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A STUDY ON SCREENING OF OSTEOSARCOMA U2OS CELL INHIBITING ACTIVE COMPONENTS FROM *NIDUS VESPAE*

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Abstract

This paper mainly examined the anti-osteosarcoma activity of total flavonoids extract from traditional Chinese medicine, *nidus vespae*. Orthogonal design was used to design the extraction process of total flavonoids. L(3^4) orthogonal test was performed and the extracts obtained by three optimal extraction processes were used for anti-tumour activity screening in order to determine the optimal anti-tumour effective component of *nidus vespae*. MTT assay was used to investigate the effect of *nidus vespae* extract on proliferation activity of osteosarcoma cells. Meanwhile, U2OS cell inhibitory capacities of extracts in three groups with higher total flavonoid contents were investigated and compared, and inhibition rates were calculated. The results showed that the optimal extraction process was ethanol concentration of 95%, 12-fold amount of ethanol relative to the weight of medicinal material, extraction times of 3 times, and extraction time of 2 hours. 9 extraction processes all showed proportional trend of cancer cell inhibition rate to extract concentration.

Keywords: *Nidus vespae*; U2OS; Inhibiting Actuve; MTT

Introduction

Nidus vespae is the nest of Polistes mandarinus Saussure (Vespidae family) or various closely related species. Its main constituents include volatile oil, beeswax, resin, a variety of saccharides, vitamins, inorganic salts, etc. It has good anti-inflammatory, analgesic, anti-tumour, and anti-bacterial activities, and at the same time is also helpful in the treatment of digestive, cardiovascular, and urinary system diseases (Wu, 2001; Lu, 1998). Especially the proteins extracted from *nidus vespae*, such as the water-soluble NV-PP-4 and NVP (1), the extracts have very good anti-hepatoma activities and leukaemia cell proliferation inhibitory effects (Wang, 2008; Xu et al., 2000; Jing and Xin, 2005). Therefore, in order to further study the anti-cancer activity of *nidus vespae* extract, especially its efficacy in human bone cancer, this paper designed and studied the optimal extraction process of total flavonoids from *nidus vespae*, and made an initial analysis on the inhibitory capacity of the extract on osteosarcoma U2OS cells.

Materials and Methods

Materials used in the study included the following: osteosarcoma U2OS cells, purchased from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences; traditional Chinese medicine *nidus vespae*, purchased from Anguo Lijian Chinese Medicinal Materials Co., Ltd.; MTT, Amresco; foetal bovine serum, GIBICO; DMSO, purchased from Beijing Yaanda Biotechnology Co., Ltd.; ethanol, Tianjin Kemiou Chemical Reagent Co., Ltd.; HS-1300-U clean bench, Suzhou Antai Air Tech Co., Ltd.; 3110 CO_2 incubator, Thermo, USA; BIORAD680-680 microplate reader, Bio-Rad; HH-S6 digital thermostat water bath, Jintan Antou Liangyou Experimental Instrument Factory.

Extraction Process (Wu H.X., 2001)

Ethanol solution extraction method was used here. On the basis of single factor experiment, orthogonal test method was used to study the

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effect of ethanol concentration, extraction temperature, and extraction time these three factors on extraction rate of total flavonoids from *nidus vespae*. UV-visible spectrophotometer was used to determine total flavonoids content in *nidus vespae* to obtain the optimal extraction process, of which ethanol concentrations were 95%, 75%, 50% (ethanol-water ratio). Extraction temperatures were set as 35 ℃, 55 ℃, 75 ℃; and extraction times were 1h, 2h, 3h respectively. The factors and levels table are shown in Table 1.

Table 1: Factors and levels table

Level	A Ethanol concentration %	B Ethanol amount times	C Extraction times ℃	D Extraction time h
1	50	8	4	3
2	75	12	3	2
3	95	16	2	1

Determination of Total Flavonoids Content

It was known from the literature that the rutin was selected as the standard substance for determining total flavonoids. It was also learned that the rutin standard solution and *nidus vespae* sample liquid both had gentle absorption peak at 511nm. Therefore, 511nm was chosen to determine total flavonoids. The results are shown in Table 2.

Plotting of Rutin Standard Curve (Xiong et al., 2010)

15.22 mg of dried rutin standard was precisely weighed, dissolved in 75% ethanol to make the volume 50 mL, shaken well, and rutin standard solution with a concentration of 0.304 mg/mL was obtained for later use. Then, the sample extraction fluids obtained from the orthogonal experiment were added with the corresponding concentration of ethanol to make the volume 500 ml to serve as sample solutions for later use. 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mL of rutin standard solutions were precisely weighed and placed in 25 mL volumetric flasks respectively. Each flask was added with water to make the volume 6.0 mL. Each was then added with 1 mL of 5% sodium nitrite solution, mixed well, and allowed to stand for 7 min. 1 mL of 10% aluminium nitrate solution was added to each flask, mixed well, and allowed to stand for 7 min. Next, each flask was added with 10 mL of 4% NaOH solution, added with water, shaken well, and allowed to stand for 13 min. The absorbance was measured at 511 nm wavelength against the corresponding reagent as blank. Standard curve was plotted with rutin content as the abscissa, and absorbance as the ordinate. Linear regression was performed. The regression equation is A = 0.0115C + 0.013, r = 0.9992, indicating that the rutin was in a good linear relationship with the absorbance within the concentration range of 12.16~72.96 μg/mL. UV absorbance of sample solutions was measured at 511 nm wavelength respectively, and total flavonoids contents in the samples were calculated from the regression equation.

Cell Culturing

Osteosarcoma U2OS cells were cultured in DMEM medium containing 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The incubator conditions were 37℃, 5% CO_2. Cells were grown adherently, digested with trypsin, and the second-generation cells in the logarithmic growth phase were used for screening.

Activity Screening

Logarithmic growth phase cells were taken, and the cell concentration was adjusted to 1×10^5 cells/ml. In 96-well plates, 190 μl of the cell suspension was added to each well and 10 μl of sample was added. Before the experiment, the resulting 3 extracts were uniformly prepared as 150 mg/ml. Negative control, positive control, and experimental groups were set up, and each group had five replicate wells. The sample addition amount was 10 μl. According to the literature, cell culture time of 48 hours was the optimal cell culture time for activity investigation (Yuan et al., 2012). 4 h before the end of cultivation, 10 μl of MTT was added to each well. After cultivation, each well was added with 100 μl of DMSO. Absorbance values were measured at 570 nm by a microplate reader, and inhibition rates were calculated (see **Table 3** and **Figure 1**):

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Inhibition rate = 1 - OD value of treatment group / OD value of control group

Results

Results for the Determination of Total Flavonoids Content

Table 2: Optimal extraction process and extraction rate

Experiment No.	A Ethanol concentration%	B Ethanol amount (times)	C Extraction times ℃	D Extraction time h	Total flavonoids extraction rate (%)
1	95	8	4	3	1.45
2	95	12	3	2	1.63
3	95	16	2	1	1.02
4	75	8	3	1	0.85
5	75	12	2	3	0.97
6	75	16	4	2	1.05
7	50	8	2	2	0.72
8	50	12	4	1	0.75
9	50	16	3	3	0.83
Mean 1	1.367	1.007	1.083	1.083	
Mean 2	0.957	1.117	1.103	1.133	
Mean 3	0.767	0.967	0.903	0.873	
Range	0.600	0.150	0.200	0.260	

It can be seen from the results of total flavonoids content that the group with highest total flavonoids content had the ethanol concentration of 95%, ethanol amount of 12 times of the weight of medicinal material, extraction times of 3 times, and extraction time of 2 hours. The next two highest groups were experiment group No. 1 and No. 6 respectively, which had ethanol concentration of 95%, ethanol amount of 8 times of the weight of medicinal material, extraction times of 4 times, and extraction time of 3 hours for number 1, and ethanol concentration of 75%, ethanol amount of 16 times of the weight of medicinal material, extraction times of 4 times, and extraction time of 2 hours for number 2.

Therefore, extracts in three groups with higher total flavonoids contents were selected as the samples for anti-cancer activity screening. Experiment numbers in the order of extraction rates were No.2, No.1, and No.6.

Table 3: U2OS cell inhibition rate in each group under different concentrations *100

Concentration (mg/ml)	No.2 group	No.1 group	No.6 group
16.25	14.9	11.2	7.5
32.5	26.4	14.5	13.2
75	35.2	23.7	22.1
150	56.1	38.3	35.2

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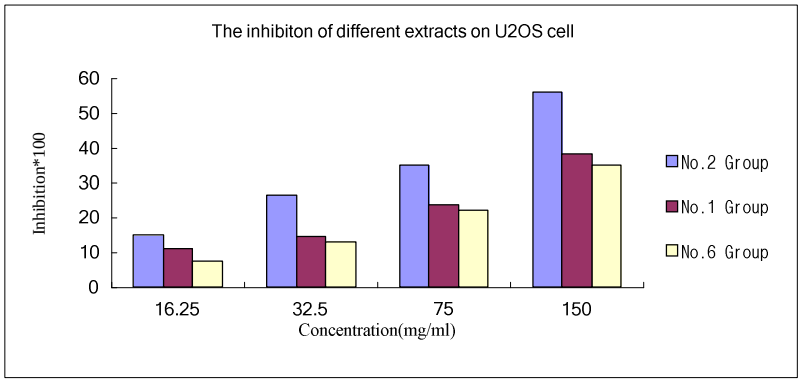

Figure 1: U2OS cell inhibition rates of samples in three groups under 4 different concentrations

It can be seen from the combined results of Table 3 and Figure 1 that the U2OS cell inhibition rates of three extracts increased with the increase of total flavonoids content. The sample in No.2 group, which had the highest total flavonoids content, had the maximum inhibition rate. Its inhibition rate reached 56.1% when concentration was 150 mg/ml. The sample of No.3 group demonstrated the minimum inhibition rate; its value was 7.5%.

Discussion

Bone cancer means that, like other organs, the skeletal system may suffer from any tissue element tumour or metastatic lesions from other organs. Tumours affecting bones can occur in bone cells, haematopoietic elements of bone, cartilage, as well as fibrous or synovial elements. So far, there have been a lot of research findings on the treatment of osteosarcoma by single traditional Chinese medicines, such as: melittin, tripterygium wilfordii extract, venenum bufonis water extract, ursodeoxycholic acid, matrine, curcumin, ginsenoside, baicalein, quercetin, tanshinone Ⅱ A, rhein derivatives, croton alkaloids, psoralen, allicin, etc. These medicines can make the tumour cells unable to carry out enough DNA repair and make them enter the division phase by up-regulating the expression of Fas protein, thereby hastening apoptosis, and arresting the cells in the G0/G1 phase. It is also probable that they exert anti-osteosarcoma effects through the mitochondrial apoptosis pathway and other means (Wang et al., 2006).

There are a lot of traditional Chinese medicines commonly used in the external treatment of tumours, which also have a wide range of anti-cancer pharmacological activities, such as *polyphaga sp.*, *nidus vespae*, *strychnos nux-vomica*, and *rhizoma curcumae*, and these are regarded as the sources of drugs for the treatment of bone tumours (Gu, 2005). For example, the curcumin isolated from *rhizoma curcumae longae* can induce apoptosis through a variety of molecular channels, which includes the anti-apoptotic Bcl-2 family proteins, mitochondrial membrane potential, mitochondrial cytochrome c, as well as caspase -c, (Jin et al., 2009). Another example is the gambogic acid from *garcinia hanburyi*, which induces the G0/G1 phase arrest of U2OS by down-regulating phospho-GSK3-β (Ser9) and promoting the expression of cyclin D1 (Zhao et al., 2011). Although the traditional Chinese medicine *nidus vespae* used in this study has anti-osteosarcoma effects, the inhibitory activity of its extract on proliferation of osteosarcoma U2OS cells has never been reported (Zhang and Zhao, 2012). Therefore, this paper designed the experiment of optimal extraction process of total flavonoids from *nidus vespae*, in combination with screening of human osteosarcoma U2OS cell inhibitory activity, and concluded the extraction process with highest total flavonoids yield, and its maximum inhibition rate on U2OS cells. Moreover, it is found from the result analysis that the cancer cell inhibition rate is proportional to the concentration of extract. Although the efficacy of constituents extracted from traditional Chinese medicine is not very satisfactory compared with the western medicine, from the perspective of doctors of traditional Chinese medicine, in addition to the disease treatment effect, traditional Chinese medicine also has the body function conditioning effect. It also plays a positive role in promoting the recovery of diseases. Therefore, the optimal process for the extraction of total flavonoids from traditional Chinese medicine provides a valuable experimental basis for future anti-osteosarcoma research of *nidus vespae*.

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