

Apoptosis in Human Hepatoma Cell Line SMMC-7721 Induced by Water-soluble Macromolecular Components of *Artemisia capillaris* Thunberg

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The aim of this study was to investigate the effect of water-soluble macromolecular components of *Artemisia capillaris* Thunberg (ACT) on human hepatoma cell line SMMC-7721 (SMMC-7721). The morphological changes of SMMC-7721 were observed under a light microscope and an electron microscope. Inhibition of proliferation was measured with a colorimetric MTT assay. It was discovered that ACT extract-treated cells exhibit morphological changes typical of apoptosis, including condensed chromatin and a reduction in volume. ACT extract at 25–200 $\mu\text{g/ml}$ dose-dependently inhibited the proliferation of SMMC-7721. The 50% effective dose, evaluated on day 3 of exposure to the extract, was $64.52 \pm 3.53 \mu\text{g/ml}$. Upon gel electrophoresis, the fragmented DNA showed a characteristic ladder pattern. Cell cycle analyses revealed that ACT induced cell cycle arrest at the G₀/G₁ phase.

Key words: *Artemisia capillaris* Thunberg — Apoptosis — Human hepatoma cell line SMMC-7721

Artemisia capillaris Thunberg (ACT) is an important ingredient of Chinese traditional medicines which are used as remedies for jaundice, hepatitis, and cholecystitis. Extensive phytochemical, pharmacological and clinical work on the plant has been reported.^{1,2)} The cancer-preventive and antitumor effects of the plant have also been reported by several groups.^{3,4)} However, to our knowledge, the pharmacological activities of macromolecular components of ACT have hardly been studied, though the Kitasato Institute⁵⁾ reported that peptides (molecular weight of 10^5 – 3×10^6) isolated from the plant were inducers of interferons in animals. In the present research, water-soluble components with a molecular weight over 3000 were extracted from the plant and the effects of the extract on human hepatoma cell line SMMC-7721 (SMMC-7721) were investigated.

MATERIALS AND METHODS

Reagents Tetrazolium salt (MTT) was provided by Fluka Co. Proteinase K and RNase A were supplied by Shanghai Co. of Huamei Biotechnology (Shanghai, China). All other chemicals were of analytical grade, and were used as supplied.

Preparation of culture medium The fresh plant material of ACT was collected from Jurong, Jiangsu province in China in April 1997 and identified with the help of a botanist at Nanjing University. The aerial parts of the plant

was cut into pieces about 3 cm long, homogenized for 5 min and soaked in distilled water at 4°C overnight. After filtration and centrifugation, the supernatant was saturated to 40% with ammonium sulfate (244 g/liter) and then centrifuged at 1500 rpm for 30 min. The precipitate was redissolved in distilled water, placed on a Sephadex-G50 column and eluted from the column with distilled water. The eluate with absorbance at 280 nm was collected, filtered through cellulose tubes (cut-off : 3000, Beijing Filter Instrument Inc., Beijing, China) and dried by lyophilizing. SDS-PAGE analysis showed that the extract mainly contained 2 kinds of polypeptides with molecular weights of 6000 and 9000.

The extract was dissolved in RPMI 1640 medium (GIBCO, Gaithersburg, MD). After centrifugation the supernatant was passed through 0.45 and 0.22 μm filters and diluted with RPMI 1640 medium to different concentrations (pH 7.4). Osmotic pressures of cultures either with or without ACT were within physiological ranges. In addition, to compare the effects on the cells, culture medium with a control drug, *Caihu* injection (Xingyi Pharmaceutical Co., Shanghai, China), was also prepared, using the same procedures. *Caihu* injection is a popular herbal medicine for the treatment of various chronic liver diseases in China and mainly contains water-soluble components of the plant *Bupleuri radix*.

Cell lines SMMC-7721 was supplied by Shanghai Institute of Cell Biology (Shanghai, China) and was maintained in RPMI 1640 supplemented with 10% fetal calf serum (Shanghai Sijiqing Biotechnique Co., Shanghai, China), penicillin 100 U/liter and streptomycin 100 U/liter.

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Cells were kept in 5% CO₂ + 95% air at 37°C and used at the exponential growth stage.

MTT assay Cells were plated in 96-well plates at the density of 4×10⁴ cells/ml (100 μl/well). After 24 h incubation, the culture medium was replaced with medium containing one of six different concentrations of ACT extract or medium alone as a control. The supernatant was replaced with 30 μl of stock MTT (5 mg/ml) after incubation with ACT extract or *Caihu* for 24, 48, 72 or 96 h. The cells were maintained at 37°C for 4 h, then the supernatants were gently removed and 100 μl/well of 0.04 mol HCl in isopropanol was added. The absorbance was measured on an EL340 ELISA reader at 570 nm. The data from 2–4 experiments were expressed $\bar{x} \pm s$.⁶⁾

Analysis of DNA DNA analysis was performed after 96 h culture with ACT extract.⁷⁾ Floating cells and attached cells were collected and washed together twice with cold phosphate-buffered saline (PBS). After centrifugation, the pellet was suspended in solution (6×10⁶ cells/500 μl, 5 mmol/liter Tris-HCl, 20 mmol/liter EDTA, 5% Triton X-100, pH 8.0), digested with RNase for 40 min at 37°C and incubated with proteinase K (200 μg/ml; Sigma, St. Louis, MO) at 55°C for 2 h. The supernatant was treated with phenol/chloroform/isoamyl alcohol(25/24/1). DNA was collected by ethanol precipitation, loaded onto a 1.8% agarose gel and visualized under UV light after staining with ethidium bromide.

Morphological assessment of apoptosis ACT extract (100 μg/ml)-treated cells were observed under a phase-contrast microscope (Olympus, Melville, NY) at 24–96 h

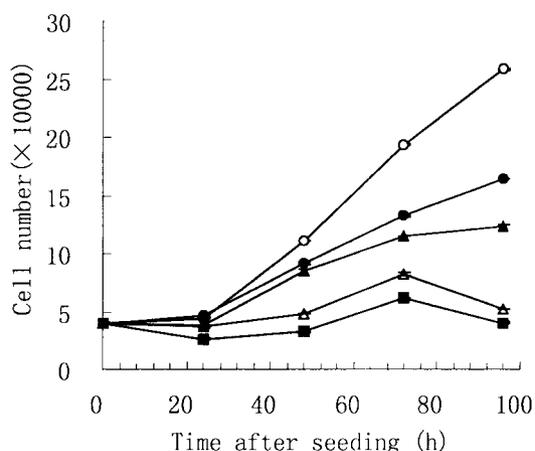


Fig. 1. Inhibitory effects of ACT on the growth of SMMC-7721. Cells were cultured without ACT extract (○) or with ACT extract 25 μg/ml (●), 50 μg/ml (▲), 100 μg/ml (△) and 200 μg/ml (■). There was a significant difference in the number of viable cells between ACT-treated cells and control cells. Values represent the average±SD of 2–4 independent experiments with duplicate wells (4×10⁴ cells/ml, 100 μl/well).

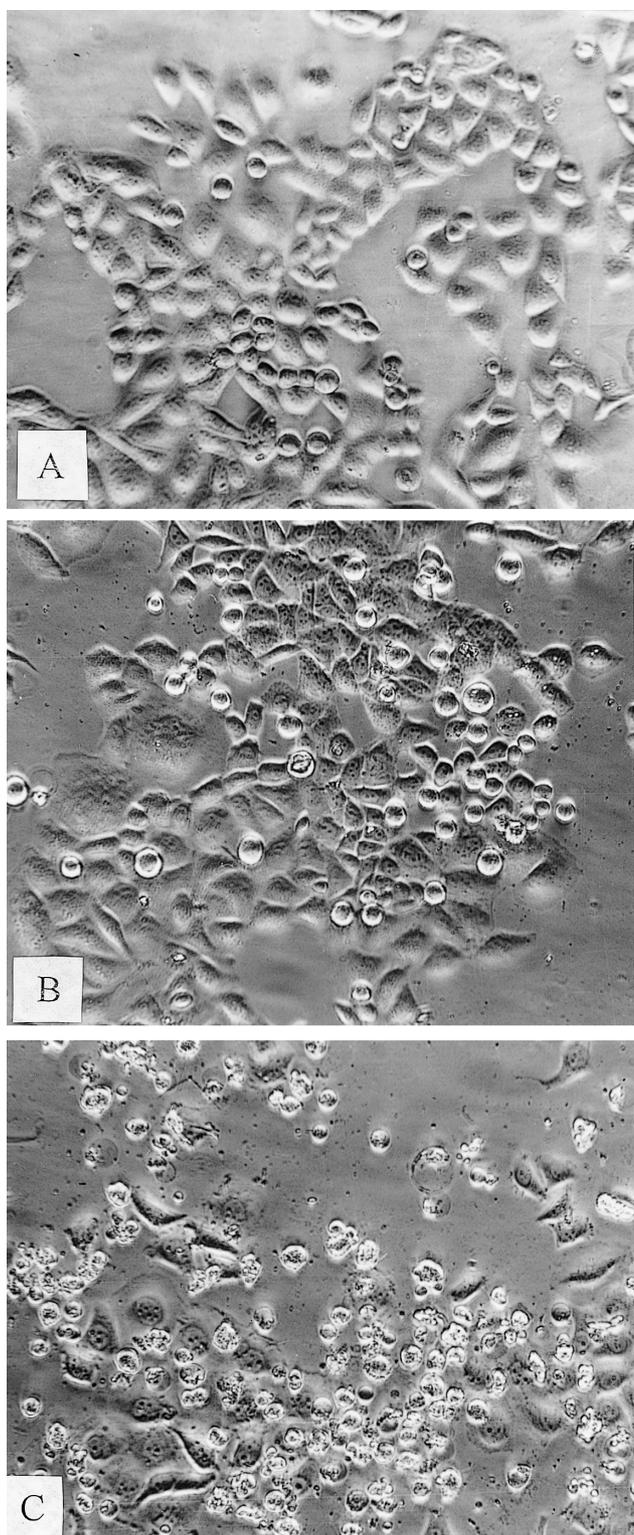


Fig. 2. Light microscopic observation (×400) of ACT-treated SMMC-7721 (100 μg/ml). A: cells without ACT. B and C: cells treated with ACT for 24 h and 96 h.

after the treatment, while RPMI 1640 medium was used as the control. The cells were also observed under a Hitachi 600 electron microscope. After 96 h incubation, cells were trypsinized, washed with PBS, pelleted by centrifugation, and fixed with glutaraldehyde. The embedded cells were thin-sectioned, stained with toluidine blue, and observed under an electron microscope.⁸⁾

Detection of apoptosis by flow cytometry Flow cytometry was used to identify apoptotic cells.⁹⁾ Briefly, cells were incubated with citrate buffer at 37°C for 1 h, suspended in PBS containing ribonuclease A (RNase A, free DNase, Sigma) at 37°C for 20 min, and stained with pro-

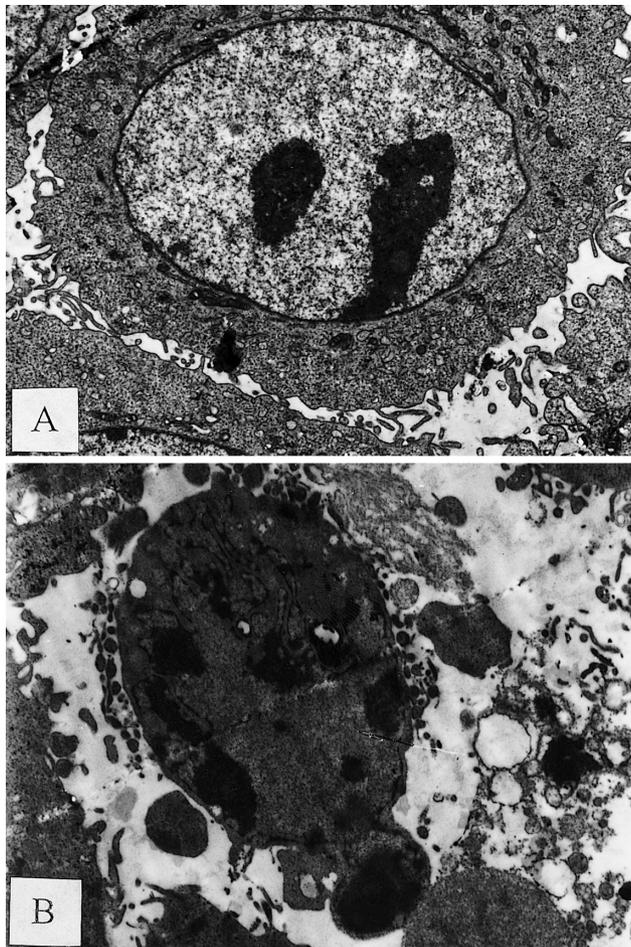


Fig. 3. Electron microscopic observation of SMMC-7721 treated with ACT extract. After 48 h incubation, cells were trypsinized, washed with PBS, pelleted by centrifugation, and fixed with glutaraldehyde. The embedded cells were thin-sectioned, stained with toluidine blue, and observed under an electron microscope. A: control, $\times 5000$. B: treated with ACT (100 $\mu\text{g}/\text{ml}$) for 48 h, $\times 5000$. Margination of compacted nuclear chromatin and deeply convoluted nuclear membrane are visible.

pidium iodide at 4°C for 1–2 h. All the samples were measured with a FACS 420 flow cytometer (Becton Dickinson Ltd., Franklin Lakes, NJ). The results were analyzed by CELLOUEST MODFIT LT computer systems (Becton Dickinson Ltd.).

RESULTS

Effects of ACT on cell proliferation ACT extract showed concentration-dependent inhibitory effects on the growth of SMMC-7721, as seen in Fig. 1. The 50% effective dose (day 3) was $64.52 \pm 3.53 \mu\text{g}/\text{ml}$. However, the effective dose 50 (ED_{50}) on day 3 of culture with *Caihu* was $10000 \pm 461.2 \mu\text{g}/\text{ml}$. The ED_{50} value for *Caihu* injection was much higher than that of ATC extract ($P < 0.001$).

Effects of ACT on morphological changes The morphology of the ACT extract-treated SMMC-7721 was examined under a phase-contrast microscopy. The ACT extract-treated cells floated in the incubation medium, while the control cells were attached to the wall of the containers. The number of floating cells increased with increase of the ACT extract concentration and incubation time. After 24 h of ACT extract treatment, cell shrinkage, membrane blebbing and reduction in cell volume were observed.

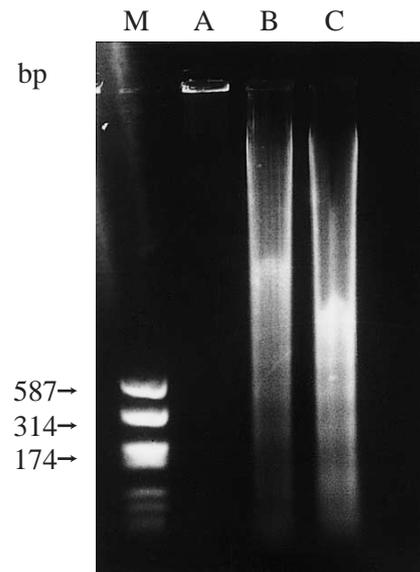


Fig. 4. Agarose gel electrophoresis of DNA extracted from cells without ACT (A) and cells treated with ACT extract 100 $\mu\text{g}/\text{ml}$ (B) or 200 $\mu\text{g}/\text{ml}$ (C) for 48 h. M: molecular weight markers. DNA was electrophoresed in an agarose gel, stained with ethidium bromide, and photographed under UV illumination. The DNA ladder characteristic of apoptosis was observed in lanes B and C.

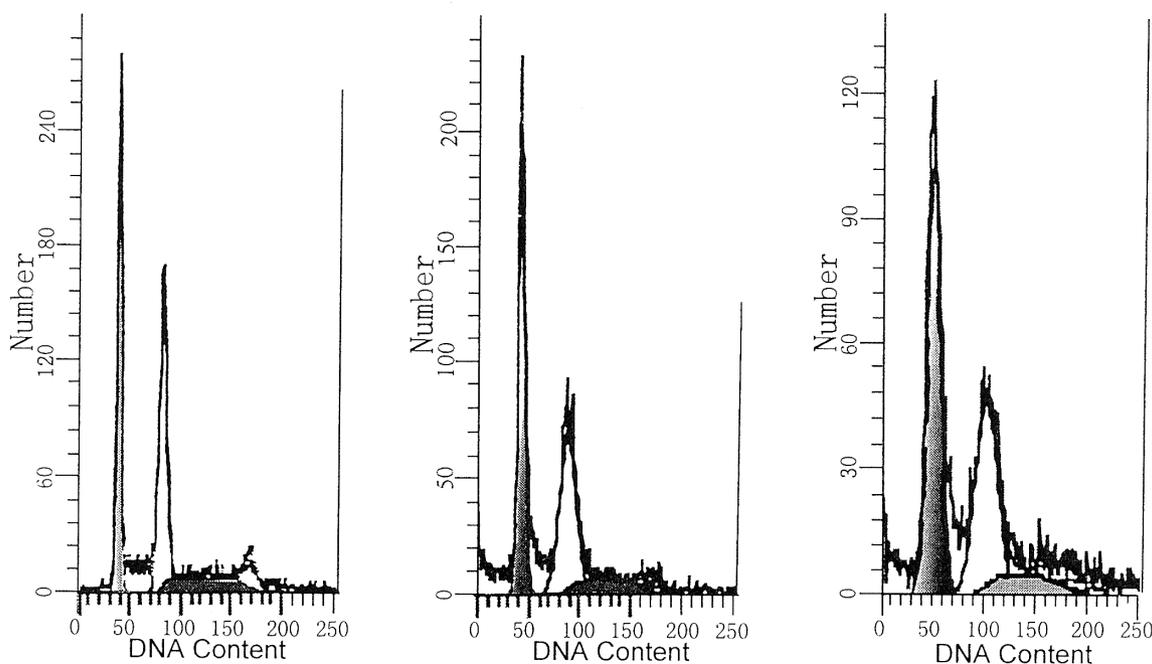


Fig. 5. Flow cytometric analyses of SMMC-7721 24 h after culture with 25 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ ACT extract, using propidium iodide staining. A: control (G_0/G_1 , 78.98%; G_2/M , 6.81%; S, 15.15%). B: treated with 25 $\mu\text{g/ml}$ ACT extract for 24 h (G_0/G_1 , 83.07%; G_2/M , 4.60%; S, 12.30%). C: treated with 50 $\mu\text{g/ml}$ ACT extract for 24 h (G_0/G_1 , 81.57%; G_2/M , 6.29%; S, 12.21%).

After 96 h, a majority of the cells had degraded and the remaining cells showed evident degenerative changes or signs of death. In contrast, the control cells were viable and showed normal morphology (Fig. 2).

The morphology of the ACT extract-treated SMMC-7721 was examined under an electron microscope. After 48 h of ACT extract treatment, apoptotic cells could be observed (Fig. 3), characterized by shrinkage of cells and chromatin condensation. Membrane-bound apoptotic bodies were visible. Other organelles, such as mitochondria and endoplasmic reticulum, were still structurally well-preserved. In contrast, the control cells were viable and showed normal morphology (Fig. 3).

DNA agarose gel electrophoresis The cells treated with ACT extract (100 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$) for 48 h exhibited a "ladder" of DNA bands representing integer multiples of the internucleosomal DNA length (about 200 base pairs, bp) in a time-dependent manner, whereas the control group did not (Fig. 4).

Cell cycle Fig. 5 shows a histogram of the integrated fluorescence signal area of SMMC-7721 treated with ACT extract (25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$) for 24 h. Compared with the control cells, the G_0/G_1 region was increased. After 24 h, 78.98% of control cells were in the G_0/G_1 phase, whereas cells in this phase accounted for 83.07% and 81.57% in cultures with 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ of the extract,

respectively. This indicated cell cycle arrest in the G_0/G_1 phase.

DISCUSSION

ACT has long been used in China and Japan as an anti-inflammatory, antipyretic, choleric, and diuretic agent to treat liver disorder and jaundice. Some investigators have reported antitumor effects of ACT extracts.^{3,4} Jiang *et al.* reported that cirsimaritin, capilarisin and three fractions of ACT showed cytotoxic activity against HeLa cells.³ Luo *et al.* found that water-soluble components of ACT had a remarkable inhibitory effect on mutagenic activity induced by aflatoxin B1 in strains TA98 and TA100.⁴ However, our research was focused on the water-soluble macromolecular components of ACT. In the preparation of the extract, cellulose tubing (molecular weight cut-off : 3000) and a Sephadex G-50 column were used in order to remove low-molecular-weight components. SDS-PAGE analysis showed the extract mainly contains 2 kinds of polypeptides with molecular weights of 6000 and 9000.

The study revealed that the water-soluble macromolecular components of ACT exerted a concentration-dependent, growth-inhibitory effect on SMMC-7721. The extract caused the death of SMMC-7721 cells by a process that involved morphological change and DNA fragmenta-

tion characteristic of apoptosis. During the apoptosis process, the integrity of cell membrane was still preserved, and membrane function remained unchanged. Cell cycle analysis revealed the extract mainly blocked the progression of SMMC-7721 through the S-phase and caused arrest at the G₀/G₁ phase. In comparison with *Caihu* injection the extract of ACT produced significantly stronger inhibitory effects on tumor cells. However, several studies reported that some *Caihu* medicines, such as sho-saiko-to, inhibit proliferation of cancer cell lines by inducing apoptosis.^{10, 11} The reasons might be (1) the concentration of active ingredients in the injection is not as high as that labeled by the company, and (2) the active components in the injection are different from those in sho-saiko-to. Nevertheless, this result supported the presence of substances which suppress tumor cell growth in ACT, and at the same time argues against the presence of killing effects attributable to other factors, e.g., osmotic pressure or pH.

Apoptosis, first described by Kerr *et al.*,¹² occurs via the activation of a cell-intrinsic suicide program, and is

morphologically distinct from necrosis. Since then, various inducers of apoptosis and mechanisms of apoptosis have been reported.^{13, 14} Apoptosis inducers for cancer cells may be useful for cancer chemotherapy. Results in this study demonstrated that the extract of ACT-treated SMMC-7721 exhibited typical apoptotic morphological changes including shrinkage of the cytoplasm, condensation of chromatin and a "ladder" of DNA bands. This anti-tumor effect of the plant is reported here for the first time. Yamamoto *et al.* reported that aqueous extracts of ACT had potent inhibitory effects on transforming growth factor β 1-induced apoptosis in rat hepatocytes.¹⁵ It is possible that the plant induces tumor cell apoptosis and inhibits normal liver cell apoptosis as well.

In conclusion, the water-soluble macromolecular components of ACT could trigger apoptosis of SMMC-7721 at an appropriate concentration.

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