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In Vitro Screening for Compounds Derived from Traditional Chinese Medicines with Antiviral Activities Against Porcine Reproductive and Respiratory Syndrome Virus

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Seventeen compounds derived from traditional Chinese medicines (TCMs) were tested for their antiviral activity against porcine reproductive and respiratory syndrome virus (PRRSV) *in vitro*. Visualization with the cytopathologic effect (CPE) assay and the 3-(4, 5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide test were used to determine the 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) in cultured Marc-145 cells. Among the tested compounds, chlorogenic acid and scutellarin showed potential anti-PRRSV activity. The EC_{50} values were 270.8 ± 14.6 μg/ml and 28.21 ± 26.0 μg/ml and the selectivity indexes were >5.54 and 35.5, respectively. The time-of-addition and virucidal assay indicated that the anti-PRRSV activity of the two compounds could be due to their inhibiting the early stage of virus replication and/or inactivating the virus directly. The inhibition of the virus attachment was not observed in the adsorption inhibition assay. The inhibition ratios of chlorogenic acid and scutellarin were, respectively, 90.8% and 61.1% at the maximum non-cytotoxic concentrations. The results have provided a basis for further exploration of their antiviral properties and mechanisms *in vivo*. We believe that the chlorogenic acid and scutellarin have a great potential to be developed as new anti-PRRSV drugs for clinical application.

Keywords: Natural compound, traditional Chinese medicine, antiviral activity, PRRSV, Marc-145 cell

Introduction

It is well known that the infection of porcine reproductive and respiratory syndrome virus (PRRSV) can cause immunosuppression, and at present, the prevalence of porcine reproductive and respiratory syndrome (PRRS) has brought about enormous financial losses to the world-wide swine industry, especially in China. Vaccination is the main tool for controlling and preventing PRRS, but the current vaccines do not provide a 100% protection because of their limited efficacy and the frequent emergence of genetic variant viruses. Some cytokines, such as IFN-α, IFN-β, IFN-γ, and IFN-λ, have an antiviral effect against PRRSV [1, 3, 16, 19], but they still stay in the laboratory research or clinical trial stage. A few chemical drugs have been used only as adjunctive therapy for PRRSV. Therefore, it becomes urgent to develop new cost-effective anti-PRRSV drugs.

Traditional Chinese medicines (TCMs) are considered as excellent pools of bioactive compounds for the discovery of new drugs because of their long-time clinical test and reliable therapeutic efficacy. Mukhtar *et al*. [18] documented the potential antiviral properties of medicinal plants against a diverse group of viruses. Previous studies have proved that a number of bioactive compounds derived from TCMs possess broad-spectrum antiviral effects [10, 20, 22]. Therefore,

medicinal plants have offered a rich source for extracting drugs against infectious diseases. Currently, many studies have been focusing on the antivral activity of crude extracts from TCMs, which possess a lot of complicated constituents, and it is difficult to define the active constituent from the crude extracts. In the present study, 17 compounds proven to possess multipharmacological activities, such as antivirus, anti-inflammation, and immunity regulation, were screened to assess their anti-PRRSV activity. All these tested compounds have a definite source of supply from TCMs.

Materials and Methods

Natural Compounds and Reagents

Seventeen compounds derived from TCMs (Fig. 1) and ribavirin was purchased from National Institutes for Food and Drug Control (China). Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% or 2% heat-inactivated fetal calf serum (FCS; Hyclone, USA), 100 IU/ml Penicillin G and 100 μg/ml Streptomycin was used for cell growth or maintenance medium. A 0.25% trypsin (Amresco, USA) was prepared in pH 7.2 PBS. A 0.5% 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco) was prepared in PBS (pH 7.4). These solutions were sterilized by a 0.22 μm Millipore membrane filter and aliquots made for future use. Dimethyl sulfoxide (DMSO) was the product of Beijing Solarbio Science & Technology Co., Ltd (China). The DMEM and MM were stored at 4°C, whereas MTT and trypsin were stored at -20°C. DMSO was stored at room temperature.

Virus and Cells

Marc-145 cells, obtained from China Institute of Veterinary Drug Control (China), were diluted to 2×10^5 cells/ml with 10% DMEM, seeded in 96-well plates, and incubated at 37°C in a 5% CO_2 atmosphere.

PRRSV vaccine (JXAI-R, No. 1012001; Guangdong Dahuanong Animal Health Products Co., Ltd, China) was propagated in Marc-145 cells. The tissue culture infectious dose 50 ($TCID_{50}$) for the virus was determined by the Reed-Muench assay. The virus was diluted to $1\times10^{-4.5}$ (100 $TCID_{50}$) with MM and stored at -80°C for future use.

Cytotoxicity Assay

The cytotoxicity of the 17 compounds was measured by MTT assay [17]. Each compound and ribavirin were 2-fold serially diluted with DMEM, containing 2% FCS and matching lytic agent to 8 gradients, respectively. Marc-145 cells were seeded into 96-well plates at a density of 2×10^4 cells/well, and incubated for 24–36 h. When the cells were at least 90% confluent, the medium was removed and the diluted compounds or ribavirin were added to the wells and incubated for 72 h. Then, the medium was discarded and 20 μl of MTT solution was added to each well. The plates were then further incubated at 37°C for 4 h. Subsequently, the supernatant was removed and 150 μl of DMSO was added to each well in order to dissolve the formazan crystals. After gently shaking the plates for 10 min, the absorbance was read on an ELISA microplate reader (ELx808, Gene Co., Ltd., China) with a 490 nm wavelength and a 630 nm reference wavelength. For each compound, the percentage of cell viability was calculated as [(A-B)/A×100], where A and B correspond to the absorbance of control and treated cells, respectively. The 50% cytotoxic concentration (CC_{50}) value was defined as the concentration of each compound that reduced the absorbance of treated cells by 50% when compared with the cell control. The maximum non-cytotoxic concentration (MNTC) was calculated as the concentration required to retain cell viability by 90% [5].

Antiviral Assay

The anti-PRRSV activity of the 17 compounds was evaluated as previously described by Li *et al*. [12] with minor modifications. Briefly; a confluent monolayer of Marc-145 cells was prepared as described above. After removal of the culture medium, the maximum non-cytotoxic concentration of each compound and a constant amount of 100 $TCID_{50}$ PRRSV were added. Cells control, PRRSV negative control, and ribavirin positive control were set up simultaneously. The plates were then incubated at 37°C. When CPE in the negative control reached 80%-90% compared with Marc-145 cells control, the cell viability was determined by the MTT method, as described previously. The inhibition ratio (%I) was calculated based on the formula [9]

$$\%I = \frac{(OD_T)_{PRRSV} - (OD_C)_{PRRSV}}{(OD_C)_{mock} - (OD_C)_{PRRSV}} \times 100\%$$

where $(OD_T)_{PRRSV}$ represents the optical density (OD) of cells infected with PRRSV and treated with the compounds (Index: T=treated), $(OD_C)_{PRRSV}$ corresponds to the OD of the untreated PRRSV-infected cells (Index: C=control), and $(OD_C)_{mock}$ is the OD of untreated mock-infected cells. The compound with the inhibition ratio exceeding 50% was selected and 8 dilutions of each compound were made by a 2-fold serial dilution with MM, and the procedures as described above were repeated. The 50% effective concentration (EC_{50}) of the compounds was determined as 50% cytoprotection against PRRSV infection. The selectivity index (SI) was calculated as the ratio of CC_{50} to EC_{50}. When EC_{50} could not be calculated owing to lower inhibition ratio of CPE, the results were counted as the maximum inhibition ratio.

Virucidal Assay

Each compound with the maximum non-cytotoxic concentration and 100 $TCID_{50}$ PRRSV were mixed and interacted at 37°C for 0.5, 1, 1.5, 2, or 2.5 h, respectively. After incubation, 100 μl of virus/compound suspension was added to a cell plate and incubated at 37°C in a 5% CO_2 humidified atmosphere. The plate was then

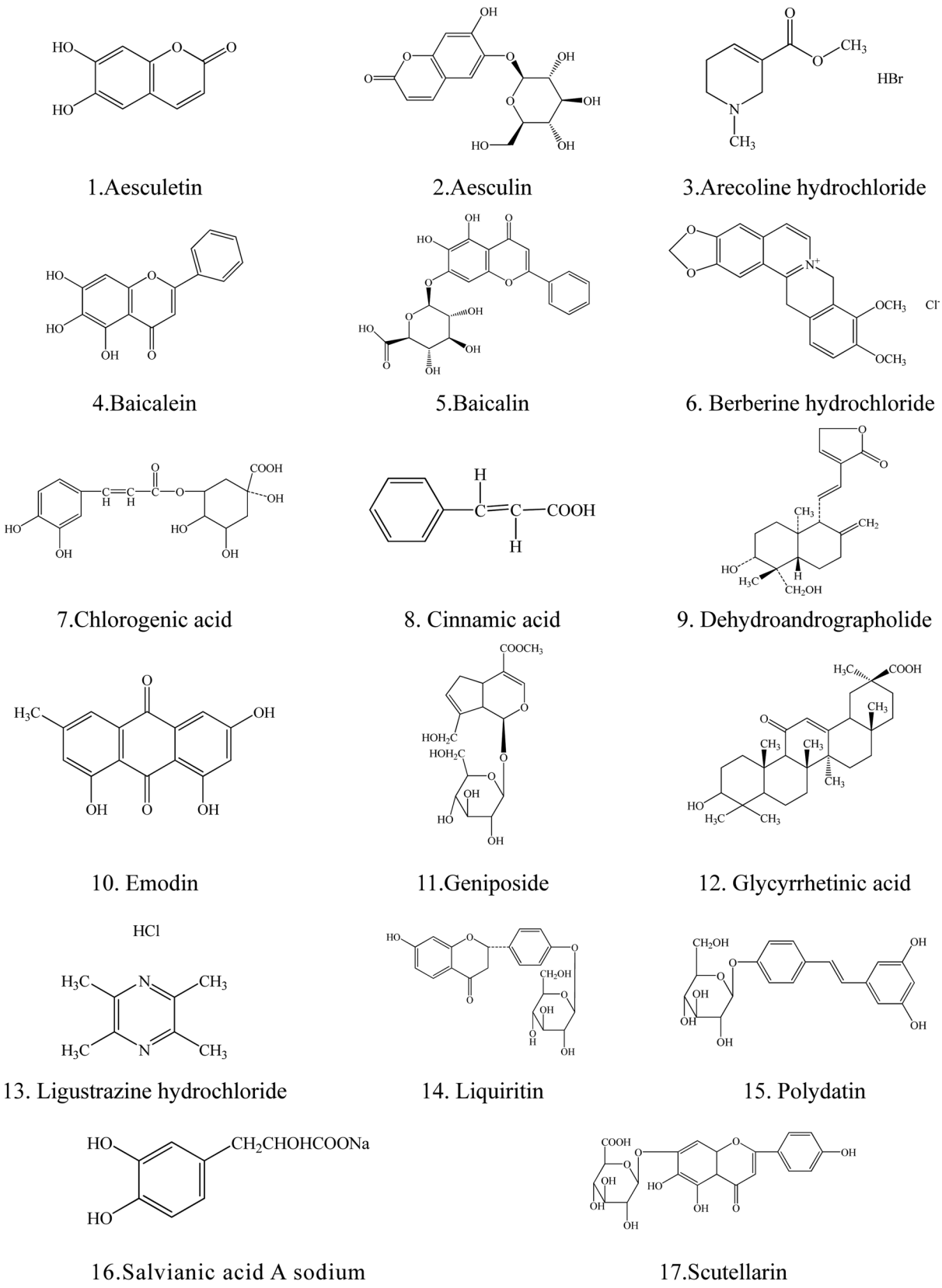

Fig. 1. The structure of the compounds used in this study.

observed under a microscope daily until the CPE of the PRRSV negative control reached 80%–90% compared with Marc-145 cell control, and the MTT test was performed [7].

Time-of-Addition Assay

A time-of-addition assay was done dynamically following previous methods with some modifications [2]. The maximum non-cytotoxic concentration of each compound and 100 $TCID_{50}$ PRRSV were used in the assay. Marc-145 cells in 96-well plates were pre-incubated with PRRSV for 1, 2, 4, 6, 8, 10, 12, and 14 h, respectively. Subsequently, the medium was removed and the cells were washed twice with PBS, and then fresh medium containing the compounds was added. The plates were further incubated at 37°C in 5% CO_2 atmosphere. The CPE was recorded at a time interval of 12 h under the microscope. When the CPE of the PRRSV negative control reached 80%–90% compared with Marc-145 cells control, the anti-PRRSV activity of all phases was assessed by MTT test and the viral inhibition ratio was calculated.

Adsorption Inhibition Assay

This assay was done by following two different approaches. First, Marc-145 cells grown in 96-well plates were pre-chilled at 4°C for 1 h, and then the medium was discarded. Subsequently, four dilutions of each compound, starting from the maximum non-cytotoxic concentration, were mixed with 100 $TCID_{50}$ PRRSV. The mixture of compound solution and virus was added to each plate well, and the plates were incubated at 4°C for 2.5 h to allow PRRSV adsorption. The cell monolayer was gently washed with cold PBS and then MM was added to the plates. The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere until 80–90% CPE was observed in PRRSV negative control compared with Marc-145 cells control. The MTT test and viral inhibition ratio were then determined as above [27].

Second, confluent monolayers of Marc-145 cells grown in 96-well plates were incubated with the compounds at 37°C in a 5% CO_2 humidified atmosphere for 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h respectively, and then the plates were incubated at 4°C for 1 h. Subsequently, the medium was removed and 100 $TCID_{50}$ PRRSV was added to each well. This was followed by the same procedure as with the first approach.

Statistical Analysis

The statistical analysis was performed using the SPSS17.0 software (SPSS Inc., Chicago, IL, USA). Data are represented as the means for four replicate samples of four independent experiments and expressed as the mean ± SD. A student's *t*-test and one-way ANOVA were used. A value of $P < 0.05$ was considered statistically significant. The CC_{50} was calculated by regression analysis of the dose-response curves for the MTT assay. The EC_{50} was determined using the GraphPad Prism version 5 software (USA).

Table 1. Summary of cytotoxicity and antiviral assays.

	Compounds	Chemical family	Dissolution medium	MNTC (μg/ml)	CC_{50}[a] (μg/ml)	%I[b]	EC_{50}[a] (μg/ml)	SI[c]
1	Aesculetin	Coumarin	1% DMSO	3.906	27.00±5.3	28.4	ND	ND
2	Aesculin	Coumarin	MM	≥1,500	>1,500	<15.0	ND	ND
3	Arecoline hydrochloride	Alkaloid	MM	62.50	1,14.2 ± 14	<15.0	ND	ND
4	Baicalein	Flavonoid	1% DMSO	12.50	>50.0	<15.0	ND	ND
5	Baicalin	Flavonoid	MM	187.5	985.6 ± 60	<15.0	ND	ND
6	Berberine hydrochloride	Alkaloid	3% Alc[d]	15.63	67.30 ± 6.3	41.7	ND	ND
7	Chlorogenic acid	Phenolic acid	MM	750.0	>1,500	90.8	270.8 ± 14.6	>5.54
8	Cinnamic acid	Phenylpropanoid	3% Alc[d]	62.50	>500	18.4	ND	ND
9	Dehydroandrographolide	Diterpenoid	1% DMSO	6.250	24.81 ± 5.2	<15.0	ND	ND
10	Emodin	Anthraquinone	1% DMSO	0.9766	34.07 ± 11	<15.0	ND	ND
11	Geniposide	Monoterpenoid	MM	≥1,500	>1,500	<15.0	ND	ND
12	Glycurrhetinic acid	Triterpenoid	1% DMSO	7.813	27.09 ± 2.0	35.0	ND	ND
13	Ligustrazine hydrochloride	Alkaloid	MM	187.5	>1,500	<15.0	ND	ND
14	Liquiritin	Flavonoid	1% DMSO	1,000	>1,000	25.9	ND	ND
15	Polydatin	Glycoside	3% Alc[d]	1,000	>1,000	<15.0	ND	ND
16	Salvianic acid A sodium	Phenolic acid	MM	31.25	126.7 ± 25	<15.0	ND	ND
17	Scutellarin	Flavonoid	MM	125.0	>1,000	61.1	28.21 ± 17.02	>35.5
18	Ribavirin		MM	125.0	4,466 ± 212	61.4	50.41 ± 6.67	88.6

[a]CC_{50} and EC_{50} represent the mean ± SD of four independent experiments. [b]Percentage of viral cytopathic inhibition compared with cells control, when the maximum non-cytotoxic concentration (MNTC) of compounds was used .[c]Selectivity index. [d]Absolute alcohol. ND: not detected owing to the low inhibition ratio.

Results and Discussion

Cytotoxicity of Tested Compounds

Cytotoxicity assays are essential for the initial phases of antiviral drug development. Therefore, the maximum non-cytotoxic concentration (MNTC) and CC_{50} values for each tested compound are listed in Table 1. It was observed that aesculin, geniposide, liquiritin, and polydatin did not exhibit cytotoxicity to Marc-145 cells with the concentrations employed. The other compounds showed CC_{50} values ranging from 24.81 to 1,500 μg/ml, and the cytotoxicity of these compounds on Marc-145 cells was in a dose-dependent manner. The MNTC ranged from 0.9766 to 1,500 μg/ml, which clearly indicated that the cytotoxicity of different compounds on the same cell varied remarkably. With the higher concentration of compound, cells underwent more morphological changes such as lyses, granulation, pyknosis, condensation, vacuolization in the cytoplasm, darkening of cell boundaries, and cell detachment.

Chlorogenic Acid and Scutellarin Possessed Potential Anti-PRRSV Activity

The results obtained from the antiviral assay demonstrated that chlorogenic acid and scutellarin had potent anti-PRRSV activity in a dose-dependent manner (Fig. 2). The inhibition ratio of chlorogenic acid was 90.8%, which was

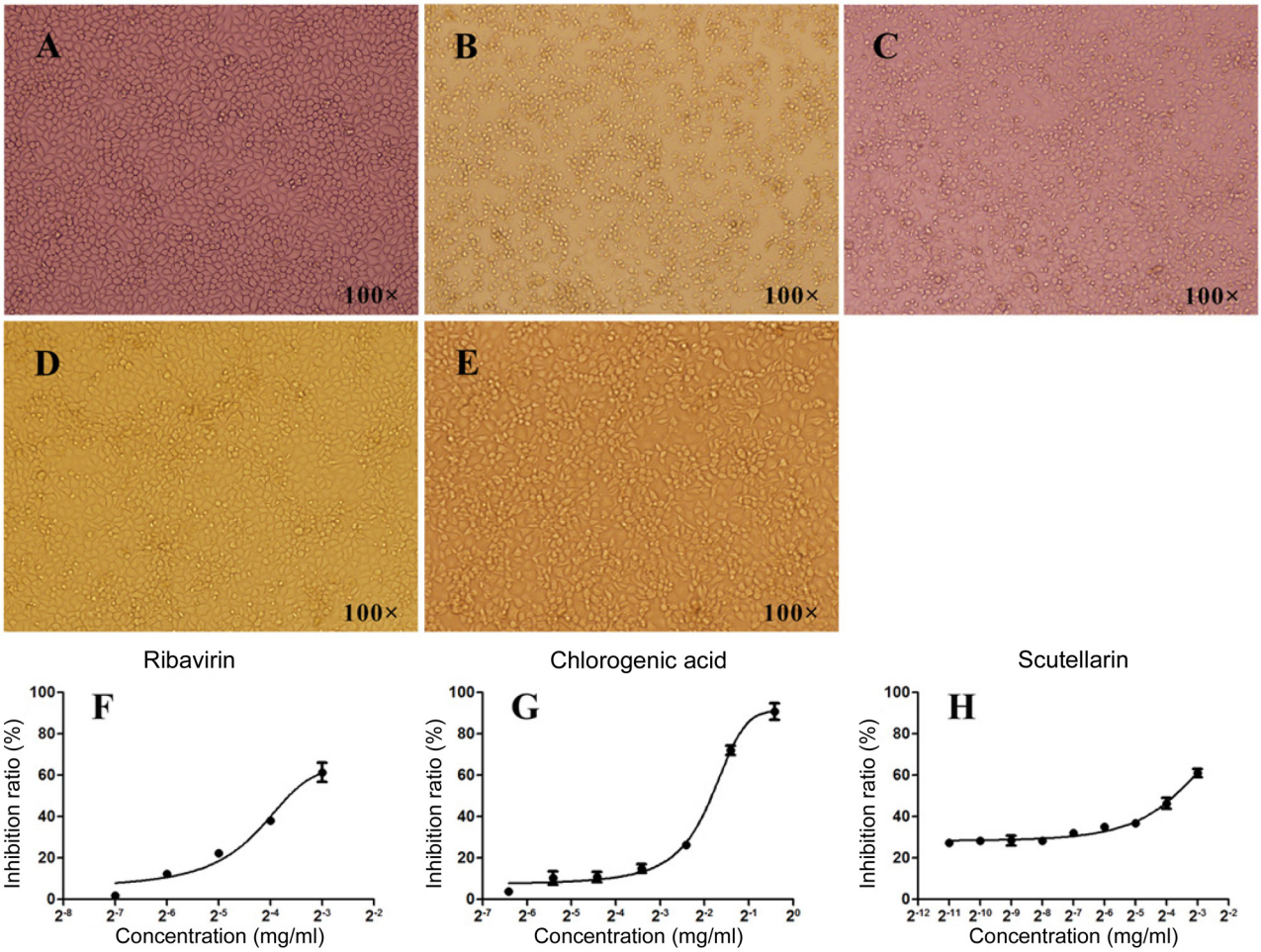

Fig. 2. Anti-PRRSV activity of the two compounds on Marc-145 cells.

Panel 1. Representation of viral replication inhibition by compounds (**A**-**E**). Panel 2. Inhibitory effects of ribavirin and two compounds on PRRSV (**F**-**H**). A: Cell control; B: PRRSV negative control; after 72 h infection with PRRSV, the Marc-145 cells showed some morphological changes, including loss of monolayer integrity, lyses, granulation, pyknosis, condensation, vacuolization in the cytoplasm, and darkening of cell boundaries. C to E: after 72 h co-incubation with PRRSV and ribavirin (**C**), chlorogenic acid (**D**), and scutellarin (**E**), respectively, the morphological changes in Marc-145 cells were significantly light, especially in D. In panel 2, the curves indicated that two compounds and ribavirin had significant inhibitory effects on PRRSV in a dose-dependent pattern.

higher than ribavirin, but the SI of chlorogenic acid was lower. However, the inhibition ratio of scutellarin was similar to ribavirin, and its SI was higher than chlorogenic acid (Table 1). Compared with ribavirin, chlorogenic acid showed stronger anti-PRRSV activity and a limited safe concentration. Scutellarin possessed moderate antiviral activity but a relatively wider safe scope. The inhibition ratios of the other compounds were lower than 50%, and no SI was found.

Chlorogenic acid is an important plant polyphenol and previous studies have demonstrated that chlorogenic acid and its derivaties have multi-antiviral activities against H_5N_1, HBV, ADV, HSV-1, and HSV-2 [6, 8, 11, 14, 24]. Wang *et al*. [25] reported that chlorogenic acid had strong anti-PRRSV effect *in vitro*. Our results confirmed the inhibitory activity of chlorogenic acid against PRRSV.

Scutellarin is a known flavone glucuronide with comprehensive pharmacological actions. It has anti-inflammation effect and inhibit several strains of HIV-1 replication with different potencies [15, 28]. In this study, for the first time, we demonstrated that scutellarin possesses moderate anti-PRRSV activity *in vitro*.

Anti-PRRSV Mechanisms of Chlorogenic Acid and Scutellarin

The principal events involved in any viral infection of host cells are attachment, absorption, uncoating, nucleic acid/protein synthesis, assembly, and release [21]. In spite of the evidence accumulated about the antiviral activity of the two active compounds, little is known about their mechanism of action. Therefore, whether they act on the intracellular stage of viral replication or directly inactivate PRRSV particles was investigated through a series of specific experiments, including virucidal assays, time-of-addition assay, and adsorption inhibition assay. In our study, both of the two compounds produced positive results in the virucidal activity test and a non-time-dependent reaction was observed. As shown in Fig. 3, when each of the two compounds was mixed with PRRSV and interacted for 0.5, 1, 1.5, 2, or 2.5 h at 37°C, respectively, the inhibition ratios of chlorogenic acid at each time point were larger than 85% in a non-time-dependent manner, and with no significant difference at different time points ($p > 0.05$). Scutellarin showed a moderate inhibition ratio with the range of 56%–59% at the maximum non-cytotoxic concentration. From the results, we can speculate that both compounds may directly inactivate PRRSV within 30 min. In our previous studies, sodium tanshinone IIA sulfonate had strong anti-PRRSV activity and may directly inactivate PRRSV within 30 min [23]. However, Wolkerstorfer *et al*. [26] found that glycyrrhizin had no direct inhibitory action on IAV particles and did not interact with virus receptor binding either.

In order to determine whether the two compounds inhibited viral replication at a specific step in the virus cycle, they were applied to Marc-145 cells infected with PRRSV at different time intervals. We found that the inhibition ratios of chlorogenic acid and scutellarin decreased from 77% and 75% at 1 h to 36% and 29% at 14 h. The inhibition ratio was lower than 50% when adding chlorogenic acid after 4 h post-infection. When cells were infected with PRRSV for 1 or 2 h, and then respectively treated with chlorogenic acid and scutellarin, both of the compounds showed significant anti-PRRSV activities. The results demonstrated that chlorogenic acid and scutellarin may only inhibit the early stage of the PRRSV replication cycle

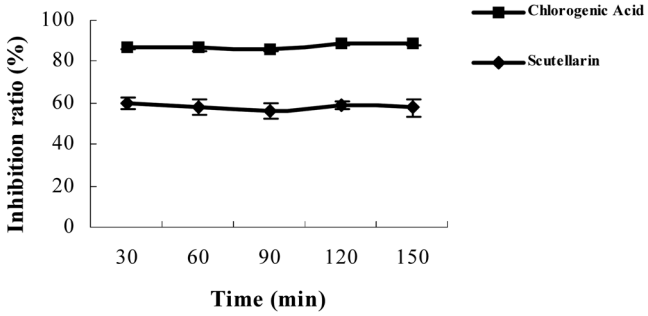

Fig. 3. Virucidal activity of the two compounds tested at different incubations.

Each value represents the mean ± SD ($n = 4$). No significant difference for each compound was detected at the different time points ($p > 0.05$).

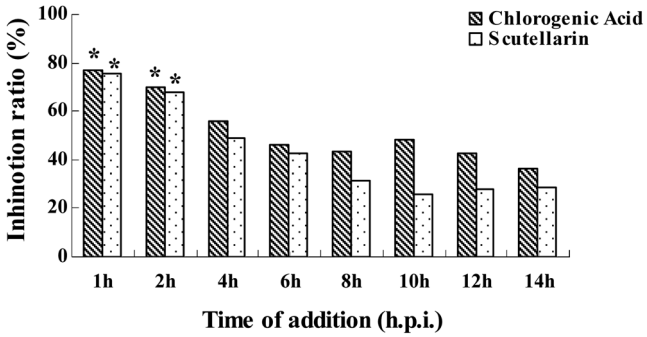

Fig. 4. Effects on the antiviral activities of adding two compounds at different incubation points within a single replication cycle.

Marc-145 cells were infected with PRRSV at 100 $TCID_{50}$. The compounds were added during viral infection from 1 to 14 h post-infection (h.p.i.). The inhibition ratio was determined by the MTT method. Data represent the mean ± SD ($n = 4$). The asterisk indicate statistical significance, $P < 0.05$.

(Fig. 4). Lin [13] reported that glycyrrhizic acid interfered with an early step of the EBV replication cycle, and had no effect on viral adsorption, nor did it inactivate EBV particles. Wang *et al.* [24] demonstrated that chlorogenic acid possessed a substantial inhibitory activity against HBV-DNA replication. Coxsackievirus B5 and herpes simplex virus-1 replication were consistently inhibited by hyaluronic acid, regardless of the time of addition [4]. Our previous study also demonstrated that sodium tanshinone IIA sulfonate could inhibit PRRSV replication during a single replication cycle [23].

In order to evaluate the effect of the two compounds on the virus adsorption, we pre-incubated the Marc-145 cells with chlorogenic acid and scutellarin, respectively, or co-incubated the cells with each of the two compounds and PRRSV simultaneously. The inhibition ratios with the two assays for both compounds was lower than 20%, suggesting that chlorogenic acid and scutellarin did not interfere with the virus ability to attach to the cells. However, Zhang *et al.* [28] found that the anti-HIV-1 action of scutellarin may be related to its interference with the entry of HIV-1 into cells.

Generally, both chlorogenic acid and scutellarin can inhibit PRRSV infection effectively *in vitro*, and their antiviral activity is most likely due to directly inactivating and/or disturbing the early stage of PRRSV replication. It is encouraged to further explore the antiviral properties and mechanism of these compounds *in vitro* and *in vivo*. They have a great potential to become the candidate for anti-PRRSV drugs in clinical application.

Acknowledgments

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