# Combined Effects of Protocatechuic Acid and 5-Fluorouracil on p53 Gene Expression and Apoptosis in Gastric Adenocarcinoma Cells

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## ABSTRACT

**Objectives:** This study evaluates the combined effects of protocatechuic acid (PCA) and 5-fluorouracil (5-FU) on gastric adenocarcinoma (AGS) cells.

**Materials and Methods:** MTT assay test, colony formation assay, flow cytometry technique, Real-Time qPCR, and western blotting were used to investigate cytotoxic effects, colony formation, apoptosis, p53 gene expression, and Bcl-2 level in AGS cells co-treated with 5-FU and PCA, respectively.

**Results:** Our results demonstrated that PCA alone, or in combination with 5-FU (10 µM, 5-FU and 500 µM, PCA) inhibited AGS cell proliferation, forms a colony, and increased apoptosis compared with untreated control cells. Also, the combined 5-FU/PCA led to up-regulation of p53 and down-regulation of Bcl-2 protein as compared to untreated control cells.

**Conclusion:** The results demonstrate that the combined 5-FU/PCA may promote anti-proliferative and pro-apoptotic effects with the inhibition of colony formation in AGS cells. The mechanisms by which the combined 5-FU/PCA exerts its effects are associated with the up-regulation of p53 gene expression and down-regulation of Bcl-2 level. Therefore, the combination of 5-FU with PCA not only could be a promising approach to potentially reduce the dose requirements of 5-FU but also it can promote apoptosis *via* p53 and Bcl-2 signaling pathways.

***Keywords*:** Apoptosis, 5-fluorouracil, protocatechuic acid, gastric cancer, combination, colony formation

## INTRODUCTION

Gastric cancer is the fourth most common cancer depending on its incidence and mortality rate, stays a major health problem worldwide. The five-year survival rate for this disease is only about 20 %.[1] Gastric cancer's prevalence varies in different geographic regions.[2] Gastric cancer is a progressive disease which can be increased due to genetic family background and environmental factors such as H.pylori, free radicals, smoking, high salt intake, and lack of adequate antioxidants. Also, disorders such as gastritis, intestinal metaplasia, dysplasia, paraneoplastic lesion, and chronic atrophic gastritis are among the underlying causes of gastric cancer.[2]

Gastric cancer treatment methods are primarily surgery, radiotherapy, and chemotherapy.[3] 5-FU, a heterocyclic aromatic compound widely used to treat cancers such as colon cancer, breast cancer, gastric cancer, and prostate cancer, blocks thymidylate synthase activity and thus stops the production of DNA.[4,5] 5-FU, however, has several side effects such as neutropenia, stomatitis, diarrhea, emesis, inflammation of the mouth, loss of appetite, low blood cell counts, hair loss, and skin inflammation.[6] Also, administration of 5-FU is frequently limited by dose-limiting toxicities. Interaction of chemotherapy with natural compounds may present a new perspective and an innovative strategy in cancer therapy. Interestingly, herbal compounds in tandem with 5-FU amplify the synergistic effects of both administered therapeutics and exert cytotoxic effects specifically in tumor cells. Combined therapy with synergistic effects not only reduces the drugs doses and resistance in chemotherapy but also it decreases metastasis, raises efficacy of 5-FU, and induces apoptosis.[7] Apoptosis in cells is a type of programmed cell death under the control of factors such as p53 gene expression which is mutated in most cancer cells. This gene plays a crucial role in genome stability, tumor suppression, induction of apoptosis, cell cycle stopping, and aging.[8]

Also, p53 acts as a transcription factor for pre-apoptotic proteins, including Bcl-2. By increasing the expression of Bcl-2, mitochondria become permeable and apoptosis begins with the release of cytochromes.[9]

Various experiments have been conducted to discover and use natural compounds for induction of apoptosis in cancer cells. Epidemiological studies have shown that a diet rich in phytochemical compounds is effective in inducing apoptosis in some cancers.[1,10] Phytochemicals with antioxidant activity can inhibit carcinogenic processes in several models due to the expression of key proteins in signal transduction pathways and induction of apoptosis.[11] Also, it is reported that many polyphenols can reduce the adverse effects of chemical therapies.[1] Protocatechuic acid (PCA, also known as 3,4-dihydroxybenzoic acid) is a herbal phenolic acid mainly present in fruits, vegetables and nuts with anti-inflammatory, antibacterial, anti-hyperglycemic, anticancer, antiulcer and antispasmodic properties.[12-14] Therefore, the purpose of this study was to evaluate the combined effects of 5-FU and PCA on apoptosis, colony formation, p53 gene expression and Bcl-2 signaling protein level in gastric adenocarcinoma (AGS) cells cell line.

## MATERIALS AND METHODS

### *Chemicals and antibodies*

AGS human gastric adenocarcinoma cells was purchased from Pasteur Institute (Tehran, Iran). RPMI 1640 medium, trypsin 0.25%, penicillin/streptomycin (pen/strep), and fetal bovine serum (FBS) were supplied from Gibco (Rockville, MD, USA). Bcl-2 and β-actin primary antibodies were purchased from Elabscience Biotechnology Co. (Wuhan, China). PCA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5-FU (50 mg/ml solution) was prepared from Haupt Pharma (Wolfratshausen GmbH Co, Germany). Roti®ZOL total RNA extraction kit was obtained from Carl Roth GmbH, Germany. Annexin V-PI staining kit was purchased from BD Bioscience (California, USA). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)* and p53 primers were purchased from Macrogen Company (South Korea). All other chemicals used were of analytical grade.

### *Cell viability assay*

AGS cells (5000 cells/per well) were seeded in 96-well plates in RPMI 1640 medium supplement with 10% FBS, 1% PEN/STREP at 37ºC in 98% humidity with 5% $CO_2$ for an overnight. Subsequently, cells were exposed to 5-FU (0-55 µM), PCA (0-1100 µM, solution in DMSO with 0.1% final concentration), and the combination of 5-FU with PCA (10 µM and 500 µM respectively) for 24h. Then, the medium was removed and the cells were incubated with MTT solution (5 mg/ml) for 4 h at 37˚C. Afterwards, dimethyl sulfoxide (DMSO) was subjoined to each well of 96-well plates. The absorbance of each well was measured with a microplate reader (Stat Fax-2100, USA) at 490-570 nm. The percentage of cell viability was assessed as follows: viability = A (sample) / A (control) × 100.[15] At least three independent experiments were carried out.

*Determination of synergistic effects of 5-FU and PCA*

The $IC_{50}$ of 5-FU and PCA were used to determine synergistic effects between 5-FU and PCA through combination index (CI) by using the CI equation[16]. $CI = A/A_{50} + B/B_{50}$, where A is the concentration of 5-FU in combination with B and B is the concentration of PCA in combination with A. $A_{50}$ is the $IC_{50}$ of 5-FU and the $B_{50}$ is the $IC_{50}$ of PCA. A CI value of 1 represents an additive effect, CI<1 indicates synergism and CI>1 represents antagonism.[17] Therefore, a combination of 5-FU (10 µM, $IC_{20}$) and PCA (500 µM, $IC_{30}$) was used based on the results of MTT assay.

*Colony formation assay*

For colony formation assay $3\times10^5$ AGS cells were seeded in 6-well plates overnight. The cells were then treated with 5-FU per se (10µM), PCA alone (500µM), and the combination of 5-FU and PCA (10µM and 500µM respectively) incubated at 37 ºC in an atmosphere of 5% $CO_2$ for 24 h. Then, the medium discarded and the cell culture medium was changed every 2 days for 14 days. Subsequently, the cells were washed with PBS and fixed with 70% ethanol and colonies were stained with 0.5% crystal violet. Then, the number of colonies was counted and plating efficiency (PE) was measured using the following formula: PE= (number of colonies/number of seeded cells) ×100 and surviving fraction (SF) was determined by SF= (number of colonies/number of seeded cells ×PE control) ×100.[18]

*Apoptosis detection assay*

The percentage of apoptosis and necrosis of cells was conducted by flow cytometry using the AnnexinV-FITC Apoptosis Detection Kit (BD bioscience, Franklin Lakes,NJ). Briefly, AGS cells ($2\times10^5$ per well) were cultured in 6-well plates and incubated overnight. Cells were treated with 5-FU (10μM) and PCA (500 μM) or combination of 5-FU and PCA (10μM and 500 μM respectively) for 24 h. Then, cells were harvested by trypsinization, washed with PBS, and stained with Annexin V for 20 min according to the manufacture's protocol at room temperature in a dark place.[19] Cells were analyzed using a FACScan system (Becton-Dickinson and Company, San Jose, CA, USA).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

RT-q PCR was used to determine the p53 gene expression in AGS cells. In summary, total RNA was extracted from each of the untreated control cells, 5-FU (10μM) and PCA (500 μM) or the combination of 5FU and PCA (10μM and 500 μM respectively) after 24 h of treatment using Roti®ZOL reagent according to the manufacturer's instructions. The total mRNA concentration and quality of RNA were assessed by OD measurements at 260/280 ratio using Nanodrop 2000 spectrophotometr (Thermo-USA). 1 μg RNA were used for cDNA synthesis using a synthesis kit (Takara Bio Inc., Japan) according to the manufacturer's instructions and stored at -20˚C for subsequent use. The procedure of cDNA reverse transcription was carried out using Prime Script ™ reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. Then, cDNA was expanded by RT-qPCR using SYBR ® Green PCR Master Mix (Takara Bio Inc., Japan) in the presence of specific primers. The sequences of the primers for the reaction were as follows: H-*p53*-F, forward 5'-CCCATCCTCACCATCATCACAC-3' and reverse 5'-GCACAAACACGCACCTCAAAG3' and H-*GAPDH*-F, forward 5′ACACCCACTCCTCCACCCTTTG3′ and reverse 5′GTCCACCACCCTGTTGCTGTA-3′. The primers were designed with Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA) and confirmed by the blast (NCBI). *GAPDH* gene was used as a reference gene for the normalization. Enzyme activation was conducted for 10 min at 95˚C, followed by 40 cycles of initial denaturation at 95˚C for 10 sec and annealing/extension at 62˚C for 15 sec and melting at 72°C for 20 sec respectively in a 3000 Rotor Gene (Corbett, Australia) Real time PCR system.[20]

*Western blotting*

The AGS cells were grown into 6-cm dishes at the density of $6\times10^5$. After 24 h of treatment, protein extraction were carried out for control, 5-FU (10μM), PCA (500 μM), and the combination of 5-FU and PCA (10μM and 500 μM respectively)) using RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton 100 X, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 0.1% sodium azide, 50 mmol/L NaF ,1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 50μL protease and 250μL phosphatase inhibitor).[21] The supernatants were collected and protein concentrations were determined using Bradford procedure.[22] The western blot procedure was described previously [21] and primary antibodies Bcl-2 and β-actin were used according to the manufacturer's protocols. β-Actin was determined as an internal control. Then blots were washed with TBS-Tween buffer 3 times for 10 min and they were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h and washed again as described above. Band intensity was evaluated using chemiluminescent reagents (ECL; Thermo Fisher scientific, USA) and analyzed using Image J software.[20]

*Data Analysis*

All experiments were expressed as mean ± standard deviation (SD) and were done at least three times. SPSS software (Version 20, SPSS Inc., Chicago, IL, USA) or GraphPad Prism6 (Graphpad Software, San Diego, CA) was used to perform the statistical analysis. Kruskal–Wallis analyses were used to assess between-group differences for MTT assay, clonogenic assay, Annexin V assay, and RT- PCR. For expression analysis, the relative levels of quantitative gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method and data were expressed as fold change. A melting curve analysis was performed after amplification to verify product identity. Western blot were repeated 3 times. *P* values less than 0.05 were considered statistically significant for the differences between the groups. Combination index (CI) was calculated using experimental CompuSyn software (Combo SynInc, City, State, USA), and CI <1, =1 and >1 indicated synergism, additive effect, and antagonism, respectively.

## RESULTS

*Effects of 5-FU, PCA and their combination on AGS cell viability*

The result of MTT assay showed that 5-FU, PCA and their combination can inhibit the proliferation of AGS cells after 24 h (Figure 1). The $IC_{50}$ values of 5-FU and PCA alone were 40 μM and 700 μM respectively (Figure 1A and B). The combination of 5-FU and PCA (10μM and 500 μM respectively) led to a synergistic CI equal to 0.6 with strong effects on AGS cell proliferation (Table 1). Also, the number of living cells decreased in the combination of 5-FU with PCA (10 μM and 500 μM respectively) related to untreated control cells and each agent per se (Figure 1C).

*Clonogenic assay of AGS cells*

The results of colony formation assay demonstrated that the combination of 5-FU with PCA (10 μM and 500 μM respectively) significantly decreased colony numbers of the AGS cells and the proliferation rate compared with those of the untreated control cells and 5-FU treated cells (Figure 2). After 14 days of cell culture, the number of colonies consisting of 63, 46, 30, and 22 for control, 5-FU, PCA, and 5-FU/PCA combinations, respectively (Figure 2B). Surviving fraction (SF) for 5-FU, PCA, and 5-FU/PCA combination were 71%, 49%, and 34%, respectively. Also, the results demonstrated plating efficiency (PE) in control and treated experimental cells (Figure 2A).

*Effects of 5-FU and PCA on the apoptosis*

The results of flow cytometry technique showed the percentage of apoptosis and necrosis of 5-FU and PCA on AGS cells (Figure 3). Apoptosis in AGS cells slightly induced 17% at 10 μM 5FU, 23% at 500 μM PCA, and 27% at the combination of 5-FU and PCA (10μM and 500 μM respectively). Apoptosis significantly increased ($P < 0.05$) in the combination of 5-FU and PCA treatment when compared to those of control and 5-FU treated cells (Figure 3).

*Expression of p53 in AGS cells*

The results of RT‑qPCR showed the p53 gene expression in the combination of 5-FU and PCA (10 µM and 500 µM respectively). PCA and 5-FU/PCA combination led to a significant increase ($P < 0.05$) in the p53 gene expression by almost 5.5 and 11.6 fold respectively in comparison with those of control cells (Figure 4). No significant change was observed between 5-FU and untreated control cells.

*Effects of 5-FU, PCA and 5-FU/PCA combination on Bcl-2 signaling protein in AGS cells*

The result of western blotting demonstrated that the protein expression level of Bcl-2 markedly decreased after treatment with 5-FU/PCA combination in AGS cells when compared to those of untreated control cells and 5-FU treated cells (Figure 5).

## DISCUSSION

The prevalence of cancer is increasing worldwide, and the growing rate of mortality is quite alarming. Nowadays, 5-FU-based chemotherapy is a widespread procedure in the treatment of a wide range of cancers, including gastric, colorectal, and breast cancers due to its effect in inhibition of thymidylate synthase.[23,27] Combination therapy not only amplifies chemotherapy effects on tumor cells at lower concentrations but also it causes little toxicity to normal cells.[24] In the present study, the combined treatment of 5-FU with PCA had a stronger anti-proliferation effects than either agents alone (Figure 1). Several previous study have shown that PCA alone can decrease cell proliferation and viability in some cancer cell lines such as breast, lung, liver, cervix, and prostate cancer cells[25,26] which is in line with findings in the present study. On the other hand, many studies have demonstrated that the combination of natural compounds with chemotherapeutic drugs would enhance their anti-tumor efficacy through various mechanisms, including, cell sensitization, induction of apoptosis, inhibition of cell proliferation, invasion, metastasis, and angiogenesis[28] which is in agreement with findings of our study. It has been reported that natural compounds can disperse vimentin, an epithelial-mesenchymal transition factor, and cause loss of cytoplasmic integrity. These compounds can make changes in cellular morphology through destabilization of the nucleus, cytoskeleton, the mitotic spindle, and cell flexibility.[7,29] Also, previous studies have shown that some antioxidants such as curcumin, resveratrol, and epigallocatechin-3-gallate not only have chemo-preventive or chemotherapeutic

effects but also they act as chemo-sensitizers on tumor cells.[7,30-32] Therefore, in present study it seems that PCA, at least partly, sensitize the AGS tumor cells to 5-FU which led to increase the anti-proliferation and cytotoxic efficiency of 5-FU.

Our data demonstrated that the combined 5-FU and PCA resulted in a decrease in the number of colonies when compared to untreated control cells and each agent per se (Figure 2) which was in agreement with previous studies results.[33,34] A published study showed that combined 5-FU, cisplatin, and curcumin enhance the anticancer effects of 5-FU in human gastric cancer MGC-803 cells by decreasing cell viability, inhibiting colony formation, and through inducing apoptosis which is in line with the present study findings.[33] Also, it has been reported in an investigation that glabridin, the major isoflavan in licorice root, may inhibit the malignant proliferation of the human gastric cancer MKN-45 cell line and enhance the efficiency of 5-FU.[34] The reduction of the number of colonies in the present study through combined 5-FU and PCA, at least partly, may be resulted from synergistic effects due to loss of sensitized cytoplasmic integrity and cellular changes such as Bcl-2 reduction, p53 elevation, and cell morphology.

Our flow cytometry results demonstrated that co-treatment of PCA and 5-FU resulted in increasing of apoptosis in AGS cells compared to that of the untreated control cells (Figure 3). Antioxidants are used as anti-cancer compounds which can lead to cell death by activating the internal or external pathways of apoptosis.[1] It has been demonstrated in several studies that PCA can induce apoptosis in cancer cells, which is in line with the results of the present study. [35-37] Also, PCA-induced apoptosis was found to be associated with the inhibition of Bcl-2, the mitochondrial translocation of Bax and Bid, and the cytosolic release of cytochrome C, which is in agreement with our study findings. [38] On the other hand, it has been found that 5-FU induces apoptosis in cancer cells though p53.[27, 39-41] Also, it has been reported that co-administration of 5-FU with troxerutin, a flavonoid, results in a dose-dependent suppression of the cell proliferation and induces apoptosis which is in line with our findings (Figures 1 and 3).[42] In addition, it is reported that antioxidants can reduce side effects and potential harmful impact of medications,[43] influence multidrug resistance (MDRs) gene, which is responsible for resistance to different cytotoxic drugs, and enhance the residence time of chemotherapeutic drugs in cancer cells.[7] Therefore, in the present study the elevated efficacy of 5-FU for AGS apoptosis in presence of PCA, at least partly, is due to PCA antioxidant capacity through sensitized cytoplasmic integrity and cellular changes

such as Bcl-2 reduction, p53 elevation, and can trigger other internal or external signaling pathways of apoptosis.

p53 acts as a transcription factor for a series of pro-apoptotic proteins such as Bad, Bax, Bid, anti-apoptotic Bcl-2 signaling protein and induce apoptosis by releasing cytochrome C.[44] Natural anti-oxidants can cause cell death by controlling members of the Bcl-2 family and promoting DNA damage.[45] Besides, it has been demonstrated that Bcl-2, which encodes an inner mitochondrial protein, can antagonize apoptosis in many tumor cells.[38] Our results showed that the combined 5-FU/PCA increased the p53 gene expression and decreased cellular Bcl-2 signaling protein (Figures 4 and 5). In many previous studies, it has been found that PCA has the potentials to induce apoptosis, increase p53 gene expression, and cause a decline in Bcl-2 protein which is in agreement with our findings.[38,46] Nevertheless, in the present study, it seems that the combination of PCA with 5-FU can strongly increase p53 gene expression (Figure 4). In a study, it was demonstrated that PCA acted as an apoptotic inducer of leukemia by decreasing the phosphorylation of retinoblastoma and decreasing the expression of Bcl-2, which is in line with the present study.[38] On the other hand, researchers determined that *Hibiscus* polyphenol-rich extract, containing PCA caused apoptosis in human gastric carcinoma cells *via* p53 phosphorylation and p38 MAPK/FasL cascade pathway.[46] In addition, it has been demonstrated that natural antioxidants such as forbesione, lupeol, luteolin and myricetin can induce the synergistic, apoptotic, and anti-proliferative effects with 5-FU through the elevation of p53 gene expression and decreasing of the cellular Bcl-2 signaling protein in some cancer cells, which is in line with our findings.[47-50] Therefore, in the present study, the elevation of p53 gene expression and the reduction of Bcl2 protein level in presence of PCA, at least partly, may result from the potential of PCA in cell sensitization to 5-FU by activating intracellular signaling pathways.

In this study, we did not investigate the effects of the combined 5-FU/PCA treatment on other cellular signaling pathways such as FAK, MAPK, MMP, COX, JNK, Akt, ERK, Nf-κb, and caspases modulating factors which influence invasion, metastasis, and apoptosis. We also did not study cell survival factors such as Bcl-xL and cFLIP. Thus, we suggest that prospective researchers investigate the above factors in combined 5-FU/PCA in future studies.

## CONCLUSIONS

Our data indicate that the combined 5-FU/PCA treatment may promote anti-proliferative and pro-apoptotic effects plus inhibition of colony formation in AGS cells. Some mechanisms by which the combined 5-FU/PCA treatment exerts its effects are associated with the up-regulation of p53 and down-regulation of Bcl-2 expression. Therefore, the combination of 5-FU with PCA not only could be a promising approach to potential reduction of dose requirements of 5-FU treatment but also promote apoptosis *via* p53 and Bcl-2 signaling pathways.

*Conflicts of Interest: The authors report no conflict of interest.*

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**Figure legends**

**Figure 1.** Inhibition of cell proliferation by 5-FU (A) and PCA (B) for 24 h. The cells were cultured at the density of $5 \times 10^3$ cells per well for 24 h. At the end of treatment time, cell viability was measured by MTT assay. Data are expressed as mean±SD of 3 independent experiments. Also Figure 1C demonstrates cell morphological changes in AGS cells after treatment with 5-FU, PCA and their combination. 1C-a: Control, 1C-b: 5-FU, 1C-c: PCA, 1C-d: 5-FU plus PCA.

**Figure 2.** Colonies produced by AGS cell line in the absence or presence of 5-FU, PCA and 5-FU/PCA combination. A: histogram plot demonstrates plating efficiency (PE) in control and treated experimental groups. B: The colony formation in the control, 5-FU, PCA, and combination of 5-FU with PCA.
[a]$P < 0.05$ *vs.* control cells.
[b]$P < 0.05$ *vs.* 5-FU treated cells.

**Figure 3**. Induction of apoptosis after 24h exposure to 5-FU (10 μM), PCA (500 μM) and combination of 5-FU and PCA (10 μM and 500 μM respectively). Data were analyzed by FACScan and represent the mean of duplicate determinations. A: flow cytometry charts of Annexin V-FITC/PI staining in untreated (control) AGS cells and cells treated with 5-FU, PCA and combination of 5-FU and PCA. B: The percentage of apoptotic AGS cells. The results are expressed as mean ± SD of three separate experiments.
[a]$P < 0.05$ *vs.* control cells.
[b]$P < 0.05$ *vs.* 5-FU treated cells.

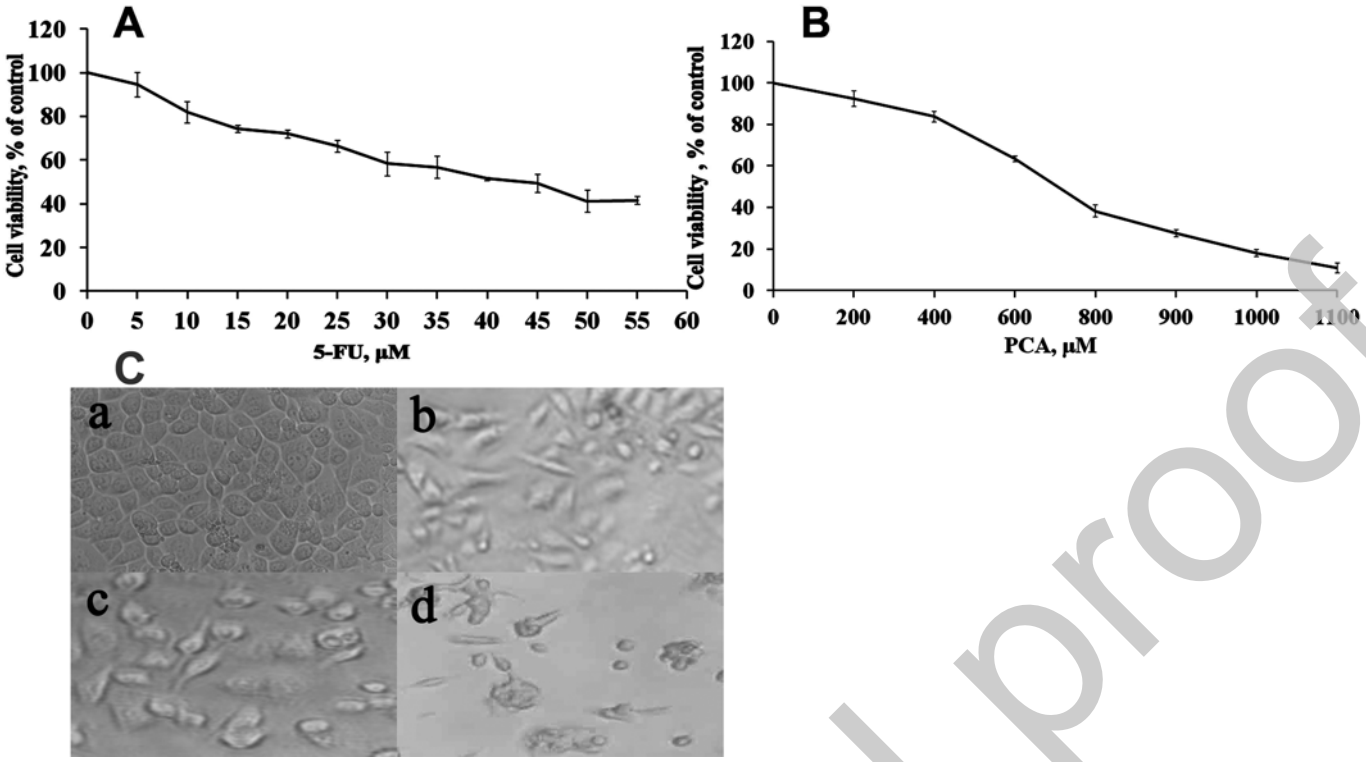
**Figure 4.** The gene expression of p53 in the presence or absence of 5-FU, PCA, and 5FU/PCA combination on the AGS cells. Cells were exposed to combination of 5-FU and PCA (10 μM and 500 μM respectively). The expression of p53 was normalized with GAPDH as an internal standard.
[a]$P < 0.05$ *vs.* control cells.
[b]$P < 0.05$ *vs.* 5-FU treated cells.

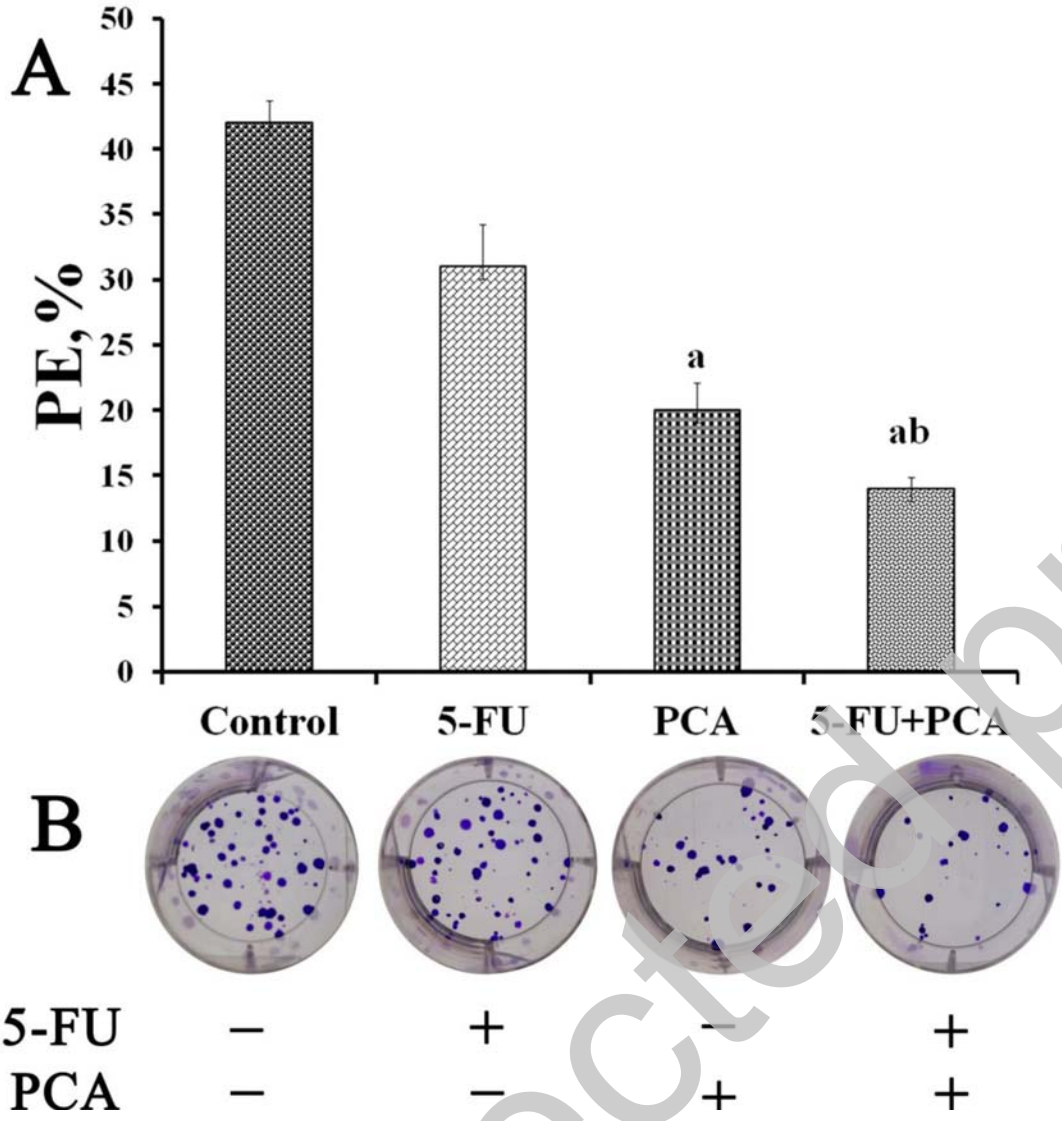
**Figure 5.** The Bcl-2 level of signaling pathway proteins in AGS cell line. Cells were treated with 5-FU (10 μM), PCA (500 μM) and 5-FU/PCA combination (10 μM and 500 μM respectively) for 24h. (A); Density chart of Bcl-2/ β-actin and (B); Western blots bands.

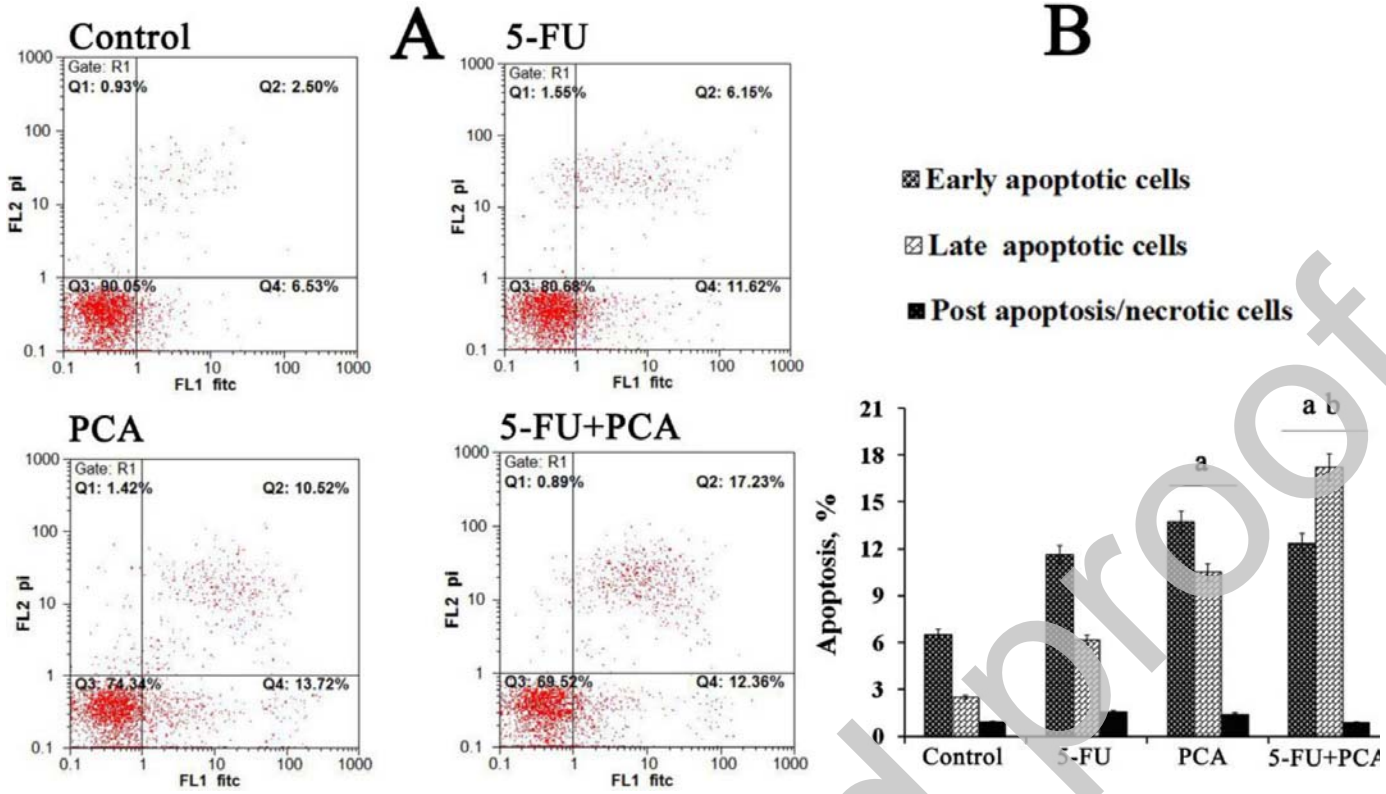
[a]$P < 0.05$ *vs.* control cells.
[b]$P < 0.05$ *vs.* 5-FU treated cells.

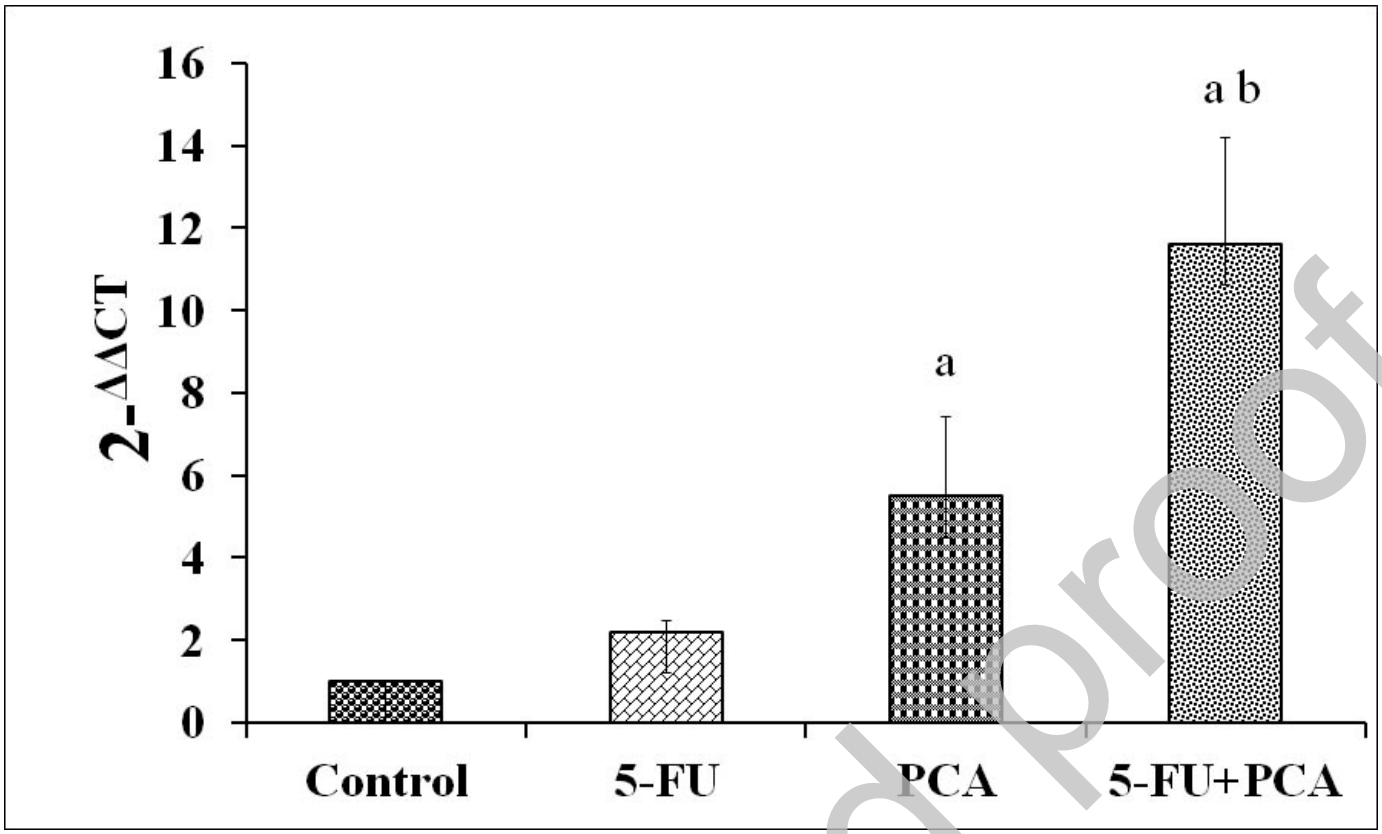
**Table 1. The viability percentage of AGS cell line treated with combination of PCA and 5-FU after 24 h.**

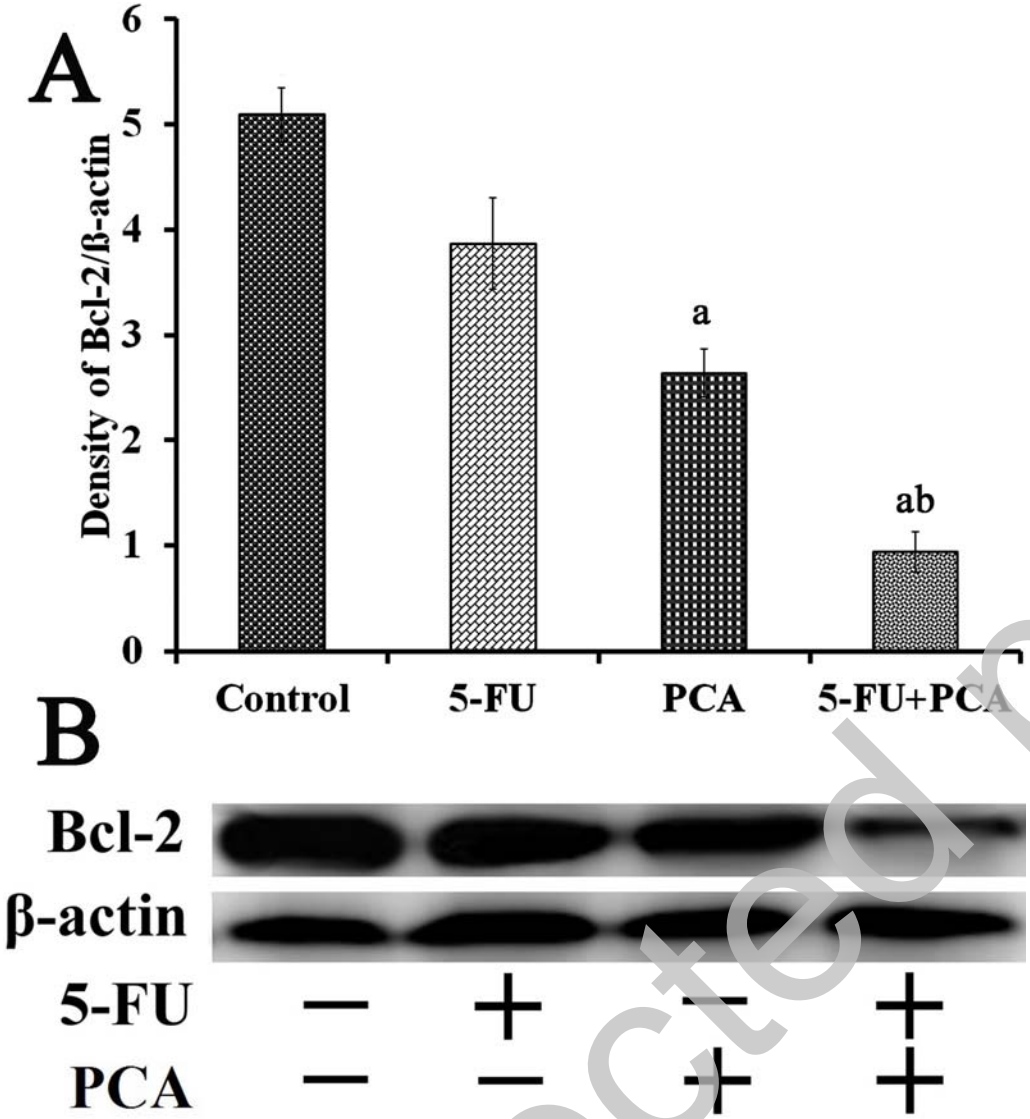
| Combination number | Dose combination, μM | | Cell viability, % | CI |
|---|---|---|---|---|
| | 5-FU (IC value) | PCA (IC value) | | |
| No. 1 | 5 ($IC_{10}$) | 600 ($IC_{40}$) | 39±4.4 | 0.72 |
| No. 2 | 10 ($IC_{20}$) | 500 ($IC_{30}$) | 20±2.7 | 0.60 |
| No. 3 | 20 ($IC_{30}$) | 400 ($IC_{20}$) | 45±4.1 | 0.81 |
| No. 4 | 30 ($IC_{40}$) | 200 ($IC_{10}$) | 45±2.9 | 0.79 |











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