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

Short Title: Genistein enhances TRAIL-mediated apoptosis

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ABSTRACT
Objectives: Colorectal cancer is one of the most common cancers in the world. However, surgical intervention and chemotherapy provide only limited benefits for the recovery and survival of patients. The anti-carcinogenic effect of genistein has attracted attention due to epidemiological studies showing that soybean consumption is associated with a decrease in 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 TRAIL ligand, the mediator of apoptosis, both alone and in combination.
Materials and Methods: The cytotoxicity and genotoxicity were determined by MTT assay and comet assay, respectively. The apoptotic effects were evaluated by RT-PCR assay, with the additional use of Annexin V FITC, mitochondrial membrane potential, caspase 3, 8 and 9 activity, reactive oxygen species assay kits.
Results: According to our findings, genistein, 5-fluorouracil and TRAIL had synergistic apoptotic effects as a result of DR5 up-regulation, ROS production, and DNA damage, which was mediated by increased caspase 3, 8 and 9 activity and decreased mitochondrial membrane potential.
Conclusion: The applied combinations of these compounds may contribute to the resistance problem that may occur in the treatment of colorectal cancer, with the decrease in DcR1 and XIAP genes.
Key Words: Genistein; 5-fluorouracil; TRAIL; Apoptosis; colorectal cancer

1- INTRODUCTION

According to the International Agency for Research on Cancer (IARC), 23 million new cancer cases are expected annually by 2030. Colorectal cancer is among the most diagnosed cancers in the world, along with breast cancer and lung cancer (1). Although the incidence and mortality of colorectal cancer vary by gender, age and race, it ranks third among cancers diagnosed in men and second in women according to GLOBOCAN (2018) data (2, 3). In Turkey, colorectal cancer ranks third and fourth 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 only diagnosed 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 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 and has necessitated the development of different treatment principles in cancers (5, 7). For this reason, 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 that was first isolated from *Genista tinctoria* L., a phytoestrogenic compound that is particularly abundant in *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 G2/M phase with the affecting of cyclin-dependent kinases (Cdk), the induction of apoptosis, the attenuation of multiple drug resistance through various signaling pathways (Akt, MAPK, EGFR), the 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 super family and is synthesized in tissues such as 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. The DcR4, DcR5 and osteoprotegrin inhibit apoptosis by acting as decoy receptors (16). Although the induction of the extrinsic pathway via 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.

2- MATERIALS AND METHODS

2.1 Cell Culture

The SW480 and SW620 cell lines were derived from a single patient at primary and secondary stages. For this reason, 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 (Department of Molecular Biology and Genetics, Bilkent University, Turkey) 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 DMEM (Wisent) containing 1% penicillin-streptomycin (Wisent) and 10% fetal bovine serum (Capricorn). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in an incubator from Heraeus Instruments.

2.2 Cell Viability Assay

The thiazolyl blue tetrazolium bromide (MTT, Sigma) assay was used for assessing cell viability and determining 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 et al. (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 h. 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 h 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 h 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 by microplate reader (SpectraMax M2) and IC_{50} values were calculated using concentration-response curves to express the effects of test materials on cell viability. The combination index (CI) for drug interaction was calculated using the 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 synergistic effects of test compounds by MTT assay, the cells were exposed to the double and triple combination of these compounds for 48 h and the same procedure was followed as described before. The results were given as the mean ± standard deviation from three independent experiments.

2.3 Cell Recovery Assay

The cell recovery assay was performed as described in 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 ng/ml, 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 h. After incubation, the cells were exposed to several concentrations of genistein, 5-fluorouracil, TRAIL, and their combinations in a medium

for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. Then, the cells for each treatment group were trypsinized (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 h incubation. The MTT assay was used for evaluating cell recovery. The results were given as the mean ± standard deviation from three independent experiments.

2.4 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 h. Then, the cells were exposed to different concentrations of genistein (0.125 µM, 0.25 µM, 0.5 µM), 5-fluorouracil (1 µM), TRAIL (5 ng/ml, 10 ng/ml) and their combinations for 48h. A negative control (0.5% DMSO) and a positive control (15 µM H_2O_2 (Merck)) were included. After incubation, the cells were re-suspended in a 0.75% low melting point agarose (Boehringer Mannheim) and this suspension was spread on pre-coated slides which were 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 h. 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 electrophores equipment (Biometra Analytical). Then, the slides were washed in a neutralizing solution (0.4 M Tris-HCl (Sigma), pH 7.5) for 15 min and incubated in 50%, 75%, and 98% of 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 by a Leica® fluorescence microscope. A computer-based analysis system (Comet Analysis Software, version 4.0, Kinetic Imaging Ltd., Liverpool, UK) was used to measure the DNA damage. In order to visualize DNA damage, 100 nuclei per slide were examined at 400× magnification. DNA damage was expressed as the percent of DNA in tail (tail intensity). The values are expressed as the mean ± standard deviation from three independent experiments.

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

Cells were cultivated at a density of 6x10^6 cells/25 cm^2 cell culture flask (Nest) for 24 h and treated with genistein (0.5 µM for SW620; 1 µM for SW480 cells), 5-fluorouracil (1 µM) and their combinations for 48 h. After incubation, the cells were trypsinized and suspended in a serum free medium (Wisent) at a density of 1x10^6 cell/ml. Then, the cells were washed twice with 2 ml cell staining buffer (Biolegend) and suspended in cell staining buffer at a density of 1x10^6 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 minutes. After staining, the cells were washed with 2 ml cell staining buffer twice and suspended in 0.5 ml cell staining buffer to analyze by flow cytometry (Beckman Coulter, Cytoflex USA).

2.6 Cell cycle analysis by flow cytometry

The cells were cultivated at a density of 2x10^6 cells/4 ml in 25 cm^2 cell culture flasks. After incubation for 24 h, 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 h. The cells were trypsinized and suspended in 1 ml PBS (Wisent) at a density of 1.5 x 10^6 cells/ml. Then, cells were fixed with 2 ml 99% ethanol and incubated for 24 h. After washing the cells with PBS twice, 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 minutes. Then, cell cycle analysis was performed using a flow cytometer (Beckman Coulter, Cytoflex USA). The results were given as the mean ± standard error from three independent experiments.

2.7 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 h and washed with PBS twice. Then, 1X binding buffer was added (10^6 cells/ml) to cells and 100 µl of the cell suspensions was transferred to 5 ml culture tubes. The 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 by a flow cytometer after adding 400 µl 1X binding buffer. The results were given as the mean ± standard error from three independent experiments.

2.8 Measurement of intracellular ROS levels

the intracellular ROS level was determined using an 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), TRAIL (10 ng/ml for SW480, 5 ng/ml for SW620) for 48 h. The fluorescence was measured at the desired time intervals by a microplate reader (SpectraMax M2) at an agitation of 495 nm and emission of 529 nm (Ex/Em= 495/529 nm).

2.9 Measurement of mitochondrial membrane potential

The effect of test compounds on mitochondrial membrane potential was determined using a JC-1 Mitochondrial Membrane Potential 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), TRAIL (10 ng/ml for SW480, 5 ng/ml for SW620) for 48 h and then JC-1 staining solution was added to the cells. Then, the cells were incubated at 37 °C for 15 min in a humidified atmosphere of 5% CO_2 and washed twice with assay buffer. Measurement was performed using a fluorescent plate reader (SpectraMax M2) - for healthy cell detection (Ex/Em= 535/595 nm), and for apoptotic cells (Ex/Em= 485/535 nm).

2.10 Determination of Activities of Caspase 3, 8 and 9

Caspase 3, 8, and 9 activities were determined using the Caspase 3, Caspase 8 and Caspase 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), TRAIL (10 ng/ml for SW480, 5 ng/ml for SW620) for 48 h. Afterwards, 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 agitation of 535 nm and emission of 620 nm for caspase 3, agitation of 490 nm and emission of 525 nm for caspase 8, and at agitation of 370 nm and emission of 450 nm for caspase 9.

2.11 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene expression

The reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine the effects of test compounds on apoptotic and antiapoptotic gene expressions. First, total RNA was isolated from cells 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) for 48 h using a 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, the reverse transcription was performed using 500 ng of total RNA at 42 °C (15 min), 95°C (5 min) in a thermal cycler (Corbett). Afterwards, 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 *GAPDH* (PPH00150F) were purchased from Qiagen. Each cycle was performed at the conditions of hold 95 °C 15 min, cycle 95 °C 15 sec and 60 °C 30 sec for 40 cycles. The relative changes in the amount of transcripts in each treatment were calculated by normalizing with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. The values indicate the mean ± standard error.

2.12 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 homogeneity of the variance was verified by the Levene test. The differences among the means of data with normal distribution were evaluated by the one-way variance analysis (ANOVA) test and *post hoc* analyses of group differences were performed by the Tukey test for homogeneous variance and Dunnett's T3 test for non-homogenous variance. The differences among the groups without normal distribution were evaluated by the Kruskal Wallis test followed by the Mann-Whitney U test. The p-values of less than 0.05 and 0.001 were considered as statistically significant.

Statistics of the Ct values for RT-PCR analysis were performed with the RT^2 Profiler PCR Data Analysis program of QIAGEN. Significance was determined based on fold change from the control ΔΔCt value, and p value of less than 0.05 was considered to be statistically significant.

3- RESULTS

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

Genistein, 5-fluorouracil and TRAIL inhibited the growth of SW480 and SW620 cells in a dose-dependent manner for 24 h, 48 h, and 72 h. Also, 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 h and 72 h, but IC_{50} of TRAIL was determined to be 93.35 ng/ml, 138.4 ng/ml and 192.9 ng/ml for 24 h, 48 h and 72 h respectively in SW480 cells. Furthermore, IC_{50} of genistein was 375.8 µM for 48 h and had significant inhibitory effect at above 200 µM in SW480 cells when compared to the negative control (0.5% DMSO). The IC_{50} values of genistein were 351.1 µM and 190.6 µM for 48 h and 72 h respectively in SW620 cells. Additionally, IC_{50} of 5-fluorouracil treated SW620 cells was 794.4 µM for 48 h. The IC_{50} values of TRAIL for 24, 48 and 72 h in SW620 cells were determined to be 20 ng/ml, 19.43 ng/ml and 50.16 ng/ml, respectively (Fig.1 a-f).

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

[insert Figures 1A, 1B, 1C and 1D around here].

Figure. 1 MTT assay results of SW480 and SW620 cells incubated with Genistein, 5- fluorouracil (5-FU) and TRAIL. Effects of Genistein (a), 5 – fluorouracil (5-FU) (b) and TRAIL (c) on SW480 and Genistein (d), 5 – fluorouracil (5-FU) (e) and TRAIL (f) on SW620 cell viability for 24 h, 48 h and 72 h. SW480 (g) and SW620 (h) cell viability after incubation with G, 5-FU, TRAIL and their combinations for 48 h. (*$p < 0.05$, **$p < 0.001$,

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.
The combination index (CI) based analysis was used to determine the synergistic effects of compounds (Fig. 2). 1 μM genistein for SW480 cells and 0.5 μM genistein for SW620 cells were selected according to 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 respectively in SW620 cells. Furthermore, the reversibility of cell growth inhibition was evaluated by a recovery MTT assay. It was found that TRAIL had the most effect on the loss of ability to recover in SW480 and SW620 cells (Fig. 3). The triple combinations were more effective than double and single concentrations of the compounds. The results showed that there were irreversible changes like apoptosis in SW480 cells.
[insert Figure 2 around here].
Figure. 2 MTT assay results of SW480 and SW620 cells incubated with Genistein (G), 5-fluorouracil (F) and TRAIL (T) used for calculating Combination index (CI) by CompuSyn software. CI versus factor affected (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 effects and antagonistic effects, respectively.
[insert Figure 3 is around here].
Figure. 3 Effects of genistein (G), 5-fluorouracil (5-FU) and TRAIL on proliferation / survival in SW480 and SW620 cells using cell recovery assay. (*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.
3.2 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 to the negative control in SW480 and SW620 cells (Fig. 4-5). The increases in DNA damage in SW480 cells treated with double and triple compounds were more than 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 when compared to the negative control (0.5% DMSO).
[insert Figure 4 around here].
Figure. 4 Tail intensity from the comet assay of SW480 and SW620 cells treated with genistein (G), 5-fluorouracil, TRAIL and their combinations for 48 h (*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.
[insert Figure 5 around here].
Figure. 5 The comet images of SW480 (A-B) and SW620 cells (C-D)
3.3 Genistein and 5-fluorouracil sensitizes TRAIL mediated apoptosis
The effects of genistein, 5-fluorouracil, and TRAIL on cell cycles were examined by flow cytometry (Supplementary Material 1). When compared to 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 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 caused an increase in accumulation of the G2/M phase of SW480 and SW620 cells, respectively.
[insert **Supplementary Material 1** around here].
Supplementary Material 1. The effects of genistein, 5-fluorouracil and TRAIL on the cell cycle in SW480 and SW620 cells examined by flow cytometry
In order 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 induction of the expression of DR5 surface protein (Fig. 6 and Supplementary material 2-3). The proportion of surface DR5+ s in the cells treated with 5-fluorouracil was higher than both the control and genistein treated cells. Also, SW480 cells were more sensitive to the apoptotic effects of genistein and 5-fluorouracil than were SW620 cells.
Figure 6. DR4 and DR5 surface expressions of SW480 and SW620 cells incubated with genistein (G) and 5 – fluorouracil (5-FU) by flow cytometry assay.
[insert **Supplementary material 2** around here].
Supplementary material 2. Effects of genistein (G) and 5-fluorouracil (5-FU) on surface expression levels of DR4 and DR5 in SW480 cells by flow cytometry**.**
[insert **Supplementary material 3** around here].
Supplementary material 3. Effects of genistein (G) and 5-fluorouracil (5-FU) on surface expression levels of DR4 and DR5 in SW620 cells by flow cytometry**.**
The results of 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 increased apoptosis significantly in SW620 cells when compared to the negative control (Fig. 7).
[insert Figure 7 around here].
Figure. 7 The results of Annexin V FITC apoptosis assay showing apoptotic effects of genistein, 5-fluorouracil and TRAIL in SW480 and SW620 cells. Results were given as the mean ± standard error. (*p < 0.05, **p < 0.001, indicates significant difference from the negative control).
Additionally, the apoptotic mechanism was evaluated by the changes in the apoptotic (DR4, DR5) and antiapoptotic (Bcl-XL, Bcl-2, XIAP, DcR1, DcR2) gene expressions. Genistein reduced DcR2 gene expression in SW480 cells and increased Bcl-XL, Bcl-2, and DR4 gene expressions in SW620 cells. When genistein was applied in combination with TRAIL ligand in SW480 cells, it was determined that Bcl-XL, XIAP, and DR5 gene expressions increased when compared to 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 receptor compared to the groups in which they were administered alone. When 5-fluorouracil was administered alone, it caused a significant reduction of XIAP genes in SW480 cells. However, it caused significant increases in Bcl-XL, Bcl-2, DR4, and DcR2 gene expressions in SW620 cells.
Bcl-XL, Bcl- 2 gene expressions increased in SW480 cells treated with double and triple combinations of 5-fluorouracil, genistein, and TRAIL ligand, however a significant decrease in XIAP gene expression was observed in all combinations when compared to the groups in which they were administered alone. Similarly, in SW620 cells, Bcl-XL, Bcl-2 expressions increased and there was a decrease in XIAP gene expression when compared to the group where TRAIL ligand was applied alone (Fig. 8-9).
[insert Figure 8 around here].
Figure. 8 Relative gene expressions of SW480 cells treated with genistein (G), 5-fluorouracil (5-FU), TRAIL and their combinations by RT-PCR. Results were given as 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 significant difference from the negative control).
[insert Figure 9 around here].
Figure. 9 Relative gene expressions of SW620 cells treated with genistein (G), 5-fluorouracil (5-FU), TRAIL and their combinations by RT-PCR. Results were given as mean of fold change compared to controls (negative and 0.5% DMSO control). The values indicate the mean ± standard error and normalized with GAPDH (*p < 0.05, indicates significant difference from the negative control).

3.4 Effects of genistein, 5-fluorouracil, and TRAIL on caspase 3-8-9 activities, mitochondrial membrane potential, 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 greatest increase in caspase activities when compared to the negative control and to the groups in which they were administered seperately. Additionally, the genistein and TRAIL combination was found to be the most effective on increasing caspase 3-8-9 activities (Fig.10).
[insert Figure 10 around here].
Figure. 10 Caspase 3 – 8 – 9 activities relative to control in SW480 and in SW620 cells by Multiplex Activity Assay Kit (Abcam). Results were presented as the mean ± standard deviation. (*p < 0.05, **p < 0.001, indicates significant difference from the negative control).
Mitochondrial membrane potential (MMP) was expressed by the JC-1 fluorescence ratio showing the rate 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 when compared to the negative control and single treatments. However, genistein and TRAIL were the most effective at reducing MMP in SW620 cells (Supplementary Material 4).
[insert Supplementary Material 4 around here].
Supplementary Material 4. The changes of mitochondrial membrane potential of SW480 and SW620 cells were presented as the mean ± standard deviation of ratio between aggregates and monomeric forms of JC-1 by JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman).
ROS levels in the cells treated with genistein, 5-fluorouracil, and TRAIL were evaluated to clarify the role of ROS production in apoptotic mechanism. The percentages of ROS production increased in all studied groups compared to the negative control in 48 h incubation. 5-fluorouracil caused the highest production of ROS in SW480 cells when compared to 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).
[insert Supplementary material 5 around here].
Supplementary material 5. Effects of genistein, 5-fluorouracil, TRAIL and their combinations on ROS level in SW480 and SW620 cells for 48 h using ROS detection assay kit (Biovision). Results were given as mean ± standard deviation of ROS level % compared to negative and DMSO controls. (*p < 0.05, **p < 0.001, indicates significant difference from the negative control).

4- 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 about 5 to 6 percent, patients with familial risk comprise about 20 percent 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 an 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 in vegetables and fruits (35, 36). Despite the improvements in systemic treatment, the 5-year survival rate is 12.5% and the chemotherapeutic combination applied in the treatment of metastatic colorectal cancer fails due to the 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 resistence 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 in order to determine if they had synergistic apoptotic effects.

Genistein, a major phytoestrogen in soybeans, has attracted attention as an anticarcinogen, due to epidemiological studies showing that soybean consumption is associated with reduced cancer incidence. Additionally, genistein has a place in phase II clinical trials of 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, while 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 expressions as transcription factors binding 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 to normal cells (43). Erβ expression in sw480 cells, Erα and Erβ expression both in sw620 cells (44). In a study conducted by Hartman et al. in SW480 cells, it was determined that the proliferation of Erβ-transduced cells decreased compared to 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 the treatment of colon cancer in terms of the anticancer effect of genistein.

Previous studies have shown that genistein increased 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 regulating phosphorylation of proteins and is effective in processes such as differentation, angiogenesis, metastasis, and apoptosis through pathways such as Akt, NFκB and ERK1/2 (11, 48, 49).). In a study by Wentao et al., genistein inhibits EGF induced loss of FOXO3 that leads to increased p27kip1 (cell cycle inhibitor) expression activity by targeting the PI3K/Akt pathway (50). Also, genistein inhibits 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 takes place in steps such as DNA replication and recombination (52). Genistein inhibits adipogenesis by inducing Peroxisome proliferator-activated receptor-γ (PPARγ), transcriptional factor for adipogenesis, through activating Erα or Erβ (53). The increased PPARγ expression leads to anti-inflammatory effect by decreasing prostaglandin E2 and cyclooxygenase-2 (COX). Also, genistein induces apoptotic pathway via PPARγ, including Bcl-2, phosphatase and tensin homolog (PTEN), survivin, and cyclin B1 (54).

A study of NCM460 colon mucosa epithelial cells and HT29, SW620, LoVo, HCT116 colon cancer cell lines by Zhu et al. found that genistein inhibits cell vitality proportionally to concentration and incubation time, reducing HT29 cell viability by 38% after 72 hours of incubation (55). In other studies of the literature 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 IC50 value was 50 μM for 48 hours, showing that the effects of genistein depends on the properties of cell lines (50, 56-58). The cytotoxic effects of 5-fluorouracil in colorectal cancer cells were also known (59, 60). In our study, the effects of genistein, 5-fluorouracil, and TRAIL were evaluated in SW480 and SW620 cells. They reduced the cell viabilities 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 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 viabilities 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 5-fluorouracil + TRAIL combination. There are studies showing the synergistic effects for double combinations of these compounds for various types of cancer(65-68). However, the effects of triple combinations of subtoxic concentrations and the double combinations in the 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 known to be the check points for DNA damage (69) and prevent entering mitosis by the inactivation of cyclin dependent kinases (14, 15, 57, 70). Also, combinations of genistein, 5-fluorouracil and TRAIL caused increased S cycle arrest, known to cause DNA damage compared with in 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 cycles and apoptosis.
We demonstrated in our study 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 to the group where TRAIL was applied alone in both cell lines. When caspase 3,8,9 activities and mitochondrial membrane potential were investigated to explain apoptotic mechanism, it was also found that triple combinations of the compounds were the most effective in reducing mitochondrial membrane potential 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 activities and the loss of mitochondrial membrane potential, decreasing XIAP gene expressions which play a critical role in suppression of apoptosis (76). It is also known that ROS production leads to mitochondria-derived apoptosis induction which leads to cytochrome c release interacting with caspase 9 and that the binding of TRAIL to death receptors initiates 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.

5- 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 the treatment of colorectal cancer with increase in ROS, decrease in mitochondrial membrane potential and increase in caspase 3, 8, 9. However, the combination concentrations should be chosen from a slightly higher range in order to see the expected synergistic effect statistically significant.

6- CONCLUSION

In conclusion, genistein and 5-fluorouracil sensitized TRAIL induced apoptosis via DR5 surface receptor protein in SW480 and SW620 cells. The induction of DNA damage and ROS production, increased caspase activities, decreased mitochondrial membrane potential, 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 the combinations of these compounds at subtoxic dosage levels may contribute to the resistance problem for colorectal cancer treatment.

7- ACKNOWLEDGMENT

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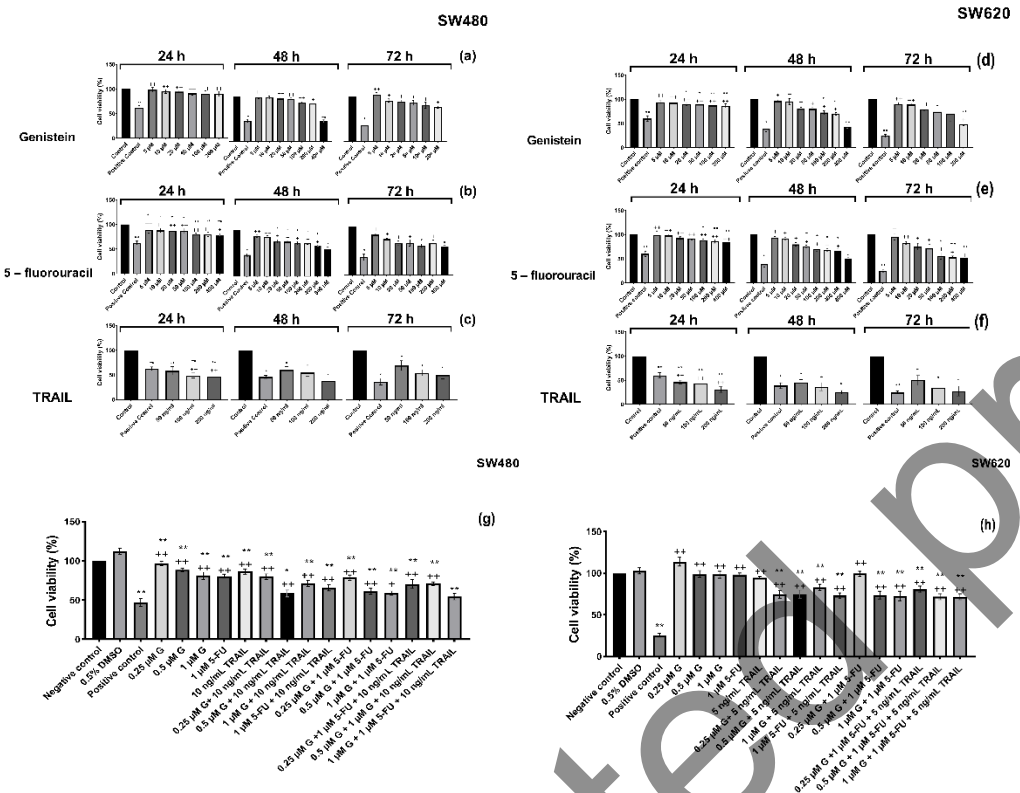


Figure. 1 MTT assay results of SW480 and SW620 cells incubated with Genistein, 5- fluorouracil (5-FU) and TRAIL. Effects of Genistein (a), 5 – fluorouracil (5-FU) (b) and TRAIL (c) on SW480 and Genistein (d), 5 – fluorouracil (5-FU) (e) and TRAIL (f) on SW620 cell viability for 24 h, 48 h and 72 h. SW480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

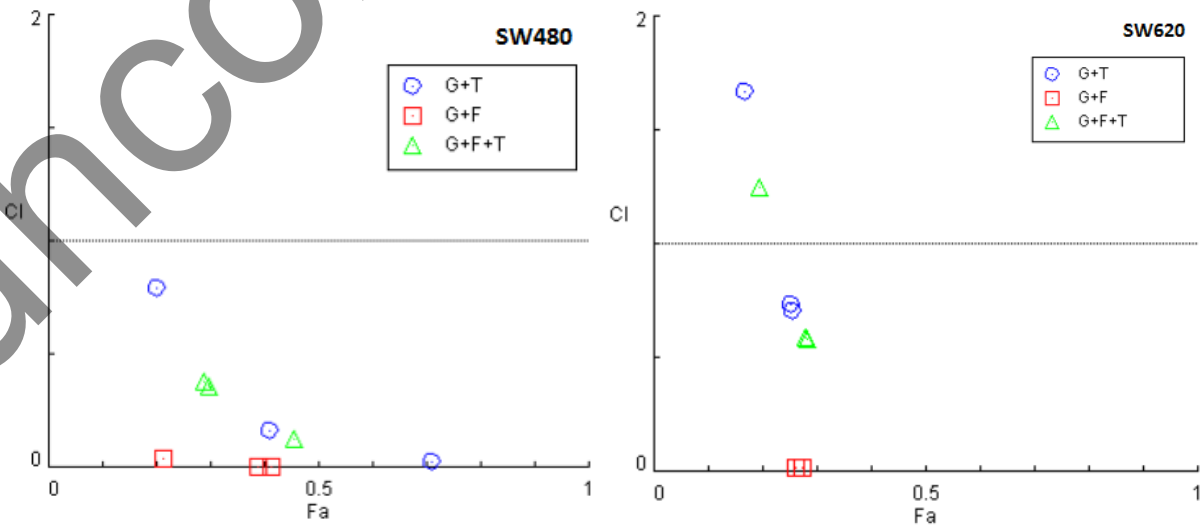


Figure. 2 MTT assay results of SW480 and SW620 cells incubated with Genistein (G), 5-fluorouracil (F) and TRAIL (T) used for calculating Combination index (CI) by CompuSyn software. CI versus factor affected (Fa) graphs of double and triple combinations for interaction of G, F and T in SW80 and SW620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

expressed in the mean ± standard deviation.

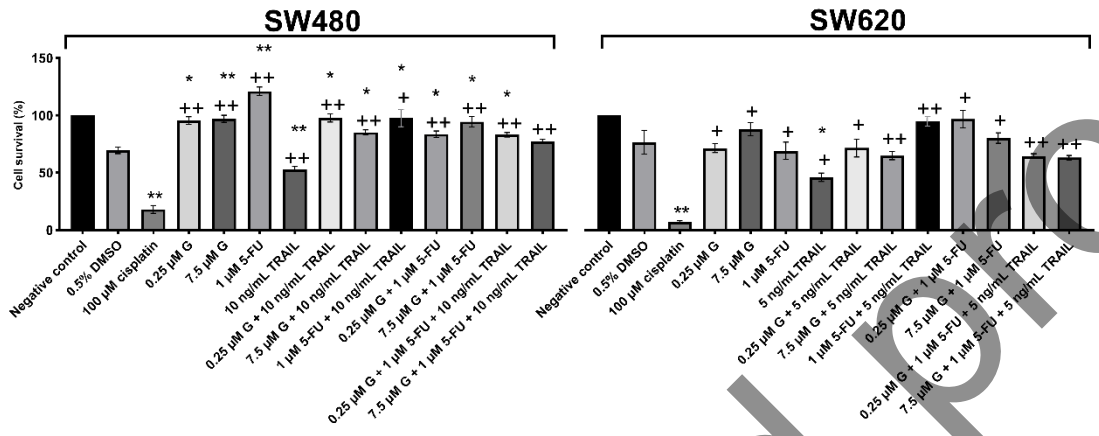


Figure. 3 Effects of genistein (G), 5-fluorouracil (5-FU) and TRAIL on proliferation / survival in SW480 and SW620 cells using cell recovery assay. (*p < 0.05, **p < 0.001, indicates significant difference from the negative control. +p < 0.05, ++p < 0.001, indicates significant difference from the positive control). Results are

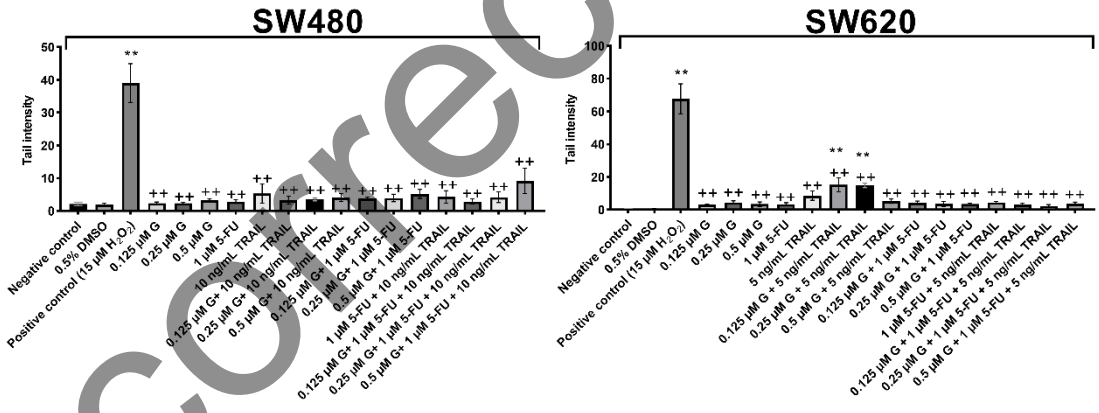


Figure. 4 Tail intensity from the comet assay of SW480 and SW620 cells treated with genistein (G), 5-fluorouracil, TRAIL and their combinations for 48 h (*p < 0.05, **p < 0.001, indicates significant difference from the negative control. +p < 0.05, ++p < 0.001, indicates significant difference from the positive control). The values are expressed in the mean ± standard deviation.

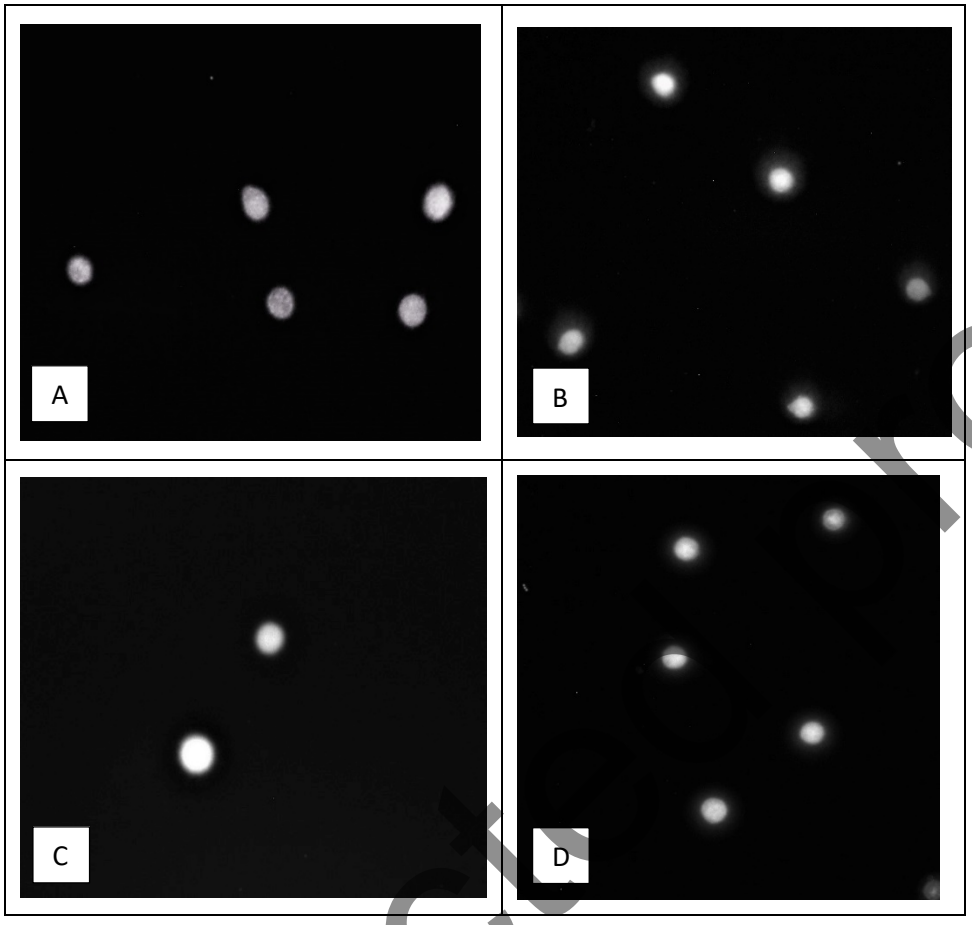


Figure. 5 The comet images of SW480 (A-B) and SW620 cells (C-D)

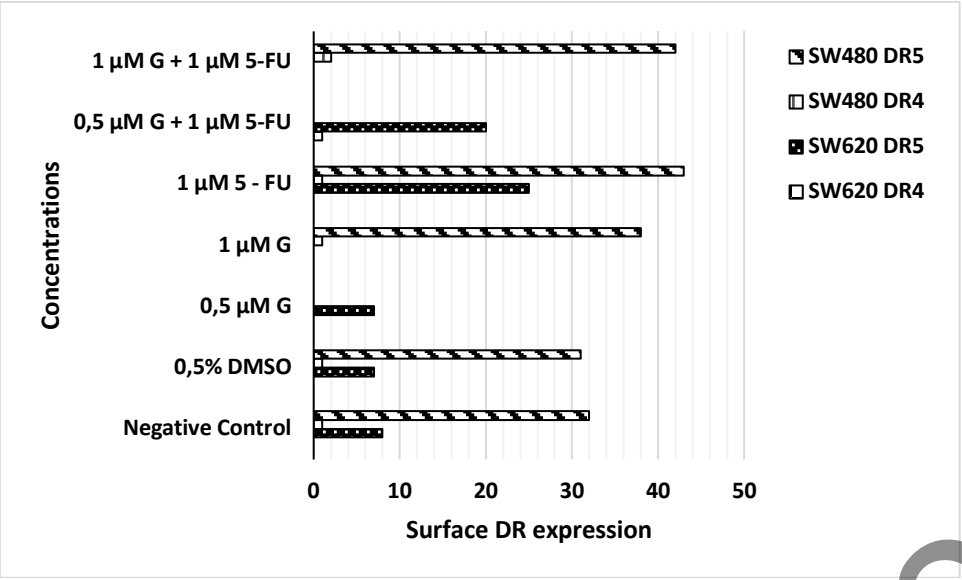


Figure 6. DR4 and DR5 surface expressions of SW480 and SW620 cells incubated with genistein (G) and 5 – fluorouracil (5-FU) by flow cytometry assay.

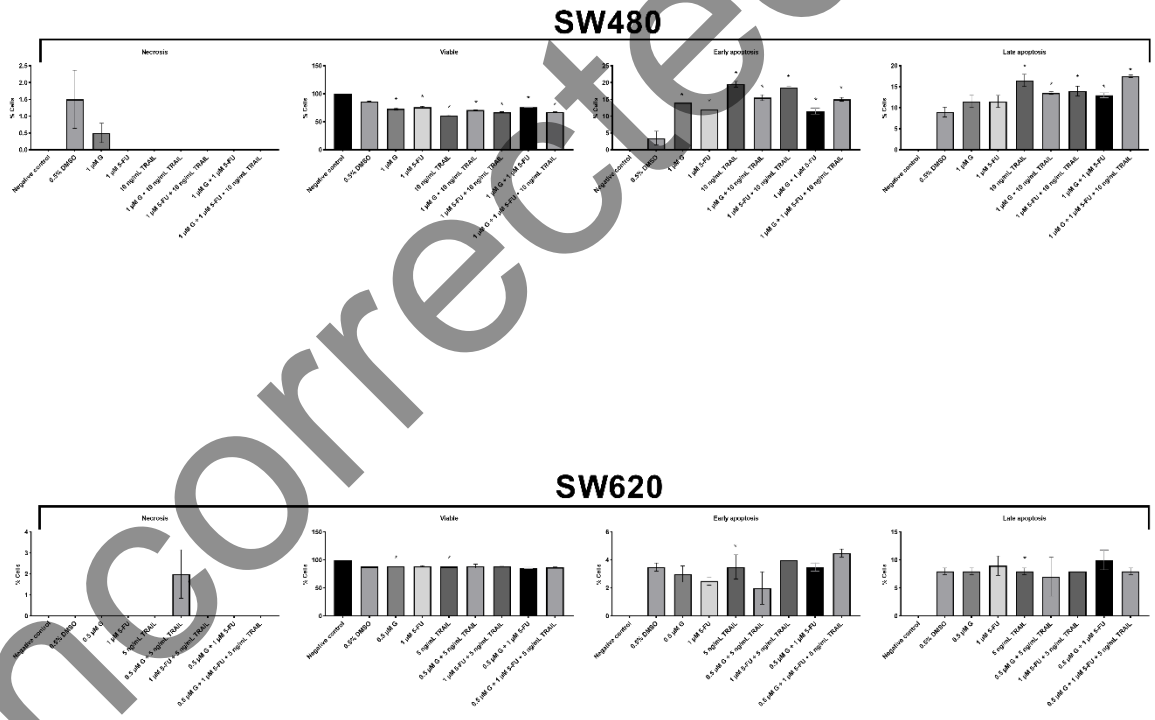


Figure. 7 The results of Annexin V FITC apoptosis assay showing apoptotic effects of genistein, 5-fluorouracil and TRAIL in SW480 and SW620 cells. Results were given as the mean ± standard error. (*p < 0.05, **p < 0.001, indicates significant difference from the negative control).

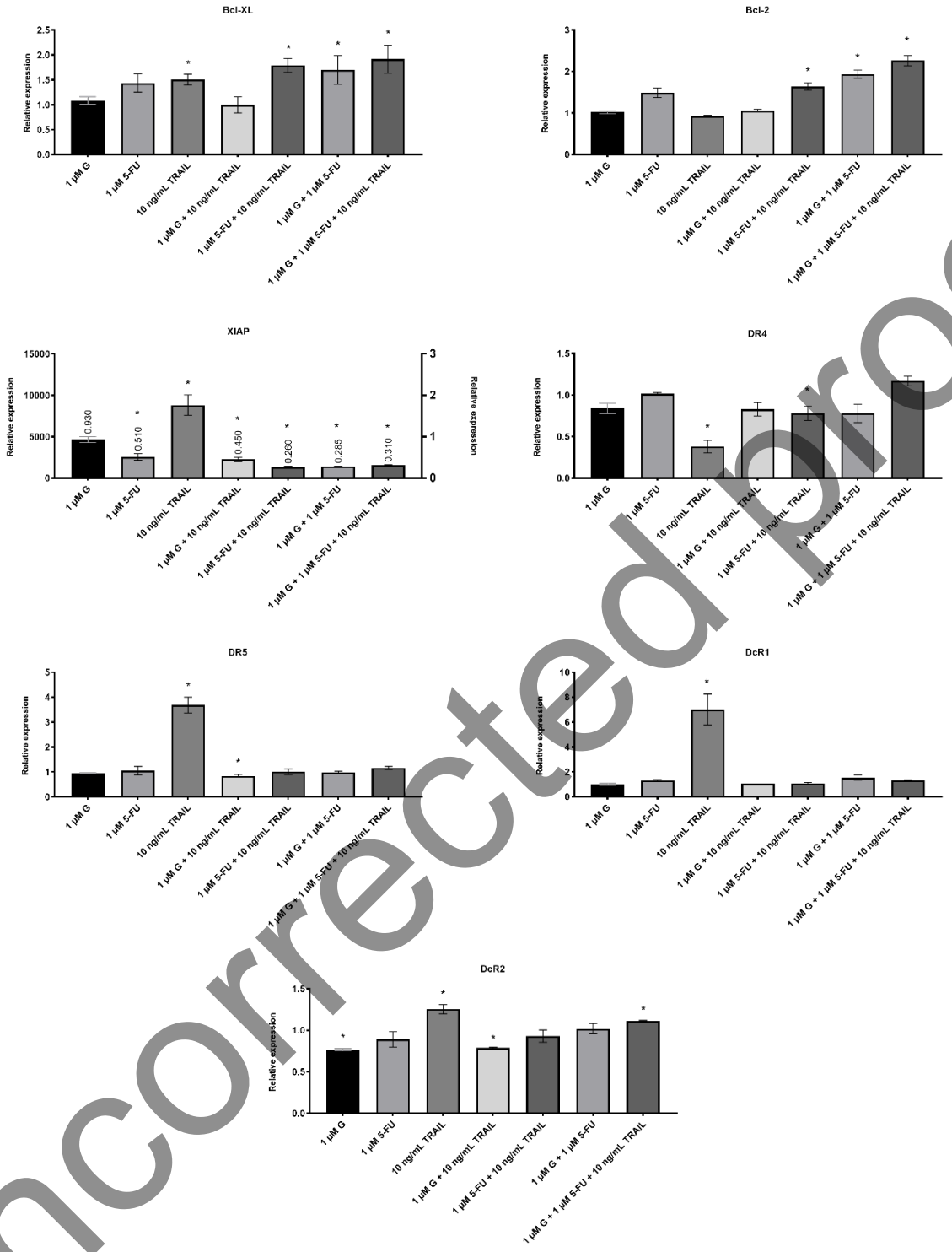


Figure. 8 Relative gene expressions of SW480 cells treated with genistein (G), 5-fluorouracil (5-FU), TRAIL and their combinations by RT-PCR. Results were given as mean of fold change compared to control (negative and 0.5% DMSO control). The values indicate the mean ± standard error and normalized with GAPDH (*p < 0.05, indicates significant difference from the negative control).

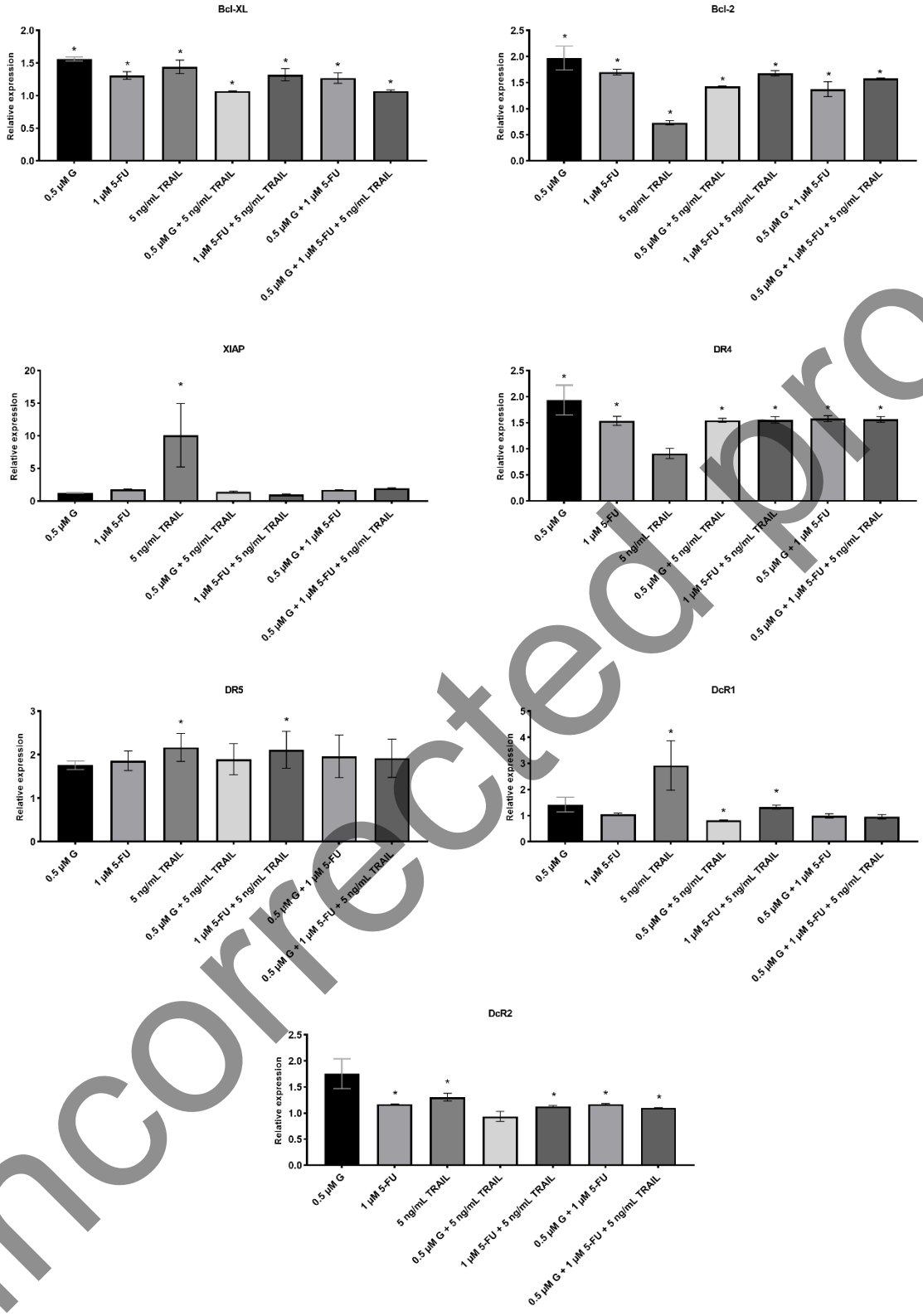


Figure. 10 Caspase 3 – 8 – 9 activities relative to control in SW480 and in SW620 cells by Multiplex Activity Assay Kit (Abcam). Results were presented as the mean ± standard deviation. (*$p < 0.05$, **$p < 0.001$, indicates significant difference from the negative control).

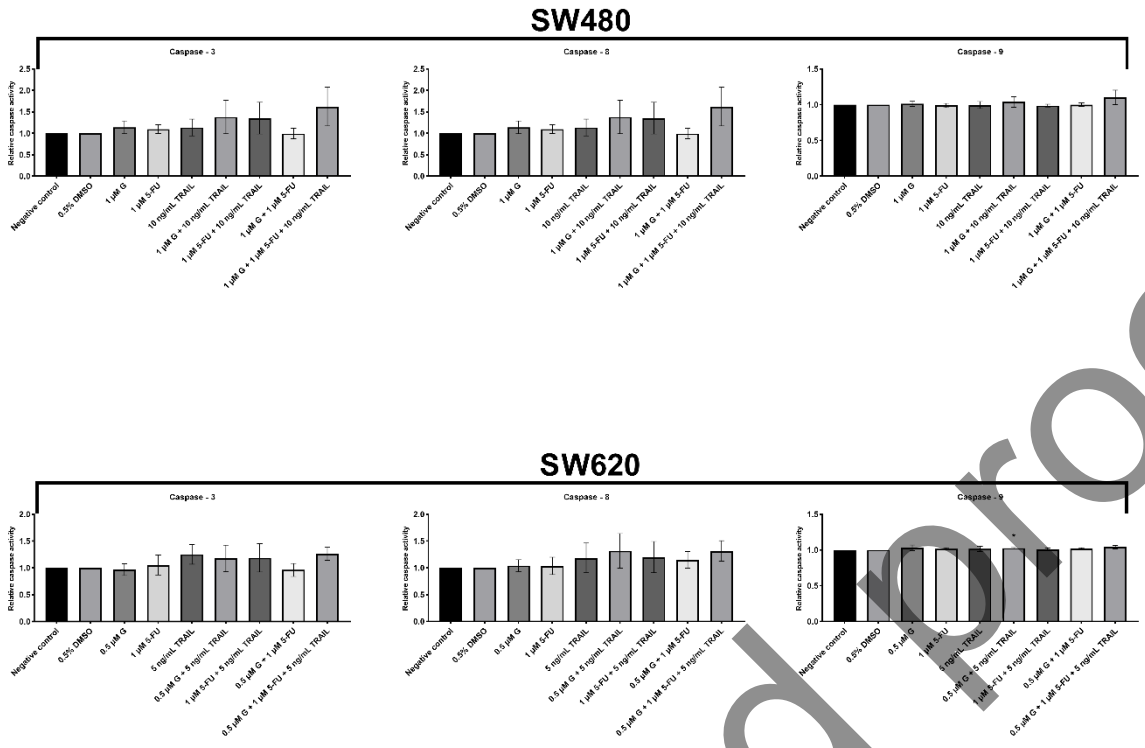
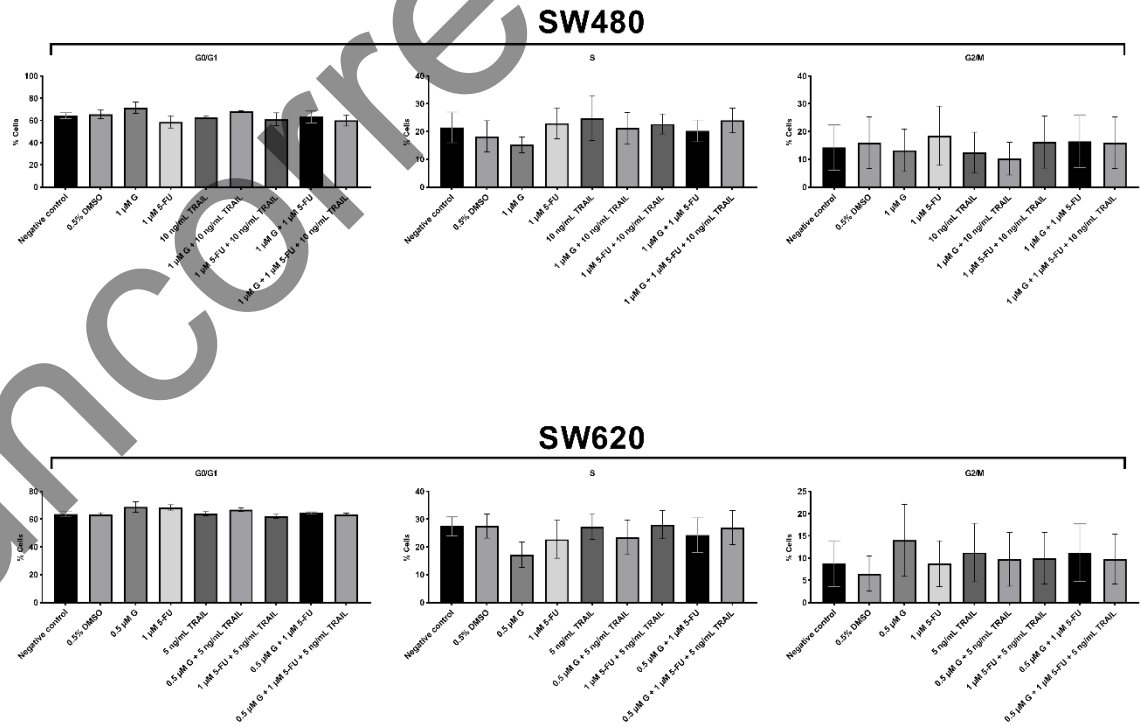
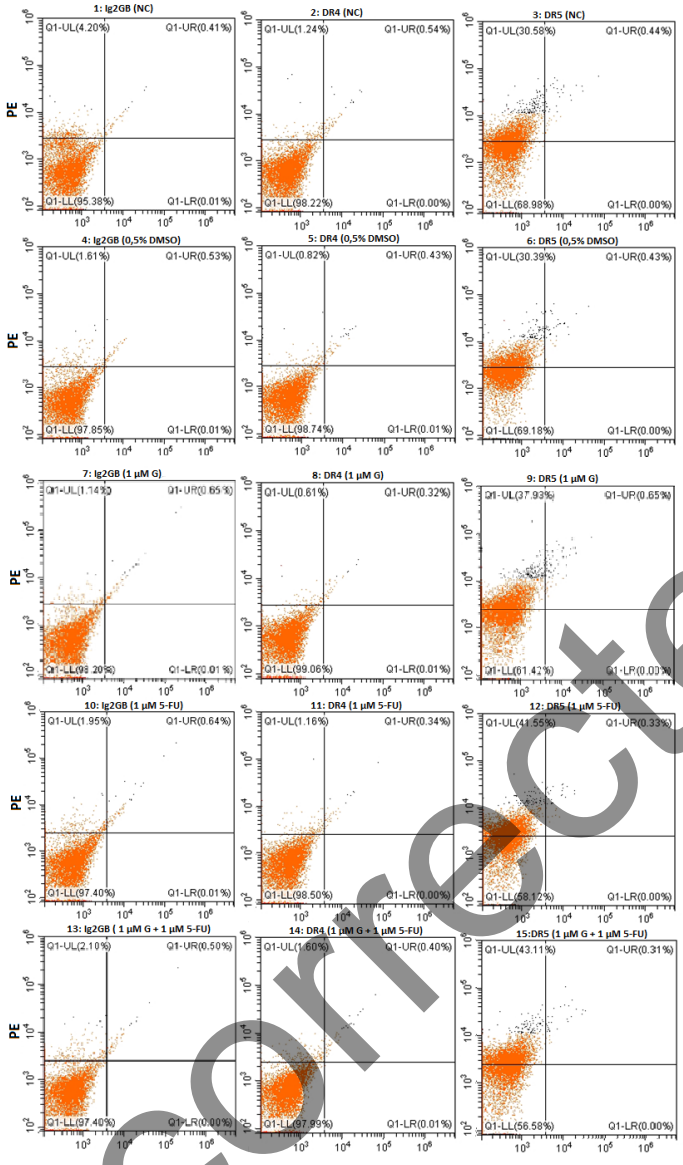


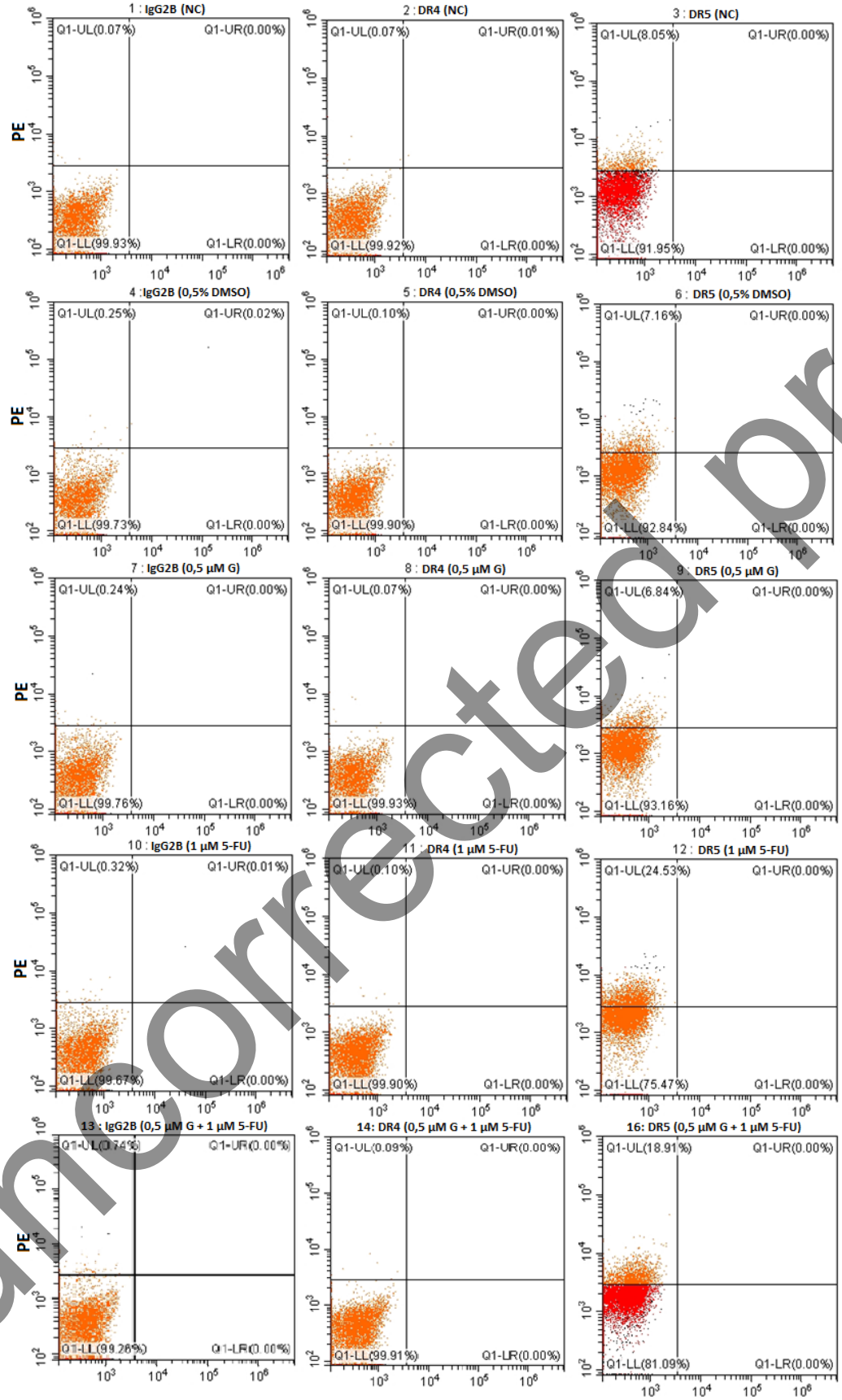
Figure. 9 Relative gene expressions of SW620 cells treated with genistein (G), 5-fluorouracil (5-FU), TRAIL and their combinations by RT-PCR. Results were given as mean of fold change compared to controls (negative and 0.5% DMSO control). The values indicate the mean ± standard error and normalized with GAPDH (*p < 0.05, indicates significant difference from the negative control).



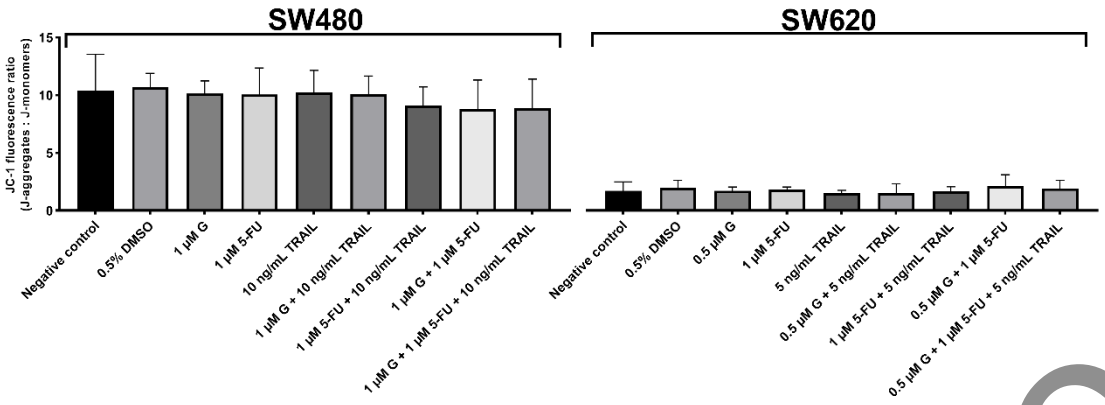
Supplementary Material 1. The effects of genistein, 5-fluorouracil and TRAIL on the cell cycle in SW480 and SW620 cells examined by flow cytometry.



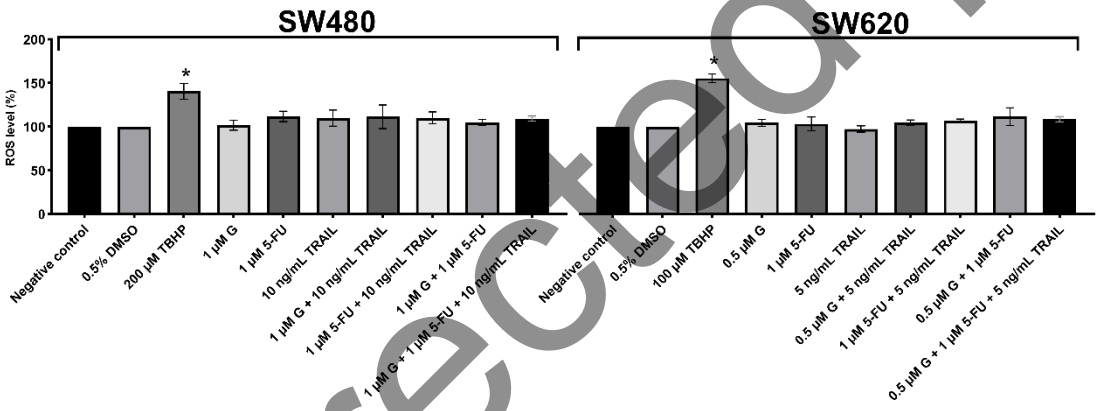
Supplementary material 2. Effects of genistein (G) and 5-fluorouracil (5-FU) on surface expression levels of DR4 and DR5 in SW480 cells by flow cytometry**.**



Supplementary material 3. Effects of genistein (G) and 5-fluorouracil (5-FU) on surface expression levels of DR4 and DR5 in SW620 cells by flow cytometry**.**



Supplementary Material 4. The changes of mitochondrial membrane potential of SW480 and SW620 cells were presented as the mean ± standard deviation of ratio between aggregates and monomeric forms of JC-1 by JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman).



Supplementary material 5. Effects of genistein, 5-fluorouracil, TRAIL and their combinations on ROS level in SW480 and SW620 cells for 48 h using ROS detection assay kit (Biovision). Results were given as mean ± standard deviation of ROS level % compared to negative and DMSO controls. (*$p < 0.05$, **$p < 0.001$, indicates significant difference from the negative control).