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Research Article

The Combination of Zerumbone and 5-FU: A Significant Therapeutic Strategy in Sensitizing Colorectal Cancer Cells to Treatment

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Objectives. Chemotherapy is considered to be essential in the treatment of patients with colorectal cancer (CRC), but drug resistance reduces its efficacy. Many patients with advanced CRC eventually show resistance to 5-fluorouracil (5-FU) therapy. Synergistic and potentiating effects of combination therapy, using herbal and chemical drugs, can improve patients' response. Zerumbone (ZER), which is derived from ginger, has been studied for its growth inhibitory function in various types of cancer. *Methods*. The cytotoxic effects of ZER and 5-FU alone and their combination, on the SW48 and HCT-116 cells, were examined, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The mRNA and protein levels of β-catenin, survivin, and vimentin were measured in treated CRC cells, using qRT-PCR and western blot. Colony formation assay, scratch test, and flow cytometry were performed to detect the changes of proliferation, migration, and apoptosis. *Key Findings*. In HCT-116- and SW48-treated cells, the proliferation, the gene and protein expression levels of the markers, the migration, the colony formation, and the survival rates were all significantly reduced compared to the control groups, and the sharpest decline was observed in the 5-FU+ZER treatment groups. *Conclusions*. Combination therapy has shown promising results in CRC cells, especially in drug-resistant cells.

1. Introduction

CRC is one of the most common diseases in industrialized countries and is currently the third most common cause of cancer-related deaths in males and the second most in females worldwide [1]. 5-FU is commonly used as a chemotherapeutic drug in cancer treatments and in combination with other drugs to treat many types of cancers including breast, anal, stomach, head, and neck cancer [2]. However, the response rate of 5-FU for advanced CRC is only 20%; whereas, combining 5-FU with other chemotherapeutic drugs has improved the response rates in these patients to 40–50% [3]. As drug resistance remains a major clinical problem for the clinical application of 5-FU and related chemotherapeutic drugs, investigating the molecular pathways and genes, responsible for therapeutic resistance to 5-FU in CRC, offers insights into mechanisms of cell survival, thus developing more responsive therapeutic targets [2, 4].

Epithelial-mesenchymal transition (EMT) is a very complex yet well-known process in cancer cells that is an essential stage in tumor metastasis and invasion [5, 6]. A critical step of this transition is the lack of expression of epithelial markers such as E-cadherin, claudin, occludin, desmoplakin, type IV collagen, and laminin 1 and upregulation of mesenchymal markers such as N-cadherin, intregrin, vimentin, type I collagen, laminin 5, and fibronectin [7]. EMT is associated with several signaling pathways, including the transforming growth factor-β (TGF-β), Wnt, Hedgehog, and Notch pathways, and it can affect the involved genes such as β-catenin which is activated in the Wnt pathway [8]. The Wnt/β-catenin signaling pathway, also called the canonical Wnt pathway, is a major regulating signaling pathway in

several cancers due to its effect on the transcription of the targeted genes [9]. Inhibition of Wnt/β-catenin signaling could increase sensitivity to chemotherapeutic agents in cancers [10]. β-Catenin is the main mediator of this pathway that is widely expressed in many tissues.

Vimentin is a widely expressed and highly conserved gene. Vimentin is constitutively expressed in normal mesenchymal cells and is the cytoskeletal component, responsible for maintaining cell integrity, supporting and anchoring the organelles, and providing resistance to avoid cell damage [11]. A previous study has shown the overexpression of vimentin in a wide range of epithelial cancers including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, lung cancer, and malignant melanoma [12]. Upregulation of vimentin in EMT and the signaling pathways, contributing to the metastasis, invasion, tumorigenesis, and chemoresistance of various tumors, plays an important role in the progression and prognosis of cancer [13].

Survivin is the smallest bifunctional protein and a member of the inhibitor of apoptosis (IAP) family of proteins that can inhibit apoptosis and promote cell division [14]. Survivin is expressed at low levels in normal cells, but it has also been found to be prominently expressed in many solid and aggressive tumors [15]. In various tumors, high expression of survivin is associated with resistance to chemotherapy, poor prognosis, and increased angiogenesis. Therefore, survivin is an important target in cancer treatment [14].

Overcoming the resistance to intrinsic and therapeutic agents is one of the most important challenges in the treatment of cancer patients, as chemoresistance causes disease relapse and metastasis and remains the main barrier in cancer therapy. Therefore, it is very important to identify the molecular mechanisms of chemoresistance [16]. In chemotherapy, the use of the combination of nontoxic or less toxic phytochemicals with chemotherapy agents may reduce the toxicity, especially toxicity to normal tissues. Moreover, the lower dose of drugs, used in combination therapy, reduces the drug resistance in cancer cells. Therefore, the use of less-toxic agents, such as those used in herbal therapy, could be a promising therapeutic approach in cancer treatment [17].

Most therapeutic approaches in cancer treatment are associated with toxicity, side effects, lack of selectivity, high cost, and chemoresistance. Herbs, plants, and plant-based compounds that are commonly referred as safe compounds have been demonstrated to exert chemopreventive features and mediate anticancer roles in diverse cells [18]. One such herbal compound is ZER with the chemical name (2E,6E,10E)-2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one that is extracted from the rhizomes of traditional plant, Zingiber zerumbet Smith [19]. ZER is known for its biomedical properties such as having antioxidant, antibacterial, antihypersensitive, and anti-inflammatory activities and exhibiting its diverse effects on proliferation, angiogenesis, and apoptosis against a wide variety of tumor cells including colon, liver, myeloid, breast, and gastric cancer [20]. Recent research has shown that ZER also mediates antiproliferative properties against various cancers such as skin, lung, liver, brain, breast, pancreas, and colon cancer [18, 20].

The current study suggests, for the first time, the synergistic and potentiating effects of the combination of both ZER and 5-FU in increasing the sensitivity of CRC cell lines to 5-FU treatment. As EMT, metastasis, and chemoresistance are closely related to tumor progression, we attempted to establish that ZER treatment may mediate 5-FU resistance by targeting the important genes involved in cancer chemoresistance such as vimentin, survivin, and β-catenin. Consequently, combination treatment of ZER with 5-FU is expected to prove more beneficial for CRC patients.

2. Material and Method

2.1. Reagents. Zerumbone (z3902-50M),HPLC grade ≥ 98%purity , and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; ZER was prepared in stock solution of 1 mM (MW 218.23 g/mol) using DMSO; the final concentration of DMSO for in vitro study was less than 0.01%; the different concentrations of ZER ranging from 0 to 100 μM were prepared from 1 mM stock. Trypan blue was purchased from Sigma Chemical (St. Louis, MO, USA). 5-FU was purchased from Sigma-Aldrich. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (molecular weight: 335.43) was purchased from Sigma-Aldrich. High Pure RNA Isolation Kit was obtained from Roche Applied Science (Germany), and SYBR Green QPCR Master Mix and DNA ladder 1 kb and 50 bp were products of Thermo Fisher Scientific (USA). The first-strand cDNA synthesis kit was obtained from Thermo Scientific (USA). Acrylamide and bis-acrylamide were purchased from Sigma-Aldrich. TEMED, APS, and isopropanol were purchased from Merck. Tris base was purchased from Sigma-Aldrich; anti-rabbit secondary antibody was purchased from Santa Cruz; anti-β-catenin antibody (ab223075), anti-vimentin antibody (ab20346), and anti-survivin antibody (ab76424) were purchased from Abcam; ECL Kit was purchased from Amersham; protein extraction kit was purchased from Bio Basic.

2.2. Cell Culture. The SW48 and HCT-116 (human colon cancer) cell lines were purchased from the National Cell Bank of Pasteur Institute (Tehran, Iran). Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco), containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco), and were maintained at 37° in a humidified incubator containing 5% CO_2. Cells were treated with ZER, 5-FU, and 5-FU+ZER. Untreated cells were used as the control groups.

2.3. Cell Viability Assay. The inhibitory effects of ZER and 5-FU alone and together on cell viability were determined by the analysis of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were seeded at 7000 cells per well (triplicates) in 96-well tissue culture plates. After 24 h, the cells were treated with the increasing concentrations of ZER (0–100 μM) and 5-FU (0-700 μM) and cotreated with 5-FU and ZER based on their concentration and treatment time. After 24, 48, and 72 h of incubation, 10 μl of MTT solution was added to each well of the plate and the mixture was incubated for 3-4 h at 37°C. Then, the medium was removed, and 100 μl of dimethyl sulfoxide

(DMSO) was added to dissolve the formazan compound. Absorbance at 570 nm was then measured with a microplate reader.

2.4. Treatment of CRC Cells with ZER, 5-FU, and 5-FU+ZER. In the present study, the CRC cells were treated with ZER and 5-FU in concentrations (IC50) that were reported from the MTT test (14 μM and 19 μM of ZER and 20 μM and 158 μM of 5-FU for HCT-116 and SW48 cell lines, respectively). In each treatment, three different IC50 were used for CRC cells (13, 14, and 15 μM of ZER and 18, 19, and 20 μM of 5-FU in HCT-116 and 19, 20, and 21 μM of ZER and 157, 158, and 159 μM of 5-FU in SW48). Later, the optimum IC50 was chosen. The combination of both drugs may induce cytotoxicity in both cell lines; therefore, we first treated cells with ZER for 7 h, and after that, the cells were treated with 5-FU over the optimum period of time (24 h in HCT-116 and 72 h in SW48). In the next part, the conditioned media of cells was removed and replaced by complete media. After 24, 48, and 72 h, cells were trypsinized and used for further analyses.

2.5. RNA Isolation and qRT-PCR. Total RNA was extracted from HCT-116 and SW48 cells using a first-strand cDNA Synthesis Kit. According to the manufacturer's directions, RNA quality and concentration were assessed by using gel electrophoresis which is frequently used to visually assess the quality of RNA, and the 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample, and NanoDrop (Thermo Fisher Scientific, Waltham, MA) spectrophotometer was used for assessing the concentration of RNAs. The complementary DNA (cDNA) was synthesized with a first-strand cDNA Synthesis Kit according to the manufacturer's protocol.

Real-time PCR was performed with 1 μl cDNA, 3.6 μl H_2O, 5 μl SYBR Green QPCR Master Mix, and 0.2 μl of specific primers in the Roche LightCycler machine (Roche Diagnostics). 18srRNA was applied as an internal control gene. Duplicate reactions were run for each cDNA sample, and relative expression of genes was determined by using the $2^{-\Delta\Delta CT}$ method. Sequences for gene-specific primers are provided in Table 1.

2.6. Protein Extraction and Western Blot Analysis. The HCT-116 and SW48 cells with different treatment groups (5-FU, ZER, and 5-FU+ZER) were trypsinized and then washed twice with cold PBS followed by centrifugation at 1500 RPM for 5 minutes. Then, the extraction of protein was performed using the Extraction Kit. Finally, the total protein concentration was measured by Bradford assay.

SDS-PAGE was then performed to resolve the equivalent proteins which were then transferred to nitrocellulose membrane. After that, blocking was done using 5% nonfat dry milk. The membrane was incubated with primary antibodies which include anti-β-catenin antibody (ab223075), anti-vimentin antibody (ab20346), and anti-survivin antibody (ab76424) overnight at 4°C. Membranes were then incubated with secondary antibody (anti-rabbit secondary antibody conjugated with horseradish peroxidase) for 1 h at 4°C. The proteins of interest were visualized using the chemiluminescence detection kit, and bands were quantified by ImageJ software.

2.7. Colony Formation Assay. Clonogenic assay is the method of choice to determine cell reproductive death after treatment with cytotoxic agents. HCT-116 and SW48 cells were seeded in a 6-well plate at a density of 3×10^5 for each group and kept in the incubator. After 24 h, the medium was pulled out from a 6-well plate and cells were treated with optimum concentrations of ZER, 5-FU alone, and together. Then, conditioned media of cells was removed and replaced with complete media. The plates were incubated again overnight. After a specified time (the optimum time that was chosen for HCT-116 was 24 h and for SW48 was 72 h), the cells were resuspended with trypsin and counted. 450 cells were double-replicated in another 6-well plate, and the plates were incubated for 3-4 days without shaking. After 8-10 days, and observing the colonies (with at least 50 cells) on the plates, the colonies were washed with PBS and then crystal violet was used to stain them. The formed colonies were counted, and then, their photos were taken for further analysis.

2.8. Migration Assay. Migration assay of HCT-116 and SW48 cells was performed by scratch assay. First, 2×10^5 HCT-116 and SW48 cells were seeded into a 24-well tissue culture plate. Then, a scratch was made in the monolayer using a yellow pipette tip across the center of the well. Detached cells were washed twice with medium, and then, cells were treated with optimum concentrations of ZER, 5-FU, and 5-FU+ZER for 24 and 48 h. Finally, cell migration to the gap was examined by recording images at the beginning of treatment and at intervals of 24 and 48 h during cell transfer to close the scratch.

2.9. Flow Cytometry Analysis of Apoptosis. HCT-116 and SW48 cells (1×10^6) were seeded into a 12-well culture plate and incubated with 5-FU, ZER, and 5-FU+ZER, in optimum concentration for prime time. A total of cells were trypsinized, washed, and resuspended in 1 ml PBS with 5% fetal bovine serum. After centrifugation, cells were stained with Annexin V-FITC/propidium iodide (PI) detection kit (MabTag, Germany) that was used to explore several phases of apoptosis and cell death in SW48 and HCT-116 cell lines. Finally, the apoptosis ratio was analyzed using Attune NxT acoustic focusing cytometer (Life Technology, USA).

2.10. Statistical Analysis. The data were analyzed using GraphPad Prism 5.0 software using the one-way analysis of variance test for comparison between three groups. Data were presented as the mean ± standard error of the mean. The p value of <0.05 was considered statistically significant.

3. Results

3.1. ZER Augments Cytotoxicity Effects of 5-FU in HCT-116 and SW48 Cells. The effect of different concentrations of ZER, 5-FU treatment, and a combination of both on the viability of HCT-116 and SW48 cells compared to untreated cells at 24, 48, and 72 h was evaluated, using MTT assay.

Table 1: Primer sequences for real-time PCR.

Gene name	Forward primer	Reverse primer	Accession number
Survivin	TGGGAAGGGTTGTGAATGAG	GCTGTCTCTACTTTCCAGGATG	NM_001012270.2
Vimentin	CATTGAGATTGCCACCTAC	CGTTGATAACCTGTCCATC	NM_003380
β-Catenin	CTTCACCTGACAGATCCAAGTC	CCTTCCATCCCTTCCTGTTTAG	NM_001330729.2

The cytotoxic effect of ZER treatment on CRC cells was also determined by calculating 50% of cell death, which was about 14 μM and 19 μM in HCT-116 and SW48 cells, respectively (Figure 1). The cytotoxic effect of 5-FU in HCT-116 cells was calculated to be 20 μM, and in SW48 cells was about 158 μM (Figure 2). Moreover, the cytotoxic effects of combination treatments were 9.5 μM in HCT-116 cells and 100 μM in SW48 cells (Figure 3) (in combination treatment, first cells were treated with ZER in 14 μM and 19 μM concentration in HCT-116 and SW48 cells, respectively, and then, 5-FU with different concentrations was added (0-700 μM)). In this study, the dose-dependent effect of ZER, 5-FU, and the combination of these two agents was observed in both cell lines at 24, 48, and 72 h. As a precaution, a concentration lower than and above the IC50 concentration was chosen for treating the cells. The best result was observed in the cells that were treated with IC50 concentrations; hence, the IC50 concentrations were used for further experiments.

3.2. The Expression of Vimentin, Survivin, and β-Catenin Genes Was Reduced in 5-FU-, ZER-, and 5-FU+ZER-Treated Cells

3.2.1. Treatment of HCT-116 and SW48 Cells with ZER. As illustrated in Figure 4, treatment with ZER in HCT-116 and SW48 cells significantly reduced the expression of survivin, vimentin, and β-catenin mRNA level compared to the control groups in different times (vimentin 0.46-fold for 24 h, survivin 0.42-fold for 24 h, and β-catenin 0.5-fold for 24 h in HCT-116 and β-catenin 0.48-fold for 48 h and 0.29-fold for 72 h, survivin 0.49-fold for 72 h, and vimentin 0.60-fold for 72 h in SW48).

3.2.2. Treatment of HCT-116 and SW48 Cells with 5-FU. Figure 5 shows the reduced expression of survivin, vimentin, and β-catenin mRNA level in the 5-FU treatment in HCT-116 cells (vimentin 0.27-fold for 24 h, 0.81-fold for 48 h, and 0.85-fold for 72 h, survivin 0.35-fold for 24 h, 0.570-fold for 48 h, and 0.73-fold for 72 h, and β-catenin 0.75-fold for 24 h in HCT-116), and no significant changes of gene expression were observed in SW48 cells treated with 5-FU.

3.3. Treatment with ZER+5-FU Reduced the Vimentin Gene Expression in CRC Cell Lines.

The mRNA level of genes in different groups was determined by qRT-PCR, and results showed a significant drop in the expression of vimentin mRNA level in the 5-FU+ZER treatment group in HCT-116 cells for 24, 48, and 72 h (0.21-fold for 24 h, 0.39-fold for 48 h, and 0.51-fold for 72 h). Treatment of CRC cells with ZER alone had inhibitory effect on expression of vimentin mRNA level in 24 h but no significant inhibitory effect on expression of vimentin mRNA level in 48 and 72 h. This shows the strong inhibitory effect of 5-FU over ZER at 48 and 72 h time (Figure 6(a)).

Similar treatment of SW48 cells showed that 5-FU and ZER alone or in combination has no significant inhibitory effect for 24 and 48 h. However, the treatment with ZER+5-FU for 72 h dramatically reduced the expression of vimentin mRNA level. This significant drop in the expression of vimentin mRNA level in ZER+5-FU-treated cells was due to potentiating effect of ZER in 72 h (0.47-fold for 72 h) (Figure 6(b)).

3.3.1. Treatment with ZER+5-FU Reduced the Survivin Gene Expression in CRC Cell Lines. The mRNA level of genes in different groups was determined by qRT-PCR, and results showed the significant drop in expression of survivin mRNA level in HCT-116 cells treated with ZER+5-FU in 24, 48, and 72 h (0.34-fold for 24 h, 0.48-fold for 48 h, and 0.76-fold for 72 h). Treatment of CRC cells with ZER alone had inhibitory effect on the expression of the survivin mRNA level in 24 h but no significant inhibitory effect on expression of survivin mRNA level in 48 and 72 h, showing the strong inhibitory effect of 5-FU over ZER at 48 and 72 h time (Figure 7(a)).

Similar treatment of SW48 cells showed that 5-FU and ZER alone or in combination has no significant inhibitory effect for 24 and 48 h. However, the treatment with ZER+5-FU for 72 h dramatically reduced the expression of survivin mRNA level. This significant reduction in the expression of survivin mRNA level in ZER+5-FU-treated cells was due to the potentiating effect of ZER for 72 h (0.4-fold for 72 h) (Figure 7(b)).

3.3.2. Treatment with ZER+5-FU Reduced the β-Catenin Gene Expression in CRC Cell Lines. As shown in Figure 8, the substantial decrease in expression of β-catenin mRNA level was detected in HCT-116 cells treated with ZER+5-FU for 24 h. This significant reduction in β-catenin gene expression in ZER+5-FU treatment at 24 h is stemmed from both inhibitory effects of ZER and 5-FU (0.48-fold for 24 h) (Figure 8(a)).

Treatment of SW48 cells revealed that there was no significant change in the mRNA level of β-catenin in all treatments in 24 h. Moreover, the combination of both ZER+5-FU treatment could significantly decrease the expression of β-catenin mRNA level at 48 and 72 h, which was due to the potentiating effect of ZER (0.46-fold for 48 h and 0.16-fold for 72 h) (Figure 8(b)).

3.3.3. Decreased Expression of Vimentin Protein in Cells Treated with ZER, 5-FU, and 5-FU+ZER. The protein expression of vimentin was assessed using western blot analysis. Results of western blot showed that the level of protein was

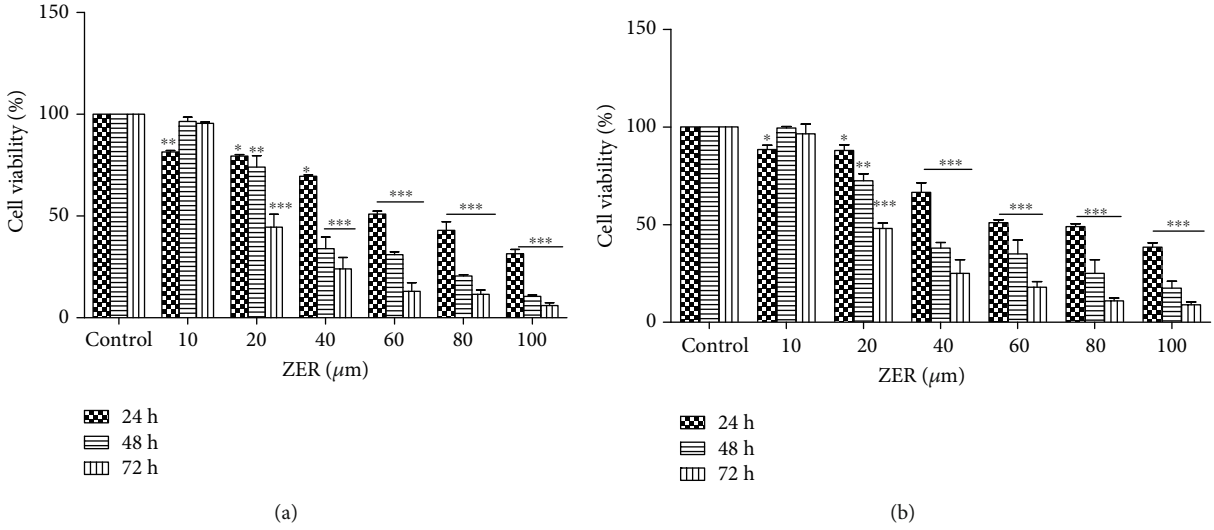


Figure 1: Effect of ZER on colorectal cancer cell viability. (a) HCT-116 and (b) SW48 were treated with various concentrations of ZER at 24, 48, and 72 h, and their viability was examined by MTT assay. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 3$).

significantly downregulated due to the effect of ZER, 5-FU, and 5-FU+ZER in HCT-116 at optimum times. The optimum time was 24 h for all treatments in HCT-116 cells (37.8%, 20%, and 40% reduction in ZER-, 5-FU-, and 5-FU +ZER-treated cells, respectively) (Figure 9(a)).

No significant changes were observed in the protein levels of vimentin in SW48 cells treated with 5-FU, while considerable reduction was observed in other treated groups including 5-FU+ZER and ZER. The optimum time was 72 h for all treatments in SW48 cells (70% and 73.3% reduction in ZER- and 5-FU+ZER-treated cells, respectively) (Figure 9(b)). Treatment with 5-FU+ZER caused the sharpest decline in all groups in both cell lines.

3.3.4. Decreased Expression of Survivin Protein in Cells Treated with ZER, 5-FU, and 5-FU+ZER. The results showed that the protein level of survivin was significantly downregulated due to the effect of ZER, 5-FU, and 5-FU+ZER in HCT-116 in 24 h after treatment (48.8%, 10%, and 80.4% reduction in ZER-, 5-FU-, and 5-FU+ZER-treated cells, respectively) (Figure 10(a)).

In the SW48 cell line with the exception of 5-FU treatment, in other treatments, significant decline was observed in the optimum time (72 h) (46.6% and 58.5% reduction in ZER- and 5-FU+ZER-treated cells, respectively) (Figure 10(b)).

Taken together, the results showed that the 5-FU+ZER treatment groups demonstrated a sharper decline than each treatment alone, in both cell lines.

3.3.5. Decreased Expression of β-Catenin Protein in Cells Treated with ZER, 5-FU, and 5-FU+ZER. Here, we found that β-catenin protein level was significantly downregulated in the HCT-116-treated groups, especially in 5-FU+ZER-treated cells for 24 h (74%, 39%, and 74.6% in ZER-, 5-FU-, and 5-FU+ZER-treated cells, respectively) (Figure 11(a)).

In the SW48 cell line, no reduction in β-catenin protein level in cells treated with 5-FU was observed. A significant decrease in protein levels especially in the 5FU+ZER-treated group was detected (35%, 4.4%, and 61.7% in ZER-, 5-FU-, and 5-FU+ZER-treated cells) (Figure 11(b)).

3.3.6. Proliferation of CRC Cells Was Inhibited by the Effect of 5-FU, ZER, and 5-FU+ZER Treatment. The clonogenic assay was performed to compare the effects of ZER and 5-FU treatment alone or together on colorectal cancer cell lines. Cells were counted by ImageJ software after staining. Figure 12 shows a significant decrease in the number of HCT-116 and SW48 cells. All three groups showed a significant reduction in the number of cells in HCT-116 cells; however, the combination therapy exerted the most inhibitory effect, followed by cells treated with ZER. Treatment with 5-FU alone showed the least inhibitory effect (5-FU: 63%, ZER: 23%, and 5-FU+ZER: 82% reduction in the number of colonies). The reduction in the number of colonies in SW48 cells was due to the effect of ZER and 5-FU+ZER treatments, and no inhibition was observed in the 5-FU-treated group (ZER: 19% and 5-FU+ZER: 39% reduction in the number of colonies).

3.3.7. Migration of HCT-116 and SW48 Cells Was Significantly Reduced by the Effect of 5-FU, ZER, and 5-FU+ZER Treatment. The wound healing assay was applied to evaluate the potential role of 5-FU, ZER, and 5-FU+ZER treatment on migration in SW48 and HCT-116 cells. The results showed that the wound widths were significantly reduced after 24 and 48 h in the control groups, while 5-FU-, ZER-, and 5-FU+ZER-treated cells demonstrated less migration in these time points (Figure 13), suggesting that 5-FU, ZER, and 5-FU+ZER markedly inhibited SW48 and HCT-116 cell migration with the exception of 5-FU treatment in the SW48 cell line. Moreover, the inhibitory effect on migration in these two cell lines was more noticeable in the 5-FU+ZER treatment group (ZER: 53%, 5-FU: 30%, and 5-FU+ZER: 70% increase of cell migration-free area in HCT-116 for 48 h) and (ZER: 55% and 5-FU

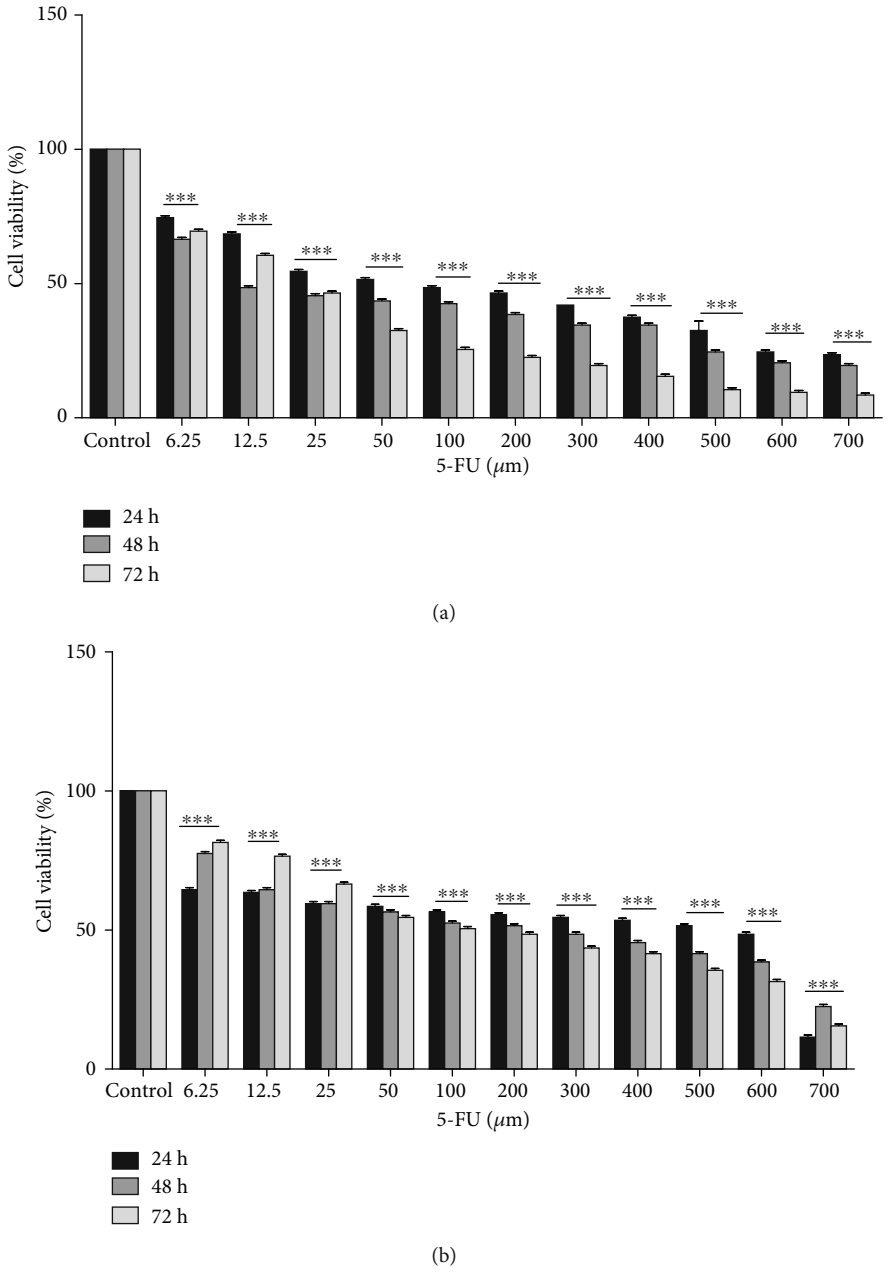


Figure 2: Effect of 5-FU on colorectal cancer cell viability. (a) HCT-116 and (b) SW48 were treated with various concentrations of 5-FU at 24, 48, and 72 h, and their viability was examined by MTT assay. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 3$).

+ZER: 65% increase for 48 h and 5-FU+ZER: 40% increase of cell migration-free area in SW48 for 24 h).

3.3.8. Apoptosis of HCT-116 and SW48 Cell Was Significantly Reduced by the Effect of 5-FU, ZER, and 5-FU+ZER Treatment. The apoptotic effect of 5-FU, ZER, and 5-FU +ZER treatment on CRC cells was evaluated using the Annexin V/PI assay. The results showed that the percentage of the apoptotic cells in the treatment groups of HCT-116 and SW48 cell lines was considerably increased (Figure 14). The highest rate of apoptosis was observed in the combination of 5-FU and ZER treatment.

4. Discussion

CRC is a major cause of morbidity and mortality all over the world. CRC is generally diagnosed as a malignant disease in both men and women [21]. CRC is most commonly diagnosed in patients of 50 years of age and older. Earlier onset is observed in hereditary and familial CRC. In patients with stage III CRC, common therapies such as surgery, chemotherapy, and radiation therapy could result in complete remission or increase life expectancy [21].

Chemotherapy resistance is a major barrier to cancer treatment that is primarily associated with EMT process and leads to cancer progression. Oncogenes and tumor

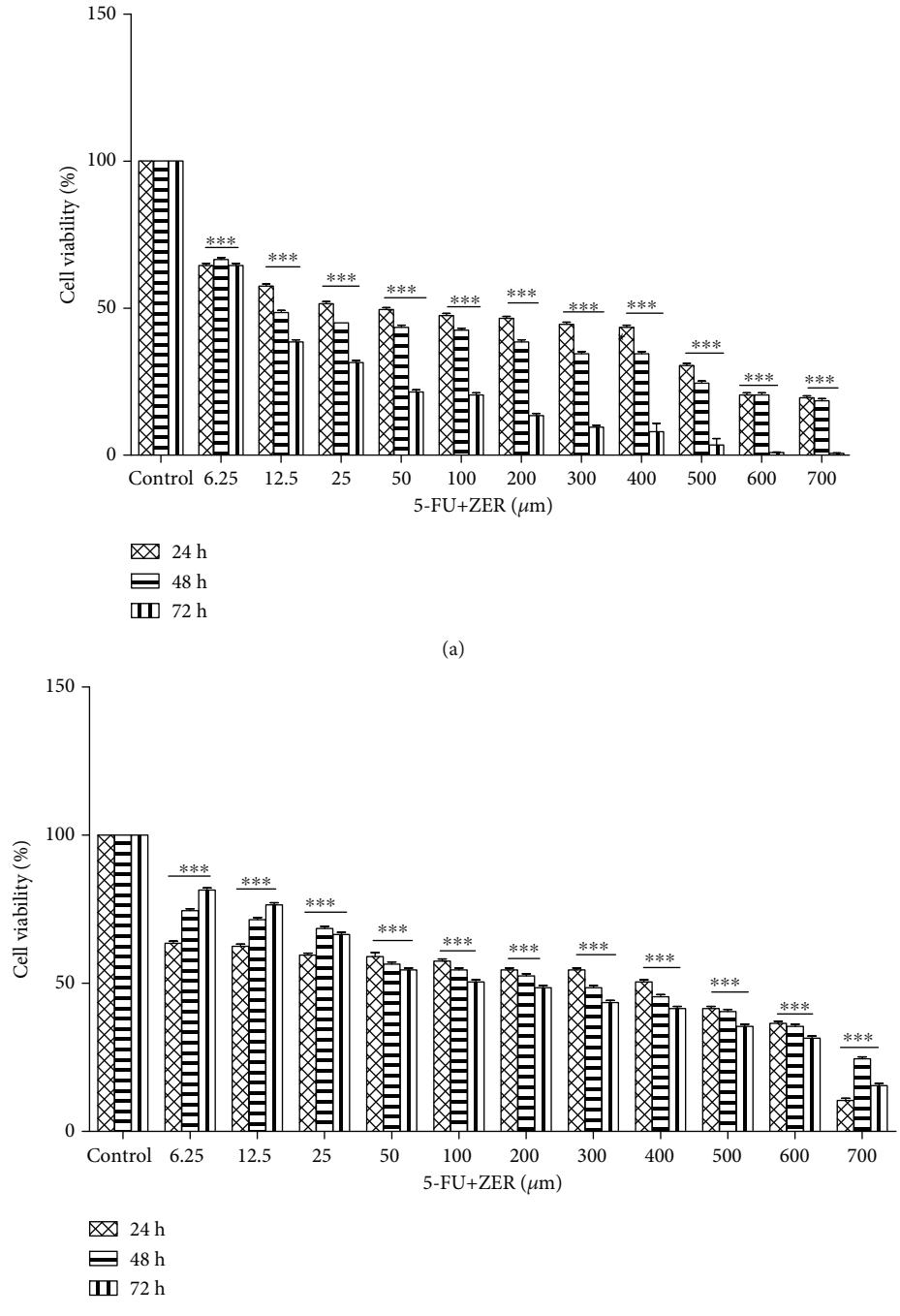


Figure 3: Effect of 5-FU+ZER on colorectal cancer cell viability. (a) HCT-116 and (b) SW48 were treated with various concentrations of 5-FU +ZER (ZER concentration was about 14 μM and 19 μM in HCT-116 and SW48 cells, respectively) at 24, 48, and 72 h, and their viability was examined by MTT assay. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 3$).

suppressors, which also play a role in inducing EMT, are major contributing factors to chemoresistance. In chemotherapy, often, a combination of two anticancer agents is more effective than each agent alone [17]. 5-FU, a fluoropyrimidine analog, is a chemotherapeutic agent widely used for treating colorectal cancer, but resistance remains a major obstacle to 5-FU clinical efficacy. Recent studies have suggested that EMT is associated with chemoresistance in animal models of lung and pancreatic cancers [22, 23]. Zhang et al. showed that downregulation of snail, an EMT marker, might be a potential therapeutic approach to solve chemoresistance and prevent metastasis during 5-FU chemotherapy in breast cancer [24].

Ginger, a key component in functional foods, has been used for thousands of years as a medicinal herb to treat a variety of chronic diseases [25]. ZER is a cyclic sesquiterpene from the rhizomes of ginger plant (Zingiber zerumbet Smith)

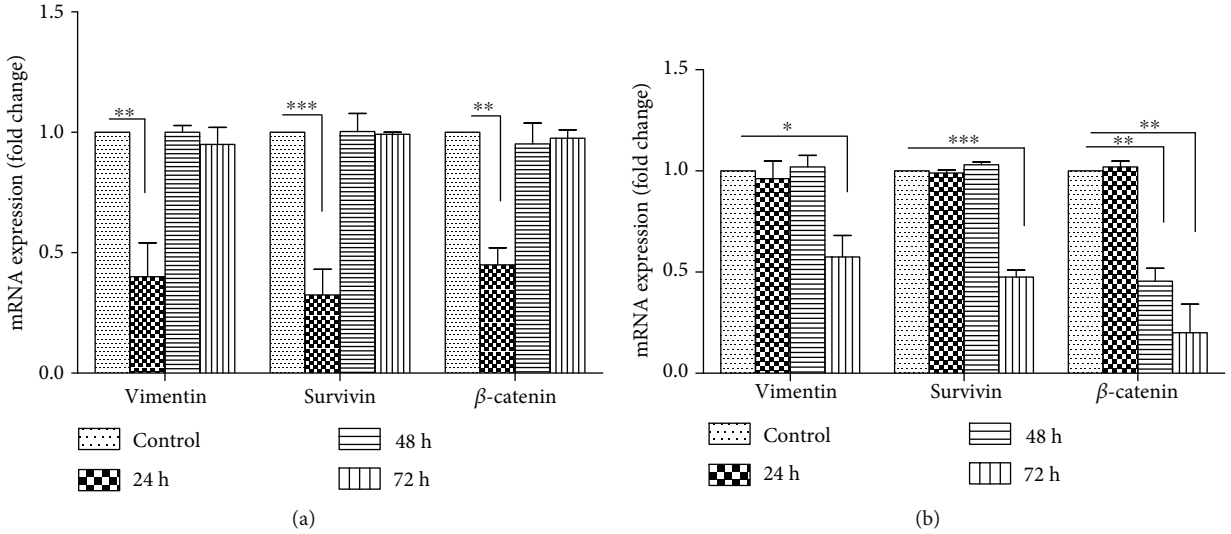


Figure 4: The effect of ZER on the expression of survivin, vimentin, and β-catenin genes in CRC cell lines. Relative expression of (a) HCT-116 and (b) SW48 cells was determined using real-time PCR technique. The expression of survivin, vimentin, and β-catenin mRNA level was considerably reduced after 24 h in HCT-116 cells (a). The expression of survivin and vimentin mRNA level was considerably reduced after 72 h; however, β-catenin mRNA level was inhibited after 48 and 72 h in SW48 cells (b). 18srRNA was used as a normalizer. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

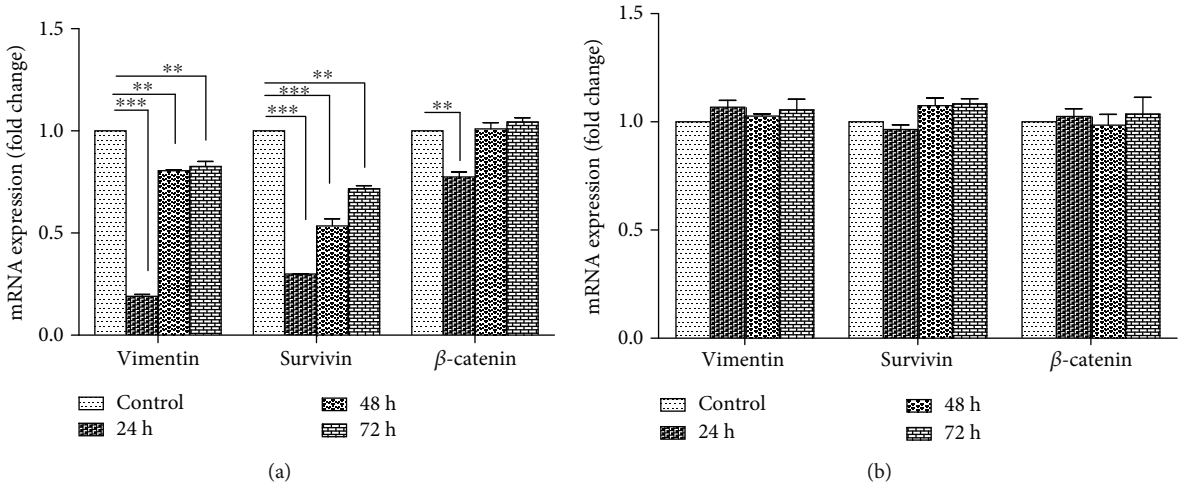


Figure 5: Effect of 5-FU on expression of survivin, vimentin, and β-catenin genes in colorectal cancer cell lines. The relative expression of (a) HCT-116 and (b) SW48 cells was determined using real-time PCR technique. The expression of target genes in treated cells versus control in SW48 cells did not significantly change at all times. However, a significant decrease was observed in expression of vimentin and survivin mRNA level in HCT-116 cells in all three time points, while the expression of β-catenin mRNA level was reduced in just 24 h. 18srRNA was used as a reference gene. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

which is known for its biomedical properties such as having antioxidant, antibacterial, anti-inflammatory, and immunomodulatory activities [26].

There are a number of mechanisms, such as the Wnt signaling pathway, that are suggested to be responsible for drug resistance. As high as 50% of metastatic CRC, patients are resistant to 5-FU-based chemotherapy [27]. Studies suggest that 5-FU combination therapies may be more beneficial [18, 28]. Some studies found that the 5-FU-resistant CRC cells demonstrate high expression of TCF4 and β-catenin, indicating an upregulated Wnt pathway. Subsequently, β-catenin-silenced CRC cells were relatively more sensitive to chemotherapy reagents [2, 10]. Furthermore, survivin has been proven to play an important role in colorectal carcinogenesis. Virrey et al. have shown that chemoresistance in tumor cells might be correlated with an overexpression of the inhibitor of bifunctional proteins, like survivin which inhibits apoptosis [15]. Moreover, in 2018, Chung and colleagues revealed that the 5-FU treatment could decrease the gene and protein expression of vimentin as an EMT marker in HCT-116 and DLD1 cells [20].

Here, we investigated the treatment effect of ZER alone and with 5-FU on the proliferation, gene and protein

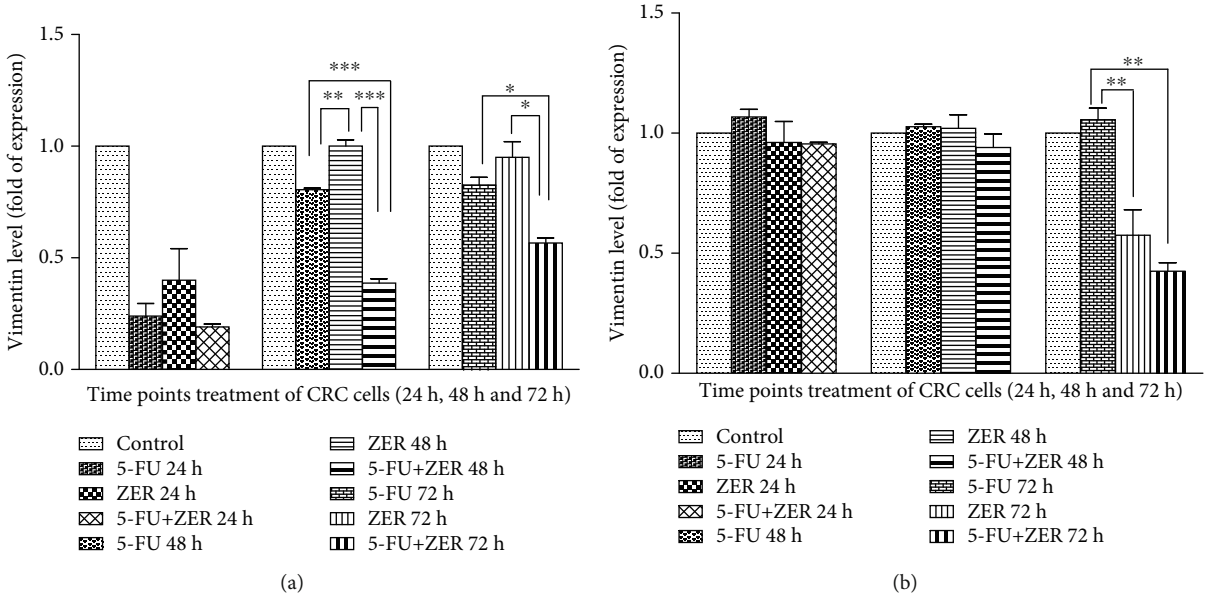


Figure 6: Synergic effect of ZER and 5-FU on mRNA level of vimentin in HCT-116 (a) and SW48 (b) cells. CRC cells were treated with ZER and 5-FU alone and together for 24, 48 h, and 72 h followed by total RNA extraction for reverse transcription to cDNA. The cDNAs were used to assess expression levels of selected genes by SYBR green-based real-time quantitative PCR. The fold changes were derived using the comparative $2^{-\Delta\Delta CT}$ method. Each data point is presented as the mean ± SD ($n = 3$). All data were normalized to levels of 18srRNA ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus nontreated cells).

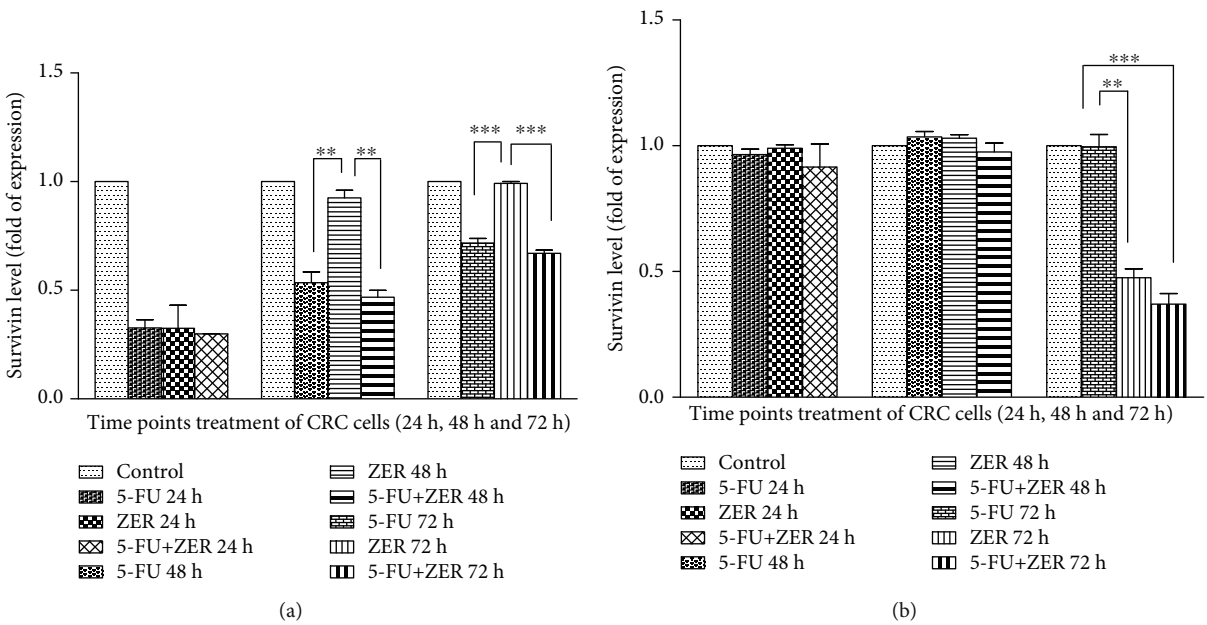


Figure 7: Synergic effect of ZER and 5-FU on mRNA level of survivin in HCT-116 (a) and SW48 (b) cells. CRC cells were treated with ZER and 5-FU alone and together for 24, 48 h, and 72 h followed by total RNA extraction for reverse transcription to cDNA. The cDNAs were used to assess expression levels of selected genes by SYBR green-based real-time quantitative PCR. The fold changes were derived using the comparative $2^{-\Delta\Delta CT}$ method. Each data point is presented as the mean ± SD ($n = 3$). All data were normalized to levels of 18srRNA ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus nontreated cells).

expression, migration, and apoptosis of CRC cells. Our aim in this research was to determine whether the combination of ZER with 5-FU-based chemotherapy regimen can increase its inhibitory effect. In the present study, the CRC cells were treated with ZER and 5-FU in IC50 concentrations, determined by the MTT test (14 μM and 19 μM of ZER and 20 μM and 158 μM of 5-FU for HCT-116 and SW48 cell lines, respectively). The cotreatment showed a lower IC50 (100 μl and 9.5 μl for HCT-116 and SW48 cell lines, respectively) than each agent alone. In treated cells, the optimum

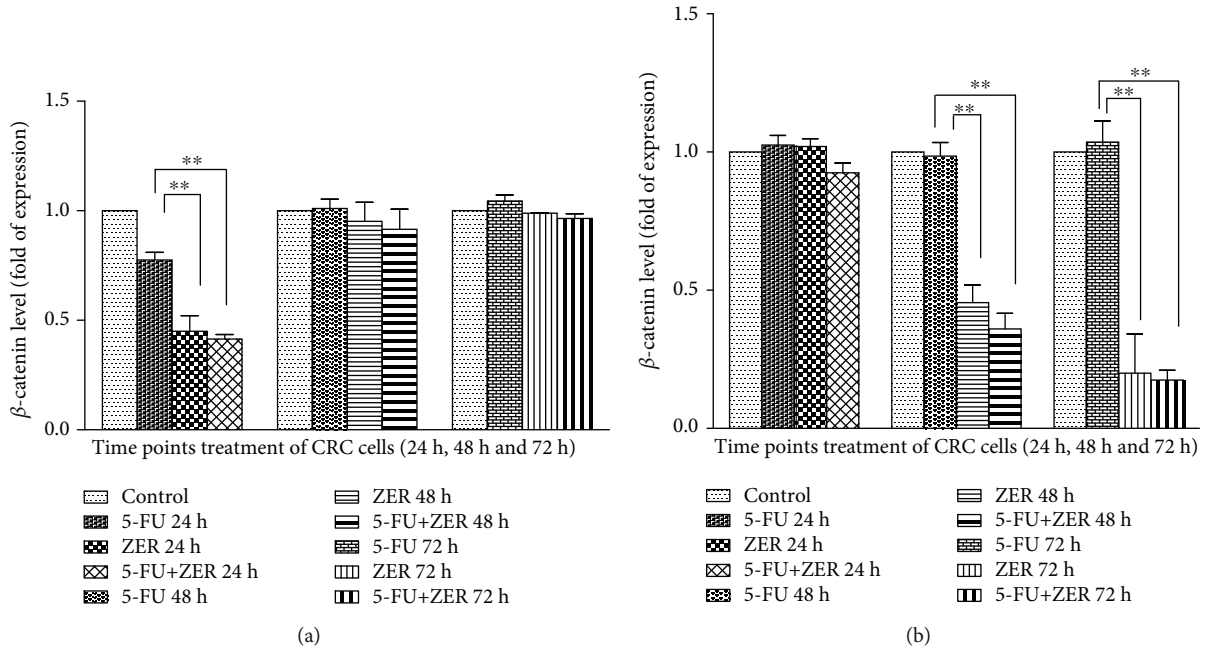


Figure 8: Synergic effect of ZER and 5-FU on mRNA level of β-catenin in HCT-116 (a) and SW48 (b) cells. CRC cells were treated with ZER and 5-FU alone and together for 24, 48 h, and 72 h followed by total RNA extraction for reverse transcription to cDNA. The cDNAs were used to assess expression levels of selected genes by SYBR green-based real-time quantitative PCR. The expression fold changes were derived using the comparative $2^{-\Delta\Delta CT}$ method. Each data point is presented as the mean ± SD ($n = 3$). All data were normalized to levels of 18srRNA. ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus nontreated cells).

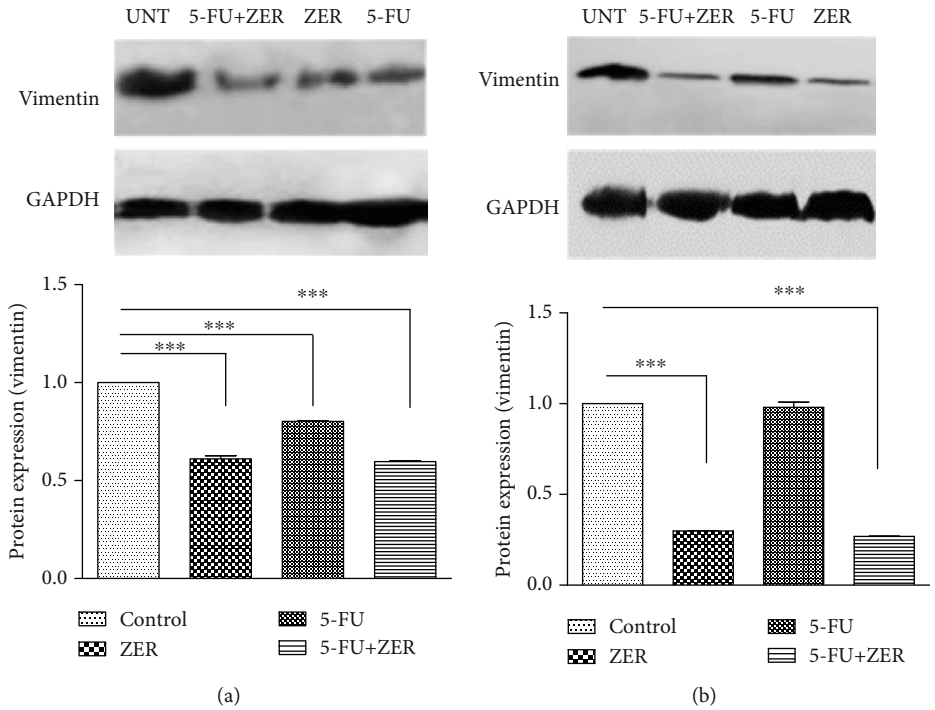


Figure 9: Synergistic effect of ZER and 5-FU on vimentin protein level in HCT-116 (a) and SW48 (b) cells. Semiquantitative evaluation of protein level by western blotting analysis showed lower intensity of vimentin band in the treated cells compared with the control groups at the optimum time (24 h in HCT-116 and 72 h in SW48 cell lines). The relative expression of proteins was measured against the GAPDH as a reference. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

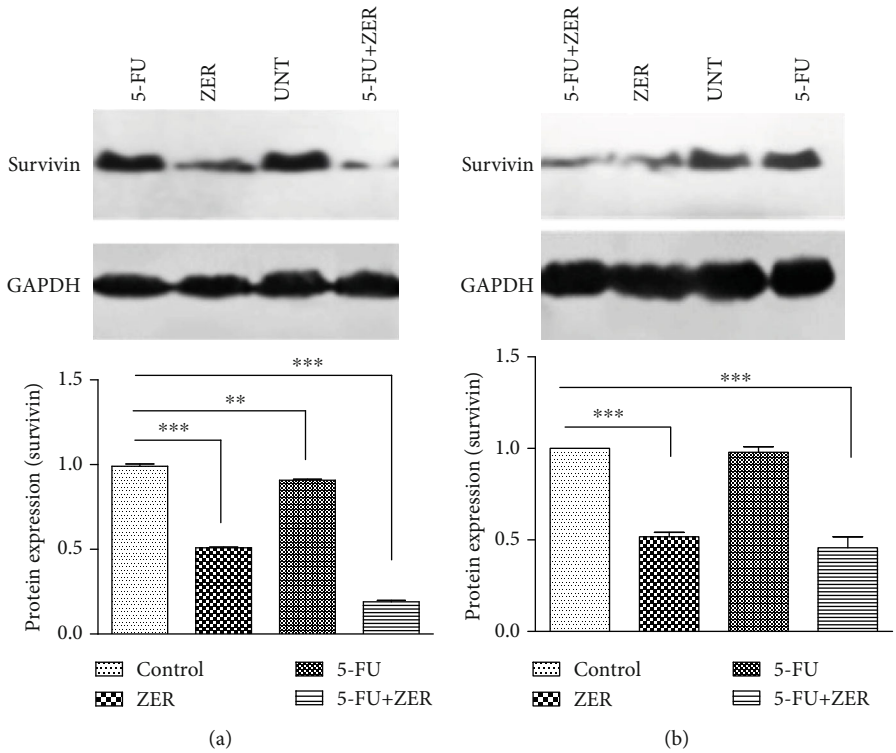


Figure 10: Synergistic effect of ZER and 5-FU on survivin protein level in HCT-116 (a) and SW48 (b) cells. Semiquantitative evaluation of protein level by western blotting analysis showed lower intensity of survivin band in the treated cells compared with the control groups at the optimum time (24 h in HCT-116 and 72 h in SW48 cell lines). The relative expression of proteins was measured against the GAPDH as a reference. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

time for each treatment varied based on the type of cells. Due to the greater sensitivity of HCT-116 cells, the treatment time in HCT-116 cells is shorter than SW48 cells (24 h and 72 in HCT-116 and SW48 cells, respectively). Our results demonstrated that treating HCT-116 and SW48 cells with ZER significantly reduce the expression of survivin, vimentin, and β-catenin mRNA levels compared to the control groups (vimentin 0.46-fold for 24 h, survivin 0.42-fold for 24 h, and β-catenin 0.5-fold for 24 h in HCT-116 and β-catenin 0.48-fold for 48 h and 0.29-fold for 72 h, survivin 0.49-fold for 72 h, and vimentin 0.60-fold for 72 h in SW48). In accordance to our findings, other studies have previously reported that ZER can reduce the gene and protein expression of survivin, vimentin, and β-catenin genes [29, 30].

In contrast, the results of another study in 2009 by Yodkeeree et al. showed that ZER has little or no effect on survivin gene expression [31].

We found no significant inhibition of the interested genes in SW48 cells treated with 5-FU in all time points. However, in HCT-116 cells, a significant decrease was observed in all three time points, except for β-catenin which inhibitory effect was only seen in the 24 h time point (vimentin 0.27-fold for 24 h, 0.81-fold for 48 h, and 0.85-fold for 72 h; survivin 0.35-fold for 24 h, 0.570-fold for 48 h, and 0.73-fold for 72 h; and β-catenin 0.75-fold for 24 h in HCT-116). Therefore, we argue that the SW48 cells are more resistant to 5-FU treatment than HCT-116 cells. In line with our findings, studies by Urushibara et al. and He et al. in 2017 and 2018 reported high expression levels of β-catenin in 5-FU-resistant CRC cells. Furthermore, the use of Wnt/β-catenin inhibitor can increase sensitivity to treatment with 5-FU in CRC cells [2, 32]. Chung et al. in 2018 examined the effect of 5-FU on colorectal cells by investigating the EMT process. They found that 5-FU only downregulated the expression of vimentin as an EMT marker in HCT-116 and DLD1 cells [20]. In another study in 2017, an aptamer containing the survivin RNAi was used, in order to increase sensitivity of HT-29 CRC cells to 5-FU and oxaliplatin [33].

Here for the first time, we evaluated the synergistic effect of ZER and 5-FU treatment in CRC cell lines (HCT-116 and SW48). We found a significant drop in expression of vimentin and survivin mRNA level in HCT-116 cells that were treated with ZER+5-FU in 24, 48, and 72 h, while treatment of cells with ZER alone could markedly inhibit the expression of vimentin and survivin mRNA level in 24 h. Therefore, it could be suggested that in combination therapy, the potentiating effect of 5-FU could make the CRC cells more sensitive to the combination of 5-FU and ZER compared to using each alone (Figures 6 and 7(a)).

In line with our results, Buhrmann et al. in 2015 and Chung et al. in 2018 reported the synergistic effects of resveratrol, a plant compound, and 5-FU in HCT-116, SW48, and DLD1 cell lines. They demonstrated that resveratrol increased the sensitivity of colorectal cancer CRC cells to 5-FU by inhibiting EMT and reducing the expression of vimentin [20, 34]. Pandey et al. in 2015 studied three substances,

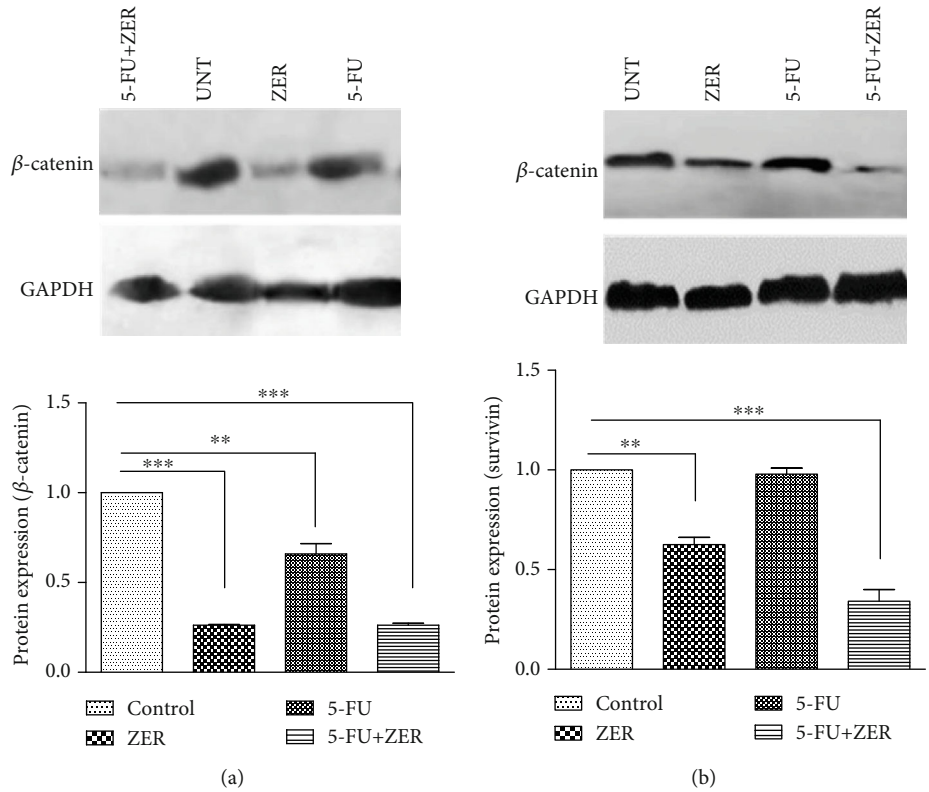


Figure 11: Synergistic effect of ZER and 5-FU on β-catenin protein level in HCT-116 (a) and SW48 (b) cells. Semiquantitative evaluation of protein level by western blotting analysis showed lower intensity of β-catenin band in the treated cells compared with the control groups at the optimum time (24 h in HCT-116 and 72 h in SW48 cell lines). The relative expression of proteins was measured against the GAPDH as a reference. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

namely, berberine (a chemical found in several plants), curcumin (another member of the ginger family), and quercetin (a plant pigment) alone and in combination with 5-FU on gastric cancer cells. They reported that the synergistic effect of each of these substances with 5-FU decreased the expression of survivin and STAT3 levels resulting in an increase in cell death in gastric cancer cells [35].

Similarly, we found that in SW48 cells at some time points, 5-FU and ZER alone did not have a significant inhibitory effect on the markers of interest, and when used in combination, they can be more effective by having a complementary effect. As shown in Figures 6 and 7, a significant drop in the expression of genes in ZER+5-FU-treated cells was due to the potential effect of ZER at 72 h (Figures 6 and 7(b)).

As illustrated in Figure 8(a), a significant decrease in expression of β-catenin mRNA level was detected in HCT-116 cells which were treated with ZER+5-FU for 24 h. This significant decrease in the ZER+5-FU treatment group at 24 h is related to both inhibitory effects of ZER and 5-FU (Figure 8(a)).

Similar to our study, the synergic effects of 5-FU with EPLE (from salvia plant) on HCT-116 and SW480 CRC cells have been demonstrated by Ye et al. in 2015. Their results revealed that EPLE alone and along with 5-FU suppressed the Wnt/β-catenin pathway, thus reducing the gene expression of survivin [36].

Regarding SW48 cells, we found that the combination of ZER and 5-FU treatment could significantly decrease the expression of β-catenin mRNA level in 48 and 72 h which was due to the potentiating effect of ZER treatment (Figure 8(b)).

Protein levels of the mentioned genes were evaluated by western blot. The results demonstrated that treatment with ZER reduced the protein level in both cell lines (in HCT-116 cells: β-catenin: 74%, survivin: 48.8%, and vimentin: 37.48%; and in SW48 cells: β-catenin: 35%, survivin: 46.6%, and vimentin: 70%). In SW48 cells that were treated with 5-FU, no significant reduction in protein levels was observed; nevertheless, in HCT-116 cells, a significant reduction in protein levels was seen (β-catenin: 39%, survivin: 10%, and vimentin: 20%). Our results demonstrated that in both cell lines in 5-FU +ZER-treated cells, the protein levels were decreased significantly more than each agent alone (in HCT-116: β-catenin: 74.6%, survivin: 80.4%, and vimentin: 40%) and (in SW48 cells: β-catenin: 61.7%, survivin: 58.5%, and vimentin: 73.3%).

Metastasis is the leading cause of cancer mortality and accounts for about 11% of cancer-related deaths. Metastasis consists of separation, migration, invasion, and adhesion. It is regulated by different signaling pathways and affected by the surrounding extracellular matrix (ECM) [37]. Studying cell migration and the involved factors provides valuable insights into cancer diagnosis, prognosis, treatment, and drug development. The inhibition of migration in HCT-116

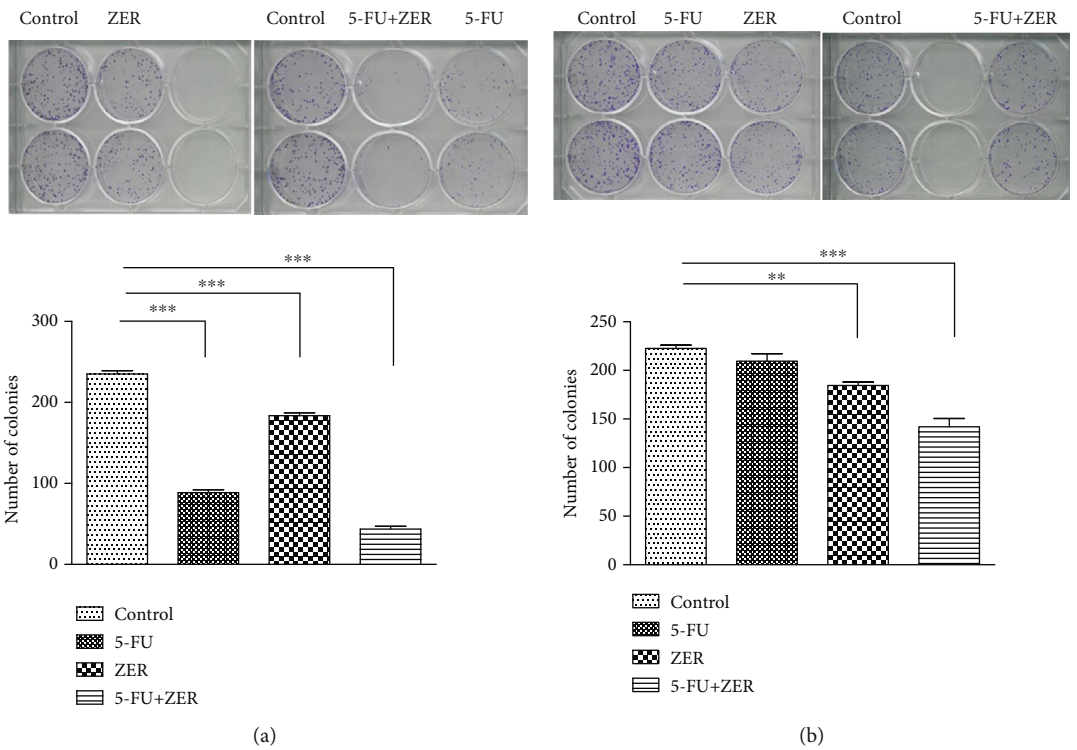


Figure 12: The inhibitory effect of ZER, 5-FU, and 5-FU+ZER on colony formation of (a) HCT-116 and (b) SW48 cells. In both cell lines, the number of colonies in the treated group showed a significant decrease compared to the control group with the exception of the 5-FU-treated group in SW48 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

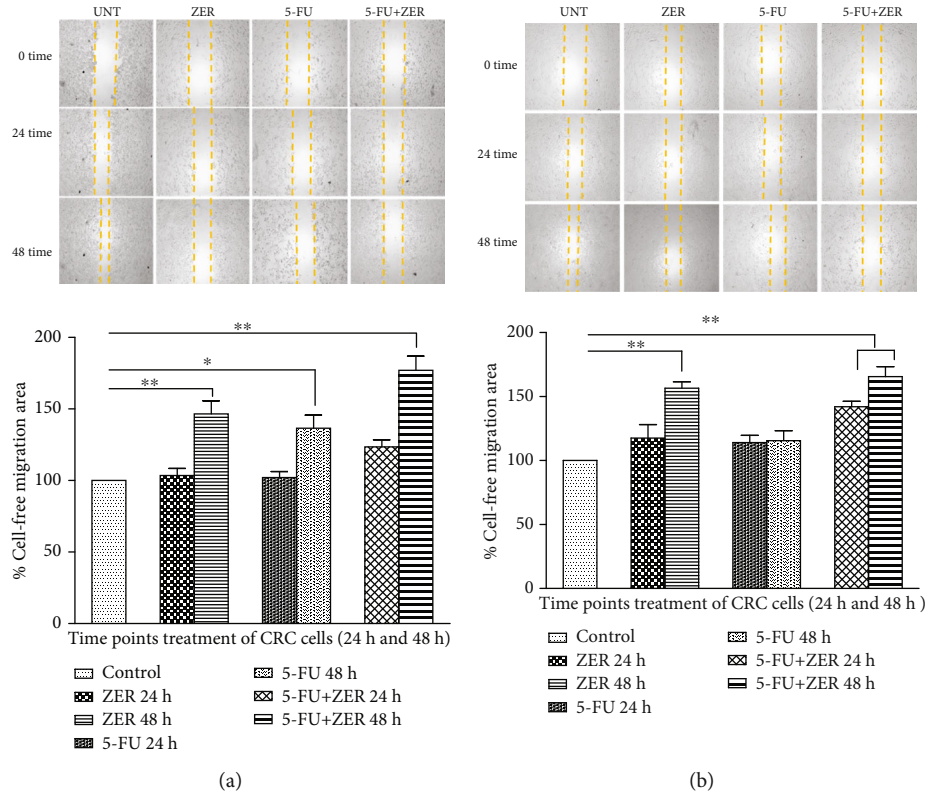
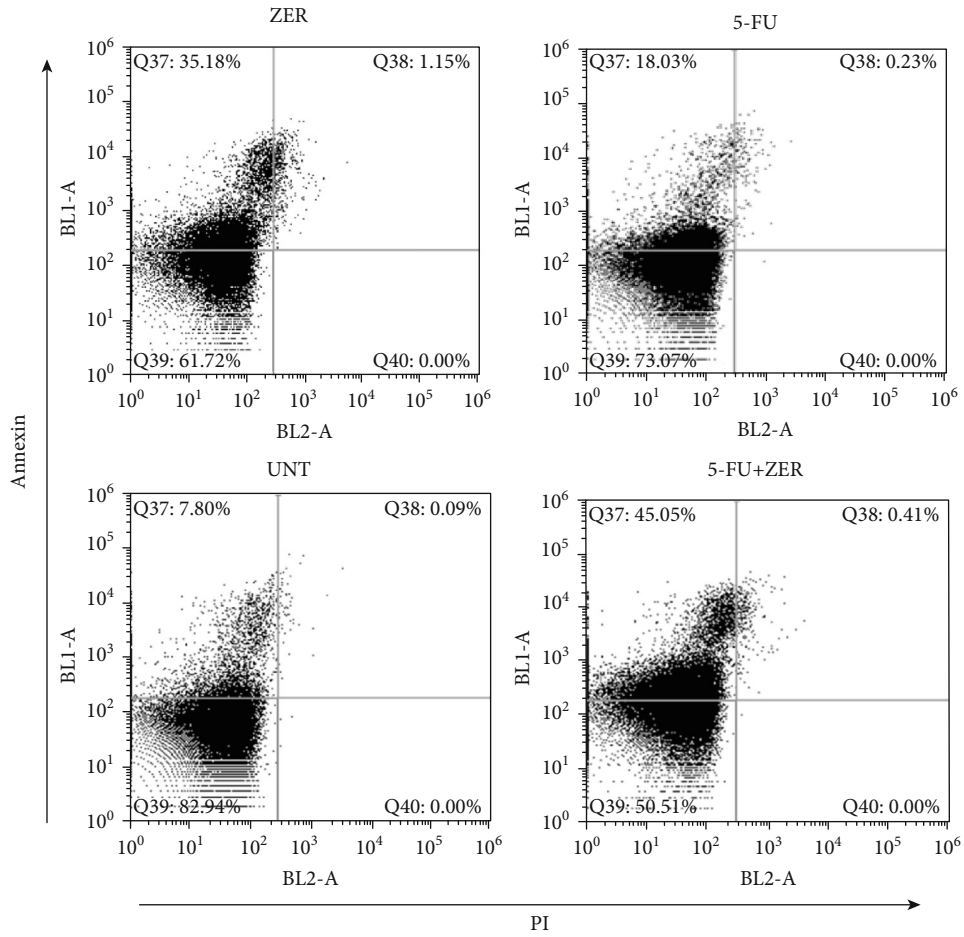
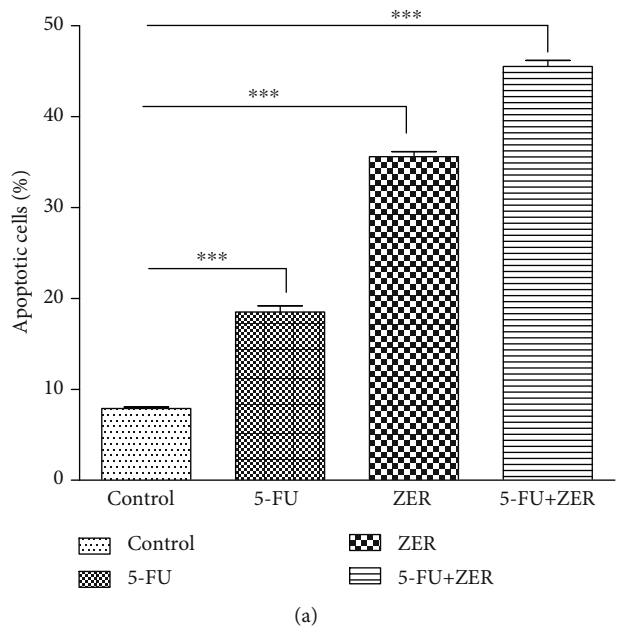


Figure 13: The inhibitory effect of 5-FU, ZER, and 5-FU+ZER on cell migration abilities of CRC cells. (a) HCT-116 and (b) SW48 cells were scratched using a 100 μl tip, and the wound widths were recorded at 0, 24, and 48 h postscratch. The widths were measured using ImageJ software, and the data were analyzed using Prism 5.0. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).





(a)

Figure 14: Continued.

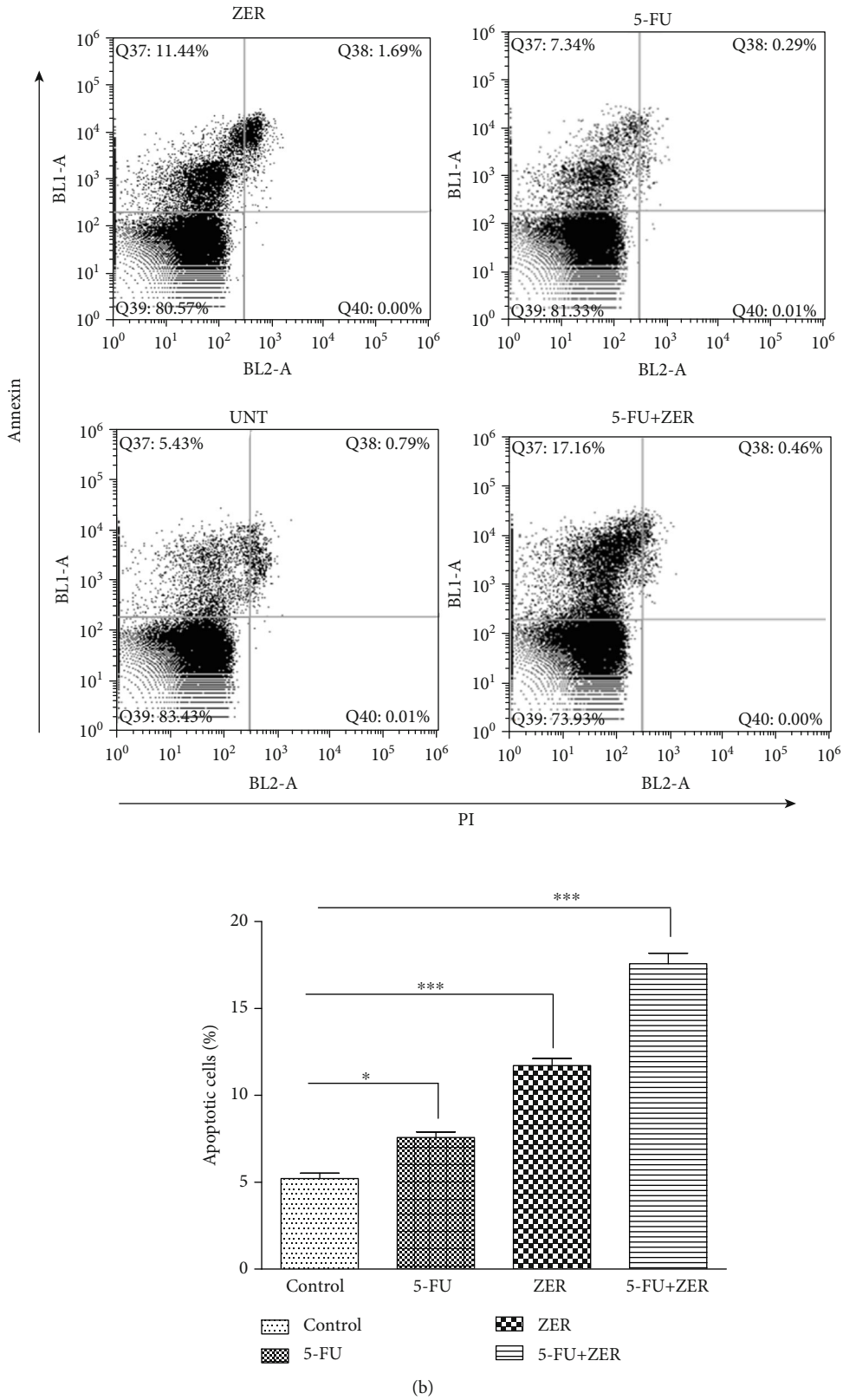


Figure 14: The inhibitory effect of 5-FU, ZER, and 5-FU+ZER treatment on cell apoptosis of CRC cells. (a) HCT-116 and (b) SW48 cells were stained with Annexin V-FITC and propidium iodide and analyzed using the flow cytometry system. The percent of apoptotic cells was increased in all treatments in both cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group ($n = 2$).

cells was due to the effect of ZER, 5-FU, and 5-FU+ZER treatment, while in SW48 cells, no inhibition was seen in the 5-FU treatment group. Similar to our findings, Manmuan et al. in 2018 reported the combinatory effects of oxymatrine (alkaloid compound derived from Sophora flavescens root) along with 5-FU markedly reduced migration of CRC cells in 24 and 48 h [38]. Colony formation is a method used to assess the independent cell proliferation of a cancer cell, during which a single tumor cell with a high proliferation rate forms a colony in the plate within a few weeks [39]. Our results demonstrated a significant reduction in all three groups with a further inhibitory effect of combination in HCT-116 cells, while in 5-FU-treated SW48 cells, no significant inhibitory effect was observed. Liu et al. in 2018 examined the effect of HQGGT (a Chinese herbal compound) along with 5-FU in H630R1 and MC38 cells of CRC. Their result revealed that the combination of HQGGT and 5-FU reduced the number of colonies more effectively than 5-FU alone [40].

Apoptosis is a physiological process in which cell death is caused by a cascade of events. It leads to the programmed removal of specific cells, without harming neighbor cells. Any changes in this process could result in a variety of diseases. In a study by Fang et al. in 2019, ethanolic extract of Spica Prunellae (EESP) (a Chinese drug) along with 5-FU increased the sensitivity of 5-FU-resistant cells (HCT-8/5-FU) and increased the apoptosis rate compared to the individual use of each component [41].

EGFR is a transmembrane protein receptor that is involved in the pathogenesis and progression of many malignancies. They are generally activated once ligands bind to the extracellular domain. After binding to their ligands, intracellular cascade reactions occur, which mainly cause cell proliferation [42]. Several studies have found various genetic changes, for example, the mutations in EGFR family in many types of tumors, including colon cancer. The mutations of EGFR, the low expression of EGFR, and the changes in its ligands have been shown to be related to drug resistance [43]. In this regard Gu et al. in 2019 demonstrated that EGFR contributed to 5-FU resistance in colon cancer cells through autophagy induction. Therefore, their results highlight the potential clinical utility of targeting autophagy genes [44]. Dysregulation of the autophagy pathway and the various signaling pathways involved in this process in cancer cells is closely related to drug resistance of tumors. The PI3K/AKT/mTOR and mitogen-activated protein kinase (MAPK) pathways are the main regulators of autophagy [45]. As mentioned earlier, mutations in the EGFR pathway could be one of the factors that contribute to drug resistance. As HCT-116 and SW48 cell lines have different molecular profiles of EGFR, SW48 cells are bearing a mutation of EGFR, but the HCT-116 cells have wild-type EGFR; it could be assumed that the observed drug resistance in SW48 cells is associated with the EGFR pathway.

Therefore, the main purpose of this study was to compare the effects of the combination of ZER and the chemotherapeutic agent, 5-FU alone, and together on the expression of important markers involved in progression, migration, proliferation, and apoptosis of CRC cells. Altogether, our findings suggest that ZER may be a promising compound to be used in combination treatment regimens to induce chemosensitization to 5-FU in CRC cell lines through downregulation of EMT marker (vimentin), apoptosis marker (survivin), and the Wnt/β-catenin pathway in CRC cells.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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