

Ethanol extract of *Antrodia camphorata* inhibits proliferation of HCT-8 human colorectal cancer cells by arresting cell cycle progression and inducing apoptosis

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Abstract. *Antrodia camphorata* (AC) is well known in Taiwan as a traditional Chinese medicine, with a long history of use in treating cancer and inflammation. Previous studies have revealed that AC exhibits anticancer effects in various cancer cell lines. However, the inhibitory influence of AC on colorectal cancer (CRC) cell growth and survival remains unknown. The present study investigated the effects of AC on the proliferation, survival, and cell cycle- and apoptosis-associated gene and protein expression in the HCT-8 human CRC cell line *in vitro*. The antitumor activity of AC against HCT-8 cells was assessed using cell viability and colony formation assays. Cell cycle distribution was analyzed by flow cytometry. Cell apoptosis and morphological alterations were assessed by Hoechst 33258 staining and microscopy. The mRNA expression of cell cycle- and apoptosis-associated genes was determined by reverse transcription-quantitative polymerase chain reaction, and protein expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2 X associated protein (Bax) cyclin D1, cyclin dependent kinase 4 (CDK4) and MYC proto-oncogene bHLH transcription factor (c-Myc) were determined by western blotting. Treatment of HCT-8 cells with various concentrations of AC (0.4-1.2 mg/ml) resulted in dose- and time-dependent reductions in cell viability. HCT-8 cell cycle was arrested in the G0/G1 phase or G0/G1 and G2/M phases following AC treatment, compared with untreated cells. Furthermore, AC markedly inhibited HCT-8 cell growth with induction of apoptotic alterations and inhibition of proliferation. AC treatment induced HCT-8 cell apoptosis, upregulated expression

of the apoptosis gene Bax, and downregulated Bcl-2, cMyc, cyclin D1 and CDK4 protein expression levels. The present data demonstrated that AC exhibited antiproliferative and growth inhibition effects on HCT-8 cells via induction of apoptosis and blocking of cell cycle progression, thus suggesting that it may have anticancer properties valuable for potential future therapeutic application for the treatment of CRC.

Introduction

Colorectal cancer (CRC) is the fourth most common cause of cancer mortality with ~9.4% of cancer cases worldwide, irrespective of gender, and an increasing CRC incidence in Asia (1-3). Cancer prevention is the most cost-effective approach to cancer control; however, many cancers are only diagnosed at advanced stages when symptoms are present. As such, treatment to slow, stop or regress tumors is the most common approach. One of the most common antitumor therapeutic techniques is the inhibition of proliferation and induction of apoptosis in tumor cells (4,5).

Despite improvements in surgical intervention and chemotherapy, the five-year survival rate for CRC patients remains poor (6). In recent years, a movement toward finding and developing improved therapeutic strategies and novel pharmaceutical compounds, particularly those with a natural origin, has been underway. A major focus has been on gathering information and understanding the mechanistic treatment properties and therapeutic potential for traditional medicines to reduce mortality in afflicted patients (7,8).

Fungi, and specifically mushrooms, have long been used in traditional medicine to treat various diseases, including many types of cancers. *Antrodia camphorata* (AC) is a native fungus to Taiwan, and is rare and expensive as it grows only on the inner heartwood wall of the endemic evergreen *Cinnamomum kanehirai* and cannot be cultivated (9). AC has a long history of use as a traditional Chinese medicine for the treatment of numerous diseases and disorders in the past few decades, including liver diseases, food detoxification and various cardiovascular diseases (10,11).

Previous studies have illustrated that AC exhibits a broad spectrum of antitumor activities, including apoptotic, antioxidative, and anti-inflammatory effects on human hepatocellular

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carcinomas and leukocytes (12-15). In addition, AC has been reported to induce apoptosis via death receptor-mediated and mitochondrial apoptotic signaling pathways, in hepatocellular carcinoma cells (16,17).

Despite these findings, no documented effects of AC in the treatment of colon cancers have been described thus far. To identify whether AC has therapeutic potential in CRC, the present study examined the AC extract effects on cell viability, proliferation and apoptosis in the widely used HCT-8 human CRC cell line. In addition, the underlying molecular mechanisms of AC influence on these cancer cells were investigated. The present findings may provide an important step toward the application of AC extract in the clinic for therapeutic use in human CRC.

Materials and methods

Reagents and antibodies. Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), trypsin-EDTA, fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI)-1640 medium, and penicillin-streptomycin were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A cell cycle assay kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Mouse or rabbit monoclonal antibodies specific for cyclin dependent kinase 4 (CDK4; 2906S), cyclin D1 (2978), c-myc (9402S), B-cell lymphoma 2 (Bcl-2; 2876S), Bcl-2 associated X protein (BAX; 2772S) and α -actin (4967L) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Bicinchoninic acid (BCA) protein assay and Enhanced Chemiluminescence (ECL) kits were obtained from Pierce; Thermo Fisher Scientific, Inc. All other chemicals used were of the highest grade commercially available.

Preparation of ethanol extracts from *A. camphorata*. *A. camphorata* used in this study was obtained from Well Shine Biotechnology Development Co., Ltd. (Taipei, Taiwan). For preparation of the extract, 500 g *A. camphorata* were extracted with 70% ethanol using a refluxing method and filtered (18). The AC extract stock solution was dissolved in DMSO to a final concentration of 500 mg/ml and stored at -20°C until use. Prior to use, the stock was thawed and appropriate amounts of the AC stock solution were filter sterilized through 0.22- μ m disc filters. Control cultures received the carrier solvent (0.1% DMSO).

Cell culture and AC treatment. The HCT-8 human CRC cell line was obtained from Nanjing KeyGen Biotech Co., Ltd. and maintained at 37°C in a humidified incubator at 5% CO₂. HCT-8 cells undergoing exponential growth were routinely cultured in 25 cm² plastic flasks containing 5 ml RPMI-1640 medium supplemented with 10% (v/v) FBS and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). For AC treatment experiments, cells were incubated with 0.4, 0.8 and 1.2 mg/ml AC, by diluting the stock with DMEM, for 24 h. Control cells were cultured in medium containing an equal amount of DMSO. Cells were washed with 2 ml PBS once and harvested with 0.25% trypsin. The cells were then centrifuged at 800 x g for 3 min at 25°C, and the isolated cells were used in subsequent experiments.

MTT assay. Various concentrations (0, 0.4, 0.8 and 1.2 mg/ml) of AC were added to 96-well plates seeded with 8x10³ HCT-8 cells/well. Following treatment for the times indicated, 10 μ l of MTT solution (5 mg/ml stock; Merck KGaA, Darmstadt, Germany) was added to each well and the plate was incubated at 37°C for 4 h. Following removal of medium, 100 μ l DMSO was added to each well and the plate was gently shaken for 5 min. Absorbance was then measured at a wavelength of 570 nm using an ELISA plate reader (Model ELX800; BioTek Instruments, Inc., Winooski, VT, USA). The percentage of viable cells was determined through comparison with untreated control cells. Each experiment was replicated 3-5 times and all data are represented as mean \pm standard deviation.

Colony formation assay. To assess the AC effect on cell colony formation, cells were seeded into a 6-well-plate at a density of 2x10⁵ cells/well. After 24 h, cells were exposed 0, 0.4 or 0.8 mg/ml AC or drug-free medium for 24 h. The drug-containing or drug-free media was then removed, cells were plated at a density of 1x10³/well into 60-mm dishes, and cells were incubated for 10 days under standard culture conditions as described above. Formed colonies were fixed in 4% paraformaldehyde for 30 min at 25°C and stained with 0.01% crystal violet for 15 min. Colony-forming efficiency was determined and cell survival was calculated by normalizing against control cell survival, from five random fields per well for three independent experiments.

Cell cycle assay. A flow cytometer was used for cell cycle analysis (FACSCalibur; BD Biosciences, San Jose, CA, USA). HCT-8 cells were cultured in 6-well culture plates at a density of 1.5x10⁵ cells/well and treated with 0, 0.4 or 0.8 mg/ml AC for 24 h. Following trypsinization, floating and adherent cells were collected by centrifuged at 800 x g for 5 min at 25°C, washed with ice-cold PBS and then incubated with 30 μ g/ml propidium iodide and 100 μ g/ml RNase for 24 h in the dark at room temperature. Data acquisition and calculation of the percentage cells in the sub-G1, G0/G1, S, and G2/M phases of the cell cycle were performed using the accompanying flow cytometer software ModfitLT version 3.3.11 (CellQuest; BD Biosciences). Twenty thousand events per sample were counted and a minimum of three assessments were performed to assure each cell cycle distribution.

Hoechst 33258 nuclear staining. HCT-8 cells were plated at low density (1.5x10⁵ cells/well) into a 6-well plate and treated with AC for 24 or 48 h. At the end of the experiment, cells were washed twice with PBS at room temperature and then fixed with ice-cold 4% paraformaldehyde for 10 min. Fixed cells were then rinsed with PBS and stained with Giemsa (0.75 mg/ml) or Hoechst 33258 (10 μ g/ml) for 15 min. Finally, cells were washed with PBS and analyzed under a fluorescence microscope with a UV light filter.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from treated HCT-8 cells was extracted using a phenol/chloroform method with RNAiso reagent (Takara Biotechnology Co., Ltd., Dalian, China). The absorbance ratio at 260/280 nm of all samples was

Table I. List of primers used for quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')
GAPDH	F: CTGCCTTCTCTTGTGACA R: TGTAGACCATGTAGTTGAGG
Cyclin D1	F: GCGTTGTACCTGTAGGACTC R: TAAGCGTGAGCCGTGTTC
CDK4	F: TGTGGAGTGTGGCTGTATC R: AGGCAGAGATTGCTTGTG
c-Myc	F: CGGAAACGACGAGAACAGT R: AGACTCAGCCAAGGTTGTG
Bax	F: AGGATGCGTCCACCAAGAAGC R: TGGCAAAGTAGAAAAGGGCGA
Bcl-2	F: TCGCCCTGTGGATGACTGAG R: CAGAGTCTTCAGAGACAGCCAGGA

CDK4, cyclin dependent kinase 4; c-Myc, MYC proto-oncogene bHLH transcription factor; Bax, B-cell lymphoma 2 X associated protein; Bcl-2, B-cell lymphoma 2; F, forward; R, reverse.

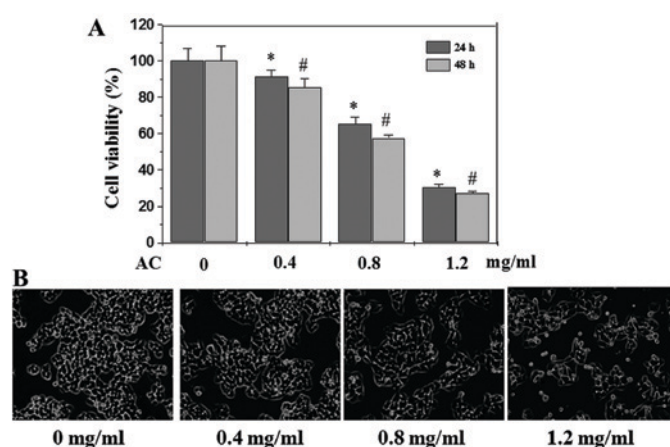


Figure 1. AC inhibits human colorectal cell viability. (A) HCT-8 cells were treated with 0, 0.4, 0.8 or 1.2 mg/ml AC for 24 and 48 h. Following treatment, cell viability was measured by MTT assay. Data are expressed as the mean \pm standard deviation. * $P < 0.05$ and # $P < 0.05$ vs. 0 mg/ml AC, for 24 or 48 h respectively. (B) Morphological alterations of HCT-8 cells following treatment with AC. The photomicrographs were captured under a phase-contrast microscope (magnification, $\times 200$). AC, *Antrodia camphorata* ethanol extract.

determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription was performed using 1 μ g total RNA with the PrimeScript™ II First-Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. qPCR was performed using SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) with an ABI 7500 Fast instrument according to the manufacturer's instructions. The reaction contained 10 μ l 2X SYBR Premix Ex Taq II, 0.8 μ l forward primer (10 μ M), 0.8 μ l reverse primer (10 μ M), 0.4 μ l 50X ROX dye II, 2.0 μ l cDNA and 6 μ l dH₂O in a total volume of 20 μ l. The qPCR reaction conditions were as follows: 30 sec at 94°C, followed by 40 cycles of 3 sec at 94°C and 30 sec at 60°C. The

primers used for qPCR are listed in Table I. Relative amount of target mRNA was calculated using the $2^{-\Delta\Delta C_q}$ method (19).

Western blotting assay. Following treatment as indicated above, HCT-8 cells were harvested and incubated in ice-cold radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) with freshly added protease and phosphatase inhibitor cocktails (Roche Applied Science, Penzberg, Germany) on ice for 30 min. The cells were scraped from the plate and the lysate was collected in a centrifuge tube and centrifuged at 12,000 \times g for 20 min at 4°C. The supernatant containing protein was collected, aliquoted, and stored at -80°C. The lysate protein concentrations were measured using a BCA protein assay kit, following the manufacturer's instructions. Total cell lysates (50 μ g) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk for 2 h at 25°C and incubated with primary antibodies (1:1,000 dilution) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution) at room temperature. Membranes were subsequently visualized using an ECL detection kit.

Statistical analysis. All data are presented as means \pm standard deviation of three independent experiments and analyzed by one-way analysis of variance followed by Tukey's post hoc test, using SPSS Statistics version 20 software (IBM SPSS, Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AC treatment downregulates cell growth and viability in HCT-8 cells. To investigate the potential effects of AC treatment on proliferation of HCT-8 cells, the cells were exposed to 0-1.2 mg/ml AC for 24 and 48 h. Fig. 1A demonstrates that AC inhibited cell proliferation in a dose- and time-dependent manner ($P < 0.05$). When treated with 0.4, 0.8 and 1.2 mg/ml AC for 24 and 48 h, HCT-8 cell viabilities were 91 and 85%, 65 and 57%, and 31 and 27%, respectively, compared with control untreated cells. Morphological changes in HCT-8 cells following AC treatment for 24 h were also evident (Fig. 1B). Based on phase-contrast inverse microscopy, the typical morphological apoptotic characteristics were observed post-treatment, including decreased cell number and an increase in apoptotic body formation, accompanied by an increased abundance of dead floating cells.

Effect of AC treatment on colony formation in HCT-8 cells. The ability of HCT-8 cells to form colonies in the presence of AC was analyzed using a flat plate colony formation assay. The results illustrated in Fig. 2A suggest that AC effectively inhibited colony formation in a dose-dependent manner. Control untreated cells demonstrated increased colony formation ability compared with cells treated with AC ($P < 0.05$; Fig. 2B). This evidence suggests that AC may have significantly affected HCT-8 cell proliferation.

AC inhibits cell cycle progression in HCT-8 cells. As AC appeared to inhibit HCT-8 cell proliferation *in vitro*, the

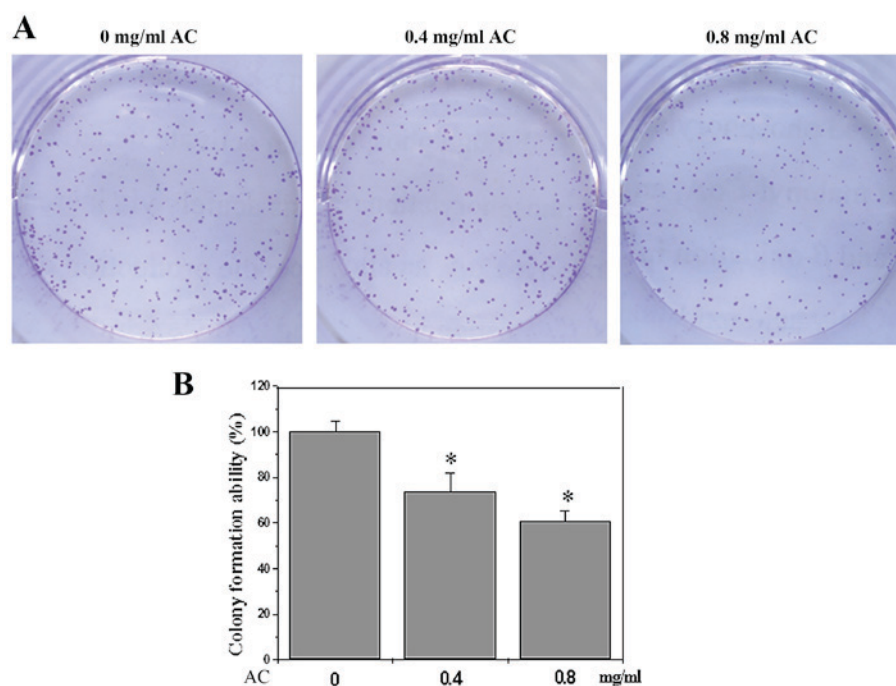


Figure 2. AC treatment inhibits HCT-8 cell colony formation. (A) HCT-8 cells were treated with 0, 0.4 or 0.8 mg/ml AC and allowed to form colonies in soft agar plates for 14 days. Representative plates are shown. (B) Quantification of the number of colonies per treatment group is presented as mean \pm standard deviation of three independent experiments. *P<0.05 vs. 0 mg/ml AC. AC, *Antrodia camphorata* ethanol extract.

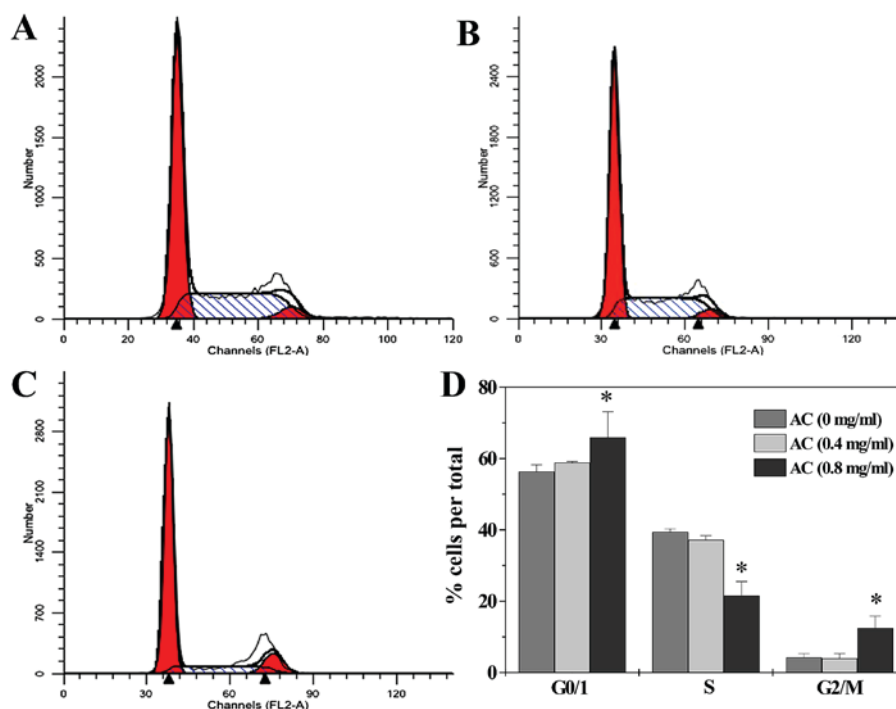


Figure 3. Flow cytometric analysis for HCT-8 cell phase distribution following AC treatment. HCT-8 cells were treated with (A) 0, (B) 0.4 or (C) 0.8 mg/ml AC for 24 h. Representative plots of cell cycle distribution per group are shown. (D) Quantification of the percentage of cells in the G0/G1, S and G2/M phases of the cell cycle. Data are presented as the mean \pm standard deviation of three independent experiments. *P<0.05 vs. 0 mg/ml AC. AC, *Antrodia camphorata* ethanol extract.

hypothesis that AC may influence cell cycle progression was tested via flow cytometry. Following treatment with AC for 24 h, the percentage of cells in the S phase in the 0.8 mg/ml AC treatment group was significantly decreased compared

with the control group (21.5 and 39.4%, respectively; Fig. 3). In addition, the percentage of cells in the G0/G1 phase was 56.4% in the control group and 66.0% in the 0.8 mg/ml AC treatment group. AC treatment significantly increased the

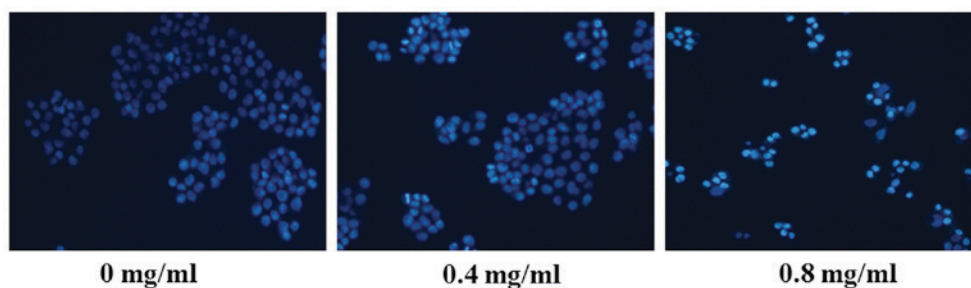


Figure 4. AC induces apoptotic nuclear morphological alterations in HCT-8 cells. Cells were treated with 0, 0.4 or 0.8 mg/ml AC for 24 h. Hoechst 33258 staining revealed AC-induced condensation of the nuclei. Original magnification, x200. AC, *Antrodia camphorata* ethanol extract.

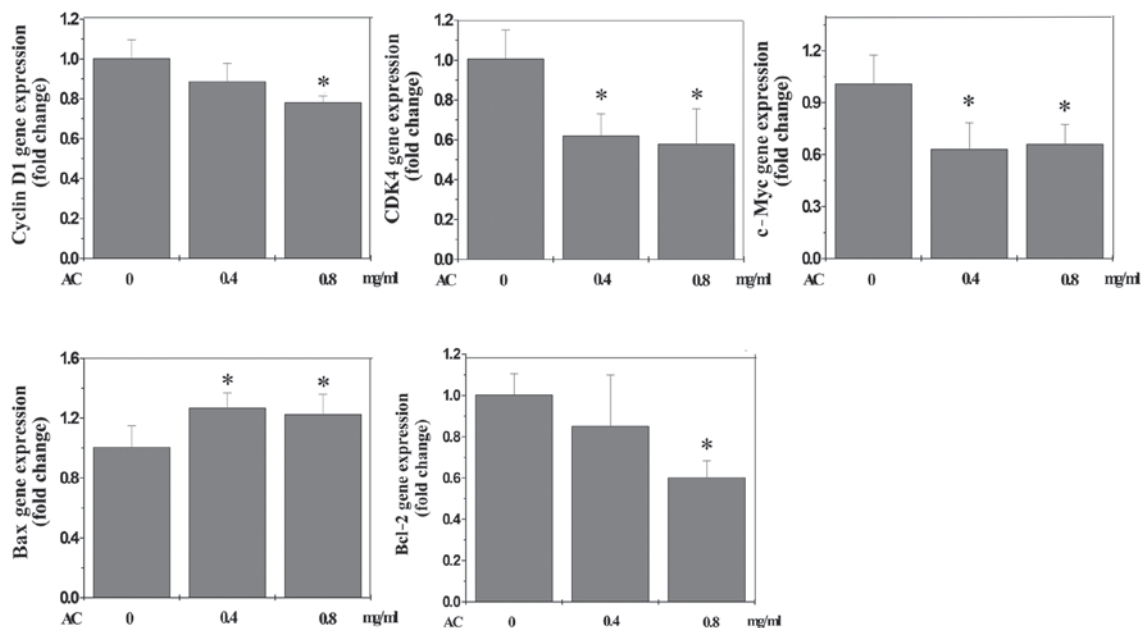


Figure 5. mRNA expression of cell cycle- and apoptosis-associated genes following AC treatment in HCT-8 cells. Cells were treated with AC for 24 h, and total RNA was extracted. mRNA expression levels of cyclin D1, CDK4, c-Myc, Bax and Bcl-2 was evaluated by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean \pm standard deviation of at least three independent experiments. * $P < 0.05$ vs. 0 mg/ml AC. AC, *Antrodia camphorata* ethanol extract; CDK4, cyclin dependent kinase 4; c-Myc, MYC proto-oncogene bHLH transcription factor; Bax, B-cell lymphoma 2 X associated protein; Bcl-2, B-cell lymphoma 2.

percentage of cells in the G0/G1 phase ($P < 0.05$; Fig. 3). These results suggested that AC arrested the cell cycle at the G0/G1 phase.

AC induces apoptosis-associated morphological alterations in HCT-8 cells. To visualize apoptotic body formation and nuclear morphological alterations characteristic of apoptosis, Hoechst 33258 staining was performed after 24 h treatment with 0.4 and 0.8 mg/ml AC in HCT-8 cells. Hoechst 33258 reagent is a membrane-permeable blue fluorescent dye that stains the cell nucleus and allows visualization of nuclear morphology. Control untreated cells exhibited characteristics of healthy cells with intact oval shape and uniform nuclei that stained less brightly with Hoechst (Fig. 4). However, cells treated with AC exhibited more brightly stained nuclei as treatment concentration increased (Fig. 4).

Effect of AC treatment on cell cycle- and apoptosis-associated mRNA expression. Having demonstrated that AC affects colon

cancer cell proliferation and apoptosis, the effect of AC treatment on associated gene expression was examined. The results demonstrated that the mRNA expression levels of cyclin D1, CDK4, cMyc and Bcl-2 were markedly downregulated in the AC treatment groups compared with the control group ($P < 0.05$; Fig. 5). In contrast, the mRNA expression levels of Bax were increased following treatment with AC for 24 h compared with the control group ($P < 0.05$; Fig. 5). These findings suggested that AC may inhibit proliferation and apoptosis of HCT-8 cells by regulating expression of cell division and apoptosis-associated genes.

Effect of AC treatment on cell cycle- and apoptosis-associated protein expression. To characterize the differential expression of Bax, cMyc, Bcl-2, cMyc, cyclin D1 and CDK4 proteins, western blot analysis was performed. As demonstrated in Fig. 6, incubation of HCT-8 cells with AC reduced Bcl-2 protein expression levels, a potent cell-death inhibitor, whereas it increased Bax protein levels, compared with untreated cells.

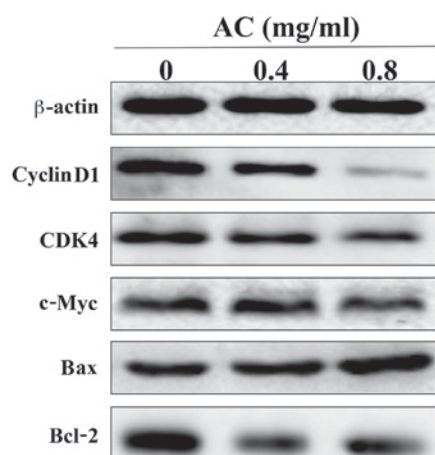


Figure 6. Expression of cell cycle- and apoptosis-associated proteins following AC treatment in HCT-8 cells. Representative western blot images of protein expression levels of cyclinD1, CDK4, cMyc, Bax and Bcl-2 following 24 h AC treatment. β -actin served as a loading control. The results are representative of three independent experiments. AC, *Antrodia camphorata* ethanol extract; CDK4, cyclin dependent kinase 4; c-Myc, MYC proto-oncogene bHLH transcription factor; Bax, B-cell lymphoma 2 X associated protein; Bcl-2, B-cell lymphoma 2.

These findings suggested that the AC-induced apoptosis may be associated with an AC-induced dysregulation of the Bcl-2/Bax ratio in HCT-8 cells. In addition, the results demonstrated that AC treatment downregulated the protein expression levels of cyclin D1, CDK4 and cMyc in HCT-8 cells following AC treatment, compared with untreated cells (Fig. 6).

Discussion

AC is considered a physiologically beneficial fungus used in China for 2,000 years to improve health and enhance longevity. The mushrooms of this species are reported to possess potent antitumor, immune modulating, cardiovascular and hypercholesterolemia, antiviral, and antiparasitic activities (20-22). Notably, AC has been reported to exhibit cytotoxic effects against various cancer types, and therefore may have potential value for translational research in CRC. To provide additional valuable information for the efficacy of AC in clinical application, the present study examined the effect of AC ethanol extract on the growth and viability of CRC cells.

Assessing the extent of proliferation and apoptosis in cells and tissues provides important clues into the efficacy of potential anticancer therapies, and many chemotherapeutic agents are reported to exert antitumor effects by inhibiting proliferation and inducing apoptosis in cancer cells (23-25). The present results demonstrated that AC treatment inhibited cell growth in HCT-8 CRC cells, potentially via blocking cell cycle progression and inducing apoptotic cell death.

Cyclin D1, a recognized oncogene, affects the G1/S phase transition of the cell cycle (26). Overexpression of cyclin D1 causes dysregulation of the cell cycle and induces abnormal cell proliferation (27). Cell cycle progression involves a sequential activation of CDKs, including CDK4, which are associated with G1/S phase cell cycle progression (28,29). The findings from the present study demonstrated that CDK4 and cyclinD1 expression was decreased in AC-treated HCT-8 cells

compared with untreated cells, which provides evidence of the mechanism by which AC reduces cell division. In addition, c-myc is a key transcription factor involved in controlling proliferation, differentiation and apoptosis. It heterodimerizes with the abundant helix-loop-helix molecule, MYC associated factor X, and is important for normal cell proliferation (30). C-myc has been reported to regulate cell proliferation via inhibiting the transcription of cell cycle checkpoint genes and CDK inhibitors, and to promote cell cycle progression via tactivation of cyclin D1, D2, E1, A2, CDK4, cyclin dependent kinase inhibitor 1A, cell division cycle 25A, E2 transcription factor (E2F) 1 and E2F2 (31). In the present study, AC significantly downregulated the expression of c-myc, suggesting that AC may inhibit HCT-8 cell proliferation by regulating the expression of CDK4, cyclinD1 and c-myc.

Apoptosis is an important cellular process during the cell cycle, which triggers characteristic morphological and biological alterations (32). Prior studies have demonstrated that Bcl-2 and Bax proteins are important in regulating apoptotic cell death (33-35). Of the Bcl-2 family members, the Bcl-2/Bax protein ratio has been recognized as a key indicator for regulation of apoptosis. As demonstrated in the present study, AC treatment resulted in a reduction of Bcl-2 and an increase in Bax protein expression levels. Therefore, AC may shift the Bcl-2/Bax ratio toward a pro-apoptotic effect in HCT-8 cells, which is a promising finding for potential translation of this compound toward human cancer treatment application.

In conclusion, the present findings demonstrated that AC inhibited growth and induced apoptosis in human CRC cells *in vitro*, via suppression of the cell cycle, suggesting that AC may be potentially useful as a therapeutic cytotoxic drug in CRC. Follow up studies are required to evaluate the efficacy of this drug *in vivo* in tumor-bearing animal models.

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