Periplocin from *Cortex periplocae* inhibits cell growth and down-regulates survivin and c-myc expression in colon cancer *in vitro* and *in vivo* via ß-catenin/TCF signaling

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Abstract. Cancer of the colon and rectum is the third most commonly diagnosed cancer and accounts for approximately 10% of all cancer-related deaths. Although surgical resection or radiotherapy are potentially curative for localized disease, advanced colon cancer is currently associated with poor prognosis. Therefore, the development of a new and effective chemotherapeutic agent is required to target critical pathways to induce responsiveness of colon cancer cells to death signals. Dysregulation of the ß-catenin/TCF pathway plays a central role in early activities of colorectal carcinogenesis. In this study, human colon cancer SW480 cells were used to investigate the effect of CPP (periplocin from *Cortex periplocae*) on the modulation of the ß-catenin/TCF signaling pathway. Our research results showed that CPP caused a dose- and time-dependent inhibition of cell growth as assessed by MTT assay and an induction in apoptosis as measured by flow cytometry and transmission electron microscopy. Furthermore, the CPP- treated cells were characterized by a decreased expression of ß-catenin protein in the total cell lysates and cytosolic and nuclear extracts. This expression alleviates the binding activity of T-cell factor (Tcf) complexes to its specific DNA-binding sites. Thus, the protein expression of the downstream elements survivin and c-myc was down-regulated. To determine the precise inhibitory mechanisms involved, further in-depth *in vivo* studies of CPP are warranted. In conclusion, our data suggest that CPP wields a multi-prong strategy to target the ß-catenin/Tcf signaling pathway, leading to the induction of apoptosis and inhibition of growth of colon cancer cells *in vitro* and *in vivo*. Therefore, CPP may become a potential agent against colon cancer.

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Introduction

The Wnt/ß-catenin pathway, one of the most crucial signaling pathways in cell proliferation and apoptosis, is highly conserved throughout cell evolution and regulates the expression of Wnt target genes through the ß-catenin/T-cell factor (Tcf) (1,2). Abnormal Wnt/ß-catenin signaling is associated with a number of human diseases, including cancer, osteoporosis, aging and degenerative disorders (2,3). ß-catenin plays a dual role in this pathway in that when it translocates into the nucleus, it forms a complex with transcriptional factor TCF. TCF forms a complex with Groucho in the absence of ß-catenin, and their interaction is thought to repress transcriptional activity. ß-catenin interferes with the interaction between TCF and Groucho and induces the expression of downstream target genes such as survivin, c-myc and cyclin D1 (4). Activated ß-catenin/Tcf signaling by accumulation of ß-catenin in the nucleus plays a role in human carcinogenesis. More recently, approximately half of the largest samples of sporadic colorectal cancers and colorectal cancer cell lines lacking adenomatous polyposis coli (APC) mutations were shown to possess a somatic mutation in the ß-catenin gene (5,6). However, no therapeutic agents currently exist that selectively modulate the Wnt/ß-catenin signaling pathway, although certain existing drugs (e.g., non-steroidal anti-inflammatory drugs, vitamins and imatinib mesylate) have been confirmed to inhibit this pathway (7). Recently, the screening of anti-tumor components from Chinese medical herbs attracted interest (8-10).

Cortex periplocae (CP) is the dry root of the traditional Chinese herb *Periploca Sepium* Bunge, referred to as *Xiangjiapi* in Chinese. It is a traditional type of medicine commonly used for a variety of clinical effects, including anti-inflammation, enhancing bones and muscles and stimulating the nervous system (11). Itokawa *et al* (12) first found that periplocoside A from CP markedly inhibited the growth of ascite cancer S180 cells. In a previous study, an ethanolic extract from CP was isolated, verified as periplocin (CPP; Fig. 1) and found to significantly inhibit the growth of esophageal cancer, leukemia and colon cancer cells (13-15). However, the molecular mechanism of this extract has yet to be elucidated. The present study confirmed the induction of apoptosis of CPP in colon cancer cells via the ß-catenin/Tcf

signaling pathway and demonstrated that the downstream elements survivin and c-myc are involved in the apoptotic process of CPP. Furthermore, an in-depth *in vivo* study confirmed the efficacy of CPP against colon cancer mediated by the ß-catenin/Tcf pathway.

Materials and methods

Chemical substances. Periplocin extracted from CP (>96% purity) (New Drug Research and Development Co., Ltd., North China Pharmaceutical Corp., Shijiazhuang, China) was dissolved in dimethylsulfoxide (DMSO) (Sigma Co., Germany) and diluted to final concentrations in each culture medium used as described below. Cells cultured in medium only were considered as the vehicle control group.

Cell lines and cell culture. Human colon carcinoma SW480 cells (Cellular Biology Institute of the Shanghai Academy of Sciences, Shanghai, China) were maintained in RPMI-1640 (Gibco) containing 10% fetal calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin, 100 μg/ml phytomycin (Invitrogen, USA) at 37˚C in an incubator with a humidified atmosphere of 5% CO_2. After ~80% confluence, the cultured cells were digested using 0.25% trypsin (Amresco, USA) and subcultured.

Cell viability assay. The effect of CPP on cancer cell viability was determined by MTT assay. The cells were plated at $1x10^4$ per well in 100 μl of complete culture medium and treated with 0.125, 0.25, 0.5, 1.0 and 2.0 μg/ml concentrations of CPP in 96-well microtiter plates (Costar, USA). Each concentration of CPP was repeated in 10 wells. After incubation for 24, 48 and 72 h at 37˚C in a humidified incubator, MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well, and cells were incubated for 4 h. The plate was then centrifuged at 1,500 x g for 5 min. After careful removal of the medium, 0.1 ml of buffered DMSO was added to each well, and the plates were shaken. The absorbance was recorded on a microplate reader (Titertek Multiskan, North Ryde, Austria) at a wavelength of 570 nm. The effect of CPP on growth inhibition was assessed as the percentage of cell viability when the vehicle-treated cells were concurrently 100% viable.

Cell-morphology transmission electron microscopy. The apoptotic morphology of SW480 cells was determined by transmission electronic microscopy (TEM), as previously described (16,17). Exponentially growing SW480 cells were seeded at $1.5x10^6$ cells per 25-cm^2 flask and exposed to a CPP dilution (0.5 μg/ml) for 24 h. Cells were fixed in 2.5% glutaraldehyde and washed with 0.075 M phosphate buffer (pH 7.4-7.6). The cells were then fixed in 0.25% aqueous osmium, dehydrated with increasing concentrations of ethanol (30, 50, 70, 90, 100, 100 and 100%) and embedded in quetol resin. Ultra-thin sections were prepared with a microtome and mounted on a copper grid. The samples were contrasted with 4% uranyl acetate and Reynolds' lead citrate. Samples were viewed with a multi-purpose Philips 301 TEM at the Electron Microscopy Unit of the Hebei Medical University (Shijiazhuang, China).

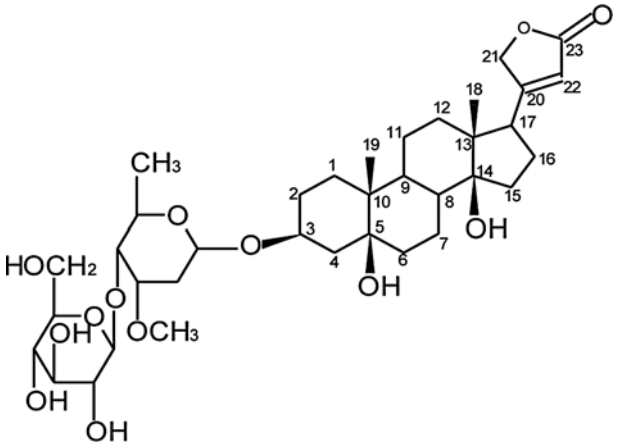

Figure 1. Structure of periplocin from Cortex periplocae (CPP) (identified by Professor Ren, New Drug Research and Development Co., Ltd. of North China Pharmaceutical Corporation, Shijiazhuang, China).

Analysis of apoptosis. Quantification of apoptotic cells was performed using propidium iodide (PI) (Sigma, St. Louis, MO, USA) staining according to the manufacturer's instructions. Briefly, cells were treated with CPP (0.5 μg/ml) for 0, 6, 12 and 24 h, then collected and resuspended in 500 μl PBS containing 50 mg/ml PI, 0.1% Triton X-100, 0.1 mmol/l $EDTA(Na)_2$ and 50 μg/ml RNase. After incubation in the dark for 30 min, analysis was performed with a FACS flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA) with Ex = 488 nm and Em = 530 nm, using Cell Quest software. Cells in sub-G0 peak were regarded as apoptotic.

Confocal microscopy. Exponentially growing SW480 cells were seeded at $1x10^5$/ml cells per well in a 6-well plate which was loaded on coverslips in advance. After 24 h of attachment, the medium was discarded and the cells were exposed to vehicle medium or to a dilution of CPP (0.5 μg/ml) and incubation was carried out for 24 h. The cells were washed twice with PBS, permeabilized with 0.5% Triton X-100 for 10 min and blocked by goat blood serum at a room temperature for 30 min. Rabbit anti-ß-catenin monoclonal antibody (sc-70509; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubation was carried out overnight at 4˚C. Rabbit secondary antibody (Zhongshan, Beijing, China) was then added and incubation was carried out for 60 min in the dark. The distribution of ß-catenin protein was observed using Leica confocal laser scanning microscope (Zeiss, Germany).

Isolation of cellular and nuclear extracts. Proteins were obtained as previously described (18). Briefly, cells were trypsinized, and the whole cell protein was obtained by lysing the cells on ice for 20 min in 700 μl lysis buffer [0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate]. The lysate was then sonicated for 20 sec, spun at 1,500 x g/min for 10 min, and the supernatant was collected. The nuclear pellets were prepared by resuspending cells in 800 μl lysis buffer [10 mM

Table I. Primers and conditions used in the reverse transcriptase-polymerase chain reaction.

Name	Sequence	Conditions	No. of cycles
ß-catenin	F: 5'-CGCATGGAGGAGATAGTTG-3' R: 5'-CGAAAGCCGTTTCTTGTAG-3'	90˚C for 60 sec, 55˚C for 60 sec, 72˚C for 60 sec, 55˚C for 60 sec and 72˚C for 60 sec	35
Survivin	F: 5'-AGCCCTTTCTCAAGGACCAC-3' R: 5'-GCACTTTCTTCGCAGTTTCC-3'	90˚C for 60 sec, 55˚C for 60 sec, 72˚C for 60 sec, 55˚C for 60 sec and 72˚C for 60 sec	38
c-myc	F: 5'-CCTACCCTCTCAACGACAGC-3' R: 5'-GTTGTGTGTTCGCCTCTTGA-3'	90˚C for 60 sec, 55˚C for 60 sec, 72˚C for 60 sec, 55˚C for 60 sec and 72˚C for 60 sec	35
ß-actin	F: 5'-TGAGACCTTCAACACCCCAG-3' R: 5'-GCCATCTCTTGCTCGAAGTC-3'	90˚C for 60 sec, 55˚C for 60 sec, 72˚C for 60 sec, 55˚C for 60 sec and 72˚C for 60 sec	35

HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$ and 0.1 mM EDTA], placing them on ice for 15 min and then vigorously mixing after the addition of 50 μl of 10% Nonidet P-40. Following a 30-sec centrifugation (12,000 x g, 4˚C), the pelleted nuclei were resuspended in 120 μl extraction buffer [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA and 10% glycerol] and incubated on ice for 30 min. Nuclear extracts were stored at -70˚C. For the preparation of cytoplasmic proteins, cell pellets were suspended in 800 μl lysis buffer (see whole cell protocol) without Tween-20 detergent, the pulse was sonicated (1 sec x 30) on ice and then spun at 10,000 x g for 1 h. The supernatant (cytoplasmic fraction) was collected as usual.

Western blot analysis. Western blot analysis was performed on SW480 cells for the presence of ß-catenin on the whole extract, cytosol or nuclear proteins, survivin and c-myc. Whole cell proteins, nuclear extracts and cytoplasmic proteins were isolated as described above. Equal loading of the protein groups on the blots was evaluated using BCA assay. Protein samples (20-100 μg) were loaded onto a 10% SDS-PAGE gel and run at 120 V for 2 h and transferred to a nitrocellulose filter (NC filter; Amersham, Arlington Heights, IL, USA) for 2 h at 135 mA. The membranes were blocked with 5% milk in TBST overnight, washed three times and incubated with the primary Ab (anti-ß-catenin, anti-survivin and anti-c-myc; Santa Cruz Biotechnology) for 2 h at room temperature. The membranes were washed three times with TBST and incubated for 1 h with fluorochrome-labeled secondary anti-rabbit IgG (IRDye 800-LI-COR; Odyssey). After four washings with TBST, the membrane was imaged with a LI-COR Odyssey infrared imager (application note http://www.licor.com/bio/Odyssey2/Odyssey13.jsp).

Electrophoretic mobility shift assay (EMSA). To detect ß-catenin/Tcf-DNA binding, EMSA was performed using the Lightshift™ chemiluminiscent kit (Pierce, Rockford, IL, USA) following the manufacturer's protocol. DNA was biotin-labeled using the biotin 3' end-labeling kit (Pierce). Briefly, in a 50-μl reaction buffer, 5 pmol of double-stranded Tcf oligonucleotide 5'-CCCTTTGATCTTACC-3' and 3'-GGGAAA CTAGAATGG-5', 10 μl of 5X terminal deoxynucleotidyl transferase (TdT) buffer, 5 μl of 5 μM biotin-N4-CTP, 10 units of diluted TdT and 25 μl of ultra-pure water were incubated at 37˚C for 30 min. Each binding reaction contained 1X binding buffer, 0.05% NP-40, 5 μg of nuclear extract and 20-50 fmol of biotin end-labeled target DNA. The content was incubated at room temperature for 20 min. A measurement of 5 ml of 5X loading buffer was added to this reaction mixture, subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. When the transfer was complete, DNA was cross-linked to the membrane at 120 mJ/cm^2 using a UV cross-linker equipped with 254-nm bulbs. The biotin end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film and developed using a Kodak film processor.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from the treated cells using TRI Reagent (Sigma, USA) according to the manufacturer's instructions. RT-PCR was conducted as previously described (20), with some modifications. In brief, cDNA was prepared using RNA samples (3-5 μg) to which 1 μg oligo(dT)18, 0.5 mM dNTP and 200 units of Revert Aid™ H-Minus M-MuLV RT enzyme were added (MBI Fermentas, USA). PCR analysis was performed using selective primers (Table I) (synthesized at Sangon, Shanghai, China), and 2 μl of RT product was incubated with 1 unit of Taq DNA polymerase in a 50-μl reaction mixture containing 1 mM dNTP and 1.5 mM $MgCl_2$ (Promega, USA). The amplified fragments were detected in 1.5% (w/v) agarose gel and analyzed using an IS1000 image analysis system (Alpha Innotech, San Leandro, CA, USA).

In vivo experiments using nude mice. Forty nude Balb/c mice, 4 to 6 weeks old, were obtained from the Animal Research Center of the Chinese Academy of Medical Science (Beijing, China), quarantined for 1 week and housed in an animal holding room under controlled conditions. After each mouse was intraperitoneally implanted with 0.2 ml of $5x10^5$ SW480 cells on Day 0, the mice were randomly divided into two groups (n=20 per group). Mice intended for CPP treatment were intraperitoneally injected with CPP at a dose rate of 30 mg/kg/day for 12 days, whereas the 20 mice intended for vehicle treatment were administered an equal volume of normal saline. During the course of the study, tumor volumes of all mice were measured according to the formula (1/2 x length x $width^2$) every 3 days, until all mice were sacrificed. The animals were sacrificed 12 days after

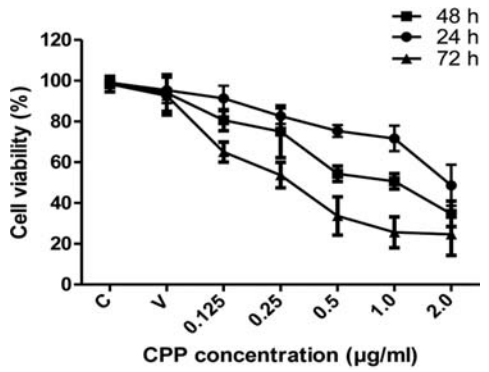

Figure 2. Effects of CPP on the viability of SW480 cells. Cells were treated with specific concentrations of CPP for 24, 48 and 72 h, and cell viability was determined by MTT assay. The values are represented as the percentage of viable cells when control cells were regarded as 100% viable. Data represent the mean percentage of viable cells ± SD of three independent experiments.

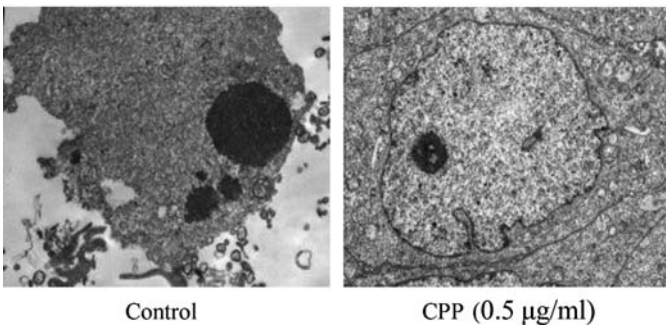

Figure 3. Apoptotic morphology in CPP-treated SW480 cells. SW480 cells were stimulated without or with 0.5 μg/ml CPP for 24 h, and the intracellular vacuole formation and cytoplasmic shrinking were observed with TEM.

tumor cell injection. The peritoneally disseminated tumor masses were collected and weighed together. Tumor tissues from 10 mice of each group were fixed in 10% buffered formalin and processed for histopathological evaluation by routine procedures (21). The expression of ß-catenin, survivin and c-myc in the tumor tissues of the other 10 mice in each group was measured by an immunohistochemistry assay as previously described (22). Specifically, tumor tissue sections were subjected to routine methods. Antigen retrieval was achieved by microwaving in 0.01 mol/l citrate buffer. The endogenous peroxidase activity was inhibited by incubating with 3% hydrogen peroxide in methanol, and non-specific binding was blocked. After washing three times with PBS, the specimens were left to react overnight at 4˚C with anti-ß-catenin, anti-survivin and anti-c-myc antibodies (Santa Cruz Biotechnology). Following incubation with peroxidase-conjugated secondary antibody (Zhongshan), the signal was developed with 3,3-diaminobenzidine tetrahydrochloride in Tris-HCl buffer (pH 7.6) containing 0.02% hydrogen peroxide. The sections were then counterstained with hematoxylin and mounted. The experimental protocol was approved by the Animal Care and Use Committee of the Hebei Medical University.

Statistical analysis. One-way analysis of variance (ANOVA) was performed to determine the significance between the two groups, and the t-test was used to compare two independent samples. Fisher's probability was used to analyze the difference in protein expression between the two groups. $P<0.05$ or 0.01 was considered to be statistically significant, and the results represent the mean ± SD. Results shown in the figures were obtained from at least three independent experiments with a similar pattern. All data analyses were performed using SPSS 13.0 software.

Results

CPP inhibits the growth of SW480 cells. As shown in Fig. 2, CPP significantly inhibited the growth of colon cancer SW480 cells in a dose- and time-dependent manner. Cells hardly grew in the presence of 1.0 μg/ml CPP. IC_{50} values for 24, 48 and 72 h were 0.59, 0.28 and 0.15 μg/ml, respectively. The results demonstrated the potency of CPP in inhibiting the growth of colon cancer cells *in vitro*.

Apoptotic morphology of SW480 cells induced by CPP. To understand the mechanism of CPP-induced cell death, morphological changes of SW480 cells induced by CPP were observed by TEM. Evidence of hypercondensed chromatin and cytoplasmic shrinking were observed in the 0.5 μg/ml CPP-treated SW480 cells (although not conclusive) revealing that CPP induced cell death through apoptosis in the SW480 cells (Fig. 3).

CPP treatment increases the rate of apoptosis in SW480 cells. To further detect apoptotic processes, the level of apoptosis in the CPP-treated SW480 cells was determined using PI staining. A time-dependent increase in the cell population in the sub-G1 phase of the cell cycle was noted (Fig. 4). In the vehicle medium, only 3.46% of the total cell population was apoptotic. In contrast, 13.6, 20.22 and 36.52% of the cell population were apoptotic when the cells were exposed to 0.50 μg/ml CPP for 6, 12 and 24 h, respectively.

CPP affects the localization and expression of ß-catenin. As the contribution of ß-catenin to tumorigenesis increases, it mediates the signal transduction cascades that play an important role in regulating apoptosis. Previous findings suggest that nuclear ß-catenin accumulation plays an important role in Wnt/ß-catenin pathway activation and transcription of downstream target genes. We investigated the effect of CPP on the location and expression level of ß-catenin. Following immunofluorescence staining and confocal microscopy analysis, we found that the majority of the ß-catenin protein was expressed in the nucleus and cytoplasm of the control cells (Fig. 5A-a), while after treatment with CPP (0.5 μg/ml) for 24 h, the expression level of ß-catenin decreased significantly in that its expression level was higher in the cytoplasm than that expressed in the cytoplasts (Fig. 5A-b). Based on this result, we hypothesized that the expression level of ß-catenin was modified. Therefore, we detected the expression level of the ß-catenin protein in total cell lysates, endochylema and cytoblasts, and its mRNA expression in the cells. Our results showed that after SW480 cells were treated with CPP (0.125, 0.5 and 2.0 μg/ml) for

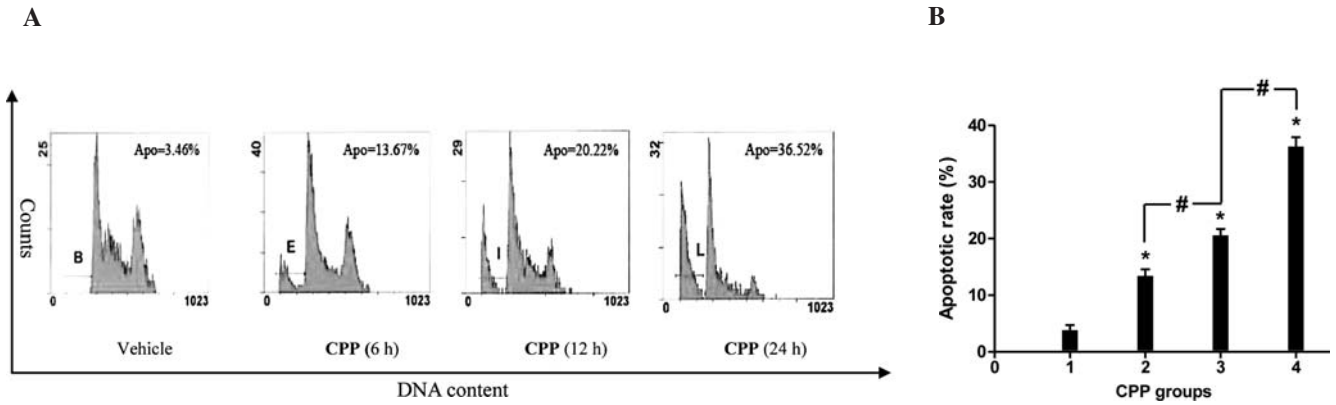

Figure 4. Effect of CPP on the apoptosis of SW480 cells treated with 0.5 μg/ml CPP for 6, 12 and 24 h. (A) The cells were stained with PI, and the total DNA content was measured to analyze the apoptotic rate (in the sub-G1 peak) by flow cytometry. (B) The apoptotic rate of the cells was analyzed by Mod Fit LT 3.0. Bars, mean of three independent plates. *P<0.01, compared to the control group (0 h); #P<0.01, compared between the two groups. Lane 1, control; 2-4, CPP treatment for 6, 12 and 24 h.

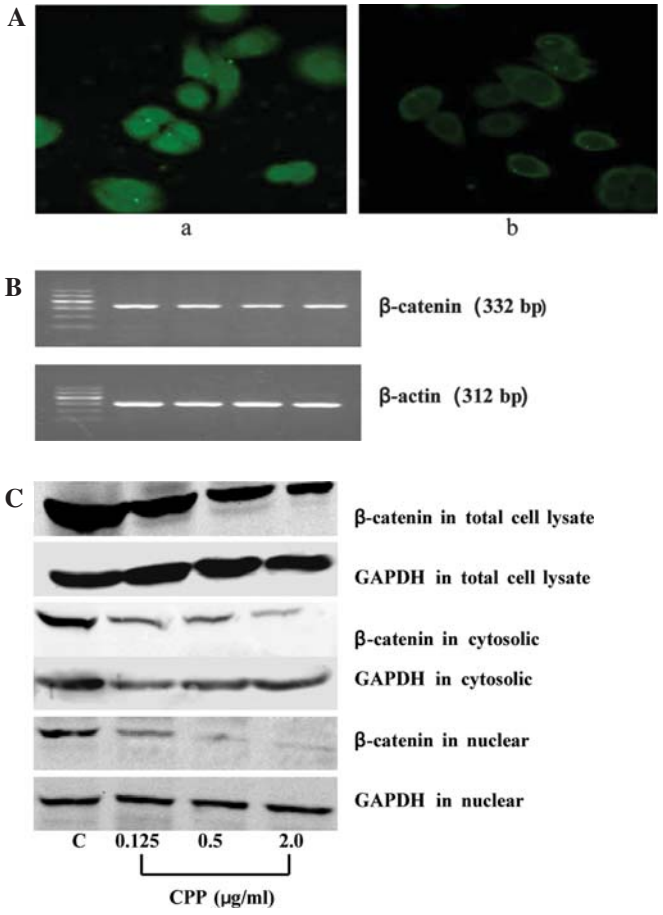

Figure 5. Subcellular distribution of ß-catenin in SW480 cells. (A) Control cells (a), and cells after exposure to CPP (0.5 μg/ml) for 24 h (b). Cells were subjected to confocal immunofluorescence assay (x400) using anti-ß-catenin antibody staining. Effects of CPP on (B) the transcriptional level and (C) protein expression level of ß-catenin in SW480 cells. Expression levels of ß-catenin mRNA and protein were measured by RT-PCR and Western blotting as described in Materials and methods. Representative results from three independent experiments are shown.

24 h, the protein expression of ß-catenin in the total protein, endochylema and cytoblasts decreased significantly in a dose-dependent manner (Fig. 5C), while the expression of ß-catenin

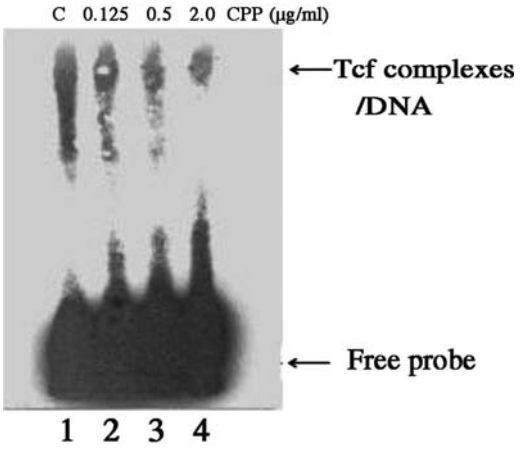

Figure 6. The binding activity of the ß-catenin/Tcf complex to DNA decreased with CPP. SW480 cells were treated with CPP for 24 h, and nuclear extracts were isolated. EMSA was performed with a mixture of 5 μg nuclear extracts from the cells treated without (lane 1) or with CPP at different concentrations (lanes 2-4) and biotin-labeled DNA strands as described in Materials and methods.

mRNA did not markedly change (Fig. 5B), indicating that CPP influenced the expression of ß-catenin at the protein but not at the transcriptional level.

CPP diminishes the binding activity of the TCF compound with its DNA sequence. ß-catenin binds with the transcription factor of lymphocyte enhancer-binding factor (LEF)/TCF in the nucleus to form a complex which stimulates the expression of downstream genes (23,24). Therefore, we considered the possibility that the decreased expression of ß-catenin in the nucleus impedes the binding of the ß-catenin/Tcf complex to its specific DNA strands. Nuclear extracts derived from CPP-treated cells were analyzed by EMSA assay for their ability to associate with biotin-labeled oligonucleotide-containing Tcf response elements. It was found that the nuclear protein extracted from the vehicle-treated cells was able to sufficiently bind with specific DNA, and the retarded band was prominent (Fig. 6, lane 1). On the other hand, the binding ability of the Tcf complex from the CPP-

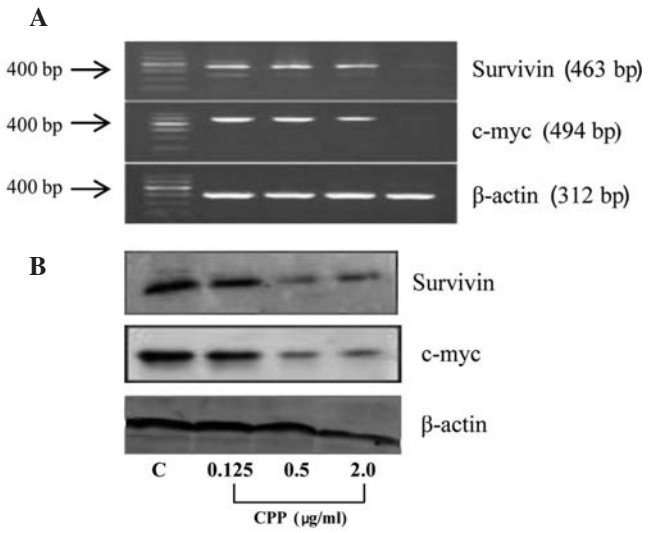

Figure 7. (A) ß-catenin/Tcf target gene survivin and c-myc mRNA and (B) protein expression in SW480 cells treated with CPP were detected by RT-PCR and Western blot analysis. The images are representative of three independent experiments. Lane 1, control; 2, 0.125 μg/ml CPP; 3, 0.5 μg/ml CPP and 4, 2.0 μg/ml CPP.

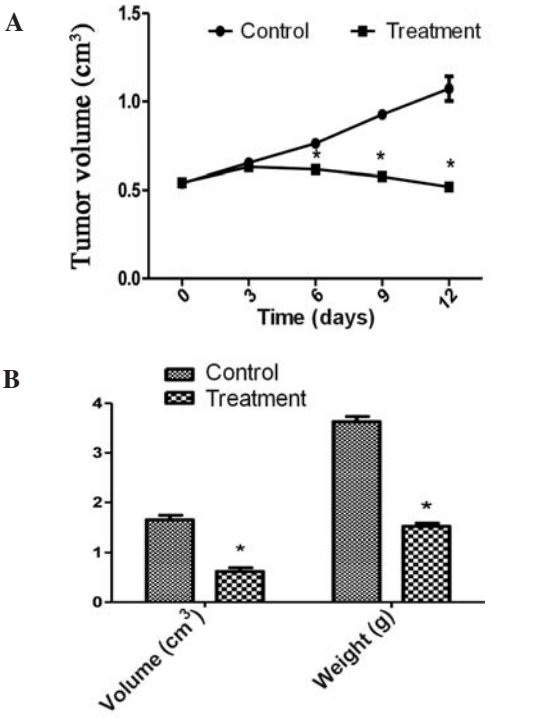

Figure 8. Effect of CPP (30 mg/kg/day) on tumor growth in nude mice. CPP treatment was started when tumors had grown to 40-45 mm^3 and was continued for 12 days. (A) From Day 3, the tumor volume of the treated mice was more than that of the control group, and the difference was significant. (B) On Day 12 of the experiment, tumor weight and volume in the CPP-treated animals were significantly less compared to those of the vehicle-treated mice. $^*P<0.01$, compared to the vehicle group.

treated cell nucleus with its DNA strand was weak, and the retarded band became superficial (Fig. 6, lanes 2-4) in a dose-dependent manner. This demonstrated that CPP interfered with the binding activity of Tcf complexes to its specific DNA.

mRNA and protein expression of survivin and c-myc. ß-catenin/Tcf-mediated signaling regulates a diverse set of genes responsible for cell proliferation, differentiation and homeostasis (25-29). To scrutinize which downstream factor accounts for the cell apoptosis induced by CPP, we examined the expression levels of the factors, including survivin and c-myc. As shown in Fig. 7, the expression levels of survivin and c-myc mRNA and proteins significantly decreased in CPP-treated cells compared to the vehicle-treated cells, which was consistent with the apoptotic process induced by CPP.

Effect of CPP on tumor growth in vivo. To further investigate whether CPP has a growth inhibitory effect on tumors *in vivo*, SW480 cells were implanted intraperitoneally into nude Balb/c mice. We examined the suppressing effects of a CPP intraperitoneal injection on peritoneally disseminated tumor masses of SW480 cells. The tumor volume in each mouse was recorded at an interval of 3 days to evaluate its retardation. The tumor volume data of the experimental groups are shown in Fig. 8A. After inoculation of colon cells, the tumor volume increased from Day 3 in the vehicle group until sacrifice, while the tumor volume did not significantly increase in the CPP mice. At the end of the experiment, tumor weight and volume in the CPP-treated animals were significantly less compared to those of the vehicle-treated mice (Fig. 8B). Average tumor volumes and weights were 1.763±0.300 cm^3 and 3.550±0.675 mg in the vehicle group and 0.515±0.184 cm^3 and 1.367±0.398 mg in the CPP-treated mice, a decrease of 71.01 and 61.49%, respectively. These results suggest that CPP has an *in vivo* anti-tumor effect.

Effect of CPP on histocyte morphosis of transplanted tumors and the expression level of ß-catenin, survivin and c-myc in tumor tissue. Tumor tissue was widespread in the vehicle-treated mice, but few tumor tissues reached the glandular tube in the CPP-treated mice. In contrast, tumor cell density in the carcinoma organism of the CPP group was lower, and the stroma was more than that of the vehicle group. Furthermore, compared to the vehicle group, there were more inflammatory cells and necrotic tissue in the CPP-treated mice (Fig. 9A, hematoxylin and eosin staining). The *in vitro* experiment showed that CPP affected the expression of ß-catenin protein and its downstream factors survivin and c-myc in the tumor tissue by immunohistochemical staining assay. Results showed that the expression of ß-catenin, survivin and c-myc in the tumor tissue of CPP-treated mice decreased compared to that expressed in the tumor tissue of the vehicle-treated mice (Fig. 9B), which was consistent with our data *in vitro*.

Discussion

This study confirmed the ethanolic extract periplocin (Fig. 1) to be one of the anti-tumor components of *Cortex periplocae*, and we elucidated the mechanism involved in the inhibition of proliferation of colon cancer cells. A time-dependent investigation was conducted over 72 h, with intervals of 24, 48 and 72 h, using human colon carcinoma treated with 0.125-2.0 μg/ml CPP. A significant growth inhibition of the SW480 cells was shown over the entire period of the experiment compared to cells propagated in the growth

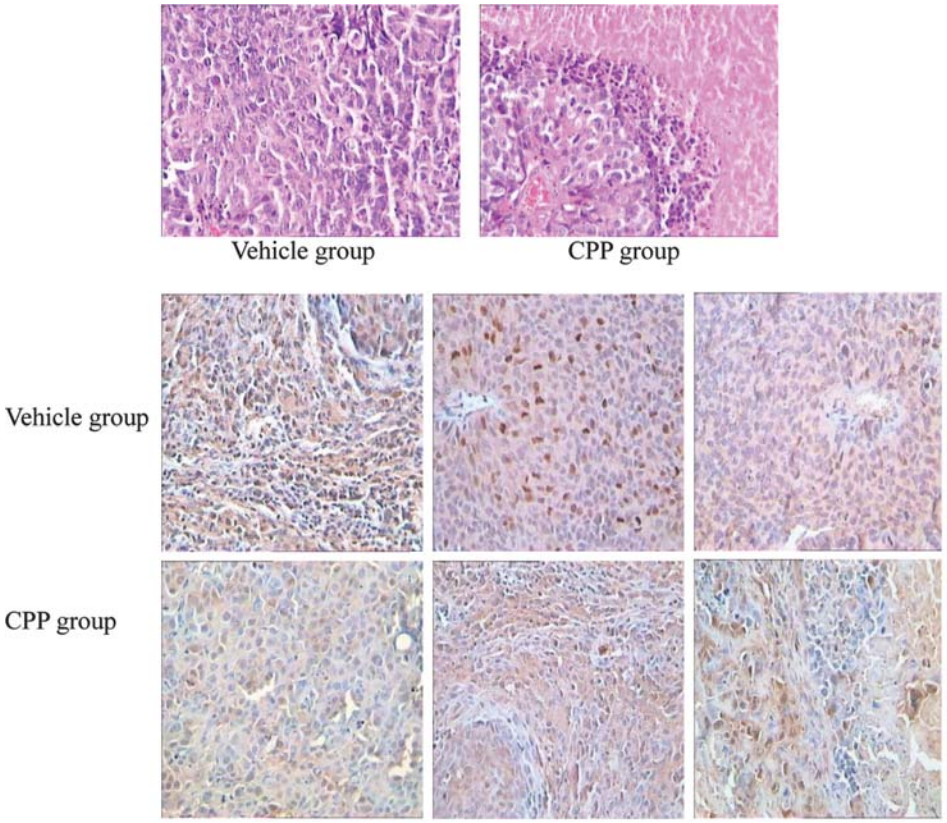

Figure 9. Histological appearance of SW480 human colon cancer cells injected into nude mice (A; streptavidin-peroxidase, x400) and expression of ß-catenin, survivin and c-myc protein in transplanted tumor organism (B; streptavidin-peroxidase, x400). Tumor tissue from CPP-treated and control mice was subjected to immunohistochemical staining with anti-ß-catenin, survivin and c-myc.

medium. Morphological and flow cytometry analysis for the CPP-treated cells demonstrated an increase in apoptotic cells, suggesting that apoptosis plays a key role in the growth inhibitory effects of CPP.

It was reported that the activation and accumulation of the oncogenic ß-catenin signaling pathway is not only an initiating event, but also plays a pivotal role in the promotion stage of colorectal carcinogenesis (30-34). In the event of no stimulation from the Wnt signal, ß-catenin in the cytoplasm binds with proteins such as APC, Axin, casein kinase (CK)1a, 1e and GSK-3ß to form a complex that regulates tumor development. GSK-3ß phosphorylates ß-catenin on serine 41, 37 and 33, and CK1 further phosphorylates ß-catenin on the 45 serine/threonine to promote degradation of the ß-catenin protein based on ubiquitin (35,36). Non-phosphorylated ß-catenin is displaced to the nucleolus and facilitates LEF/TCF to activate the expression of downstream target genes, including survivin, c-myc, cyclin D1 and FGF18 (26-29), which are responsible for cell proliferation and apoptosis. Moreover, it is reported that elevated ß-catenin levels promote early neoplastic change through oncogenic signaling within cells (23,24). ß-catenin signal transduction activity is abnormal in 90% of human colon carcinomas (34). The present study showed that CPP suppresses the Wnt/ß-catenin signaling pathway by inhibiting nuclear ß-catenin localization and by regulating the transcription of target genes.

Our previous studies and other studies (18,37) showed that quercetin, one of the most common flavonoids, inhibits human SW480 colon cancer growth in association with the regulation of the ß-catenin/Tcf signaling pathway. Aspirin, one of the non-steroidal anti-inflammatory drugs, was reported to inactivate ß-catenin/Tcf signaling and inhibit the growth of colorectal cancer cells (38,39). The present study demonstrated that CPP induces the inhibition of proliferation and apoptosis of SW480 cells by modulating the ß-catenin/TCF signaling pathway. First, our results showed that CPP significantly reduces ß-catenin expression at the protein, but not at the transcriptional level, suggesting that CPP influences the ß-catenin/TCF signaling pathway via ß-catenin, but has no effect on the upstream factors of the pathway. Moreover, the ß-catenin expression decreased in total protein, endochylema and the cytoblast protein of the CPP-treated cells. In addition, following treatment with CPP, the decreased expression of ß-catenin in the cytoplasm resulted in a reduction in the ß-catenin translocation to the nucleus, leading to the reduction of binding activity between the ß-catenin/TCF complex and its specific DNA strands, thus its downstream target genes were not effectively transcripted. Down-regulation of the survivin and c-myc mRNA and protein expression, which was the final mechanism contributing to the anti-proliferation effects of CPP, was also noted. It has been reported that suvivin and c-myc are associated with colon cancer cell apoptosis (28,32). We also demonstrated that c-myc is involved in the apoptosis induction mechanism of CPP. Survivin, a member of the inhibitor of apoptosis family proteins, is thought to be a bifunctional regulator of cell death and proliferation expressed during embryonic development, while it is undetectable in healthy adult tissues and re-expressed in a number of types of

cancer, including colorectal (40-43). Studies of colon cancer cells suggest that the regulation of survivin expression is at least partially ß-catenin/TCF-dependent (43,44). Our results suggest that the down-regulation of the survivin expression plays a role in the apoptosis of SW480 cells induced by CPP. To further investigate the effect of CPP on tumor growth *in vivo*, athymic nude mice models engrafted with SW480 cells were established. Data demonstrated that CPP inhibited the growth of the tumors *in vivo*. The volume and weight of transplanted tumors in the treatment groups were less than those of the vehicle group, and the percentage of inhibition was 71.01 and 61.49%, respectively. Furthermore, the down-regulation of the ß-catenin/Tcf signaling pathway target genes, survivin and c-myc, contributed to the anti-proliferation of the tumors, consistent with the results of the *in vitro* experiment.

Based on the results of this study, periplocin from *Cortex periplocae* (CPP) highlights a multi-prong beneficial strategy for targeting the ß-catenin/Tcf signaling pathway leading to apoptosis and the inhibition of growth of colon cancer cells. This may be explained by the modulation of ß-catenin/Tcf mediated by down-regulation of its downstream elements, survivin and c-myc. Since colon cancer is resistant to conventional chemotherapeutic regimens, CPP is a potential agent that can overcome this resistance, which our in-depth *in vivo* study confirms. In conclusion, our results provided a firm basis for further research on this novel anti-tumor medicinal plant.

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