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Anti−HSV−1 activity *in vitro* of extracellular polysaccharides purification of *Paecilomyces lilacinus* on isolated from Hainan mangrove

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ABSTRACT

Objective: To explore the antiviral activity on HSV-1 of the extracellular polysaccharides (EPS) purification of *Paecilomyces lilacinus* (*P. lilacinus*) isolated from mangrove in Hainan province. **Methods:** The toxicity of the EPS purification on Vero cells and its anti-HSV-1 activity were assessed by cytopathic effect(CPE) and MTT assay. The Vero cells survival rates, HSV-1 inhibition rates by the purification and virus titer were calculated. **Results:** The purification showed little cytotoxic effect on Vero with a CC_{50} value of 735.49 μg/mL. It could inhibit HSV-1 absorption on Vero cells, and there was a significant difference (P<0.01) compared with control group (virus group), and the highest inhibition ratio was 35.0% at dose of 400 μg/mL; The biosynthesis of HSV-1 could be inhibited by the extract with dose-dependent manner, and the IC_{50} value to the viruses was 387.26 μg/mL, and the highest inhibition ratio was 61.3% at dose of 400 μg/mL; but the purification couldn′t inactivate HSV-1 directly. **Conclusion:** The EPS purification had certain antiviral effect, it could inhibit HSV-1 absorption and biosynthesis with a dose effect relationship.

1. Introduction

Herpes simplex virus type 1 (HSV-1) which belongs to herpes virinae is a ball type linear double stranded DNA virus. It is one of the most common human viral disease virus causing infection of skin and mucous membrane and nerve tissue and other parts of body, and is the first reason cause blindness in corneal inflammation of corneal disease[1,2]. The commonly used anti HSV-1 drugs mainly are nucleoside capable of interfering virus DNA polymerase activity, but this kind of drug could induce side effects, the recurrence rate is high, drug resistance is easy to produce thus the treatment effect is limited[3,4], so looking for antiviral drugs with low toxicity and high efficiency is of particular importance.

Our previous research isolated a strain of *Paecilomyces lilacinus* (*P. lilacinus*)[5] from mangrove forest in Hainan. Those extracellular polysaccharide (EPS) has certain antiviral action *in vitro*[6,7], but the effect is limited, when it shows good antiviral effect at the final concentration of polysaccharide reached 1 000 g/mL or above which is probably related to lower purity of polysaccharide. Further research on has lead to purification with sugar content of 63.5%. This study intends to model in vitro further detection of the purified anti HSV effect of HSV -1 in Vero cells and to explore its antiviral mechanism, screening for new antiviral drugs from the bioactive substances of marine origin in basic research.

2. Materials and methods

2.1. Materials

2.1.1 Drug

P. lilacinus EPS purified by our lab preparation, double distilled water with 10 g/L 0.22 μm mother liquor, membrane filtration, packaging, -20 ℃ stored at standby. In the presence of DMEM, the

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solution is diluted to the required concentration.

2.1.2 Kit and cell line

African green monkey kidney cell (Vero cells, ATCC CCL81), DMEM medium (Hyclone), fetal bovine serum (Hangzhou Sijiqing), trypsin (AMERSCO), four methyl thiazolyl tetrazolium salt (MTT, Sigma).

2.1.3 Virus strain

HSV-1 virus strains were stored in our laboratory.

2.1.4 Equipment

Biological safety cabinet (SG403A-HE, USA), enzyme immunoassay (Shanghai Techcomp Instrument Co. Ltd), CO_2 incubator (NU-4750E, USA), inverted microscope (Shenzhen tuotian Instrument Equipment Co., Ltd), electronic balance (Saiduoli Adams Scientific Instruments Co. Ltd. (Shanghai), low speed centrifuge Anting scientific instrument factory), 6 hole and 96 hole cell culture plate (Corning, USA).

2.2. Method

2.2.1 HSV-1

Vero cells which has grown into a monolayer were fetched from cultured liquid at 6 well plates, then added 0.5 mL HSV-1, and stored at incubator at the atmosphere of 37 ℃, 5% CO_2 for 2 h. After virus liquid were abandoned, the cultured liquid were washed with PBS rinse and then DMEM was added and it was maintained at incubator (37 ℃, 5% CO_2) to culture, the medium was changed once per day and CPE were observed under an inverted microscope. When cells were reached at +++~++++ put it at -80 ℃ freezing and thawed 3 times for cell lysis, finally put it at 1 000 r/min centrifugation for 10 min, and Torikami Kiyo was cultured to 3 generations. Last passage of virus was loaded to 0.5 mL vials, cryopreserved at -80 ℃ for further use.

2.2.2 Determination of virus infection

Took 100 L HSV-1 strains for determination and then maintained with DMEM for 10 fold dilution series (10-1-10-9), and inoculated in 96 pore plate Vero cell for growth, 100 μL in each hole . 6 wells was set for each dilution concentration. Meanwhile normal cells without virus liquid was set as control, and stored at 37 ℃, 5% CO_2 incubator for 2 h. After discarded the supernatant in each hole, added complete medium l00 L. Observed CPE daily, when the cell CPE was no longer progress (about D 5-6) stopped titration, calculate the virus half infection ($TCID_{50}$) according to-Muench Reed formula[8] in order to determine the virus titer.

The logarithm of the $lgTCID_{50}$= distance ratio difference of logarithm of the dilution degree + logarithm of dilution degree higher than the 50% lesion rate.

Range ratio = (percentage of cells with abnormal rate higher than 50% -50%) / (percentage of cells with abnormal rate higher than 50% - percentage of cells with abnormal rate lower than 50%)

2.2.3 Drug cytotoxicity determination

EPS purification were diluted with DMEM (600, 500, 400, 300, 200, 100, 50, 25, 12.50 μg/mL), and then added to Vero cells of good growth state, 100 μL per hole, 6 holes were set for each concentration, liquid without drugs was set as control which was stored at 37 ℃ and 5% CO_2 incubator training for 72 h. Four hours before the end of experiment, 20 μL MTT at concentration of 5.0 mg/mL were added to each hole and cultured for 4 h,

100 μL DMSO was added into each hole, and shaped for 5 min. OD was measured at 570 nm. The survival rate of Vero cells under the action of each concentration and the concentration induce 50% toxic in cells (CC_{50}) was calculated.

Cell viability = The average absorbance value of the treatment group/The average absorbance value of the control group* 100%;
Inhibition rate =100%- Cell viability
CC_{50}=Antilog [log (<50% concentration)+[(50-<50% cell death rate)/ (>50% cell death rate -<50% cell death rate)]* [log (>50% drug concentration -log (<50% drug concentration)]

2.2.4 Drug

Direct inactivation of virus Various concentrations of EPS and purified 100TCID50 virus liquid were mixed and stored at 37 ℃ for 2 h, then after the virus solution and purified EPS mixture was treated with 10 fold serial dilutions, Vero cells were planted into 96 hole plate, grown into a single layer, each hole 100 L infection, 2 after H in culture medium, 37 ℃, 5% CO_2 incubator training, daily observation of CPE, CPE +++~++++ for virus control hole and cell control, suction change medium, the virus detection method of MTT inhibition rate. The concentration of each drug is provided with 6 multiple holes, while the virus control group and normal cell control group were set up. The experiment was repeated 2 times.

2.2.5 Medicine

Virus adsorption Purified EPS at different concentrations were added to Vero cells infected with 100 TCID50 HSV-1, and then were incubated for 2 h at 37 ℃, virus and drug that were not adsorbed were removed, added maintenance liquid, and then cultured at 37 ℃ , 5% CO_2 incubator, observed CEP every other day. When CPE grew to be +++~++++ and the control virus remained normal discarded the culture liquid. Measured the OD with MTT method, each concentration of CEP was cultured in 6 multiple holes, and virus control group and normal cell control group were set up. All the experiment was repeated twice.

Inhibition rate (%) = (A value of the test group - A value of the virus control group)/ (A value of the normal cell control group - A value of the virus control group)

2.2.6 Effects of drugs biosynthesis

value of MTT method. Each drug concentration is provided with 6 multiple holes, a virus control group and a normal cell control group. The experiment was repeated 2 times.

2.2.7 Effect of drug release

EPS at different concentrations were added to Vero monolayer infected with 100 TCID50 HSV-1 cultured in 96 holes plate, and then stored in incubator at for 2 h 5% CO_2 at 37 ℃. When CPE grew to be +++~++++ and the control virus remained normal, terminated the culture and separated the culture liquid which was further stored at -70 ℃. Measured the OD with MTT method, each concentration of CEP was cultured in 6 multiple holes, and virus control group and normal cell control group were set up. All the experiment was repeated twice. After 3 times of melting, virus titers in the cell and culture liquid were determined by PFU. The virus control group and normal control group were also established. The experiment was repeated twice.

Percentage inhibition = Intracellular virus/(intracellular virus + culture fluid)*100%

2.3 Statistical treatment

SPSS 11.5 software was applied for statistical analysis, EPS purification of the cell toxicity test and antiviral experiment MTT method were expressed as $\bar{x}\pm s$ the measurement data is compared with the t test, the test level a=0.05.

3. Results

3.1 Determination of virus titer

According to Reed-Meuench method to calculate the virus TCID50 of $10^{-5.4}$, the inoculation titer of $10^{-5.4}$ 100 μL of the virus can make 50% of the Vero cells significant lesions, the experimental virus with 100 $TCID_{50}$.

3.2 Drug toxicity of Vero cells

Figure Purified EPS showed no significant proliferation on Vero cells, and vero cells treated with EPS purified at 400 μg/mL or less showed no morphological changes under light microscope. The cell viability was more than 90%. When the concentration reached 600 μg/mL, cell morphology of the experimental group rounded down, part of the cells shedding death, so concentration of EPS purification using for experiment should be below 400 μg/mL (Figure 1, 2). Cell viability was determined by MTT assay. The half-dose concentration (VCC) of Vero cells was calculated to be 735.49 μg/mL.

3.3 The direct effect of drugs on the virus

The $TCID_{50}$ of HSV-1 was $10^{-4.1}$, indicating that the purified product had no direct inactivation effect on HSV-1 in the same concentration as the virus control.

3.4 Drugs on the role of virus adsorption

The results are shown in Table 1. It can be seen that different concentrations of EPS purification have different degrees of cytopathic effect, compared with the virus control group (P<0.01), and the inhibition rated increased as the concentration of purified substances increased showing a certain dose-effect relationship. In particular, after treatment of 100-400 μg/mL of purified EPS, Vero cell viability can be more than 30%, indicating that it has a certain interference HSV-1 adsorption, but can not produce complete inhibition.

3.5 Drugs on the impact of viral biosynthesis

The HSV-1 biosynthesis of HSV-1 was inhibited to a certain extent in the concentration range, and the inhibitory rate increased gradually with the increase of the concentration. The CPE of the HSV-1 cells was gradually reduced, showing a certain dose-response relationship (HSV-1 regression equation Y = 0.000 86X + 0.167, R = 0.979, P<0.01), the IC_{50} was 387.26 μg/mL, the inhibitory rate was above 50% in the concentration range of 100-400 μg/mL indicating that it can inhibit virus biosynthesis, but still did not produce complete inhibition.

3.6 Drugs on the impact of viral release

No significant difference was found in the concentration of EPS purified from the control group. The results showed that the ratio of virus to total virus in the drug group was not significantly different from that of the control group. The experiment was repeated twice and the results were the same.

Table 1
Effects of EPS purification on HSV-1 adsorption and biosynthesis.

Groups	Drug concentration (μg/mL)	Drug on the adsorption of the virus			Effect of drugs on virus biosynthesis		
		CPE	OD value	Inhibition rate (%)	CPE	OD value	Inhibition rate (%)
Polysaccharide	6.25	++++	0.432±0.030	5.6	++++	0.446±0.010	6.5
	12.50	++++	0.455±0.050	10.2	++++	0.512±0.040	19.1
	25.00	++++	0.500±0.110	18.9	+++	0.582±0.080	32.4
	50.00	++++	0.527±0.080	24.2	+++	0.623±0.010	40.3
	100.00	+++	0.562±0.010	31.2	++	0.687±0.040	52.3
	200.00	+++	0.574±0.120	33.4	++	0.714±0.110	57.6
Virus control	400.00	+++	0.582±0.150	35.0	++	0.733±0.130	61.3
Cell control	–	++++	0.403±0.120	–	++++	0.412±0.080	–
	–	–	0.914±0.060	–	–	0.936±0.060	–

Table 2
Effect of EPS Purification on HSV-1 Release.

Purification concentration (μg/mL)	Virus		Percentage (%)
	In the cell	Culture medium	
6.25	8.7×10^6	4.2×10^5	95.4
12.50	7.9×10^6	3.5×10^5	95.8
25.00	8.1×10^6	5.2×10^5	94.0
50.00	8.7×10^6	4.9×10^5	93.6
100.00	7.8×10^6	3.6×10^5	95.6
200.00	8.3×10^6	4.1×10^5	95.3
400.00	8.6×10^6	4.8×10^5	94.7

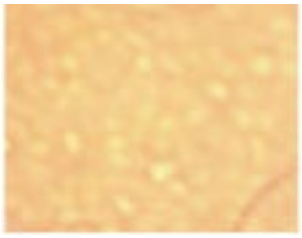

Figure 1. Normal Vero cell.

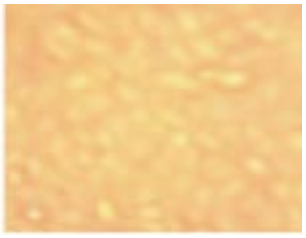

Figure 2. 400 μg/mL EPS purified Vero cells .

4. Discussion

In addition to energy substances and cell components, polysaccharides also involved in all cell activities, such as cell information transferring, sensation, division, and regeneration, it is considered to be the third life chain following protein and nucleic acid. The use of similar structure and function of different polysaccharides intervene in cell metabolism and pathogen multiplication in order to achieve anti-tumor, anti-viral effect is feasible[9,10]. Mangrove fungi are the second largest microbial resources in the mangrove ecosystem[11]. They have long lived in an oligotrophic, weakly alkaline salt-bearing marine environment, forming a unique resistance to hunger, alkali and salt and other living mechanisms, thus it can produce completely different from the terrestrial fungi polysaccharides bioactive substances[12,13]. Studies have shown that mangrove fungi exopolysaccharide mainly heteropolysaccharide, has good antiviral, antitumor and immune regulation and other biological activities[14,15].

In this study, Vero cells were used as virus infecting target cells to study the anti-HSV-1 activity of EPS purified from *P. lilacinus* in vitro and the results showed that the purified product had no obvious proliferative effects on Vero cells. At concentration of 400 μg/mL or lower, its toxicity to Vero cells was slight, and the half toxic concentration was 735.49 μg/mL. The purification of HSV-1 and HSV-1 in different concentrations showed no direct killing effects on the HSV-1 infection, but it could interfere with the virus adsorption, compared with the virus control group, the difference was statistically significant ($P<0.01$). Purified Vero cells at concentration of 1 001-400 μg/mL can make the survival rate of 30% or more, indicating that the polysaccharide may have a certain effects of blocking, changing the cell surface of the virus receptor or compete with other virus receptor, or activating the cells to be in anti-virus status. It is also shown that purified EPS has inhibitory effects on HSV-1 biosynthesis with a half inhibitory concentration of about 387.26 μg/mL. Especially at the concentration range of 100-400 μg/mL, the half inhibition rate could be 50% or more, indicating that the purified product has a good inhibition of viral biosynthesis, but the suppression targets need to be further studied.

In conclusion, we can see that the EPS purified from the mangrove-derived mangrove in Hainan has anti-HSV-1 effects, which can inhibit the virus adsorption and biosynthesis to some extent. When the concentration of the purified EPS reached 400 μg/mL, the inhibitory rate was 35.0% and 61.3%, respectively. The inhibition of HSV-1 biosynthesis was stronger than that of inhibition of sorption, but both effects could not be completely inhibite. This study is only the preliminary study on the antiviral effect of EPS purified from *P. lilacinus*, and its structure and action mechanism still need further study.

Reference

[1] Leopardi R, Roizman B. Functional interaction and colocalization of the herpes simplex virus 1 major regulatory protein ICP4 with EAP, a nucleolar-ribosomal protein. *Proc Natl Acad Sci USA* 2014; **93**(10): 4572-4576.

[2] Szpara ML, Parsons L, Enquist LW. Sequence variability in clinical and laboratory isolates of herpes simplex virus 1 reveals new mutations. *J Virol* 2010; **84**(10): 5303-5313.

[3] Antoine TE, Park PJ, Shukla D. Glycoprotein targeted therapeutics: a new era of anti-herpes simplex virus-1 therapeutics. *Rev Med Virol* 2013; **23**(3): 194-208.

[4] Fan Jianyong, Zhao Yang, Yang Huilan. The biological characteristics of herpes simplex virus and its latent recurrence mechanism. *J Diagn Ther Dermato-venereol* 2015(1): 82-85.

[5] Rao Lang-yu, Chen Zheng-liang, Pei Hua. Screening and identification of a mangrove fungus possessing immunoenhancement activity. *China Trop Med* 2011; **11**(6): 683-685.

[6] Pei Hua, Lin Ying-zi, Rao Lang-Yu, Wang Yong-xia, Niu Li-na, Wang Hua-min. Antiviral activities of exopolysaccharide from one fungi strain isolated from mangrove in Hainan province *in vitro*. *J Hainan Med Univ* 2012; **18**(2): 152-154.

[7] Pei Hua, Lin Ying-zi, Rao Lang-yu, Wang Yong-xia, Niu Li-na, Wang Hua-min. Study on antiviral activities of a fungi strain isolated from mangrove to herpes simplex virus type 2. *Hainan Med J* 2012; **23**(3): 1-3.

[8] Fu Jihua. *Virology practical experimental technology*. Jinan: Shandong Science and Technology Press; 2001, p. 61-63.

[9] Zhang N, Wardwell PR, Bader RA. Polysaccharide-based micelles for drug delivery. *Pharmaceutics* 2013; **5**(2): 329-352.

[10] Jin F, Zhuo C, He Z. Anti-herpes simplex virus activity of polysaccharides from *Eucheuma gelatinae*. *World J Microbiol Biotechnol* 2015; **31**(3): 453-460.

[11] Chen YL, Mao WJ, Tao HW. Preparation and characterization of a novel extracellular polysaccharide with antioxidant activity, from the mangrove-associated fungus fusarium oxysporum. *Marine Biotechnol* 2015; **17**(2): 219-228.

[12] Ameen F, Moslem M, Hadi S. Biodegradation of diesel fuel hydrocarbons by mangrove fungi from Red Sea Coast of Saudi Arabia. *Saudi J Biological Sci* 2015; **21**(2): 21-28.

[13] Wang Y, Barth D, Tamminen A. Growth of marine fungi on polymeric substrates. *BMC Biotechnol* 2016; **16**(1): 1-9.

[14] Kumaran S, Ramaprasanna D, Murugaiyan K. Production and characterization of exopolysaccharides (EPS) from mangrove filamentous fungus, *Syncephalastrum* sp. *Afr J Microbiol Res* 2014; **8**(21): 2155-2161.

[15] Yan M, Mao W, Chen C. Structural elucidation of the exopolysaccharide produced by the mangrove fungus *Penicillium solitum*. *Carbohydrate Polymers* 2014; 111C(1): 485-491.