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Analysis of the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation and its relevant mechanism.

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Abstract

Objective: To explore the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation and its relevant mechanism.

Method: MTT assay was used to detect the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation. Morphology of colorectal cancer cell apoptosis was observed. Cell cycle and apoptosis was assayed using Annexin V and PI double staining flow cytometry. Caspase-3 protein expression was detected by Western blot.

Results: The inhibitory rate in every experimental group with different concentration was significantly higher than that in control group ($p<0.05$). The inhibitory rate was more significant along with the increasing concentration of the drug dose-dependently. IC_{50}=201.908 mg/L. The concentration to HT29 cells was respectively 160 mg/L, 320 mg/L and 640 mg/L in experimental group. The HT29 cell cycle was affected. The main block was in HT29 cells G2/M phase, S phase. The apoptosis rate in experimental group with 160 mg/L, 320 mg/L and 640 mg/L was significantly higher than control group dose-dependently ($p<0.05$). Caspase-3 protein expression in experimental group with 160 mg/L, 320 mg/L and 640 mg/L was significantly higher than control group ($p<0.05$). The expression increased along with the increasing concentration. This demonstrated that safflower polysaccharide could significantly up-regulate Caspase-3 protein. Conclusion: Safflower polysaccharide could significantly inhibit HT29 colorectal cancer cell. The mechanism of it inducing HT29 cell apoptosis might have correlation with blocking cells in G2/M phase, S phase, and up-regulating Caspases-3 protein expression.

Keywords Safflower polysaccharide, HT29 colorectal cancer cell, proliferation, inhibition, mechanism.

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Introduction

Colorectal cancer is a common malignancy. Epidemiological studies found that with the modern lifestyle changes and increasing population aging process, the incidence of colon cancer showed an increasing trend [1-6]. It has now become the third malignant tumor after lung and breast cancer, which is a serious threat to people's lives. Safflower is a traditional Chinese medicine, with the effect of promoting blood circulation, dispersing blood stasis and analgesic. Modern pharmacological studies have shown it had anti-tumor and improving immunity effect, but its exact mechanism of anti-tumor is not very clear [7-9]. In this study, HT29 cells from colon cancer tissues were selected as the experimental study. The aim of this study was to investigate the inhibitory effect of safflower polysaccharide on HT29 cell proliferation and its related mechanism. Further studies on HT29 cell lines were needed for a certain experimental basis.

Materials and Methods

Experimental cells and experimental drugs

HT29 human colon cancer cells (purchased from the National Laboratory of Biology Treatment Sichuan University). Safflower (purchased from Zhongnuo Biotechnology Co., Ltd., Shanxi), safflower polysaccharide was extracted by water extraction and alcohol precipitation. The identification of purchased drug was in line with the 2010 edition of "Chinese Pharmacopoeia" regulations.

Reagents and instruments

DMEM medium (Gibco Company, US), 0.25% trypsin (Hyclone, US), dimethyl sulfoxide (DMSO, Kermel Chemical Reagent Co., Ltd., Tianjin), Annexin V-FITC apoptosis detection kit (KGI Biological Production Co., Ltd., Nanjing). Microplate reader (Thermo Company, US), FACS Canto II flow cytometer (BD Biosciences, USA), inverted microscope (Olympus, Japan).

Grouping

The cells were divided into control group and experimental group. Cells in experimental group were divided into 6 groups according to the drug concentration, namely 20 mg/L, 40 mg/L, 80 mg/L, 160 mg/L, 320 mg/L, 640 mg/L.

Methods

HT29 colon carcinoma cell culture: HT29 colon cancer cells were cultured using 10% DMEM medium containing fetal bovine serum seeded to culture flasks and placed in 37°C, 5% CO_2 incubator. The medium was changed every day. Cells were subcultured every 2 to 3 days. Cells in logarithmic growth phase were used for experiments.

Detection of the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation using MTT assay: The above HT29 colon cancer cells in logarithmic growth phase were seeded on to a 96 well plate at 2×10^3 cells/well. After incubation for 24 h, the sample culture medium was added to each group with five wells. After cultured for 48h, 20 μL 5 mg/mL MTT solution was added to each well. After 4 h of incubation at 37°C, 5% CO_2 incubator, the supernatant was discarded. 150 μL of DMSO solution was added to each well to dissolve the purple crystal precipitate, with oscillation for 10min. The above processes were carried out for 3 times. The optical density (OD) was measured using a microplate reader at 570 nm to compute cell survival rate and median lethal concentration (IC50). Cell survival rate (%)=A sample-A blank/(A control-A blank) × 100%. A sample was the OD after different concentration of safflower polysaccharide was added. A control was the optical density of the negative control whole cells. A blank was the optical density of the blank control group.

Morphological examination of colon cancer cell apoptosis: The progress of cell apoptosis was assessed using the nuclear chromatin morphology as an index. Hochest 33258/PI double staining was used to identify apoptotic and necrotic cells. The HT29colon carcinoma cells to be seeded in 6-well plates at 3×10^5. After incubation for 24 h, 160 mg/L, 320 mg/L, 640 mg/L safflower polysaccharide culture medium was added, then placed in a 37°C, 5% CO_2 incubator. After incubation 48 h, Hochest 33258 stock solution was added to a final concentration of 1 μg/mL, and then incubated at 37°C, 5% CO_2 incubator for 10 min. Then it was centrifuged at 1000 r/min for 5 min. Staining solution was discarded. 1 mL 5 μg/mL PI staining solution was added to stain for 15 min at 4°C away from light. The cells were collected and coated on a slide to 10 μL drop anti-fluorescein mounting liquid. The coverslip was covered immediately. Cells were observed under an inverted fluorescence microscope. Cell morphology between the groups was compared.

Detection of cell cycle and apoptosis using Annexin V and PI double staining flow cytometry: HT29 cells were seeded in 6-well plates at 3×10^5 for 24 h of incubation. Then 160 mg/L, 320 mg/L, 640 mg/L safflower polysaccharide culture medium was added, then placed in a 37°C, 5% CO_2 incubator

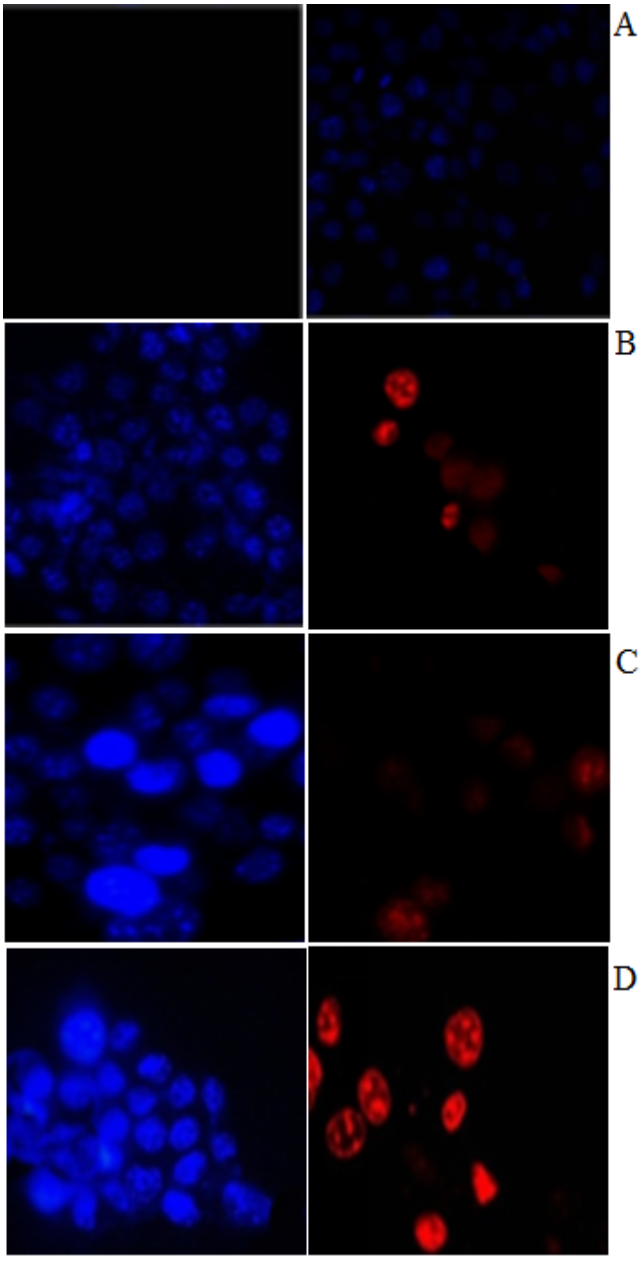

Figure 1. *A) Control group; B) Experimental group (160 mg/L); C) Experimental group (320 mg/L); D) Experimental group (640 mg/L).*

for 48 h. The supernatant was discarded, and trypsin was used to digest and cell collected. Cells were washed with cold PBS three times and centrifuged at 1200 r/min for 5 min. Cells were resuspended, counted, to make each tube reach $1 \times 10^6 \sim 5 \times 10^6$/mL. Cell pellet was collected by centrifugation, and then resuspended in 200 μL binding buffer. Annexin V-FITC 5 μL was added and then 5 μL PI for 10 min of reaction at room temperature away from light. 300 μL binding buffer was used to mix. Flow cytometry was used for detection.

Detection of Caspase-3 protein by Western blot: The above cultured HT29 cells and collected HT29 cells that treated by 160 mg/L, 320 mg/L, 640 mg/L safflower polysaccharide for 48 h were digested by trypsin and centrifuged. Total proteins

were extracted using PIRA lysate. BCA assay was sued for detecting protein concentration. After SDS- polyacrylamide gel electrophoresis, it was semi-dry blotted to a PVDF membrane and sealed in 5% skim milk for 1.5 h. Caspase-3 primary antibody was added at 4°C overnight. Diluted secondary antibody was added after washing the membrane (1: 500) and incubated at room temperature for 1h. ECL coloration was used for exposure. Gel imaging system was sued for scan analysis. β-actin protein expression were detected, as an internal reference.

Statistical analysis

SPSS 22.0 statistical software was used to process data. Measurement data were represented as ($\bar{x} \pm s$). LSD-t test was used for pairwise comparison within groups. ANOVA was used for comparison between groups. $P<0.05$ indicates a statistically significant difference.

Results

Detection of the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation using MTT assay

As illustrated in Table 1. The inhibitory rate in every experimental group with different concentration was significantly higher than that in control group ($p<0.05$). The inhibitory rate was more significant along with the increasing concentration of the drug dose-dependently. IC_{50}=201.908 mg/L.

Morphology test results of colon cancer cell apoptosis

As illustrated in Figures 1A-1D, According to IC_{50}=201.908 mg/L, we selected the concentration of safflower polysaccharide at 160 mg/L, 320 mg/L, 640 mg/L. PI cannot pass through normal cell, Hoechst is a fluorescent dye with membrane permeability. Therefore, when in late apoptosis or necrosis, cell membrane is damaged, and then the cells can be dyed red by PI. Cells in control group were not stained red fluorescence, with the increasing concentration of safflower polysaccharide, some cells were colored by PI. In late apoptosis and necrosis, when safflower polysaccharide concentration increased to 640 mg/L, blue and red light appear superimposed. The chromatin of nuclei was highly coagulated and marginalized, with obvious apoptotic bodies, belonging to late apoptosis.

Table 1. *Detection of the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation using MTT assay ($\bar{x} \pm s$).*

Groups	n	Inhibitory rate (%)
Control group	5	0.00 ± 0.00
Experimental groups		
20mg/L	5	9.48 ± 2.34*
40mg/L	5	18.79 ± 3.49*
80mg/L	5	30.14 ± 4.78*
160mg/L	5	43.29 ± 5.42*
320mg/L	5	58.82 ± 8.93*
640mg/L	5	74.39 ± 11.34*

Note: Compared with control group, * $p<0.05$.

Detection of cell cycle using flow cytometry

As illustrated in Table 2 and Figure 2, treated by safflower polysaccharide at various concentrations, cell cycle of the HT29 was affected, mainly blocked at G2 / M phase, S phase.

Table 2. *Detection of cell cycle using flow cytometry ($\bar{x} \pm s$).*

Groups	G0-G1phase	G2-M phase	S phase
Control group	77.83 ± 5.42	12.16 ± 4.13	10.01 ± 2.14
Experimental groups			
160 mg/L	39.65 ± 5.23*	14.49 ± 4.07	44.86 ± 5.24*
320 mg/L	44.61 ± 3.63*	17.30 ± 4.58*	38.09 ± 3.97*
640 mg/L	45.49 ± 4.10*	19.79 ± 3.65*	34.72 ± 4.25*

Note: Compared with control group, *$p<0.05$.

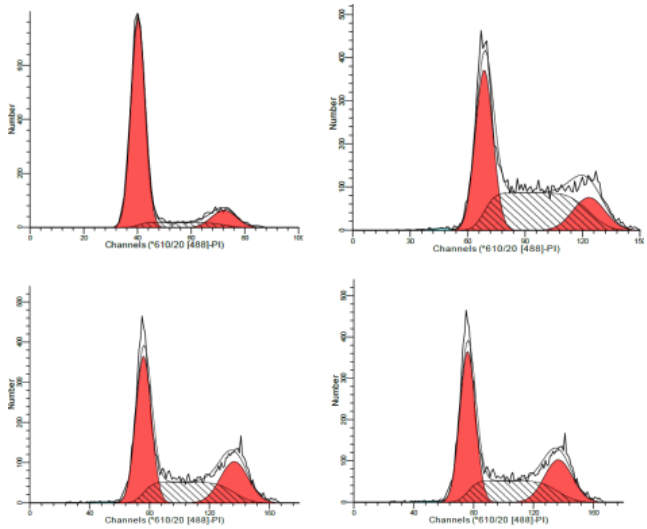

Figure 2. *Detection of cell cycle using flow cytometry.*

Detection of cell apoptosis using flow cytometry

As illustrated in Table 3 and Figure 3, The apoptosis rate in experimental group with 160mg/L, 320mg/L and 640mg/L was significantly higher than control group dose-dependently ($p<0.05$).

Table 3. Detection of cell apoptosis using flow cytometry ($\bar{x} \pm s$).

Groups	Apoptosis rate (%)
Control group	6.92 ± 1.32
Experimental groups	

160 mg/L	34.99 ± 4.25*
320 mg/L	61.89 ± 6.78*
640 mg/L	71.91 ± 9.32*
Note: Compared with control group, * p <0.05.	

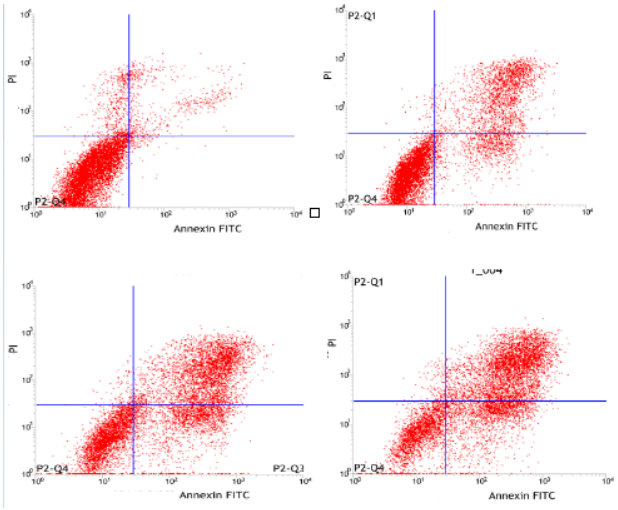

Figure 3. *Detection of cell apoptosis using flow cytometry.*

Detection of Caspase-3 protein by Western blot

As illustrated in Figure 4, Caspase-3 protein expression at different concentration safflower polysaccharide was significantly higher than that in control group. The expression increased along with the increasing concentration, demonstrating that safflower polysaccharide could significantly up-regulate Caspase-3 protein.

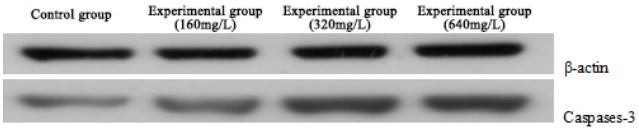

Figure 4. *Detection of Caspase-3 protein by Western blot.*

Discussion

At present, the clinical treatment of colon cancer was comprehensive treatment based on surgery, with 60% to 70% of surgical resection rate. But recurrence and metastasis is a major cause of mortality after surgery, and most chemotherapy drugs will bring a lot of side effect [10,11]. Recent studies found that traditional Chinese medicine had achieved good results in anti-tumor, with little side effect. Safflower, also known as gold saffron, Du safflower, thorn safflower, is a traditional Chinese medicine, with the effect of promoting blood circulation, dispersing blood stasis and warming the channels to relieve pain. Pharmacological studies have shown it to have anti-cancer, sterilization and other effects, with extensive clinical applications [12]. Yan et al. [13] reported that safflower polysaccharide could inhibit the expression of cell cycle-related genes, and induced liver SMMC-7721 cell blocked, so as to achieve anti-tumor effects. In this study, the inhibitory rate in every experimental group with different concentration was significantly higher than that in control group. The inhibitory rate was more significant along with the increasing concentration of the drug dose-dependently. This demonstrated that safflower polysaccharide had significant tumor inhibitory effect.

Cancers are a class of cell cycle diseases, most of them are uncontrolled proliferative diseases, with a number of external regulation factors including growth factors, drugs, nutrients etc., affecting cell cycle progression, and further inducing cell proliferation, cell cycle arrest or apoptosis [14,15]. Cell cycle, the basic process of tumor cell life activities, is divided into four periods: G1 phase, S phase, G2 phase and M phase. Wherein the G1 and G2 phases are respectively the early and late DNA synthesis, S phase is DNA synthesis phase [16]. Apoptosis of tumor cells, also known as programmed cell death, is an important part of cell life cycle which is regulated by cell-intrinsic gene coding. Cell suicide is promoted by active biochemical processes. The process is a process of cell-autonomous death regulated by multiple genes [17]. Apoptosis is an important part of body regulating growth and development and maintaining a stable internal environment. Treated by safflower polysaccharide at various concentrations, cell cycle of the HT29 was affected, mainly blocked at G2/M phase, S phase. The apoptosis rate in experimental group with 160mg/L, 320mg/L and 640 mg/L was significantly higher than control group dose-dependently. In this study, we have shown that G2/M phase and S phase of HT29 cells are mainly blocked by the preliminary study, but we need further study in the follow-up to provide a reliable reference value for the effect of safflower polysaccharide on cell cycle of HT29 tumor cells.

Caspase family is a large class of apoptosis regulating genes. As the effector molecule of apoptosis, Caspase-3 is one of the key hydrolysis protease presented in downstream of the apoptotic pathway, known as apoptosis "performer" [18,19]. The activation of Caspase-3 expression can promote cytoskeletal damage, destruct some proteases, eventually leading to characteristic DNA fragmentation, promoting apoptosis [20,21]. In this study, caspase-3 protein expression at different concentration safflower polysaccharide was significantly higher than that in control group. The expression increased along with the increasing concentration, demonstrating that safflower polysaccharide could significantly up-regulate Caspase-3 protein.

In summary, safflower polysaccharide could significantly inhibit HT29 colorectal cancer cell. The mechanism of it inducing HT29 cell apoptosis might have correlation with blocking cells in G2/M phase, S phase, and up-regulating Caspases-3 protein expression.

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