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Effect of *Rhizoma Curcumae* oil on proliferation of human cervical carcinoma CASKI cells.

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Abstract

The objective is to study the extraction and refinement processes of Rhizoma Curcumae oil and its inhibitory effect on cervical cancer. Orthogonal test, MTT assay and flow cytometry are used, respectively, to optimize the extraction process of Rhizoma Curcumae oil, and to study its inhibitory effect on CASKI cells. Orthogonal test results show that favorable extraction and refinement effects can be achieved if Rhizoma Curcumae is crushed, passed through an 80-mesh sieve, added with 30-fold amount of water, and steam distilled for 2 h. In the industrial production, the above conditions can easily be met, which can minimize production costs as well. MTT assay and flow cytometry results show that the medium- and high-dose (100, 150 µg/ml) Rhizoma Curcumae oil groups have significantly different growth inhibition rates against CASKI cells, indicating that Rhizoma Curcumae oil can inhibit CASKI cell growth to varying degrees, and in a somewhat dose-dependent manner under the experimental conditions. Rhizoma Curcumae oil can arrest CASKI cell cycle at the G2/M phase.

Keywords: Rhizoma Curcumae oil, CASKI cell, MTT assay

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Introduction

ARhizoma Curcumae is the dried rhizome of Curcuma phaeocaulis Valeton, Curcuma kwangsiensis S. G. Lee et C. F. Liang or Curcuma wenyujin Y.H. Chen et C. Ling [1]. It is acrid, bitter and warm, and enters the liver and spleen meridians, which has qi circulation promoting, blood stasis dissipating, food retention removing and pain relieving functions. Rhizoma Curcumae is used in the treatment of blood stasis, amenorrhea, indigestion, swelling pain and abdominal mass and lump; meanwhile, it also has anti-early pregnancy, anticoagulant, antioxidant and hepatoprotective activities [2-3]. Main active constituent of Rhizoma Curcumae is volatile oil, which has complex composition, and is comprised primarily of sesquiterpenoids and sesquiterpenes [4-6].

Modern pharmacological studies have shown that Rhizoma Curcumae oil has good anti-tumor, anti-inflammatory and antiviral effects [7-10]. Since Rhizoma Curcumae has rather definite efficacy, a variety of preparations (including injections, suppositories, soft capsules, etc.) containing main constituents of Rhizoma Curcumae have now been launched for use, which have gain wide trust clinically. This paper focuses on studying the extraction process and anticancer activity of Rhizoma Curcumae oil, in order to provide the basis for its industrial production and clinical application.

Materials

Instruments and reagents

Methyl thiazolyl tetrazolium (MTT) and DMSO were purchased from AMRESCO company; PI was purchased from Sigma; SW-CJ-2F clean bench (Suzhou Purification Equipment Plant); volatile oil extractor; heating mantle; mini pulverizer.

Drug and cell

Rhizoma Curcumae was purchased from Anguo medicine market, which was identified as the rhizome of Curcuma kwangsiensis S. G. Lee et C. F. Liang. Rhizoma Curcumae oil was self-prepared. Cervical carcinoma cell line CASKI was purchased from Shanghai Xiangf Biotechnology Co., Ltd.

Methods

Determination of extraction process parameters

Based on the pre-experimental results and relevant litera-

ture, Rhizoma Curcumae medicinal material was hard in texture, so active constituents cannot be easily extracted. Therefore, three factors, i.e. crushing fineness, water addition and steam distillation time were selected for orthogonal test in this paper. Experimental factors and levels are shown in Table 1.

Table 1. *Experimental factors and levels*

Level	A Water addition (fold)	B Crushing fineness (mesh)	C Distillation time
1	10	20	2
2	20	40	4
3	30	80	6

Cell culturing

CASKI cell lines were cultured in RPMI 1640 medium containing 10% FBS, and subcultured routinely in a 37℃, 5% CO2 incubator. Logarithmic phase cells were collected for later experimental use.

MTT assay of cytostatic effect of Rhizoma Curcumae oil on CASKI cells

Logarithmic phase CASKI cells were trypsinized, and seeded in 96-well culture plates at a concentration of 5×104/mL; total reaction system was 200 μL per well. After culturing in a 37℃, 5% CO2 incubator for 24 h, supernatant was discarded, and each 100 μL of different concentrations (25, 50, 100 and 150μg/ml) of Rhizoma Curcumae oil was added separately. Six parallel wells were set up for each concentration, and PBS was used as the negative control. The cells were cultured for additional 24, 48 and 72 h in a 37℃, 5% CO2 incubator. 4 h before termination of the experiment, 20 μL of MTT solution (concentration of 0.5 mg/mL) was added to each well, and then supernatant was discarded. Afterwards, each well was added with 150 μL of DMSO, and shaken with a shaker. Finally, absorbance (A) values were measured at a 490 nm wavelength using a microplate reader, and cell inhibition rate was calculated.

Inhibition rate = (A value of control group - A value of treatment group) / A value of control group

Flow cytometric analysis

CASKI cells were seeded in culture flasks at 1×106/mL, and cultured in a 37℃, 5% CO2 incubator for 24 h. Then the medium was replaced, and different concentrations of (50, 100 and 150μg/ml) of Rhizoma Curcumae oil were added for treatment for 48 h. Triplicate wells were set up for each group. Next, cells were harvested, made into single cell suspension, centrifuged for 5 min, washed three times with PBS (pH 7.4), fixed in 70% precooled ethanol, and stored overnight at 4℃. After removing ethanol by centrifugation, the cells were washed twice with PBS, and DNA stained with 50 mg/L propidium iodide (PI), followed by determination of cell cycle by flow cytometry.

Statistical methods

Statistical analysis software SPSS version 15.0 was used, normal distribution test was performed on the experimental data, values were expressed as $\bar{x} \pm s$, and one-way ANOVA was used. a<0.05 was chosen as the significance level, p<0.05 was considered statistically significant, and p<0.01 was considered significantly different.

Results

Optimization results of Rhizoma Curcumae oil extraction process

Experiment was designed according to the L9(33) orthogonal array, Rhizoma Curcumae was crushed into specified mesh sizes, added with 4-, 6- and 8-fold amounts of water, respectively, and extracted as per the volatile oil extraction method in the Pharmacopoeia of the People's Republic of China. After setting aside for 1 h, volatile oil amount was measured, and extraction yield was calculated. The results are shown in Tables 2 and 3.

Table 2. *Optimization results of Rhizoma Curcumae oil extraction process*

Experiment No.	**A**	**B**	**C**	**Rhizoma Curcumae oil yield (%)**
1	1	1	1	0.44
2	1	2	2	0.98
3	1	3	3	1.46
4	2	1	2	0.57
5	2	2	3	0.92
6	2	3	1	1.96
7	3	1	3	0.57
8	3	2	1	1.47
9	3	3	2	1.76

K_1	0.960	0.527	1.286
K_2	1.150	1.123	1.101
K_3	1.267	1.727	0.981
R	0.307	1.200	0.305

Table 3. *ANOVA results of Rhizoma Curcumae oil yield*

Factor	Sum of squared deviations	Degree of freedom	F ratio	F critical value	Significance
Water addition	0. 144	2	1.007	19	
Crushing fineness	2. 160	2	15.105	19	
Distillation time	0. 143	2	1.000	19	
Error	0. 14	2			

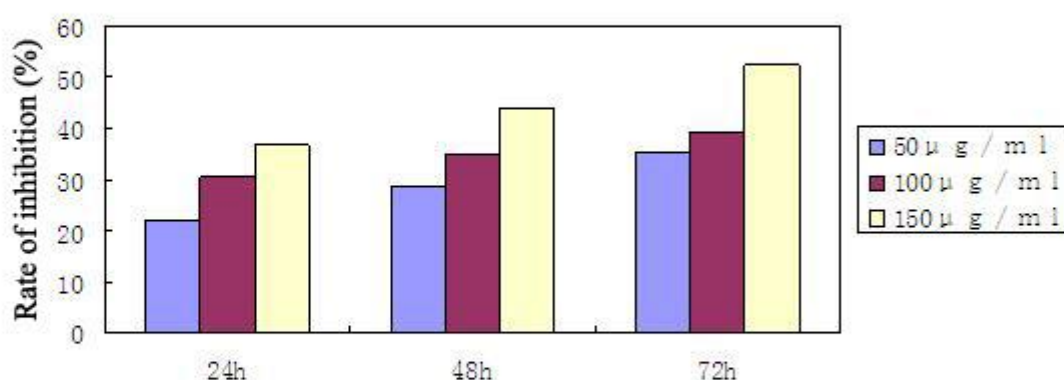

Figure 1. *Growth inhibition rate against CASKI cells by Rhizoma Curcumae oil*

Table 4. *Effects of Rhizoma Curcumae oil on cell cycle distribution and apoptosis rate of CASKI cells (n = 4, $\bar{x}$±s)*

Group	**Concentration** (μg / ml)	**G0/G1 phase**	**S phase**	**G2/M phase**
Control group		58.36±0.13	41.46±0.16	0.18±1.05
Rhizoma Curcumae oil	50	56.54±0.14	40.58±0.15	2.88±0.66
	100	49.63±0.02	38.43±0.13	11.94±0.34
	150	45.82±0.07	30.51±0.07	23.67±0.27

Orthogonal range analysis showed that factors influencing the extraction efficiency of Rhizoma Curcumae oil were: B> A> C, i.e. crushing fineness > water addition > extraction time, in a descending order. Among them, water addition and extraction time had relatively small influences on the experiment. As can be seen from the orthogonal test and ANOVA results, the optimal extraction process regarding Rhizoma Curcumae oil was A3B3C1, i.e. addition of a 30-fold amount of water, crushing fineness of 80 mesh, and steam distillation time of 2 h .

MTT assay of the effect of Rhizoma Curcumae oil on CASKI cell proliferation Compared with the control group, the inhibition rate did not change much in the low-dose group (50 μg/ml), which was not statistically significant. Medium- and high-dose (100, 150 μg/ml) Rhizoma Curcumae oil groups exhibited significantly different growth inhibition rates against CASKI cells, of which the high-dose group (150 μg/ml) had an inhibition rate reaching 52.3%. This indicates that Rhizoma Curcumae oil can inhibit CASKI cell growth to varying degrees, and in a somewhat dose-dependent manner under the experimental conditions (See Figure 1).

Flow cytometric determination of the effects of Rhizoma Curcumae oil on cell cycle distribution and apoptosis rate

The effect of Rhizoma Curcumae oil on cell cycle distribution was observed by flow cytometry 48 h after treatment of CASKI cells by different concentrations of hizoma Curcumae oil. Experimental results revealed reduced proportion of cells in G0/G1 and S phases, and increased proportion of cells in G2/M phase in a somewhat

dose-dependent manner, suggesting that Rhizoma Curcumae oil can arrest CASKI cell cycle at the G2/M phase.

Discussion

Main active constituents of Rhizoma Curcumae are volatile oil and curcumin. Rhizoma Curcumae oil primarily consists of sesquiterpenes, which are readily soluble in organic solvents and hardly soluble in water; and its traditional extraction method is steam distillation. In recent years, CO2 supercritical extraction technology has been widely applied in the pharmaceutical industry, which has played an important role in accelerating the development of the industry. However, supercritical extraction technology has limitations such as high cost, easy residue retention and high threshold for industrial production, and thus is not applied substantially in the traditional pharmaceutical industry.

So this paper stills uses the conventional steam distillation method for refinement of Rhizoma Curcumae oil, and employs orthogonal test design for determination of factors influencing the yield of Rhizoma Curcumae oil. The experimental results demonstrate that favorable extraction and refinement effects can be achieved if Rhizoma Curcumae is crushed, passed through an 80-mesh sieve, added with 30-fold amount of water, and steam distilled for 2 h based on the pre-experiment. In the industrial production, the above conditions can easily be met, which can also minimize production costs, and help companies improve efficiency and optimize competitive situation.

Among the most common female malignancies, cervical cancer ranks second only to breast cancer. Its new incidence is about 500,000 cases yearly, of which China accounts for up to 28.8%, that is, more than 130,000 cases. Early prevention and treatment of cervical cancer, as well as relief of physical and physiological stresses of women have become the major concern in society, and bounden duty of researchers. After a lot of research, scientists have found that the main antitumor mechanisms of action of Rhizoma Curcumae oil include direct cytotoxic effect, induction of tumor cell apoptosis, inhibition of abnormal tumor cell proliferation, effects on nucleic acid metabolism and membrane potential of cancer cells, enhancement of immune function, etc. [11-12]

In this study, the effects of Rhizoma Curcumae oil on CASKI cells are determined by MTT assay and flow cytometry. The results show that the medium- and high-dose (100, 150 μg/ml) Rhizoma Curcumae oil groups have significantly different growth inhibition rates against CASKI cells, indicating that Rhizoma Curcumae oil can inhibit CASKI cell growth to varying degrees, and in a somewhat dose-dependent manner under the experimental conditions. Rhizoma Curcumae oil can arrest CASKI cell cycle at the G2/M phase.

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