

*Full Length Research Paper*

# Rhein induces apoptosis of HCT-116 human colon cancer cells via activation of the intrinsic apoptotic pathway

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**Rhein, a major compound in rhubarb, has been found to have anti-tumor properties in many human cancer cells. However, the details about rhein suppressing the growth of human colon cancer cells remained elusive. In this paper, we explored the potential of rhein as a chemotherapeutic agent on HCT-116 cells and demonstrated significant inhibition of HCT-116 cells proliferation in both concentration (0, 10, 30, and 100  $\mu$ M) and time (24, 48, and 72 h) dependent manners. The anti-tumor effects were associated with the introduction of cellular apoptosis, which was relative with reduction of Bcl-2, NF- $\kappa$ B and activation of caspase-9 and 3. In conclusion, these findings suggested that rhein inhibited the growth of HCT-116 through the intrinsic apoptotic pathway and might be a useful strategy of chemotherapeutics of colon cancers.**

**Key words:** Rhein, apoptosis, HCT-116 human colon cancer cells, anti-tumor properties, caspases, Bcl-2 family proteins, NF- $\kappa$ B.

## INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide. Chemotherapy, which is the most important adjuvant therapy, has been used for many decades. However, the traditional chemotherapeutic drugs have many adverse effects and are restricted in clinical application. In recent years, many compounds in the natural plants have been found to have anti-tumor effects with limited side effects. Rhein, a component of rhizome of rhubarb, has been found to have extensive biological

And pharmacological activities including anti-atherosclerosis (Heo et al., 2010), anti-angiogenesis (Fernand et al., 2010) and anti-tumor properties. The anti-tumor properties of rhein have been reported in many types of cancer cells, such as SCC-4 human tongue cancer cells (Lai et al., 2009; Chen et al., 2010a; Chen et al., 2010b), Caco-2 human adenocarcinoma cells (Aviello et al., 2010), breast cancer cells (Lin et al., 2009a), nasopharyngeal carcinoma cells (Lin and Zhen, 2009b), A-549 human lung cells (Hsia et al., 2009), human hepatocellular carcinoma BEL-7402 cells (Shi et al., 2008), and human cervical cancer Ca Ski cells (Ip et al., 2007).

The antitumor properties of rhein are supported by various observations:

(1) Disruption of cell cycle. Rhein induced G1/S and G0/G1 cell-cycle arrest through inhibition of cyclin D3, Cdk4 and Cdk6, and then it would increase the effects of cancer chemotherapy (Hsia et al., 2009).

(2) Induction of apoptosis. It inhibited the uptake of

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**Abbreviations:** GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMP, matrix metalloproteinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

glucose in Ehrlich ascites tumor cells by altering the membrane-associated functions and induced apoptosis in HL-60 cells through the mitochondrial death pathway by causing the loss of mitochondrial membrane potential, cytochrome c release, and cleavage of Bid protein (Castiglione et al., 1993). Other reports showed that rhein induces apoptosis through induction of endoplasmic reticulum stress and  $\text{Ca}^{2+}$ -dependent mitochondrial death pathway (Hsia et al., 2009; Lin et al., 2003; Ip et al., 2007; Lai et al., 2009).

(3) Inhibiting migration and invasion. It was found that rhein inhibited the protein expression of activity of matrix metalloproteinase-2 (MMP-2) and the gene expression of MMP-9 by modulation of NF- $\kappa$ B activation pathway, and decreased the expression of vascular endothelial growth factor (Chen et al., 2010b; Lin et al., 2009a).

(4) Sensitization activity. Rhein showed synergistic inhibitory effect with mitomycin, although the mechanism is not yet known (Huang and Zhen, 2001).

However little is known on whether rhein can inhibit the proliferation in HCT-116 colon cancer cells via apoptotic pathways. In this paper, we tested the effect of rhein on apoptosis via Bcl-2/BAX and NF- $\kappa$ B mediated caspase-activated pathways in HCT-116 cells.

## MATERIALS AND METHODS

Rhein was purchased from Sigma-Aldrich (St Louis, MO). Annexin V-FITC/propidium iodide (PI) was purchased from Biosea (Beijing, China). The TUNEL staining kit and nucleoprotein extraction kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The primers for real-time PCR were purchased from Genscript (Nanjing, China). M-MLV reverse transcriptase and relevant reagents for RT-PCR were purchased from Promega Corporation (USA). Antibodies against Pro-caspase-3, caspase-3, caspase-8, caspase-9, Bcl-2, Bax, NF- $\kappa$ B,  $\beta$ -actin, GAPDH and Histone H3 were purchased from Santa Cruz Biotechnology (CA, USA). The trizol reagent kit and fluorescence-conjugated secondary antibodies were purchased from Invitrogen (USA). Other reagents were of analytical purity.

### Cell culture

The HCT 116 cells were obtained from the cell line bank of Chinese Academy of Sciences (CAS). Cells were cultured in complete RPMI-1640 medium (Hyclone, USA) supplemented with 10% heat-inactivated bovine serum (Gibco, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C and 5%  $\text{CO}_2$ , and routinely passaged every other day.

### Cell proliferation assay

The cells were seeded into a 96-well culture plate at the density of 5000 cells/well. Cells were incubated with rhein (0, 10, 30, and 100  $\mu$ M) for 24, 48, and 72 h and cell viability was measured with MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. MTT was added to each well for 4 h, the supernatant was then discarded and the purple-colored precipitates of formazan were dissolved in 150  $\mu$ l of dimethyl sulfoxide. The optical density was measured at a wavelength of 490 nm on a multiwell plate reader. Each concentration of rhein was repeated in 6 wells. The

effect of rhein on growth inhibition was assessed as the percentage of inhibition in cell growth where untreated cells were taken as 100% viable. Percent viability was calculated as (Absorbance of drug-tested group/Absorbance of control group)  $\times$  100%.

### TUNEL staining

To detect the apoptosis of HCT 116 cells, TUNEL assay was performed with *in-situ* Nick-End Labeling kit. Cells were treated for 48 h with rhein (0 to 100  $\mu$ M) in 96-well plates. After being washed twice with PBS, the attached cells were fixed in freshly prepared 4% paraformaldehyde for 30 min. Then the cells were washed twice with PBS and incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase-catalyzed reaction for 1 h at 37°C in a humidified atmosphere. The cells were immersed in a stop/wash buffer for 10 min at room temperature, and then incubated with an anti-digoxigenin antibody conjugating peroxidase for 30 min. The nuclei fragments were stained using 3, 3'-diaminobenzidine as a substrate of the peroxidase for 5 min, which stains brown.

### Flow cytometry measurement

For quantification of apoptotic HCT 116 cells, Annexin V-FITC and PI staining were performed followed by flow cytometry. After treatment of cells for 48 h with rhein (0 to 100  $\mu$ M), both suspended and attached cells were collected and washed twice with PBS, and then were subjected to Annexin V and PI staining using an apoptosis assay kit following the protocol provided by the manufacturer. Stained cells were analyzed by fluorescence activated cell sorting (Becton Dickinson Corporation, USA). The early apoptotic cells stained with Annexin V-FITC are presented in the lower right quadrant of the histogram. The late apoptotic cells stained with both Annexin V-FITC and PI are presented in the upper right quadrant of the histogram.

### Quantitative real-time PCR analysis

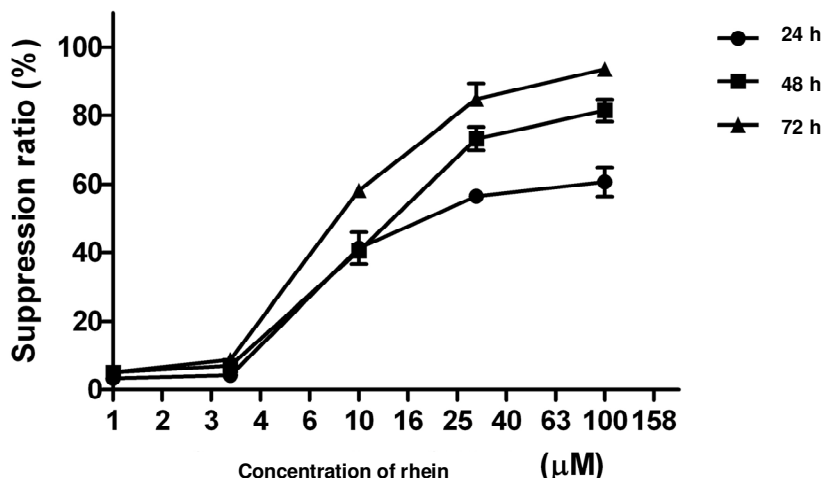
Total RNA of cells was extracted by Trizol reagent kit. The quality of each total RNA sample (including its concentration and purity) was checked and controlled by measurement of the optical density. Each sample RNA (1  $\mu$ g total RNA) was used to generate cDNA by using M-MLV reverse transcriptase according to manufacturer's specifications. After an initial denaturation step at 95°C for 10 min using SYBR Green PCR Master Mix (Applied Biosystems, USA), real-time PCR was cycled between 95°C/15 s and 60°C/1 min for 40 times. Amplification was performed by using 7500 Fast Real-Time PCR Systems (Applied Biosystems, USA) and the products were routinely checked by using dissociation curve software. Transcript quantities were compared by using the relative Ct method, where the amount of caspase-3 and caspase-9 was normalized to the amount of endogenous control (GAPDH). The relative value to the control sample is given by  $2^{-\Delta\Delta\text{CT}}$ . Real-time PCR primer sequences for caspases measurement was shown in Table 1.

### Western blot analysis

Following desired treatments, HCT 116 cells were washed twice with ice-cold PBS and collected by scraping in the specified media. The supernatant was obtained by centrifuging for 20 min at 13,500 rpm. Nucleoprotein was extracted following manufacturer's specifications. The concentration of total protein and nucleoprotein was quantified by Bradford assay (Bradford MM 1976). For

**Table 1.** Real-time PCR primer sequences for caspases measurement.

Caspase	Sense primer	Anti-sense primer
Caspase-3	5' TGGTTCATCCAGTCGCTTTG 3'	5' TAGCCCTCTGCTCCATCCTG 3'
Caspase-8	5' GTGGAGGAAAGCAATCTGTC 3'	5' TATTAGCCCTGCCTGGTGTCT 3'
Caspase-9	5' CGAACTAACAGGCAAGCAGC 3'	5' ACCTACCAAATCCTCCAGAAC 3'

**Figure 1.** Rhein inhibited the proliferation of HCT-116 cells.

immunoblot analysis, the samples were subjected to electrophoresis in 12% SDS-PAGE gels and the separated proteins were transferred onto nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% non-fat milk powder (w/v) for 2 h at room temperature, then incubated with the primary antibodies against pro-caspase-3 (1:200), cleaved caspase-3 (1:200), Bcl-2 (1:200), Bax (1:200), NF- $\kappa$ B (1:500),  $\beta$ -actin (1:200), Histone H3 (1:1000), GAPDH (1:1000) overnight at 4°C, respectively. After being washed these membranes were incubated with fluorescence-conjugated secondary antibodies (anti-rabbit or anti-mouse) (invitrogen, USA) at dilution 1:10000 for 50 min. Western blot bands were quantified by using Odyssey infrared imaging system (LICOR, Lincoln, NE). Western blot experiments were repeated three times.

#### Statistical analysis

All data were reported as the means ( $\pm$ SEM) of at least three separate experiments. Statistical analysis used the *t* test, with significant differences determined at the level of  $p < 0.05$  or highly significant differences at  $p < 0.01$ .

## RESULTS AND DISCUSSION

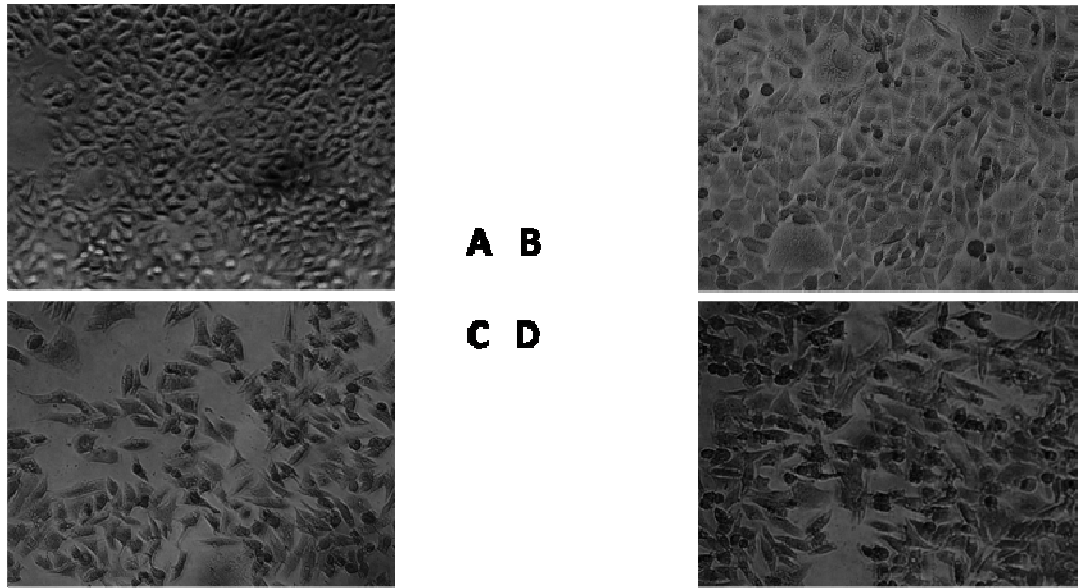
### Cell Proliferation Assay

The viability of the HCT-116 cells incubated with rhein was decreased in a dose and time dependent manner. With rhein at concentration of 0, 3, 10, 30, 100  $\mu$ M, the viability of HCT-116 cells, which was carried out by MTT assays, was decreased from 100% to  $42.45 \pm 1.42\%$  for

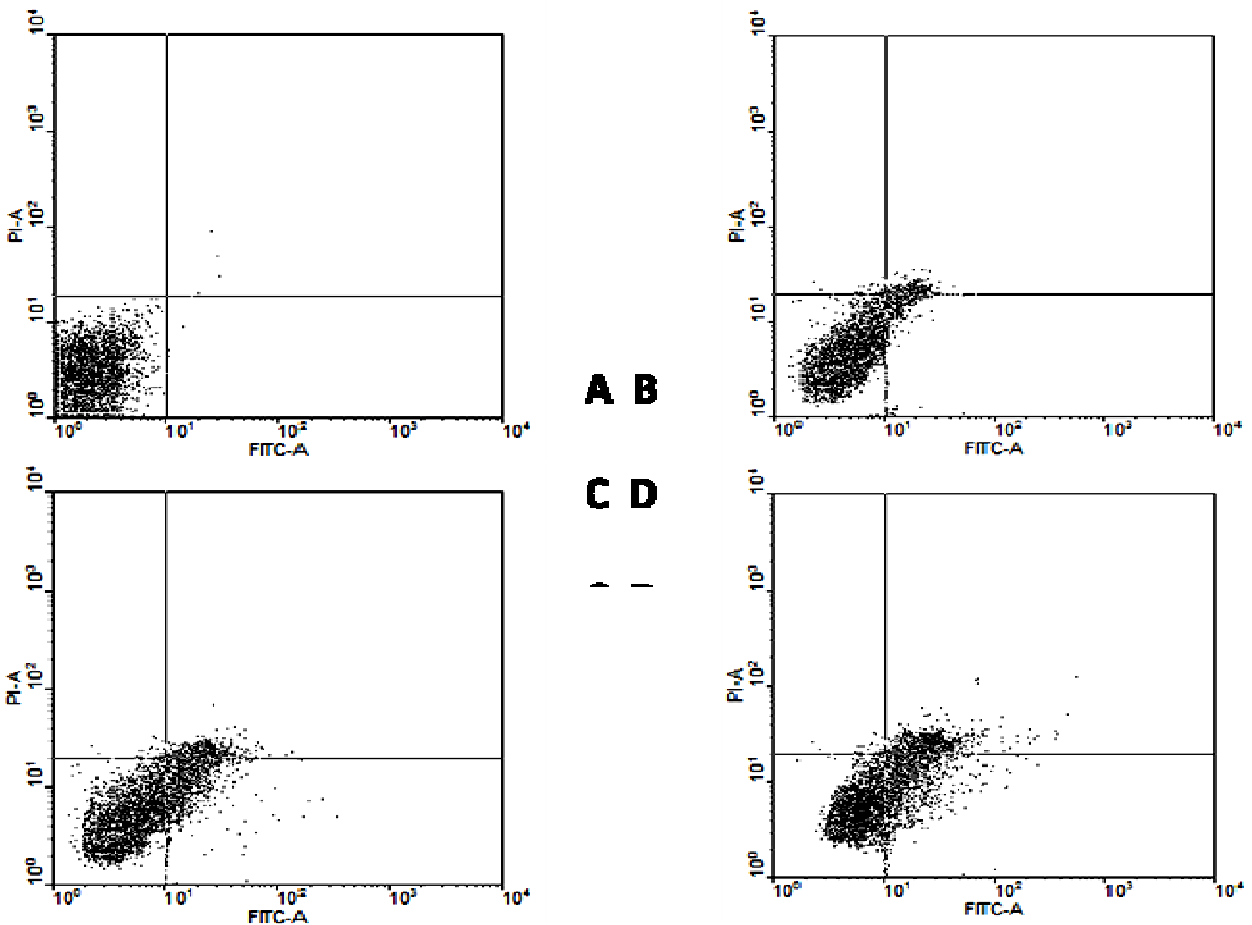
24 h, from 100% to  $21.62 \pm 1.78\%$  for 48 h, from 100% to  $16.42 \pm 1.9\%$  for 72 h ( $n = 18$ ) (Figure 1). We also observed with invert microscopy and found HCT-116 cells appeared round with small wrinkle and broken debris after incubation with rhein compared with normal HCT-116 cells, which indicated that rhein exerted cytotoxicity on HCT-116 Cells. Our results were in accordance with other reports (Hsia et al., 2009; Lai et al., 2009; Lin et al., 2003).

### TUNEL Staining and Flow cytometry

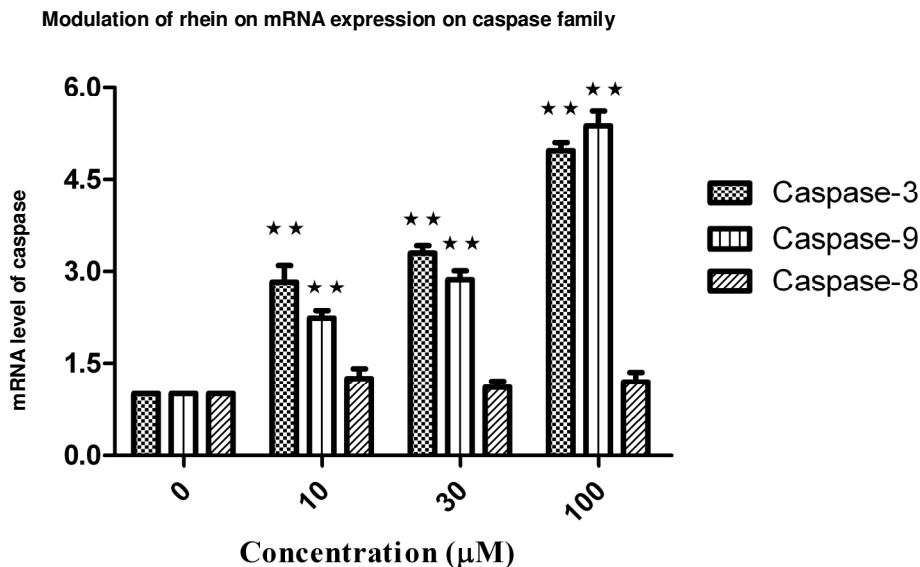
To explore the effect of rhein on apoptosis in HCT-116 cells, the TUNEL apoptosis detection kit was used to identify the apoptotic cells after 48 h of exposure to rhein. The amount of brown staining and nuclear material pycnosis of HCT-116 cells increased in a dose dependent manner (Figure 2). Flow cytometry measurement was used for the quantification of apoptotic HCT-116 cells. As shown in Figure 3, the percentage of apoptotic cells treated with rhein (0, 10, 30 and 100  $\mu$ M) for 48 h were 0%, 8.2%, 18.7% and 34.6%, respectively. So the data showed that rhein induced apoptosis of HCT-116 cells, and had a potential as a chemotherapeutic agent. Maybe the strategy of enhancing the efficacy of anti-cancer therapy with natural compounds such rhein by itself or in combination will be a rather novel approach, which deserves further investigations.



**Figure 2.** Morphological changes in the nuclei (typical of apoptosis) of HCT-116 cells induced by rhein. (A) Control; (B) HCT-116 cells treated with 10  $\mu$ M rhein; (C) HCT-116 cells treated with 30  $\mu$ M rhein; (D) HCT-116 cells treated with 100  $\mu$ M rhein.



**Figure 3.** Rhein-induced apoptosis in HCT-116 cells was determined by the flow cytometry using AnnexinV-PI staining method. Panel A indicated the control cells. The cells in panels B, C, and D were treated with 10, 30, 100  $\mu$ M rhein for 48 h



**Figure 4.** Treatment of rhein increased the gene expression of caspase-3 and caspase-9 in HCT-116 cells but caspase-8 remained unchanged. \*\*:  $p < 0.01$  versus control group.

### Quantitative real-time PCR analysis

To detect the possible mechanisms of rhein on apoptosis in HCT-116 cells, real-time quantitative PCR was utilized to detect the mRNA expression of caspase-3, caspase-8 and caspase-9 in HCT-116 cells after being treated with different concentrations of rhein (0, 10, 30 and 100 μM) for 48 h. Then the expression of mRNAs was normalized by GAPDH expression. The results showed that the mRNA expression of caspase-3, caspase-9 increased while the expression of caspase-8 remained unchanged (Figure 4). It is known that apoptosis plays a crucial role in prevention of cancer progression and apoptosis is governed by a complex network of anti-apoptotic and pro-apoptotic effector molecules. There are two major apoptotic pathways which have been identified. One is the extrinsic cell death pathway and the other is the intrinsic pathway (Dicou and Perez-Polo, 2009). In the extrinsic pathway, the activation of caspase-8 plays a key role. In our paper, the gene expression of caspase-8 remained unchanged after treatment of rhein, that showed the apoptosis induction by rhein wasn't related with the extrinsic pathway. So the intrinsic apoptotic pathway was the main mechanism through which rhein induced apoptosis in HCT-116 cells.

### Western blot analysis

The proteins of the Bcl-2 family play an important role in intrinsic apoptosis and are now considered as a target for anticancer therapy (Kang and Reynolds, 2009). Bcl-2 functions as a suppressor of apoptosis, while Bax is a pro-apoptotic protein of the Bcl-2 family (Watanabe et al.,

2001). After forming transmembrane pores across the outer mitochondrial membrane, Bax leads to loss of membrane potential and efflux of cytochrome c which is the apoptosis inducing factors (Portier and Tagliatela, 2006). Cytochrome c together with Apaf-1, ATP and pro-caspase-9 forms a complex (apoptosome) and finally leads to the activation of caspase-3, at the same time the expression level of Pro-caspase-3 descends (Acehan et al., 2002). When the ratio of Bax/Bcl-2 protein expression increases, the caspases will be fragmented and induce cell apoptosis (Mantena et al., 2006).

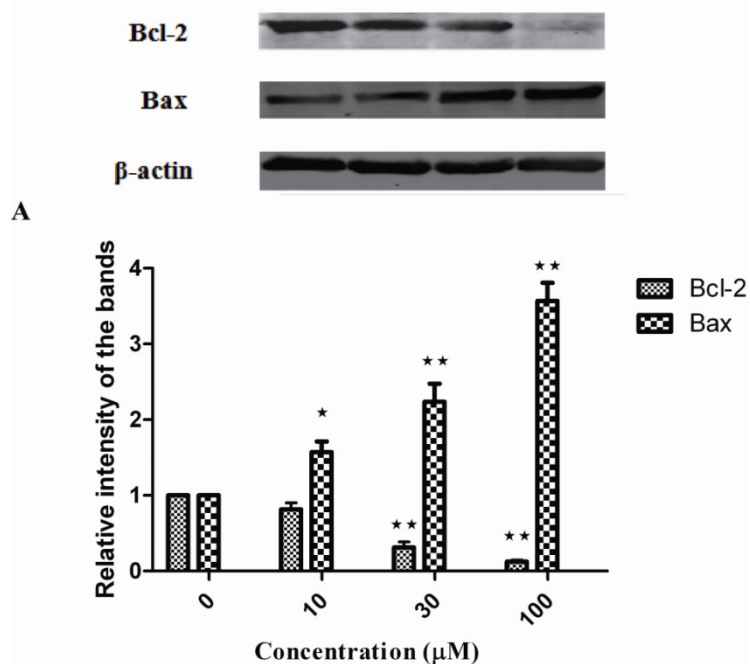
Our data showed that the Pro-caspase-3 and Bcl-2 proteins was down-regulated, while the pro-apoptotic Bax and the cleaved caspase-3 proteins were up-regulated after incubation with different concentrations of rhein (0, 10, 30 and 100 μM) for 48 h by Western blot analysis. The results indicated that the protein production of Bcl-2 and Pro-caspase-3 decreased, but the production of Bax and cleaved caspase-3 increased (Figure 5-6).

### Nuclear NF-κB Transcription Factor in HCT-116 Cells

The nucleoprotein of HCT-116 cells was extracted following the manufacturer's instruction. After incubation with rhein (0, 10, 30 and 100 μM) for 48 h, the expression of NF-κB was calculated via Western blot assay. β-actin was used as an internal control to monitor the equal loading and transfer of proteins from the gels to the membranes. Western blot analysis indicated that the protein production of NF-κB decreased in a dose dependent manner (Figure 7).

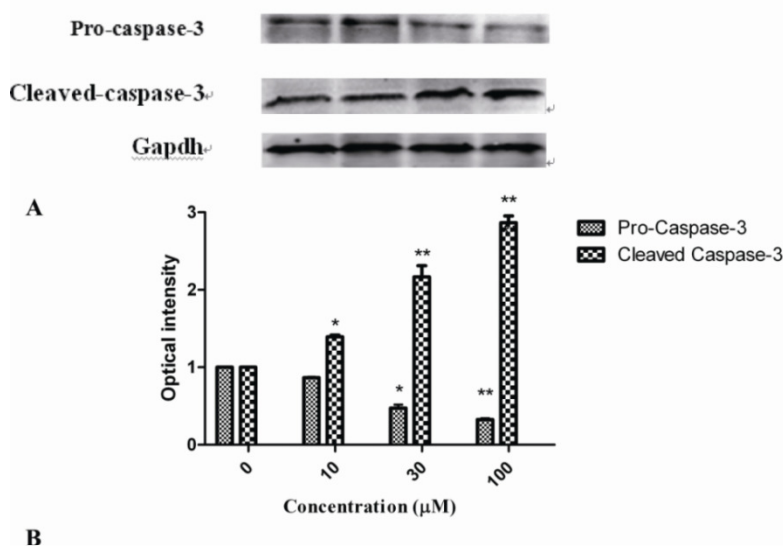
NF-κB is an important transcription factor known to play an important role in autoimmune, inflammation, cell proli-

### Modulation of Rhein on Bcl-2, Bax protein



**Figure 5.** Rhein decreased the expressions of Bcl-2 and increased the expression of Bax in HCT-116 cells in a dose dependent manner. \* and \*\*:  $p < 0.05$  and  $p < 0.01$  with respect to control, respectively.

### Modulation of Rhein on Pro-Caspase-3 and Cleaved Caspase-3



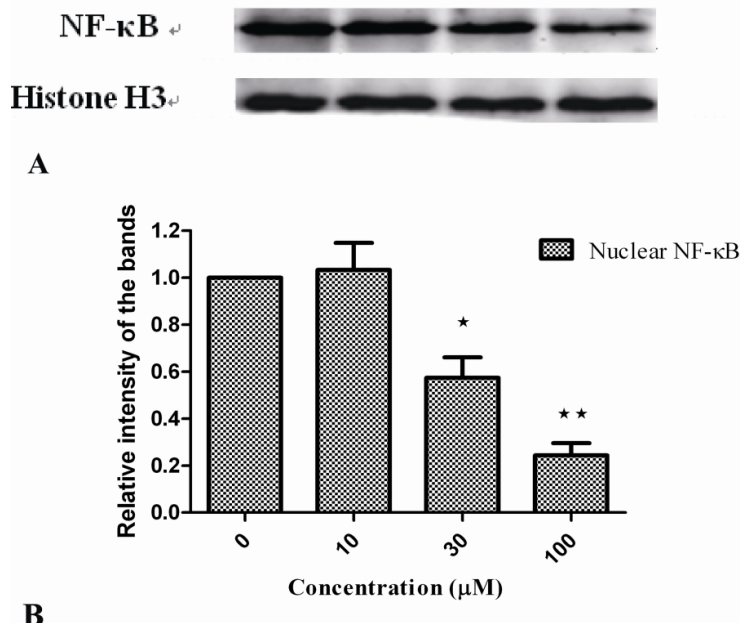
**Figure 6.** Rhein decreased the expressions of pro-caspase-3 and increased cleaved caspase-3 in HCT-116 cells in a dose dependent manner. \* and \*\*:  $P < 0.05$  and  $P < 0.01$  with respect to control, respectively.

feration, and apoptosis by regulating the expression of genes involved in many processes (Kuttan et al., 2007). When NF- $\kappa$ B is inactivated by I $\kappa$ B, cells are more sensitive to TNF- $\alpha$ -induced apoptosis (Liu et al., 1996). In

colon cancer, It is reported that constitutive NF- $\kappa$ B was activated and over-expressed (Sakamoto and Maeda, 2010). Moreover, recent researches on the anti-carcinogenic mechanism of rhein focuses on some



### Modulation of Rhein on protein expression on nuclear NF- $\kappa$ B



**Figure 7.** Rhein decreased the expressions of NF- $\kappa$ B in HCT-116 cells in a dose dependent manner. \* and \*\*:  $p < 0.05$  and  $p < 0.01$  with respect to control, respectively.

molecular targets, such as NF- $\kappa$ B, mitogen-activated protein kinases, PI3K/Akt, caspases, etc (Fernand et al., 2011; Hsia et al., 2009). In addition, the effect of NF- $\kappa$ B mediated inhibition of apoptosis, which are induced by DNA damaging agents, could be due to the regulation of members of Bcl-2 family (Zong et al., 1999). NF- $\kappa$ B also regulates the expression of the Bcl-2 family (Chen et al., 2000). Thus, it is possible that Bcl-2 may be involved in anti-apoptotic activity of NF- $\kappa$ B. Hence we investigated whether NF- $\kappa$ B was involved in rhein-induced apoptosis in HCT-116 cells. The result showed that the expression of NF- $\kappa$ B in HCT116 cells, which were treated with rhein, was reduced in a dose-dependent manner after rhein treatment.

### Conclusions

Rhein had exerted cytotoxicity on human colon carcinoma HCT-116 cells, mainly through Bcl-2/BAX and NF- $\kappa$ B mediated caspase-activated apoptotic pathways. Our results showed that rhein has the potential to be developed as a chemotherapeutic or adjuvant agent on human colon cancers.

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