

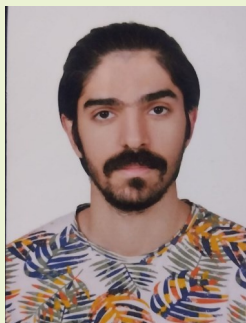
Investigating the synergistic effects of Peiminine in combination with 5-FU on the expression profile of *miR-181* and *miR-106* genes in colorectal cancer cells.

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Abstract

Introduction

This study aims to investigate the synergistic effects of Peiminine in combination with 5-Fluorouracil (5-FU) on the expression profiles of *miR-181* and *miR-106* in colorectal cancer cells.

Materials and Methods

Colorectal cancer cell lines (HT-29) were treated with Peiminine, 5-FU (1 and 0.5 µg/ml), and their combination at optimized concentrations (1,5,10,50,100,150,200 and 400 µg/ml). Cell viability was assessed using MTT to evaluate the combined cytotoxic effects. Quantitative real-time PCR (qRT-PCR) was performed to determine the expression levels of *miR-181* and *miR-106* following each treatment. Flow cytometry was used to examine programmed cell death (apoptosis). Statistical significance was determined using ANOVA and post hoc tests.

Results:

The IC₅₀ value of 5-FU was calculated as 2.902 µg/mL at 24 hours, while Peiminine as a single treatment exhibited an IC₅₀ of 0.431 µg/mL at 24 hours. In the combined treatment, the IC₅₀ of Peiminine at 24 hours was determined to be 359.4 µg/mL and 78.42 µg/mL when combined with 0.5 µg/mL and 1 µg/mL of 5-FU, respectively. The combination of 5-FU and Peiminine in HT-29 cells increased the relative expression of the *miR-181* gene at the RNA level, although this increase was not statistically significant. In contrast, the relative expression of the *miR-106* gene at the RNA level was significantly reduced ($p < 0.005$). Furthermore, the combination treatment induced apoptosis in 92% of HT-29 cells.

Conclusion:

Peiminine enhances the anticancer efficacy of 5-FU in colorectal cancer cells by modulating the expression of *miR-181* and *miR-106*. The observed synergistic interaction presents a promising therapeutic approach to improve CRC treatment outcomes. Further studies are required to elucidate the underlying molecular mechanisms and validate these findings in vivo.

1. Introduction

According to the World Health Organization (WHO), cancer is known as the main cause of death worldwide in the 21st century. In 2015, can-

cer was the first or second cause of death in most countries before the age of 70. According to the expectations, the number of new cancer cases and deaths will reach 21.4 million people per year and 13.2 million people per year by 2030, respectively [1].

Colorectal cancer (CRC) is the third most common cancer in men and women, accounting for 8% of new cancer cases in the United States and responsible for 8–9% of estimated cancer deaths[2].

Peiminine is a bioactive alkaloid primarily derived from *Fritillaria imperialis*. Known for its unique pharmacological properties, peiminine has garnered significant attention in recent years for its potential therapeutic applications[3]. According to previous studies, pprevents mast cells from producing pro-inflammatory cytokines and MAPKs[4]. Peiminine has been shown to prevent LPS-induced mastitis by decreasing the *AKT/NF-κB*, *ERK1/2*, and *p38 MAPK* signalling pathways[5]. Peiminine has been shown to have a neuroprotective effect on Parkinson's disease rats by lowering the production of reactive oxygen species (ROS) and so preventing the degeneration of dopaminergic neurones[6].

5-FU is a chemotherapeutic agent that is commonly used to treat various types of malignant tumors such as breast, pancreatic, skin, stomach, lung, head and neck cancer. In the treatment of colorectal cancer, 5-FU, administered intravenously or orally or together with other fluoropyrimidines (FPs), has been used as the main systemic therapy since the 1990s. Due to unpredictable absorption in the gastrointestinal tract and significant changes in pharmacokinetics, the use of oral 5-FU alone has declined. Since then, research has focused on biomodulation of 5-FU to improve its therapeutic efficacy and cytotoxicity[7].

miRNAs (microRNAs) are small non-coding RNAs with a length of 20-24 nucleotides that inhibit or degrade translational targets by recognizing the untranslated region (3'-UTR). As regulators of gene expression, miRNAs control about 30% of human genes. In recent years, several studies have shown that miRNAs play a key role in tumor growth, metastasis and immune response in CRC (colorectal cancer) and can act as oncogenes (tumor genes) or tumor suppressors[8, 9]. For example, MiR-196a-5p, a proto-oncogene, is an epithelial-mesenchymal regulator of CRC by binding to IκBα and promoting CRC proliferation, metastasis and invasion[10]. It has also been confirmed that MiR-500a-P5 is a tumor suppressor that is significantly decreased in CRC and inhibits the growth and metastasis of CRC tumors by regulating the histone deacetylase 2

(HDAC2) and being negatively regulated by the Yin Yang 1(YY1). Following recent advances in cancer therapy, they have become an effective and indicative treatment for CRC. In other words, serum miRNAs have been shown to be able to predict CRC tumor recurrence and treatment outcomes[11].

miR-181 has been shown to promote angiogenesis in CRC by directly suppressing the SRC kinase signaling inhibitor 1 (SRCIN1) expression and promoting the SRC/VEGF signaling pathway, which is critical for tumor angiogenesis. In addition, miR-181a has been identified as a novel angiogenic upstream target of VEGF, and its downregulation may be a potential strategy for the treatment of CRC.

miR-106 is associated with the formation and development of CRC. It is involved in the regulation of cell cycle and apoptosis factors, and its target genes are enriched in several important Gene Ontology (GO) categories and signaling pathways in cancer, such as the *Wnt*, *PI3K-Akt* and *FoxO* pathways. In addition, miR-106 has been shown to suppress tumor cell death in CRC by targeting ATG7.

both *miR-181* and *miR-106* play a crucial role in CRC, with miR-181 promoting angiogenesis and miR-106 suppressing tumor cell death. Their potential as diagnostic and therapeutic biomarkers in CRC is currently being investigated with promising results. The aim of this study is to investigate the effect of Peiminine on the chemosensitivity of colorectal cancer cells to 5-FU and on the expression of the miR101 and miR181 genes.

2. Material and methods

2.1 Preparation of Peiminine and 5FU

Peiminine is obtained from the company Sigma with the catalog number 274623 and the drug 5FU from the company Sigma with the catalog number 818505.

2.2 Cell cultures

The HT-29 cells (Human colorectal adenocarcinoma, IBRC C10097) were purchased from the National Cell Bank in the Iranian Biological Resource Center. Cells were incubated in DMEM culture medium containing 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics at 37°C in a humidified atmosphere with 5% carbon dioxide (CO₂).

The morphology and number of cells were checked with an inverted microscope. After the density reached 70%, the cells were passaged. Excess cells were also frozen for the next steps.

2.3 Determination of the percentage of surviving cells

To investigate the effects of 5-fu, Peiminine and of 5-fu+Peiminine on the proliferation of HT-29 cancer cells, the MTT method was used. The MTT reagent (4,5-dimethyltetrazolyl-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt that absorbs the mitochondria of metabolically active cells and the activity of dehydrogenase enzymes, producing purple formazan crystals. 10.000 cells were transferred to 96-well plates containing the complete culture medium to determine the percentage of survival, and the IC50 was measured using the MTT assay. To allow cell death to occur uncontrollably and the excessive concentration of the drugs not to be a limiting factor, and to compare the effects of the different drugs, the IC50 value was calculated after treating the HT-29 cells with different concentrations of Peiminine(1,5,10,50,100,150,200,400ug/ml) and 5-FU (0.05, 0.1, 0.5, 1,2,4,8,10 ug/ml) for 24 hours. After the incubation period, 20ul of MTT solution at a concentration of 0.5 mg/ml was added to each well. The plates were kept in an incubator protected from light for 4 hours. The supernatant solution was then discarded and 150 microliters of dimethyl sulfoxide DMSO was added to convert the insoluble formazan crystals into soluble color. In less than 20 minutes, the absorbance of the generated dye was measured with an ELISA reader at a wavelength of 570 nm.

2.4 Determination of cell apoptosis using Annexin-V-FITC/PI staining technique

To verify whether the decrease in the survival rate of these cell lines after treatment of the cells with the desired 5-FU, Peiminine and 5-FU+Peiminine was due to apoptosis, the amount of phosphatidylserine release on the surface of the cells and the amount of induction after 48 hours of treatment by annexin-V-FITC/PI were analyzed by flow cytometry. Annexin-V conjugated to fluorescein isothiocyanate (FITC) was used to quantify the abundance of apoptotic cells, and propidium iodide

(PI) was used to separate primary apoptotic cells from necrotic cells. The cells were treated for 48 hours. After trypsinization and washing twice with cold PBS, the cells were transferred to sterile microtubes and centrifuged at 1500 rpm for 5 minutes, and the supernatant was discarded. Then, they were dissolved in binding buffer containing 5 µL of FITC-conjugated Annexin-V and 5 µL (250 mg/ml) PI, and after 15 minutes of incubation in the dark, they were read by flow cytometry.

2.5 Evaluation of gene expression by real-time PCR

RNA extraction

RNA extraction is the first step in the investigation of gene expression. After treating HT-29 cells with the IC50 concentration of the 5-FU and Peiminine in 6-well plates and after the incubation period, RNA extraction was performed using the Trizol method and according to the protocol.

the quality and quantity of the extracted RNA

Agarose gel electrophoresis

To examine the extracted RNA, the RNA sample was electrophoresed on a 1% agarose gel. The analysis of the agarose gel with Gel Doc showed clear RNA bands.

Spectrophotometry

To quantitatively determine the concentration and purity of the samples, a Nanodrop device was used at wavelengths of 260 and 280 nm.

cDNA synthesis

After preparing equal concentrations of RNA, cDNA was synthesized using the Revert Aid RT Reverse Transcription Kit - Thermo Fisher according to the instructions.

Examination of the expression changes of the mir181 and mir106 genes using the qRT-PCR method

To detect and compare gene expression, qRT-PCR was performed using the SYBR-Green PCR Master Mix and the Real-Time PCR Rotor gene 6000. The primers were designed using the primer3 plus software (Table 1). The relative expression level of the genes was checked by comparison with the source gene of U6 as a control.

Table1. Primers used in this study

Primer	Forward	Reverse
<i>Mir-181</i>	CAGTGAACATTCAACGCTGTC	GCTGATGGTTGGCCATAGG
<i>MiR-106</i>	AAAGTGCTGACAGTGCAGA	GAACATGTCTGCGTATCTC
U6	GCTCGCTTCGGCAGCACATATAC	GGTCCGAGGTATTTCGCACTGGATA

First, HT-29 cells were cultured in a 6-well plate and Time PCR preparations were performed as shown in placed under the influence of IC50 concentration lower Table 2. The reaction conditions are also listed in Table and higher than the IC50 of 5-FU and Peiminine for 24 3. hours. After RNA extraction and cDNA synthesis, Real

Table 2. Component used for Real Time PCR reaction

No	Component	20ul reaction
1	qPCR Master	10ul
2	Forward primer	1ul
3	Reverse primer	1ul
4	Template DNA	1ul
5	Water, RNase-Free	Up to 20ul

Table 3. Real time PCR reaction conditions

Step	Temp (°C)	Time	Cycle
Initial Denaturation	95	5 minutes	1
Denature	95	30 Seconds	40
Annealing	60	30 Seconds	
Extension			

2.6 Statistical analysis

The data were presented as mean values with standard error of the mean (SD). Statistical analysis and graphing were performed using Prism GraphPad 8.0 software. The T-TEST was used for the significance level. The number of cycles or Ct obtained by the formula ($2^{-\Delta\Delta Ct}$) was calculated and used. The analyzes were performed using the Relative Expression Software Tool.

3. Results

3.1 Optimizing the appropriate concentration of Peiminine and 5FU for treatment with HT-29 by MTT method

The percentage of viable HT-29 cells after treatment with different concentrations 5-FU (0.05, 0.1, 0.5, 1, 2, 4, 8, 10 $\mu\text{g/ml}$) is shown in Figure 1. The IC₅₀ of the drug 5FU as monotherapy was 2.902 $\mu\text{g/ml}$ in 24 hours

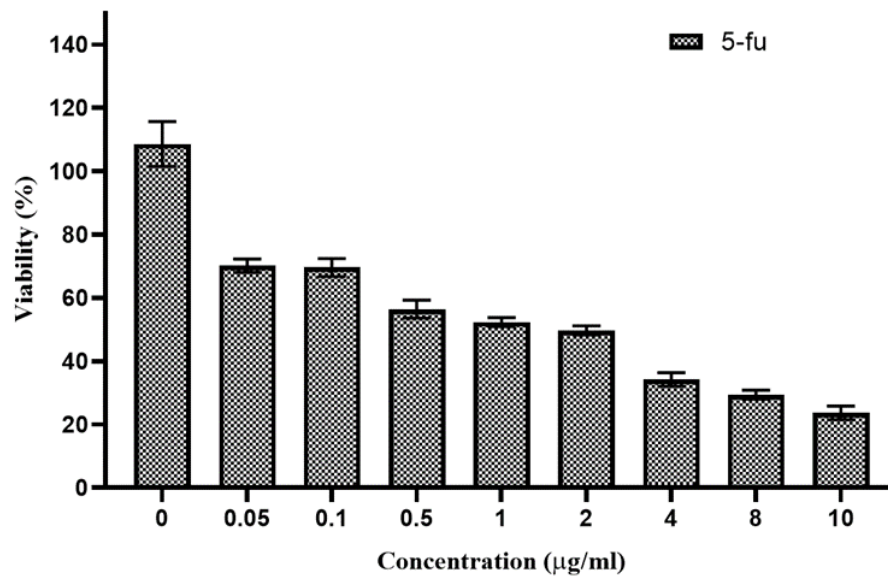


Figure 1. Cell growth inhibitory effect of 5-FU as monotherapy in HT-29 cells in 24

The percentage of viable HT-29 cells after treatment IC₅₀ of Peiminine as monotherapy was 0.431 $\mu\text{g/ml}$ in 24 hours (Figure 2). The percentage of viable HT-29 cells after treatment with different concentrations Peiminine (1, 5, 10, 50, 100, 150, 200, 400 $\mu\text{g/ml}$) is shown in Figure 1. The

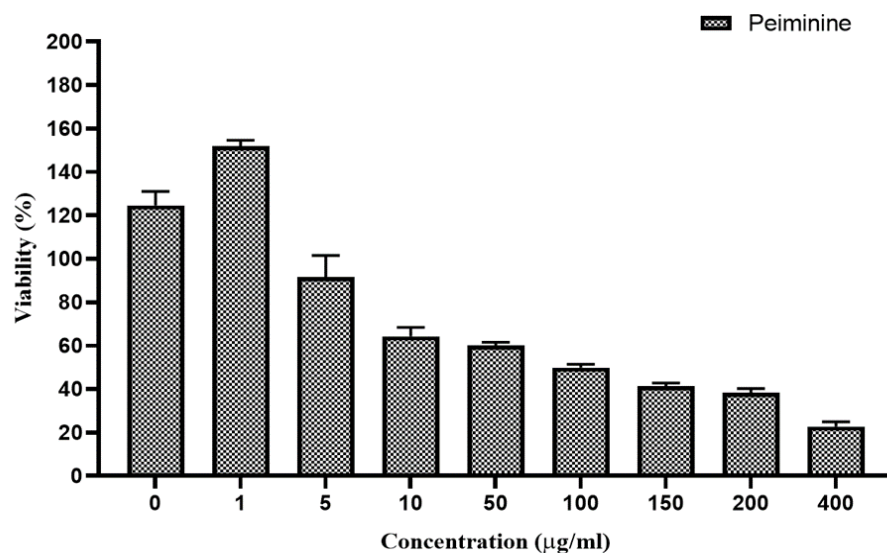


Figure 2. Cell growth inhibitory effect of Peiminine as monotherapy in HT-29 cells at 24 hours.

In the combined treatment, to reduce the side effects of 5-FU, concentrations of 1 and 5 $\mu\text{g/ml}$ of 5-FU were used together with different concentrations of Peiminine (5, 50, 100, 200 $\mu\text{g/ml}$). The IC_{50} value of Peiminine in combination with 5-FU was measured over 24 hours. For the concentration of 1 $\mu\text{g/ml}$ 5-FU, the IC_{50} value was equal to 78.42 $\mu\text{g/ml}$ Peiminine. An IC_{50} value of 359.4 $\mu\text{g/ml}$ Peiminine was also determined for the concentration of 0.5 $\mu\text{g/ml}$ 5-FU. The results show that the IC_{50} concentration decreases with the combination of Peiminine and 5-FU. (Figure 3).

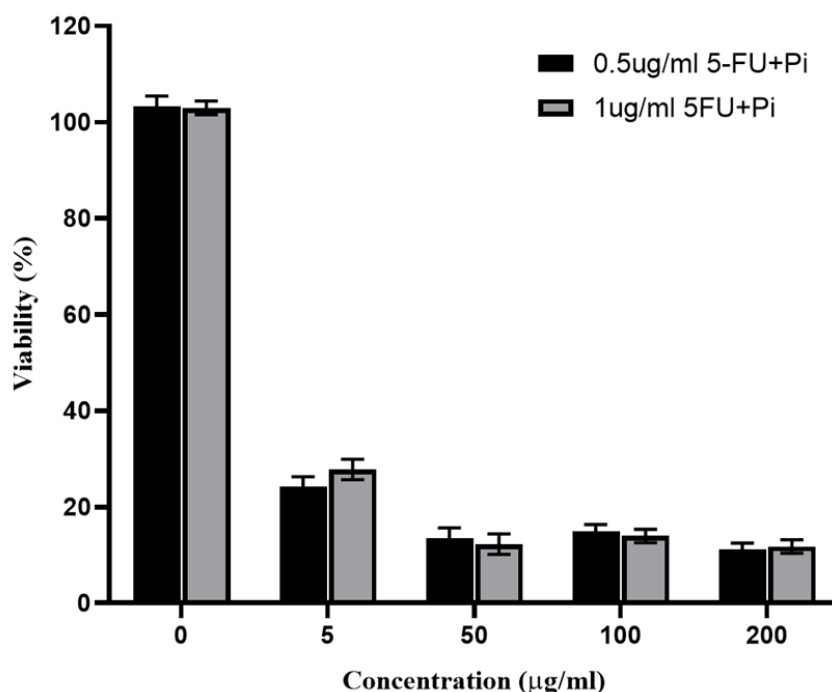


Figure 3. Combination treatment in HT-29 cells. Data were expressed as mean $\pm$ standard deviation.

3.2 Investigation of the phenotype of HT-29 cancer cells after combined treatment with Peiminine and 5-FU

Morphologically, the results were consistent with the

MTT assay. At a concentration of 1 $\mu\text{g/ml}$ 5-FU together with 78.42 $\mu\text{g/ml}$ Peiminine, there was a decrease in cell number, smaller and rounder cells compared to untreated control cells (Figure 4), indicating cell death.

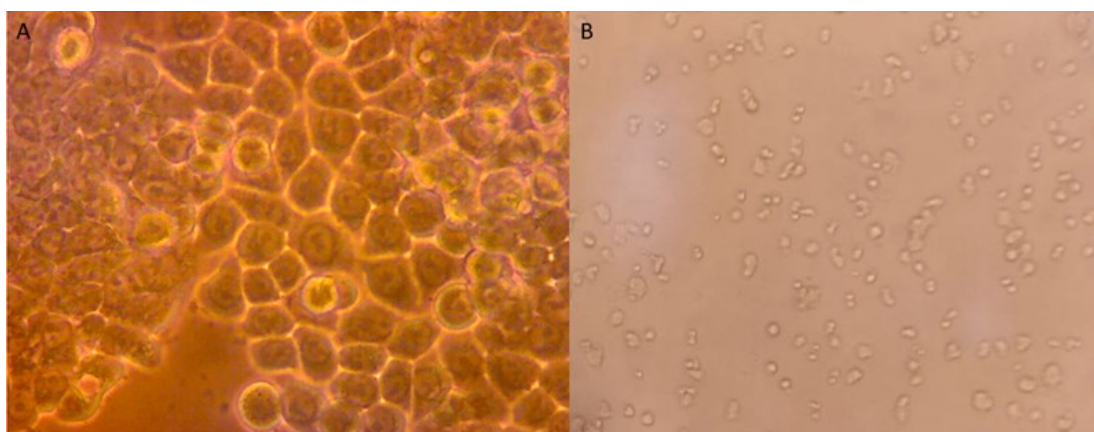


Figure 4; A; Control cell, B; HT-29 cells after 24 hours of incubation with piminin+5-FU

3.3 Flow cytometry results in cells treated with Peiminine and 5-FU and their combination

The results of the study of HT-29 cells showed that after 24 hours of exposure to Peiminine, 5-FU, Peiminine with 5-FU, about 0.743%, 1.42% and 12.8% of the cells underwent necrosis and 28%, 36% and 91% of the cells

underwent apoptosis. This shows that the Peiminine together with the drug 5FU was able to kill a percentage of cells with a significant difference of $P < 0.05$ compared to the control group (Figure 5). This result shows that the combination of the drug with Peiminine has a stronger lethal effect on the HT-29 cells.

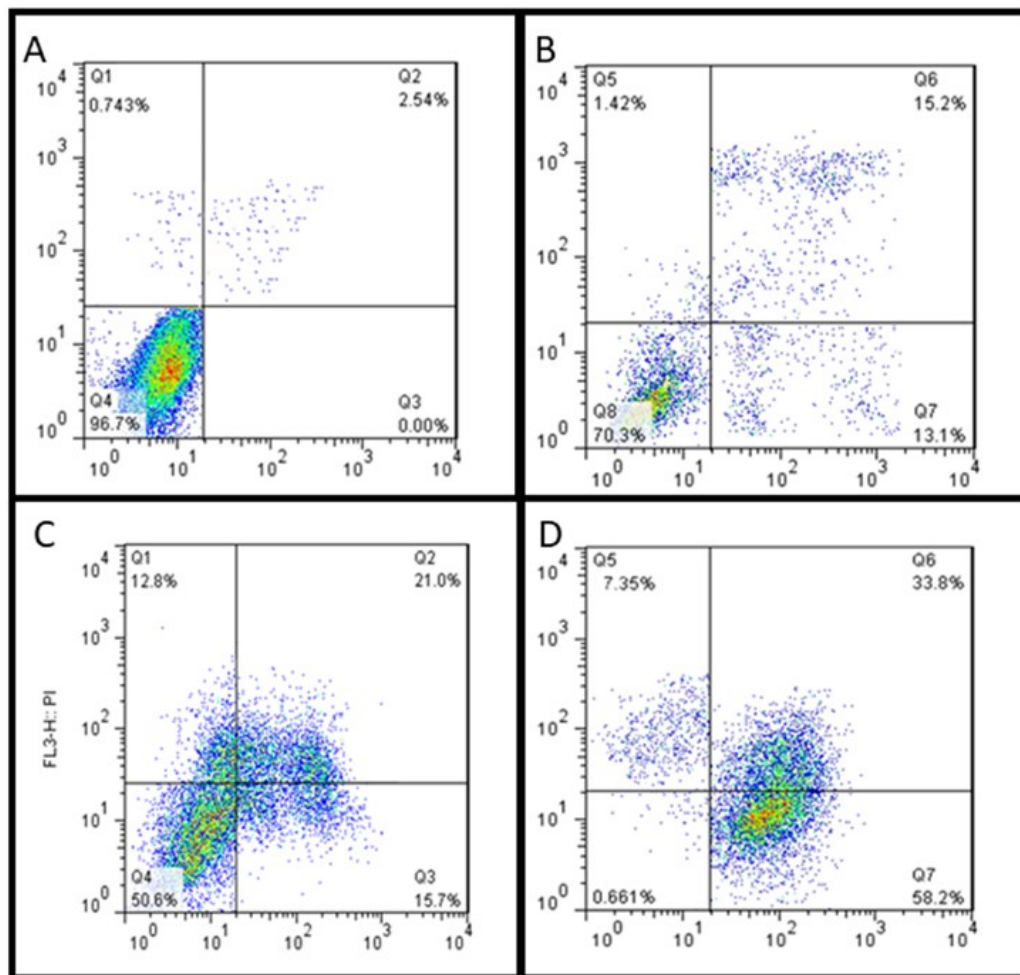


Figure 5. The result of flow cytometry. A; cells in the control group, B; Peiminine, C; 5-FU and D; 5-FU + Peiminine.

3.4 Investigating the expression level of mir181, mir106 gene

Using the PCR-qRT assay, it was found that after 24 hours of treatment of HT-29 cells with a concentration of 1 $\mu\text{g/ml}$ 5-FU together with 78.42 $\mu\text{g/ml}$ Peiminine, the relative expression of the miRNA-181 gene at the RNA level increased, but was not significant. In other words, at this time point, it was observed that the drug

combination of 5FU and Peiminine ($P < 0.005$) did not significantly increase the induction of miRNA-181 gene expression in HT-29 cells (Figure 6).

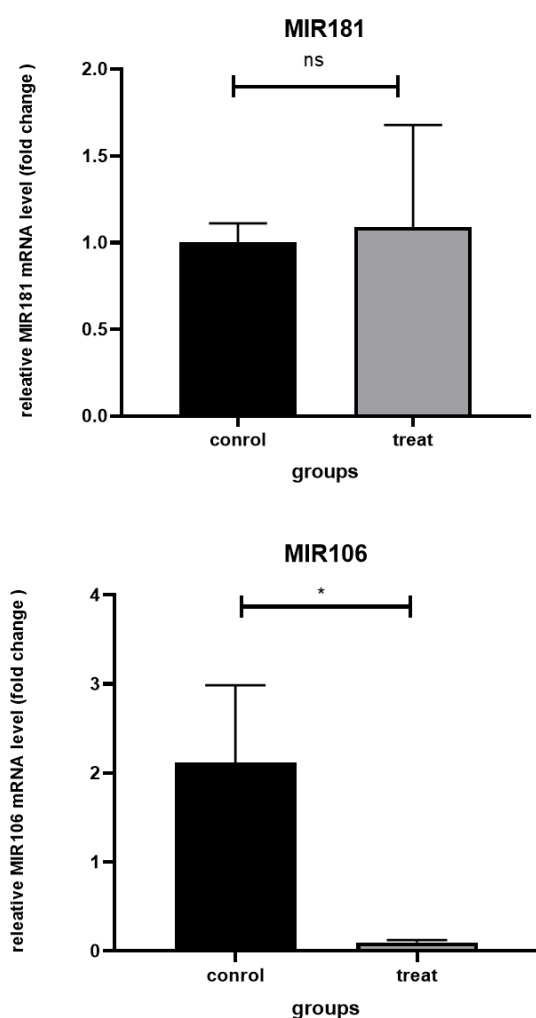


Figure6. The expression level of miRNA-181 and miRNA-106 after treatment with 5-FU and Peiminine.

4. Discussion

Impaired expression of natural microRNAs (miRNAs) is frequently observed in cancer initiation and development [12]. miRNAs are small, non-coding, single-stranded RNA sequences that can post-transcriptionally regulate the expression of their target genes by directly binding to complementary 3'UTR sequences[13]. Depending on the situation, miRNAs act as tumor suppressors or oncogenes [14]. Overexpression of oncogenic miRNAs and under expression of tumor suppressor miRNAs alters cell signaling and promotes cancer progression. Several studies have shown that the expression of some specific miRNAs is associated with response to chemotherapeutic agents in CRC and may serve as a predictor for CRC patients receiving chemotherapy[15].

Peiminine is an isosteroid alkaloid with multiple biological activities, such as anti-cancer and anti-inflammatory effects, but its cellular uptake and pharmacodynamics are unclear.

Peiminine significantly inhibits the secretion of pro-inflammatory cytokines (such as TNF- α , IL-6 and IL-1 β), increases anti-inflammatory cytokines (such as IL-10) and the production of inflammatory cytokines produced by It inhibits lipopolysaccharide (LPS). It blocks the extracellular signal-regulated kinase (MAPK) and nuclear factor kappa B (NF- κ B) signaling pathway[16]. Peiminine has been reported to have potent anti-inflammatory effects on a variety of diseases, making it a potential therapeutic agent for lung diseases[17], osteoarthritis (OA)[18], Parkinson's disease (PD)[19] and allergic inflammatory diseases[20]. The aim of this study is to investigate the synergistic effects of Peiminine in combination with 5-FU on the RNA expression pattern of miR-181 and miR-106 in colorectal cancer cell lines.

For this purpose, colon cancer cells were treated with different concentrations of Peiminine and 5FU alone and in combination. The expression level of miR-181 and miR-106 was measured by real-time PCR and the percentage of cell viability was also assessed using the MTT method. The combination therapy reduced cell viability more than the individual drugs. The drug combination of 5-FU and Peiminine (1 μ g/ml 5-FU+78.42 μ g/ml Peiminine) did not significantly increase the expression of the miRNA-181 gene in HT-29 cells. The drug combination of 5-FU and Peiminine caused the strongest decrease in the induction of miRNA-106 gene expression in HT-29 cells ($p < 0.005$). In addition, the combination of Peiminine with the drug 5-FU induced more significant apoptosis than the control group in colorectal cancer cells It was HT-29.

Ghada's study investigated the antiproliferative effect of 5-FU on colon cancer cells by targeting the miR-27a/WNT/ β -catenin signaling pathway. The results showed that the combination of quercetin and 5-FU had a greater cytotoxic effect than 5-FU alone. This compound increased apoptosis in colon cancer cells and inhibited the expression of miR-27a, resulting in suppression of the Wnt/ β -catenin signaling pathway.

Down-regulation of cyclin D1 expression further supported the efficacy of the combination therapy[21]. Our study is similar to the study conducted by Ghada, which indicates an increase in the efficacy of 5-FU with herbs. Another study investigated the synergistic effect of diosmetin and 5-FU on colorectal cancer cells and examined the apoptotic activity of this compound. The combination of 5-FU and diosmetin showed a synergistic effect on HCT116 cells and an additive effect on HT29 cells. The combination treatment reduced the dose of 5-FU in HCT116 cells three-fold compared to monotherapy. The cells treated with the combination had more apoptotic cells than the cells treated with the monotherapy, which were mainly activated via the intrinsic apoptotic pathway. Cell death of HCT116 was confirmed by mitotic arrest in the G2/M phase[22]. In our study, HT-29 cells treated with the combination of 5-FU and Peiminine had more apoptotic cells than those treated with uni therapy.

In the study by Gustavo and colleagues, a series of 5-FU-genistein hybrids were synthesized and tested for their chemopreventive potential in colorectal cancer cells. The most active compound, 4a, exhibited cytotoxicity against SW480 and SW620 cells, induced cell cycle arrest and apoptosis, and showed stable binding to human caspase 8/3 and human Tp53[23]. Similar to our study, it can be concluded that the increased toxicity is associated with the induction of apoptosis. The study by Lindsey et al. compared the transcriptome and cytokine profiles of colorectal cancer cells treated with 5-fluorouracil (5-FU), irinotecan (CPT-11), oxaliplatin and their paid cisplatin. It identifies drug-general, drug-specific, drug-class-specific, p53-independent and p53-dependent markers and elucidates the mechanisms of efficacy and toxicity of these drugs. Down-regulation of histone genes by 5-FU and up-regulation of FOS and ATF3 by oxaliplatin were observed. BTG2 was identified as one of the most important genes regulated by all four drugs. This study also shows differential effects of these drugs on the expression of TRAILR2, IL-8 and ferritin, which may contribute to cancer cell survival[24]. The results of

our and Lindsey's study provide insights into the molecular basis of chemotherapy in colorectal cancer.

Conclusion

Peiminine in combination with 5FU has a synergistic effect on HT-29 colon cancer cells. The combination therapy increases the efficacy of 5FU and reduces its side effects and activates apoptosis mainly via the intrinsic pathway. This combination also played a role in decreasing the expression of miRNA106 and increasing the expression of miRNA181. Further studies are needed to evaluate the mechanism of action of this compound and to confirm the antitumorogenic activities of this compound in an appropriate animal model.

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