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• BASIC RESEARCH •

In vitro anti-coxsackievirus B_3 effect of ethyl acetate extract of Tian-hua-fen

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Abstract

AIM: To investigation the anti-coxsackievirus B_3 (CVB_{3m}) effect of the ethyl acetate extract of Tian-hua-fen on HeLa cells infected with CVB_{3m}.

METHODS: HeLa cells were infected with CVB_{3m} and the cytopathic effects (CPE) were observed through light microscope and crystal violet staining on 96-well plate and A_{600} was detected using spectrophotometer. The protective effect of the extract to HeLa cells and the mechanism of the effect were also evaluated through the change of CPE and value of A_{600}.

RESULTS: The extract had some toxicity to HeLa cells at a higher concentration while had a marked inhibitory effect on cell pathological changes at a lower concentration. Consistent results were got through these two methods. We also investigated the mechanism of its anti-CVB_{3m} effect and the results indicated that the extract represented an inhibitory effect through all the processes of CVB_{3m} attachment, entry, biosynthesis and assemble in cells.

CONCLUSION: The results demonstrate that the ethyl acetate extract of Tian-hua-fen has a significant protective effect on HeLa cells infected with CVB_{3m} in a dose-dependent manner and this effect exists through the process of CVB_{3m} attachment, entry, biosynthesis and assemble in cells, suggesting that the ethyl acetate extract of Tian-hua-fen can be developed as an anti-virus agent.

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INTRODUCTION

Tian-hua-fen is the dried root of *Trichosanthes kirilowii Maxim* or *Trichosanthes japonica Regel*. The major component of it is mass starch, various amino acids, phytohemagglutinin, saccharide, saponin and some other things[1]. Tian-hua-fen was mentioned in Compendium of Materia Medica written by Li Shizhen in the late 14th Century as a drug to reset menstruation and facilitating the expulsion of retained placenta. For a long time, Tian-hua-fen had been used in the powdered form in conjunction with other Chinese herbal medicines to induce abortion[2]. Clinical applications over the years proved that Tian-hua-fen had multiple pharmacological effects, such as termination of pregnancy, anti-tumor, anti-inflammation, anti-virus and immunoregulation and so on[3-5]. The anti-virus, especially anti-HIV-1, effect of trichosanthin (TCS) has been known to us. But to the author's knowledge research about the non-protein parts of Tian-hua-fen is rare. Coxsackievirus B is the major pathogen of viral myocarditis and now there is no effective therapeutic drug. In the process of screening anti-virus agents from Chinese medicinal herb we found that the nonprotein parts of Tian-hua-fen had a notable anti-virus effect *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of the ethyl acetate extract of Tian-hua-fen

A total amount of 200 g Tian-hua-fen powder (Shanghai drug store) was macerated with 2 L 750 mL/L ethanol overnight, then was boiled in water under reflux for 3 h and the boiled fluid was filtered. The filtrate was evaporated under reduced pressure to gain a residue. The residue was suspended in water and partitioned with petroleum ether, ethyl acetate and n-BuOH (Analytical pure, Shanghai chemical company Ltd) successively. The four fractions were evaporated under reduced pressure to give petroleum ether fraction (0.56 g), ethyl acetate fraction (0.82 g), n-BuOH fraction (0.85 g) and aqueous fraction (12.7 g), respectively.

Preparation of HeLa cells and titration of virus titer

HeLa cells were stored in liquid nitrogen with 100 g/L dimethyl sulphoxide (DMSO) and 900 mL/L fatal calf serum (FCS) and maintained in culture flasks in complete RPMI 1640 medium (Gibcol, BRL America). Subculture was carried out every 2-3 d after it had formed a confluent monolayer. CVB_{3m} (Stored by our laboratory) was serially diluted to 10^{-10} with non-FCS RPMI1640 culture medium. On 40-well plate, 0.025 mL CVB_{3m} with variable dilution and 0.025 mL non-FCS RPMI1640 culture medium were added to each well. Finally, 0.05 mL viable HeLa cells (3×10^5/mL) were added. Each dilution was quadrupled and normal HeLa cells co-cultured only with RPMI1640 containing 10 mL/L FCS were prepared as negative control at the same time. Then the cells were incubated at 37 ℃ with 50 mL/L CO_2 for 72 h. The cytopathic effects (CPE) were observed under light microscope. The titer at which cells appeared 50% CPE was designated 1 $TCID_{50}$ (50% tissue culture infectious doses). A 100 $TCID_{50}$ was used as the infectious titer in the following experiment.

Assay of the toxicity of the extract to HeLa cells

A total of 5 mg extract was dissolved in 5 μL DMSO, then 2.5 mL deionized water was added and the liquid was sterilized at 115 ℃ for 20 min. After cooling to 55 ℃, 2.5 mL 2×RPMI 1640 culture medium (without FCS) was added to make the end concentration of the extract (1 mg/mL). The original liquid

was diluted with non-FCS RPMI 1640 culture medium serially from 1:2 to 1:1 024. A total of the 0.025 mL sample with various concentration and 0.025 mL of the same culture medium were added to each well on 96-well plate. Finally 0.05 mL viable HeLa cells (3×10^5/mL) were added. Each concentration of the sample was quadrupled. Two controls, HeLa cells co-cultured only with RPMI1640 (containing 100 mL/L FCS) and 1 g/L DMSO respectively were prepared synchronously. Cells were incubated at 37 ℃ with 50 mL/L CO_2 for 72 h. CPE was observed under light microscope and the concentration at which cells appeared <50% CPE (compared with that of extract-free cultures) was regarded as the lowest toxic concentration. In addition, cells were stained with 5 g/L crystal violet (Ameresco) and A_{600} was detected using spectrophotometer.

Assay of the anti-CVB_{3m} effect of the extract

The extract was diluted serially from 1:256 at which it had no toxicity to HeLa cells to 1:8 192 with non-FCS RPMI 1640 culture medium. Then 0.025 mL extract with variable concentration was added to each well of 96-well plate. Then 0.025 mL 100 $TCID_{50}CVB_{3m}$ was overlaid. After incubation at 37 ℃ with 50 mL/L CO_2 for 1 h, 0.05 mL viable HeLa cells (3×10^5/mL) were added. Each concentration was quadrupled and three controls, normal HeLa cells co-cultured only with RPMI1640 (containing 100 mL/L FCS), 100 $TCID_{50}CVB_{3m}$ and the extract (1:256 mg/mL), respectively were prepared synchronously. Cells were grown for 3 days and then CPE was observed under light microscope. Later, the cells were stained with 5 g/L crystal violet and A_{600} was measured using spectrophotometer.

Primary study on the mechanism of anti- CVB_{3m} effect of the extract[6]

The extract was diluted with non-FCS RPMI1640 culture medium from 1:256 to 1:8 192. HeLa cells (1.5×10^4/well) were seeded onto three 96-well plates and allowed to attach to the well bottom. When the cells were confluent the culture medium was discarded and the cells were rinsed twice with the same culture medium. The cells on three plates were treated respectively as follows: The first plate: 0.025 mL 100$TCID_{50}CVB_{3m}$ and 0.025 mL extract of variable concentration were added to each well. After incubation at 37 ℃ with 50 mL/L CO_2 for 1 h, the mixture was substituted with 0.1 mL non-FCS RPMI1640 culture medium; the second plate: 0.025 mL 100$TCID_{50}$ CVB_{3m} and 0.025 mL extract of variable concentration were added. The plate was incubated at 37 ℃ with 50 mL/L CO_2 for 1 h. Then the mixture was substituted with 0.025 mL extract of variable concentration and 0.075 mL non-FCS RPMI1640 culture medium; the third plate: 0.025 mL 100$TCID_{50}CVB_{3m}$ and 0.025 mL non-FCS RPMI1640 culture medium were added first. After incubation at 37 ℃ with 50 mL/L CO_2 for 1 h the mixture was discarded and 0.025 mL extract of variable concentration and 0.075 mL non-FCS RPMI1640 culture medium were overlaid. Three controls, HeLa cells co-cultured only with RPMI1640 containing 100 mL/L FCS, 100$TCID_{50}$ CVB_3m and extract (1:256 mg/mL) respectively were prepared synchronously and each extract concentration was quadrupled. At last all plates were incubated at 37 ℃ with 50 mL/L CO_2. When the cells treated with CVB_{3m} appeared 100% CPE, the cells were stained with 5 g/L crystal violet and A_{600} was detected using spectrophotometer.

RESULTS

Titration of CVB3m titers

The incubation was terminated after 72 h and CPE was observed under light microscope. The results showed the cells in all quadrupled wells treated with CVB_{3m} at titer of 10^{-1}-10^{-6} appeared 100% CPE. About the cells treated with CVB_{3m} at titer of 10^{-7}, the cells in two wells appeared 100% CPE while the others appeared 50% CPE. In regards to the cells co-cultured with CVB_{3m} at titer of 10^{-8}-10^{-10}, the cells in four wells appeared no CPE completely. Then $TCID_{50}$ was calculated as 10^{-7} according to Reed-Muench method[7].

Assay of toxicity of ethyl acetate extract to HeLa cells

After incubation for 72 h, CPE induced by the extract was observed under light microscope. The results showed the cells co-cultured with extract at concentration from 1:2-1:128 mg/mL appeared CPE of different degrees, while the cells co-cultured with extract at concentration from 1:256 to 1:8 192 mg/mL appeared no CPE. The 50% toxic concentration was 1:128 mg/mL according to Reed-Muench method. The value of A_{600} also indicated that the extract had no toxicity to HeLa cells from 1:256 mg/mL. The cells co-cultured with 1 g/L DMSO appeared no CPE, which showed that DMSO at this concentration had no toxicity to HeLa cells (Table 1).

Assay of the anti-CVB_{3m} effect of the extract

From 1:256 to 1:8 192 mg/mL the extract showed various protective effects to HeLa cells and the effect was decreased with the increased dilution. The protective effect was best at concentration from 1:256 to 1:1 024 mg/mL. The minimal effective inhibitory concentration (EIC) was 1:8 192. The value

Table 1 Cytotoxity of ethyl acetate extract to HeLa cells shown in the value of A_{600}

Dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1 024
Value of A_{600}	0.20±0.029	0.22±0.030	0.32±0.022	0.29±0.069	0.38±0.043	0.59±0.035	0.69±0.075	1.30±0.069	1.23±0.061	1.29±0.056

The A_{600} value of the cells co-cultured with the extract at concentration from 1:2 to 1:128 mg/mL was lower than that of normal HeLa cells (1.32±0.034), which showed that the extract had some toxicity to HeLa cells at these concentrations. From 1:256 mg/mL the A_{600} value became close to that of normal HeLa cells, which indicated that from 1:256 mg/mL the extract had no toxicity to HeLa cells again.

Table 2 Protective effect of ethyl acetate extract on HeLa cells shown in the value of A_{600}

Dilution	1:256	1:512	1:1 024	1:2 048	1:4 096	1:8 192
Value of A_{600}	1.09±0.017	1.02±0.122	0.89±0.060	0.91±0.039	0.83±0.048	0.77±0.028

The A_{600} value of the cells co-cultured only with RPMI1640 was 1.32±0.02; the A_{600} value of the cells co-cultured only with 10^{-5} CVB_{3m} was 0.22±0.026; and the A_{600} value of the cells co-cultured only with extract at concentration of 1:256 mg/mL was 1.21±0.042. As shown in the table the value of A_{600} of the cells treated with extract is higher than that of the cells infected with CVB_{3m} while not treated with the extract ($^bP<0.01$), indicating the protective effect of the extract on' HeLa cells from CVB_{3m} infection.

Table 3 Protective effect of ethyl acetate extract on HeLa cells shown in percentage (A_{600} value of cells protected with the extract / A_{600} value of normal HeLa cells

Dilution	1:256	1:512	1:1 024	1:2 048	1:4 096	1:8 192	10^{-5} CVB_{3m}
The ratio of A_{600}	88.56±0.032	79.46±0.008	73.07±0.057	65.57±0.023	61.83±0.038	56.1±0.015	16.7±0.027

The percentage of vital cells protected with the extract accounted for compared with normal HeLa cells decreased with the increased extract dilution, and it was preferable from concentration 1:256 mg / mL to 1:1 024 mg / mL. $^bP<0.01$ *vs* group of 10^{-5} CVB_{3m}.

Table 4 The value of A_{600} obtained from various infective phase

Dilution / A_{600}	1:256	1:512	1:1 024	1:2 048	1:4 096	1:8 192
First group	0.45±0.041	0.56±0.011	0.63±0.036	0.65±0.043	0.37±0.076	0.38±0.029
Second group	0.72±0.022	0.96±0.013	0.69±0.009	65.00±0.025	0.78±0.056	0.73±0.054
Third group	0.53±0.030	0.48±0.036	0.40±0.049	0.39±0.066	0.39±0.077	0.32±0.015

The A_{600} value of the cells co-cultured only with RPMI1640, cells co-cultured only with 10^{-5} CVB_{3m} and cells co-cultured only with extract (1:256 mg / mL) was 1.29±0.011, 0.24±0.023 and 1.20±0.029, respectively. The value of A_{600} of the cells treated with extract of variable concentration at different time of CVB_{3m} infection was higher than that of the cells infected with CVB_{3m} and not treated with the extract, which indicated the protective effect of the extract existed through all the processes of CVB_{3m} attachment, entry, biosynthesis and assemble in cells.

of A_{600} obtained using crystal violet staining showed that starting from 1:256 mg/mL, the value of A_{600} decreased with the increased dilution. There was a negative correlation between them (r=0.8525, P<0.01, Tables 2-3, Figure 1).

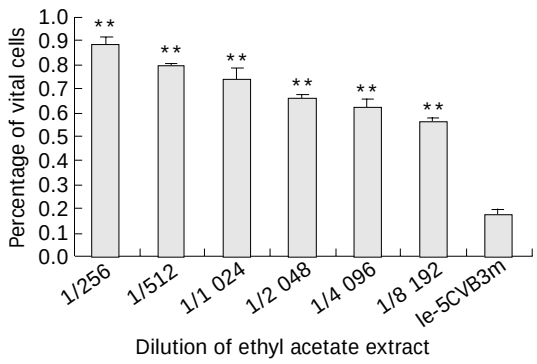

Figure 1 The protective effect of the extract on HeLa cells expressed in column figure. The value of A_{600} obtained using crystal violet staining decreased with the increased dilution of the extract. From 1:256 to 1:8 192 mg / mL the extract showed various protective effects on HeLa cells and the effect was decreased with the increased dilution of the extract. The protective effect was best at concentration of 1:256 to 1:1 024 mg / mL.

Primary study on the mechanism of anti-CVB_{3m} effect of the extract in vitro

In the first group, the extract was added in the process of virus adsorption. In second group, the extract was added in the process of virus adsorption and also after adsorption while in the third one, the extract was added after virus adsorption. The value of A_{600} of the three groups were obtained. Among all three groups, A_{600} obtained from the cells treated with extract from 1:256 to 1:8 192 mg/mL was higher than that treated with 100$TCID_{50}$ CVB_{3m}, and that of the second group was the highest. This suggested the extract exerted anti-CVB_{3m} effect through all the processes of the virus infection (Table 4).

DISCUSSION

Proliferation of virus primarily depends on the biosynthesis system of host cells because virus deficit the enzymes needed for their proliferation. Virus proliferation was similar to nucleotide replication of host cells and there is also great similarity between its products such as nucleotide and proteins and that of host cells. So the demand for killing virus while not infecting the normal physiological function of host cells brings us great difficulty to design and synthesize effective anti-virus drug. The development of anti-virus drugs is very slow compared with that of anti-bacteria drugs in the last one hundred years since the discovery of virus. Though it is an important way to synthesize anti-virus drugs using chemical methods, it is very slow and time-consuming. In recent years, many countries are paying more and more attention to look for anti-virus agents from Chinese medicinal herbs. Tian-hua-fen is a traditional Chinese medicinal herb. It has been used in Chinese for centuries to induce mid-term abortion. And there were many records about its biological efficacy. In the 70 s, the active ingredient of Tian-hua-fen, TCS, a protein from the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii Maxim* was found and purified. It is a monomeric protein with a pI (isoelectric point) of 9.4 and an apparent molecular weight of 24 kDa. There are no cysteine residues in the molecule[8]. TCS is a member of type 1 ribosome-inactivating protein (RIP) family[7-9]. RIP is a group of cytotoxic proteins acting on eukaryotic ribosomes. They can inactivate 60S ribosomal subunits by only hydrolyzing a single phosphodiester bond between the guanosine residue at position 4 325(G4 325) and the adenosine residue at position 4 326(A4 326) in 28S rRNA. TCS can inactivate eukaryotic ribosomes through its N-glycosidase activity by hydrolyzing the N-C glycosidic bond of adenylic acid at 4 324 site in 28S rRNA of rat liver[10]. Thus cell protein synthesis was inhibited. Clinical application over the years showed TCS had multiple pharmacological effects. The research about TCS was once popular since McGrath *et al.*[11,12] reported it could inhibit HIV-1 for the first time. But the sever side effect prevented its more extensive clinical application[13]. There are some other components such as polysaccharide, phytohemagglutinin, sterol and palmitic acid and so on in Tian-hua-fen besides TCS[14]. Polysaccharide in Tian-hua-fen had marked immunoregulation effect[1]. The galactose-binding lectin from Tian-hua-fen stimulated the incorporation of D-[3-3H]glucose into lipids in isolated rat epididymal adipocytes[15]. In the process of screening anti-virus agents, we found the ethyl acetate extract of Tian-hua-fen had obvious preventive effect on HeLa cells from CVB_{3m} infection. This showed the potential anti-virus effect of the ethyl acetate extract of Tian-hua-fen. In this study, Tian-hua-fen was treated with chemical reagents and then was extracted with organic reagents. Finally, four kinds of extract were obtained. The anti-CVB_{3m} effect of the four fractions was tested and the results showed

ethyl acetate fraction had the best preventive effect on HeLa cells from CVB_{3m} infection. The preventive effect decreased as the extract dilution increased. There was an obvious correlation between them. The ethyl acetate fraction was further chromatographed on silica gel column and their anti-virus effect was also tested. The results suggested that toxicity of the further extract to HeLa cells was decreased compared with the former one and there also existed a negative correlation between the preventive effect and the dilution.

Many methods have been developed for determining the antiviral activities of compounds in cell culture. For viruses that cause discernable cytopathic effects (CPE) microscopically in cells, visual scoring of CPE inhibition is performed most frequently because it is rapid, and allows a number of compounds to be evaluated using 96-well microplates[16]. Since solely relying on visual scoring was inaccurate for assessing the cytotoxicities, the use of a dye or stain is very important. The results indicated that methods using bisbenzimide, crystal violet, fluorescein diacetate, MTT, neutral red, or rhodamine 6G were similar to visual scoring for determining anti-influenza virus activity in cell culture. Rapid staining (15 min) methods could be done with crystal violet and rhodamine 6G, and rhodamine 6G gave a high background in microwells containing only water (no cells or virus) which had to be subtracted[17], whereas other methods for determining antiviral activity of test substances may be much more tedious, requiring more microplates, compound, and/or time than the above methods. These include the plaque reduction assay[18], virus yield reduction assay[19], determining drug effect by counting the number of infected cells stained by fluorescent antibody[20] and [^{3}H]TdR incorpration[21]. For this reason, we preferred cystal violet staining in our study in order to quantitatively screen anti-virus agents.

We also did some primary study on the anti-CVB_{3m} mechanism of the extract. The extract was added before virus adsorption, during adsorption and after adsorption and then the cells were stained with crystal violet and A_{600} was measured. The value of A_{600} of the three groups was higher than that of the cells infected with virus. This indicated the three kinds of treatment had preventive effect on HeLa cells from CVB_{3m} infection at different levels and suggested that the extract could act through virus adsorption, penetration and synthesis in cells.

Our results showed that the ethyl acetate extract of Tian-hua-fen had marked anti-CVB_{3m} effect *in vitro*. Some questions still remained to be answered. Which component play key role in the process of anti-virus? Which has the better anti-virus effect between the crude extract and the further extract? What is the anti-virus mechanism of it? To answer these questions needs more and further research. Anyway it is sure that the ethyl acetate extract of Tian-hua-fen has marked anti-virus effect. The answers to the above questions will help to wide the range of virus that Tian-hua-fen resists and also help to make Tian-hua-fen a clinical-used drug.

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