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Anti-hepatocarcinoma effects of *Aconitum coreanum* polysaccharides

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ABSTRACT

Crude polysaccharides (CACP) were extracted from the stems of *Aconitum coreanum* by water extraction and ethanol precipitation. In the present investigation, the chemical properties and antitumor activities of CACP were valuated. The results showed the oral administration of CACP could specifically inhibit the growth of H22 tumor cells by *in vitro* and *in vivo* model, and exhibited a significant lower cytotoxicity to nontumorous cell lines. Moreover, CACP treatment greatly prolonged the survival period in H22 ascites tumor-bearing mice. After CACP treated, most of H22 cells were arrested in G0/G1 phage determined by flow cytometry. These results suggested CACP exhibited significant antitumor activity, and it possessed a great potential for developing a novel antitumor drug.

1. Introduction

Hepatocellular carcinoma is one of the most prevalent adult malignancies worldwide. In last decades, most patients diagnosed with hepatoma have low recovery rates, and conventional and modified therapies currently available are rarely beneficial (Sheu, 1997; Thomas & Zhu, 2005). Moreover, the limited responses of hepatoma, mainly hepatocellular carcinoma, to these agents are often due to its multidrug resistance (MDR) to them. Thus, developing new therapeutic agents for hepatocellular cancer becomes an urgent need to reduce the mortality caused by this disease (Deng et al., 2006). At present, the demands for more effective and safer therapeutic agents for cancer have greatly increased. Natural products from medical plants are valued as an important source to find innovative agents for treatment of cancer (Guribi-Fakim, 2006). Importantly, many researches identified that medical plant polysaccharides could significantly inhibit the growth of various tumors in experimental animals and increase the survival rate (Shin et al., 2004; Sun, 2011; Yang, Guo, Zhang, & Wu, 2007). In addition, most polysaccharides extracted from medical plants have relatively low toxicity, which usually could rival the defect of chemotherapeutic drugs. Thus, polysaccharides from traditional Chinese medical plants have been widely used in Asian countries as therapeutic agents for cancer due to their significant antitumor effects with lower toxicity.

The roots of *Aconitum coreanum* (Lèvl.) Rapaics is a well-known traditional Chinese medicines documented in ancient Chinese medicinal literature (Bisset, 1981). It has been used to treat various kinds of disorders over centuries, such as cardialgia, facial distortion, epilepsia, migraine headache, vertigo, tetanus, infantile convulsion and rheumatic arthralgia (Alessandra et al., 2003; Liou, Liu, Lai, & Cheng, 2005). Previous chemical studies of this herb have led to the isolation of more than 30 diterpenoid alkaloids, several sitosterols, and some organic acid (Yang, Wang, Tang, Liu, & Liu, 2008). The pharmacological functions of the roots of *A. coreanum* have been investigated widely. However, the stems of *A. coreanum* have been constantly considered as waste discarded. So far, there is no literature focusing on the antitumor activity of polysaccharide from the stems of *A. coreanum*. In order to fully develop the wild resources and extend the potential use of *A. coreanum* in antitumor biomedicine, the present study was carried out to investigate antitumor effects of polysaccharide from the stems of *A. coreanum* through *in vivo* and *in vitro* models, and clarified the antitumor mechanism of *A. coreanum* stem polysaccharide.

2. Materials and methods

2.1. Materials

The stems of *A. coreanum* was purchased from local drugstore, and identified according to the identification standard of

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Pharmacopeia of the People's Republic of China. Standard sugars, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cyclophosphamide, propidium iodide (PI) and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Medium RPMI-1640 was purchased from Gibco Invitrogen Co. Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. All other chemical reagents used were analytical grade.

2.2. Preparation of polysaccharides and chemical properties analysis

Briefly, the dried *A. coreanum* was grinded into powder. The powder was then defatted twice with ethanol. After centrifugation, the residue were dispersed in distilled water at 3% (w/v) and kept under continuous stirring for 2 h at a temperature of 90–100 °C for three times. After filtration through fine cloth, the combined extraction solution was centrifuged at 17,000 × *g* for 20 min at 20 °C. The supernatant was filtered through a G3 vacuum funnel and concentrated in a rotary evaporator to small volumes under reduced pressure. The polysaccharide was precipitated by addition of ethanol (1:4 (v/v)), washed with ethanol/acetone, dried at 40 °C. The isolated polysaccharide was referred to as CACP.

The total carbohydrate content was determined by the phenol–H_2SO_4 method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was quantified according to the Bradford's method (Bradford, 1976). Total uronic acid contents were measured by m-hydroxydiphenyl method using galacturonic acid or glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991).

2.3. In vitro antitumor activity

The *in vitro* antitumor activity of CACP on tumor cells was determined using the colorimetric MTT assay (Qiu, Huang, Huang, Pan, & Zhang, 2010). Briefly, all cell lines, including mouse hepatocellular carcinoma cell (H22), mouse melanoma cell (B16), mouse mammary cancer cell (MA-891), mouse embryonic fibroblast cell (NIH 3T3) and mouse macrophage cell (ANA-1), were suspended in complete RPMI 1640 medium, and then seeded at a density of 1×10^4 cells/well in 100 μl into 96-well plates and incubated overnight. Sterilized sample solutions were added to 96-well plate, while the negative control was treated with the complete RPMI1640 medium only. After cultivation for 48 h, MTT (5 mg/ml) 20 μl was added. After incubated for another 4 h, the supernatant was aspirated, and 100 μl dimethyl sulphoxide was added to each well. Absorbance was measured at 570 nm by a 96-well microplate reader (BIO-RAD 680, American), and the IC_{50} values were calculated according to the inhibition ratios.

2.4. In vivo antitumor activity

2.4.1. Animals

Female BALB/c mice (18–22 g) were purchased from Animal Experimental Center of Harbin Medical University. The mice were housed in plastic cages in a room and kept under standardized conditions at a temperature of 22–24 °C, and 20% humidity with a 12 h light/dark cycle, and they had free access to tap water and food throughout the study. They were allowed to acclimatize for 3 days before the experiments were started. Animal experiments were conducted under principles in good laboratory animal care, and approved by ethical committee for Laboratory Animals Care and Use of Harbin Medical University.

2.4.2. H22 hepatoma carcinoma cell lines

H22 cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and used for evaluating *in vivo* antitumor activity. H22 cells were maintained as ascites in mice by weekly passage. H22 cells, obtained from the peritoneal cavity of the tumor inoculated mice, were washed twice with phosphate buffered saline (PBS) and resuspended and adjusted to 1×10^6 cells/ml in PBS. The cells were detached and adjusted to 1×10^7 cells/ml in PBS for further assays.

2.4.3. Solid tumor-bearing mice model

The H22 cell suspension was inoculated to the right armpit of the mice subcutaneously for 0.2 ml per mouse on day 0. The tumor-bearing mice were divided into five groups (ten mice each group), including negative control group (normal saline), positive control group [cyclophosphamide (CTX) 50 mg/kg], and three groups for CACP administration with dosages of 25, 50, 100 mg/kg, respectively. After administered orally by gastric intubation once a day for continuous 10 days, the mice were sacrificed, and solid tumors were excised and weighed. The antitumor activity of CACP was expressed as following formula:

$$\text{Inhibition rate}\,(\%) = 1 - \left(\frac{\text{tumor weight of experimental group}}{\text{tumor weight of control group}}\right) \times 100$$

2.4.4. Ascites tumor-bearing mice model

The effect of administration of CACP on survival and progression of tumor on ascites H22-bearing mice was investigated as described (Chen et al., 2011). Mice were inoculated intraperitoneally (i.p.) for 0.2 ml H22 cell suspension on day 0. After 3 days of tumor transplantation, CACP was administrated intragastrically to H22-bearing mice at a dose of 100 mg/kg body weight once a day for consecutive 10 days, and recorded the survival days of mice. Median survival time (MST) and increase in life span (ILS) were calculated accordingly to the mortality data within the observation period. The ILS was calculated by the following equation:

$$\text{ILS value} = \frac{(\text{MST of the treated group} - \text{MST of the control group})}{\text{MST of the control group}} \times 100\%$$

The ILS value of greater than 25% is considered for significantly improving survival rate.

2.4.5. Cell cycle analysis by flow cytometry

Flow cytometry analysis was performed as described previously (Shi, Nie, Chen, Liu, & Tao, 2007). H22 cells were seeded into six-well plates at a density of 4×10^5 per well. After overnight incubation, cells were treated with CACP (50 μg/ml). After 48 h treatment, the cultured cells in the logarithmic phase were harvested and fixed with 70% ethanol phosphate buffer at −20 °C overnight. Then the cells were washed twice with PBS, and incubated with 1 mg/ml RNase A and 50 μg/ml proteinase inhibitor at room temperature in dark for 30 min. Then, treated cells were stained with propidium iodide (50 mg/ml) for 30 min, avoiding light at room temperature. The cell cycle progression was measured by flow cytometry (FACScan, Becton Dickinson, USA).

2.5. Statistical analysis

Results were expressed as mean ± SD and statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student's *t*-test. $p < 0.05$ implied significance.

3. Results and discussion

Polysaccharides from traditional Chinese medical plants have shown significant antitumor activities by attacking the cancer cell directly, enhancing the host's immune function, blocking cell cycles and inducing apoptosis of tumor cells. Thus, many kinds of polysaccharides from medical herbs have been applied for clinical treatment for cancer in Asia countries, especially in China (Sakarkar & Deshmukh, 2011). *A. coreanum*, as a frequently used Chinese folk medicine, has been used for cancer treatment for a long time. However, the specific active chemical compositions and the possible mechanism have been still unclear. Therefore, in the present investigation, a water-soluble polysaccharide CACP was extracted from the stems of *A. coreanum*, and then the antitumor activities of CACP were evaluated by *in vivo* and *in vitro* models.

3.1. Preparation of A. coreanum polysaccharides and its chemical properties

The crude polysaccharide from the stems of *A. coreanum* was extracted by hot water and ethanol precipitation with a yield of 5.7%. The contents of total sugar, protein and uronic acid of CACP are summarized in Table 1. CACP appeared as a white powder, and it had a positive response to Bradford assay. In addition, absorption at 280 nm was detected by UV spectrophotometer, which indicated the presence of protein. Results from phenol-sulfuric acid assay showed that CACP contained 61.2% carbohydrate, 12.5% uronic acid evaluated by m-hydroxydiphenyl method and 38.5% protein determined by Bradford's method.

3.2. Antiproliferative activity assay

In vitro antiproliferative activity against three tumor cell lines and two nontumorous cell lines, including H22, B16, MA-891, NIH 3T3 and ANA-1, was evaluated by MTT method. The results from Table 2 showed CACP exhibited a significant antiproliferative activity against H22 with the IC50 values of 25.3 μg/ml. While, CACP could not effectively inhibit the growth of B16 and MA-891 with a higher IC50 values over 200 μg/ml. In addition, CACP exhibited a significant lower cytotoxicity to NIH 3T3 and ANA-1 with highest IC50 values over 2300 μg/ml, which showed CACP did not have negative side-effects for inhibiting proliferation of normal cells.

The above results demonstrated that CACP could only inhibit proliferation of tumor cells, and it showed a discriminate antiproliferative activity against different type of tumor cell. Moreover, no antiproliferative effect of CACP on NIH 3T3 and ANA-1 was observed, implying that CACP had no direct cytotoxicity to noncancerous cells, and it was safer than other chemotherapeutical drugs.

Table 1
Physicochemical properties of CACP.

Sample	Total sugar (%)	Protein (%)	Uronic acid (%)
CACP	61.2	38.5	12.5

Table 2
Antiproliferative activity of CACP against different cell lines.

Sample	IC50 (μg/ml)				
	H22	B16	MA-891	NIH 3T3	ANA-1
CACP	25.3	214.2	274.2	2312.3	2943.2

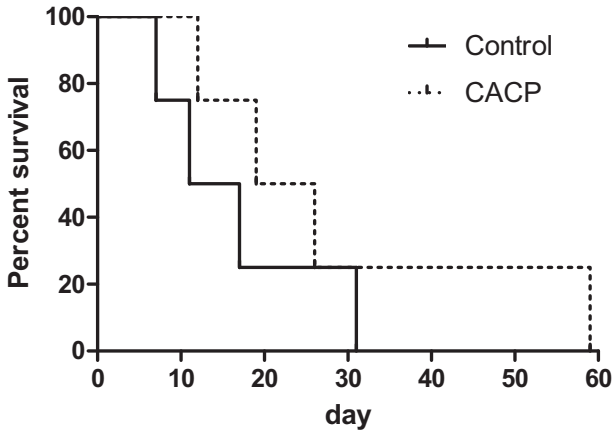

Fig. 1. Survival curve of ascites H22-bearing mice treated with CACP.

3.3. Antitumor activity of solid tumor-bearing mice

H22 tumor-bearing mice were used to evaluate the antitumor activity of CACP *in vivo*. Due to the fast growth of tumor, the transplanted tumor model mice in the control group gradually exhibited a series of weak appearance, such as the lost of appetite, the reduced activity and the body weight with dim hairs. At later stages, ulceration in some tumors was observed as a result of the tumor perforation out of the skin, and two mice died at day 9 and day 10. After treated orally with CACP, the growth of H22 tumors in the model mice was significantly suppressed compared with control group ($p < 0.01$). Inhibiting ratios of H22 tumor cells were 27.2%, 46.3% and 55.6% at concentrations of 25, 50 and 100 mg/kg body weight, respectively (Table 3), which indicated CACP possessed excellent antitumor activity. Furthermore, the body weights of CACP-treated group were increased significantly when compared with the negative control group during the 10 day-experimental period. The frequently used chemotherapy drug, cyclophosphamide (CTX), exhibited a high antitumor activity (60%). However, CTX considerably reduced the body weight of tumor-bearing mice, indicating the strong side effect to the body.

3.4. Survival assay of ascites tumor-bearing mice

To determine the survival effect of CACP, the ascites H22-bearing mice were treated with 100 mg/kg body weight for 10 days starting from 3 days after inoculation of tumor cells and left until death. When compared against the control group, the CACP-treated group showed a significant higher survival rate (Fig. 1), and the mean survival days of the treated group were prolonged compared with the mean survival days of the control group. All the animals ($n = 10$) in controls developed tumor and died within 7–31 days, whereas the CACP-treated group with restricted tumor growth died in with in 12–59 days. The ILS value was 68.2% far greater than 25%, which showed significantly improving survival rate by CACP (Table 4). These data demonstrated that CACP treatment greatly prolonged the survival period in H22 tumor-bearing mice.

3.5. Flow cytometry analysis of H22 tumor cells treated with CACP

Subsequently, the cell cycle stages of the H22 tumor cell treated with CACP was analyzed by flow cytometry. As shown in Fig. 2, CACP significantly caused H22 cells arrest at G0/G1 phase for marked increase of the percentage of G0/G1 stage cells, while that of G2/M stage cells decreased. Besides, there was no significant change in the percentage of S stage cells. The intervention of cell cycle is most

Table 3
In vivo antitumor activities of CACP on H22-bearing mice.

Sample	Dose (mg/kg)	Tumor weight (g)	Inhibition rate (%)	Body weight (g)	
				Before treatment	After treatment
Control		3.22 ± 0.54		21.42 ± 1.78	26.58 ± 3.21
CACP	25	2.35 ± 0.49	27.2	21.23 ± 1.53	28.78 ± 2.68
	50	1.73 ± 0.52*	46.3	22.03 ± 1.94	29.75 ± 3.63
	100	1.43 ± 0.56**	55.6	21.68 ± 1.88	30.11 ± 3.45
CTX	50	1.36 ± 0.31**	57.8	21.53 ± 1.95	25.57 ± 2.94

Values are expressed as mean ± SD ($n = 10$).
* $p < 0.05$.
** $p < 0.01$ *vs.* control.

Table 4
Effect of CACP on survival rates of H22-bearing mice.

Group	MST (days)	AST (days)	ILS (%)
Negative control	17.9 ± 7.97	18.4 ± 6.83	–
CACP (100 mg/kg)	30.1 ± 16.41**	30.6 ± 14.62**	68.2

Values are expressed as mean ± SD ($n = 10$).
** $p < 0.01$ *vs.* control.

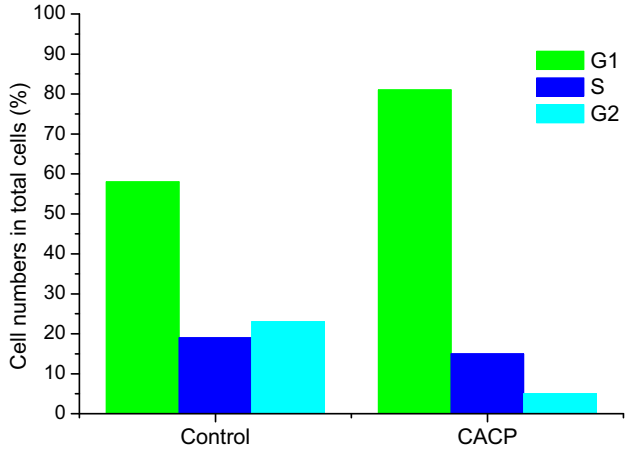

Fig. 2. Cell cycle stages of H22 tumor cell treated with CACP.

likely the main reason for antiproliferative and antitumor activity of CACP.

4. Conclusion

In the present study, we have confirmed that the polysaccharide CACP isolated from the stems of *A. coreanum* has significant antitumor effects by *in vivo* and *in vitro* models, and exhibited a significant lower cytotoxicity to nontumorous cell lines. Moreover, CACP greatly prolonged the life span of tumor-bearing mice. The mechanism of antitumor activity was achieved by intervention of cell cycle. Therefore, CACP could be explored as a novel potential antitumor agent for the functional food and pharmaceutical purpose. This study also provided evidences to support the therapeutic effects of this herb for treatment of cancer in China.

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