, which could be considered a significant factor in developing a new combination chemotherapy strategy. Therefore, we evaluate whether silibinin can synergize with the therapeutic efficacy of vinblastine in human TNBC cell models.

MATERIALS AND METHODS

Cell culture condition

Highly metastatic human breast cancer TNBC cell line (MDA-MB-231) was provided from the Pasteur Institute Cell Bank of Iran. Complete RPMI-1640 medium containing 10 % FBS, 2 mg/ml sodium bicarbonate, 100 µg/ml streptomycin and 0.05 mg/ml penicillin G were used for cell culture. Standard 25 cm^2 flasks were used for cell culture in a humidified incubator containing 5 % CO2 at 37 °C.

MTT cytotoxicity assays and drug combinations analysis

The IC50 value (half-maximal inhibitory concentration) of silibinin and vinblastine for MDA-MB-231 cell line was assessed by the MTT cytotoxic assay. MDA-MB-231 cells at a density of 5,000 cells per well were seeded into 96-well plates and incubated in normal cell culture conditions to reach 80 % confluency. The next day, cells were exposed to single agents at increasing concentrations (from 5 mM - 640 µM for silibinin and 2.5 µM-160 µM for vinblastine). After 48 hours of incubation, the supernatant culture medium was discarded and 50 µL of tetrazolium dye (Sigma) (0.5 mg/ml) was added to each well and incubated for 4 hours at 37 °C in a CO_2 incubator. After incubation period, to dissolve the formazan crystals, 25 µl of Sorenson's buffer and 200 µl of DMSO were added to the each well. Finally, by using an ELISA plate reader (Stat-Fax-3200; Awareness Technologies, Westport, CT/USA), absorbance at 570 nm wavelength was read. Cell viability was determined using the following formula:

Cell viability (%) = Absorbance Test/ Absorbance Control × 100.

For experiment with silibinin and vinblastine according to the strongest concentration-dependent effects of the single agents, cells were simultaneously exposed to both agents (silibinin 5-640 µM and vinblastine 1.9-30 µM) with the combined ratio of 1: 25, each diluted 1: 2 within the given ranges were used. CompuSyn software (Version 1.0, Combo-Syn Inc., US) was used to determine the drug interaction between fixed ratios of silibinin and vinblastine based on the Chou-Talalay method for drug combinations. CI (combination Index) value indicated the mechanism of drugs interactions. CI<0.9, CI= 0.9–1.1, and CI>1.1 indicating synergistic, additive mode and antagonistic manners, respectively [14].

Flowcytometry YO-PRO-1/PI double staining

The Yo-Pro-1 assay kit (Invitrogen, CA, USA) was used to quantify apoptotic cells, and the procedure was performed according to the manufacturer's protocol. Briefly, 250000 cells/well were seeded in a six-well plate and drugs were adde after 24 hours of incubation in a humidified atmosphere. After the incubation period, cells were exposed for 48 hours to silibinin and vinblastine (equal to IC50 values), and their combination. Later, cells were detached by trypsin and resuspended in one milliliter of cold PBS. Then, one microliter of Yo-Pro-1 solution and one microliter of propidium iodide (PI) solution were added to the cell suspension. Cells were kept on ice in a dark place for 30 minutes, and fluorescence results were measured using the flow cytometer apparatus (Becton Dickinson, CA, USA) and evaluated by CellQuest Software. (Becton Dickinson, CA, USA).

Western blot

After treatment with silibinin, vinblastine (equal to IC50 values) and their combination for 48 hours, cells were washed three times with PBS and lysed in RIPA cell lysis buffer containing 50 µL 0.1 mol/L PMSF and100 µL proteinase inhibitor cocktails. The cell lysate was centrifuged at 12,000 rpm for 10 min at 4 °C. Protein in each treated sample was separated on SDS-PAGE and electrically transferred to PVDF membrane. The PVDF membrane was blocked with 5% nonfat dry milk for 1 hour and followed by the incubation with 1:1000 dilution of primary antibody at 4 °C overnight. (Primary antibodies: anti-BCL-2(1:1,000, Bioworld Technology Inc., Minneapolis, USA), rabbit anti-BAX (1:1,000, Bioworld Technology Inc.), anti-CASPASE-3 (1:1,000, Santa Cruz Biotechnology Inc.), and anti β-ACTIN (1:1,000, Santa Cruz Biotechnology Inc.)). Then, the PVDF membrane was washed and incubated with HRP-conjugated secondary antibody (1:2,000, Santa Cruz Biotechnology Inc.) at room temperature for one h. ECL kit were used for the immunoreactive proteins detection. Densitometric calculations were performed using ImageJ software. The intensity of each protein band was compared to the intensity of the corresponding β-actin binding, and relative intensity ratios were calculated.

Real-Time PCR

Following 48 hours of treatment of the MDA-MB-231 cell line with concentrations equal to IC50 values of both agents and their combination, total RNA

extraction was performed using the RNXPlus Solution (SinaClon Co., Tehran, Iran). The synthesis of cDNA was conducted using the Pars Tous RT Reagent kit (Pars tous, Iran) as described by the manufacturer.

For RT-PCR, three replicates of each sample were amplified in a 20 µL reaction mixture containing 1 µL of 0.5 mM of primer, 4 µL of diluted cDNA template, 10 µL SYBR Green reaction mix and 5 µL DW in PCR micro tube. Table 1 showed the specific primers. The rotor gene 6000 system (corrbet, Australia) were used for PCR amplification. β-actin was used as endogenous control for normalizing RNA concentration, and relative expression in mRNA levels of desired genes was calculated using the comparative $2^{-\Delta\Delta Ct}$ method [15].

Statistical analysis

GraphPad Prism 6.01 software was used for analyzing data (mean ± SD). Unpaired student-t test was used for assessing statistical difference. P value less than 0.05 was considered statistically significant.

Tabel 1: Primer sequences

Gene name	Primer sequence
B-Actin	Forward: 5'- TCCCTGGAGAAGAGCTACG -3' Reverse: 5'- GTAGTTTCGTGGATGCCACA -3'
BAX	Forward: 5'-CGTGGTTGCCCTCTTCTACTTT -3' Reverse: 5'- GATCAGCTCGGGCACTTTAGTG -3
BCL2	Forward: 5'- GTCATCCACAGAGAGCGATGTT -3' Reverse: 5'- GATGACTTCTCTCGTCGCTA -3
CASPASE-3	Forward: 5' -CTCGGTCTGGTACAGATGTCGATG-3' Reverse: 5' -GGTTAACCCGGGTAAGAATGTGCA-3'

RESULTS

Effects of silibinin and its combination with vinblastine on MDA-MB-231 cell line

MTT assay were used for cytotoxic effect determination of two agents alone and their combination. (Figures 1A, B, and C). Within the described ranges, both agents evidently decreased the viability of MDA-MB-231 cells in a concentration-dependent manner. The IC50 values of silibinin and vinblastine calculated by CompuSyn software were 192.249 µM and 29.819 µM for MDA-MB-231 cells, respectively. Furthermore, MTT results also indicated that combining two agents mediated synergistic effects on MDA-MB-231 cells (CI<0.9). (Figures 1D, E, and F). The combination of 105 µM of silibinin with 4 µM of vinblastine (total concentration 110 µM) caused a decrease in the viability of cells to 50% viability (the IC50 value of combination agents) (CI=0.69). The dose reduction index (DRI) for the combination of silibinin and vinblastine, which affected 50% of the cells after 48h treatment, was 1.8 (the dose of silibinin was reduced from 190 to 110 µM) and 7.01 (the dose of vinblastine was reduced from ~30 µM to 4 µM), respectively. All subsequent experiments were performed with IC50 concentrations.

Combination of silibinin and vinblastine induced marked apoptosis of MDA-MB-231 cells

Apoptosis plays a crucial role in the regulation of the killing of cancer cells by anticancer agents. To evaluate whether the cell death observed in MDA-MB-231 cells after silibinin and vinblastine combination treatment might occur through apoptosis, YO-PRO-1/PI double staining was utilized. YO-PRO-1/PI double staining results indicated that total apoptotic cell of MDA-MB-231cells treated with silibinin, vinblastine and their combination were 17.46 %, 34.72 %, and 47.60 %, respectively (Figure 2). These results indicated that a combination of two agents could effectively induce apoptosis in MDA-MB-231 cells.

Combination of silibinin and vinblastine altered Bcl2/Bax ratio and cleaved caspase-3 in protein level

After observing the flow cytometry results, we measured Bcl-2, Bax, and cleaved caspase-3 proteins levels after the MDA-MB-231 cells were treated with IC50 concentration of silibinin, vinblastine, and their combination for 48 hours. We found that Bax and cleaved caspase-3 were significantly upregulated while the expression of Bcl-2 protein was downregulated. Furthermore, the ratio of Bcl-2/Bax was decreased considerably (Figure 3).

Combination of silibinin and vinblastine alter mRNA expression of Bcl2, Bax and caspase3

Following apoptosis-induced changes in protein expression, we evaluated the effects of silibinin and vinblastine on the gene expression levels of Bcl2, Bax, and caspase-3 by quantitative RT-PCR. As shown in Figure 4, after 48 hours of treatment, the

expression levels of Bax (2.96-fold) and caspase 3 (3.46-fold) showed significant upregulation, while Bcl2 mRNA expression level revealed a 2-fold decrease in the combined treatment as compared to the control.

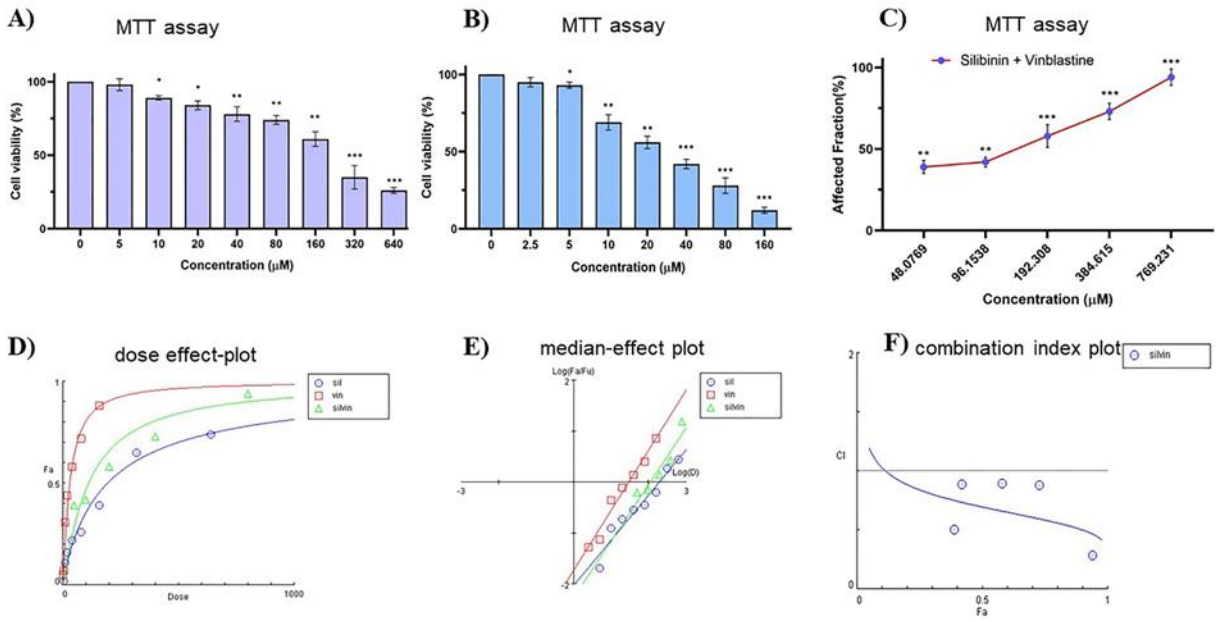

Figure 1: Concentration-response effects of Silibinin (A) and Vinblastine (B) separately and in combination (C) on MDA-MB-231 cells using MTT assay following 48-hours treatment with both agents. Analysis of synergy between Silibinin and Vinblastine. (D), (E) and (F) represent dose effect-plot, median-effect plot and combination index plot respectively for MDA-MB-231 cells. Data are presented as mean ± SD (* P < 0.05, ** P < 0.01, *** P < 0.001 vs. untreated control).

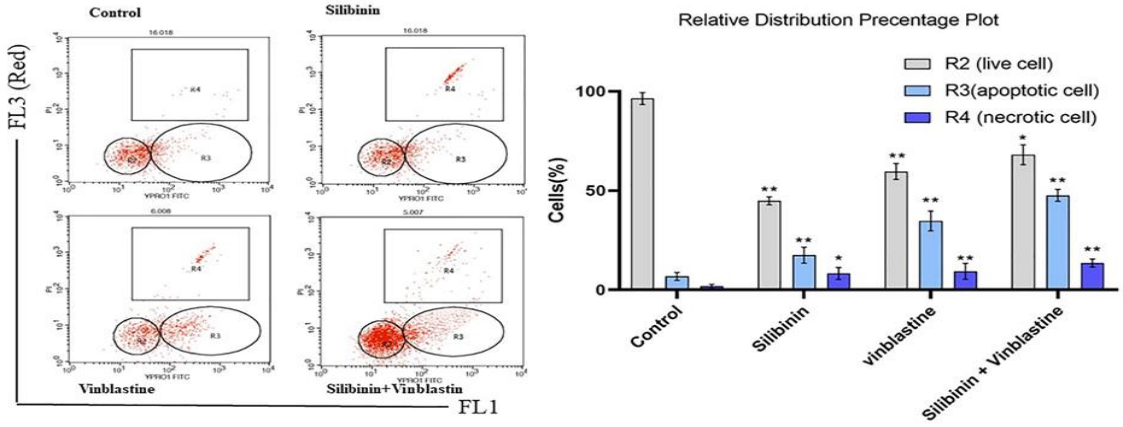

Figure 2. Combination of silibinin and vinblastine induced apoptosis in MDA-MB-231 cells. Cells were treated with silibinin (190 μM), vinblastine (30 μM) and combination (110 μM) at 37 °C for 48 h, stained with YO-PRO-1/PI, and then subjected to flow cytometry analysis. Results on the left representative flow cytometric dot plots showing the increase in apoptotic cells. Results on the right showed the relative distribution percentage of each stage. Bars presented are the mean± standard deviation (n=3) with single and double asterisks showing the significant difference from the controls at P<0.05 and P<0.01, respectively.

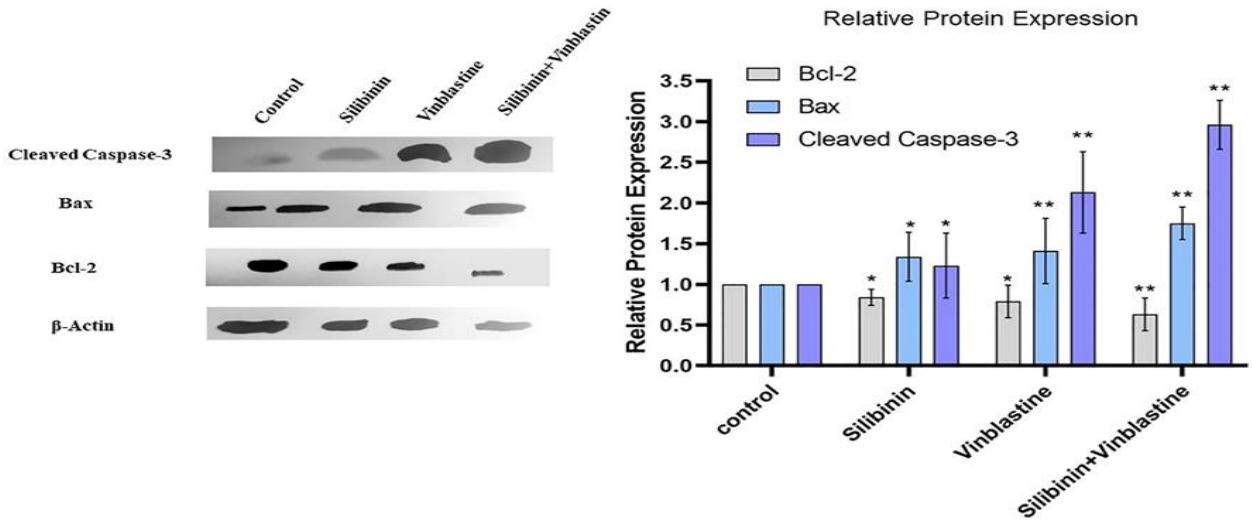

Figure 3. Western blot analysis of protein levels of Cleaved Caspase-3 Bcl-2, Bax, and in the control group and in the groups treated for 48 h with silibinin (190 µM), vinblastine (30 µM) and combination (110 µM). Results on the left were representative gel images exhibiting bands for Cleaved Caspase-3, Bcl-2 and Bax with corresponding β-Actin bands. Results on the right showed relative protein levels of Cleaved Caspase-3, Bcl-2 and Bax which were normalized to control. These data were representative of results obtained from the analysis of three independent experiments. Data were expressed as mean ± standard deviation. *P < 0.05 and **P < 0.01 as compared with control.

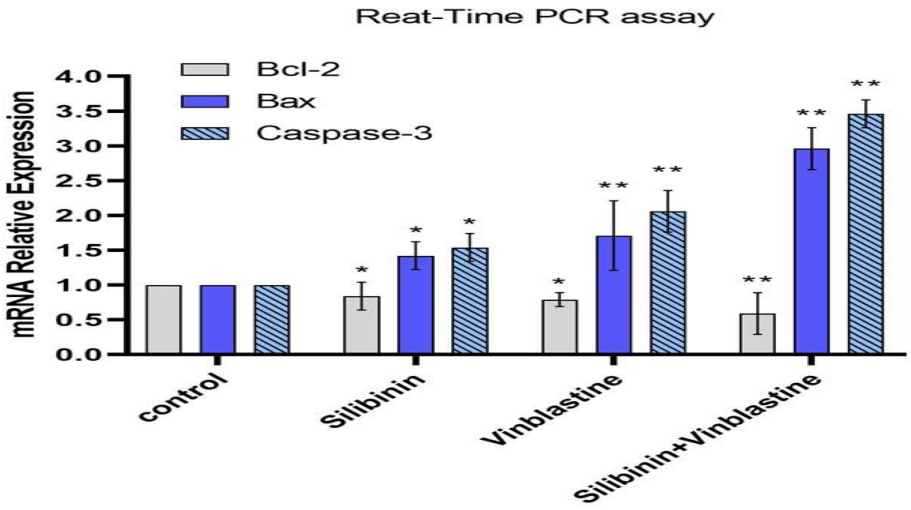

Figure 4. Inhibitory effects of silibinin, vinblastine and their combination on apoptotic mRNA (Bax, Bcl-2 and Caspase-3) expression determined by qPCR assay. MDA-MB-231 cells were treated with silibinin (190 µM), vinblastine (30 µM) and combination (110 µM) for 48h. Data of qPCR were normalized against β- actin. Bars represent fold differences of the mean of normalized expression values ± SD. (* P < 0.05, ** P < 0.01 vs. untreated control).

DISCUSSION

Triple-negative breast cancer (TNBC) has been poor associated with survival, particularly in younger patients, with reduced progression-free and overall survival. The lack of critical molecular markers of breast cancer makes this subtype unresponsive to targeted therapy, making the development of new and effective treatments crucial to improving the outcome of TNBC patients [16, 17].

Although the use of chemotherapy agents is effective in controlling TNBC, widespread side effects limit their use. In such situations, combinatorial therapeutic methodologies involving two or more synthetic anticancer agents and flavonoids are applied as an alternative solution to enhance the efficacy and minimize side effects [18].

Vinblastine is a tubulin-targeting agent belonging to the vinca alkaloids family and, since its introduction, has been responsible for many successes in cancer chemotherapy in the clinic. Vinblastine perturbs tubulin polymerization and induces apoptosis and cell death, but its side effects are one of the obstacles to its widespread use in treatment [19, 20].

To reduce the side effects and improve the therapeutic efficacy of vinblastine, the exploitation of a vinblastine combination strategy with silibinin appears to be effective. Silibinin has wide spread biological application, including chemopreventative, anticancer activity, an d antioxidant properties. It is evident from the published studies in the last couple of decades that silibinin exhibits its anticancer activity through targeting proliferation, apoptosis, cancer cell metabolism, and angiogenesis. We previously described the role of silibinin in breast cancer metastasis by targeting Rac1 expression and its anticancer effect on the luminal A subtype of breast cancer by reducing Leptin, OB-Ra, and OB-Rb gene expression [21, 22]. It is also reported that most of the silibinin's biological effects are performed by molecular alterations and a targeting a range of cellular signaling cascades, including NF-kappa B transcription factor, epidermal growth factor receptor, phosphoinositide 3-kinase, β-catenin, and cyclooxygenase [23].

Data from this study revealed the synergy of silibinin and vinblastine in an in vitro model. The combination significantly improved apoptosis induction and reduced antiapoptotic protein Bcl-2 expression, while increasing Bax and caspase-3 expression compared to the single use of silibinin or vinblastine. The toxicity of silibinin and vinblastine was assessed by MTT assay, which exhibited a significant decrease ($p < 0.01$) in cell viability compared to individual treatment.

The results presented here correspond with the study of Tyagi et al., in which they studied the synergistic anticancer effects of silibinin with conventional cytotoxic agents, including doxorubicin, cisplatin, and carboplatin, against human breast carcinoma MCF-7 and MDA-MB-468 cells. Combinations of silibinin with these three agents showed strong synergistic effects on cell growth inhibition in both cell lines [24].

Apoptosis plays a crucial role in protecting organisms against tumor formation. Many anti-cancer drugs act by inducing apoptosis, eliminating cells that have genetic damage or are divide inappropriately. In mammalian cells, apoptosis has been is divided into two major pathways: the extrinsic pathway, which is activated by pro-apoptotic receptor signals on the surface, and the intrinsic pathway, which regulates apoptotic cascades through the convergence of signaling in the mitochondrion, which leads to the modification of the mitochondrial membrane potential (MMP), the release of C into the cytosol and the activation of caspase cascades[25].

One way to trigger the apoptosis machinery is to downregulate the expression of the apoptosis inhibitor Bcl-2 and upregulate the expression of the apoptosis-promoting factor Bax[26]. Our results indicated that, after treating MDA-MB-231 cells with a combination of two agents, the expression of Bcl-2 was significantly downregulated and Bax protein upregulated in both protein and mRNA levels. This expression alteration led to a decrease in the Bcl-2/Bax ratio, increasing the mitochondrial membrane permeability and releasing cytochrome C, thus inducing caspase-3 (a pro-apoptotic gene) and initiating the apoptosis program. Results demonstrated that the cleaved caspase-3 protein was significantly increased after combination treatment. In addition to the flow cytometry results, these findings lead us to conclude

that the induction of apoptosis occurs through the activation of the intrinsic pathway. Flow cytometry results showed that the combination had a more significant effect on inducing apoptosis, likely due to the sharing of pathways leading to apoptosis of the two common drugs. Previous studies declared that vinblastine induced apoptosis via various signaling molecules, including NF-kB, c-myc, JNK, and Erk[27]. Therefore, silibinin could affect the vinblastine-mediated caspase-3-dependent apoptotic pathway [28].

Another important molecule in apoptosis machinery is p53. P53 is a tumor suppressor gene involved in a variety of cellular stress and response processes by interacting with regulatory factors and genes. p53 has several functions, such as mediating cell cycle arrest, promoting cell apoptosis, maintaining promoting cell apoptosis, maintaining genome stability and inhibiting tumour angiogenesis [29]. There is evidence that silibinin can impose its effect through p53 alteration[30]. For this reason, we suggest that the p53 signaling pathway be investigated in future studies and its role in the combination between silbinin and vinblastine be investigated.

To investigate the role of Bcl-2 and Bax, RT-PCR was employed. These proteins play an apparent key role in mitochondria in silibinin and vinblastine-mediated apoptosis. For the regulation of the mitochondrial pathway, the pro and anti-apoptotic activities of the Bcl-2 family are necessary[31]. MDA-MB-231 cells treated with a combination of silibinin and vinblastine down-regulated Bcl-2 and up-regulated Bax. Furthermore, the combination significantly up-regulated caspase-3, indicating that apoptosis enhancement occurred via the Bcl-2/Bax and caspase-3 pathway.

CONCLUSION

There is an urgent need to develop new and effective combination treatments to improve the outcomes of patients with TNBC. Here, by combining silibinin with vinblastine, our findings revealed outstanding synergistic cytotoxicity on the MDA-MB-231 cells. These data led us that a better treatment outcome could be gained for TNBC therapy, and treatment could be administered at a lower dose to reduce the side effects of TNBC patients.

Altogether, this study corroborated silibiin enhanced the apoptotic effect of vinblastine via alteration of the Bcl-2/Bax ratio, which may offer novel insights into uncovering new therapeutic methods for breast cancer.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Ethical approval

This research was approved by The Ethics Committee of Lorestan University of Medical Sciences (IR.LUMS.REC.1398.095).

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