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Original Research

Extracts of *Scrophularia frigida* Boiss display potent antitumor effects in human breast cancer cells by inducing apoptosis and inhibition of expression of the human epidermal growth factor receptor 2

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Abstract: Some species of the *Scrophularia* genus have been extensively used as a natural remedy for treatment of various medical conditions. The objective of this study was to evaluate the growth inhibitory activity of *Scrophularia frigida* Boiss extracts as well as to study the effect of the potent extracts on the induction of apoptosis and cell cycle arrest on human breast cancer cells. *S. frigida* Boiss extracts exhibited obvious inhibitory effects on the growth of cancer cells and induced apoptosis. It is suggested that the extracts exert their anti-proliferative effect through multiple implications such as suppressing growth, arresting the cell cycle, increased DNA fragmentation, downregulation of the expression of human epidermal growth factor receptor 2 and myeloid cell Leukemia-1, and upregulation of pro-apoptotic messenger RNAs like caspase-3 and caspase-9. Taken together, the results obtained indicate that *S. frigida* Boiss extracts may contain effective compounds that can be used as a therapeutic anticancer agent.

Key words: Antitumor, *Scrophularia frigida* Boiss, apoptosis, HER2 expression, SKBR3 cells.

Introduction

Breast cancer is the most common cancer, representing nearly one-third of the cancers diagnosed in females and the second leading cause of cancer-related deaths (15 %) (1, 2). In a subset of breast cancers, human epidermal growth factor receptor 2 (HER2) is overexpressed, which controls an oncogenic signaling network that inhibits tumor cell death through a specific biochemical regulation of apoptotic pathways (3). HER2 is a 185- kDa receptor tyrosine kinase that belongs to the epidermal growth factor receptor family. Its overexpression is found in approximately 25-30% of breast cancers and is associated with a high risk for cancer metastasis, reduced effectiveness of antitumor therapies and poor prognosis (4). For that reason, inhibition of HER2 expression or activity may be an effective approach for the treatment of HER2-overexpressing cancers. Although treatment of HER2-overexpressing cancers with novel anticancer drugs such as lapatinib, a synthetic small molecular reversible inhibitor of HER2 tyrosine kinase, and trastuzumab, a humanized monoclonal antibody, have had promising results (5), severe side effects and resistance during long-term use have limited their efficiency. Thus, new therapeutic agents are still needed (6).

Currently, the field of herbal medicines as cancer therapeutic agents is strongly debated. Such drugs have gained popularity as they are inexpensive and have limited side effects (7). Some natural products derived from medicinal plants have been identified to inhibit cancer cell proliferation and appear to arrest cancer cell cycle and induce apoptosis (8, 9). *Scrophularia* is a genus of the large angiosperm family *Scrophulariaceae*, which consists of about 250 genera and 5000 species (9). The genus *Scrophularia* is known for the rich presence of phytochemicals such as iridoid diglycoside (10), sugar esters (11), alkaloids (13), flavonoids, cinnamic acid, phenylpropanoid glycosides (14) and phenylethanoid glycosides (15). Numerous reports have been published claiming bioactive compounds in the *Scrophularia* genus that promote physical and mental health, ranging from anti-inflammatory (16), antiprotozoal (15), antioxidant (17), antibacterial (18), hepatoprotective (19), and neuroprotective (20, 21) to anticancer activity (22-25). In the present study we aimed to investigate cytotoxic effects of extracts of *S. frigida* Boiss (SFB) against HER2/neu-overexpressing SKBR3 cells as well as on the cell cycle and mRNA expression of several apoptotic-related genes that might provide experimental evidence for clinical applicability.

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Materials and Methods

Chemicals

RPMI-1640 medium, fetal calf serum (FCS), penicillin-streptomycin, 3-(4,5-dimethylethiazol-2-yl)-2,5 diphenyltetrazolium (MTT), and propidium iodide (PI) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol, trypan-blue, and dimethyl sulphoxide (DMSO) were purchased from Merck (Darmstadt, Germany). The terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay kit was obtained from Roche Diagnostics, (Penzberg, Germany). SYBR Green PCR Master Mix was purchased from Takara (Takara, Bio, Otsu, Shiga, Japan).

Preparation of SFB extracts

SFB plant was collected from around Taleghan, located in Alborz province, Iran, in April 2010 and was identified by Y. Ajani (Department of Botany, Institute of Medicinal Plants (IMP) of Karaj, Iran). Voucher specimens (NO. 1462) were deposited in the herbarium. Following the collection of the plants, the aerial parts were washed, dried and ground into a fine powder. The powdery material was extracted with different solvents using a Soxhlet apparatus (Cambridge, UK). First, a 100 g sample of dried powder was placed into the thimble. Then, the flask was filled with 500 mL of each solvent. After redistilling the solvent, *S. frigida* Boiss extracts in ethyl acetate (SFBEAE), ethanol extract (SFBEE), and petroleum ether extract (SFBPEE) were obtained. The resulting extracts were concentrated under reduced pressure by means of a rotary evaporator (Heildolph, Schwabach, Germany) at 45ºC, and the dried extracts were stored at –20ºC for future assays. Sterilization of test solutions was performed using 0.22 µm syringe filters (Nalge Nunc; Rochester, NY, USA). Of each extract, 10 mg was dissolved in 100 µL DMSO. For all treatments, the final DMSO concentration was kept to less than 1% in RPMI-1640 medium supplemented with 10% FCS.

Cell line and cell culture

SKBR3 cells (human breast adenocarcinoma cell line) were originally obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and grown in RPMI-1640 medium supplemented with 10% FCS, 100 µg/mL streptomycin and 100 U/mL penicillin. Cells were incubated at standard cell culture conditions in a humidified incubator containing 5% CO_2 at 37ºC. The experiments were performed in triplicate.

Cytotoxicity assay

The inhibitory effect of SFB extracts on the proliferation of SKBR3 cells as well as normal fibroblastic cells were determined in the absence and presence of diverse concentrations of the extracts (0 - 300 µg/ml) by MTT assay as previously described (23). The effects of different SFB extracts on growth were assessed as percent cell growth as compared to DMSO-treated cells. The IC_{50} values of SFB extracts on SKBR3 cell line were determined using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) and used in subsequent experiments. Briefly, values were log transformed and normalized, and nonlinear regression analysis was used to generate a sigmoidal dose-response curve to calculate IC_{50} values. Alternatively, cell viability was accessed by the trypan-blue exclusion test. SKBR3 cells were seeded in 24-well plates at 5×10^4 cells/well (1 mL). After 24 h of incubation, the cells were treated with extracts at IC_{50} concentrations and then incubated for 5 days. Some wells were treated with the same final concentration of DMSO as negative controls. At different time points, the cells were harvested and then stained with 0.4% trypan-blue solution for 2 min. Following the number of viable cells was counted by a haemocytometer under an inverted light microscope (OPTICA, California, USA).

Cell morphological assessment by inverted microscopy

Cells grown on 6-well plates and treated with extracts at IC_{50} concentrations for 24 and 48 h were photographed under an inverted light microscope; 1% DMSO served as negative control.

DNA fragmentation assay

DNA fragmentation of tumor cells was analyzed by TUNEL assay. Briefly, SKBR3 cells (6×10^3 cells/well) were plated onto 96 well-plates and exposed to 112 µg/ml of SFBEAE and 173 µg/ml of SFBEE for 48 h. After treatment, cells were fixed in freshly prepared 4% paraformaldehyde solution in PBS (pH 7.4) for 1 h at room temperature. After one hour incubation, cells were washed with 200 µL of PBS and the fixed cells were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min at 25°C and rinsed with PBS. Cells were then incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, and then rinsed with PBS. An aliquot of 50 µL of the reaction mixture containing terminal deoxy transferase enzyme and nucleotide was added to the cells and they were all incubated for 1 h at 37°C. After washing three times with PBS, the slides were incubated with 50 µL converter-POD streptavidin HRP solution for 30 min, and rinsed three times with PBS. Finally, the cells were incubated with DAB and the stained cells were analyzed and photographed using a light microscope.

Alternatively, DNA fragmentation was assessed by electrophoresis; 1×10^6 cells/well of SKBR3 cells were seeded and treated by SFB extracts in IC_{50} concentrations for 48 h. The genomic DNA was extracted from the cells, processed and analyzed as previously described (26).

Cell cycle analysis

For the cell cycle analysis, SKBR3 cells (6×10^5 cells/well) were treated with SFB extracts (with IC_{50} values obtained from MTT assay) or DMSO for 48 h. Following treatment, the cells were trypsinized and fixed in ice-cold 70% ethanol 1 h at 4 °C, washed with PBS and incubated in 0.1 mL 0.5% Triton X-100/PBS at 37°C for 30 min and incubated with RNase at 37°C and PI was added for 10-30 min in the dark. The cell cycle was analyzed by flow cytometry (BD FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ, USA). The data was analyzed using the Flowing software.

Quantitative RT-PCR

After treatment of SKBR3 cells with 48 h IC_{50}

Table 1. Primer sequences used in real-time PCR analysis.

Gene	Sequence	Product size (bp)
Caspase-3	F-TGTCATCTCGCTCTGGTACG R-AAATGACCCCTTCATCACCA	142
Caspase-9	F-GCA GGC TCT GGA TCT CGG C R-GCT GCT TGC CTG TTA GTT CGC	152
HER2	F-AGA CCC GCT GAA CAA TAC CAC R-CCT TCC ACA AAA TCG TGT CC	152
Mcl-1	F-TAA GGA CAA AAC GGG ACT GG R-ACC AGC TCC TAC TCC AGC AA	137
B-actin	F-TCCCTGGAGAAGAGCTACG R-GTAGTTTCGTGGATGCCACA	131

Table 2. IC_{50} value (µg/ml) of SFB extracts for SKBR3 and normal cells lines.

Sample	24 h treatment		48 h treatment	
	SKBR3 cells	Normal cells	SKBR3 cells	Normal cells
SFBEAE	90	364	112	436
SFBEE	324	324	173	-

concentration of extracts, total cellular RNA was isolated by RNX^{Tm}- plus solution (CinnaGen, Tehran, Iran) as described by the manufacturer's protocol. The RNA concentration was verified using Nanodrop spectrophotometer (ND-1000, Thermo Fisher, Wilmington, DE, USA). The purified RNA was used as template for cDNA synthesis. The cDNA construction was carried out by Revert Aid TM Kit (Fermentas, Helsinki, Finland) following the manufacturer's protocol. Primer sequences are shown in table 1. Quantitative PCR (qPCR) was performed in the LightCycler® 96 Real-Time PCR System according to the protocols. Amplification was carried out in a total volume of 12 µL for 40 cycles of 10 s at 95 °C, 30 s at 58.5 °C and 20s at 72 °C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target B-actin. These were then compared with the normalized expression in control untreated sample to calculate a change value (%): ΔΔCt = ΔCt (Treated) - ΔCt (Control). The value was used to plot the expression of apoptotic genes using the expression $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

All data in this study were presented as mean ± standard deviation (SD). Data was analyzed by analysis of variance (ANOVA) and Bonferroni's test using the GraphPad Prism 6 software program. A value of $P < 0.05$ was statistically significant (shown as *).

Results

Cytotoxic effect of SFB extracts on the growth of SKBR3 cells

The cytotoxic effects of SFB extracts on the growth of SKBR3 cells which were determined by MTT and trypan blue assays are shown in Figures 1A and B. As shown in Figure 1A, SFBEE inhibited the proliferation of SKBR3 cells in a dose- and time-dependent manner ($P < 0.05$), while SFBPEE did not exert anti-proliferative effects on the viability of SKBR3 cells (data not shown). The inhibitory effect of SFBEAE on cell growth of SKBR3 cells was demonstrated in a dose-dependent manner; however, this extract did not show further anti-proliferative effects at a later time point (Figure 1A). Among the three extracts analyzed, the greatest anti-proliferative response was obtained with SFBEAE as evidenced by the lowest IC_{50} values 90.13 ± 0.73 µg/ml (Table 2). Moreover, MTT assay was also carried out under the same experimental conditions in normal fibroblastic cells. Interestingly, the growth of normal cells was not influenced by SFB extracts after 24 and 48 h exposure (Table 2). For negative control, MTT assay was carried out by treating SKBR3 cells with 1% DMSO; no significant decrease in cell viability was observed.

Furthermore, trypan-blue exclusion counts were conducted on the cells treated with SFB extracts for 5 days. As shown in Figure 1B, a substantial time- and dose-dependent decrease in live cell number ($P < 0.05$) was observed in SKBR3 cells that were treated with the SFBEE compared to the control (Figure 1B). The SFBEAE extract did not show significant differences in cytotoxicity of SKBR3 cells line in next four days

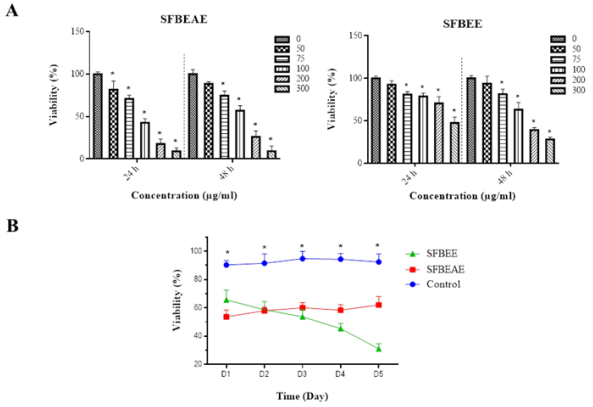

Figure 1. MTT analysis and trypan-blue staining in SFB extracts treated SKBR3 cells. Panel A - MTT proliferation assays in SKBR3 cells treated with DMSO as negative control or the indicated SFB extracts (50-300 µg/ml) for 24 h and 48 h. Panel B- Cell viability was determined by trypan-blue assay over a period of 5 days. The results are expressed as mean ± SD of triplicate, * $P < 0.05$ for all three extracts versus control.

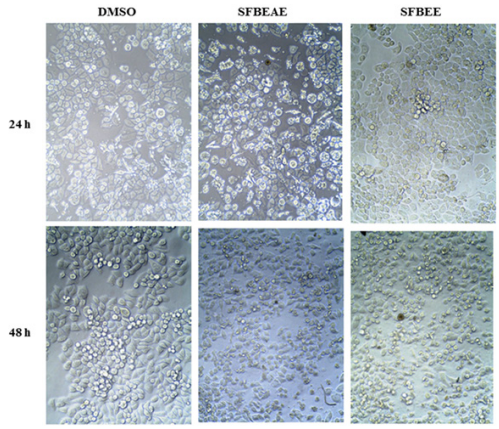

Figure 2. SKBR3 cells were treated with 90 µg/ml SFBEAE, 173 µg/ml SFBEE and 1% DMSO as control for 24 and 48 h, and observed under an inverted microscope (20 × magnification).

treatment.

Morphology of apoptotic SKBR3 cells induced by SFB extracts observed under light microscope

The morphological study suggests that after 24 and 48 h treatment with the extracts of SFB, cell morphology became abnormal with features of apoptosis in SKBR3 cells in contrast to control treatment. As shown in Figure 2, the SFB extracts treatment induced the majority of cells to shrink and float, as well as exhibit many cytoplasmic vacuoles, which is a typical apoptotic appearance.

DNA fragmentation assay

To determine if exposure to SFB extracts causes cell death by apoptosis in SKBR3 cells, DNA fragmentation and TUNEL assays were performed. As shown in Figure 3A, after exposure to extracts, SKBR3 cells began undergoing apoptosis. The TUNEL-positive cells produced brown stained nuclei, while the negative control cells didn't get any stain. The results of extracted DNA on agarose gel electrophoresis showed typical DNA ladder in SKBR3 treated cells, while a sharp DNA band with no laddering-like pattern was seen in normal treated cells (Figure 3B). Therefore, selectivity of the extracts is based on their ability to induce extensive DNA damage in cancer cells but not in normal cells.

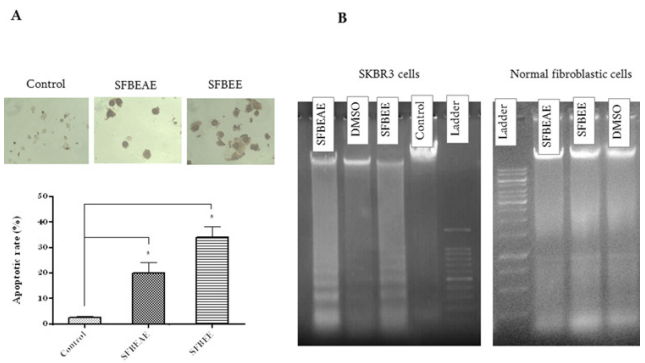

Figure 3. DNA degradation in SFB extracts treated SKBR3 cells. Panel A - the SKBR3 cells were treated with IC_{50} concentration of extracts or 1% DMSO as control for 48 h. The columns represent the percentage of TUNEL positive cells from one experiment performed in triplicate. The data represents the mean ± SD, *p < 0.05 treated vs control cells. Panel B - DNA fragmentation assay was performed in SKBR3 cells and Normal fibroblastic cells treated with extracts at IC_{50} concentrations. The untreated cells and cells treated with 1% DMSO served as the negative control. DNA was extracted and analyzed by 1.8% agarose gel electrophoresis. One of two similar experiments is presented.

Cell cycle

Due to the promising cytotoxicity of SFB extracts on SKBR3 cells, it was therefore chosen to further investigate the effects of these extracts on cell cycle distribution in SKBR3 cells by flow cytometry. As shown in Figure 4, the results indicated that, compared with the control, a significant sub G0/G1 (apoptotic cells) phase arrest was observed following SFB extracts treatment in SKBR3 cells ($P < 0.05$).

Effects of diverse extracts of SFB on the levels of HER2, Caspase 3, Caspase 9 and myeloid cell leukemia-1 in SkBR3 cells

To investigate the molecular mechanisms by which SFB extracts induce apoptosis in SKBR3 cells, the mRNA expression levels of several apoptosis-related genes were examined. In comparison to the untreated control cells, the expression levels of HER2 in SKBR3 cells treated with SFB extracts were decreased (Figure 5A). To determine whether SFB extracts affect apoptosis cascade activation in SKBR3 cells, we next measured the mRNA expressions of caspase-3, caspase-9 genes with real time PCR. As shown in Figure 5B, caspase-3 expression was significantly upregulated by 17.32 fold compared to controls in SKBR3 cells treated with SFBEE for 48 h. Similar to the results for caspase-3 expression, the mRNA expressions of caspase-9 were upregulated in SKBR3 cells treated with SFB extracts (Figure 5C). Moreover, myeloid cell leukemia-1 (Mcl-1) mRNA levels were measured by qPCR. As shown in Figure 5D, treatment of SKBR3 cells with SFB extracts led to the decreasing expression of Mcl-1 in mRNA level.

Discussion

Apoptosis is a cellular procedure that is closely regu-

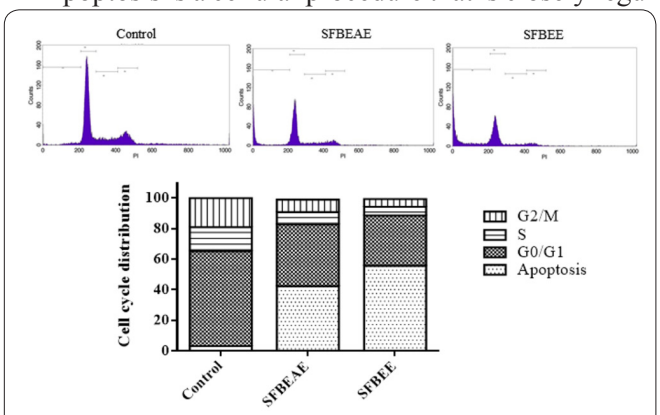

Figure 4. Cell cycle analysis of SKBR3 cells treated with SFB extracts. SKBR3 cells were treated with IC_{50} concentration of extracts for 48 h, and the cell cycle distribution was evaluated after PI staining.

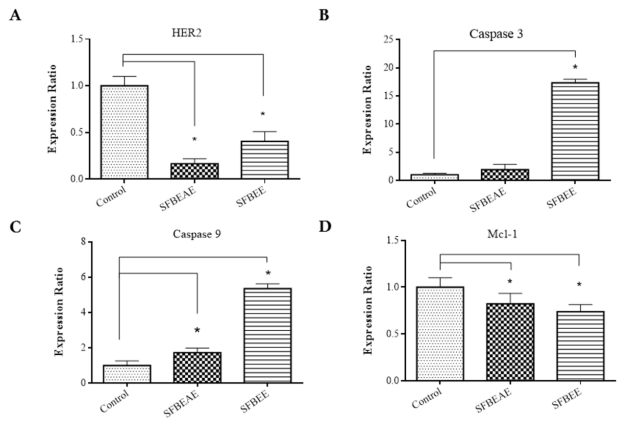

Figure 5. The effect of SFB extracts on mRNA expression of HER2 (A), Caspase-3 (B), Caspase-9 (C) and Mcl-1 (D), which were determined by qRT-PCR. B-actin was used as internal control. The results are expressed as mean ± SD of triplicate, * P < 0.05 vs. control.

lated by diverse groups of regulatory molecules and is characterized by specific morphological and biochemical features in which activation of caspase plays a crucial role. One of the fundamental features of human cancers is evasion of apoptosis, which contributes to both tumor progression and resistance to cancer treatments (27). Several studies have shown that a number of herbal extracts and isolated compounds possess antitumor activity through induction of apoptosis (28).

The data presented here demonstrate that SFB extracts inhibited proliferation and induced apoptotic cell death in HER2-overexpressing SKBR3 cells. As a measure of cytotoxicity, we found that SFBEE dose- and time-dependently increased cell death in breast cancer cells but not normal cells (Figure 1A) (Table 2). The result gained by trypan-blue exclusion assay further confirmed the inhibitory effect of SFB on SKBR3 cell proliferation analyzed using MTT assay (Figure 1B). Furthermore, the morphological characteristics of apoptosis, such as shrinkage of the cell and formation of apoptotic bodies, have been observed under an inverted microscope as presented in Figure 2.

The apoptosis induced by SFB extracts was confirmed by visualizing the fragmented DNA (Figure 3B), which is characteristic of cells undergoing apoptotic cell death. DNA damage-induced apoptosis was also observed in TUNEL assay in which SFB extracts increased TUNEL staining and the percentage of TUNEL-positive cells in HER2-overexpressing SKBR3 cells compared to the control (Figure 3A). These results showed that apoptosis is the main mechanism for cell killing in the presence of SFB extracts.

In addition to apoptosis, cell cycle arrest is another way for cell growth inhibition by natural products that target rapidly cycling tumor cells (29). The flow cytometry results of the present study showed that SFB extracts increased the sub G0/G1 phase population after 48 h exposure ($p< 0.05$) (Figure 4). Similarly, numerous studies have shown that HER2 monoclonal antibodies lead to the accumulation of HER2 overexpressing cells in the G_0/G_1 phase and inhibit them from advancing to S-phase (30, 31). This result suggested that the growth inhibitory effect of SFB might be started by arresting the cells at G0/G1 phase before undergoing apoptosis.

To understand the potential anti-cancer mechanism of SFB, the mRNA expression levels of several apoptotic-related genes were investigated. As mentioned above, approximately 25-30% of women diagnosed with breast cancer exhibit HER2 overexpression. The overexpression of HER2 confers many of the characteristics of a cancerous cell, including the inhibition of apoptosis through downregulation of some caspases, an increase in anti-apoptotic Bcl-2 proteins (32) and induction of downstream signaling cascade(s), like the ERK1/2 and PI3K/AKT/mTOR pathways, which control cell growth, cell cycle, motility and differentiation (33, 34). Various studies showed that downregulation of HER2/neu expression in SKBR3 resulted in an anti-proliferative and apoptotic response by G0/G1 cell cycle arrest (35, 36). Therefore, HER2 is a tremendous therapeutic target for breast cancers (37). Recently, some natural components have been found which can specifically inhibit HER2/neu expression and are toxic against cancer cells, but nontoxic for normal cells (38, 39). In the present study, it was observed that SFB extracts decreased the expression of HER2 (Figure 5A). This result suggests that the apoptotic induction by SFB extracts in SKBR3 cells may be due to their inhibition of the expression of HER2. In the current study, SFBEE significantly increased mRNA expression of caspase-3 and caspase-9 (Figure 5B, Figure 5C), so this expression shows that the extract induces apoptosis through the intrinsic pathway. Interestingly, despite a significant increase in expression of caspase-9, no significant mRNA overexpression of caspase-3 was detected in cells treated with SFBEAE. It has been proposed that caspase-3 activation is an important initial event in the apoptosis enactment caspase cascade (40). That SKBR3 cells treated with SFBEAE undergo morphological and physiological apoptosis, such as DNA fragmentation, suggests the presence of an alternative pathway. It has, in fact, been suggested that initiator caspase (such as caspase-9) can result in apoptosis enactment via activation not only of caspase-3, but of caspase-7 as well (41). Furthermore, Mcl-1 is a highly expressed anti-apoptotic gene for which inhibition of its expression induces apoptosis in cancer cells and increases sensitivity to cancer treatment (42). As shown in Figure 5D, the results confirmed that SFB extracts inhibited Mcl-1 gene expression, and this inhibition may contribute to induction of apoptosis in SKBR3 cells. In conclusion, the present study demonstrated that SFB extracts induced apoptosis and arrested the cell cycle in SKBR3 cells. Bioassay-directed isolation of the chemical constituents might provide the basis for future structural and mechanistic studies for designing new anticancer drugs to be used alone or as adjuvant therapy.

Acknowledgements

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