Short Communication

Activation of p53 Gene Expression and Synergistic Antiproliferative Effects of 5-Fluorouracil and β-escin on MCF7 Cells

Abstract:

One of the most common malignancies in women is breast cancer. β-escin has pharmacological anticancer effects. 5-fluorouracil (5-FU) has antimetabolite and antiproliferative properties. The purpose of this study was to investigate the combined effects of 5-FU and β-escin on apoptosis, colony formation, Bcl-2 signaling protein, and p53 gene expression in MCF7 breast cancer cell line. The cytotoxic effects, the number of colonies, apoptosis, p53 gene expression, and Bcl-2 signaling protein of the combined 5-FU and β-escin on MCF7 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, clonogenic assay, flow cytometry, real-time quantitative polymerase chain reaction, and western blotting methods, respectively. Half-maximal inhibitory concentration values of β-escin and 5-FU were 80 μg/ml and 2 μM, respectively. The combination of 5-FU and β-escin on MCF7 cell viability showed a combination index equal to 0.5. The expression of p53 and apoptosis increased in the combination of 5-FU and β-escin on MCF7 cells compared to that of control group ($P < 0.05$). In addition, the number of colonies and Bcl-2 signaling protein in combination of 5-FU and β-escin decreased with respect to untreated control cells or single treatment of 5-FU and β-escin. The combination of 5-FU and β-escin not only has synergistic effects by increasing cell apoptosis and p53 gene expression but also decreases Bcl-2 signaling protein in MCF7 cell lines.

Keywords: *5-fluorouracil, apoptosis, MCF7, p53,* β-*escin*

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Introduction

Cancer is one of the major health problems and a leading cause of death in the world. Breast cancer is the most common malignancy in women and the second cancer in many populations after lung cancer.[1] Cancer therapy would be more feasible in case of instant cancer diagnosis by physical examination, blood test, magnetic resonance imaging, computerized tomography, mammography, and biopsy.[2] Nevertheless, the routine cancer treatment is radiotherapy, surgery, and chemotherapy.

Application of antiproliferative agents such as antioxidants can be beneficial for cancer therapy. β-escin is a triterpenoid saponin antioxidant which is widely used as a herbaceous compound in China. β-escin has therapeutic efficacy in chronic venous insufficiency, postsurgery edema, hemorrhoids,[3] traumatic brain edema,[4] inflammation, and clinical trials in patients with HIV infection.[5] Recently, β-escin has attracted attention due to its anticancer

and antiproliferative properties.[6] β-escin induces apoptosis in cancer cells through its antioxidant activities. In addition, it expresses apoptosis genes and stops cell-cycle progression.[7] β-escin has multiple mechanisms such as inducing apoptosis,[8,9] the prostaglandin release, nitric oxide synthesis, antihistamine, and antiproliferative activities.[10]

5-Fluorouracil (5-FU), a chemotherapeutic agent, is a pyrimidine analog with antimetabolite and antiproliferative properties. 5-FU has anticancer effects in most cancers such as breast cancer. 5-fluoro-2'-deoxyuridylate, the main metabolite of 5-FU, leads to inhibition of thymidylate synthase. Furthermore, misincorporation of fluoronucleotide to DNA and RNA inhibits DNA and RNA synthesis and leads to cellular death.[6] Nevertheless, 5-FU has limited clinical applications through its side effects[6] such as neutropenia, mucositis, diffusion in all body fluids, diarrhea, and vomiting.[11]

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Apoptosis is a process of programmed cell death and many medications and agents can cause apoptosis in most cancer cell lines. P53 is a tumor-suppressor gene[12] with many anticancer properties such as cell cycle arrest and inducing apoptosis.[13] P53 can cause apoptosis through regulation and transcription-independent signaling pathways, that lead to the activation of proapoptotic and repressing antiapoptotic proteins such as Bcl-2.[14] Bcl-2 protein inhibits apoptosis through an intrinsic apoptotic pathway.[15]

It has been reported that combined therapy can not only reduce drug intake and chemotherapy side effects but also it can increase the respective therapeutic efficacy. Moreover, combined therapy can reduce or delay the development of drug resistance through multiple targeting mechanisms of the drug combination.[16] Combined therapy with synergistic effects reduces the drugs doses, resistance in chemotherapy, metastasis, raises efficacy of 5-FU, and inducing apoptosis. Therefore, the aim of this study was to investigate the combined impact of 5-FU and β-escin on Bcl-2 signaling protein, apoptosis, colony formation, and p53 gene expression in MCF7 breast cancer cell line.

Methods

Chemicals

MCF7 human breast adenocarcinoma cells were obtained from Pasteur Institute (Tehran, Iran). β-escin, sodium orthovanadate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium, penicillin/streptomycin (pen/strep), trypsin 0.25%, and fetal bovine serum (FBS) were obtained from Gibco (Rockville, MD, USA). Antibodies were purchased from Elabscience Biotechnology Co. (Wuhan, China). Roti®ZOL total RNA extraction kit was prepared from Carl Roth GmbH (Germany). 5-FU (50 mg/ml solution) was prepared from Haupt Pharma (Wolfratshausen GmbH Co, Germany). Annexin V kit was purchased from BD Bioscience (California, USA). All other chemicals used were of analytical grade.

Cell culture

MCF7 cells were grown in RPMI 1640 medium supplement with 10% FBS and 1% pen/strep at 37°C in 98% humidity including 5% CO_2.[17]

Cell viability assay

The cytotoxic effects of 5-FU and β-escin alone on MCF7 cells were determined using MTT assay. MCF7 cells (5000 cells/per well) were grown in RPMI 1640 medium overnight. Then, the cells were exposed to 5-FU (0–11 μM) and β-escin (0–100 μg/ml, solution in dimethyl sulfoxide (DMSO) with 0.1% final concentration) for 72 h. Then, the medium was removed, and the cells were incubated with 10 μL MTT solution (5 mg/mL) for 4 h at 37°C. Subsequently, the medium removed

and 150 μL of DMSO was added to each well, and the absorbance of each well was measured with a microplate reader (Stat Fax-2100, USA) at 490 nm. Then, the percentage of cell viability was expressed as follows: viability = A (sample)/A (control) × 100.[18] These experiments were performed three times.

Determination of 5-fluorouracil and β-escin synergism

Based on the results of cell viability assay for 5-FU and β-escin alone, a combination of 5-FU and β-escin in different concentrations (0.25 μM 5-FU and 60 μg/ml β-escin; 0.5 μM 5-FU and 45 μg/ml β-escin; 1 μM5-FU and 20 μg/ml β-escin; and 1.5 μM5-FU and 10 μg/ml β-escin) was used to evaluate synergetic effects between 5-FU and β-escin by the combination index (CI) theorem in a way that CI <1, =1, and >1 indicated synergism, additive effect, and antagonism, respectively.[19] Therefore, a combination of 5-FU (1 μM, IC_{30}) and β-escin (20 μg/ml, IC_{20}) was used based on MTT assay for the combined impact of 5-FU and β-escin in other experiments.

Colony formation assay

MCF7 cells were seeded in 6-well plates at a density of 3×10^5 cells per well. After overnight incubation, the cells were treated with 5-FU *per se* (1 μM) or β-escin alone (20 μg/ml) or the combination of 5-FU with β-escin (1 μM and 20 μg/ml respectively) for 72 h. Then, the medium discarded and the cells were incubated in a CO_2 incubator (5% CO_2 and 98% humidity) in the absence of 5-FU and β-escin treatment at 37°C for an additional 14 days to obtain visible colonies. The culture medium was changed every 2 days. Colonies were fixed with 70% ethanol and stained with 0.5% crystal violet.[20,21] In the next step, the image of the colonies was taken, and plating efficiency (PE) was measured using the following formula; (number of colonies/number of seeded cells) × 100 and surviving fraction (SF) was determined by (number of colonies/number of seeded cells × PE control) × 100.[22]

Apoptosis assay

Cells (2×10^5 per well) were seeded into a 6-well plate and incubated overnight. The cells were treated with 5-FU (1 μM) and β-escin (20 μg/ml) or the combination of 5-FU and β-escin (1 μM and 20 μg/ml, respectively) for 72 h. Then, cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and stained with Annexin V (BD Bioscience) for 20 min at room temperature in a dark place. Cells were analyzed by flow cytometry with a FACScan system (Becton-Dickinson and Company, San Jose, CA, USA) according to the manufacturer's protocol in three independent experiments.[23]

Real-time quantitative polymerase chain reaction

MCF7 breast cancer cells were harvested after treatment with 5-FU (1 μM) and β-escin (20 μg/ml) or a combination of 5-FU and β-escin (1 μM and 20 μg/ml, respectively)

in 6-cm dishes after 72 h. Then, the total mRNA of the cells was extracted with Roti®ZOL reagent based on manufacturer's instructions. In the next step, cDNA was prepared from RNA using a synthesis kit (Takara Bio Inc., Japan) and 1 µg total RNA was used according to the manufacturer's instructions. Reverse transcription was carried out using Prime Script™ reagent Kit (Takara Bio Inc., Japan) for cDNA synthesis based on the manufacturer's instructions. Subsequently, cDNA was expanded by real-time quantitative polymerase chain reaction (RT-qPCR) using SYBR® Green PCR Master Mix (Takara Bio Inc., Japan) in the presence of specific primers for *p53* (forward: 5'-CCCATCCTCACCATCATCACAC-3'; reverse: 5'-GCACAAACACGCACCTCAAAG3'), and, *GAPDH* (forward: 5'ACACCCACTCCTCCACCCTTTG3'; reverse: 5'GTCCACCACCCTGTTGCTGTA-3'). Oligo 6.0 software (Molecular Biology Insights, Cascade, Co, USA) was used to design the primers and confirmed by the blast (NCBI). The primers were purchased from Macrogen Company (South Korea). Expression of p53 gene was detected using Rotor-Gene 3000 (Corbett, Australia) for each of the above concentrations. The temperature profile for the reaction was an initial denaturation stage of 95°C at 10 min, and then a three-step program was developed for 40 cycles including 95°C for 10 S, 62°C for 15 S, and 72°C for 20 S, respectively. The glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene, was amplified separately for normalizing the data.[24]

Western blotting

The MCF7 cells were seeded into 6-cm dishes (6×10^5 cells) and incubated overnight. The MCF7 treated with 5-FU (1 µM) and β-escin (20 µg/ml) or the combination of 5-FU with β-escin (1 µM and 20 µg/ml, respectively) in 6-cm dishes for 72 h. Later, the cells were washed with PBS and lysed in ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, and 1% Triton X-100, 0.5% sodium deoxycholate, sodium dodecyl sulfate

0.1%, 1 mM ethylenediaminetetraacetic acid, 0.1% sodium azide, 50 mmol/L NaF,1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and phosphatase inhibitor).[9] The total protein concentration was quantified using the Bradford procedure.[25] The western blot procedure was conducted as described previously,[18] and primary antibodies Bcl-2 and β-actin were used based on the manufacturer's protocols. β-actin was determined as an internal control. The bands were detected by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, USA).

Data analysis

All results were expressed as mean ± standard deviation. SPSS software (SPSS, version 20, Inc, Chicago, IL, USA) or GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used to perform the statistical analysis. Kruskal–Wallis test was used to evaluate the statistical significance of the differences between the treated and the untreated control cells for MTT assay, clonogenic assay, Annexin V assay, and real-time PCR. The relative levels of quantitative gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method and data were expressed as fold change. Furthermore, melting curves were generated to ensure the purity of the amplification product of each reaction. Western blot experiments were repeated three times. $P < 0.05$ was considered statistically significant for the differences between the experimental groups. For combination treatment, CI was calculated using CompuSyn software (Combo SynInc, City, State, USA) and CI <1, =1, and >1 indicated synergism, additive effect, and antagonism, respectively.

Results

Cell viability assay

Figure 1A and B shows the treated MCF7 cells with different concentrations of β-escin and 5-FU. Half-maximal inhibitory concentration values of β-escin and 5-FU were 80 µg/ml and 2 µM, respectively. Furthermore, Figure 1C
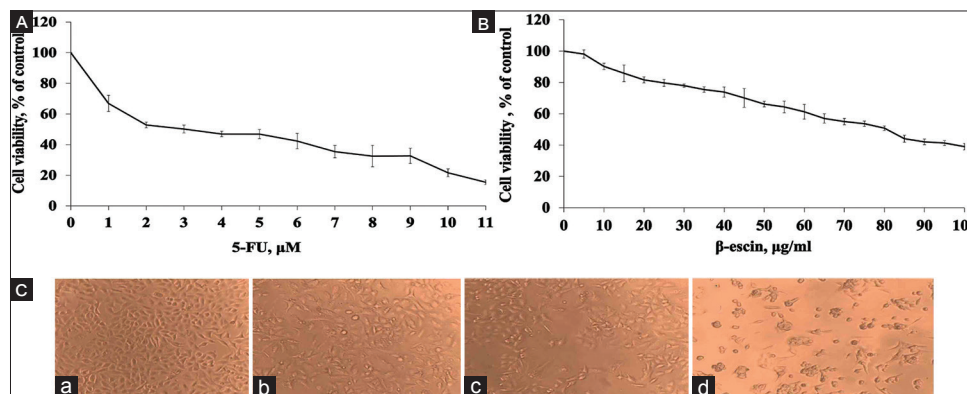
Figure 1: Inhibition of MCF7 cells proliferation. (A) 5-fluorouracil treatment; (B) β-escin treatment; and (C) morphological changes of cell by 5-fluorouracil and β-escin after treatment with various concentrations of 5-fluorouracil and β-escin (a: control, b: 1 µM 5-fluorouracil, c: 20 µg/ml β-escin, and d: 1 µM 5-fluorouracil plus 20 µg/ml β-escin). The cells were cultured at the density of 5 × 10³ cells per well for 72 h. At the end of treatment times, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data were expressed as the mean ± standard deviation for three independent experiments

shows morphological changes in treated MCF7 cells at concentrations of 5-FU (1 μM) and β-escin (20 μg/ml) or a combination of 5-FU and β-escin (1 μM and 20 μg/ml, respectively). In addition, Table 1 shows the combination of 5-FU and β-escin that led to a synergistic CI equal to 0.5.

Effect of β-escin and 5-fluorouracil on apoptosis in MCF7 cells

Figure 2 shows flow cytometric analysis of β-escin and 5-FU on apoptosis in MCF7 cells. The rate of apoptosis in MCF7 cells with single treatment of 5-FU, β-escin, and combination of 5-FU and β-escin were 37%, 31%, and 49.77%, respectively. 5-FU in combination with β-escin resulted in a significant increase ($P < 0.05$) in apoptosis compared with untreated control cells.

The effects of β-escin, 5-fluorouracil, and their combination on p53 expression in MCF7 cells

Figure 3 shows the treated MCF7 cells with 5-FU, β-escin, and their combination for investigating p53 expression using RT-qPCR. 5-FU and the combination of 5-FU and β-escin led to a significant increase ($P < 0.05$) in p53 gene expression in comparison with that of the control cells by almost 2.2 and 5.6 fold, respectively. No significant change was observed between β-escin and untreated control cells.

Clonogenic assay of MCF7 cells

Figure 4a shows the clonogenic assay of MCF7 cell line. The number of MCF7 cell colonies, after 14 days, consisting of 81, 63, 46, and 9 colonies for control, β-escin, 5-FU, and combination of 5-FU with β-escin, respectively. Furthermore, Figure 4 shows a remarkable decrease in the number of colonies for combination therapy of 5-FU and β-escin compared with those of control or single treatment of 5FU and β-escin. SF for β-escin, 5-FU, and combination of 5-FU and β-escin colonies were 78%, 57%, and 11%, respectively. Furthermore, Figure 4b demonstrates PE in control and treated experimental groups. PE showed a significant decrease ($P < 0.05$) in combination therapy when compared with the control cells.

Effects of β-escin, 5-fluorouracil, and their combination on the cellular Bcl-2

Figure 5 shows the effect of single and combined therapy of β-escin and 5-FU on the cellular level of Bcl-2. Combination of 5-FU and β-escin resulted in an additional decrease in the level of Bcl-2 in comparison with control or single treatment of 5-FU and β-escin.
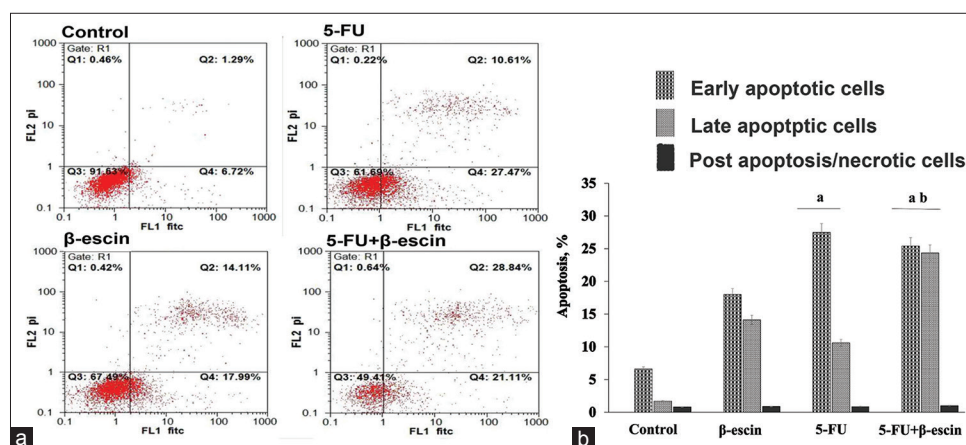
Discussion

Chemotherapy is a major treatment for most cancers, especially breast cancer. Nevertheless, chemotherapy is accompanied with many side effects. Therefore, many studies have been conducted to discover the natural compounds which can prevent and treat cancer.[26,27] Nowadays, changes in the usage or combinations of confirmed anticancer agents can lead

Table 1: The viability percentage of MCF7 cell line treated with a combination of β-escin and 5-fluorouracil after 72-h incubation

Combination number	Dose combination		Cell viability (%)	CI
	β-escin (IC value), μg/ml	5-FU (IC value), μM		
1	10 (IC10)	1.5 (IC40)	31±0.61	0.3
2	20 (IC20)	1 (IC30)	43±0.46	0.5
3	45 (IC30)	0.5 (IC20)	66±1.2	1.4
4	60 (IC40)	0.25 (IC10)	80±0.9	2.8

The results were expressed as mean±SD of three separate experiments. IC – Inhibitory concentration; 5-FU – 5-fluorouracil; SD – Standard deviation; CI – Combination index

Figure 2: β-escin and 5-fluorouracil potentiate on apoptosis in MCF7 cell line. Cells were grown in the absence or presence of 5-fluorouracil (1 μM) and β-escin (20 μg/ml) or the combination of 5-fluorouracil and β-escin (1 μM and 20 μg/ml, respectively) for 72 h. (a) Dot plot analysis of treated and untreated control cells. (b) Percentage of live/apoptotic/necrotic MCF-7 cells treated with 5-fluorouracil, β-escin, and the combination of 5-fluorouracil and β-escin as compared to control cells. The results were expressed as mean ± standard deviation of three separate experiments. [a]$P < 0.05$ versus control, [b]$P < 0.05$ versus β-escin

to a new strategy for identification and development of advanced therapeutic producers for cancer treatment.[16] One of the cancer treatment approaches is the utilization of multicomponent combinations of anticancer drugs with antioxidants.[28] This procedure not only increases potent antiproliferative effects of combination therapy through apoptosis but also it reduces the dose of anticancer drugs and their side effects.

Results in this study showed that the combined effects of 5-FU and β-escin (1 μM and 20 μg/ml, respectively) had stronger antiproliferative effects than that of each agent *per se* [Figure 1 and Table 1]. Nevertheless, several studies have shown that β-escin *per se* can decrease cell proliferation in some cancer cell lines.[5,7,9] Our results showed, at least partly, that β-escin has a synergistic

Figure 3: The effects of β-escin, 5-fluorouracil, and combination of 5-fluorouracil and β-escin on the gene expression of p53. The cells were cultured with β-escin (20 μg/ml), 5-fluorouracil (1 μM), and their combination (1 μM 5-fluorouracil and 20 μg/ml β-escin) for 72 h. Columns and bars represent the mean ± standard deviation of three independent experiments. The expression of p53 was normalized with glyceraldehyde-3-phosphate dehydrogenase as an internal standard. [a]$P < 0.05$ versus control, [b]$P < 0.05$ versus β-escin

effect in combination with 5-FU [Table 1]. A recent study by Ming *et al.* reported anticancer synergistic combined effects of β-escin and 5-FU on human hepatocellular carcinoma (HCC) SMMC-7721 cells.[6] They showed synergistic effects of β-escin and 5-FU occurred depending on the ratio and concentration of the drugs which is consistent with findings in our study on MCF7 cells.[6] They used the different ratio of β-escin and 5-FU in their experiments, whereas we used the different IC values of combined β-escin and 5-FU in our experiments. The present study and Ming's study showed the similar potential effects of combined β-escin and 5-FU to reduce Bcl-2 protein expression, cell proliferation, and inducing cell apoptosis in SMMC-7721 and MCF7 cells with respect to untreated control cells or single treatment of 5-FU and β-escin. SMMC-7721 and MCF7 have different tissue origin; however, they have some similar properties such as positive telomerase activity[29,30] which may be influenced in their response to the effects of combined β-escin and 5-FU.[31] It seems that the combination of β-escin and 5-FU may be effective, at least partly, on other cancer cell lines in special ratio and concentration. Nevertheless, the results of the combined effects of 5-FU and β-escin may be influenced by different media, experimental conditions, cancer cell lines, and the source preparation of antioxidant and anticancer drug. Furthermore, a published study showed that combined 5-FU, cisplatin, and curcumin enhance the anticancer effects of 5-FU in human gastric cancer MGC-803 cells by decreasing cell viability, inhibiting colony formation, and through inducing apoptosis[32] which is in line with the present study findings. In addition, recent studies have demonstrated that combination of antioxidants such as rutin with anticancer drugs can reduce the proliferation of many cancer cells through downregulation of some genes such as Bcl-2, Bcl-XL, XIAP, and surviving of mediators expression.[33,34] On the other hand, it has been reported that the combined effects of 5-FU and gambogic
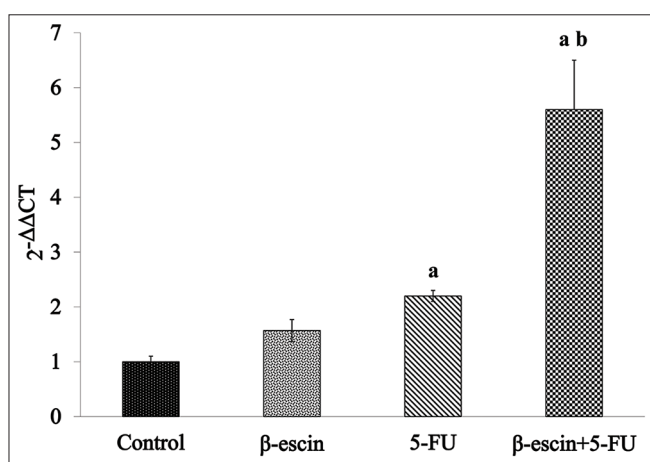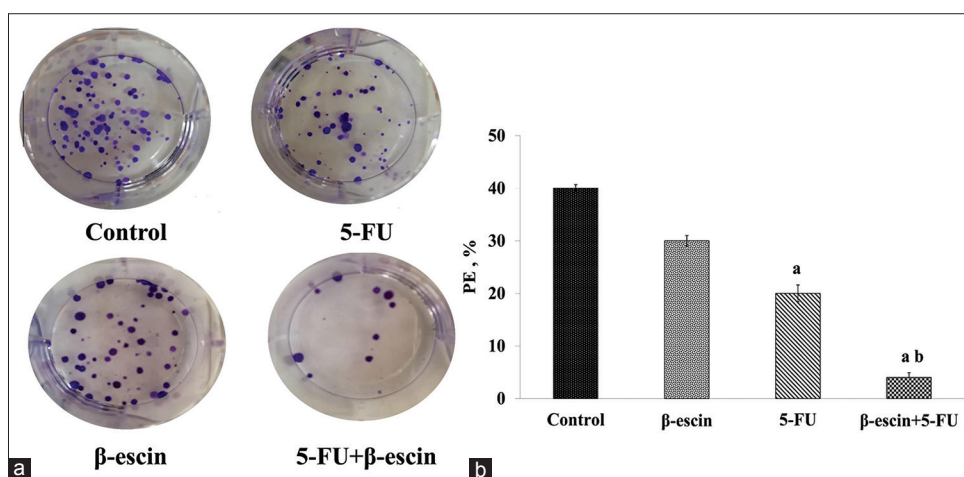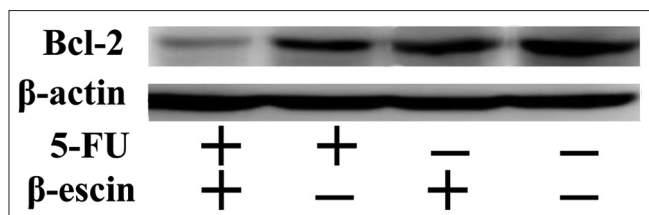

Figure 4: Photographs of colonies in the absence or presence of 5-fluorouracil and β-escin on MCF7 cell line. (a) The colony formation in the control, β-escin, 5-fluorouracil, and combination of 5-fluorouracil with β-escin, (b) histogram plot demonstrates plating efficiency in control and treated experimental cells. [a]$P < 0.05$ versus control, [b]$P < 0.05$ versus β-escin

Figure 5: The effects of β-escin, 5-fluorouracil, and combination of 5-fluorouracil with β-escin on the Bcl-2 level of signaling pathway protein. MCF7 cell line was treated with 5-fluorouracil (1 μM), β-escin (20 μg/ml), and a combination of 5-fluorouracil and β-escin (1 μM and 20 μg/ml, respectively) for 72 h

acid, an antioxidant, on BGC-823 human gastric carcinoma cells lead to Bcl-2 downregulation expression[19] which confirms the findings in our study. Gambogic acid led to increased apoptosis through regulating metabolic enzymes of 5-FU.[19] Therefore, in our study, it is possible that β-escin influences metabolic enzymes of 5-FU which leads to increased apoptosis. In addition, our flow cytometry results showed that apoptosis increased in combination with 5-FU and β-escin on MCF7 cells compared to that of the control cells [Figure 2]. In addition, the number of colonies in a combination of 5-FU and β-escin decreased in comparison with those of control or single treatment of 5-FU and β-escin [Figure 4]. It has been reported that the combined effects of 5-FU and genistein, a flavonoid, on HT-29 colon cancer were indicative of apoptosis and inhibition of cell growth,[35] which is in line with findings in our study. Therefore, it seems that, in the present study, the cotreated 5-FU with β-escin not only leads a decrease in the required dose of 5-FU but also it results in the increased apoptosis and inhibition of cell proliferation.

In this study, an increase in p53 gene expression along with a decrease in cellular Bcl-2 signaling protein was observed in the combination of 5-FU with β-escin [Figures 3 and 5]. A previous study demonstrated that downregulation of Bcl-2 expression by the combination of 5-FU with apigenin on HCC led to cancer cellular apoptosis[36] which is consistent with findings in our study. In addition, the previous studies have demonstrated that the antioxidants alone or in combination with chemotherapy significantly inhibit cell proliferation and induce caspase-dependent apoptosis through activating tumor-suppressor protein p53 and downregulating MDM2. Furthermore, antioxidants can decrease the expression of antiapoptotic proteins such as Bcl-2 in cancer cells.[37] In cancer cell lines, p53 induces apoptosis through the regulation of Bcl-2 and Bax gene expression, which leads to the downregulation of antiapoptotic proteins such as Bcl-2.[13] Bcl-2 is an antiapoptotic protein belonging to Bcl-2 family which contributes to intrinsic pathways of apoptosis.[15] Many reports have indicated that drug-induced apoptosis in most cancer cell lines is through the downregulation of Bcl-2 expression.[9,15,17,38] Furthermore, it is reported that Bcl-2 family regulates apoptosis by mitochondrial pathway.[36]

Therefore, in this study the apoptosis of MCF7 cells, due to the combination of 5-FU with β-escin, may be induced by Bcl-2 through mitochondrial pathways. In addition, it was demonstrated that the combination of quercetin with 5-FU-induced apoptosis in colorectal cancer cells through he elevation of p53 and decreasing of Bcl-2 protein expression[39] which is in line with our findings. Moreover, it has been reported that 5-FU leads to apoptosis in gastric cancer cell through p53 gene expression accompanied by an increased Bax/Bcl-2 ratio which results in arrest cell proliferation.[38] In addition, the previous studies have demonstrated that β-escin can induce apoptosis[9,40] by activating caspase-8 and caspase-3[41] which regulate the expression levels of Bcl-xL and Bcl-2 family members.[17] Therefore, in the present study, apoptosis was seen in MCF7 cells to result from, at least in part, increased p53 and decreased Bcl-2 protein expression.

We did not evaluate the combined effects of 5-FU with β-escin on other cellular signaling pathways or gene expressions such as p65, p21, MAPK-p38, and caspase activation. These factors can influence apoptosis and cell survival. Therefore, we suggest that future studies focus on these parameters.

Conclusions

Our findings indicate that antiproliferative effects of 5-FU and β-escin combination were higher than each of them *per se*. Furthermore, the combination of 5-FU and β-escin not only has synergistic effects by increasing cell apoptosis and p53 gene expression but also it decreases Bcl-2 signaling protein in MCF7 cell line.

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Conflicts of interest

There are no conflicts of interest.

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