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Extraction process of ginger Pinellia and its anti-proliferative and proapoptotic activities on human gastric cancer SGC7901 cells.

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Abstract

To screen and identify the optimal extraction process of ginger Pinellia, and to observe the effects of ethanol extracts of ginger Pinellia on proliferation and apoptosis of human gastric cancer SGC7901 cells. Extraction process of ginger Pinellia was optimized through orthogonal experiment employing integrated weighted scoring with licorice concentrating volume, lime water addition and extraction temperature as investigation indices. After treating with different concentrations of ginger Pinellia ethanol extracts, morphological changes of SGC7901 cells were observed under inverted phase contrast microscope. Cell proliferation was determined by MTT assay, and the proapoptotic activity of ginger Pinellia ethanol extracts on SGC7901 cells was detected by flow cytometry. The degrees of influence of factors on extraction efficiency of ginger Pinellia were lime water addition>licorice concentrating volume>extraction temperature in descending order. Optimal extraction process of ginger Pinellia was licorice concentrating volume of 300 ml, lime water addition of 80, and extraction temperature of 50°C. MTT assay demonstrated that different doses of ginger Pinellia ethanol extracts (1 g/L, 2 g/L, 3 g/L) could all inhibit the proliferation of SGC7901 cells after acting for 24 ~ 72 h. Flow cytometry revealed that with the increasing concentration of ginger Pinellia ethanol extract, the proportion of S phase SGC-7901 cells increased from 18.46% in the control group to 36.35%, whereas the proportion of G2/M phase cells decreased significantly from 29.27% in the control group to 11.93%. At high magnification, after culturing with medium containing ginger Pinellia ethanol extract (concentration of 3 g/L) for 24 h, the SGC-7901 cells in the ginger Pinellia ethanol extract groups were rounded, with unclear boundary. Secretory granules increased, cellular refraction decreased, and cellular glycogen lowered. Organelle swelling and liquefaction degeneration of cytoplasm were present. Mitochondria decreased, with only residual organelle fragments visible. The optimized extraction process is simple, convenient, reproducible and operable, which facilitates the quality control of ginger Pinellia. Ginger Pinellia ethanol extract can inhibit the proliferation of human gastric cancer SGC7901 cells.

Keywords: Ginger Pinellia, SGC7901 cell, Flow cytometry.

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Introduction

Banxia, also known as sanyebanxia, banyuelian, sanbutiao, dibadou, shoutian, shuiyu and yangyan, is the dried tuber of *Pinellia ternata* (*Thunb*.) *Breit*. in the genus *Pinellia* of the family Araceae, which was originally recorded in the Shen Nong's Herbal Classic. It is pungent, warm, toxic, and enters the spleen, stomach and lung meridians, with dampness-eliminating, phlegm-reducing, adverse qi-lowering, vomiting-arresting, lump-dissolving and mass-dissipating effects. Major active constituent of Pinellia tubers is volatile oil; besides, it also contains alkaloids that have similar pharmacological actions to coniine and nicotine, and substances with similar skin irritating effect to protoanemonin [1, 13, 10].

Traditional pharmacological actions of *Pinellia ternata* include dampness elimination, phlegm reduction, adverse qi lowering, vomiting arrest, lump dissolution and mass dissipation; as well as external treatment of acute mastitis, acute & chronic suppurative otitis media and other diseases. Due to differences in extraction technology, medication regimen and compatibility, *Pinellia ternata* may exhibit different pharmacological effects. Modern pharmacological studies have shown that *Pinellia ternata* also has anti-tumor [14, 3, 11] anti-fertility, hypolipidemic, hepatoprotective and coronary heart disease curing [4, 7].

Crude *Pinellia ternata* is toxic, which may cause vomiting and even death in severe cases. Extraction of *Pinellia ternata* with a variety of materials can effectively weaken

and eliminate its toxicity. In the thousands of years of clinical application history of *Pinellia ternata*, in order to fully exert its clear clinical efficacy while reducing toxic side effects, numerous physicians and scholars have made extensive attempts to explore the extraction technology of *Pinellia ternata* from various perspectives, so as to achieve the best attenuated synergistic effect. This study optimizes the extraction process of ginger Pinellia, and studies its anti-gastric cancer effect.

Materials

Instruments

751G UV-Vis spectrophotometer (Shanghai Analytical Instrument Factory); CO-150 CO_2 incubator (NBS, USA); inverted fluorescent microscope (OLYMPUS, Japan); FACSAria Flow Cytometer (BD, USA); ELX 800 microplate reader (BioTEK, USA).

Drugs, Reagents and Cells

Pinellia ternata and licorice were purchased from Bozhou medicine market, which were identified as the dried tubers of Araceae plant *Pinellia ternata (Thunb.) Breit*. and the roots and rhizomes of Leguminosae plant *Glycyrrhiza uralensis Fisch*., respectively. Alum was purchased from a pharmacy. Quality of the three drugs was all in line with the current Chinese Pharmacopoeia requirements. RPMI 1640 was product of Gibco; FBS was product of Hangzhou Shijiqing Bioengineering Materials Company; and MTT, DMSO and PI were products of Nanjing Keygen Biotech Company. Human gastric cancer SGC7901 cells were purchased from China Medical University.

Methods and Results

Sample Preparation

Nine aliquots of 200 g of uniformly sized *Pinellia ternata* were soaked separately in water (with level 3 cm higher than *Pinellia ternata*) until completely moist, then taken out, shaken to remove surface water, and set aside for orthogonal experiment.

Orthogonal Factors and Levels

During the extraction of *Pinellia ternata*, licorice concentrating volume, lime water addition and extraction temperature had comparatively great influences on the extraction efficiency. On the basis of single factor design, orthogonal experiment was conducted according to $L^9(3)^3$ array selecting the above three factors by determining the monoammonium glycyrrhizinate content in each extract using UV spectrophotometry and HPLC as the index, as shown in Table 1.

Orthogonal experiment showed that during the extraction of ginger Pinellia, the degrees of influence of factors on ginger Pinellia extraction efficiency were: B>A>C, i.e. lime water addition>licorice concentrating volume>extraction temperature in descending order. Based on the orthogonal results and range analysis, optimal extraction process of ginger Pinellia was identified as: A3B3C3, i.e., licorice concentrating volume of 300 ml, lime water addition of 80, and extraction temperature of 50°C, as shown in Table 2.

Cell cultivation

Gastric cancer SGC-7901 cells were seeded in culture flasks with RPMI 1640 medium containing 10% FBS, penicillin (100 mL/L) and streptomycin (1 mg/ml), cultured statically in a 37°C, 5% CO_2 incubator, and digested and passaged with 0.25% trypsin. Cells passaged three times were used in the experiment.

SGC-7901 Cell Growth Inhibition Experiment by MTT Assay

After trypsinization for 2 min, exponential phase cells were prepared into a 5×10^4 cells/ml cell suspension, seeded in 96-well plates at 180 μl per well, cultured for 24 h, and then

Figure 1: *Effect of ginger Pinellia ethanol extracts on proliferation of SGC-7901 cells*

Level	Factor		
	A Licorice concentrating volume (ml)	B Lime water addition (ml)	C Extraction temperature (°C)
1	100	40	30
2	200	60	40
3	300	80	50

Table 1: *Orthogonal factor level table*

No.	A	B	C	Monoammonium glycyrrhizinate yield (%)
Experiment 1	1	1	1	0.15
Experiment 2	1	2	2	0.17
Experiment 3	1	3	3	0.35
Experiment 4	2	1	2	0.14
Experiment 5	2	2	3	0.25
Experiment 6	2	3	1	0.17
Experiment 7	3	1	3	0.18
Experiment 8	3	2	1	0.28
Experiment 9	3	3	2	0.34
Mean 1	0.223	0.157	0.2	
Mean 2	0.187	0.233	0.217	
Mean 3	0.267	0.287	0.26	
Range	0.08	0.13	0.06	

added with different concentrations (1g/L, 2 g/L, 3 g/L) of ginger Pinellia ethanol extracts. Five replicate wells were set up for each concentration. Meanwhile, control group was added with an equivalent volume of culture medium. After culturing in 5% CO_2 incubator for additional 24 h, 48 h and 72 h, 30 μl of MTT solution (5g/L) was added to each well on the third day, and the cultivation was continued for 4 more h. Then supernatant was discarded, each well was added with 150 μl of DMSO, and shaken for 10 min. Absorbance of each well was measured at 490 nm with microplate reader, and inhibition rate was calculated for cells in each group.

Inhibition rate = (A value of negative control group - A value of treatment group) / A value of negative control group × 100%.

MTT assay results showed that different doses of ginger Pinellia ethanol extracts (1 g/L, 2 g/L, 3 g/L) could all inhibit the proliferation of SGC7901 cells after acting for 24 ~ 72 h. Moreover, the anti-proliferative effect of the ginger Pinellia ethanol extracts became increasingly evident with the increase of drug concentration and prolongation of incubation time. At test doses, cell viability declined continuously with increasing dose; and at the same concentration, cell viability declined continuously over time. In particular, after 72 h of drug intervention, SGC7901 cell inhibition rate reached 88.6% in the high dose group, which was statistically different from the control group, as shown Figure 1.

Effect of Ginger Pinellia Ethanol Extracts on Cell Cycle Distribution of SGC-7901 Cells

Logarithmic phase SGC-7901 cells were trypsinized, prepared into a 5 × 10^4 cell suspension, and cultured under 37°C, 5% CO_2 and saturated humidity conditions for 24 h. Then cells were collected, and added with different concentrations (1 g/L, 2 g/L, 3 g/L) of ginger Pinellia ethanol extracts and treated for 72 h. Five replicate wells were set up for each group. Next, cells were collected, prepared into single cell suspension, and centrifuged at 2,000 r/min for 5 min. Afterwards, supernatant was discarded, and the remaining cells were washed 3 times with PBS, and fixed in 70% precooled ethanol at 4°C for 12 h, then washed with PBS twice, and PI stained in an ice bath for 30 min. Finally, changes in the cell cycle after drug intervention were analyzed by flow cytometry.

Flow cytometry results revealed that the ginger Pinellia ethanol extracts had very significant effects on cell cycle distribution of SGC-7901 cells. Compared with the control group, with the increasing concentration of ginger Pinellia ethanol extracts, the proportion of S phase SGC-7901 cells increased from 18.46% in the control group to 36.35%, while the proportion of G2/ M phase cells decreased significantly from 29.27% in the control group to 11.93%. This suggests that the ethanol extracts of ginger Pinellia can arrest SGC-7901 cells in S phase. Compared with the control group, G2/M phase cells decreased, while the percentage of S phase cells increased, presenting a marked time-dose-response relationship, as shown Table 3.

Morphological Changes in SGC7901 Cells after 72 h of Intervention with Ginger Pinellia Ethanol Extracts

Logarithmic phase SGC-7901 cells were seeded in 6-well plates at 5 × 10^4/ml for growth on slides. Well growing, rapidly dividing, morphologically normal cells without cytoplasmic granules were collected, and cultured with medium containing ginger Pinellia ethanol extract (concentration of 3 g/L) for 24 h, then observed under an inverted microscope.

At high magnification, after culturing with medium containing ginger Pinellia ethanol extract (concentration of 3 g/L) for 24 h, the SGC-7901 cells in the control group: were grown firmly adherent, which were bright, clear, refractive and fully stretched, with intact membrane and uniform cytoplasmic distribution. In comparison, cells in the ginger Pinellia ethanol extract groups: were rounded, with unclear boundary. Secretory granules increased, cellular refraction decreased, and cellular glycogen lowered. Organelle swelling and liquefaction degeneration of cytoplasm were present. In addition, mitochondria decreased, with only residual organelle fragments visible.

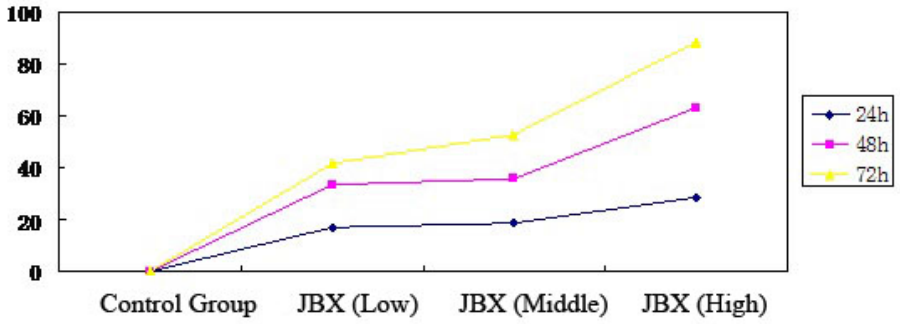

Figure 1: *Effect of ginger Pinellia ethanol extracts on proliferation of SGC-7901 cells*

Table 3: *Effect of ginger Pinellia ethanol extracts on cell cycle of SGC-7901 cells*

Group	Concentration (g/L)	G_0/G_1 phase	S phase	G_2/M phase
Control group	-	52.27±0.54	18.46±0.23	29.27±0.15
Ginger Pinellia ethanol extracts	1	49.54±0.51	25.52±0.24	24.94±0.29
	2	55.87±0.36	32.37±0.28	22.61±0.37
	3	51.72±0.41	36.35±0.22	11.93±0.28

Discussion

Traditional Chinese medicine *Pinellia ternata*, as an antiemetic, plays an important role in clinical practice in China [1].

In the Chinese Pharmacopoeia 2010 edition, four extract varieties of *Pinellia ternata* are included, they are: unprocessed Pinellia, liquorice processed Pinellia, ginger processed Pinellia, and alum processed Pinellia. Extraction procedure of ginger processed Pinellia is as follows: soak clean *Pinellia ternata* in water until completely moist, then remove; decoct ginger slices, add alum and *Pinellia ternata* and boil out, then remove and air dry, or air dry half and dry; or slice and dry [2].

In this study, the degrees of influence of licorice concentrating volume, lime water addition and extraction temperature on ginger Pinellia extraction efficiency are given full consideration. The influence of each level of each factor on ginger Pinellia extraction efficiency is determined through orthogonal experiment. The experimental results show that the degrees of influence of factors on ginger Pinellia extraction results are lime water addition>licorice concentrating volume>extraction temperature in descending order. Based on the orthogonal results and range analysis, the optimal extraction process of ginger Pinellia is identified as licorice concentrating volume of 300 ml, lime water addition of 80, and extraction temperature of 50°C. In addition to the toxicity to mucous membranes, *Pinellia ternata* mainly acts on organs of liver, intestines and kidneys; besides, it also has strong toxicity and teratogenicity to embryos. After extraction, *Pinellia ternata's* mucous membrane irritating effect decreases significantly, which has thus gained broader clinical application.

Cell cycle refers to the process of cell proliferation in which cells produce colonies that resemble themselves through division. The period from the beginning of one cell division to the beginning of the next is called a cell cycle, which is divided into four phases: G1 phase, the phase before DNA synthesis; S phase, DNA synthesis phase; G2 phase, the phase after DNA synthesis; and M phase, mitotic phase [12]. According to modern oncology studies, an increasing number of tumors cause excessive cell growth and reproduction by unbalancing the cell cycle, thereby resulting in dissimilation [5, 9, 8].

In the experiment on anti-proliferative activity of ginger Pinellia on SGC7901 cells, different doses of ginger Pinellia ethanol extracts (1 g/L, 2 g/L, 3 g/L) can all inhibit the proliferation of SGC7901 cells after acting for 24 ~ 72 h. Moreover, the anti-proliferative effect becomes increasingly evident with the increase of drug concentration and prolongation of incubation time. At test doses, cell viability declines continuously with increasing dose.

In addition, ginger Pinellia ethanol extracts also have very significant effects on cell cycle distribution of SGC-7901 cells. Compared with the control group, with the increasing concentration of ginger Pinellia ethanol extracts, the proportion of S phase SGC-7901 cells increases from 18.46% in the control group to 36.35%, while the proportion of G2/M phase cells decreases significantly from 29.27% in the control group to 11.93%. This suggests that the ethanol extracts of ginger Pinellia can arrest SGC-7901 cells in S phase. Clearly, ginger Pinellia ethanol extracts can achieve the therapeutic goal of suppressing tumor activity by affecting cell cycle progression.

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