Anti-tumor activity of *Hedyotis diffusa* Willd. in mice

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Abstract: The whole plant with root of *Hedyotis diffusa* Willd. (AE) has been demonstrated to have anti-inflammatory, antioxidant, and anti-bacterial activities. In our study, we aim to examine the anti-tumor effect of alcoholic extract of AE in mice implanted with sarcoma S180 cells (SBT mice). We also compared the immune system function and the life span of SBT mice with that of control mice. We found that AE displayed a significant inhibitory effect on solid tumor growth, as well as increased the life span. At the dose of 10 mg/kg body weight, both tumor weight and volume were decreased significantly. We also measured several immune function markers, inlcuding spleen index (SI), thymus index (TI) and parameters of hematological. We observed no reduce in these markers, indicating that AE could inhibit tumor growth without affecting immune function in SBT mice.
Keywords: *Hedyotis diffusa* Willd.; Anti-tumor; Immunomodulatory; Iridoid; E-6-O-*p*-coumaroyl scandoside methyl ester

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1. Introduction

Currently, tumor has become one of the three major killers of human health, posing a serious threat to our health. Cancer is now the leading cause of death in China. The current treatment for cancer in modern medicine includes chemotherapy, radiotherapy, surgery, immunotherapy, etc. The major drawback of these modern methods in cancer treatment is broken down or suppression of the body's immune system[1], which may ultimately affect normal cells in cancer patients[2]. Therefore, the application of natural products has become a research hotspot in recent years for cancer treatment[3].

The traditional Chinese medicine has a long history of medicinal used in China and elsewhere, and it has been widely used for cancer[4]. Several natural medicines such as flavonoids[6,7] and terpenoids[8], have become important sources of new drugs[5]. Some studies demonstrated that such natural anti-cancer drugs not only have the effect of anti-tumor, but also play a role in regulation of immune system[9–14].

The whole plant with root of *Hedyotis diffusa* Willd. (AE) has been shown to have anti-inflammatory, antioxidant, and anti-bacterial activities, and has been used as a traditional Chinese medicine to enhance immune function and liver and gallbladder function in patients with various cancers[15]. Both the alcoholic extract of AE and iridoids in the extraction displayed significant anti-tumor activity[16,17]. We have previously investigated the anti-tumor effect of AE[18]. In the present study, we aim to examine the anti-tumor activity of AE in a mice model.

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2. Materials and methods

2.1. Experimental animals

STB male mice (Grade II, 5 weeks old) weighing 18–22 g were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd (Beijing, China). All mice were kept for a week under environmentally controlled conditions with free access to standard food and water.

2.2. Materials

Pure reference compound E-6-O-*p*-coumaroyl scandoside methyl ester was isolated from *Hedyotis diffusa* Willd. as described previously[18]. Cyclophosphamide (CY, No. 100234-200502) was provided by Zhongxi Reagent Co., Ltd., Beijing, China. All other chemicals and reagents used were of analytical grade.

2.3. Cell lines

Mouse sarcoma S180 cell lines were provided by Dingguo Biological Technology Co., Ltd (Beijing, China). All cells were maintained in DMED supplemented with 10% FBS. Cells were incubated at 37 °C in 5% CO_2 atmosphere. Mice were injected intraperitoneally with 1×10^6 viable cells.

2.4. Preparation of the extract

The whole plant with root of *Hedyotis diffusa* Willd. was collected from Anguo county, Hebei province, China in August 2008. The specimen was identified with the guidance by Prof. Chunsheng Liu from College of Pharmacy at Beijing University of Chinese Medicine. A voucher specimen (No. 20080906) has been deposited for advanced studies at the Laboratory of Analysis and

Testing Center, Department of Chemistry, Capital Normal University, China. The whole plant of AE was dried at 35–45 °C in dark in a ventilated hood for a week, and then was cut into small pieces. The dried material (5.0 kg) was extracted with 60% (v/v) ethanol 3 times at 50 °C. The alcoholic extract was concentrated at 50 °C using rotary vacuum evaporator. The concentrated extract was suspended in water, and sequentially partitioned with petroleum ether. The water soluble extract (100 g) was absorbed onto macroporous resin (250 g) and chromatographed on a macroporous resin column (2500 g) eluted with a gradient mixture of ethanol–water (1:1→1:4, v/v). The eluted fractions were evaluated by UPLC-ESI-MS, and the eluted fraction obtained with a mixture of ethanol–water (2:3, v/v) consists mainly of iridoids.

UPLC was carried out on a Waters ACQUITY UPLC instrument equipped with a Waters TUV detector. A Waters BEH C_{18} column (2.1 mm×150 mm, 1.7 μm) was used for separation. The column was eluted with acetonitile–MeOH–H_2O (with 0.05% formic acid) at a flow of 0.25 mL/min. Peaks were assigned by comparing their retention times with that of reference compound eluted in parallel with the same mobile phase and by spiking sample with reference compounds. The iridoids compound in the alcoholic extract was found to be mainly E-6-O-*p*-coumaroyl scandoside methyl ester (70.34%)[19]. The MS analysis was detected by Waters Alliance system (Micromass Q-Tof) under the electronic-spray ionization (ESI^-) mode. The MS detection parameters were listed as follows: the capillary voltage was 3.5 kV, source temperature was 80 °C, desolation temperature was 250 °C, desolation flow was 350 L/h, collision energy (MS) was 10 eV, and collision energy (MS/MS) was 30 eV. The Quasi-molecular ion peak (*m/z*) of the iridoids compound in the alcoholic extract was 549. The corresponding fragment peaks (*m/z*) were 387, 223, 163, and 119, respectively. Fragment peaks were assigned by comparing their *m/z* with that of reference compound eluted in parallel with the same mobile phase and by spiking sample with reference compounds. The LD_{50} value of the alcoholic extract was found to be >10 g/kg body weight of mice, indicating a very low toxicity of this extract in animals.

2.5. Treatment and drug administration

2.5.1. Effect of extract administered simultaneous with tumor inoculation

Mice were divided into five groups randomly with ten mice in every group. Ascites of the S180 bearing mice were drawn out under aseptic conditions. Then the ascites was diluted at 1:5 in aseptic saline. The S180 cancer cells were prepared as suspension at 1×10^6 viable cells, and then 0.2 mL suspension was inoculated subcutaneously into the armpit in each mouse. After 24 h of tumor inoculation, the mice were administered orally with AE at the doses of 5, 10 or 20 mg/kg for 10 d (intragastric administration, i.g.), with CY at a dose of 20 mg/kg for 10 d once daily as positive control. The control group was treated with vehicle (ethanol–H_2O, 0.5%, v/v) at 0.2 mL/10 g body weight. All mice were weighted once every day. On the 11th day, the mice were sacrificed by cervical dislocation, and blood was collected. The solid tumors were removed, weighted and measured for volume. The volume was calculated as the volume of solid tumor divided by the weight of mice, where the volume of solid tumor is the corresponding volume of water. Spleen and thymus were also collected and weighted for analysis.

Then white blood cell (WBC) count[20] and red blood cell (RBC), hemoglobin (HGB) content[21] were measured using by Leishman stained blood smears[22].

The inhibitory rate (%) against the growth of tumor was calculated by the formula:

$$\text{Inhibitory rate (\%)} = [(B–D)/B] \times 100\%$$

where B is average tumor weight of the control group, and D is average tumor weight of the treated group[23].

We also assess the effect on immune organs by spleen index (SI) and thymus index (TI) calculated as described previously[24]. The spleen index (%) = the weight of spleen divided by the weight of mice; the thymus index (%) = the weight of thymus divided by the weight of mice.

2.5.2. Tumor growth response

The effects of AE on tumor growth and host survival were estimated by evaluating tumor volume and percentage increase in life span (ILS) of the STB mice. Mice were divided into three groups with five mice in each group. After 24 h of tumor inoculation, the S180 bearing-tumor STB mice were treated with AE (10 mg/kg body weight, i.g. and CY 20 mg/kg body weight, i.g.). The control group was treated with vehicle (ethanol–H_2O, 0.5%, v/v, i.g.). The mortality rate was noted in mice treated with AE or positive control, and then compared with that of control group to calculate ILS. The formula ILS % = (1–A/B) × 100 where A is mean survival time of treat group and B is mean survival time of control group[25].

2.6. Statistical analysis

Results were expressed as mean±SD. Statistical evaluation was done by Student's *t*-test and *P* value less than 0.05 was considered as significant.

3. Results

AE exhibited significant anti-tumor activity against solid tumor in STB mice (Table 1). As a positive control drug, CY showed a high inhibitory rate (20 mg/kg body weight, i.g.) in STB mice. The tumor growth in STB mice was also significantly inhibited with the treatment of AE for 24 h at the dose of 10 mg/kg body weight (41.15%) compared with the control group (P<0.001). The weight and volume of the solid tumor at this condition were both significantly lower (P<0.001) than the control group.

We have also studied the role of AE in immune organs in STB mice, and the results are shown in Figures 1–2. The SI and TI in the STB mice induced by AE was not significantly reduced at the three doses (P>0.05), and there was a modest increase at the dose of 10 mg/kg body weight comparing with control group. However, in STB mice treated with CY at dose of 20 mg/kg body weight, the SI and TI were significantly reduced compared with that in controls (P<0.01 for SI, P<0.001 for TI).

The hematological responses in STB mice treated with AE are presented in Table 2. The index was measured in blood samples obtained from STB mice treated AE at three doses (5 (I), 10 (II), 20 (III) mg/kg) and controls, and no significant difference in the index was observed between three treated groups with controls. However, in blood from CY-treated mice, we observed a decreased total HGB and total HCT counts, as well as decreased percentage of lymphocyte index (LYM, %) compared with that of control group.

We further examined the anti-tumor effect of AE in STB mice. Based on the survival time, among the three doses in the study, the best dose is 10 mg/kg body weight as shown in Table 3. The life span of AE- and CY-treated STB mice increased 33.7% and 53.7% respectively, which is significantly higher than that in the control mice.

Table 1. Inhibitory effect of AE on tumor growth in STB mice

Group	Treatment (mg/kg)	Volume of tumor (cm^3)	Weight of tumor (g)	Inhibitory rate (%)
Control	–	2.07±0.68	2.50±0.53	–
CY	20	0.95±0.49***	1.08±0.49***	57.47
Treated	5	1.53±0.72	1.86±0.51*	26.87
	10	0.97±0.50***	1.50±0.47***	41.15
	20	1.34±0.67*	1.53±0.81**	35.90

CY: cyclophosphamide (positive drug); Values are mean±SD, n = 10; Significant differences with control group were designated as *P<0.05, **P<0.01, ***P<0.001.

Table 2. Effect of AE on hematological parameters in STB mice

	Control	CY[a]	Treated[b]		
			I	II	III
Total WBC ($\times10^9$/mL)	6.26±0.63	5.30±4.38	5.46±2.37	6.06±4.48	4.88±3.65
Total RBC ($\times10^{12}$/mL)	7.41±0.97	5.37±1.42*	5.53±1.31	5.53±1.43	5.69±0.44
Total HGB (mg/mL)	130.00±9.30	99.60±15.10**	89.20±17.83	94.40±21.48	100.20±10.13
Total HCT	0.43±0.04	0.33±0.01	0.31±0.07	0.31±0.07	0.32±0.02
LYM (%)	0.83±0.01	0.42±0.12***	0.39±0.14	0.44±0.09	0.44±0.15
RDW	0.12±0.01	0.13±0.01	0.11±0.01	0.13±0.01	0.13±0.01

LYM: lymphocyte index; HCT: hematocrit; HGB: hemoglobin; RDW: Red cell distribution width; [a] Values are mean±SD, n = 5. Significant differences with control group were designated as *P<0.05, **P<0.01, ***P<0.001; [b] Statistically not significant differences with control group.

Table 3. Effect of AE on the survival time in STB mice

Group	Treatments (mg/kg)	Survival time (d)	Increase in life span (%)
Control	–	10.6±0.8	–
CY	20	16.3±3.5***	53.7
Treated	10	14.0±3.4**	33.7

CY: cyclophosphamide (positive drug); Values are mean±SD, n = 6. Significant differences with control group were designated as *P<0.05, **P< 0.01, ***P<0.001.

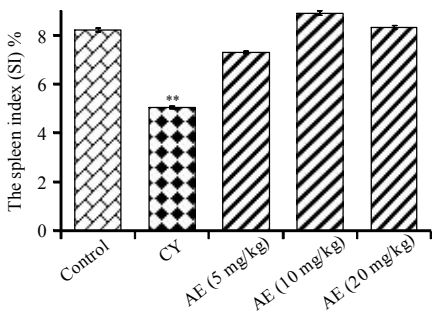

Figure 1. Effect of AE on the spleen index (SI) in STB mice. The spleen weight was expressed as SI. CY was used as a positive control. The values are mean±SD, n = 10. Significant differences with control group were designated as *P<0.05, **P<0.01, ***P<0.001.

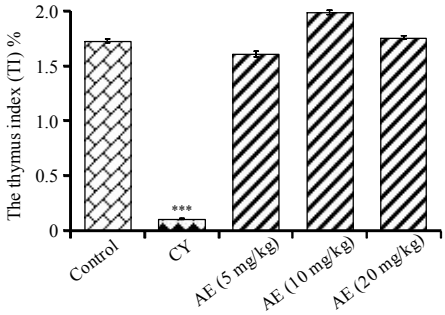

Figure 2. Effect of AE on the thymus index (TI) in the STB mice. The thymus weight was expressed as SI. CY was used as a positive control. The values are mean±SD, n = 10. Significant differences with control group were designated as *P<0.05, ***P<0.01, ***P<0.001.

4. Discussion

Traditional Chinese medicine has been used in a range of medical practices and health interventions in China for thousands of years. Nature products screening becomes an important component of exploring novel compounds. Today natural products have been widely used in a variety of diseases, including cancer. For anti-cancer treatment, even more than 60% of the approved and pre-new drug application candidates are natural products or synthetic molecules based upon the natural product molecular skeletons[26]. Compared to a variety of side effects of the current anti-tumor treatments, advantages of natural products include higher anti-tumor effect and lower toxicity. Furthermore, some extracts from natural products, such as *Piper longum* L.[27], *Cassia fistula*[28], *Phellinus rimosus*[29] and Soamsan[30] could improve the immune function, which may contribute to boost the immune system's response to cancer. Recently, the anti-tumor effect and potential immune responses of terpenoids, such as terpenoid indole alkaloids[31], betulinic acid and ceanothic acid[32] and non-glycosidic iridoids[33] have been repeatedly reported. The unsaturated bonds acting as a Michael acceptor in biological systems could be a possible mechanism underlying the anti-tumor activity. Therefore, terpenoids may preferably react with nucleophiles, especially thiol groups of proteins[34]. We have demonstrated that AE has a certain inhibitory effect on the proliferation of the small blood vessels in the chick chorioallantoic membrane experiments (unpublished data), indicating that the inhibition of tumor angiogenesis may serve as another mechanism for its anti-tumor effect.

Our main objective was to examine the anti-tumor activity of AE in vivo. The dosage of AE extract was determined based on series of pilot experiments. The LD_{50} value of AE was found to be >10 g/kg body weight of mice, suggesting its very low toxicity to the mice. In our study, AE exhibited anti-tumor activity in a dose-dependent manner in mice. At dose of 10 mg/kg body weight, AE could prevent 41.15% of solid tumor growth in the STB mice comparing with control group mice. The volume of the solid tumor was also decreased significantly compared with control mice.

Based on the SI, TI and parameters of hematological, AE could exhibit anti-tumor effect without affecting the immune system in STB mice, which is one of the most important advantages of nature products. Spleen and thymus, two important components of the immune system, can generate a large number of immune cells, such as T cells and B cells. Normal immune system could activate cytotoxic effector cells, such as T cells and NK cells[35], which may fight against tumor with cytotoxic effector cells. The parameters of hematological also reflected the situation of immune system. In STB mice treated with AE, SI, TI and parameters of hematological did not decrease significantly at dose of 5 or 10 mg/kg body weight, and there was a modest increase at the dose of 10 mg/kg body weight, suggesting that AE did not affect the function in immune system, or even slightly enhance the immune system at doses with significant anti-tumor activities. In addition, we also observed increase of life span in AE-treated STB mice.

In conclusion, AE showed significant anti-tumor activity without affecting immune function in a mice model. Further studies are needed to better understand the underlying mechanism and to confirm our conclusion.

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传统中药白花蛇舌草的抗肿瘤活性研究

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摘要: 研究了传统中药白花蛇舌草的乙醇提取有效部位对S180小鼠模型的肿瘤生长抑制作用、免疫系统及小鼠生存天数的影响。该醇提部位能有效抑制S180小鼠的肿瘤生长, 并延长荷瘤小鼠的生存天数。通过灌胃给药, 醇提有效部位在10 mg/kg的剂量下与阳性对照药物环磷酰胺 (20 mg/kg) 相比, 能够有效降低荷瘤小鼠体内的肿瘤大小与重量。对荷瘤小鼠的脾指数, 胸腺指数及血常规参数研究表明, 该醇提有效部位对荷瘤小鼠未见明显的毒副作用。总之, 研究结果表明白花蛇舌草的醇提部位具有有效的抗肿瘤活性。

关键词: 白花蛇舌草; 抗肿瘤; 免疫调节; 环烯醚萜苷; E-6-O-*p*-鸡屎藤苷甲酯