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RAPID COMMUNICATION

In vitro screening of traditionally used medicinal plants in China against Enteroviruses

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Abstract

AIM: To search for new antiviral agents from traditional Chinese medicine, specifically anti-enterovirosuses agents.

METHODS: The aqueous extracts (AE) of more than 100 traditionally used medicinal plants in China were evaluated for their *in vitro* anti-Coxsackie virus B3 activities with a MTT-based colorimetric assay.

RESULTS: The test for AE of 16 plants exhibited anti-Coxsackie virus B3 activities at different magnitudes of potency. They can inhibit three steps (inactivation, adsorption and replication) during the infection. Among the 16 plants, *Sargentodoxa cuneata* (Oliv.) *Rehd. et* Wils., *Sophora tonkinensis* Gapnep., *Paeonia veitchii* Lynch, *Spatholobus suberectus* Dunn. and *Cyrtomium fortunei* J. sm. also have activity against other enterovirus, including Coxsackie virus B5, Polio virus I, Echo virus 9 and Echo virus 29. Cell cytotoxic assay demonstrated that all tested AE had CC_{50} values higher than their EC_{50} values.

CONCLUSION: The sixteen traditionally used medicinal plants in China possessed antiviral activity, and some of them merit further investigations.

Key words: Traditional used medicinal plant; China; Antiviral activity; Enterovirus; *Sargentodoxa cuneata (Oliv) Reld. et. Wils.*; *Sophora tonkinensis Gapnep.; Paeonia veitchii Lynch.*; *Cyrtomium fortunei J. sm.*; *Spatholobus suberectus Dunn*

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INTRODUCTION

Human enteroviruses, the largest genus of the *Picornaviridae* family, are of great medical and economic importance causing a variety of clinical syndromes and diseases. Enterovirus can cause any of the syndromes and, vice versa, any syndrome or disease could be the result of infection by the enterovirus. Coxsackieviruses, part of the enterovirus genus, can cause severe diseases of the heart, liver, eyes and pancreas, as well as acute infections of the central nervous system. Nowadays, Coxsackieviruses B are the major etiological agents of human myocarditis, causing between 25% and 35% of cases for which a cause is found[1]. Transition from acute myocarditis to dilated cardiomyopathy has been suspected[2]. So far, dilated cardiomyopathy is one of the major reasons for cardiac transplantation. Coxsackieviral RNA in the myocardium can be a marker of a poor clinical outcome and might influence prognosis after heart transplantation[3]. Enteroviruses and Coxsackieviruses in particular, have been implicated in several chronic illnesses including juvenile onset diabetes mellitus, chronic fatigue syndrome, dermatomyositis and polymyositis, congenital hydrocephalus and amyotrophic lateral sclerosis[4-6]. Until recently, there were no enterovirus-specific drugs available for clinical use[7]. A great number of picornavirus replication inhibitors *in vitro* have been described but just few of them have shown effectiveness *in vivo*[8], and none has been approved for clinical use yet. Etiological therapy for enteroviral diseases still remains elusive. The main reason for that is the fast development of drug-resistant and even drug-dependent mutants[9,10]. Use of synergistic combinations of antivirals might be one of the possible efficient approaches to overcome the disadvantages of monotherapy. The same or greater effect could be achieved at lower concentrations than those required if drugs were to be used alone. Combined chemotherapy may also restrict the emergence of resistance to either or both of the partners in the combination. Also, research on the antiviral effect of combinations of picornavirus inhibitors might contribute to the better understanding of their mode of action and, in general, the mechanism of picornavirus replication. Currently, there is no specific antiviral therapy to treat or prevent enterovirus disease. Thus, new and more effective antiviral agents for future therapy in enterovirus infection are desired.

The development of a new antiviral drug is a difficult task taking into account the poor selective toxicity and fast selection of resistant viral variants with the existing

drugs. Frequencies of viral resistance to antiviral drugs are increasing and the difficulty of virus latency remains unsolved.

The screening of plants as a possible source of antiviral agents has led to the discovery of potent inhibitors of *in vitro* viral growth[11-17] and the use of the ethnopharmacological approach enhances the probability of identifying new bioactive plant compounds[18,19].

Plants have long been used as remedies against infection diseases. Many plant preparations have been used externally as disinfectants and antiseptics for wounds and pimples, as antidiarrhoeics and in the treatment of respiratory diseases. Nowdays, these are still used by rural populations. Plants considered useful against infectious diseases are interesting to test with regard to different etiological agents (viral, bacterial, fungus).

In our continuous efforts to search for novel antiviral agents from traditional medicinal plants, more than one hundred traditionally used medicinal plants in China were extracted with water and investigated for their in vitro anti-enterovirus activities. This is the first report on screening traditionally used medical plants against enterovirus.

MATERIALS AND METHODS

Preparation of the extracts

All traditionally used medicinal plants were purchased from Darentang Pharmaceutical Co. Ltd, a famous local traditional Chinese medicine provider in Tianjin city of China. All plant materials were air dried, ground and powdered. Hot water extracts of the traditionally used medicinal plants were prepared according to the procedures as described bellows: different medicinal plant materials (20 g) were boiled with 1000 mL of distilled water for 2 h. The aqueous was collected and the residual was extracted again with another 1000 mL of distilled water. The resulting aqueous extracts were collected, combined, filtered by gauze, concentrated under reduced pressure and lyophilized to dry. The AE extracts were dissolved in sterile distilled water.

Cells and virus

Vero E6 was used as target cells for virus infection. Cells were cultivated using RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, 100 μg/mL streptomycin. In the antiviral assay, the medium was supplemented with 2% FBS and the above mentioned antibiotics. All cell culture reagents and media were purchased from Gibco BRL (Grand Island, New York).

Polio virus I was obtained from the American Type Culture Collection (ATCC), Rockville, USA. Coxsackie virus B3 and Coxsackie virus B5 were provided by Professor X.M. Li (Medical University of Tianjin, Tianjin, China). Echo virus 9 and Echo virus 29 were provided by the Chinese Type Culture Collection (CTCC), Wuhan, China. All virus strains were kept in our laboratory. All viruses were prepared and quantitated on Vero E6 cells and stored in small aliquots at -70℃ until use.

Titration of viruses

Vero E6 cells were seeded in 96-wells culture plates at a density of 10^4 cells/well and then incubated at 37℃ in a humidified atmosphere containing 5 mL/L CO_2 for 24 h. A serial dilutions of virus stocks were prepared and cells were infected with the dilution of virus. After an additional 72 h of incubation, the cytopathic effect was recorded. The 50% tissue culture infective dose ($TCID_{50}$) per mL was calculated as described previously by Reed and Muench[20].

Antiviral assay using MTT method

The antiviral activity of AE extracts was evaluated by the MTT method based in the color change which occurred following the reduction of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial enzymes[21]. Vero E6 cells, treated by trypsin, were seeded in 96-well culture plates with a volume of 200 μL/well and a concentration of 10^5 cells/mL. After 24 h incubation, 100 μL of 100 $TCID_{50}$ of Coxsackie virus B3 was added, and the infected cells were incubated for another 2 h. Tested compound (200 μL) at different concentrations was then added to culture wells in triplicate. After further incubation at 37℃ with 5 mL/L CO_2 for 72 h, MTT was added. The culture was incubated for four hours to allow the production of formazan and the absorbance at 492 nm was measured using a 96-well plate ELISA reader (Multiskan EX, Labsystems).

Viral inhibition rate was calculated as follows: [(ODtv-ODcv)/(ODcd-ODcv)] × 100%.

ODtv, ODcv and ODcd indicate the absorbance of the test compounds with virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively. The 50% effectiveness concentration (EC_{50}) was defined as the concentration that achieved 50% cyto-protection against virus infection.

To confirm the results obtained with the MTT assay, the monolayers were also observed microscopically for estimating CPE (i.e. rounding and other marked morphologic changes with respect to control cells).

In addition to the post-incubation method of virus inhibition assay, pre-treatment (adding 2 h before virus infection) and co-treatment (adding at the same time of virus infection) of AE extracts were also attempted in the same protocol as mentioned above except the variation in the time of addition of AE extracts.

Cell cytotoxic effect

The cell cytotoxic effect of tested compounds toward Vero E6 cells was evaluated by MTT-based method. It was performed according to the procedures as described above with no virus added. Cell cytotoxic effect of each tested compound was calculated by the following formula: Percent of cell cytotoxic effect = [1- (ODt/ODs)] × 100%.

ODt and ODs indicate the absorbance of the test substances and the solvent control, respectively. The 50% cell cytotoxic concentration (CC_{50}) of tested compounds was calculated according to Chiang *et al*[22,23].

Statistical analysis

Data were calculated for three separate experiments. The selectivity index (SI) was calculated as the ratio of CC_{50} to EC_{50}. The Student's unpaired *t*-test was used to calculate

Table 1 *In vitro* **anti-Coxsackie virus B3 activity of 16 traditionally used medicinal plants in China**

Infusion	Used part	Antiviral activity		
		Viral inactivation	Inhibition of adsorption	Inhibition of replication
Arctium lappa L.	Seeds	+++	+	+
Belam anda chinensis (L.) DC	Stem and root	+++	+	+
Cyrtomium fortunei J. sm.	Stem	++++	+++	+++
Dictamnus dasycarpus Turcz.	Root	+++	+	+
Ephedra Sinica stapf	Stem	++++	+	++
Epimedium brevicornum Maxim.	Stem and leaf	+++	+	+
Herba patriniae.	Whole grass	+++	+	+
Lindera aggregata (Sims) Kostem.	Root	+++	+	+
Lygodium japonicum (Thunb) Sw.	Seeds	+++	+	+
Paeonia lactiflora Pall.	Root	+++	+	++
Paeonia veitchii Lynch	Root	++++	+	++
Plantago asiatica L.	Seeds	+++	+	+
Sargentodoxa cuneata (Oliv) Reld.et.Wils	Stem	++++	+++	+++
Sophora tonkinensis Gapnep.	Root	++++	+	++
Spatholobus suberectus Dunn	Stem	++++	+++	+++
Terminalia chebula Retz	Seeds	+++	+	+

++++: the strongest antiviral activity; +++: strong antiviral activity; ++: the modest antiviral activity; +: the weak antiviral activity; -: no antiviral activity.

Table 2 EC_{50} **and selectivity index of five traditionally used medicinal plants in China**

Infusion	CC_{50} (mg/L)	Viruses									
		Coxsackie virus B3		Coxsackie virus B5		Echo virus 9		Echo virus 29		Polio virus I	
		EC_{50} (mg/L)	SI	EC_{50} (mg/L)	SI	EC_{50} (mg/L)	SI	EC_{50} (mg/L)	SI	EC_{50} (mg/L)	SI
Guanidine Sargentodoxa	> 1000.0	35.4	28.2	36.7	27.3	18.6	53.8	33.2	30.1	67.8	14.7
Cuneata (Oliv.) *Rehd.et Wils*	> 500.0	51.2	9.8	29.1	17.2	12.8	39.1	28.2	17.7	43.2	11.6
Sophora tonkinensis Gapnep.	> 125.0	19.2	6.5	77.5	1.6	6.3	19.8	23.2	5.4	14.9	8.4
Paeonia veitchii Lynch.	> 500.0	115.3	4.3	86.2	5.8	43.5	11.5	65.3	7.7	136.8	3.7
Spatholobus suberectus Dunn.	> 250.0	60.8	4.1	47.1	5.3	14.8	16.9	65.5	3.8	29.1	8.6
Cyrtomium fortuntei J. sm.	> 250.0	64.9	3.9	66.2	3.8	13.6	18.4	44.2	5.7	52.8	4.7

P values of difference of means between control and the tested samples on the inhibition of virus replication. Difference of sample between tested viruses with a *P* value less than 0.05 was considered statistically significant.

RESULTS

Anti-Coxsackie virus B3 activity of traditionally used medicinal plants in China

Aqueous extracts from 151 traditionally used medicinal plants were studied to detect the activities against Coxsackie virus B3, including effect on viral replication; effect on viral adsorption and subsequent replication and *in vitro* viral inactivation. The results showed that aqueous extracts of 16 used medicinal plants in China exhibited *in vitro* anti-Coxsackie virus B3 during the three different antiviral assays (Table 1). The antiviral activity against Coxsackie virus B3 by estimating cytopathic effect. Among the 16 medicinal plants, all exhibited strong anti-Coxsackie virus B3 activity on *in vitro* viral inactivation. But the extracts from *Sargentodoxa Cuneata* (Oliv.) *Rehd.et Wils.*, *Sophora tonkinensis Gapnep.*, *Paeonia veitchii Lynch.*, *Paeonia lactiflora Pall., Ephedra Sinica stapf*, *Spatholobus suberectus Dunn*. and *Cyrtomium fortunei J. sm.* appeared to possess the strongest anti-Coxsackie virus B3 activity on viral replication; and the extract from *Cyrtomium fortunei J. sm.*, *Sargentodoxa Cuneata* (Oliv.) *Rehd.et Wils.*, and *Spatholobus suberectus Dunn*. can strongly inhibited the adsorption of Coxsackie virus B3 to the cell.

EC_{50} and selectivity index of five traditionally used medicinal plants against other enterovirus

Table 2 shows EC_{50} of AE extract of five medicinal plants. Overall, all AE extract showed CC_{50} higher than their EC_{50}. These observations indicated that the antiviral activity of AE extracts was not a result of their cytotoxic effect toward cells. The CC_{50} values ranged from 125 to 500 mg/L.

With the EC_{50} and CC_{50} data, the selectivity index (SI) was calculated by dividing CC_{50} by EC_{50}. The SI for the anti-Coxsackie virus B3 assay ranged from 3.9 to 9.8, and 1.6 to 17.2 for the anti-Coxsackie virus B5 assay. For anti-Echo virus 9, Echo virus 29 and Polio virus I assays, the SI ranged from 11.5 to 39.1, 3.8 to 17.7 and 3.7 to 11.6, respectively.

DISCUSSION

Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. Some of them are reported to exhibited antiviral activity in literature[15,18]. According to Cragg's report, approximately 60% of anti-tumor and anti-infective agents that are commercially available or in the late stages of clinical trials today are of natural product origin[24]. There is therefore,

no doubt that traditional medicinal plants can serve as a potential resource in the development of new antiviral agents in the future. Since current chemotherapy agents for enterovirus infections are either insufficient in quantity or limited in efficiency, there is thus a need to search for new and more effective antiviral agents for future therapy in enterovirus infections.

Among the 151 tested medicinal plants, five of them were found to exhibited a broad spectrum of anti-enterovirus activity. These five medicinal plants were *Sargentodoxa cuneata* (Oliv.) *Rehd. et Wils*, *Sophora tonkinensis Gapnep.*, *Spatholobus suberectus Dunn.*, *Paeonia veitchii Lynch.* and *Cyrtomium fortunei J. sm.* In China, they were not used to treat the virus infection. *Sargentodoxa Cuneata* (Oliv.) *Rehd.et Wils* is the dried vine stem of Sargentodoxa cuneata (Oliv.) Rehd. et Wils. (Fam. Lardizabalaceae), traditionally used to remove toxic heat, to promote blood circulation, and to relieve rheumatic conditions. *Sophora tonkinensis Gapnep.* is the dried root and rhizome of Sophora tonkinensis Gapnep. (Fam. Leguminosae), used to remove toxic heat, promote the subsidence of swelling, and soothe the sore throat. *Spatholobus suberectus* Dunn. is the dried stem of Spatholobus suberectus Dunn (Fam. Leguminosae), used to enrich the blood, to activate blood circulation, and to remove obstruction of the channels and collaterals. *Paeonia veitchii Lynch.* is the dried root of paeonia lactiflora Pall. or Peaonia veitchii Lynch (Fam. Ranuncullaceae), used to remove heat from blood, to eliminate blood stasis, and to relieve pain. *Dryopteris crassirhizoma Nakai* is the dried rhizome of Dryopteris crassirhizoma Nakai (Fam. Aspidiaceae), used to remove toxic heat, to expel parasites, and to stop bleeding. Our studies revealed that the five medicinal plants suppressed five entroviruses at different magnitudes of potency. Among them, the aqueous extracts of *Sargentodoxa cuneata* (Oliv.) *Rehd.et Wils* showed the strongest antiviral activity to all five viruses and noteworthy SI value (SI > 4.0). The SI of five medicinal plants to five viruses values from 1.6 to 39.1 suggested a promising future for these extracts as antiviral products. It is important to emphasize there has been no a novel drug for enteroviruses diseases.

Anti-enteroviruses of aqueous extracts of these five plants against five enteroviruses *in vitro* have been demonstrated. It is not known, however, what these results signify for *in vivo* effectiveness. The results obtained in this preliminary screening justify continuing with the purification of crude extracts and isolation of active compounds for improving their potential as antiviral drugs and/or finding of new lead molecules.

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