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EFFECTS OF ETHANOL EXTRACT OF *RADIX SOPHORAE FLAVESCENTIS* ON ACTIVITY OF COLON CANCER HT29 CELLS

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Abstract

This paper mainly studied the inhibitory effect of total ethanol extract of *Radix Sophorae Flavescentis* on proliferation of colon cancer HT29 cells. By reflux extraction method and with ethanol as extraction solvent, different extracts were obtained at different ethanol concentrations, different solid-liquid ratios, and at different times. And their inhibitory activities against HT29 cells were compared using MTT assay. The experimental results showed that the extraction processes under three conditions can all draw relatively high inhibition rates. The optimum ethanol extraction process conditions were as follows: a solid-liquid ratio of 1:9, 80 min of heat reflux extraction with 95% ethanol.

Keywords: Sophora flavescens, cancer HT29, Orthogonal experiment, inhibition

Introduction

Radix Sophorae Flavescentis (Latin name: *Sophora flavescens*), alkaloids and flavonoids are the main active constituents of *Radix Sophorae Flavescentis*, which mainly include benzoic acids, isoflavone glycosides and alkaloids（Shen & Zhang., 2012）. Studies on the biological activities of them or their derivatives have been reflected in many aspects, including antitumour, antibacterial, anti-inflammatory and analgesic effects (Jeong et al., 2010; Gi et al., 2012). So far, studies on different constituents of *Radix Sophorae Flavescentis* on colon cancer have been very abundant, such as the relatively good inhibitory activities on colon LOVO, SW1116, HT-29, SW1116, SW620 and SW480 cells. However, the effect of total ethanol extract of *Radix Sophorae Flavescentis* on HT29 cells is still not studied. The studies on its anticancer mechanism are mainly focused on the inhibition of cancer cell DNA synthesis, inhibition of certain enzyme activities, and inhibition of cancer cell proliferation by affecting cell cycle arrest. They also include inhibition of tumour metastasis by controlling the expression levels of some factors, affecting telomerase activity, induction of cell apoptosis, as well as induction of cancer cells to differentiate to normal cells (Zhou et al., 2000; Liu., 2004; Ding et al., 2007; Liang et al., 2008; Peng., 2011). Therefore, this paper is focused on the extraction rates of total ethanol extracts under different extraction methods, and studies their anticancer activities against HT29 cells.

Materials and Methods

Materials

Radix Sophorae Flavescentis was purchased from the Beijing Tongrentang Pharmacy and was identified by Professor Ming Sun of Central South University. The specimen was placed in the pharmacognosy laboratory of the university. Ethanol was purchased from the Sinopharm, water bath and rotary evaporator from Shanghai Yarong Biochemical Instrument Factory, and human colon cancer HT29 cells were purchased from the Institute of Materia Medica, CAMS. RPMI 1640 medium (Gibco); foetal bovine serum (Hangzhou Sijiqing Bioengineering Material Co., Ltd.); DMSO (Beijing Biosea Biotechnology Co., Ltd.); MTT (Sigma) were used.

Orthogonal process design (Lin et al., 2011)

Orthogonal experimental design was used. Three factors were selected, with each factor having three levels. Extraction processes of *Radix Sophorae Flavescentis* were optimised as shown in Table 1, and optimum process parameters for *Radix Sophorae Flavescentis* extraction were determined according to the concentration with highest anticancer effect.

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Table 1: Factors and levels of ethanol extraction of *Radix Sophorae Flavescentis*

Level	Solid-liquid ratio	Extraction time (min)	Ethanol percentage /%
1	1:6	40	45
2	1:9	80	75
3	1:12	120	95

Preparation of reagents

Preparation of cell culture medium: 1 pack of RPMI 1640, 10^5U of penicillin and streptomycin each, 2.0 g of NaHCO3 and 2.0 g HEPES were added with triple-distilled water. They were mixed uniformly with a glass rod. The dissolution was then accelerated with an ultrasonic cleaner, and the volume was made to 1 L with triple-distilled water. The pH was adjusted to 7.2~7.4 with 5.6% $NaHCO_3$ aqueous solution; then the solution was filtered through 0.22 μm membrane, and stored in a 4 ℃ refrigerator.

Preparation of PBS: 1.56 g of $Na_2HPO_4H_2O$, 0.20 g of KH_2PO_4, 0.20 g of KCl and 8.00 g of NaCl were accurately weighed, added with triple-distilled water to dissolve and make the volume to 1 L, pH was adjusted to 7.2. The solution was then sterilised with high-pressure steam at 121℃ for 30 min, sub-packaged as 100 mL/bottle, and stored in a 4 ℃ refrigerator.

Preparation of 0.25% trypsin cell dissociation buffer: 0.25 g of trypsin and 0.02 g of EDTA were weighed and sufficiently dissolved by addition of 100 mL of sterile PBS. The pH was adjusted to 7.2~7.4, and the solution was placed in a 4 ℃ refrigerator overnight. It was then filtered and sterilised through 0.22 μm bilayer membrane, dispensed into the bottles (10 mL/bottle), and stored in a -20 ℃ refrigerator for later use.

Preparation of MTT solution: 250.0 mg of MTT was weighed, added with 50 mL of PBS, and stirred on a magnetic stirrer for 30 min to allow the MTT to be sufficiently dissolved to give a final concentration of 5 mg/mL. The solution was then filtered and sterilised through 0.22 μm bilayer membrane, sub-packaged, and stored in a 4 ℃ refrigerator for later use.

Preparation of drug solution
Preparation of Chinese medicinal herbal ethanol extract

5 g of pulverised *Radix Sophorae Flavescentis* was weighed each, and extracted separately according to the solid-liquid ratios, with extraction durations and ethanol percentages in Table 1 in a 90 ℃ water bath. The resulting extracts were then placed in a 60 ℃, 0.08 MPa rotary evaporator and concentrated to 25 mL (drug content in the solution of 0.2 g/mL). Excess ethanol was evaporated and the remaining liquid was slowly evaporated to dryness in a water bath at 70 ℃. The resulting powder was then uniformly prepared as 5 mg/ml aqueous solutions with water, and filtered through 0.22 μm membrane for later use.

Cell cultivation (Tan et al., 2006**)**

HT29 cells were cultured in the RPMI 1640 medium containing 10% foetal bovine serum, and statically incubated under 37 ℃, 5% CO_2 and saturated humidity conditions. The logarithmic phase cells were selected and used in the group experiment.

Effects of 9 extracts on HT29 cell proliferation by MTT assay (Jiang., 2009**)**

The cells were seeded in a density of 5×10^4 ml^{-1}. After the culture plate was incubated for 48 h, 100 μl of different *Radix Sophorae Flavescentis* solutions were added to each well. Five replicate wells were set up for each sample, and the OD values were averaged. The final concentration was 2.5 mg/ml; the control group was added with 10% FBS containing RPMI 1640 medium. After culturing for 24 h respectively, 20 μl of MTT (5 mg/ml) solution was added to each well, and the culture was continued for another 4 h. Then each well was added with 100 μl of DMSO, and the plate was shaken in a decolourisation shaker for 5 min. OD value of each well was measured at 570 nm using a microplate reader. The experiment was repeated three times. The cell inhibition rate was calculated according to this formula: Cell inhibition rate (%) = (1 - $\overline{OD}$ of experimental group / $\overline{OD}$ of control group) × 100%, the readout was set to zero with the $\overline{OD}$ value of blank group.

Results

Investigation of optimum extraction process and the inhibition rate against colon cancer HT29 cells
Inhibition rates of extracts under different extraction processes against HT29 cells

As can be seen from Figure 1, extracts obtained by the extraction processes with experiment Nos. 2, 5 and 9 have relatively higher inhibition rates against HT29 cells. Experiment No.1 has the lowest inhibitory rate, the inhibition rates are generally above 20%.

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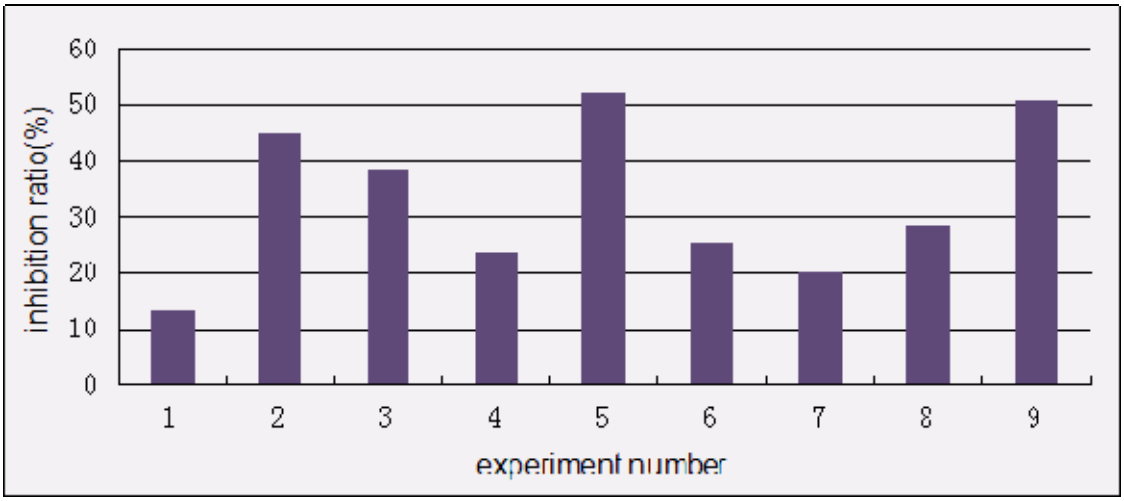

Figure 1: Inhibition rates of extracts under different extraction processes against HT29 cells

Table 2: Statistics of orthogonal experiment of *Radix Sophorae Flavescentis* ethanol extracts

Experiment	Solid-liquid ratio	Extraction time (min)	Ethanol percentage /%	Inhibition rate (%)
1	1	1	1	13.2
2	1	2	2	45.1
3	1	3	3	38.4
4	2	1	2	23.6
5	2	2	3	52.0
6	2	3	1	25.5
7	3	1	3	20.3
8	3	2	1	28.7
9	3	3	2	50.9
K1	32.000	18.667	22.000	
K2	33.333	41.667	39.333	
K3	32.667	37.667	36.667	
极差	1.333	23.000	17.333	

It can be drawn from Table 2 that the colonic cancer inhibition rate of the extract obtained by heat reflux under a solid-liquid ratio of 1:9, 80 min, and 95% ethanol is very close to that of the extract obtained by 120 min of heat reflux extraction with a solid-liquid ratio of 1:12 and 75% ethanol, which are both around 51%, but the latter has a longer reflux time. After overall consideration in the investigation experiment on colon cancer cell inhibitory activity, the optimum ethanol extraction process conditions of *Radix Sophorae Flavescentis* are as follows: a solid-liquid ratio of 1:9, 80 min of heat reflux extraction with 95% ethanol.

Discussion

In this paper, inhibition rates of various extracts against HT29 cells are comprehensively investigated through extraction processes with different concentrations of ethanol, different extraction durations, and different solid-liquid ratios. The experiment found that the extraction processes under three conditions can draw a relatively high inhibition rate, but in a comprehensive consideration of various conditions, it is concluded that the optimum ethanol extraction process conditions of *Radix Sophorae Flavescentis* are thus: a solid-liquid ratio of 1:9, 80 min of heat reflux extraction with 95% ethanol. This paper mainly measured the anti-colon cancer activities of total ethanol and aqueous extracts of *Radix Sophorae Flavescentis*. These total extracts must contain matrine and flavonoids that have been recognised in the literature. Under the circumstance that purer and more active compounds are relatively difficult to obtain, we used the method similar to boiling which is commonly used by the Chinese people to obtain a series of such extracts in treatingmany incurable diseases in a similar way. The anticancer activities of *Radix Sophorae Flavescentis* and alkaloids are reflected not only in the presence of direct cell killing activity, but also in the induction of some tumour cells to normal cell differentiation and promotion of apoptosis. Moreover, they do not produce damaging effects on normal cells, and can elevate white blood cell count and enhance immune function that other chemotherapy drugs can hardly

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compare with. At present, a total of 23 alkaloids have been isolated from the root, stem, leaves and flowers of *Sophora flavescens*, most of which are quinolizidines, and very few are dipiperidines. The majority of quinolizidine alkaloids are matrine-type alkaloids, others include three cytisine type-, three sparteine type-, and one lupine-type alkaloids (Zhao et al., 1991). Kushecarpin D (KD) is a new-type flavonoid extracted from *Sophora flavescens*, and it has been indicated that the antiangiogenic effect of KD may be related to the cell cycle arrest at the G2/M phase and the inhibition of intracellular H2O2 production (Pu., 2010).

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