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ORIGINAL ARTICLE

Genistein Enhances TRAIL-Mediated Apoptosis Through the Inhibition of *XIAP* and *DcR1* in Colon Carcinoma Cells Treated with 5-Fluorouracil

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ABSTRACT

Objectives: Colorectal cancer is one of the most common cancers worldwide. However, surgical intervention and chemotherapy provide only limited benefits for the recovery and survival of patients. The anticarcinogenic effect of genistein has attracted attention because epidemiological studies have shown that soybean consumption is associated with a decrease in the incidence of cancer. There are limited studies on the effects of genistein in colorectal carcinoma cells. We aimed to investigate the cytotoxic, genotoxic, and apoptotic effects of genistein in SW480 and SW620 colon adenocarcinoma cells treated with 5-fluorouracil, the basis of chemotherapy, and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) ligand, the mediator of apoptosis, both alone and in combination.

Materials and Methods: Cytotoxicity and genotoxicity were determined by MTT and comet assays, respectively. The apoptotic effects were evaluated by reverse transcription-polymerase chain reaction assay, with the additional use of Annexin V FITC, mitochondrial membrane potential (MMP), caspase 3, 8, and 9 activity, and reactive oxygen species (ROS) assay kits.

Results: According to our findings, genistein, 5-fluorouracil, and TRAIL had synergistic apoptotic effects because of DR5 upregulation, ROS production, and DNA damage, which were mediated by increased caspase-8, and -9 activity and decreased MMP.

Conclusion: The applied combinations of these compounds may contribute to the resistance problem that may occur in treating colorectal cancer, with a decrease in *DcR1* and *XIAP* genes.

Keywords: Genistein, 5-fluorouracil, TRAIL, apoptosis, colorectal cancer

INTRODUCTION

According to the International Agency for Research on Cancer, 23 million new cancer cases are expected annually by 2030. Colorectal cancer is among the most diagnosed cancers in the world, along with breast and lung cancers.[1] Although the incidence and mortality of colorectal cancer vary by gender, age, and race, colorectal cancer ranks third among cancers diagnosed in men and second in women according to GLOBOCAN (2018) data.[2,3] In Türkiye, colorectal cancer ranks 3rd and 4th in terms of prevalence in women and men, respectively.[4]

Colorectal cancer is characterized by the transformation of intestinal epithelial cells into carcinoma tissue due to inflammatory stress, genetic variation, and environmental factors such as modern consumption habits, smoking, and alcohol consumption.[5] Highly invasive and metastatic colorectal cancer cells, if left untreated, can spread to other organs, most often the liver and lymphoid organs.[6] However, patients are often diagnosed only at an advanced or metastatic stage. The presence of problems such as tumor regeneration and progression due to drug resistance in the applied chemotherapeutic treatment and

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the cellular damage caused by mechanisms such as apoptosis or cell cycle arrest with chemotherapeutic drugs is found not only in cancer cells but also in normal cells. It has necessitated the development of different treatment principles for cancers.[5,7] Therefore, it is important to increase the effectiveness of existing chemotherapeutic drugs by combining them with some agents and to find a solution to the drug resistance problem.[7]

Epidemiological data have shown that soy consumption reduces the risk of colon cancer and phytoestrogens may protect against colorectal cancer.[1] Genistein, which was first isolated from *Genista tinctoria* L., a phytoestrogenic isoflavonoid compound that is particularly abundant in Fabaceae (formerly Leguminosae) plants, has drawn attention with its anticarcinogenic effect.[1,8,9] It has been suggested that genistein may be beneficial in the prevention and treatment of many types of cancer, including colorectal cancer, by showing anticancer activity in several ways, including the inhibition of Nf-κB signaling, accumulation of cancer cells in the G2/M phase with the affecting of cyclin-dependent kinases (CDK), induction of apoptosis, attenuation of multiple drug resistance through various signaling pathways [protein kinase B, mitogen-activated protein kinase, epidermal growth factor receptor (EGFR)], and induction of proapoptotic caspase-3, caspase-9, and *Bax* protein expressions in the apoptotic pathway.[10-15]

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a type II transmembrane protein consisting of 281 amino acids belonging to the TNF superfamily and is synthesized in tissues such as the colon and thymus. It induces apoptosis through the extrinsic pathway by binding to 5 different membrane-bound receptors, namely *DR4, DR5, DcR1, DcR2,* and osteoprotegerin. *DcR4, DcR5,* and osteoprotegrin inhibit apoptosis by acting as decoy receptors.[16] Although the induction of the extrinsic pathway *via* the TRAIL ligand, which does not have a cytotoxic effect on normal cells, is a potential alternative in cancer treatment,[17,18] the development of resistance to TRAIL-induced apoptosis can also be seen in some cancer cells.[19,20] However, it has been determined that the combination of TRAIL and chemotherapeutics or phytotherapeutics may overcome TRAIL resistance in cancer cells.[21-23]

In this study, we aimed to investigate whether the combination of genistein and 5-fluorouracil, a backbone of colorectal cancer treatment,[24] can enhance TRAIL-mediated apoptosis in SW480 and SW620 human colorectal adenocarcinoma cells.

MATERIALS AND METHODS

Cell culture

The SW480 and SW620 cell lines were derived from a single patient at the primary and secondary stages. Therefore, these cell lines may represent a useful model for colon cancer progression. The SW480 cell line (human colorectal adenocarcinoma) was provided by Serkan İsmail Göktuna (Bilkent University Faculty of Science, Department of Molecular Biology and Genetics, Türkiye), and the SW620 (metastatic human colorectal adenocarcinoma) cell line (ATCC® CCL-227) was purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (Wisent) containing 1% penicillin-streptomycin (Wisent) and 10% fetal bovine serum (Capricorn). The cells were maintained at 37 °C in an incubator in a humidified atmosphere of 5% CO_2 (Heraeus Instruments).

Cell viability assay

Thiazolyl blue tetrazolium bromide (MTT, Sigma) assay was used to assess cell viability and determine IC_{50} values. IC_{50} value is the concentration of the compounds necessary to kill one-half of the cell population,[25] as previously described by Mosmann[26] and Ferrari et al.[27] Briefly, SW480 and SW620 cells were cultured, and 10,000 cells were seeded in the chambers of a 96 well plate and allowed to attach for 24 hours. After incubation, the cells were exposed to different concentrations of genistein LKS (5-200 µM) dissolved in dimethyl sulfoxide [DMSO (Sigma)]. The final concentration was 0.5% (*v/v*). For 5-fluorouracil (Sigma-Aldrich) (5-800 µM) dissolved in DMSO, the final concentration was 0.5% (*v/v*). TRAIL (Cell Applications) (50-200 ng/mL) dissolved in sterile distilled water containing 0.1% (*v/v*) bovine serum albumin (Capricorn) in the medium for 24, 48, and 72 hours at 37 °C in a humidified atmosphere of 5% CO_2. When the exposure time ended, the cell medium was aspirated and 10 µL of MTT (Sigma) solution [5 mg/mL in phosphate buffer saline (PBS)] was added to each well. After 4 hours of incubation, the cell medium was replaced with 100 µL DMSO and the plates were shaken for 5 min. The absorbance was determined at 570 nm using a microplate reader (SpectraMax M2), and IC_{50} values were calculated using concentration-response curves to express the effects of the test materials on cell viability. The combination index (CI) for drug interaction was calculated using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). The values of CI < 1 indicate synergistic interaction, while the values > 1 or not significantly different from 1 specify antagonistic or additive interaction, respectively.[28] After determining the synergistic effects of the test compounds by the MTT assay, the cells were exposed to the double and triple combination of these compounds for 48 hours, and the same procedure was followed as described previously. The results are presented as the mean ± standard deviation from three independent experiments.

Cell recovery assay

The cell recovery assay was performed as described by Li et al.[29] with modifications to determine the proliferative capacity of the cells after the removal of genistein (0.25 µM, 7.5 µM), 5-fluorouracil (1 µM), TRAIL (5 and 10 ng/mL), and their combinations. Briefly, SW480 and SW620 cells were cultured and 2 x 10^4 cells were seeded in the chambers of a 96 well plate and allowed to attach for 24 hours. After incubation, the cells were exposed to several concentrations of genistein, 5-fluorouracil, TRAIL, and their combinations in a medium for 48 hours at 37 °C in a humidified atmosphere of 5% CO_2. Then, the cells for each treatment group were typsinized (Trypsin-EDTA, Sigma) and counted (5 x 10^3) to seed in triplicate in a 96 well plate in test compound-free medium for 48 hours incubation. The MTT assay was used to evaluate cell recovery. The results are presented as the mean ± standard deviation from three independent experiments.

Alkaline comet assay

The basic alkaline comet assay was performed to determine DNA damage as described by Singh et al.[30] with the modifications of Hartmann et al.[31] Briefly, SW480 and SW620 cells were seeded at a density of 15000 cells/200 µL in 96 well plates and allowed to attach for 24 hours. The cells were then exposed to different concentrations of genistein (0.125 µM, 0.25 µM, 0.5 µM), 5-fluorouracil (1 µM), TRAIL (5 and 10 ng/mL), and their combinations for 48 hours. A negative control (0.5% DMSO) and a positive control [15 µM H_2O_2 (Merck)] were used. After incubation, the cells were re-suspended in 0.75% low melting point agarose (Boehringer Mannheim) and this suspension was spread on pre-coated slides coated with 1% normal melting point agarose (Sigma) and allowed to dry. After removing the coverslip, the slides (Marienfeld) were submerged in lysing solution [2.5 M NaCl (Sigma), 100 mM EDTA (Merck), 100 mM Tris (Sigma), 1% sodium sarcosinate (Sigma), 1% Triton-X 100 (Sigma), and 10% DMSO, pH 10] at 4 °C for 24 hours. Afterwards, the slides were left in an electrophoresis solution [300 mM NaOH (Merck) and 1 mM sodium EDTA (Merck), pH 13] at 4 °C for 20 min, and electrophoresis was performed at 4 °C for 20 min by applying an electrical current of 300 mA and 24 V in the electrophoresis equipment (Biometra Analytical). The slides were then washed in a neutralizing solution [0.4 M Tris-HCl (Sigma), pH 7.5] for 15 min and incubated in 50%, 75%, and 98% ethyl alcohol (Sigma-Aldrich) for 5 min successively. The dried slides were stained with EtBr (Sigma-Aldrich, 20 µg/mL in distilled water, 60 µg/slide) and examined using a Leica® fluorescence microscope. A computer-based analysis system (Comet Analysis Software, version 4.0, Kinetic Imaging Ltd., Liverpool, UK) was used to measure DNA damage. To visualize DNA damage, 100 nuclei *per* slide were examined at 400x magnification. DNA damage was expressed as the percentage of DNA in the tail (tail intensity). Values are expressed as the mean ± standard deviation from three independent experiments.

Analysis of cell surface expressions of DR4 and DR5 surface receptor proteins

Cells were cultivated at a density of 6 x 10^6 cells/25 cm^2 cell culture flask (Nest) for 24 hours and treated with genistein (0.5 µM for SW620; 1 µM for SW480 cells), 5-fluorouracil (1 µM) and their combinations for 48 hours. After incubation, the cells were trypsinized and suspended in a serum-free medium (Wisent) at a density of 1 x 10^6 cells/mL. The cells were then washed twice with 2 mL cell-staining buffer (Biolegend) and suspended in cell-staining buffer at a density of 1 x 10^6 cells/500 µL. 5 µL Phycoerythrin-conjugated mouse anti-human DR4, DR5, and IgG2B for isotype control (Biolegend) was added to cells and incubated at 4 °C for 30 min. After staining, the cells were washed twice with 2 mL cell staining buffer and suspended in 0.5 mL cell staining buffer for flow cytometry (Beckman Coulter, Cytoflex USA).

Cell cycle analysis by flow cytometry

The cells were cultivated at a density of 2 x 10^6 cells/4 mL in 25 cm^2 cell culture flasks. After incubation for 24 hours, the cells were treated with genistein (1 µM for SW480; 0.5 µM for SW620), 5-fluorouracil (1 µM), TRAIL (10 ng/mL for SW480; 5 ng/mL for SW620), and their combinations for 48 hours. The cells were trypsinized and suspended in 1 mL PBS (Wisent) at a density of 1.5 x 10^6 cells/mL. The cells were then fixed with 2 mL of ethanol (99%) and incubated for 24 hours. After washing the cells twice with PBS, 70 µL Ribonuclease A from bovine pancreas (RNase A, Sigma Aldrich) and 100 µL propidium iodide (PI, Sigma Aldrich) were added and incubated in the dark for 30 min. Cell cycle analysis was then performed using a flow cytometer (Beckman Coulter, Cytoflex USA). The results are presented as the mean ± standard error from three independent experiments.

Apoptosis detection

Apoptosis was measured with an Annexin V FITC apoptosis detection kit I (BD Pharmingen) according to the manufacturer's instructions. Briefly, the cells were collected after treatment with genistein (1 µM for SW480, 0.5 µM for SW620), 5-fluorouracil (1 µM), TRAIL (10 ng/mL for SW480, 5 ng/mL for SW620) for 48 hours and washed twice with PBS. Then, 1X binding buffer was added (10^6 cells/mL) to cells and 100 µL of the cell suspension was transferred to 5 mL culture tubes. Cells were incubated with 5 µL Annexin V FITC and 5 µL PI for 15 min at room temperature in the dark. Subsequently, apoptosis was analyzed using a flow cytometer after adding 400 µL 1X binding buffer. The results are presented as the mean **±** standard error from three independent experiments.

Measurement of intracellular reactive oxygen species (ROS) levels

The intracellular ROS level was determined using a ROS detection assay kit (Biovision), following the kit instructions. In brief, the cells were washed with 100 µL assay buffer and 100 µL 1X ROS label was added and incubated at 37 °C for 45 min in the dark. Cells were washed with PBS and treated with genistein (1 µM for SW480, 0.5 µM for SW620), 5-fluorouracil (1 µM), and TRAIL (10 ng/mL for SW480, 5 ng/mL for SW620) for 48 h. Fluorescence was measured at the desired time intervals by a microplate reader (SpectraMax M2) at an excitation of 495 nm and emission of 529 nm (Ex/Em= 495/529 nm).

Measurement of the mitochondrial membrane potential (MMP)

The effect of the test compounds on MMP was determined using a JC-1 MMP Assay Kit (Cayman). Briefly, the cells were collected after treatment with genistein (1 µM for SW480, 0.5 µM for SW620), 5-fluorouracil (1 µM), and TRAIL (10 ng/mL for SW480, 5 ng/mL for SW620) for 48 hours. Then, JC-1 staining solution was added to the cells. The cells were then incubated at 37 °C for 15 min in a humidified atmosphere of 5% CO_2 and washed twice with the assay buffer. Measurements were performed using a fluorescent plate reader (SpectraMax M2) for healthy cell detection (E_x/E_m= 535/595 nm) and for apoptotic cells (E_x/E_m= 485/535 nm).

Determination of activities of caspase 3, 8 and 9

Caspase 3, 8, and 9 activities were determined using the caspase 3, 8, and 9 Multiplex Activity Assay Kit (Abcam). In brief, the cells were collected after incubation with genistein

(1 μM for SW480, 0.5 μM for SW620), 5-fluorouracil (1 μM), and TRAIL (10 ng/mL for SW480, 5 ng/mL for SW620) for 48 hours. Next, caspase assay solution (containing caspase 3, 8, and 9 substrates) was added to the cells, followed by incubation at room temperature for 60 min. The fluorescence was measured at an excitation of 535 nm and emission of 620 nm for caspase 3, at an excitation of 490 nm and emission of 525 nm for caspase 8, and at an excitation of 370 nm and emission of 450 nm for caspase 9.

Reverse transcription-polymerase chain reaction analysis of gene expression

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine the effects of the test compounds on apoptotic and antiapoptotic gene expression. First, total RNA was isolated from cells treated with genistein (1 μM for SW480, 0.5 μM for SW620), 5-fluorouracil (1 μM), and TRAIL (10 ng/mL for SW480, 5 ng/mL for SW620) for 48 hours using an RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Then, an RT² HT First Strand Kit (Qiagen) was used to synthesize the first strand cDNA. In brief, reverse transcription was performed using 500 ng of total RNA at 42 °C (15 min) and 95 °C (5 min) in a thermal cycler (Corbett). Afterward, real-time PCR reactions were performed using RT² qPCR SYBR Green MasterMix-2 (Qiagen) and RT² qPCR Primer Assay (Qiagen). Primers *Bcl-XL* (PPH00082C), *Bcl-2* (PPH00079B), *XIAP* (PPH00323A), *DR4* (PPH00842A), *DR5* (PPH00241C), *DcR1* (PPH00837A), *DcR2* (PPH00838B), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (PPH00150F) were purchased from Qiagen. Each cycle was performed at the conditions of holding 95 °C for 15 min, cycling at 95 °C for 15 s, and cycling at 60 °C for 30 s for 40 cycles. The relative changes in the number of transcripts in each treatment were calculated by normalizing with *GAPDH* mRNA levels. The values indicate the mean ± standard error.

Statistical analysis

Statistical analysis of data was performed using SPSS 20.0 for Windows. The distribution of the data was checked for normality using the Shapiro-Wilk test. The Levene test verified the homogeneity of variance. The differences among the means of data with normal distribution were evaluated by one-way variance analysis (ANOVA), and *post-hoc* analyses of group differences were performed by Tukey's test for homogeneous variance and Dunnett's T3 test for non-homogeneous variance. Differences among the groups without normal distribution were evaluated by the Kruskal-Wallis test followed by the Mann-Whitney U test. *p* values of 0.05 and 0.001 were considered statistically significant.

Statistics of the Ct values for RT-PCR analysis were performed using the RT² Profiler PCR Data Analysis program of QIAGEN. Significance was determined on the basis of fold change from the control ΔΔCt value and $p < 0.05$ was considered statistically significant.

RESULTS

Genistein, 5-fluorouracil, and TRAIL inhibit colon cancer cell growth in vitro

Genistein, 5-fluorouracil, and TRAIL dose-dependently inhibited the growth of SW480 and SW620 cells for 24, 48, and 72 hours. In addition, 5-fluorouracil had time-dependent inhibitory effects in SW480 and SW620 cells. The IC_{50} values of genistein and 5-fluorouracil could not be determined in the studied concentration range for 24 hours and 72 hours, but IC_{50} of TRAIL was determined to be 93.35, 138.4, and 192.9 ng/mL for 24 hours, 48 hours, and 72 hours, respectively, in SW480 cells. Furthermore, IC_{50} of genistein was 375.8 μM for 48 hours and had a significant inhibitory effect above 200 μM in SW480 cells compared with the negative control (0.5% DMSO). The IC_{50} values of genistein were 351.1 μM and 190.6 μM for 48 and 72 hours, respectively, in SW620 cells. Additionally, the IC_{50} of 5-fluorouracil-treated SW620 cells was 794.4 μM for 48 hours. The IC_{50} values of TRAIL for 24, 48, and 72 hours in SW620 cells were determined to be 20, 19.43, and 50.16 ng/mL, respectively (Figure 1 a-f).

The combined effects of genistein (G), 5-fluorouracil (F), and TRAIL (T) were also evaluated, and more growth inhibition was observed in double and triple combinations than in single treatment with these compounds. In addition, triple combinations had more inhibitory effects than double combinations (Figure 1g and h).

A CI-based analysis was used to determine the synergistic effects of the compounds (Figure 2). 1 μM genistein for SW480 cells and 0.5 μM genistein for SW620 cells were selected according to the analysis. The CI values of G + T, G + F, and G + F + T were 0.02, 0.003, and 0.123 for 1 μM genistein, respectively, in SW480 cells and 0.73, 0.015, and 0.59 for 0.5 μM genistein, in SW620 cells. Furthermore, the reversibility of cell growth inhibition was evaluated using a recovery MTT assay. It was found that TRAIL had the most significant effect on the loss of ability to recover in SW480 and SW620 cells (Figure 3). Triple combinations were more effective than double and single concentrations of the compounds. The results showed that there were irreversible changes such as apoptosis in SW480 cells.

Genistein, 5-fluorouracil, and TRAIL cause DNA damage in SW480 and SW620 cells

According to the data of the comet assay, genistein, 5-fluorouracil, and TRAIL caused increases in DNA damage expressed as tail intensity, when compared with the negative control in SW480 and SW620 cells (Figures 4 and 5). The increases in DNA damage in SW480 cells treated with double and triple compounds were more significant than those in the single treatment and negative control groups (0.5% DMSO). However, the highest increases in DNA damage were observed at the concentrations of "0.125 μM G + 5 ng/μL TRAIL" and "0.25 μM G + 5 ng/μL TRAIL" in SW620 cells compared with the negative control (0.5% DMSO).

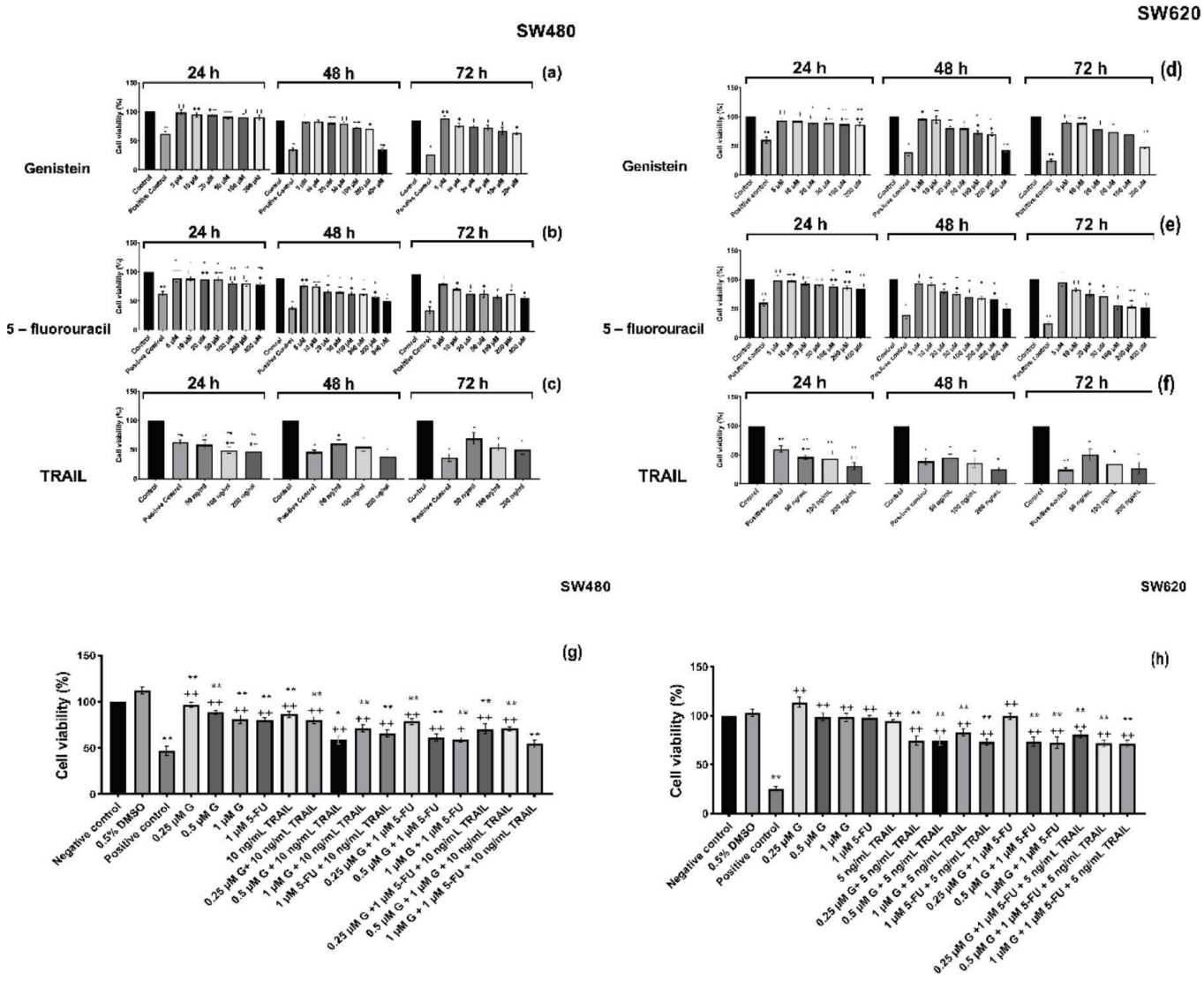

Figure 1. MTT assay results of SW480 and SW620 cells incubated with genistein, 5-fluorouracil (5-FU), and TRAIL. Effects of genistein (a), 5-FU (b), and TRAIL (c) on SW480 and genistein (d), 5-FU (e), and TRAIL (f) on SW620 cell viability for 24, 48, and 72 hours. SW480 (g) and SW620 (h) cell viability after incubation with G, 5-FU, TRAIL, and their combinations for 48 hours

*$p < 0.05$, **$p < 0.001$, indicates significant difference from the negative control. +$p < 0.05$, ++$p < 0.001$, indicates significant difference from the positive control. Results are expressed in the mean ± standard deviation, 5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

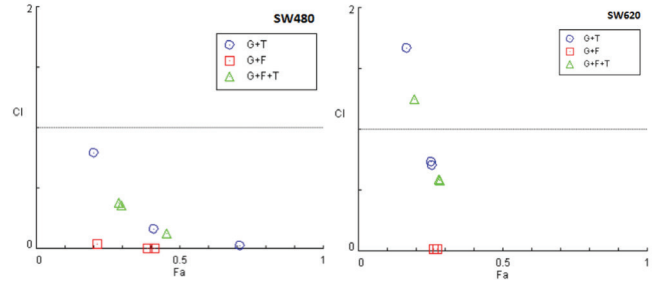

Figure 2. MTT assay results of SW480 and SW620 cells incubated with genistein **(G)**, 5-fluorouracil (5-FU) **(F)**, and TRAIL **(T)** used for calculating the CI by CompuSyn software. CI *versus* Fa graphs of double and triple combinations for interaction of G, F, and T in SW80 and SW620 cells. The values below and above the dashed line indicate synergistic and antagonistic effects, respectively

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand, CI: Combination index, Fa: Factor affected

Genistein and 5-fluorouracil sensitizes TRAIL mediated apoptosis

The effects of genistein, 5-fluorouracil, and TRAIL on the cell cycle were examined by flow cytometry (Supplementary Material 1). When compared with the negative control, the highest percentages of cells in the G0/G1 phase were the 0.5 and 1 µM genistein treated SW620 cells and SW480 cells, respectively. The highest proportions of cells in the S phase were "1 µM 5-FU + 5 ng/mL" TRAIL-treated SW620 cells and 10 ng/mL TRAIL-treated SW480 cells. Furthermore, 0.5 µM genistein and 1 µM 5-fluorouracil increased accumulation of the G2/M phase in SW480 and SW620 cells, respectively.

To examine the apoptotic mechanism of genistein and 5-fluorouracil, DR4, and DR5 surface expressions were investigated. It was found that genistein and 5-fluorouracil sensitized apoptosis *via* the induction of the expression of the

DR5 surface protein (Figure 6 and Supplementary Materials 2 and 3). The proportion of surface DR5 + s in cells treated with 5-fluorouracil was higher than that in both control and genistein-treated cells. In addition, SW480 cells were more sensitive to the apoptotic effects of genistein and 5-fluorouracil than SW620 cells.

The results of the Annexin V FITC apoptosis assay were consistent with *DR5* expression levels. The percentage of apoptotic cells significantly increased at all concentrations of genistein, 5-fluorouracil, TRAIL, and their combinations in SW480 cells. However, triple combinations of these compounds had the highest percentage of early apoptosis, and 5 ng/mL TRAIL significantly increased apoptosis in SW620 cells compared with the negative control (Figure 7).

In addition, the apoptotic mechanism was evaluated by the changes in apoptotic (*DR4* and *DR5*) and antiapoptotic (*Bcl-XL*, *Bcl-2*, *XIAP*, *DcR1*, and *DcR2*) gene expression. Genistein reduced *DcR2* expression in SW480 cells and increased *Bcl-XL*, *Bcl-2*, and *DR4* expression in SW620 cells. When genistein was applied along with the *TRAIL* ligand in SW480 cells, it was determined that *Bcl-XL*, *XIAP*, and *DR5* gene expressions increased when compared with the application of genistein and TRAIL alone. In SW620 cells, this combination was found to cause a decrease in *Bcl-XL*, *DcR1*, and *DcR2* gene expressions and an increase in *DR4* agonistic surface receptors compared with the groups in which they were administered alone. When 5-fluorouracil was administered alone, it caused a significant reduction in *XIAP* genes in SW480 cells. However, it caused significant increases in *Bcl-XL*, *Bcl-2*, *DR4*, and *DcR2* gene expression in SW620 cells.

Bcl-XL and *Bcl-2* gene expressions increased in SW480 cells treated with double and triple combinations of 5-fluorouracil,

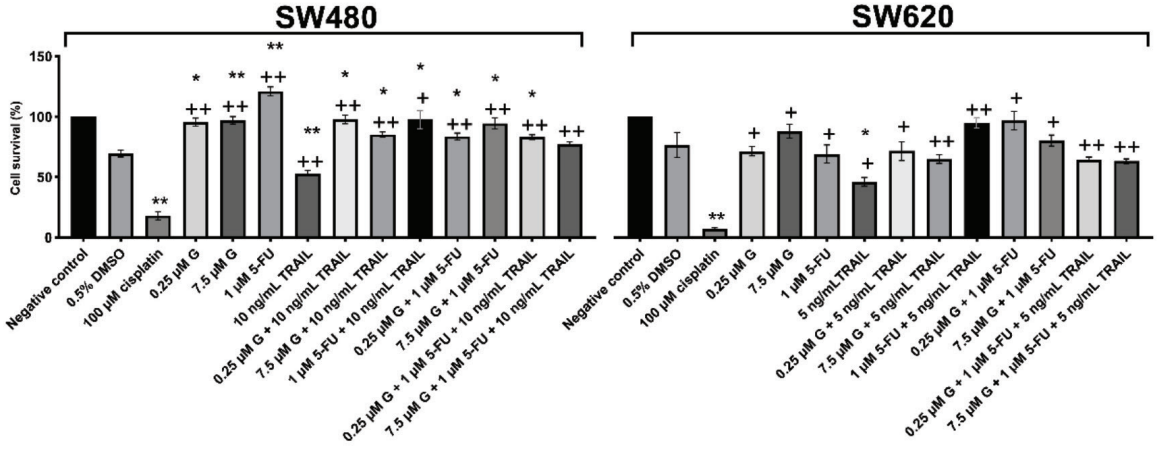

Figure 3. Effects of genistein **(G)**, 5-FU, and TRAIL on proliferation/survival in SW480 and SW620 cells using cell recovery assay

*$p < 0.05$, **$p < 0.001$, indicates a significant difference from the negative control. +$p < 0.05$, ++$p < 0.001$, indicates a significant difference from the positive control. Results are expressed in the mean ± standard deviation.

5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

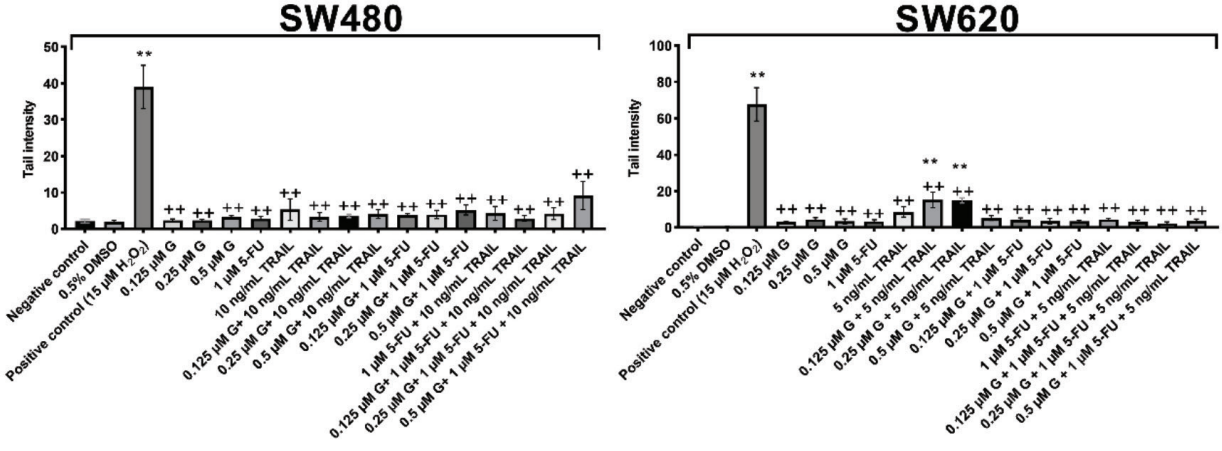

Figure 4. Tail intensity from the comet assay of SW480 and SW620 cells treated with genistein **(G)**, 5-FU, TRAIL, and their combinations for 48 hours

*$p < 0.05$, **$p < 0.001$, indicates significant difference from the negative control. +$p < 0.05$, ++$p < 0.001$, indicates significant difference from the positive control. The values are expressed in the mean ± standard deviation

5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

genistein, and TRAIL ligand; however, a significant decrease in *XIAP* gene expression was observed in all combinations when compared with the groups in which they were administered alone. Similarly, in SW620 cells, *Bcl-XL* and *Bcl-2* expressions increased and there was a decrease in *XIAP* gene expression when compared with the group in which the TRAIL ligand was applied alone (Figures 8, 9).

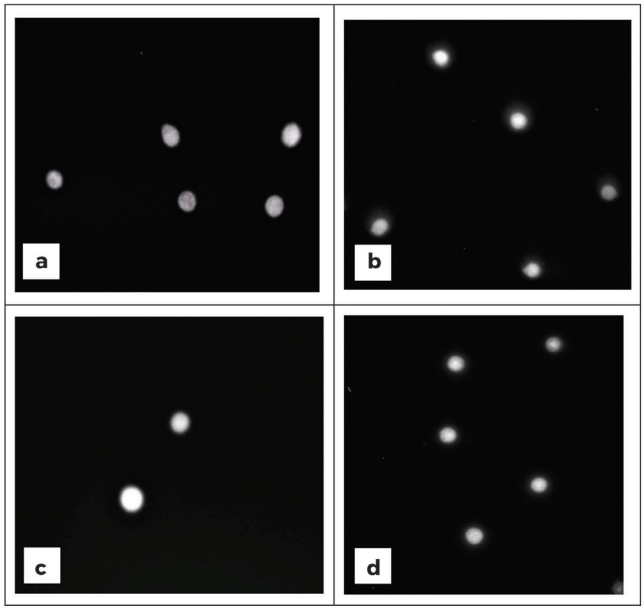

Figure 5. Comet images of SW480 (a, b) and SW620 cells (c, d)

Effects of genistein, 5-fluorouracil, and TRAIL on caspase 3-8-9 activities, MMP, and ROS levels in SW480 and SW620 cells

Caspase activities were investigated to clarify the apoptotic cell death pathway in SW480 and SW620 cells treated with genistein, 5-fluorouracil, and TRAIL. In both cell lines, the triple combination caused the most significant increase in caspase activity compared with the negative control and the groups in which they were administered separately. In addition, the genistein and TRAIL combination was found to be the most effective in increasing caspase 3, 8, and 9 activities (Figure 10).

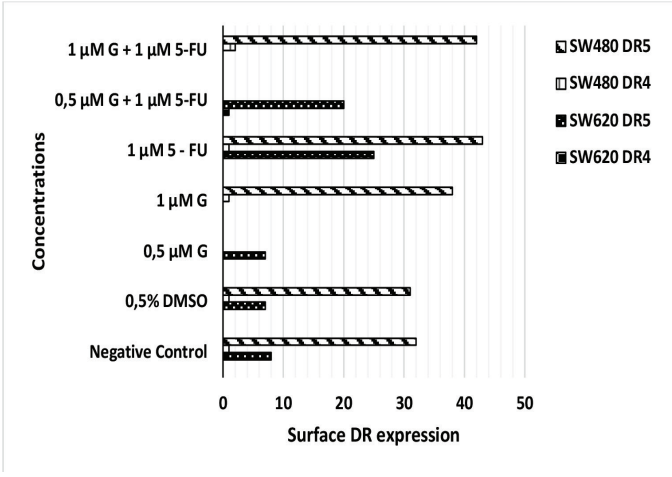

Figure 6. DR4 and DR5 surface expressions of SW480 and SW620 cells incubated with genistein **(G)** and 5-FU by flow cytometry assay

5-FU: 5-Fluorouracil

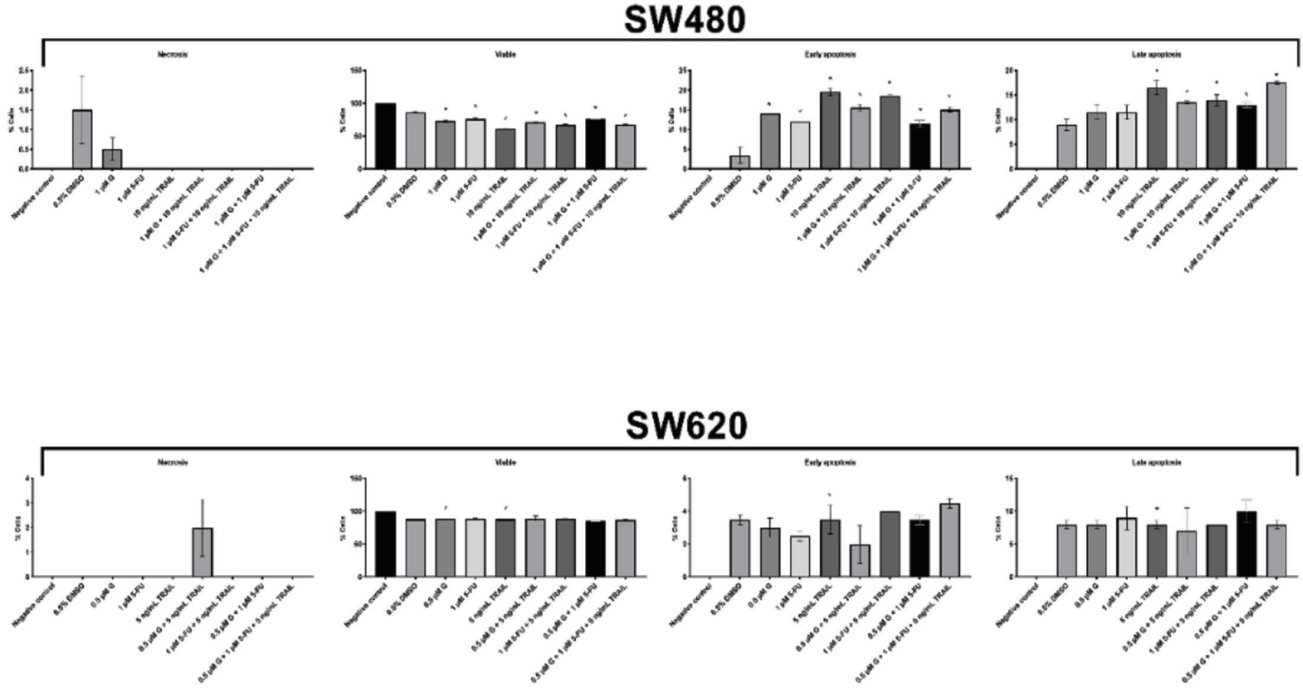

Figure. 7 Results of Annexin V FITC apoptosis assay showing apoptotic effects of genistein, 5-FU, and TRAIL in SW480 and SW620 cells. Results are presented as the mean ± standard error.

*$p < 0.05$, **$p < 0.001$, indicates a significant difference from the negative control, 5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

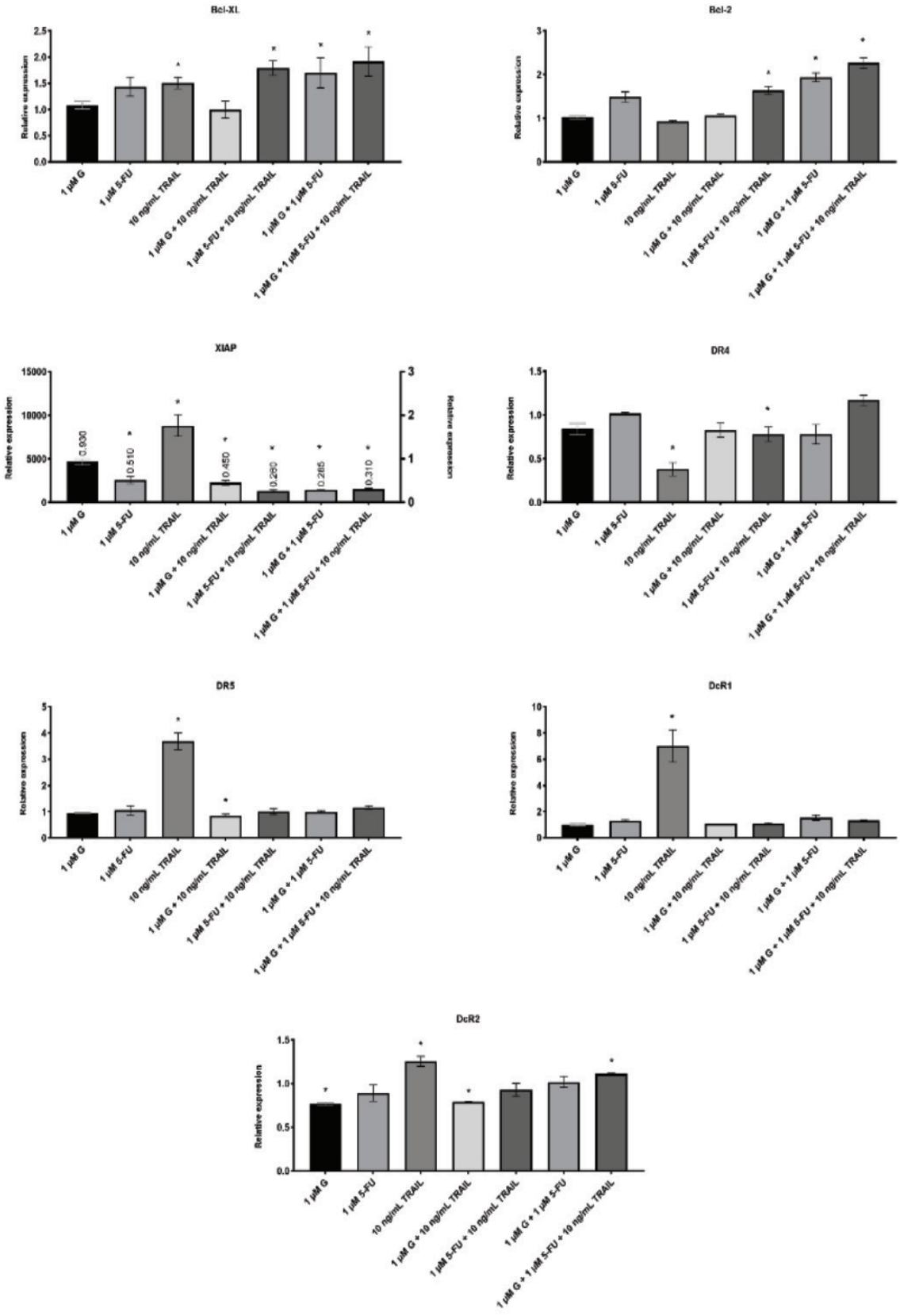

Figure. 8 Relative gene expression of SW480 cells treated with genistein **(G)**, 5-FU, TRAIL, and their combinations by RT-PCR. Results are given as the mean of fold change compared to control (negative and 0.5% DMSO control). The values indicate the mean ± standard error and normalized with *GAPDH*

*$p < 0.05$, indicates a significant difference from the negative control, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand, RT-PCR: Reverse transcription-polymerase chain reaction, DMSO: Dimethyl sulfoxide, 5-FU: 5-fluorouracil, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

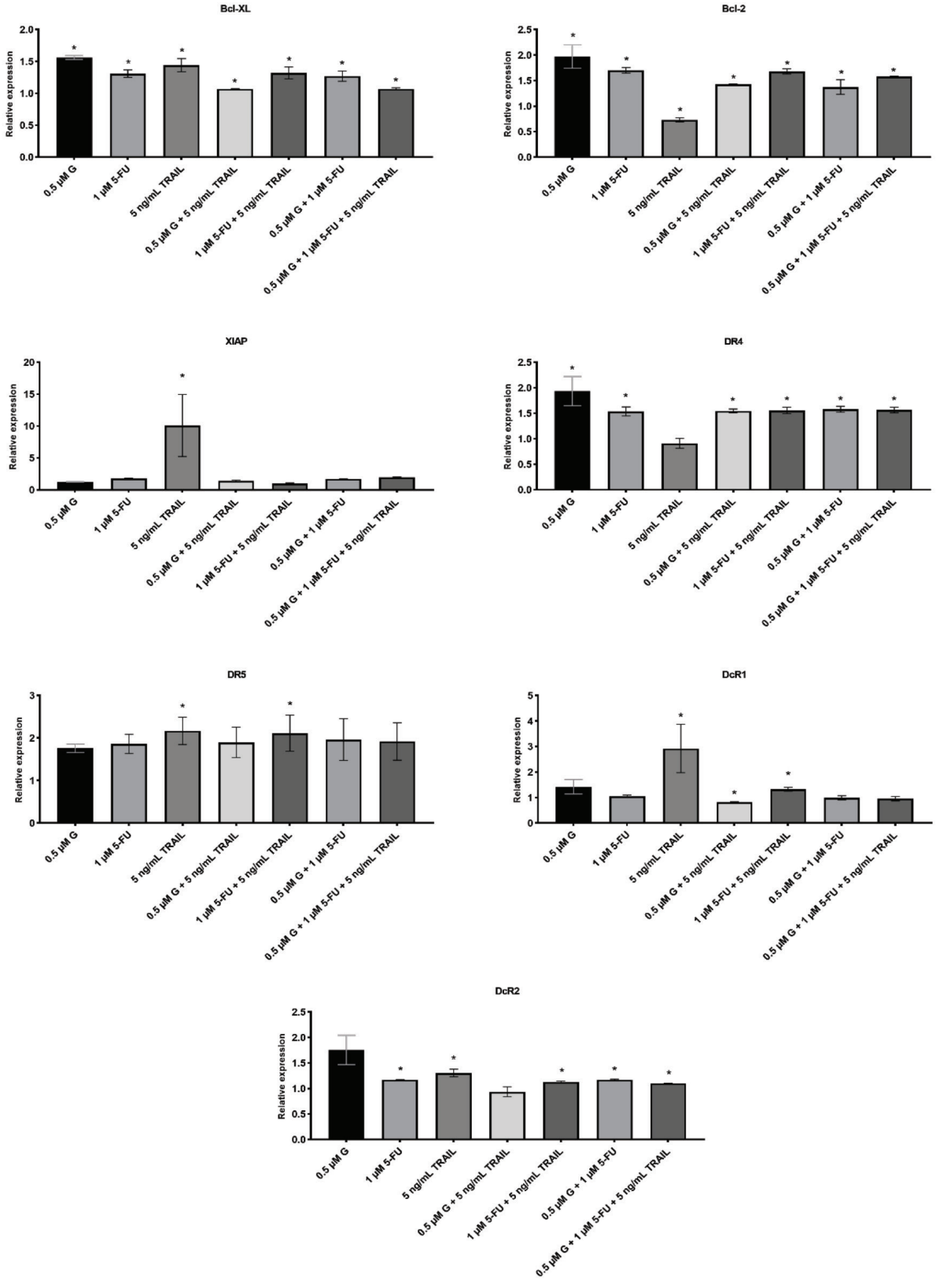

Figure 9. Relative gene expression of SW620 cells treated with genistein **(G)**, 5-FU, TRAIL, and their combinations by RT-PCR. Results are given as the mean of fold change compared with controls (negative and 0.5% DMSO control). The values indicate the mean ± standard error and are normalized with *GAPDH*

*$p < 0.05$, indicates significant difference from the negative control, 5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand, RT-PCR: Reverse transcription-polymerase chain reaction, DMSO: Dimethyl sulfoxide, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

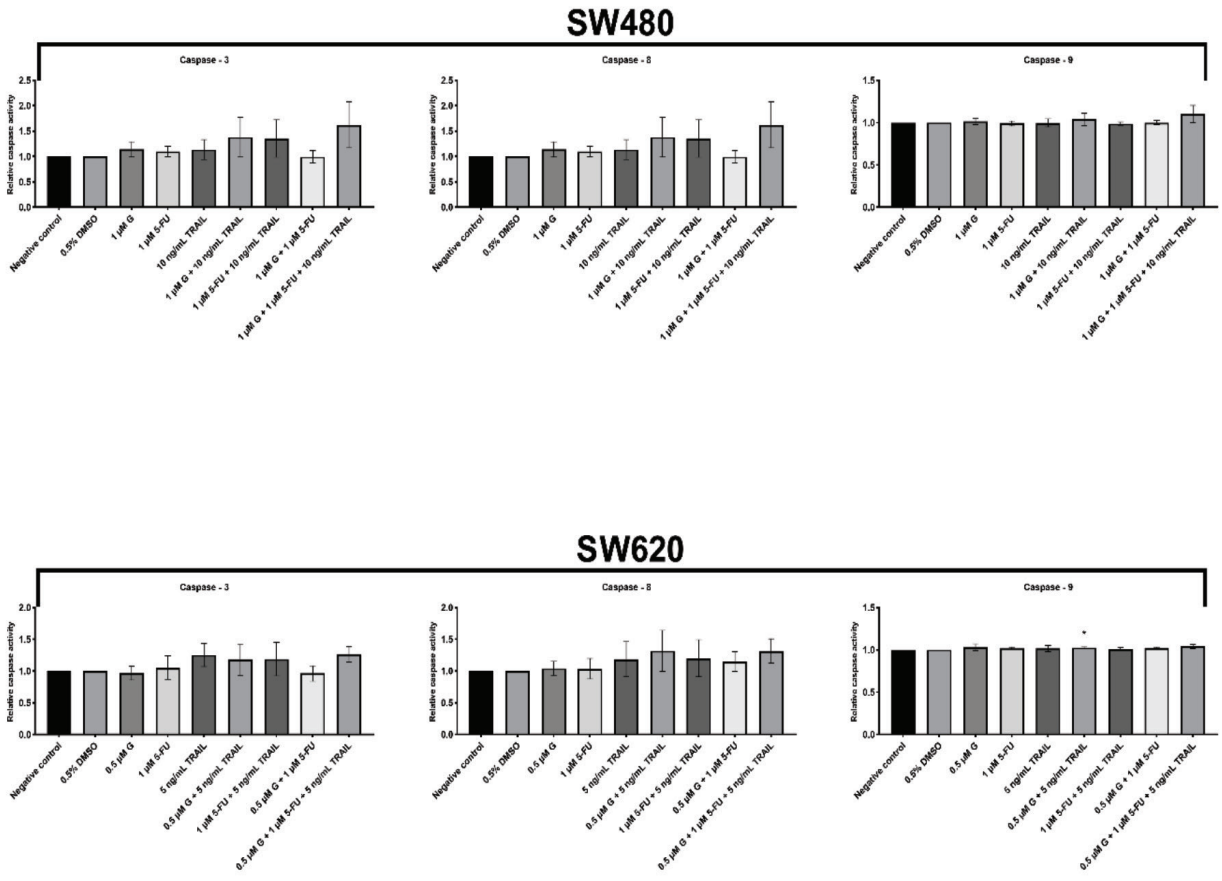

Figure 10. Caspase 3, 8, and 9 activities relative to control in SW480 and SW620 cells by Multiplex Activity Assay Kit (Abcam). Results are presented as the mean ± standard deviation

*$p < 0.05$, **$p < 0.001$, indicates significant difference from the negative control, 5-FU: 5-fluorouracil

MMP was expressed by the JC-1 fluorescence ratio showing the ratio of healthy cells to apoptotic cells. These results were statistically insignificant, but double and triple combinations of genistein and 5-fluorouracil caused the greatest reduction ratio in SW480 cells compared with the negative control and single treatments. However, genistein and TRAIL were the most effective at reducing MMP in SW620 cells (Supplementary Material 4).

ROS levels in cells treated with genistein, 5-fluorouracil, and TRAIL were evaluated to clarify the role of ROS production in the apoptotic mechanism. The percentages of ROS production increased in all studied groups compared with the negative control after 48 hours of incubation. 5-Fluorouracil caused the highest production of ROS in SW480 cells compared with the negative control. The highest ROS production was also observed in SW620 cells treated with double and triple combinations of genistein and 5-fluorouracil (Supplementary Material 5).

DISCUSSION

It is estimated that cancer is the leading cause of death today and that the incidence of cancer will continue to increase in the coming years.[32] Among the cancer types, colorectal cancer ranks second, with an estimated 881,000 deaths worldwide in 2018.[33] While the lifetime risk of colorectal cancer in the general population is approximately 5-6%, patients with familial risk comprise approximately 20% of all patients with colorectal cancer, and colorectal cancer is known to be transmitted in an autosomal dominant manner.[34] Colorectal cancer begins as a benign adenomatous polyp and progresses to invasive cancer due to inherited mechanisms such as genomic instability, DNA repair defects, tumor suppressor gene mutations, and environmental factors such as obesity, physical inactivity, and diets lacking vegetables and fruits.[35-36]

Despite the improvements in systemic treatment, the 5-year survival rate is 12.5%, and the chemotherapeutic combination applied for treating metastatic colorectal cancer fails because of treatment resistance seen in 90% of the patients.[37] Therefore, it is important to determine the treatment resistance mechanisms.[38] Based on the fact that defects in apoptotic processes also cause resistance to anticarcinogens and radiotherapy,[39] the effects of genistein, 5-fluorouracil, and TRAIL were evaluated alone or in double/triple combinations in SW480 and SW620 cell lines to determine, if they had synergistic apoptotic effects.

Genistein, a major phytoestrogen in soybeans, has attracted attention as an anticarcinogen because epidemiological studies have shown that soybean consumption is associated with reduced cancer incidence. In addition, genistein has a place in phase II clinical trials for the treatment of a variety of human cancers. Genistein has a synergistic effect on endogenous hormones such as estradiol through its metabolite formed by intestinal microbiota metabolism. In contrast, it has an antagonistic effect on estrogen receptors such as Erα and Erβ.[40] Genistein has a 30-fold greater affinity for Erβ receptors than for Erα receptors.[41] ERs regulate gene expression as transcription factors that bind to DNA, and their activities vary depending on the cell type and Erα/Erβ ratio.[41,42] Erβ inhibits cell proliferation by suppressing the activity of Erα.[41] While Erα expression is low in normal colon cells and cancer cells, Erβ level varies inversely with the stage of the disease in cancer cells compared with normal cells.[43] Erβ expression in sw480 cells, Erα and Erβ expression both in sw620 cells.[44] In a study by Hartman et al.[31] in SW480 cells, it was determined that the proliferation of Erβ-transduced cells decreased compared with that of control cells.[45] In another study, the effects of 17β estradiol and 5-fluorouracil alone and in combination were investigated in SW480 and SW620 cells. It has been determined that estradiol alone causes cell accumulation in the SubG1 phase more effectively in SW620 cells and increases the induction of apoptosis. However, the anticancer effect of the combination was higher in SW480 cells.[44] These studies show that the Erβ receptor has an important place in treating colon cancer in terms of the anticancer effect of genistein. Previous studies have shown that genistein increases the cell growth inhibition and apoptotic effects of chemotherapeutic drugs such as doxorubicin, paclitaxel, and cisplatin.[9,14,46,47] Genistein acts as a protein tyrosine kinase (EGFR, insulin receptor) inhibitor that regulates protein phosphorylation and is effective in processes such as differentiation, angiogenesis, metastasis, and apoptosis through pathways such as Akt, NFκB, and ERK1/2.[11,48,49] In a study by Qi et al.,[50] genistein inhibited EGF-induced loss of *FOXO3* that led to increased p27kip1 (cell cycle inhibitor) expression activity by targeting the PI3K/Akt pathway.[50] In addition, genistein inhibited cell invasion and migration of colon cancer cells by regulating the expression of migration-associated factors and genes (MMP9, E-cadherin, β-catenin, c-Myc, and cyclin D1).[51] Genistein causes DNA strand breaks and induction of apoptosis through topoisomerase inhibition, which occurs in steps such as DNA replication and recombination.[52] Genistein inhibits adipogenesis by inducing peroxisome proliferator-activated receptor-γ (PPARγ), a transcriptional factor for adipogenesis, through activating Erα or Erβ.[53] The increased PPARγ expression leads to an anti-inflammatory effect by decreasing prostaglandin E_2 and cyclooxygenase-2. In addition, genistein induces the apoptotic pathway *via* PPARγ, including *Bcl-2*, phosphatase and tensin homolog, survivin, and cyclin B1.[54]

A study of NCM460 colon mucosa epithelial cells and HT29, SW620, LoVo, and HCT116 colon cancer cell lines by Zhu et al.[55] found that genistein inhibits cell vitality proportionally to concentration and incubation time, reducing HT29 cell viability by 38% after 72 hours of incubation. In other studies conducted on HT29 cells, it was determined that 72 hours of incubation with 60 μM genistein resulted in 67.3% cell viability, and 47% in 200 μM and 48 hours of incubation. In another study, the IC_{50} value was 50 μM for 48 hours, showing that the effects of genistein depend on the properties of cell lines.[50,56-58] The cytotoxic effects of 5-fluorouracil in colorectal cancer cells are also known.[59,60] In our study, the effects of genistein, 5-fluorouracil, and TRAIL were evaluated in SW480 and SW620 cells. They reduced cell viability in a dose-dependent manner. As reported previously, SW480 cells were found to be less sensitive to 5-FU-induced cell growth inhibition. However, contrary to the literature,[5,61-63] SW480 cells were more resistant to the inhibitory effects of TRAIL than SW620 cells, which may be the result of more *XIAP* expression ($p < 0.05$), as the TRAIL resistance mechanism is known for.[64] Genistein, 5-fluorouracil, and TRAIL in double and triple combinations had synergistic effects on reducing the cell viability and recovery ability of SW480 and SW620 cells showing apoptotic changes.[29] Furthermore, this effect was determined by an Annexin V FITC assay and the highest early apoptotic cell percentage was observed in SW620 cells treated with the triple combination and in SW480 cells treated with the 5-fluorouracil + TRAIL combination. Studies show the synergistic effects of double combinations of these compounds for various types of cancer.[65-68] However, the effects of triple combinations of subtoxic concentrations and double combinations in the concentrations we studied were not evaluated previously in SW620 and SW480 cells. In the current study, genistein and 5-fluorouracil caused the activation of G0/G1 and G2/M cell cycle arrest, which are known to be the checkpoints for DNA damage,[69] and prevented mitosis by the inactivation of CDK.[14,15,57,70] In addition, combinations of genistein, 5-fluorouracil, and TRAIL caused increased S cycle arrest, which is known to cause DNA damage, compared with both cell lines treated with single compounds.[71] In addition, DNA damage that may be caused by ROS production[72] induced by genistein, 5-fluorouracil, and TRAIL supported the results regarding cell cycle and apoptosis.

In our study, we demonstrated that genistein and 5-fluorouracil sensitized SW480 and SW620 cells to TRAIL-induced apoptosis *via* DR5 agonistic surface receptors and DR5 gene expression in accordance with the literature.[73,74] Additionally, it was observed that *DcR1* and *XIAP* antiapoptotic gene expressions in double and triple combinations of the studied compounds decreased significantly compared with the group in which TRAIL was applied alone in both cell lines. When caspase 3, 8, and 9 activities and MMP were investigated to explain the apoptotic mechanism, it was also found that triple combinations of the compounds were the most effective in reducing MMP in SW480 cells and in increasing caspase activities in both cell lines. Previous studies have shown that genistein[73,75] and 5-fluorouracil[67,74] sensitized TRAIL-induced apoptosis by increasing caspase activity and the loss of MMP, decreasing *XIAP* gene expression, which plays a critical role in the suppression of apoptosis.[76] ROS production leads to mitochondria-derived apoptosis induction, which leads

to cytochrome c release interacting with caspase 9 and the binding of TRAIL to death receptors initiates the caspase 8-3 cascade,[19,75,77] in accordance with our results. However, it was observed that the concentrations of the compounds used in our study were insufficient for the expected reduction effect on *Bcl-XL* and *Bcl-2* gene expression.

Study limitations

It has been determined that genistein, 5-fluorouracil and TRAIL have more cytotoxic, genotoxic, and apoptotic effects in combination and show synergistic effects together. These effects may contribute to the resistance problem that may occur in treating colorectal cancer with an increase in ROS, a decrease in MMP, and an increase in caspase 3, 8, and 9. However, the combination concentrations should be chosen from a slightly higher range to see the expected synergistic effect as statistically significant.

CONCLUSION

In conclusion, genistein and 5-fluorouracil sensitized TRAIL-induced apoptosis *via* the DR5 surface receptor protein in SW480 and SW620 cells. The induction of DNA damage and ROS production, increased caspase activities, decreased MMP, and decreased *XIAP* and *DcR1* gene expression may play a role in the apoptotic mechanism of a combination of genistein, 5-fluorouracil, and TRAIL. It is thought that combinations of these compounds at subtoxic dosage levels may contribute to the resistance problem for colorectal cancer treatment.

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Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Authorship Contributions

Concept: T.Ç.D., S.A.D., H.C., Ü.Ü.B., Design: T.Ç.D., S.A.D., H.C., Ü.Ü.B., Data Collection or Processing: T.Ç.D., Analysis or Interpretation: T.Ç.D., S.A.D., Ü.Ü.B., Literature Search: T.Ç.D., Writing: T.Ç.D., Ü.Ü.B.,

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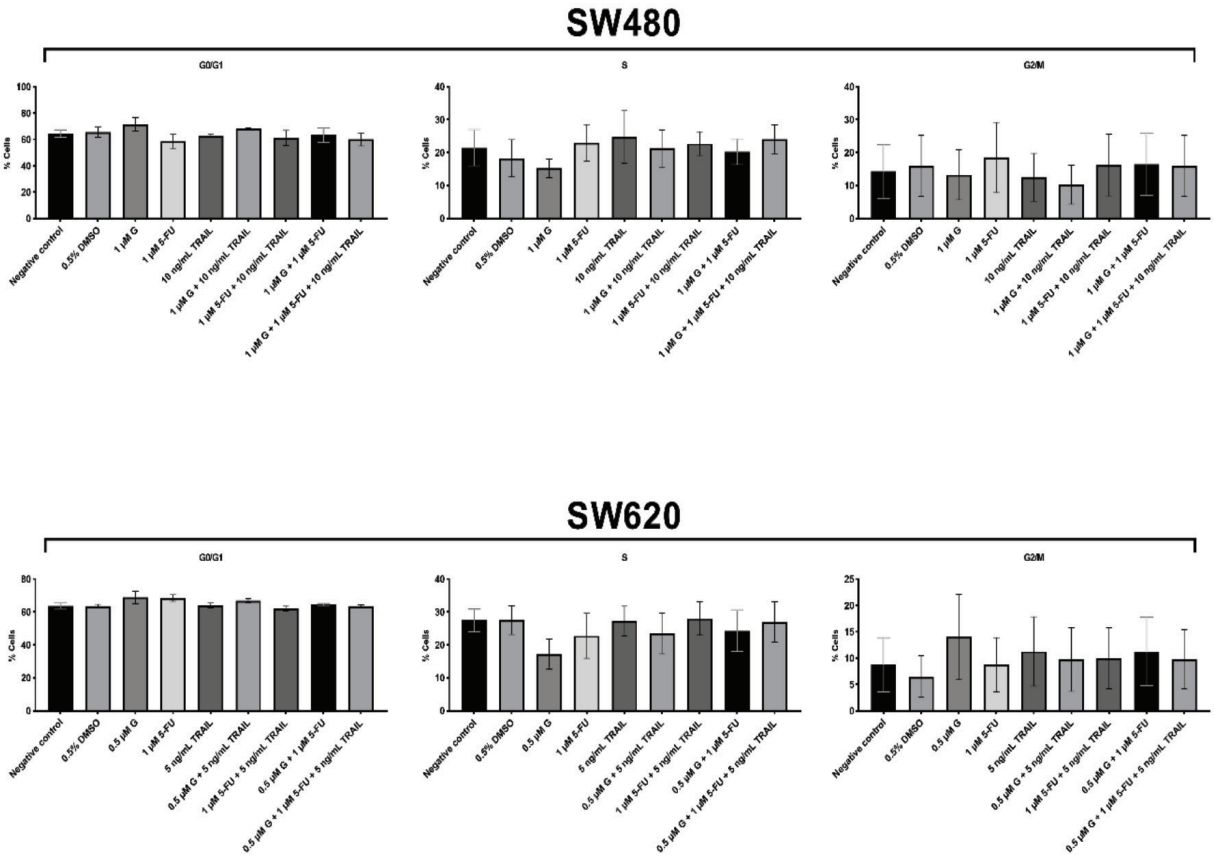
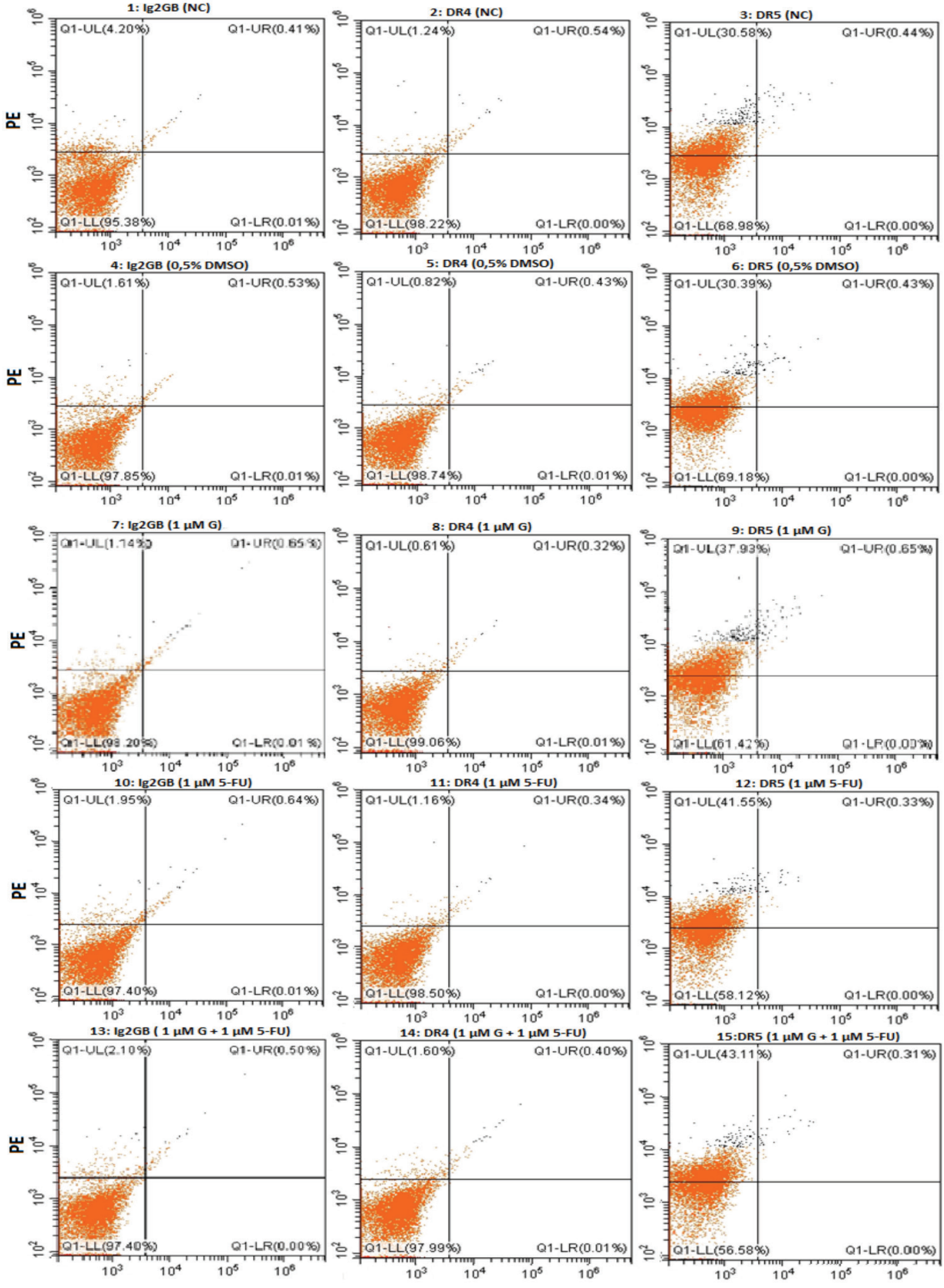
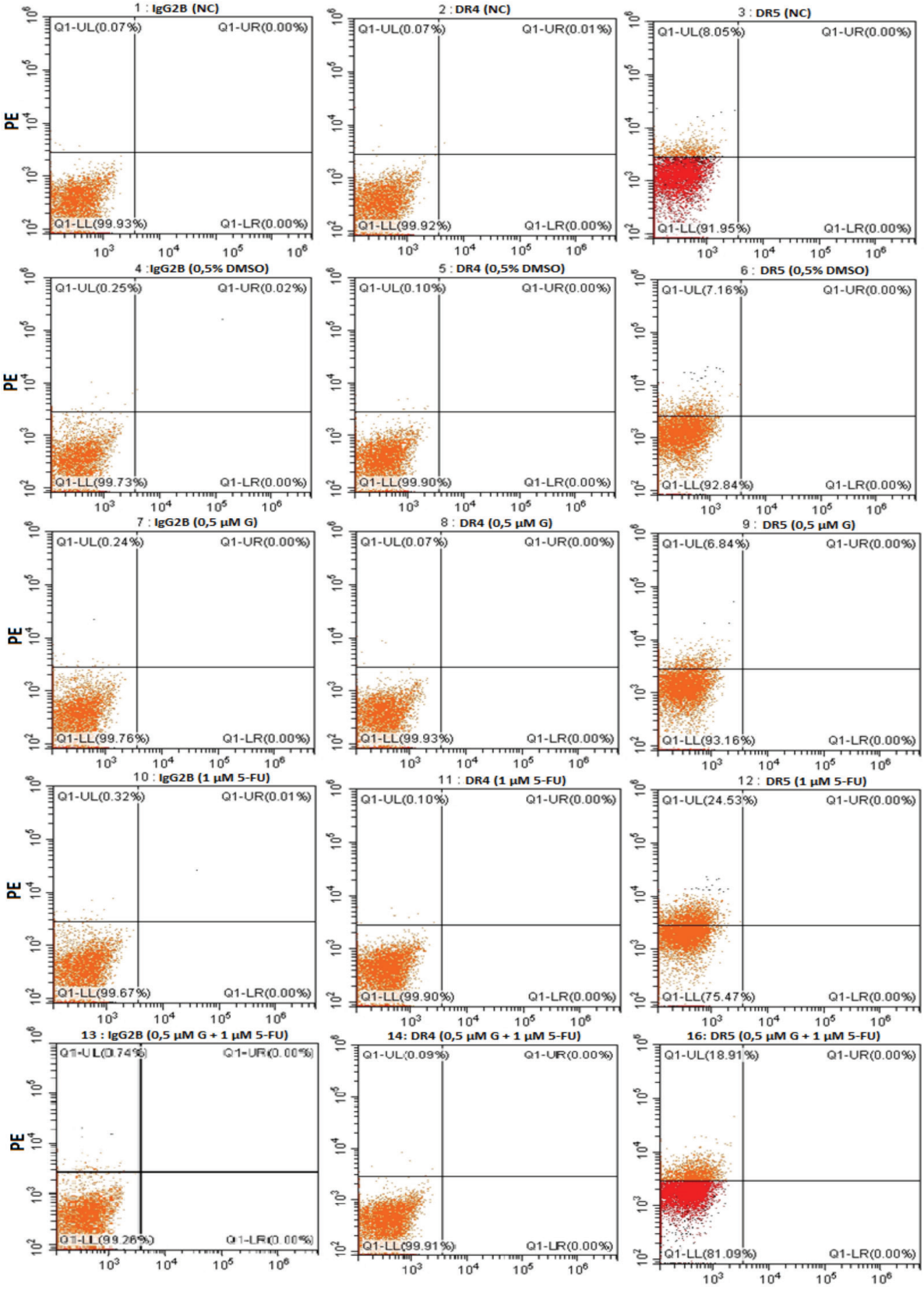


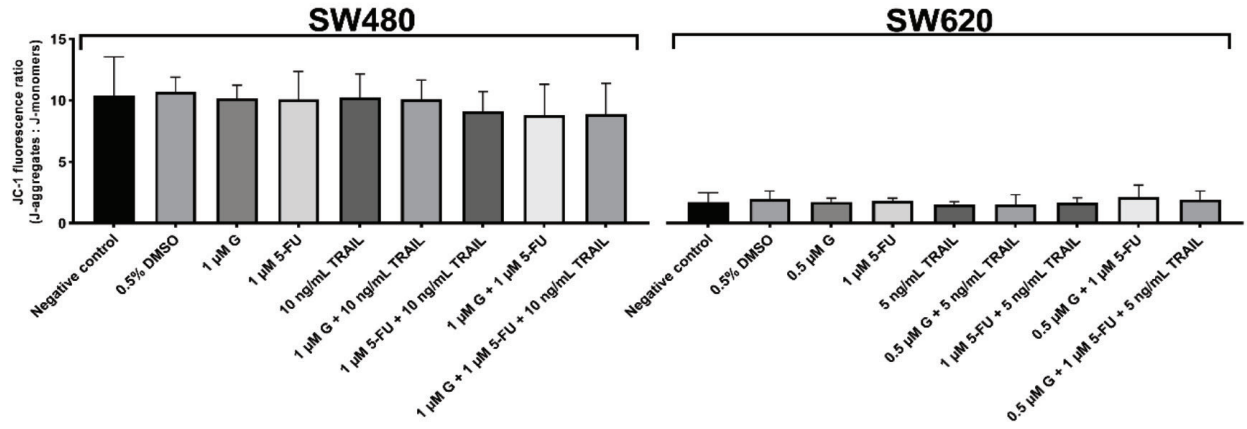
Supplementary Material 1. Effects of genistein, 5-FU, and TRAIL on the cell cycle in SW480 and SW620 cells examined by flow cytometry
5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand



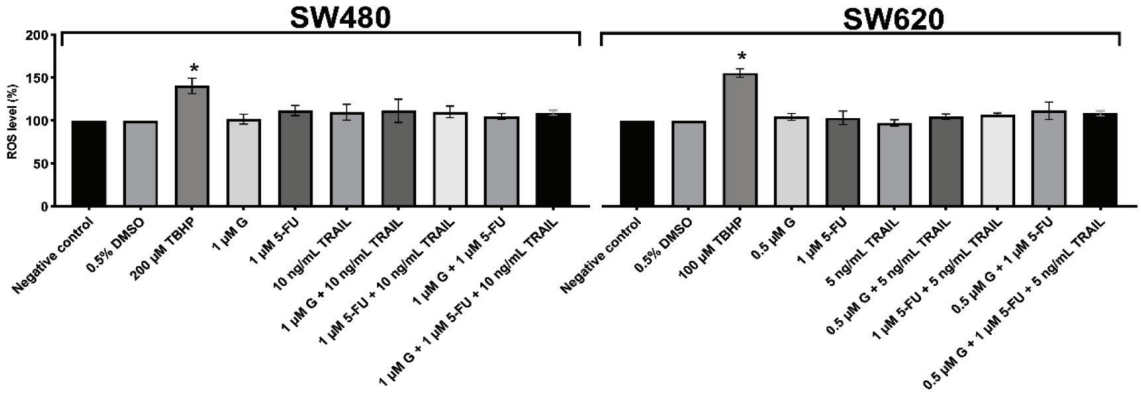
Supplementary Material 2. Effects of genistein **(G)** and 5-FU on the surface expression levels of DR4 and DR5 in SW480 cells by flow cytometry
5-FU: 5-Fluorouracil



Supplementary Material 3. Effects of genistein **(G)** and 5-FU on the surface expression levels of DR4 and DR5 in SW620 cells by flow cytometry
5-FU: 5-Fluorouracil, DMSO: Dimethyl sulfoxide



Supplementary Material 4. The changes in MMP of SW480 and SW620 cells are presented as the mean ± standard deviation of the ratio between aggregates and monomeric forms of JC-1 using the JC-1 MMP Assay Kit (Cayman)



Supplementary Material 5. Effects of genistein, 5-FU, TRAIL, and their combinations on ROS levels in SW480 and SW620 cells for 48 hours using a ROS detection assay kit (Biovision). Results are given as mean ± standard deviation of ROS level percentage compared with negative and DMSO controls

*$p < 0.05$, **$p < 0.001$, indicates a significant difference from the negative control, 5-FU: 5-Fluorouracil, TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand, ROS: Reactive oxygen species, DMSO: Dimethyl sulfoxide