

Andrographolide suppresses proliferation of human colon cancer SW620 cells through the TLR4/NF- κ B/MMP-9 signaling pathway

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Abstract. Modern pharmacological research has revealed that andrographolide has various functions, including anti-bacterial, anti-inflammatory and anti-viral effects, immunoregulation, treating cardiovascular and cerebrovascular diseases, and prevention and treatment of alcoholic liver injury. The present study investigated whether andrographolide suppresses the proliferation of human colon cancer cell through the Toll-like receptor 4 (TLR4)/nuclear factor (NF)- κ B/matrix metalloproteinase-9 (MMP-9) signaling pathway. The MTT assay and lactate dehydrogenase assay were used to evaluate the anticancer effects of andrographolide on cell proliferation and cytotoxicity in human colon cancer SW620 cells. Flow cytometry was used to analyze the anticancer effects of andrographolide on apoptosis by Annexin V-fluorescein isothiocyanate/propidium iodide kit. The effects of andrographolide on the activity of caspase-3/9 were measured using ELISA. Western blot analysis was also used to analyze the protein expression of TLR4, myeloid differentiation primary response gene 88 (MyD88), NF- κ B-p65 and MMP-9. In the present study, it was found that andrographolide suppressed the cell proliferation, augmented cytotoxicity, evoked cell apoptosis and activated caspase-3/9 activities in human colon cancer SW620 cells. The results revealed that the anti-proliferation effects of andrographolide on the SW620 cells was associated with the inhibition of TLR4, MyD88, NF- κ B-p65 and MMP-9 signaling activation. The results suggest that andrographolide is a promising drug for treatment of human colon cancer via suppression of the TLR4/NF- κ B/MMP-9 signaling pathway.

Introduction

Colon cancer poses a significant risk to the health of individuals. In recent years, with changes in dietary patterns, the morbidity and mortality rates of colon cancer have significantly increased (1). In cancer progression, the invasion and metastasis of colon cancer markedly affects the prognosis of colon cancer patients and is a common leading cause of cancer-associated mortality (2). The metastasis and infiltration of cancer are associated with various factors. Proteases secreted by cancer, including matrix metalloproteinases, (MMPs) have important roles (3).

As a nuclear transcription factor with numerous regulatory functions, nuclear factor (NF)- κ B exists in various cells in a dipolymer or heterodimer form (4). The p50/p65 form is the most common combination form (5). Various studies have shown that NF- κ B participates in the transcription of genes regulating apoptosis and hyperplasia (6). It is well-known that apoptosis and hyperplasia are closely associated with the occurrence of cancer.

Toll-like receptor (TLR) is a type I transmembrane protein with >10 family members (7). As an important bridge between innate immune defense and acquired immunity, it can trigger signal transduction, which further leads to the release of inflammatory mediators by identifying pathogen-associated molecular patterns and certain endogenous ligands (8). A previous study found that TLR may play certain roles in pathogenesis and bio-immunotherapy (9).

Andrographolide is a diterpene lactone extracted from *Andrographis paniculata* and is also present in *Jacobinia suberecta*. The molecular formula of andrographolide is C₂₀H₃₀O₅ and it is one of the active components of *A. paniculata* extract, comprising up to 1.84% of the extract (10,11). Andrographolide has been produced as a raw material with antipyretic and analgesic effects (12). Previous studies have suggested that andrographolide has various functions, including anti-bacterial, anti-inflammatory and anti-viral effects, immunoregulation, treating cardiovascular and cerebrovascular diseases, and prevention and treatment of alcoholic liver injury (13,14). The present study investigated the effects of andrographolide on the proliferation of human colon cancer cell through the TLR4/NF- κ B /MMP-9 signaling pathway.

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Materials and methods

Cell line and culture conditions. The human colon cancer SW620 cell line was procured from Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China) and cultured with Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Mediatech, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

Cell viability assay and cytotoxicity. SW620 cells were inoculated onto 96-well plates at a density of 1×10^4 cells per well and treated with dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany) or 0, 5, 10, 15, 20 or 25 μ M andrographolide (Sigma-Aldrich; Merck KGaA) for 12, 24 and 48 h. Subsequent to processing, the supernatant was assessed using an MTT assay (Beyotime Institute of Biotechnology, Haimen, China) and cultured for 4 h. The supernatant was discarded and DMSO (0.5 mg/ml) was added to each well for 20 min at 37°C. The cytotoxicity of andrographolide in SW620 cells was measured using Triton X-100 (1%) and a detergent, leading to a complete release of the LDH enzyme (Sigma-Aldrich; Merck KGaA). The absorbance values were determined using a DTX 880 Multimode Detector (Beckman Coulter, Inc., Brea, CA, USA) at 550 nm.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay for cell apoptosis examination. SW620 cells were inoculated onto 96-well plates at a density of 2×10^6 cells per well and treated with DMSO or 20 μ M andrographolide for 24 h. The supernatant of the cell culture was collected and incubated with Annexin V-FITC (BD Biosciences, San Jose, CA, USA) for 20 min in the dark. PI (BD Biosciences) was added into every well and the wells were incubated for 20 min in the dark. Apoptosis was detected using flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Activity of caspase-3/9. SW620 cells were inoculated onto 96-well plates at a density of 2×10^6 cells per well, and the cells were treated with DMSO or 20 μ M andrographolide for 24 h. The supernatant of the cell culture was collected and incubated with Ac-DEVD-pNA or Ac-LEHD-pNA for 4 h to assess the activity of caspase-3 and caspase-9, respectively. The activity of caspase-3/9 was detected using a DTX 880 Multimode Detector (Beckman Coulter) at 405 nm.

Western blot analysis. SW620 cells were inoculated onto 96-well plates at a density of 1×10^4 cells per well and treated with DMSO or 20 μ M andrographolide for 24 h. The supernatant of the cell culture was collected and harvested with lysis buffer. Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the protein content. Protein (50 μ g) from each sample was separated on a 10-12% SDS-PAGE gel (Beyotime Institute of Biotechnology) and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked with Tris-buffered saline with Tween-20 containing 5% non-fat milk and incubated with rabbit monoclonal anti-TLR4 (cat. no. sc-10741; dilution, 1:400; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-myeloid differentiation primary response gene 88 (cat. no. sc-11356,

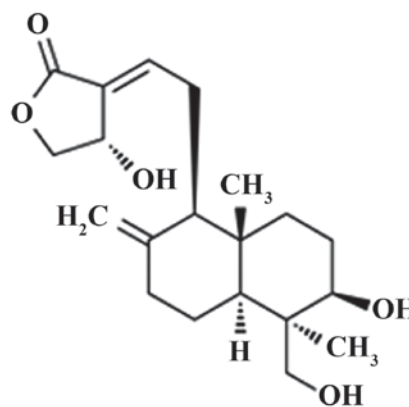


Figure 1. The chemical structure of andrographolide.

MyD88; dilution, 1:400; Santa Cruz Biotechnology, Inc.), anti-NF- κ B-p65 (cat. no. sc-109; dilution, 1:400; Santa Cruz Biotechnology, Inc.), anti-MMP-9 (cat. no. sc-10737; dilution, 1:400; Santa Cruz Biotechnology, Inc.) and β -actin (cat. no. sc-7210; dilution, 1:2,000; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2357; dilution, 1:3,000; Bio-Rad Laboratories, Inc.) at 37 °C for 1 h and detected using a BeyoECL Star enhanced chemiluminescence kit (Beyotime Institute of Biotechnology).

Statistical analysis. The data were presented as the mean \pm standard deviation and analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for multi-group comparisons with a Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Andrographolide suppresses cell proliferation of SW620 cells. The chemical structure of andrographolide is shown in Fig. 1. To examine the anticancer effect of andrographolide on SW620 cells, the cells were treated revealed with DMSO or 0, 5, 10, 15, 20 and 25 μ M andrographolide for 24 h. As shown in Fig. 2, andrographolide could suppress the cell proliferation of SW620 cell in dose-dependent manner compared to the controls or DMSO group. In particular, 15, 20 and 25 μ M andrographolide significantly inhibited the proliferation of SW620 cells compared to the control or DMSO groups.

Andrographolide promotes cytotoxicity of SW620 cells. In the present study, the cytotoxic effect of andrographolide on human colon cancer SW620 cells was analyzed. As shown in Fig. 3, 15, 20 and 25 μ M andrographolide significantly enhanced the cytotoxicity of SW620 cells compared with the control or DMSO group.

Andrographolide induces apoptosis of SW620 cells. For flow cytometry, SW620 cells treated with 20 μ M andrographolide were stained with Annexin V-FITC/PI and evaluated for the apoptosis rate. As shown in Fig. 4, 20 μ M andrographolide significantly induces the apoptosis rate of SW620 cells compared with the control or DMSO groups.

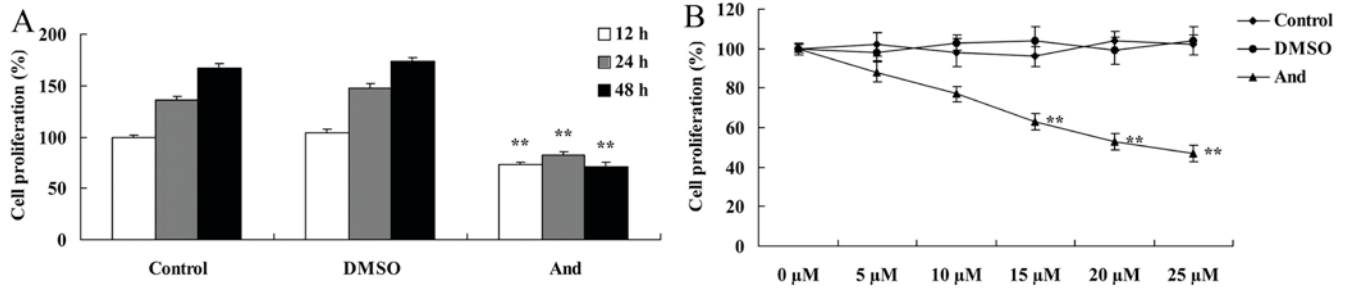


Figure 2. Andrographolide suppresses cell proliferation of human colon cancer SW620 cells. Andrographolide suppresses cell proliferation at (A) 12, 24 and 48 h time-points at an andrographolide concentration of 20 μM and (B) increasing concentrations of andrographolide at 24 h in SW620 cells. **P<0.001 compared with the control group. And, andrographolide; DMSO, dimethyl sulfoxide.

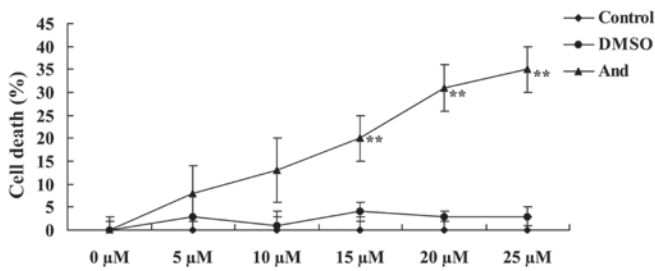


Figure 3. Andrographolide is cytotoxic in SW620 cells. **P<0.001 compared with the control group. And, andrographolide; DMSO, dimethyl sulfoxide.

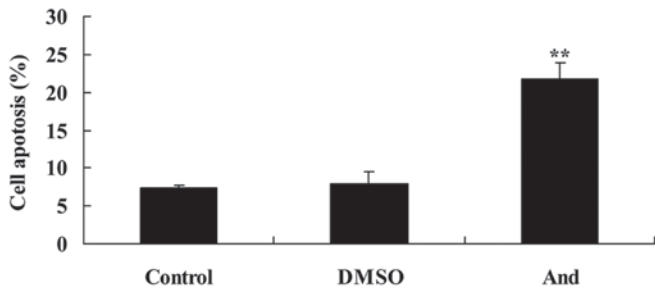


Figure 4. Andrographolide induces apoptosis of SW620 cells. **P<0.001 compared with the control group. And, andrographolide; DMSO, dimethyl sulfoxide.

Andrographolide induces caspase-3/9 activities of SW620 cells. The anticancer effect of andrographolide on SW620 cells was demonstrated by caspase-3/9 activity. As shown in Fig. 5, 20 μM andrographolide significantly increased the activity of caspase-3/9 in SW620 cells compared with the control and DMSO groups.

Andrographolide induces the protein expression of TLR4 in SW620 cells. Western blot analysis was used to detect TLR4 protein expression in SW620 cells (Fig. 6). As compared with the control or DMSO groups, the protein expression of TLR4 in SW620 cells was significantly suppressed by treatment with 20 μM andrographolide.

Andrographolide induces the protein expression of MyD88 SW620 cells. In order to explore the effect of andrographolide on MyD88 protein expression in SW620 cells, western blot analysis was performed to detect whether MyD88 participates

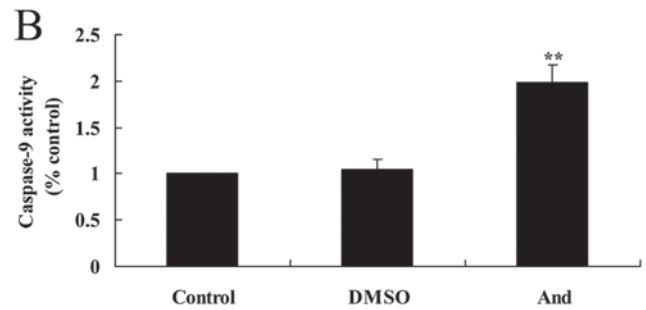
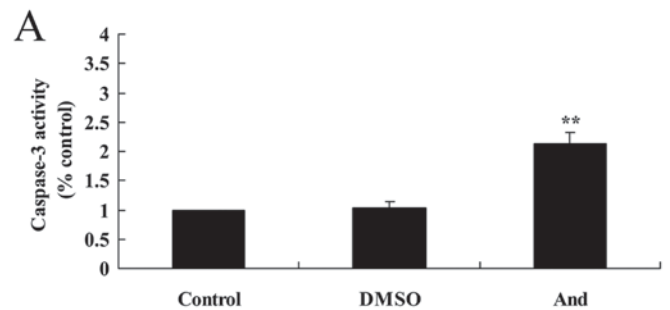


Figure 5. Andrographolide induces caspase-3/9 activity in SW620 cells. Andrographolide induces (A) caspase-3 and (B) caspase-9 activity in human colon cancer SW620 cells. **P<0.001 compared with control group. And, andrographolide; DMSO, dimethyl sulfoxide.

in the anticancer effect of andrographolide. The results showed that treatment with andrographolide significantly inhibited MyD88 protein expression in SW620 cells compared to the control or DMSO groups (Fig. 7).

Andrographolide induces the protein expression of NF-κB-p65 in SW620 cells. To research the mechanism of andrographolide on human colon cancer, NF-κB-p65 was examined using western blot analysis, as shown in Fig. 8. Treatment with 20 μM andrographolide significantly attenuated the protein expression of NF-κB-p65 in SW620 cells compared to the control or DMSO groups.

Andrographolide induces MMP-9 protein expression in SW620 cells. To further investigate the mechanism of andrographolide in human colon cancer cells, the protein expression of MMP-9 was investigated using western blot analysis. As shown in Fig. 9, 20 μM andrographolide treatment

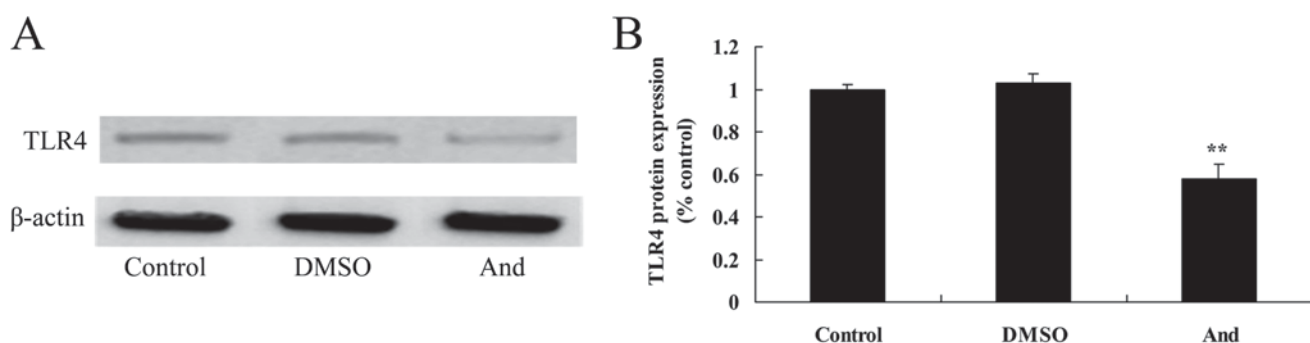


Figure 6. Andrographolide induces the protein expression of TLR4 in SW620 cells, as shown by (A) western blot analysis and (B) quantification of TLR4 protein expression in SW620 cells. ** $P < 0.001$ compared with control group. And, andrographolide; DMSO, dimethyl sulfoxide; TLR4, Toll-like receptor 4.

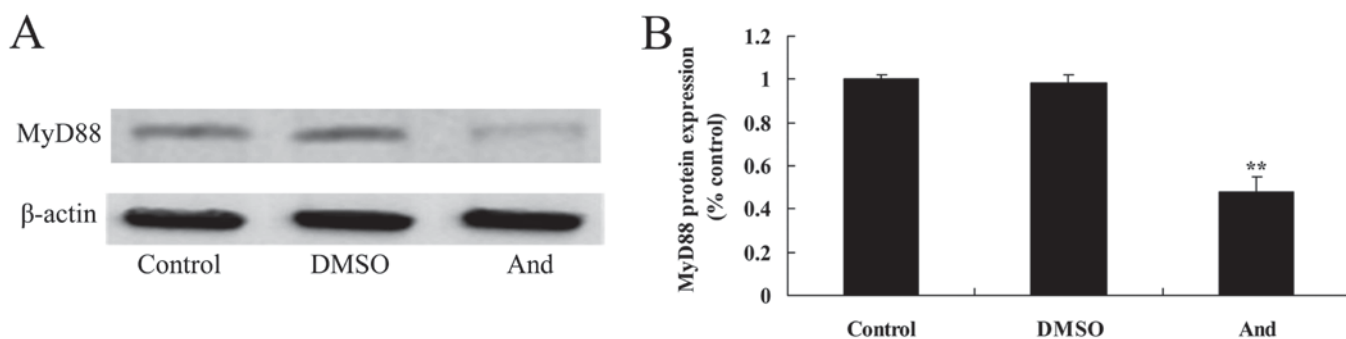


Figure 7. Andrographolide induces the protein expression of MyD88 in SW620 cells. The protein expression of MyD88 was determined using (A) western blot analysis and (B) quantification. ** $P < 0.001$ compared with control group. MyD88, myeloid differentiation primary response gene 88; DMSO, dimethyl sulfoxide; And, andrographolide.

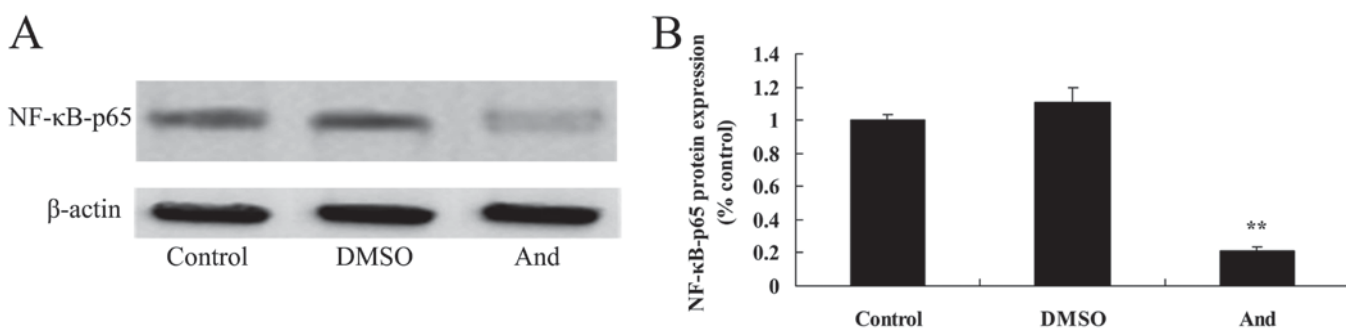


Figure 8. Andrographolide induces the protein expression of NF- κ B-p65 in SW620 cells. The protein expression of NF- κ B-p65 was determined using (A) western blot analysis and (B) quantification. ** $P < 0.001$ compared with control group. NF- κ B, nuclear factor- κ B; DMSO, dimethyl sulfoxide; And, andrographolide.

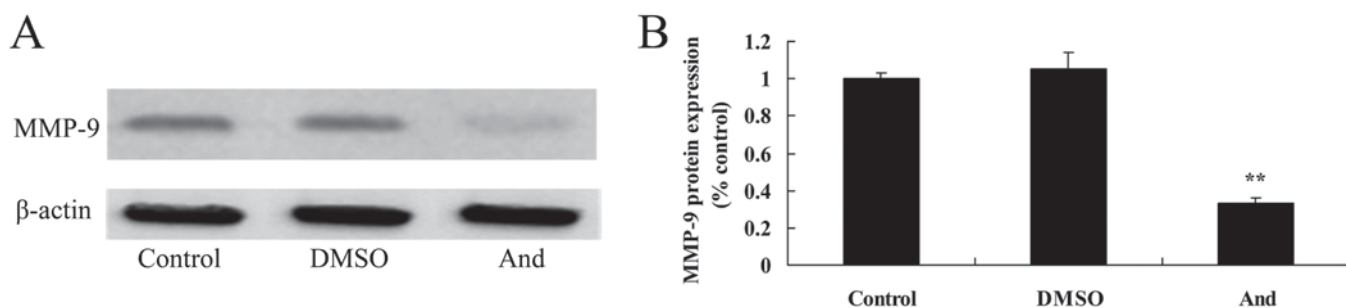


Figure 9. Andrographolide induces the protein expression of MMP-9 in SW620 cells. The protein expression of MMP-9 was determined using (A) western blot analysis and (B) quantification. ** $P < 0.001$ compared with control group. MMP-9, matrix metalloproteinase-9; DMSO, dimethyl sulfoxide; And, andrographolide.

significantly reduced MMP-9 protein expression in SW620 cells compared to the control and DMSO groups.

Discussion

Colon cancer is a common malignant cancer with increasing morbidity and mortality rates (15). During the occurrence and progression of colon cancer, the cellular mechanisms involved change continuously. Metastasis and infiltration refers to the direct and discontinuous process as well as the growth at other regions, including separation, invasion, recurrence, adhesion and growth (16). The metastasis and invasion of malignant cancers is complicated and polytropic, and is regulated by numerous genes. Different cancers have specific biological features (17). However, the degradation of extra-cellular matrix and basement membrane is prerequisite of infiltration and metastasis of cancers. Therefore, certain studies showed that andrographolide sensitizes the cytotoxicity of human colorectal carcinoma cells (12), prevents human breast cancer (18) and suppressed cell growth of A549 non-small cell lung cancer (19). The present study demonstrates that andrographolide suppresses proliferation, induces apoptosis and increases the caspase-3/9 activity of SW620 cells. Therefore, andrographolide may be a new drug for the treatment of human colon cancer.

A previous study investigating colon cancer and laryngeal carcinoma suggested that the expression of TLR2 or TLR4 in cancer cells or infiltrating inflammatory cells are likely to be closely associated with immune escape of cancer (20). Continuous activation of TLR2 and TLR4 signal pathways is considered to be an important factor in the progression of malignant cancers from chronic inflammation (21). In the present study, treatment with andrographolide significantly suppressed TLR4 and MyD88 protein expressions in SW620 cells. Zhang *et al* also showed that andrographolide suppresses the tumor growth of insulinoma through the TLR4/MyD88/NF- κ B signaling pathway (22).

NF- κ B, which locates to the cytoplasm, is a transcription factor that is most common in its p50/p65 form. Subsequent to combining with inhibitor of κ B (I κ B- α), NF- κ B locates to the cytoplasm. Under the activity of I κ B kinase, I κ B- α can be phosphorylated and separated from NF- κ B (23). NF- κ B combines with promoters of target genes and induces the synthesis of mRNA. This participates in the immune response to infection and inflammation, and immunoregulation (24). A recent study suggested that NF- κ B has is associated with the occurrence, infiltration and metastasis of cancers, such as apoptosis, cell cycle control and differentiation (25). In the present study, treatment with 20 μ M andrographolide significantly attenuated the protein expression of NF- κ B-p65 in SW620 cells. Peng *et al* also reported that andrographolide suppresses NF- κ B expression in nasopharyngeal carcinoma cells (26).

The MMP family consists of enzymes that can break down the extra-cellular matrix. MMP-9 is produced by neutrophil granulocytes, macrophages, malignant cells and capillary endothelial cells (27). MMP-9 can promote cancer cells to have infiltration invasion through the degradation and destruction of the basilar membrane or new vessels to enhance cancer growth and dissemination (28). The present study found that treatment with andrographolide

significantly reduced MMP-9 protein expression in SW620 cells. Luo *et al* (29) also suggested that andrographolide inhibits MMP-9 and NF- κ B activity in lung cancer H3255 cells.

In conclusion, the present study demonstrated that andrographolide suppresses proliferation and induces apoptosis of SW620 cells through suppression of the TLR4/MyD88/NF- κ B/MMP-9 signaling pathway. It is therefore suggested that andrographolide may be a promising drug candidate for treatment of human colon cancer.

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