



Effect of *Rhizoma paridis* total saponins on apoptosis of colorectal cancer cells and imbalance of the JAK/STAT3 molecular pathway induced by IL-6 suppression

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ABSTRACT. We observed the influence of different concentrations of *Rhizoma paridis* total saponins (RPTS) on the apoptosis of colorectal cancer cells and explored the internal mechanism involved. We determined whether RPTS influences the interleukin-6 (IL-6)/Janus kinase (JAK)-signal transducer and activator of transcription-3 (STAT3) apoptosis

molecular pathway and looked for colon cancer-related signal transduction pathways or targets inducing apoptosis. We also cultured SW480 colorectal cancer cells using different concentrations of RPTS (10, 20, 40, and 80 $\mu\text{g}/\text{mL}$), and observed the effect of RPTS on SW480 cell morphology under a fluorescence inverted microscope. We detected serum IL-6 using the polymerase chain reaction and the expression of JAK-STAT3 protein by western blot. After treating SW480 with RPTS and Hoechst 33258 dyeing, we found that the typical apoptosis morphology had changed. Secretion of IL-6 in the serum decreased significantly ($P < 0.05$), and STAT3 levels were reduced. RPTS can significantly promote apoptosis in SW480 colorectal cancer cells. The mechanism may be that it suppresses the secretion of IL-6 and inhibits the IL-6/JAK-STAT3 protein signaling pathway.

Key words: *Rhizoma paridis* total saponins (RPTS); Apoptosis; IL-6/JAK-STAT3 pathway; Colorectal cancer

INTRODUCTION

We explored the effect of *Rhizoma paridis* total saponins (RPTS) on SW480 colorectal cancer (CRC) cells and elucidated the internal mechanism by assuming that interleukin-6 (IL-6) induces an imbalance in the molecular pathway of Janus kinase (JAK)/signal transducer and activator of transcription-3 (STAT3). This may clarify the role of the Chinese herb *R. paridis* in the treatment of CRC, and provide a theoretical basis for its clinical application. Furthermore, it will promote traditional Chinese medicine in the field of tumor therapy.

Paris polyphylla is a species of liliaceous plant that grows in the Yunnan Province of China and produces rhizomes, which can be dried. It has several beneficial properties and can reduce body temperature, eliminate toxic materials, and relieve swelling and pain (Chinese Pharmacopoeia, 2005). In recent years, *P. polyphylla* has been widely used in the treatment of CRC. However, most research into its mechanism of action has focused on arresting the proliferation of tumor cells (Li et al., 2010). The effect may arise through inhibition of the compound of the tumor cell protein with P7F and inhibition of tumor cell mitosis, which reduces cell proliferation. RPTS is now the main antitumor active substance, but unfortunately it is still uncertain whether it can induce apoptosis in colorectal tumor cells. In recent years, the annual incidence of CRC has been on the rise in China, and it has been proven that its pathogenesis is related to various cytokines, especially IL-6 (Kim et al., 2014). Wu et al. (1996) found that the level of serum IL-6 in patients with rectal cancer was elevated. The probable reason is that IL-6 is a pleiotropic cytokine that, when combined with its receptor, causes the activation of STAT3 after the receptor complexes with gp130 (Yoon et al., 2010; Fahmi et al., 2013), finally inducing the expression of IL-6/STAT3 downstream molecules, such as VEGF and Bcl-2, which are closely related to inflammation and cancer (Jones et al., 2001; Chang et al., 2013). In addition, STAT3 has a dual role in IL-6-mediated signaling: its activation may result in increased IL-6, but IL-6 itself may lead to phosphorylation of STAT3 resulting in diverse biological responses (Fielding et al., 2008). Michaud-Levesque et al. (2012) provided new insight into the role of quercetin as a blocker of the STAT3 activation pathway stimulated by IL-6, with a potential role in the prevention and treatment of glioblastoma. Moreover, the studies found that RPTS can reduce intestinal mucosal barrier dysfunction, inhibit the activation of nuclear factor- κB , intervene and regulate the expres-

sion of many important cytokine genes, and inhibit the generation of IL-6, tumor necrosis factor (TNF)- α , and IL-1 at the mRNA level (Surh et al., 2001). In this study, we observed the apoptosis effect of RPTS on CRC cells, and determined whether it affects IL-6 at the molecular level, and therefore affects the expression of the IL-6/JAK-STAT3 apoptosis molecular pathway.

MATERIAL AND METHODS

Instruments and reagents

Instruments and reagents, and their suppliers, were as follows: RPMI 1640 medium (Gibco, Grand Island, NY, USA), NAPCO series 5400 CO₂ incubator, and 1:125 pancreatin (Gibco); newborn calf serum (Hangzhou Sijiqing); chain, penicillin, and TE2000-E fluorescence inversion microscope system (Nikon, Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Amresco, USA); EPICS XL flow cytometer (Coulter, USA); horseradish peroxidase labeling resistance against mouse, sheep, and rabbit IgG antibody and chemiluminescence reagent kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA); polyvinylidene difluoride membrane (The Beijing China Biological Technology Co., Ltd., Beijing, China); and GelDoc 100 Vilber Lourmat and ChemiDoc XRS chemiluminescence imaging (Bio-Rad, Hercules, CA, USA).

Medicinal materials

P. polyphylla medicinal materials were purchased from the Guangdong Zhanjiang Tianma Pharmacy. Dr. Xifeng Teng, Guangdong College of Pharmacy, has identified them as the dry roots of *P. polyphylla*, conforming to the "Chinese Pharmacopoeia" (Cui, 2005).

Cell lines

Human SW80 CRC cell lines were purchased from the Shanghai Xin Yu Biotechnology Co., Ltd. (Xinyu, China).

Methods

Preparation of RPTS

We ground 40 g dry tuber, added 400 mL 70% ethanol, and refluxed twice for 3 h each time. After filtering and combining the filtrates, we removed the ethanol under reduced pressure. We then added enough water to float and remove the lipophilic component with petroleum ether and ethyl acetate. After we had extracted the effective constituents with n-butanol, we removed the n-butanol leaving a dry powder, which was RPTS.

Cell culture

We inoculated SW480 human colon cancer cells into RPMI 1640 complete culture medium that contained 10% fetal bovine serum, and cultured them in a cell incubator (37°C and 5% CO₂). We replaced the culture medium every 2 days and used trypsin for cell dissociation.

tion every 4 days (1:2). Logarithmic-phase cells were taken for the experiments.

Influence of RPTS on the proliferation of SW480

Using the MTT assay method, we took SW480 cells in the logarithmic phase (2×10^4 /mL) to inoculate a 96-well plate, then cultivated them for 24 h and discarded the nutrient solution until the cells became adherent. We next added different concentrations of RPTS nutrient solution (10, 20, 40, and 80 $\mu\text{g}/\text{mL}$). We also set up a blank control and a negative control (fresh nutrient solution), both arranged in three duplicated wells. We cultured the cells at 37°C in 5% CO_2 for 12, 24, 36, and 48 h, and discarded the supernatant. We then added 100 μL serum-free nutrient solution and 20 μL MTT solution (5 mg/mL) to each well. After cultivating the cells for 4 h, we discarded the supernatant and added 200 mg dimethyl sulfoxide to each well. We then agitated the cells at low speed for 10 min. We determined the absorbance at 570 nm (A570) of the cells in each well to get the mean value. Finally, we calculated the inhibition rate of tumor cells using the following formula. The concentration-effect curve was drawn with the concentration of the RPTS extracting solution as the abscissa, and the A570 value of the tumor cells as the ordinate.

Tumor inhibition rate (%) = [(negative control group A570 value - test well A570 value) / (negative control wells A570 value - blank wells A570 value)] x 100%

Examination of cell morphology using a fluorescence inverted microscope

We made a cell suspension with a concentration of 2.0×10^5 /mL from SW480 cells in the logarithmic phase and inoculated a 24-well plate with it (2 mL per well). After cultivating the cells overnight at 37°C in 5% CO_2 , we extracted the nutrient solution and added the RPTS nutrient solution (20, 40, and 80 $\mu\text{g}/\text{mL}$). We also set up the negative control, to which was added the RPMI 1640 nutrient solution. After cultivation for 48 h, the cells were washed twice with precooled phosphate-buffered saline (PBS), and dyed with Hoechst 33258 (10 $\mu\text{g}/\text{mL}$) for 15 min at 37°C. After washing twice with PBS, we added propidium iodide staining fluid (50 $\mu\text{g}/\text{mL}$) to dye the cells for 15 min in ice, then washed them twice more with PBS. The cells were examined and photographed under a fluorescence inverted microscope.

Flow cytometry analysis of the effect of RPTS on apoptosis of SW480 cells

We adjusted the concentration of the suspension of SW480 cells in the logarithmic phase to 2.0×10^4 cells/mL and added 6-mL aliquots of the suspension to 50-mL culture flasks. After leaving overnight at 37°C in 5% CO_2 , we removed the culture and added RPTS culture solution (to produce final concentrations of 20, 40, and 80 $\mu\text{g}/\text{mL}$). We also set up a negative control and added the same volume of culture solution. After 24 h, the cells were trypsinized, collected by centrifugation, washed with PBS, and counted. We then took $5-10 \times 10^4$ cells for centrifugation and discarded the supernatant. Next, we added 195 μL Annexin V-fluorescein isothiocyanate (FITC) binding buffer (1X) and gently re-suspended the cells. After adding 5 μL Annexin V-FITC to avoid light incubation for 10 min at room temperature (20°-25°C), we repeated the centrifugation and added 10 μL propidium iodide staining solution. We then used flow cytometry to detect the cell apoptosis rate (laser wavelength 488 nm, power 15 mW).

The experimental data are reported as means \pm standard deviation. Mean comparisons between groups were made using analysis of variance and comparisons between two groups were made using least significant differences. The heterogeneity of variance was analyzed by Tamhane's T2 ($P < 0.05$ was considered to be statistically significant).

Detection of IL-6 expression in SW480 cells using RT-P

We took 10 mL SW480 cell suspension in the logarithmic growth phase (2.0×10^4 mL) to seed a 24-well plate over 48 h. We then replaced the nutrient solution with TNF- α (250 U/mL) and RPTS (80 μ g/mL), and continued to culture in a cryogenic refrigerator at -20°C for 24 and 48 h to collect the supernatants. The concentration of IL-6 in the supernatants was detected by enzyme-linked immunosorbent assay and the steps were carried out strictly according to manufacturer instructions.

Experimental data are reported as means \pm standard deviation. We used the SPSS 15.0 software for analysis of variance and the Student *t*-test ($P < 0.05$ was considered to be statistically significant).

Examination of STAT3 protein expression using western blot

We took 2.0×10^6 SW480 cells treated by different concentrations of RPTS nutrient solution (10, 20, 40, and 80 μ g/mL) and extracted from them total proteins, cytoplasmic proteins, and nuclear proteins using protein extraction and nuclear-cytosol extraction kits. We then mixed a certain amount of protein with sample buffer and after 7 min in a 100°C water bath, added anti-STAT3 monoclonal antibody. After maintaining the mixture overnight at 4°C , we added goat anti-mouse IgG and incubated it in the dark at room temperature for 60 min. We then used Odyssey color infrared fluorescence to detect and a grayscale scanning system for analysis. The relative quantity of the target protein was represented as the gray ratio of the target protein color stripe to the glyceraldehyde 3-phosphate dehydrogenase stripe.

RESULTS

Impact of RPTS on proliferation of SW480 cells

The test results showed that the RPTS culture solutions inhibited the proliferation of SW480 cells and there were significant dose-effect and time-effect relationships, as shown in Table 1.

Table 1. Impact of *Rhizoma paridis* total saponins (RPTS) on proliferation of SW480 (means \pm standard deviation, N = 3).

Concentration of RPTS nutrient solution (μ g/mL)	12 h		24 h		36 h		48 h	
	A570 inhibition ratio (%)		A570 inhibition ratio (%)		A570 inhibition ratio (%)		A570 inhibition ratio (%)	
Negative control	1.09 \pm 0.06	-	1.16 \pm 0.06	-	1.19 \pm 0.04	-	1.14 \pm 0.08	-
10	1.06 \pm 0.03	2.80	0.96 \pm 0.02	17.24	0.74 \pm 0.06	37.82	0.72 \pm 0.04	36.84
20	0.86 \pm 0.04	21.49	0.56 \pm 0.04	51.72	0.56 \pm 0.03	52.94	0.45 \pm 0.03	60.53
40	0.84 \pm 0.02	23.36	0.32 \pm 0.04	72.41	0.14 \pm 0.02	88.23	0.12 \pm 0.05	89.47
80	0.39 \pm 0.03	65.42	0.23 \pm 0.03	80.17	0.08 \pm 0.02	93.28	0.06 \pm 0.02	94.74

After comparing each experimental group and the negative control group (except for the 10 $\mu\text{g}/\text{mL}$ RPTS culture broth acting over 12 h), we found that the differences in the inhibition rates were statistically significant ($P < 0.05$), as shown in Figure 1.

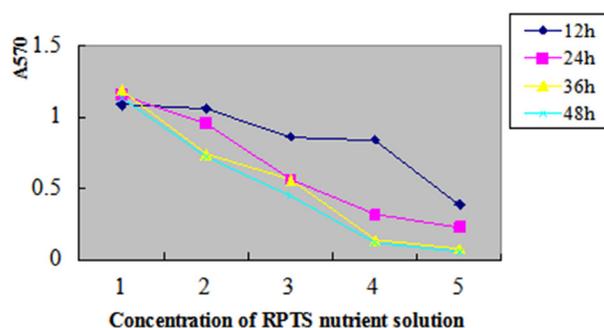


Figure 1. Relationship between the concentration of the *Rhizoma paridis* total saponins (RPTS) nutrient solution and the absorbance at 570 nm (A570) of the SW480 cells for various cultivation periods.

Observation of the effect of RPTS on apoptosis of SW480 cells under an inverted fluorescence microscope

After 24 h, we used ultraviolet excitation under a fluorescence microscope. We found that after the interventions of the different concentrations of RPTS, there were varying degrees of nuclear chromatin aggregation, nuclear fracture, cytoplasmic concentration, and bright blue fluorescence. In keeping with typical apoptotic morphological changes (Figure 2A-D, red arrows), we observed nuclear chromatin aggregation, nuclear fragmentation, cytoplasmic concentration, and the presence of crescent-shaped, irregular, round, and oval bright blue fluorescent objects. However, the negative control group showed uniform blue fluorescent nuclei.

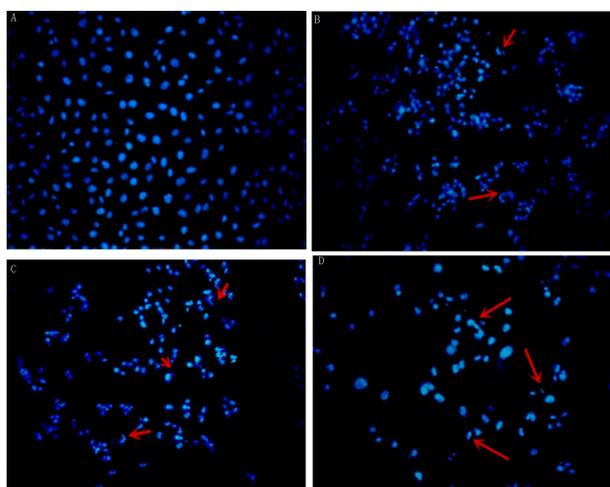


Figure 2. Apoptosis evaluation. (A) Control group; (B) Morphological changes in SW480 cells after culture in 20 $\mu\text{g}/\text{mL}$ *Rhizoma paridis* total saponins (RPTS) broth; (C) Morphological changes in SW480 cells after culture in 40 $\mu\text{g}/\text{mL}$ RPTS broth; (D) Morphological changes in SW480 cells after culture in 80 $\mu\text{g}/\text{mL}$ RPTS broth.

Results of flow cytometry analysis of the effect of different concentrations of RPTS on SW480 cells over 24- or 48-h periods

Apoptotic cells were detected by flow cytometry propidium iodide/Annexin V-FITC double staining, and the scatter plots are shown in Figure 3A-C for concentrations of RPTS of 20, 40, and 80 $\mu\text{g/mL}$, respectively.

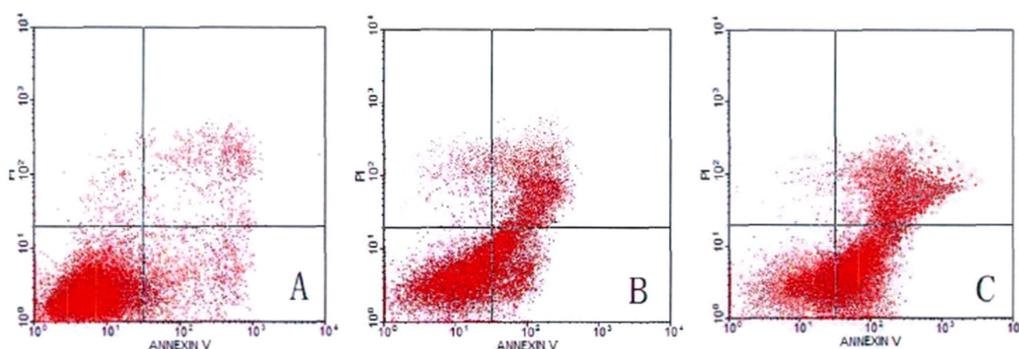


Figure 3. Apoptotic cells were detected by flow cytometry propidium iodide (PI)/Annexin V-fluorescein isothiocyanate (FITC) double staining. The scatter plots for concentrations of *Rhizoma paridis* total saponins (RPTS) of 20, 40, and 80 $\mu\text{g/mL}$ are shown in **A**, **B**, and **C**, respectively. In each plot, the upper left quadrant indicates mechanically damaged cells [Annexin V-FITC (-)/propidium iodide (PI) (+)]; the upper right quadrant represents late apoptotic or secondary dying cells [Annexin V-FITC (+)/PI (+)]; the lower left quadrant represents normal cells [Annexin V-FITC (-)/PI (-)]; and the lower right quadrant represents early apoptotic cells [Annexin V-FITC (+)/PI (-)].

The number of cells in the negative control group was larger in the third quadrant of each plot and the apoptosis rate was less than 5.0%. After treatment with the different concentrations of RPTS broth for 24 h, the cells increased in quadrants 3 and 4, and apoptosis rates increased to 19.06, 27.83, and 30.21%, respectively. There were significant differences compared with the negative control group ($P < 0.01$).

Results of PCR analysis of the effect of different concentrations of RPTS on SW480 cells

The level of serum IL-6 was significantly decreased in the RPTS groups compared with the control group ($P < 0.05$), as shown in Table 2.

Table 2. Effect of *Rhizoma paridis* total saponins (RPTS) on SW480 cells.

Concentration of RPTS ($\mu\text{g/mL}$)	24 h	36 h	48 h
	Level of IL-6 (pg/mL)	Level of IL-6 (pg/mL)	Level of IL-6 (pg/mL)
Control group	60.23 \pm 6.12	80.18 \pm 5.56	100.56 \pm 4.43
20	60.23 \pm 2.32	80.18 \pm 4.32	100.56 \pm 1.21
40	59.23 \pm 2.56	79.63 \pm 2.16	98.43 \pm 2.23
80	58.12 \pm 1.89	78.15 \pm 2.09	96.36 \pm 2.49

IL-6 = interleukin-6.

When the concentration of RPTS was greater than 20 $\mu\text{g/mL}$, there was greater inhibition of the expression of IL-6 in the SW480 cells (Figure 4).

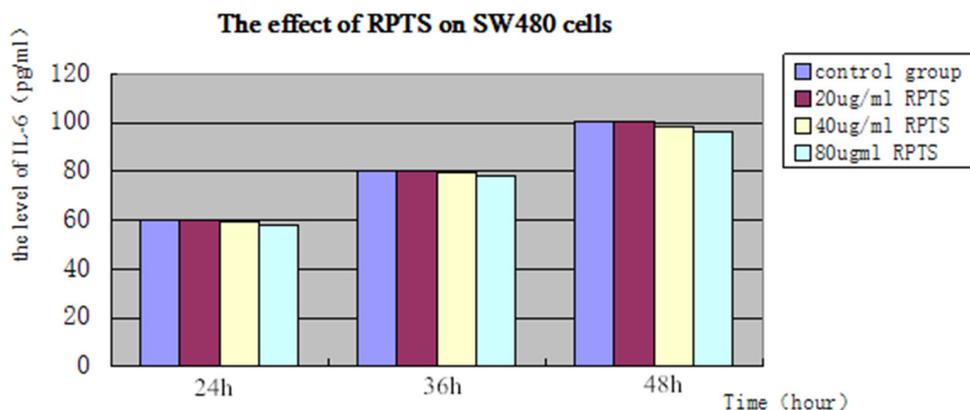


Figure 4. Effect of different concentrations of *Rhizoma paridis* total saponins (RPTS) on SW480.

Effect of different concentrations of RPTS on the expression of STAT3 in SW480 cells

Western blot analysis showed that with the increase of RPTS concentration, the expression of STAT3 protein in the total protein of the SW480 cells and in the cytoplasm and nucleus was significantly lower, as shown in Figure 5.

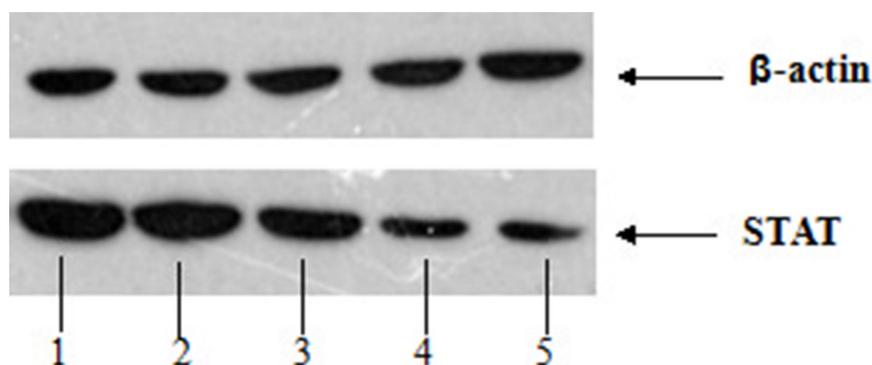


Figure 5. STAT protein levels in Control group (lane 1), SW480 cells after culture in 10 $\mu\text{g/mL}$ *Rhizoma paridis* total saponins (RPTS) broth (lane 2), SW480 cells after culture in 20 $\mu\text{g/mL}$ RPTS broth (lane 3), SW480 cells after culture in 40 $\mu\text{g/mL}$ RPTS broth (lane 4), SW480 cells after culture in 80 $\mu\text{g/mL}$ RPTS broth (lane 5).

DISCUSSION

IL-6 is widely considered to play a key role in inflammatory bowel disease (Sartor, 2006), and the rates of inflammatory bowel disease and CRC are closely related to

each other (Mantovani et al., 2008; Ullman and Itzkowitz, 2011; Yamamoto and Rose-John, 2012). A large number of experiments and clinical studies have found that the level of IL-6 increases significantly in CRC (Waldner et al., 2012), and it can cause tumor recurrence (Belluco et al., 2000; Galizia et al., 2002). At the same time, there is over-activation and dysfunction of STAT3 in CRC (Devarajan and Huang, 2009); this change in IL-6 and STAT3 ultimately induces the expression of downstream molecules closely related to cancer in the IL-6/STAT3 pathway. The tumor-promoting mechanism of STAT3 may involve the upstream STAT3 signaling pathway - the IL-6 signal transduction disorders - which cause the increase of p-STAT3, and therefore the expression of downstream molecules: anti-apoptotic genes and cell cycle-related factors. Eventually, the mechanism leads to the development of a tumor (Corvinus et al., 2005; Devarajan and Huang, 2009). Recently, a growing number of researchers have reported abnormal activation of the JAK-STAT signaling pathways in CRC (Klampfer, 2008; Xiong et al., 2008). Some scholars suggest that the expression of the STAT3 downstream molecule Bcl-x1 can be used directly as an indicator to determine the prognosis of CRC (Biroccio et al., 2001). The IL-6/STAT3 signaling pathway is obviously important in the clinical diagnosis of CRC.

The Chinese herb *P. polyphylla* has a long history of use and several beneficial properties; it can reduce body temperature, eliminate toxic materials, and relieve swelling and pain. A modern pharmacological study found that RPTS, the active ingredient in *P. polyphylla*, has antitumor properties (Xiao et al., 2012; Man et al., 2013; Liu et al., 2014; Jiang et al., 2014; Xiao et al., 2014), can act as an analgesic or hemostatic agent, and has particular effect as an antibacterial or anti-inflammatory substance (OuYang and Huang, 2000). Angus et al. (2001) reported that RPTS can reduce intestinal mucosal barrier dysfunction, which is caused by intestinal ischemia and reperfusion injury, and it can also suppress levels of IL-6 and other inflammatory markers; therefore, it may be capable of reducing the systemic damage caused by them (Ling et al., 2009). The results of our preliminary experiment prove that RPTS can induce CRC SW480 cell apoptosis. The mechanism by which this takes place may be an alteration in the STAT3 signaling pathways of SW480 cells. Blocking or inhibiting the expression of the JAK-STAT3 pathway can not only inhibit tumor cell proliferation but also promote apoptosis (Lin et al., 2005; Lassmann et al., 2007; Xiong et al., 2008). Some studies have shown that the JAK-STAT3 signaling pathway can increase the expression of anti-apoptotic proteins such as survivin, cyclin-D1, Bcl-x1, and Mcl-1. (Ma et al., 2004; Lin et al., 2005; Lassmann et al., 2007; Xiong et al., 2008). As a result, we have reason to believe that RPTS is likely to inhibit the levels of IL-6 expression and to have an impact on the expression of its downstream target STAT3, which can cause IL-6/STAT3 pathway imbalance and lead to the occurrence of CRC.

In summary, RPTS induces apoptosis in CRC. Apoptosis is closely related to the occurrence and development of tumors. Disorders in apoptosis regulation can lead to uncontrolled cell proliferation. The clinical significance of RPTS to colon cancer merits further study.

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