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A STUDY ON THE ANTICANCER ACTIVITY OF ETHANOL EXTRACT OF *ARISTOLOCHIA MOLLISSIMA HANCE*
ON OSTEOSARCOMA HOS CELLS

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Abstract

This paper mainly studied the extraction process of traditional Chinese medicine *Aristolochia mollissima Hance* (Aristolochiaceae) and the inhibitory effect of its extracts on osteosarcoma HOS cells. The extraction process included the ultrasonic extraction method, heat reflux method and decoction method to obtain three different extracts. MTT assay was used to test the effect of the extracts on proliferation of HOS cells, to compare the degree of inhibitory activity of three extracts, and to calculate cell survival rates. The results showed that among the three extracts obtained by the ultrasonic extraction method, heat reflux method and decoction method, the one obtained by ultrasonic extraction has the largest yield, but the extract obtained by heat reflux method has the strongest anticancer activity. Nevertheless, the three extracts all have a good inhibitory activity on the proliferation of osteosarcoma HOS cells.

Key words: *Aristolochia mollissima Hance*; HOS cells; MTT; Anticancer

Introduction

Aristolochia mollissima Hance is a traditional Chinese medicine with a long history of medicinal use. It is the dried whole plant of *Aristolochia mollissima Hance* (Aristolochiaceae). It is generally distributed in provinces such as Shanxi, Shaanxi and Shandong. It has anti-inflammatory, anti-tumour, analgesic and anti-rheumatic effects (Chen et al, 2001; Chen, 2003; Shen et al, 1999). Animal studies have shown that its extract has an inhibitory effect on S37 tumour cells in mice (Wang et al, 2010). The extract of this Chinese medicine extracted with a combination of methanol, ethyl acetate and n-butanol has a hepatoma BEL-7404 cell line inhibition rate of 32.26%, which indicates that the extract has a certain anticancer effect. Monomers extracted from *Aristolochia mollissima Hance* include allantoin, aristolactone, mollislactone, β -sitosterol, aristolochic acid A, 9-ethoxyaristolactam and 9-ethoxyaristo-lactone. In this paper, three different extracts are obtained by ultrasonic extraction method, heat reflux method and decoction method, and their anticancer activities are compared (Liu et al, 1993).

Materials and Methods

Identification of Chinese medicine

Aristolochia mollissima Hance used in this study was purchased from the Anguo Chang'an Chinese Medicinal Materials Co., Ltd. The medicinal herb was identified by Professor Ye Zilong of the China Medical University as the plant of *Aristolochia mollissima Hance* (Aristolochiaceae). The specimen (12-ss-08) was placed in the pharmacy centre.

Comparison of extraction processes

Heat reflux extraction method

4 g of *Aristolochia mollissima Hance* was weighed, added with 200 ml of extractant with a water-ethanol ratio of 1:1, and extracted by heat reflux at 100 °C for 1 h three times. The filtrates were combined and evaporated to dryness in water bath. Then, ethanol was removed and the remaining material was dissolved with a small amount of water. The solution was diluted to the mark in a 25 ml volumetric flask for later use. The extract yield was calculated.

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Ultrasonic extraction method (Liang, 2008)

4 g of *Aristolochia mollissima* Hance was weighed, placed in a 500 ml beaker, added with 10-fold amount of 200 ml of 75% ethanol, placed in an ultrasonic cleaner, heated to 75°C with frequency of 600 kHz, and repeatedly extracted 3 times, each time lasted 1 h. The extract yield was calculated.

Decoction method

4 g of *Aristolochia mollissima* Hance was weighed, placed in a 500 ml beaker, added with 200 ml of distilled water, and boiled for 1 h. The filtrate was recovered by filtration, then was added with new distilled water, and boiled for another 1 h. The extraction was repeated a total of 3 times. After evaporation to dryness in water bath, the extract was obtained. The extract yield was calculated.

Preparation of drug solution

0.2 g of the obtained extracts was uniformly weighed, ultrasonically dissolved and prepared as 10 mg/ml solutions. The solutions were then filtered through 0.22 µm membrane for the screening of anti-HOS cell activity.

HOS cell cultivation (Jiang, 2011; Wang et al, 2012)

HOS cells were cultured in RPMI 1640 (20% FBS) and stained positive with Gomori calcium-cobalt alkaline phosphatase staining. HOS cells were digested with 0.25% trypsin (containing 0.02% EDTA) into a single cell suspension, and 2×10^4 HOS cells were added to each well of 96-well plates. Then, the RPMI 1640 medium was added to make the volume in each well up to 200 µl. After culturing for 24 h, three different *Aristolochia mollissima* Hance extracts were added respectively. 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml extracts were added to each well respectively. Control group was added with equal volumes of 0.01 mol/L PBS. After incubation at 37 °C and 5% CO₂ for another 24 h, the plates were removed.

Blank control was set up where only the medium was added without addition of cells. The cell cultivation was divided into three groups: 12 h, 24 h and 48 h. 20 µl of 5 mg/mL MTT was added to each well and the cultivation was continued for another 4 h. Then, 100 µl of DMSO was added to each well, and the plates were shaken uniformly for 5 min. The OD value of each well was measured at the wavelength of 490 nm with an ELISA reader, and the cell survival rate was calculated according to the following formula: Cell survival rate = (OD value of the experimental group - OD value of the blank control group) / (OD value of the control group - OD value of the blank control group) x 100%. Four replicate wells were set up for each concentration at each time point, the experiment was repeated three times.

Results

Weight of extracts obtained by three extraction methods (Table 1)

Table 1: Extract yields by three extraction methods

Extraction method	Solvent	Temperature	Extraction times	Weight of extract (g)
Heat reflux extraction	50% ethanol	100□	3	2.23
Ultrasonic extraction	75% ethanol	75□	3	2.4
Decoction	Distilled water	100□	3	2.05

The weight of extract obtained by heat reflux method was 1.23 g, and the weight of extract obtained by ultrasonic extraction was 1.4 g. Compared with other methods, ultrasonic extraction method had the largest extract yield. The weight of extract obtained by decoction method was 1.05 g. The comparison of weight of extracts obtained by three extraction methods is shown in Figure 1.

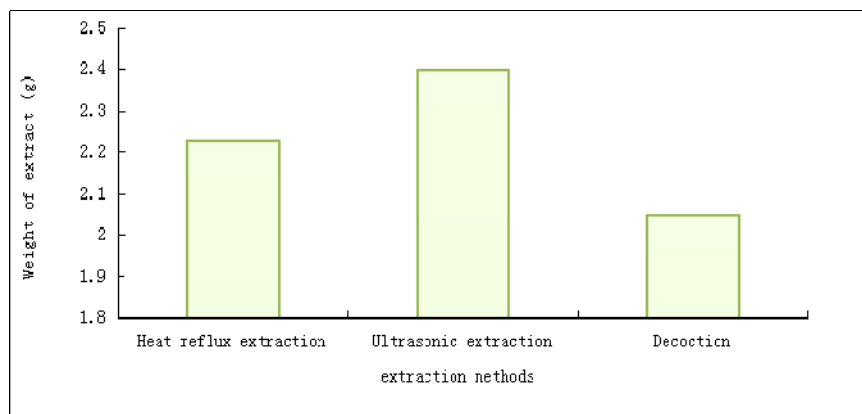


Figure 1: Comparison of weight of extracts obtained by three extraction methods

Study on HOS cell proliferation inhibitory activity by MTT assay

The MTT assay results showed that the extract obtained by heat reflux method had the most obvious inhibitory effect, and the inhibition rate increased with the increase in time and concentration. The inhibitory effects by decoction method and ultrasonic extraction method were relatively poor, yet the effects somewhat existed. The inhibitory activity of extract A obtained by heat reflux method is shown in Table 2 and Figure 2.

Table 2: Cell inhibitory effects of three extracts

	Inhibition rate %				
time (h)	0.625	1.25	2.5	5	10
Heat reflux extraction	98	85	70	64	60
Ultrasonic extraction	94	88	79	72	67
Decoction	97	87	76	74	73

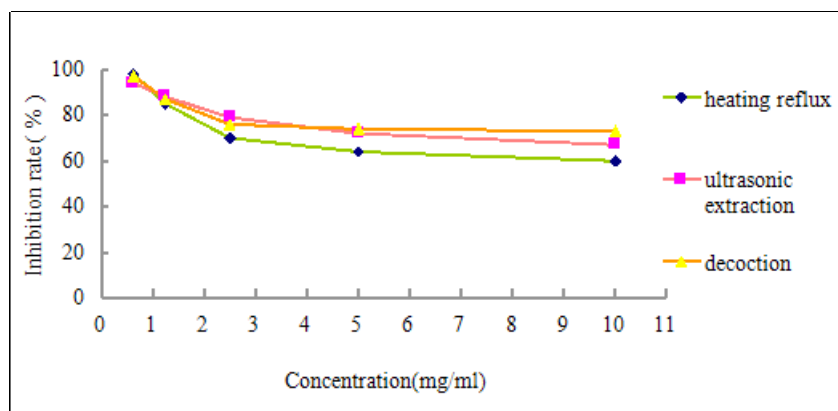


Figure 2: Inhibitory activities of different extracts after 24 h of action

As can be seen from the figure, the extract obtained by heat reflux method has the best inhibitory activity and lower cell survival rate, where cell survival rate reduced to about 60% within 24h. The HOS cell proliferation inhibitory effects of the extracts obtained by three methods were generally higher, where the inhibition rates of extracts obtained by ultrasonic extraction and decoction methods were also around 70%.

Discussion

Osteosarcoma is a malignant disease of mesenchymal tissue origin, which often occurs among young people. Pathological sites are generally in the metaphysis. It has high early incidence of haematogenous metastasis which is rapidly progressing as well. Commonly used therapeutic methods include surgery, gene therapy, immunotherapy, chemotherapy and molecularly targeted therapy. The repair and reconstruction surgery of the surgical therapy can provide a variety of repair means for limb salvage, and thus have gained wide applications.

A gene drug for the treatment of osteosarcoma is the recombinant human endostatin (rh-Endostatin), which is a specific multi-target vascular endothelial cell growth inhibitor. It has an inhibitory effect on human endothelial cell migration, and can specifically inhibit the tumour neovascular

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endothelial cell proliferation (Zhang, 2011). In China, the research status on treatment of *multicentric osteosarcoma* is the same with the present status of treatment of infection by antibiotics; both are facing the multidrug resistance problem. In order to make the chemotherapy drugs for the treatment of osteosarcoma produce lesser side effects and thus reduce the suffering of patients, researchers are committed to study the Chinese medicine extracts which have no toxicity or side effects. For instance, berberine can cause tumour cell cycle arrest and induce tumour cell apoptosis, and ginsenosides-1, -F3, -Rf, PPT and PT extracted from ginseng can also exert the cell proliferation inhibitory effects by promoting the tumour cell cycle arrest (Liu, 2009; Zhang et al, 2001). Other Chinese medicinal herbal extracts include tanshinone II A and liver or kidney supplementing medicines which also have preventive and therapeutic effects on osteosarcoma (Yi, 2011; Zheng et al, 2006). For example, ABP-Ia had an evident inhibitory effect on the growth of HOS cells mainly through induction of apoptosis, with a minor toxicity to normal human osteoblast cell (Bei et al, 2012). And flavonoids purified from *Rhus verniciflua* Stokes named RCMF (the RVS chloroform-methanol part) had inhibitory effects on HOS, being an agent which may be capable of inducing sensitive growth inhibition and apoptosis in HOS cells (Hyon-Seok et al, 2005).

From the experimental results, we learned that the *Aristolochia mollissima* Hance extracts, no matter whether they are aqueous extract or ethanol extract, all have a good inhibitory activity, and therefore may provide a new method for the osteosarcoma treatment. However, *Aristolochia mollissima* Hance is reported in the literature to have a relatively strong renal toxicity, so how to avoid renal toxicity while utilizing its tumour inhibiting effect is the next problem to be solved. Our experiment found that the extract obtained by heat reflux method has the strongest inhibitory effect on osteosarcoma HOS cells, and therefore, taking into account the very scarce studies on inhibitory effect of *Aristolochia mollissima* Hance on osteosarcoma cells, this paper has some reference significance for future studies.

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