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Extraction of Total Lignans from Radix Isatidis and Its Antiviral Activity

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Abstract

The orthogonal test method was used to investigate the effects of solvent volume fraction, extraction time and solvent on the extraction process. The results showed that the best extraction process of the total lignans in Radix Isatidis is 70% ethanol, the extraction time is 2 hrs, and the amount of solvent is 8 times. The inhibitory effect of the total lines of Radix Isatidis on RSV was determined by MTT method and cytopathic effect (CPE) method. The total lignans of Radix Isatidis significantly reduced the formation of plaques during the phase of virus adsorption and virus replication. The plaques decreased slightly when the virus entered the cell stage, indicating that the total lignans of Radix Isatidis may exert their anti-RSV during the stage of virus adsorption and virus replication. The extraction conditions of the Radix lignans obtained by orthogonal design experiments are stable and feasible.

Keywords

Radix Isatidis; Total lignans; Orthogonal design; Antiviral

Introduction

Radix Isatidis is a traditional Chinese medicine, derived from the dry roots of the cruciferous plant Isatis indigotica. It has the effect of clearing heat and detoxifying cold blood and pharynx [1,2]. Nowadays, Radix Isatidis is often used clinically for the treatment of bacterial infections and viral diseases, including influenza virus, herpes simplex virus, respiratory syncytial virus (RSV), mumps virus, hepatitis B virus, coxsackie virus and so on [3-6]. It is commonly considered a broad-spectrum natural antiviral drug [7]. However, due to the complex chemical composition of Radix Isatidis, the discovery of the potential medicinal components of Radix Isatidis and the mechanism of action is seriously restricted [8]. The view that there may be a synergistic effect of Banlangen on the human body has been reported. The theory of "pharmacy superposition" proposed by Professor Cai [9] of Peking University shows that most of the active ingredients with similar active structures act on one or more similar targets. Different types of compounds have been isolated from Radix isatidis including indirubin, clemastanin B, alkaloids, lignans, and flavonoids [10,11]. The chemical composition of Banlangen mainly includes three major categories: alkaloids, lignans and organic acids. At present, studies on Banlangen focus on the study of total alkaloids and total organic acid sites [12-14], and there are few reports on the study of lignans. However, the antiviral effect of lignans is significant [15,16], and the total lignans in Radix Isatidis occupies a larger proportion, so the antiviral effect of total lignans in Banlangen cannot be ignored. The extraction process of total lignans from Radix Isatidis was optimized, and its antiviral effect was studied. It provides a theoretical and practical basis for the effective utilization of Radix Isatidis medicinal resources and the synergistic antiviral effects of Radix Isatidis.

Materials

Cells and viruses

Human laryngeal carcinoma epithelial cells (Hep2 cells) were kindly provided by the Institute of Pediatrics, Nanjing University of Chinese Medicine; RSV type, a long strain was provided by Wuhan Institute of Virology, Chinese Academy of Sciences.

Drugs and reagents

Banlangen (Origin: Bozhou, Anhui Province, batch number:20171211), medicinal herbs were provided by the market of Anhui Bozhou medicinal herbs, were identified to meet the "People's Republic of China Pharmacopoeia" (2015 edition) standards; sodium hydroxide (batch number:150708); hydrochloric acid (batch number: 20141126);

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Methanol (analytical alcohol, batch number: 20170110); Ethanol (batch number: 20161216); Ribavirin (Shanghai Yuanye Bio, Batch No. 36791045); DMEM high-glucose medium (US HyClone, Lot No. AAK207235); Pancreatin , Fetal calf serum (US GIBCO, Lot 1801539, 20171026); MTT, DMSO, BSA (Sigma USA, batch number C10036912, 045M4080V, 10735078001); RIPA, phenylmethylsulfonyl fluoride, BCA kit, loading Buffer (Shanghai Biyuntian Biological Co., Ltd., batch number is 0123251105, 0421323721, 03161712517, 0140260519);Phosphatase inhibitor, protease inhibitor (Jiangsu Jikai Biological Technology Co., Ltd., batch number KGP602180214, KGP603024523); anti-GAPDH antibody, goat anti-rabbit fluorescent secondary antibody; Protein Marker (Runsheng Biological Co., Ltd., batch number 03018120); RSV F antibody (batch number sc-101362, purchased from Santa Cruz Biotechnology); RSV G antibody (batch ab94966, purchased from Santa Cruz Biotechnology); 1:29 bis-Acr, 10% SDS, APS (US GIBCO, Lot No. B0153K0103185, 3642A462, B016M052548); TRIS, Glycine (Beijing Soley Company, Lot 562B018, 803B0156); Skim milk powder (Shanghai Wulian Technology Co., Ltd., Lot No. 1216542).

Methods and Results

Optimization of total lignan extraction process of Radix Isatidis

Using L9 (34) orthogonal design [17,18] (Table 1), the volume fraction, solvent amount and extraction time of ethanol were investigated, and the extraction rate and total lignans content obtained under various conditions were determined. The total lignans amount was measured by ultraviolet spectrophotometry. Using larch resin alcohol as a reference substance, through full-wavelength scanning, it can be seen that the reference substance and test solution have the same maximum absorption wavelength at 280 nm, so it is determined to measure at 280 nm. Pipette precision pipette a certain volume of larch resin alcohol standard solution, place it in a 10 ml volumetric flask, add methanol to dilute to volume, and shake, to obtain concentrations of 0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.08 mg/ml, 0.16 mg/ml, 0.32 mg/ml standard solution, respectively. The methanol solution was used as a blank solution to determine the absorbance at the maximum absorption wavelength of the above prepared standard solutions with different concentrations. The concentration of the standard solution was plotted on the abscissa, and the absorbance value A was plotted on the ordinate to draw a standard curve. The total lignans content was calculated and the results are shown in (Table 2).

Level	A ethanol volume fraction/%	B extraction time/h	C solvent amount
1	70	1	6
2	80	1.5	8
3	95	2	12

Table 1: Extraction process factor level

Orthogonal test results show that the total rate of lignans in Radix Isatidis decreases with increasing ethanol volume fraction. The Table 3 variance analysis showed that the three factors of ethanol concentration, solvent doubling amount and extraction time had significant effects on the total lignan content of Radix Isatidis. According to the extreme difference of the Extraction rate, the effects of various factors on the rate of total lignans were as follows: ethanol volume fraction> extraction time> solvent amount. The pharmacological effect was determined by the total lignans content of Radix Isatidis. Therefore, the total lignan content is the main indicator in this experiment. Because the amount of solvent has little effect on the experimental results, adhering to the principle of saving resources, 8 times the amount of solvent is selected for extraction. Therefore, the preferred process conditions are 70% ethanol volume fraction, 2 h extraction time, and 8 times solvent.

Macroporous resin enrichment and purification

The total lignan content of Radix Isatidis was extracted according to the preferred process conditions as described above, the solvent was recovered in a low pressure environment, and the sample was

Test sequence	A ethanol volume fraction/%	B extraction time/h	C solvent amount	D	Total lignin content/%	Extract rate/%
1	1	1	1	1	-	24.63
2	1	2	2	2	-	24.89
3	1	3	3	3	-	25.05
4	2	1	2	3	0.07	23.75
5	2	2	3	1	0.09	23.24
6	2	3	1	2	0.06	23.46
7	3	1	3	2	0.08	23.91
8	3	2	1	3	0.07	22.45
9	3	3	2	1	0.06	22.82
Lignin content						
K1	0	0.15	0.13	0.15		
K2	0.22	0.16	0.13	0.14		
K3	0.21	0.12	0.16	0.14		
R	0.09	0.04	0.03	0.01		
Extract rate						
K1	74.57	72.29	70.54	70.69		
K2	70.45	70.58	71.46	72.26		
K3	69.18	71.33	72.20	71.25		
R	5.39	1.71	1.66	1.57		

Table 2: Orthogonal test scheme and results of extraction process

Factors	Sum of squares of deviations	df	F	P
A	8.823	2	46.44	>0.05
B	0.309	2	1.724	>0.05
C	0.693	2	3.869	>0.05
D	0.346	2		

Table 3: Analysis of variance

prepared to have a mass concentration of 1 g/ml, adjusted to pH=7 with NaOH, and the macroporous resin [19,20] was reprocessed and then wet-packed. Column, the sample was applied to the column, statically adsorbed for 12 hours, and eluted with distilled water to remove unadsorbed components and water-soluble impurities, and then eluted with different volume fractions of ethanol solution, identified by ultraviolet spectrophotometry, and identified as the eluent of the lignan component was dried under reduced pressure to obtain the total lignan of Radix isatidis.

Determination of total lignan content

Precision weighed total lignan sample 0.6 mg, placed in a 10 ml volumetric flask, diluted with methanol to determine the volume, that is, the sample solution. The total lignan content was determined under the same conditions as described above. The total lignan content in the sample after isolation and purification was 74.87%. Total lignan determination methodological investigation.

Precision inspection

1 ml of the test solution was taken and measured as described above (n=6), and the result showed that the RSD value was 0.235%. It shows that the precision of the instrument is good.

Reproducibility study

Six samples (n=6) of the total lignan sample of Radix Isatidis were taken in parallel and measured as described above. The results showed that the RSD value was 2.03%, indicating that the method has good reproducibility.

Stability investigation

Take the appropriate amount of total lignans from Radix Isatidis, dissolve with methanol, and make up to volume. Take 1 ml of the test solution, and measure the absorbance at the time of 0 min, 5 min, 15 min, 30 min, 1 h and 2 h, the absorbance was 0.2305, 0.2386, 0.2317, 0.2400, 0.2339 and 0.1538, respectively. It indicates that the sample solution has good stability within 1 h.

Recovery test

The sample was accurately weighed, and an appropriate amount of the reference substance was added and measured according to the above method. The recovery rates of the samples were calculated to be 98.59%, 103.15%, 98.76%, 99.53%, 102.58%, and 98.36%, respectively.

Antiviral Effects of Total Lignans from Radix Isatidis

Cell lines and virus

Hep2 cells were cultured in DMEM high glucose medium with 10% FBS, and virus amplification and virus virulence assay methods were described in [21].

Calculate virus TCID50=10-5.5/50ul according to Reed-Muench formula.

Cell proliferation assay

Hep2 cells were seeded at 1×10^5 in 96-well plates at 200 μL per well. DMEM (without FBS) was used to dialysis the drug to 7 different concentrations. When the cells are full of the bottom of the holes, the above-mentioned formulated drugs were added. Continue to culture in a constant temperature incubator for 24 h, then add 10 ul of 5% MTT [22] per well, incubate in the incubator for 4 hours, remove the culture solution, add 100 ul of DMSO per well, shake at low speed for 10 min, and measure the absorbance at 490 nm on a microplate reader. The results are shown in Figures 1 and 2.

Direct anti-RSV study of total lignans

Hep2 cells were seeded in 6-well plates, and different concentrations (50, 100, 200 μg/mL) of total lignans were incubated with RSV on ice. The whole process is carried out on ice to prevent the virus from entering the cell. After 2 h of incubation, the cells were washed with pre-cooled medium to remove unbound virus. The cells were then lysed and the RSV G and F proteins used to detect cell attachment were analyzed by immunoblot [23]. The result is shown in Figure 3.

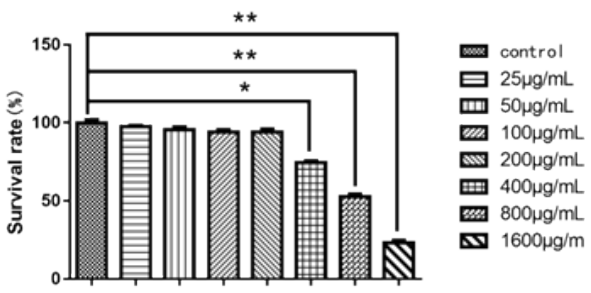
Hep2 cells were seeded in 6-well plates and incubated with RSV (500 μL virus titer of 100 TCID50/well) for 2 hours on ice, allowing virus attachment in the presence or absence of the indicated concentration of drug. The non-attached virus was removed with ice-cold medium and then allowed to adsorb at 37°C for 2 hours to allow the attached RSV to enter the host cell. At the end of the incubation period, the culture medium was removed and the culture was replaced with agarose containing 1% of 2.5% calf serum and incubated at 37°C for 6-7 days. The formation of plaques was observed daily. The result is shown in Figure 4.

Hep2 cells were inoculated into 6-well plates, 500 ul of 100 TCID50 virus solution was added to each well, and placed in a constant temperature incubator for 2 h, shaking once every 20 min. After the end of the adsorption, the virus suspension in the culture plate was discarded, and replaced with agarose containing 2.5% calf serum 1%, and different concentrations of the drug were added, and the mixture was incubated at 37°C to observe the formation of plaques every day. The result is shown in Figure 4.

The RSV F protein and RSV G protein were not expressed in the normal cell group, and the RSV F protein and RSV G protein expression levels were higher in the virus group, indicating that the virus adsorption amount was higher in 2 hours. Compared with the virus group, each drug group RSV The expression of F protein and RSV G protein decreased to varying degrees ($p<0.01$). With the increase of drug concentration, the expression of RSV F protein and RSV G protein decreased in turn.

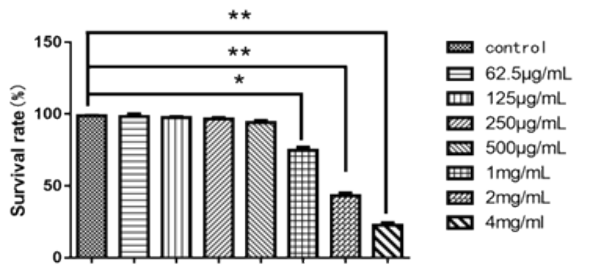
Discussion

The chemical composition of Radix Isatidis is very complicated, which seriously hinders the research process of Radix Isatidis. It has been shown in the literature that compounds with larger content may play a leading role in the overall content and efficacy of such components [9], and the total lignan content has a large proportion in Banlangen, and its antiviral effect is diverse and the mechanism of action is not clear. The study on the antiviral effect of total lignans



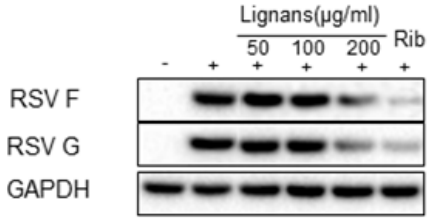
The results showed that the concentration of lignans in Radix Isatidis was less than 200 μg/ml had no effect on cell survival, and that higher than 200 μg/ml all inhibited the proliferation of Hep2 cells in a dose-dependent manner, so the maximum non-toxicity of total lignans in Radix Isatidis The concentration is about 200 μg/ml.

Figure 1: The effect of lignans of Radix Isatidis on Hep2 cells survival rate (x ± s, *n=6*)



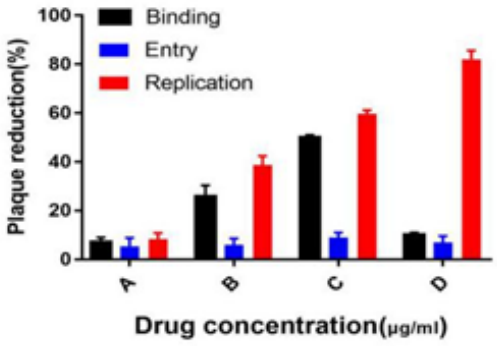
As shown in Figure 2, the concentration of ribavirin below 500 μg/ml had no effect on cell survival, and higher than 500 μg/ml dose-dependently inhibited the proliferation of Hep2 cells, so the maximum nontoxic concentration of ribavirin was about 500 μg/ml.

Figure 2: The effect of Ribavirin on Hep2 cell survival rate(x ± s, *n=6*)



The RSV F protein and RSV G protein were not expressed in the normal cell group, and the RSV F protein and RSV G protein expression levels were higher in the virus group, indicating that the virus adsorption amount was higher in 2 hours. Compared with the virus group, each drug group RSV The expression of F protein and RSV G protein decreased to varying degrees (p<0.01). With the increase of drug concentration, the expression of RSV F protein and RSV G protein decreased in turn.

Figure 3: Lignans treatment suppresses RSV F and G protein expression



(A) The total lignans low dose group (50 μg/mL); (B) The total lignans medium dose group (100 μg/mL); (C) The total lignans high dose group (200 μg/mL); (D) Ribavirin group. As shown in Figure 3, the addition of lignans only slightly reduced the number of plaques at the stage of entry of the virus into the cells, indicating that the compound does not exert its antiviral effect against RSV entering the cell stage. At the virus binding and replication stage, as shown in Figure 3 (black bars and red bars), the drugs in the total lignans of Radix is added, causing a significant reduction in plaques at this stage, and the number of plaques decreased with the increase of concentration of lignans, it indicates that the compound may exert its antiviral effect by hindering virus adsorption and inhibiting viral replication. In the stage of virus replication, total lignans inhibit viral replication less than ribavirin group.

Figure 4: Inhibitory effect of lignans on RSV infection

is of progressive significance to elucidate the antiviral mechanism of Radix Isatidis, so its antiviral effect needs to be proved. However, since the lignans are mainly polar substances and there is no specific color reaction and identification method, it is difficult to enrich, because the research in this part is still blank, so the extraction and purification of the total lignans from Radix Isatidis the process needs to be unified.

In this study, we used orthogonal test to optimize the extraction process of total lignans from Radix Isatidis. The effects of ethanol concentration, solvent dosage, and extraction time on the total lignans content of Isatis indica were investigated. The method is stable, reliable and suitable for applicability; macroporous resin adsorption separation technique was used to separate and enrich the extracted samples. For the problem of how to effectively avoid the absorption of impurities, our study uses the wavelength, which the total lignans have higher UV absorption and the impurity has no or less absorption, as the detection wavelength of this experiment to avoid the interference of impurities. For the problem of how to purify the total lignans of Radix Isatidis more effectively, this study fully considers that the research object is the headquarters with the same basic nuclear structure, according to the principle of similarity of ultraviolet absorption of the same basic nuclear structure, so Ultraviolet spectrophotometry was used to collect the lignans components in sections, and other components of the UV absorption spectrum were discarded to purify and increase the total lignan content, which provides a basis and guarantee for the study of the pharmacological effects of the total lignans in Radix Isatidis.

For the study of the pharmacological activity of the total lignans in Radix Isatidis, we first separated the total lignans from Radix isatidis and used plaque reduction experiments (CPE method is a classic method for studying viral infection experiments. This method is simple, intuitively), the anti-RSV effects were studied from the three stages of adsorption, entry and replication of virus invading cells. The results showed that the total lignans of Radix Isatidis could significantly inhibit the formation of plaques during RSV adsorption and replication. Although this study shows that the total lignans of Radix Isatidis can exert antiviral effects during the adsorption and replication stages, it is still unknown for the mechanism of how the target exerts antiviral activity. And because this article is limited to respiratory syncytial virus, it does not mean that the total lignans are effective against all viruses. So, further research is needed.

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