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Research Paper

Sambucus Nigra Synergizes Cisplatin to Improve Apoptosis and Metabolic Disorders, and Reduce Drug Resistance in Two Human Breast Cancer Cell Lines

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ABSTRACT

Background: Despite modern developments in its management, still major concerns remain about drug resistance in chemotherapy. Natural adjuvants combined with chemotherapy might be the answer. We examined the anti-cancer, anti-proliferative and synergistic effects of Sambucus nigra extract with cisplatin chemotherapy (CDDP) on MCF-7 and MDA-MB-231 human cancer cell lines.

Methods: MCF-7 and MDA-MB-231 cell lines were cultured in DMEM culture media, containing 10% FBS and 100 U/ml penicillin/streptomycin. The anti-proliferative activity of SNA, CDDP and their synergic doses were determined using MTT method. Next, the apoptotic, metabolic, and cellular resistance gene expressions were measured through real-time quantitative PCR technique. To show the apoptosis effects and to diagnose cellular damages, an annexin V/propidium iodide (AV/PI) kit and malondialdehyde level were performed, respectively.

Results: The synergic doses of SNA and CDDP in MCF-7 were 1.25µM CDDP+1.25µM SNA and on MDA-MB-231 was 2.5µM CDDP+2.5µM SNA. The results of real-time PCR showed that SNA induced apoptosis, disrupted metabolic pathways and reduced cellular drug resistance. In addition, the combination of SNA with CDDP compared with CDDP alone was able to change the expression of these genes and increase the rate of MDA and apoptosis generation ($P<0.05$).

Conclusion: The outcomes of this investigation indicate that SNA, as a herbal supplement, may be a candidate for increasing the effect of CDDP therapy in the treatment of breast cancers. This synergy was not estrogen-dependent in the MDA-MB-231 cells, promoted apoptosis, cell damages, disorders of metabolism, and reduced the drug resistance in the cancer cells.

Keywords: Apoptosis, Breast cancer, Cisplatin, Drug resistance, Sambucus nigra

Introduction

A major concern of health organizations is the spread of breast cancer in women under 40 years old, which accounts for approximately 22.9% of the world's population [1, 2]. To control and reduce this type of cancer, various strategies such as radiotherapy, surgery and chemotherapy have been suggested and implemented [3]. But so far, these methods have not provided a cure for the disease. One of the strategies that have recently been considered by researchers is the use of adjuvant agents combined with conventional chemotherapy drugs.

Cisplatin (CDDP) is an example of the most common chemotherapy drug used in the treatment of various cancer tumors [4]. A major problem of using chemotherapy for various human cancers is the drug resistance that follows

the treatment. Thus, the efficacy of the drug is gradually decreased due to the developed resistance while treating various cancer tumors [5]. Thus, the sensitivity and response of tumor cells to CDDP is greatly affected by the suppression or recurrence of cancer. Many studies have shown that the combination of CDDP with other chemotherapeutic agents has led to unsuccessful treatment of cancer cells and even causing increased toxicity in patients [6].

Sambucus nigra (SNA) is used in traditional medicine, and different species of this plant have been utilized for the treatment of various diseases [7, 8]. Many pharmacological studies have been conducted on this plant, demonstrating its antioxidant, anthocyanidin, antibiotic, and anti-inflammatory effects [8-13]. In addition, many investigations have confirmed that SNA has strong anticancer activity on various cancer cells [14, 15]. However, not many studies have been undertaken on the toxicity and therapeutic activities of SNA on human cancer cells combined with CDDP.

One of the strategies that seems to enhance the efficacy of CDDP is the use of natural compounds. Therefore, finding a new and suitable combination of potential compounds with CDDP is important to minimize the adverse effects of this drug on patients. According to previous studies [7-15], SNA may be a candidate agent to improve the cisplatin efficacy. For this purpose, the effective and synergistic doses of SNA and CDDP were determined and the necessary cellular and molecular techniques were performed to demonstrate the effect of the two drugs individually and concurrently.

Materials and Methods

Cell Culture: Human cell lines, MDA-MB-231 and MCF-7, were obtained from the Pasteur Institute in Tehran, Iran. Culture medium Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany), required for these cells contained 10% fetal bovine serum (Sigma-Aldrich), 100µg/ml streptomycin 100IU/ml penicillin, amphotericin B (Sigma, USA) and 2mM glutamine. The cells were incubated at 37°C, under 95% humidity and 5% CO2.

Drug sensitivity assays

After culturing the cell samples for 24hr, varying concentrations of CDDP and SNA (0-100µM) (Sigma-Aldrich, Germany) were added to the serum-free culture medium for 1 hour and then replaced with the usual culture medium containing FBS. The cell culture medium was drained 72hr later, and 20µL MTT (5mg/ml) was added and the incubation continued for another 4hr. The medium was removed, and 200µL DMSO was added to the culture media. Finally, the optical densities of the samples were read on an ELISA reader (Hyperion MPR4, Germany) at 570nm. The obtained values were used to plot the inhibitory concentration to produce 50% cell death (IC50) based on the dose-responses.

Determination of malondialdehyde level

To measure the malondialdehyde (MDA) levels after 24hr of incubation of the cell lines, the following cell groups were treated with varying concentrations of CDDP, SNA, and CDDP+SNA, based on the IC50 data for 1hr:

0.001% DMSO+MCF-7 cell line

10 µM SNA+MCF-7

2.5 µM CDDP+MCF-7

1.25 µM SNA+1.25 µM CDDP+MCF-7

0.001% DMSO+MDA-MB-231

10 µM SNA+MDA-MB-231

5 µM CDDP+MDA-MB-231

2.5 µM SNA+2.5 µM CDDP+MDA-MB-231

After 72hr of incubation, the MDA concentrations for both cell lines were determined based on the instructions from the supplier's kit (Kiazist, Iran). The MDA levels were measured for each of the cell samples at 532nm, using a UV-vis spectrophotometer (Jenway-6505, UK).

Real-time quantitative polymerase chain reaction

Initially, the RNA samples were extracted from the cells by TRIzol reagent (Invitrogen, USA) and the RNA concentrations were estimated on a spectrophotometer at 260-280 nm. The cDNA synthesis was also performed by the revert aid first strand cDNA synthesis kit (Takara Bio, Japan) based on the supplier's protocol. The relative expressions of the genes were measured, using GAPDH primer as the internal control and Maxima SYBR Green Reaction Kit (Takara Bio, Japan) method (2-ΔΔCt). All of the primer sequences that were used in the real-time PCR are shown in Table 1.

For each gene, three replications were used. Finally, the microtubes were transferred to the StepOne/Plus real time PCR device, using the following protocol. First, the gene denaturation was performed at 95°C for 10 min-

Table 1. Primer sequences used for the realtime PCR experiment

Gene		Primer Sequence
BAX	Forward	5′-GCGAGTGTCTCAAGCGCATC-3′
	Reverse	5′-CCAGTTGAAGTTGCCGTCAGAA -3′
BCL-2	Forward	5′-ATGTGTGTGGAGAGCGTCAACC-3′
	Reverse	5′-GCATCCCAGCCTCCGTTATC-3′
P53	Forward	5′-CCTCAGCATCTTATCCGAGTGG-3′
	Reverse	5′-TGGATGGTGGTACAGTCAGAGC-3′
GAPDH	Forward	5′-GGAGCGACATCCGTCCAAAAT-3′
	Reverse	5′-GGCTG TGTCAATCTTCTCATGG-3′
Caspase-3	Forward	5′-CAGAACTGGACTGTGGCATTG-3′
	Reverse	5′–GCTTGTCGGCATACTGTTTCA-3′
Caspase-8	Forward	5′-CATCCAGTCACTTTGCCAGA-3′
	Reverse	5′-GCATCTGTTTCCCCATGTTT-3′
ABCB4	Forward	5′-GCAGACGGTGGCCCTGGTTGG-3′
	Reverse	5′-GGATTTAGCGACAAGGAAA-3′
MCT1	Forward	5′-GTGGCTCAGCTCCGTATTGT-3′
	Reverse	5′-GAGCCGACCTAAAAGTGGTG-3′
MCT4	Forward	5′-CCATGCTCTACGGGACAGG-3′
	Reverse	5′-GCTTGCTGAAGTAGCGGTT-3′

utes. Next, the denaturation was achieved at 95°C for 15sec, followed by annealing at 60°C and extension at 72°C for one minute each. These processes were repeated for 40 cycles [16]. Finally, the melting curves were prepared and analyzed.

Apoptosis assay by flow cytometry

The apoptosis in the cell lines, MDA-MB-231 and MCF-7, was measured with flow cytometer, using Annexin V/PI Apoptosis Assay Kit (Roche Company, Switzerland).

Statistical analysis

We used SPSS software, v. 23 to statistically analyze the data. One way analysis of variance (ANOVA) and Tukey's post hoc tests were utilized for the quantitative data analyses. The data were expressed as the means (±SEM), and P-values less than 0.05 were considered as being statistically significant.

Results

Cell viability

The survival rates of the cancer cell lines after exposure to CDDP and SNA alone and the combinations were determined by MTT assay. First, we determined the IC50 levels of CDDP and SNA, and the combinations for the cell lines (Figure 1). At concentrations above 20µM SNA, the survival rate of both MDA-MB-231 and MCF-7 cell lines showed a significant decrease (Figure 1A). Accordingly, the IC50 value for SNA in both cancer cell lines, was found to be 10µM. Figure 1B shows the IC50 level for CDDP in both cancer cell lines. The IC50 for CDDP in MDA-MB-231 and MCF-7 cancer cell lines, 2.5 and 5µM were obtained, respectively. Then, we evaluated the combined effect of CDDP and SNA on the survival of the cancer cell lines. As shown in Figure 1C, the IC50 for CDDP and SNA

for MDA-MB-231 and MCF-7 cell lines, were approximately 2.5µM and 1.25µM, respectively.

SNA stimulated lipid peroxidation

The data from this study revealed that the level of MDA in various groups treated with SNA and CDDP had a meaningful increase compared to the control group (Figure 2). However, the level of MDA in MDA MB-231 cell lines was higher and linked to the MCF-7 cell lines. Also, the levels of MDA in both cell lines treated with combined SNA cand CDDP were higher than those treated with either CDDP or SNA alone. Also, this group showed significant differences in other parameters in both cell lines compared to other groups (P <0.05).

The impression of SNA on ATP binding cassette subfamily gene B member 4 (ABCB4)

We observed that the expression of this gene in both cell lines was predominantly higher in the treated groups compared to the untreated groups (Figure 3A). In addition, compared with the CDDP-treated group in both cell lines, the expression of ABCB4 decreased in both cell lines when the combined SNA and CDDP was applied. Also, the groups treated with SNA+CDDP in both cell lines significantly different from other groups with respect to the above parameters (P<0.05).

Apoptosis genes

The expression of the p53 gene in both cell lines showed overexpression (Figure 3B). The increased expression of the p53 gene in the MCF-7 cell line was higher than that of the MDA-MB-231 cell line. However, the p53 gene expression in the groups treated with SNA+CDDP showed higher values compared to the groups treated with either CDDP or SNA alone (p<0.05). The Bax gene expression was higher than the Bcl-2 gene in both cell lines (Figures 3C & 3D). However, the Bax gene expression was higher in SNA+CDDP-treated groups in both cell lines than those in other groups. However, the expression of this gene decreased in the CDDP-treated group compared to that in the SNA+CDDP-treated groups (P<0.05). Also, the expression of the Bax gene in both cell lines treated with combined SNA and CDDP was notably different from other groups (P<0.05). On the other hand, the expression of the Bcl-2 gene in the group treated with SNA+CDDP showed a lower expression than in other groups (P<0.05). However, the expression of the Bcl-2 gene in the SNA-treated group showed a higher expression compared to other groups (P<0.05).

Further, we determined the expression ratio of Bax/Bcl-2 genes (Figure 3E). The highest values for Bax/Bcl-2 ratios were observed in SNA+CDDP-treated groups in both cancer cell lines. The increased gene expression in SNA+CDDP-treated groups for both cell lines was associated to their increased apoptosis (P<0.05). The expression of caspases 3 and 8 genes in both cancer cell lines increased significantly for the SNA+CDDP-treated groups (P<0.05) (Figures 3F & 3G). On the other hand, the expression of these genes in the MCF-7 cell line was higher compared to that for MDA-MB-231 cancer cell line. However, the expressions of these genes were lower in the group treated with either SNA or CDDP alone compared to the groups treated with combined SNA and CDDP (P<0.05).

Monocarboxylate transporters 1 and 4 (MCT1 & MCT4) genes

Significant increases in the expression of MCT1 and MCT4 genes were observed for both cancer cell lines (Figures 3H & 3I). However, the expression of MCT1 genes in the MDA-MB-231 cancer cell line was much higher than that of MCF-7. However, the expression of the MCT1 gene in both cancer cell lines when treated with SNA+CDDP was lower than those of all other groups (P<0.05). The expression of the MCT4 gene was the opposite of that for MCT1, i.e., the expression of the MCF-7 gene was higher than that of MDA-MB-231 cancer cell line. The similarity in the gene expressions for MCT1 and MCT4 when treated with SNA+CDDP were lower than in other treated and untreated groups (P<0.05). Therefore, the combined SNA and CDDP had affected their metabolic pathways more than those observed in other groups.

Apoptosis assessment

The data from the flow cytometry indicated that cells, either treated or untreated with media containing different compounds, were grouped into four quarters (Q1-Q4). The percentages of the intact, early or late apoptotic/necrotic, and completely necrotic cells are illustrated as histograms for MCF-7 (Figure 4A) and MDA MB-231 cells (Figure 4B), respectively. The results showed that SNA, similarly to CDDP, had apoptotic effects on the cell lines. The two dyes, PI and Annexin V used for the flow cytometry, showed that the predominant cause of cell death in both MCF-7 and MDAMB-231 cell lines that were treated with SNA, were related to necrosis or early apoptosis (Figures 4A & B). While treatment of both cell lines with CDDP had the highest percentage of late apoptosis for MCF-7 and necrosis for MDAMB-231

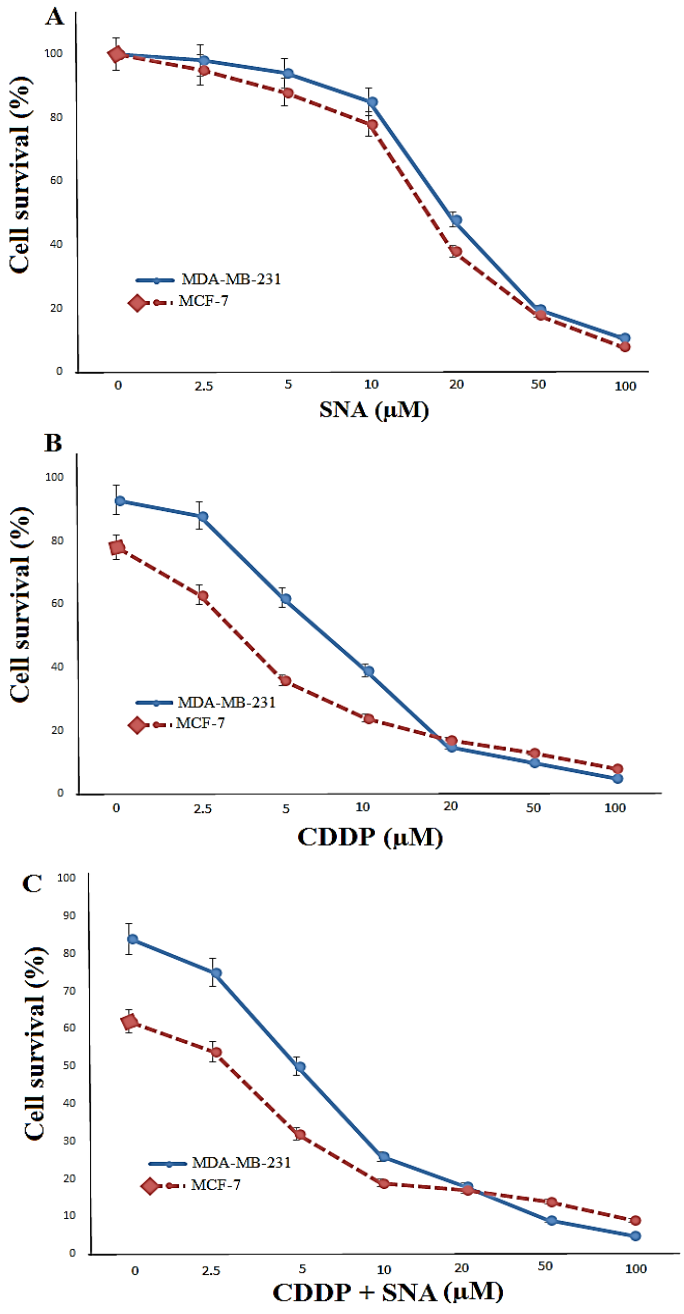


Figure 1. Evaluation of IC50 (µM) and response rate and sensitivity of breast tumor cell lines MCF-7 and MDA-MB-231

Exposed to varying concentrations for Sambucus nigra (SNA) and Cisplatin (CDDP) at 0-100µM, and 0-50µM for each of the CDDP and SNA drugs, respectively, in their combination. The IC50 was performed and the survival rate of these cells was determined by MTT assay within 72 hours. The results are as means ± standard deviations and at least three replications were performed for each group. IC50 rate and MTT tests were done as described in the Materials and Methods section.

cell lines, respectively. In both cell lines treated with SNA+CDDP, the most common types of cell death were early apoptosis and cell necrosis (Figures 4A, B).

Discussion

Based on the findings, this study demonstrated that SNA suppressed the growth of human cancer cell lines and induced cellular apoptosis, with the effect being similar to that of chemotherapy drug, cisplatin. Also, the therapeutic effects of SNA were demonstrated on both estrogen positive and negative breast cancer cells (MDA-MB-231). The SNA agent exerted its chemotherapeutic properties in a dose-dependent manner. Also, based on the IC50 data for SNA and cisplatin, we found that the combined mixture had a synergistic effect on the cispl-

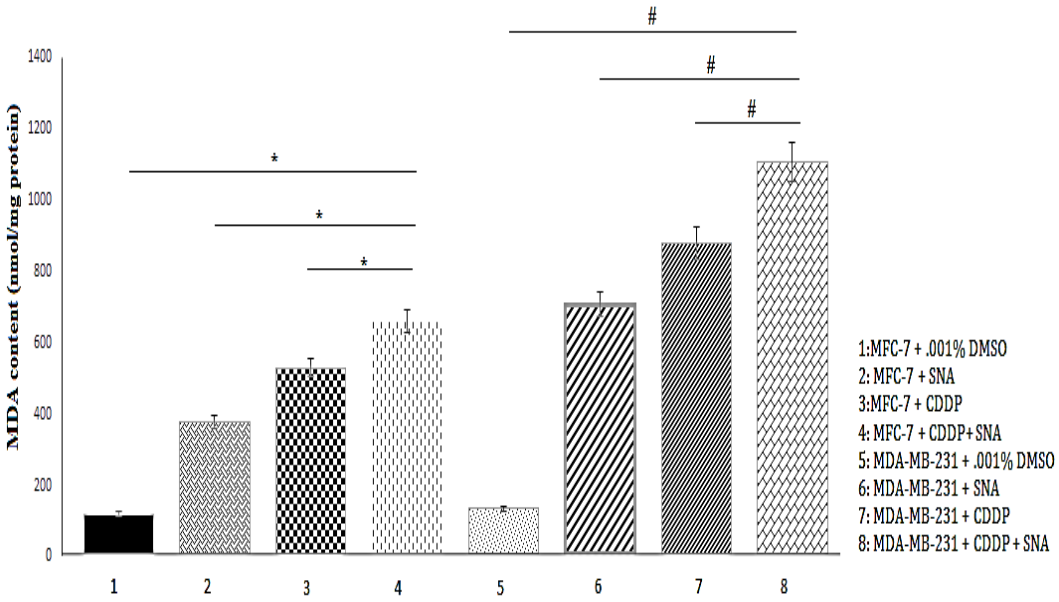


Figure 2. Effect of Sambucus nigra (SNA), Cisplatin (CDDP) and SNA+CDDP on malondialdehyde (MDA) levels in MCF-7 and MDA MB-231 breast tumor cell lines

The values presented are shown as Mean±SD for three replications for each group. The symbols * and # mean the notable variation among the groups (*P<0.05, #P <0.05).

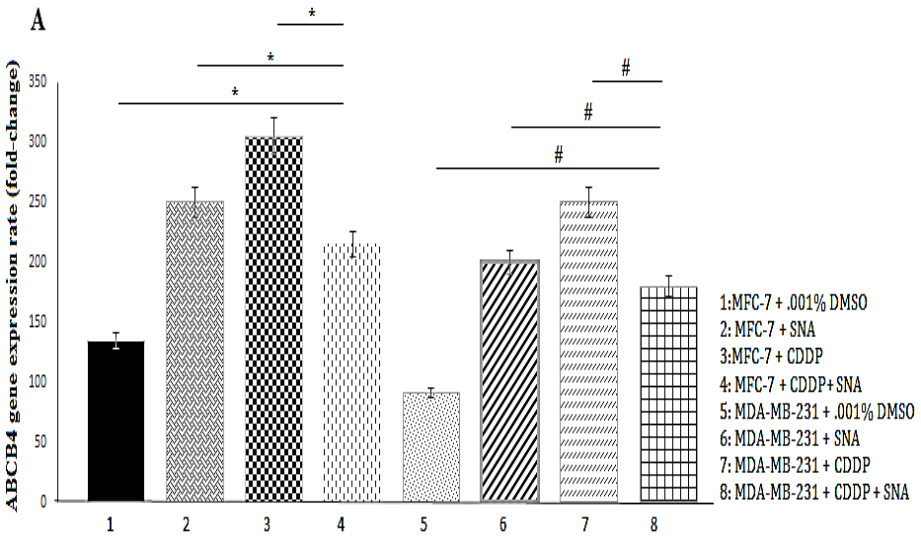
atin performance. In terms of the mechanism of SNA action, the results suggest that SNA synergizes the cisplatin's effect on human breast cancer cells, by reducing their drug resistance and metabolic processes.

The Effect of SNA and CDDP on Genes Involved in Resistance Pathways: A known reason that a variety of chemotherapy drugs, especially CDDP, cause drug resistance in cancer cells is the expression of ATP binding cassette (ABC) transporter genes [17]. In this context, various plant-derived agents and extracts have been shown to inhibit the expression of these genes [18]. Hence, the tendency to use natural compounds together with chemotherapy drugs that are suggested often for cancer research [19, 20].

There has been ample research on SNA in support of its antineoplastic properties on a variety of cancer cells [14, 15]. The results from the current study also confirmed the effect of SNA combined with CDDP on the two cell lines (MCF-7 & MDA-MB-231). Specifically, they inhibited the ABCB4 gene expression, which is the cause of drug resistance. Other studies have also shown that the increased expression of ABCB4 gene in cells treated with other chemotherapeutic drugs has led to increased drug resistance presumably regulated through the methylation of promoters [21]. In addition, the regulation of ABCB4 gene expression is also influenced by chemotherapy drugs [21].

The Effect of SNA and CDDP on Cell Death: A major mechanism of action of chemotherapeutic drugs is the induction of apoptosis in various cancer cells [22]. Therefore, examining the signaling pathways responsible for apoptosis and understanding the associated events are the necessary steps in the investigation of new and potential anti-cancer compounds [23]. Evidently, SNA exerts its effects on such molecules as p53, Bcl-2, and Bax in cancer cells that are involved in the regulation of apoptosis through interaction with caspases 3 and 8, and other associated genes [24, 25].

Studies have reported that the reduction in p53 expression leads to a rise in the growth of cancer cells [26, 27]. In the current study; however, the expression of the p53 gene was increased in both cell lines. This is likely to be due to increased oxidative stress effect of SNA on the cells. Other studies have also reported that the Bcl-2 family and pro-apoptotic proteins, such as Bax, function by altering the permeability of mitochondrial membrane by caspases 3, 8 and 9, the activation of which induces apoptosis secondary to the fatal damages [28, 29]. Therefore, it may be suggested that the mitochondrial pathway for apoptosis is applied due to the potentially therapeutic effect of SNA on human breast cancer cells. Results from a previous study that evaluated the same effect of SNA are consistent with our findings [14].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2: MFC-7 + SNA
3:MFC-7 + CDDP
4: MFC-7 + CDDP+SNA
5: MDA-MB-231 + .001% DMSO
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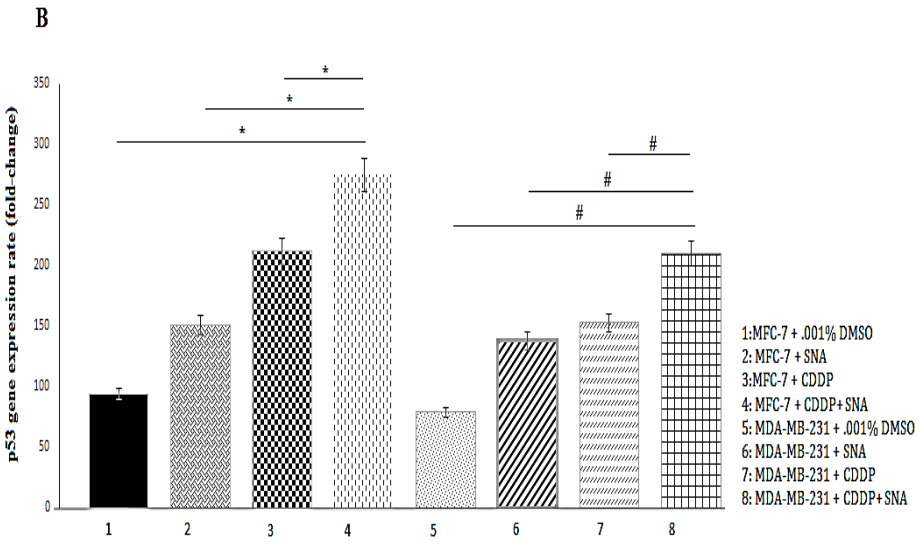
7: MDA-MB-231 + CDDP
8: MDA-MB-231 + CDDP + SNA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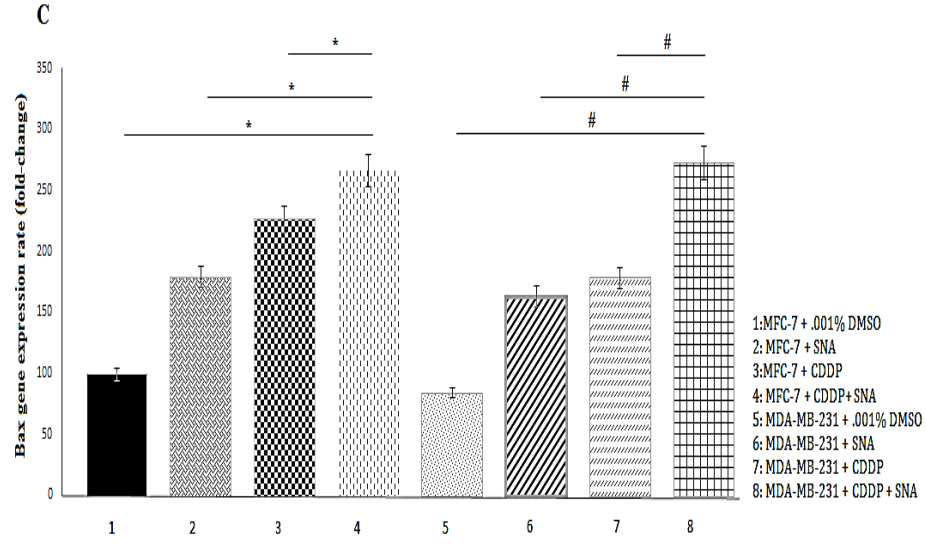
1:MFC-7 + .001% DMSO
2: MFC-7 + SNA
3:MFC-7 + CDDP
4: MFC-7 + CDDP+SNA
5: MDA-MB-231 + .001% DMSO
6: MDA-MB-231 + SNA
7: MDA-MB-231 + CDDP
8: MDA-MB-231 + CDDP+SNA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1:MFC-7 + .001% DMSO
2: MFC-7 + SNA
3:MFC-7 + CDDP
4: MFC-7 + CDDP+SNA
5: MDA-MB-231 + .001% DMSO
6: MDA-MB-231 + SNA
7: MDA-MB-231 + CDDP
8: MDA-MB-231 + CDDP + SNA

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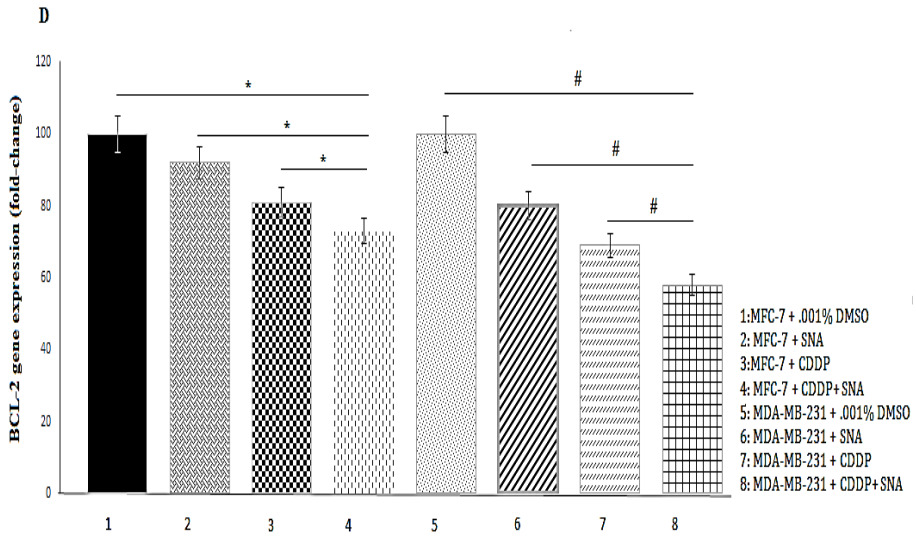

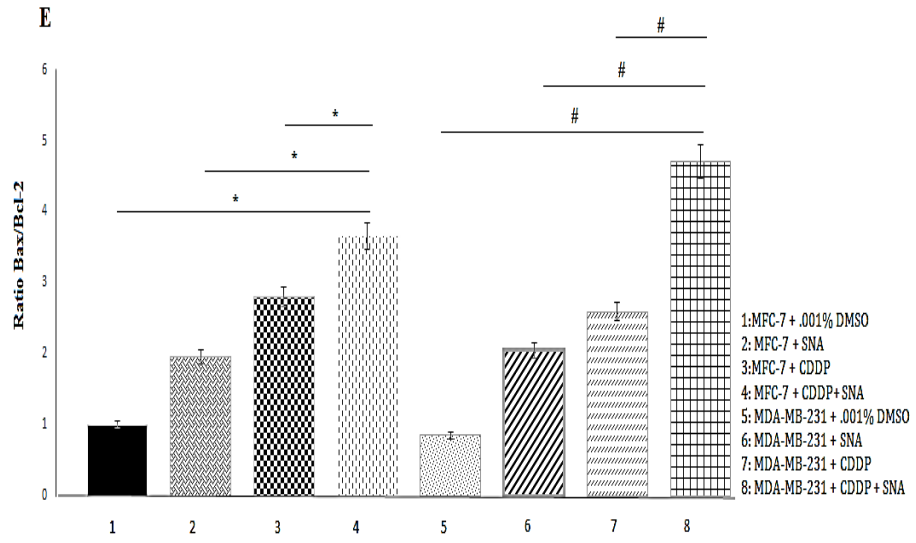

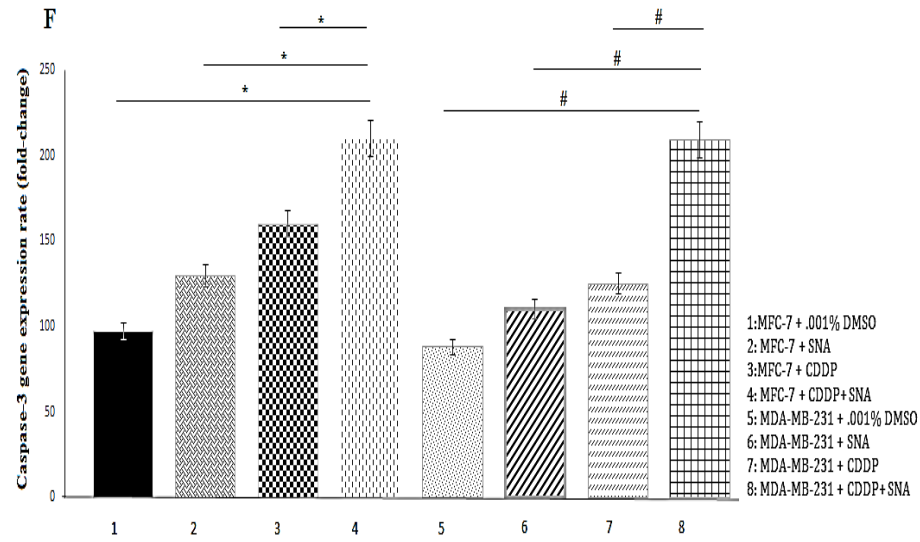

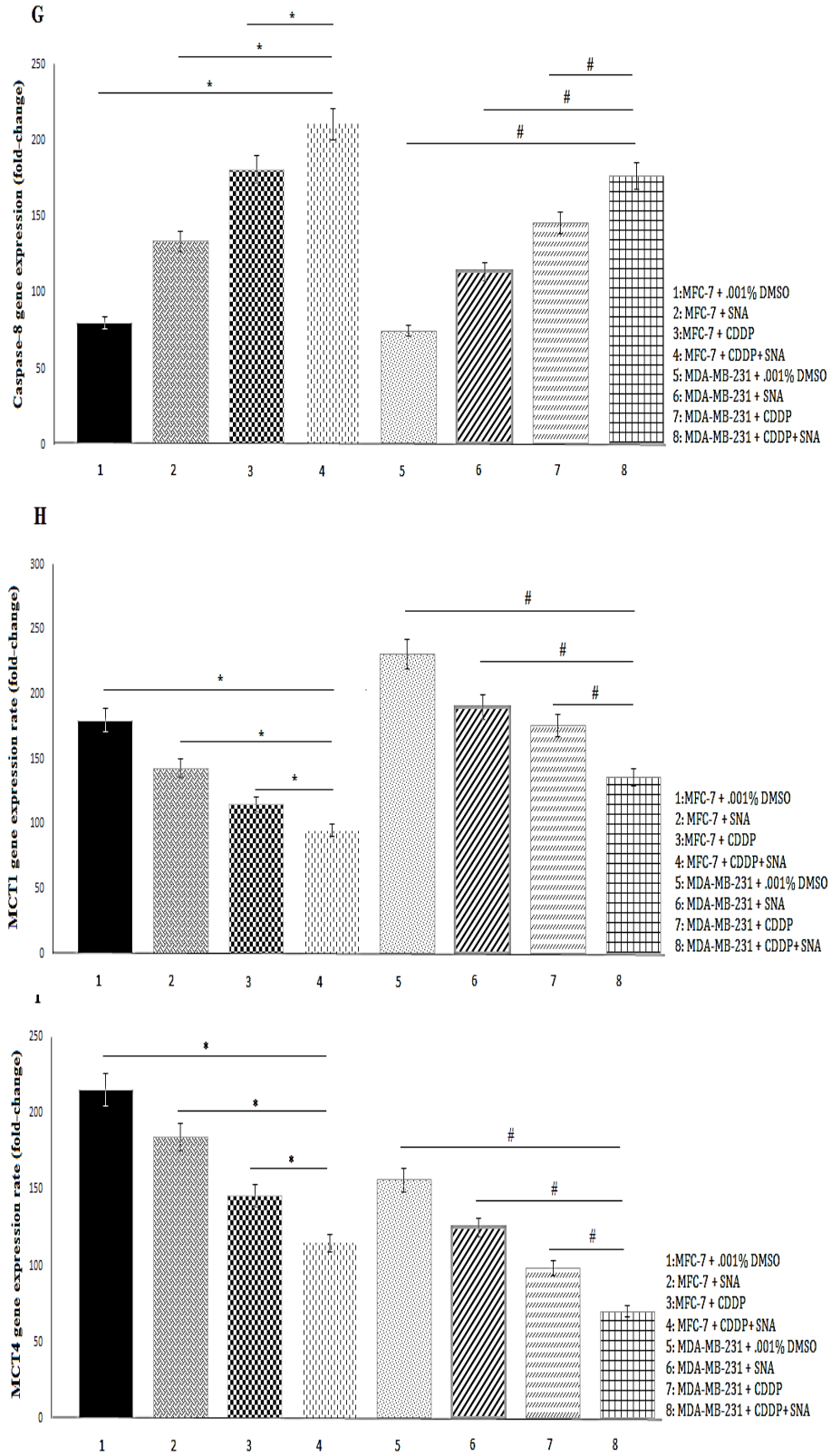

Figure 3. Effect of Sambucus nigra (SNA) on the expression of toxicity-resistant gene

ATP binding cassette subfamily B member 4 (ABCB4) (A), apoptotic genes P53, Bax, Bcl-2, Caspase-3, and 8 (B-F) and genes of the Monocarboxylate transporter 1 (MCT1) and MCT4 metabolic pathways (G-I) in Cisplatin (CDDP) -resistant MCF-7 and MDA-MB-231 cell lines in different groups. The values presented are shown as Mean±SD for three replications for each group. The symbols * and # indicate the significant difference between the groups (*P<0.05, #P<0.05).

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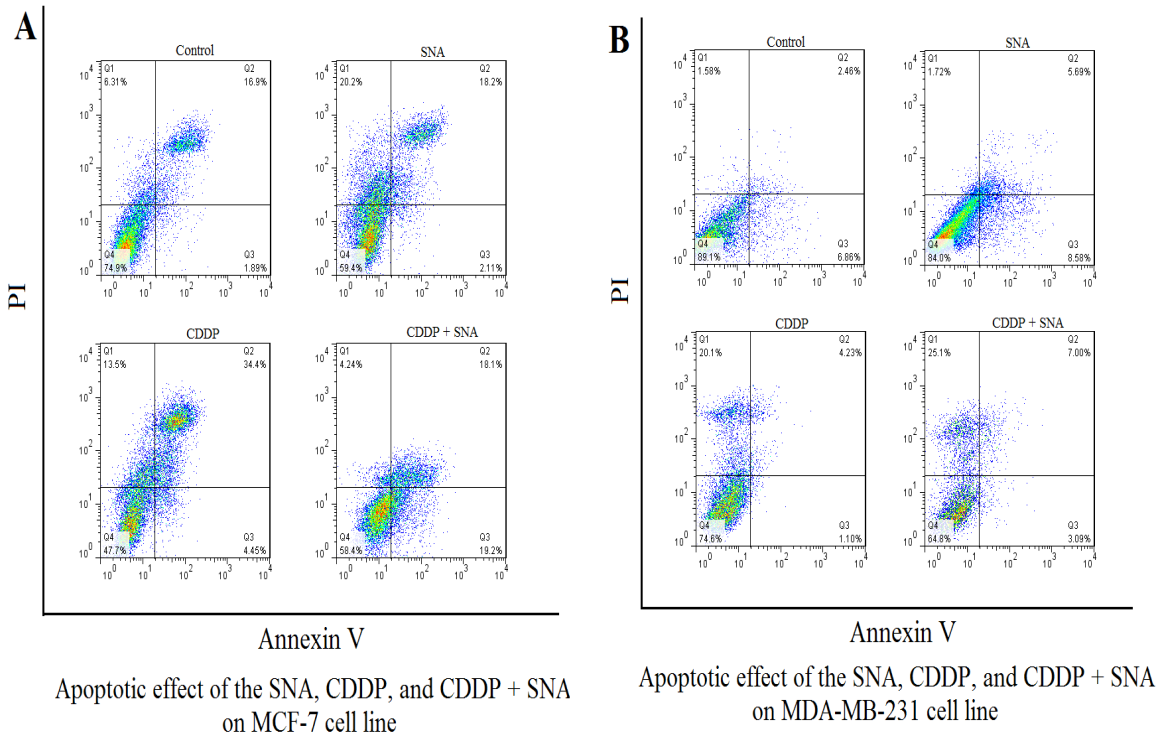

Figure 4. Flow cytometric analysis of the effects of Sambucus nigra (SNA), Cisplatin (CDDP) and SNA+CDDP

Control group on apoptosis in MCF-7 (A) and MDAMB-231 cell lines (B) within 72hr.

The Effect of SNA and CDDP Agents on Genes Involved in Metabolic Pathways: One of the roles of monocarboxylate transporters (MCTs) in cancer cells is to maintain the homeostasis, which preserves the glycolytic and acid-resistant phenotypes [30, 31]. This is an essential function responsible for the growth of malignant cells. The most common isoforms of MCT found in cancer cells include monocarboxylate transporters 1 and 4 (MCT1, MCT4) [32-36]. However, these cell lines showed significant metabolic changes, through the expression of the MCT1 and MCT4 genes, associated with SNA and CDDP treatments. The reason for the reduced gene expression may be explained by results from studies that investigated the flavonoids compounds found in SNA and their ability to inhibit the expression of cancer genes [37, 38]. It has been confirmed that estrogen-negative receptors in human breast cancer cells, such as MDA-MB-231 are more aggressive and lead to a worse prognosis than the estrogen-positive cells, such as MCF-7. Therefore, we may assume that the transfer of SNA, CDDP, and their combination in MDA-MB-231 cell line occurs through channels located in the plasma and cytosolic membranes, while in the MCF-7 cell line, it occurs through channels in the plasma membrane [39].

The Effect of SNA and CDDP Agents on Malondialdehyde (MDA) Production: Oxidative stress induces apoptosis in tumor cells and is considered an effective mechanism for the treatment of cancer cells [40, 41]. Since MDA results from lipid peroxidation, the use of SNA, CDDP, and the combination against tumor cell lines may lead to a significant rise in the oxidative stress around them. Our data also suggest that the highest rate of cell membrane damage is directly related to the MDA level, as measured post treatment.

Finally, the results of the flow cytometry clearly demonstrated that apoptosis occurred in the treated cancer cell lines, as compared to those seen in the controls. Another study on an ovarian cancer cell line has shown that SNA is likely to promote cellular apoptosis through DNA fragmentation [14]. These findings provide further mechanistic support for our results, indicating that SNA induces apoptosis rather than necrosis.

Conclusions

The outcomes of this study propose that SNA is able to cause apoptosis in the two human cancer cell lines by disrupting the various metabolic pathways. In addition, SNA may increase the permeability of cancer cells to chemotherapy drugs, such as CDDP and promote better therapeutic outcomes in the cells via opening membrane channels. Further, SNA is likely to function as an

anti-proliferative agent, especially in estrogen-negative cells, inhibiting the drug resistance effectively in various human cancer cells. Finally, it may be hypothesized that this herbal compound may be used as a therapeutic adjunct to further enhance the effects of chemotherapy drugs against resistant breast cancer cells.

Ethical Considerations

Compliance with ethical guidelines

The protocol for conducting this in-vitro research was reviewed and approved by the Office of Vice Chancellor for Research, Kermanshah University of Medical Sciences, Kermanshah (IR.KUMS.REC.1399.1075).

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Author's contributions

SR, and AG designed the experiments. ME performed the experiments and collected the data. MRS and AG performed the analyses and interpreted the results. SR supervised, directed and managed the study. All authors reviewed the various drafts of the manuscript and approved of its final version prior to submission to this journal for publication.

Conflict of interest

The authors declared no conflict of interests.

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