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Chinese herbal extracts with antiviral activity: evaluation, mechanisms, and potential for preventing PRV, PEDV and PRRSV infections

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Abstract

The rapid expansion of large-scale pig farming has brought about a surge in viral diseases with high morbidity rates and diverse manifestations. This widespread occurrence of multiple viral diseases in pig farms has inflicted severe economic losses on the global swine industry. Consequently, there is an urgent need for eco-friendly and efficient antiviral drugs that can effectively combat viruses and prevent diseases such as PEDV, PRRSV, PRV, and other viral infections. To this end, we conducted a study on the antiviral activity and cytotoxicity of eleven different Chinese herbal extracts (CHE) against PRV. In vitro testing of several extracts, namely, Echinacea, Ilex purpurea Hassk, Ganoderma lucidum Kars, Taraxacum mongolicum, and Ilex rotunda Thunb, exhibited remarkable inhibition of PRV infection without causing any cytotoxic effects. Specifically, their antiviral selectivity indexes were significantly higher, with values ranging from 6- to 144-fold. The antiviral efficacy of five CHEs was evaluated against other RNA viruses, including PRRSV and PEDV. The extracts showed substantial inhibition of PEDV and PRRSV proliferation. Echinacea and Ilex purpurea Hassk extracts exhibited the highest virus inhibitory effects. To understand the antiviral mechanisms underlying their potent activity, a time-of-addition experiment was conducted. The results indicated that these extracts effectively targeted the early infection and postinfection stages of PRV, PEDV, and PRRSV. The study found that the Chinese herbal extracts, Echinacea and Ilex purpurea Hassk, had both direct and indirect effects on virus particles and cellular targets, demonstrating broad-spectrum antiviral activity against multiple clinical strains of PRV and PEDV. These findings provide a strong foundation for the development of herbal medicines to prevent and treat infections caused by PRV, PEDV and PRRSV in the swine industry. The identified extracts show great promise for the formulation of effective and environmentally friendly antiviral interventions.

Keywords CHE, PRV, PEDV, PRRSV, Antiviral activity

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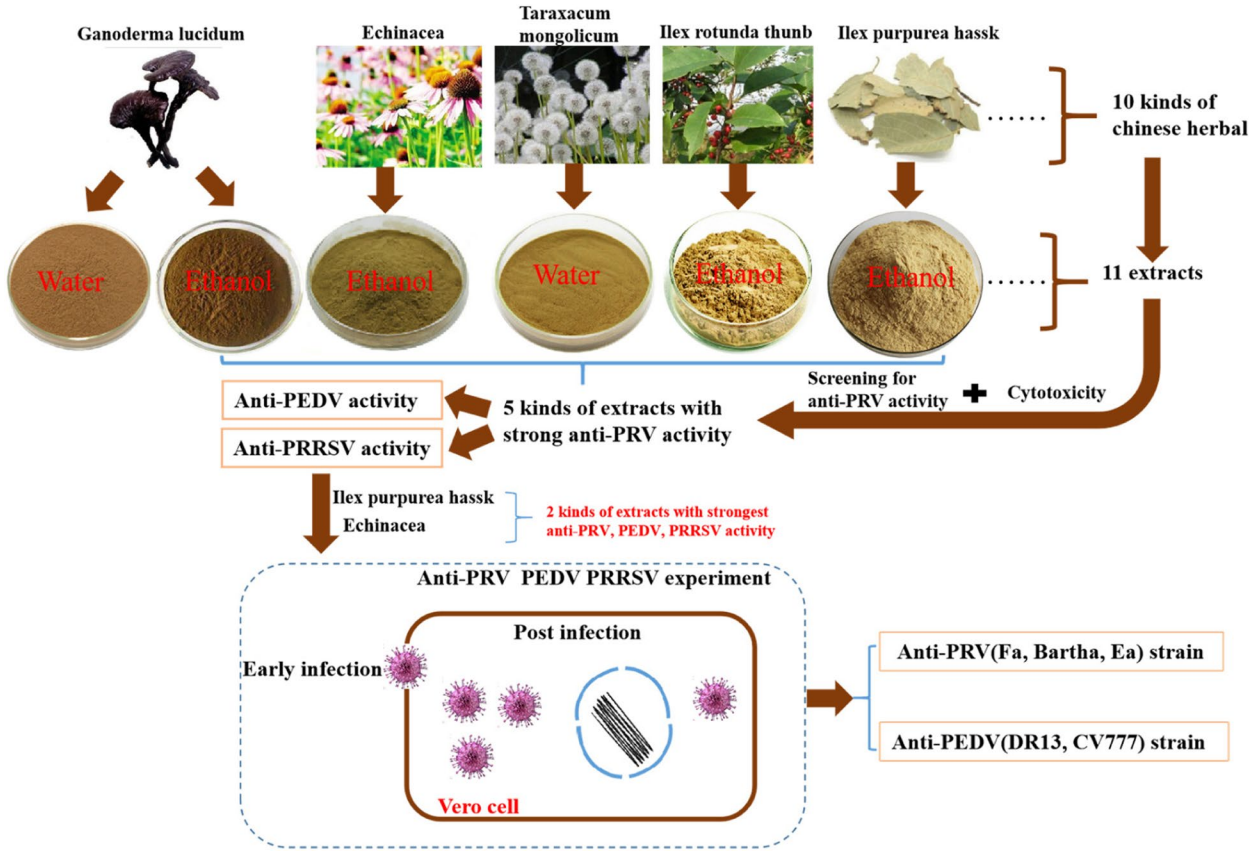
Graphical Abstract



Introduction

Despite the expansion of the pig industry, the increase in intensity and the continuous improvement of feeding and management conditions, the prevention and control of porcine viral infectious diseases such as porcine epidemic diarrhea (PED) (Lee 2016), porcine pseudorabies (PR) (Tan et al. 2021) and porcine reproductive and respiratory disorder syndrome (PRRS) (Jiang et al. 2021) remain difficult to prevent and control. These diseases cause huge economic losses to the pig industry and pose serious environmental and biosecurity threats (Liu et al. 2020; Wang et al. 2020a). Therefore, it is imperative to research and develop effective prevention and treatment strategies for swine viral infectious diseases.

Pseudorabies virus (PRV) is a double-stranded DNA virus with an envelope belonging to the *alpha-herpesvirus* subfamily (Kuhn et al. 2008). In 2011, many Bartha-K61 vaccinated pig farms in China experienced PR outbreaks (Yu et al. 2014). Porcine epidemic diarrhea virus (PEDV) is an enveloped single-stranded positive-sense RNA virus belonging to the *Coronaviridae* family (Lee 2016). Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded positive-sense RNA virus of 15 kb that belongs to the *Arteriviridae* family (Thiel et al. 1993). The high mutation rate and rapid turnover of RNA viruses, along with frequent recombination events between subtypes, contribute to the genetic diversity of PEDV and PRRSV. This diversity poses a challenge for the effective prevention and control of RNA viruses through vaccine immunization, as there is currently no vaccine that can offer protection against all serotypes. PRV, PEDV and PRRSV are common and highly damaging to the swine industry worldwide and are often accompanied by other viruses and bacteria. Therefore, there is an urgent need to develop effective, nontoxic, broad-spectrum, and environmentally friendly antiviral drugs to control viral infections.

To avoid issues of drug resistance and metastasis resulting from long-term treatment, many countries and

regions have restricted or prohibited the use of antibiotics and other drugs in animal production. As a result, natural compounds have emerged as a promising area of drug research and development. Traditional Chinese herbs, which have a long history of use, are increasingly being explored for their potential in treating viral diseases. For instance, Chinese herbal oral liquids have been utilized to treat the new coronaviruses that have impacted human health and quality of life globally (Ni et al. 2020; Wang et al. 2020a, b; Deng et al. 2020; Hong et al. 2020). Natural Chinese herbs exhibit significant advantages over chemical drus, such as low toxicity, low drug resistance, and antiviral activity through various mechanisms (Kuo et al. 2005; Bisignano et al. 2017; Li and Peng 2013). Research shows that Houttuynia cordata targets the beginning stage of herpes simplex virus (HSV) infection and suppresses HSV replication (Hung et al. 2015). Therefore, the screening of natural Chinese herbs in antiviruses could provide new options for developing antiviral drugs. Some traditional applications of Taraxacum mongolicum are supported by pharmacological research. Taraxacum mongolicum extract has anti-inflammatory, antioxidant, and anticancer activities and liver-protecting activities and has been widely used to treat a variety of human diseases, such as HBV infection (Sch et al. 2006; Sigstedt et al. 2008; Hagymási et al. 2000; Jia et al. 2014). Echinacea is one of the most popular botanicals used in dietary supplements and has a broad spectrum of pharmacological effects, including antiviral, anticancer, antibacterial, and immunomodulatory effects (Chicca et al. 2007; Kim et al. 2020; Dong et al. 2015; Hudson 2012). Moreover, Echinacea shows the ability to eliminate multiple viruses during their initial contact with host cells, including IAV, Haemophilus influenza virus, and HSV (Pleschka et al. 2009; Papers 2010; Schneider et al. 2010). JiuBiYing, also called Ilex rotunda Thunb, is highly valued for the treatment of tonsillitis, colds, eczema, diarrhea, and rheumatism and is a typical Chinese herbal (Wang et al. 2014). Modern pharmacological studies have also found that Ilex rotunda Thunb protects against cardiovascular system effects such as anti-inflammatory, antioxidation, and antibacterial effects (Kim et al. 2020; Zhao et al. 2012). Ilex purpurea Hassk is reported to be used to treat scalds, eczema, sores, lung heat cough, sore throat, hot showers, diarrhea, and trauma bleeding (Tang et al. 2006). The antiviral activity of Ilex purpurea Hassk has not been properly explored. Ganoderma lucidum is one of the most frequently used medicinal fungal species in the world and has long been used as a traditional medicine for various disorders (Chang et al. 2015; Klupp et al. 2016), such as antitumor, antimicrobial, antiatherosclerotic, anti-inflammatory, hypolipidemic, antidiabetic, antioxidative, radical-scavenging and antiaging activities (Boh et al. 2007; Cherian et al. 2009). It has antiviral activity against a wide variety of viruses, including HSV-1, HSV-2, IAV, vesicular stomatitis virus (VSV), dengue virus (DENV), and human immunodeficiency virus (HIV) (Shiv et al. 2019; Zhu et al. 2015; Eo et al. 1999).

Exploring the antiviral activity of Chinese herbs, which possess a variety of pharmacological activities, can provide potential therapeutic drugs against diseases induced by PRV, PEDV, or PRRSV. However, whether these plants have antiviral activity against pseudorabies virus has not been studied. The study aimed to find potential therapeutic drugs by testing the inhibitory PRV activities of eleven plant extracts. Additionally, the study evaluated the antiviral activity of potential PRV inhibitors (five extracts) on PEDV and PRRSV. The purpose of this study was to analyze the potential mechanisms of action of Echinacea and Ilex purpurea Hassk extracts that have the highest antiviral activity against various swine viruses, including PRV, PEDV, and PRRSV. Additionally, the study aimed to assess the inhibitory effect of extracts against PRV infection on multiple genotypes of PRV and PEDV. To accomplish this, researchers have investigated antiviral CHE against PRV, PEDV and PRRSV to offer treatment and prevention of swine viral diseases.

Results

Cytotoxicity of CHE

To assess the cytotoxicity of CHEs, we conducted the MTT assay to determine the viabilities of Vero cells treated with CHE. The CC_{50} and CC_{90} results are summarized in Table 1, indicating that eleven CHEs have low toxicity at higher concentrations. For convenience in the subsequent experiments, we selected an extract concentration that did not show any toxicity toward Vero cells, as presented in Table 1 (concentration used for antiviral experiments).

Screening of eleven CHEs with potential PRV inhibition activity

The antiviral efficacy of CHE against PRV was evaluated using IFA and plaque reduction assay to screen the inhibitory effect of extracts on PRV-induced infection in Vero cells. Of the five CHEs tested, Ganoderma lucidum Kars (ethanol), Ilex rotunda Thunb, Ilex purpurea Hassk, Taraxacum mongolicum, and Echinacea showed obvious inhibition of the range of virus infection and the number of plaques in cells infected with PRV (Fig. 1A, B). Among the three CHEs, Andrographis paniculata, Ganoderma lucidum Kars (water), and Clerodendrum cyrtophyllum Turcz also had a slight inhibitory effect on the range of virus infection and the number of plaques in cells infected with PRV (Fig. 1A, B). These results confirmed that the extracts of Ganoderma lucidum Kars (ethanol), Ilex rotunda Thunb, Ilex purpurea

Table 1 The CC_{50} and CC_{90} of extracts in Vero cells

Substance	Cytotoxicity (mg/mL)		Concentration used for antiviral experiments (mg/mL)
	CC_{90}	CC_{50}	
Codonopsis pilosula	9.9 ± 0.21	43.0 ± 0.73	6.25
Ilex rotunda thunb	2.52 ± 0.07	5.54 ± 0.20	1.67
Astragalus membranaceus	> 12.50	> 12.50	6.25
Clerodendrum cyrtophyllum turcz	7.71 ± 0.89	48.73 ± 0.71	6.25
Taraxacum mongolicum	8.51 ± 0.11	16.82 ± 0.21	6.25
Andrographis paniculata	> 13.50	> 13.50	1.69
Echinacea	9.99 ± 0.28	20.34 ± 0.73	3.58
Ilex purpurea hassk	7.02 ± 0.06	13.16 ± 0.14	3.13
Ganoderma lucidum kars (ethanol)	6.29 ± 0.33	9.25 ± 0.16	2.50
Ganoderma lucidum kars (water)	7.47 ± 0.20	11.03 ± 0.31	5.00
Dioscorea opposite thunb	4.40. ± 0.01	40.2 ± 0.02	6.25

The cytotoxic concentrations of 50% (CC_{50}) and 90% (CC_{90})

Hassk, Taraxacum mongolicum or Echinacea have antiviral activity against PRV. Meanwhile, through qPCR detection, five extracts could significantly inhibit the genome copy number/mL of PRV DNA in the cell culture supernatant (Fig. 1C). Moreover, the MTT assay was conducted to detect the CPE inhibition rate of the five extracts that showed strong inhibitory activity against PRV in PRV-infected cells. Table 2 shows the IC_{50}, IC_{90}, and SI values for Ganoderma lucidum Kars (ethanol), Ilex rotunda Thunb, Ilex purpurea Hassk, Taraxacum mongolicum and Echinacea extracts. When the concentration of these extracts was greater than 1.09 mg/mL, 2.42 mg/mL, 0.42 mg/mL, 2.96 mg/mL, and 0.22 mg/mL, the pathological changes in PRV-infected cells were significantly reduced. The selectivity index (SI) was approximately > 21, > 6, > 49, > 21, and > 144. This suggests that the anti-PEDV drug effect of these 5 extracts is Echinacea > Ilex purpurea Hassk > Ganoderma lucidum Kars (ethanol) > Taraxacum mongolicum > Ilex rotunda Thunb.

Five kinds of CHE with strong PRV inhibition activity can inhibit PEDV infection

During the screening of antiviral candidates from eleven CHEs, it was found that five of them, Ganoderma lucidum Kars (ethanol), Ilex rotunda Thunb, Ilex purpurea Hassk, Taraxacum mongolicum, and Echinacea, have antiviral activity against DNA viruses (PRV) in Vero cells. These same five CHEs may have antiviral activity against RNA viruses (PEDV). To demonstrate this presumption, we performed IFA to examine the range of PEDV infection and RT−qPCR and qPCR to examine PEDV M expression in infected cells and cell culture supernatant with or without extracts. The results displayed in Fig. 2 demonstrate that treatment with extracts from Taraxacum mongolicum, Ilex purpurea Hassk, or Echinacea significantly reduced the range of PEDV infection compared to the control group. Conversely, the extracts of Ilex rotunda Thunb and Ganoderma lucidum Kars (ethanol) showed weaker inhibitory effects on PEDV infection. The findings from RT−qPCR and qPCR were consistent with those from IFA. The study found that extracts of Taraxacum mongolicum, Ilex purpurea Hassk, Echinacea, Taraxacum mongolicum, or Ganoderma lucidum Kars (ethanol) exhibited antiviral activity against PEDV infection. The MTT assay was used to examine the inhibitory rate of the extracts on PEDV-induced CPE, and the SI values are presented in Table 3. When the concentration of Ilex rotunda Thunb, Ilex purpurea Hassk, Taraxacum mongolicum or Echinacea extract exceeded 0.42, 0.67, 0.28L and 0.13 mg/mL, respectively, the pathological changes were significantly reduced in PEDV-infected cells. The selectivity index (SI) was approximately > 13, > 19, > 60 and > 151, respectively. Therefore, the results indicated that the inhibition on PEDV infection by the four extracts was in the order of Echinacea > Taraxacum mongolicum > Ilex purpurea Hassk > Ilex rotunda Thunb.

Five kinds of CHE with strong PRV inhibition activity can inhibit PRRSV infection

This study aimed to evaluate the antiviral activity of five extracts, namely, Echinacea, Taraxacum mongolicum, Ilex rotunda Thunb, Ilex purpurea Hassk, and Ganoderma lucidum (ethanol), against PRRSV in vitro. The virus yield and gene mRNA level of the virus were determined using RT−qPCR, qPCR and IFA in the presence

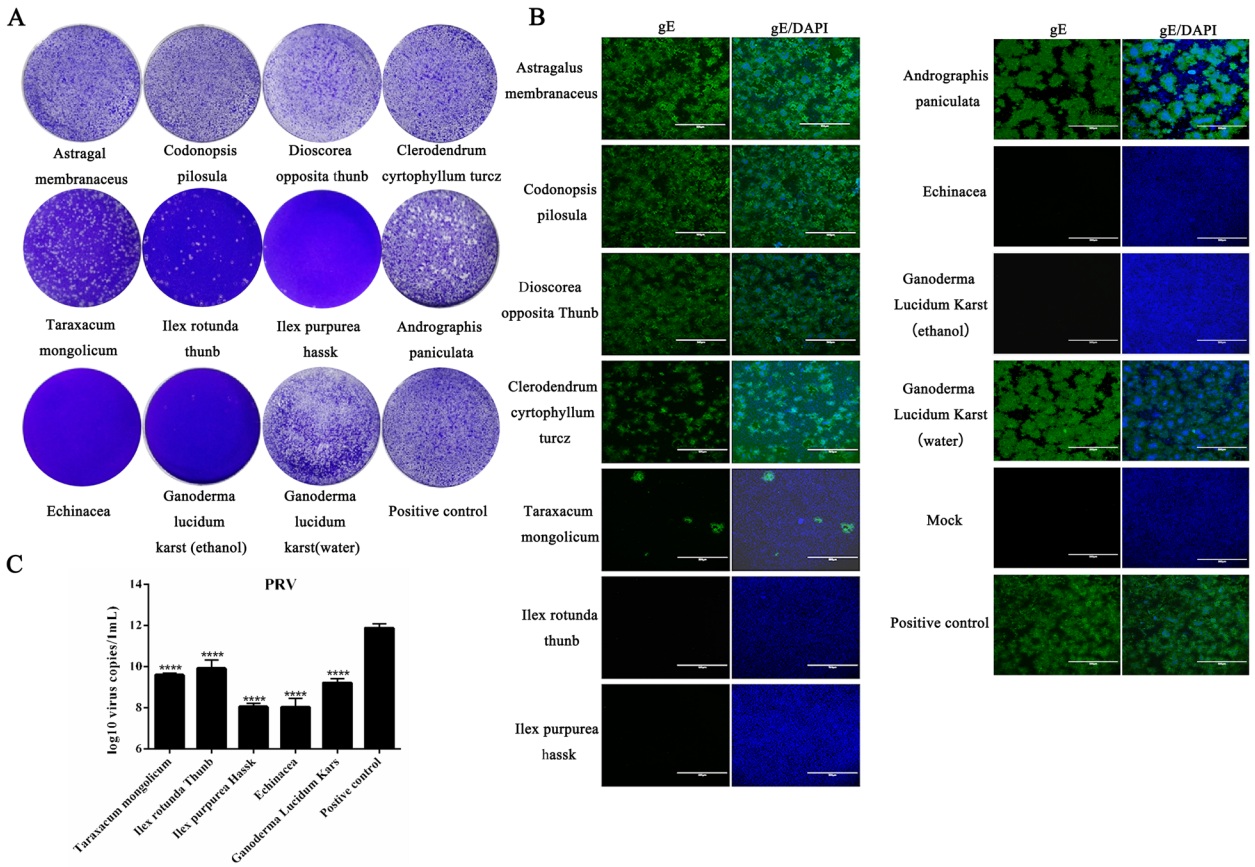


Fig. 1 Anti-PRV effect of eleven Chinese herbal extracts. Monolayer cells were incubated with CHE and the PRV HNX strain (MOI 0.01) for 1 h and washed three times with PBS. Infected cells were then incubated in medium with or without extracts for 23 h. The antiviral activity of extracts against PRV in Vero cells was detected by plaque reduction assay (**A**) and photographed by microscopy (**B**). The nucleocapsid (DAPI, blue) and the gE protein of PRV (gE, green). The viral DNA fragments in the cell culture supernatant were tested by qPCR (**C**). All data are represented as the mean ± SD, ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, vs the positive control

Table 2 IC_{50}, IC_{90} and SI of the extracts against porcine pseudorabies virus

Substance	Antiviral activity		Selectivity index	
	IC_{90} (mg/mL)	IC_{50} (mg/mL)	CC_{90}/IC_{90}	CC_{50}/IC_{50}
Echinacea	0.22	0.14	> 44	> 144
Taraxacum mongolicum	2.06	0.78	> 4	> 21
Ilex rotunda thunb	2.42	0.80	> 1	> 6
Ilex purpurea hassk	0.42 ± 0.03	0.26 ± 0.10	> 16	> 49
Ganoderma lucidum karst (ethanol)	1.09 ± 0.16	0.43 ± 0.09	> 5.79	> 21

The cytotoxic concentrations of 50% (CC_{50}) and 90% (CC_{90}); The inhibitory concentrations of 50% (IC_{50}) and 90% (IC_{90}) values of extracts

SI The selectivity index is defined as the ratio of CC_{50} to IC_{50} ($SI = CC_{50}/IC_{50}$), *N.d* not determined

or absence of the extract. The results showed that at noncytotoxic concentrations, all five extracts significantly inhibited the infection range and gene expression of PRRSV, reducing the genomic copy number of PRRSV down to 4.2, 5.13, 2.9, 3.97 and 1.5-fold of log 10 (Fig. 3C). Therefore, the study suggests that these five extracts can effectively inhibit PRRSV infection.

Time (site) of intervention

Generally, the antiviral activity of Chinese herbs has direct and indirect effects. Directly, they act on viruses by inactivating and inhibiting the proliferation of viral infections. Indirectly, they enhance the immune function in the host to suppress viruses (Li et al. 2013). Among the various extracts, Ilex purpurea Hassk and Echinacea have

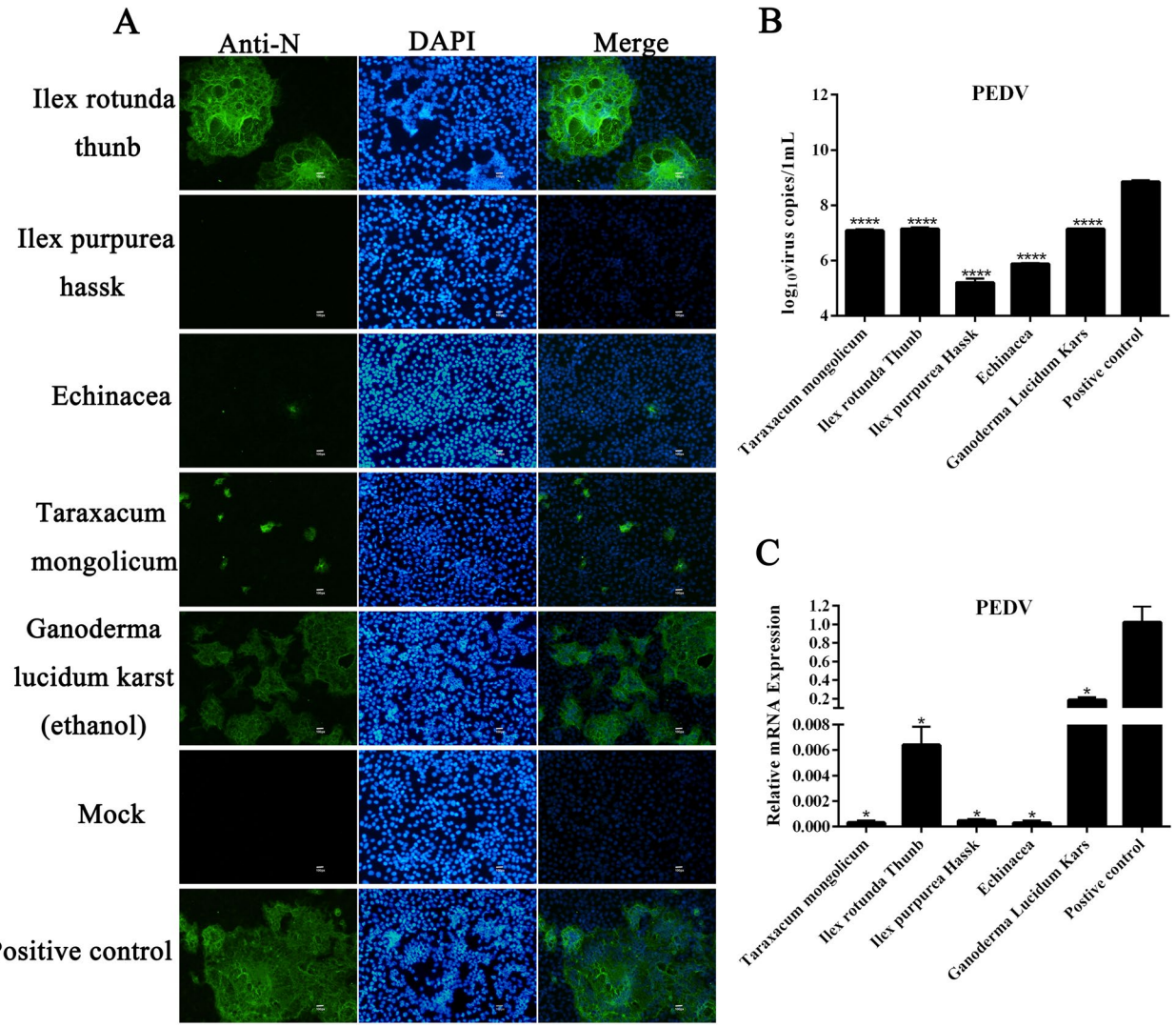


Fig. 2 The inhibitory effect of five potential antiviral extracts on PEDV infection. Vero cells were infected with YN14 PEDV (MOI = 0.01) and incubated for 1 h either with or without extracts. After 23 h of total culture, viral N protein was detected with N-specific monoclonal antibody (green); nucleocapsid (DAPI, blue) (**A**). The intracellular total RNA fragments obtained from the attached cells were measured by RT–qPCR (**B**). The viral RNA fragments in the cell culture supernatant were tested by qPCR (**C**). All data are represented as the mean ± SD, ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *vs* the positive control

Table 3 IC_{50}, IC_{90} and SI of the extracts against porcine epidemic diarrhea virus

Substance	Antiviral activity		Selectivity index	
	IC_{90} (mg/mL)	IC_{50} (mg/mL)	CC_{90}/IC_{90}	CC_{50}/IC_{50}
Echinacea	0.64	0.13	> 15	> 151
Taraxacum mongolicum	N.d	0.28	N.d	> 60
Ilex rotunda thunb	N.d	0.42 ± 0.05	N.d	> 13
Ilex purpurea hassk	1.13 ± 0.05	0.67 ± 0.05	> 6	> 19
Ganoderma lucidum Karst (ethanol)	N.d	N.d	N.d	N.d

The cytotoxic concentrations of 50% (CC_{50}) and 90% (CC_{90}); The inhibitory concentrations of 50% (IC_{50}) and 90% (IC_{90}) values of extracts

SI The selectivity index is defined as the ratio of CC_{50} to IC_{50} ($SI = CC_{50}/IC_{50}$), *N.d* not determined

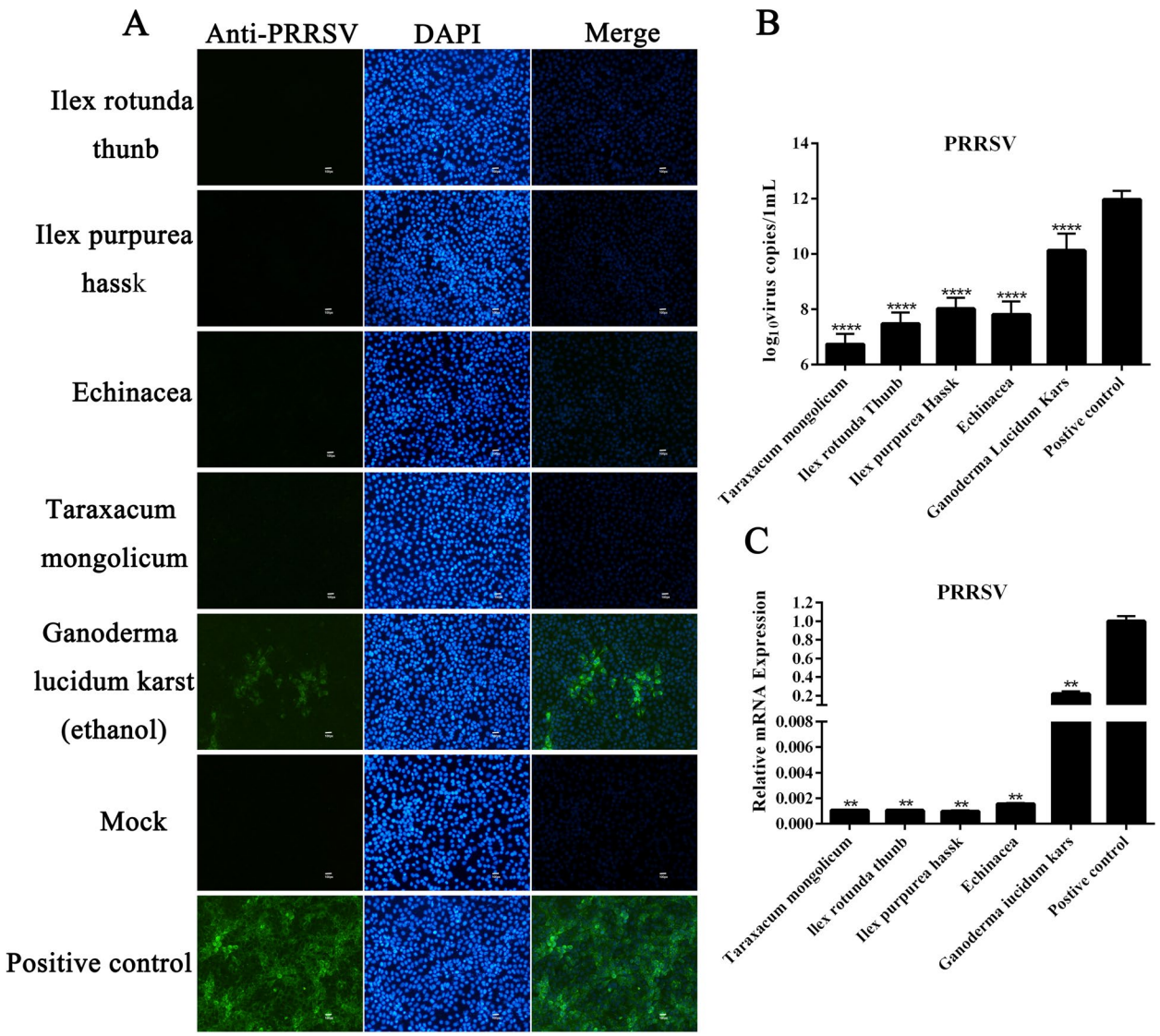


Fig. 3 The inhibitory effect of five potential antiviral extracts on PRRSV infection. Vero cells were infected with WUH3 PRRSV (MOI = 0.01) and incubated either with or without extracts. (**A**) At 23 h postinfection, the infection range of PRRSV in cells was observed by microscopy. The nucleocapsid (DAPI, blue) and the PRRSV protein (green). (**B**) The intracellular total RNA fragments obtained from the attached cells were measured by RT–qPCR. (**C**) The PRRSV RNA fragments in the supernatant were tested by qPCR. All data are represented as the mean ± SD, ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *vs* the positive control

the most significant antiviral activity against PEDV, PRV, and PRRSV infection. A time-of-addition experiment was conducted to determine the antiviral targets of the two extracts: (1) cell pretreatment, (2) virus pretreatment, (3) cotreatment, and (4) positive control (Fig. 4A). In this study, it was observed that pretreatment of the virus with Echinacea or Ilex purpurea Hassk extract before infecting cells or pretreatment of cells with Echinacea or Ilex purpurea Hassk extract before virus infection led to a significant inhibition of the quantitative genomic copies/mL of PEDV RNA, PRV DNA, and PRRSV RNA in the cell culture supernatant (Fig. 4B, C). It was found that the direct killing effect of the two extracts on the virus was greater than their preventive activity. Therefore, it can be inferred that the antiviral activity of Echinacea and Ilex purpurea Hassk extracts not only directly acts on the virus but also indirectly acts on the cells.

To investigate whether the antiviral effect of Echinacea or Ilex purpurea Hassk extract prevents virus infection or inhibits postinfection stage viral proliferation, a time-of-addition experiment was conducted. (1) early infection studies and (2) postinfection studies, (3)

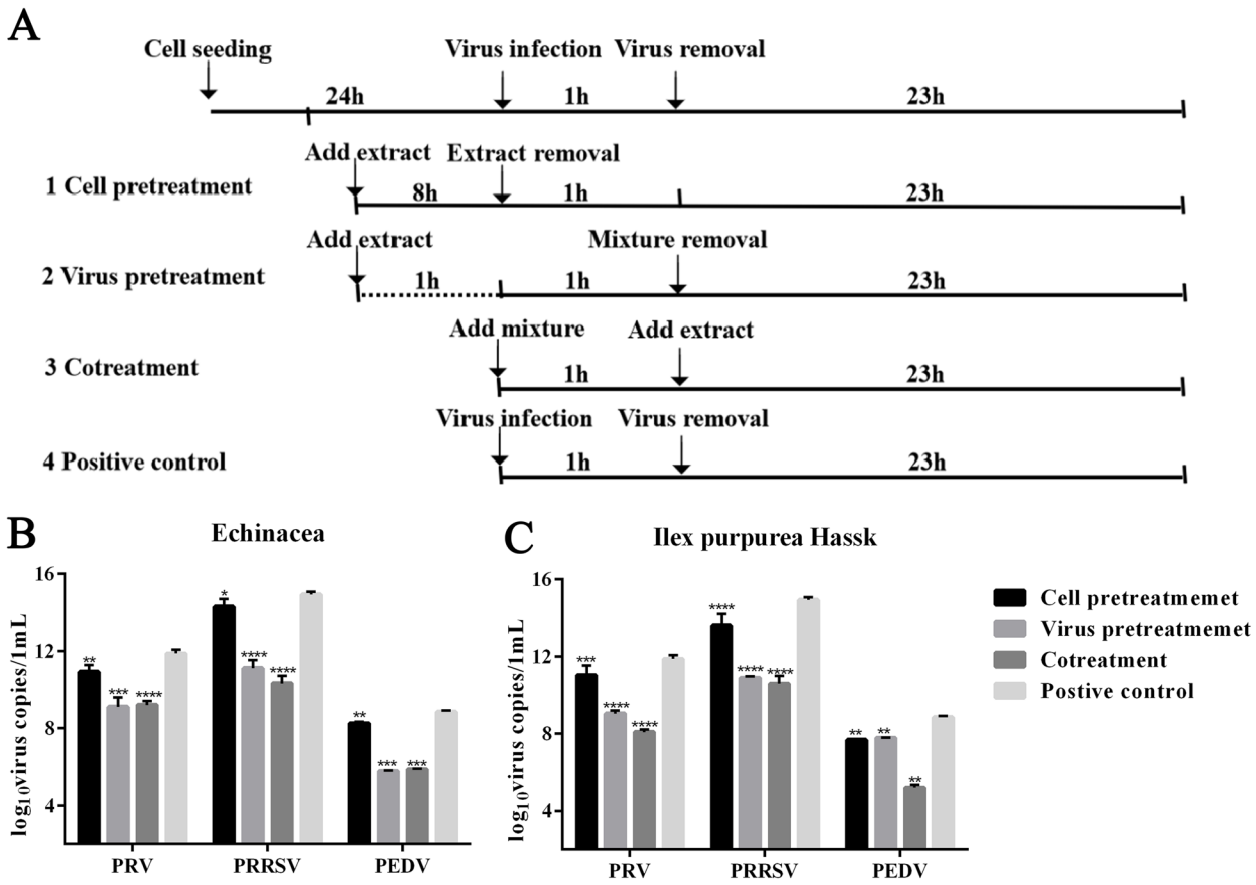


Fig. 4 Time-of-addition experiment. (**A**) Time mark of adding extract. (1) Cell pretreatment; (2) Virus pretreatment; (3) Cotreatment; (4) Positive control. The infectious virus released in the infected cell culture supernatant was quantified by qPCR. Time-of-addition assays of Echinacea (**B**) and Ilex purpurea Hassk (**C**) are shown. All data are represented as the mean ± SD, ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *vs* positive control. PRV: porcine pseudorabies virus; PEDV: porcine epidemic diarrhea virus; PRRSV: porcine reproductive and respiratory disorder syndrome

cotreatment: adding extract throughout the life cycle of the virus, and positive control where no extract was added throughout the life cycle of the virus (Fig. 5A). The number of copies of the virus released into the supernatant of the cell culture was measured by qPCR. The addition of Echinacea or Ilex purpurea Hassk extract to virus-infected cells resulted in significant inhibition of PRV, PRRSV or PEDV yields (Fig. 5B, C). Furthermore, a significant reduction was observed when the Echinacea or Ilex purpurea Hassk extract was added in virus early infection or post infection. Research indicates that Echinacea and Ilex purpurea Hassk exhibit higher antiviral activity against PRV and PEDV in the early stage of infection than in the postinfection stage. However, the antiviral activity of Echinacea against PRRSV during early infection was less than that during the postinfection stage (Fig. 5B). These results suggest that multiple steps of the viral (PRV, PEDV and PRRSV) life cycle may be affected by Echinacea or Ilex purpurea Hassk extract.

Echinacea and Ilex purpurea Hassk have antiviral activity against divergent strains of PRV and PEDV

Ilex purpurea Hassk and Echinacea extracts have demonstrated antiviral activity against PRV HNX strain and PEDV YN14 strain infection. To determine whether the antiviral effect was subtype specific, we used RT–qPCR to test whether the two extracts have antiviral activity against multiple strains of PRV (Fa, Batha, Ea) and PEDV (DR13, CV777). As shown in Fig. 6A for Ilex purpurea Hassk and Fig. 6B for Echinacea, the mRNA expression levels of divergent strains of PRV and PEDV were significantly reduced. These results confirm that the virucidal effect of Ilex purpurea Hassk or Echinacea extract is not limited to specific subtypes of PRV and PEDV.

Discussion

Cross-species transmission of viruses occurs frequently, and the protection capability of vaccines has become increasingly insufficient. In China, since 2011, multiple mutant strains of PRV have been isolated from pig farms

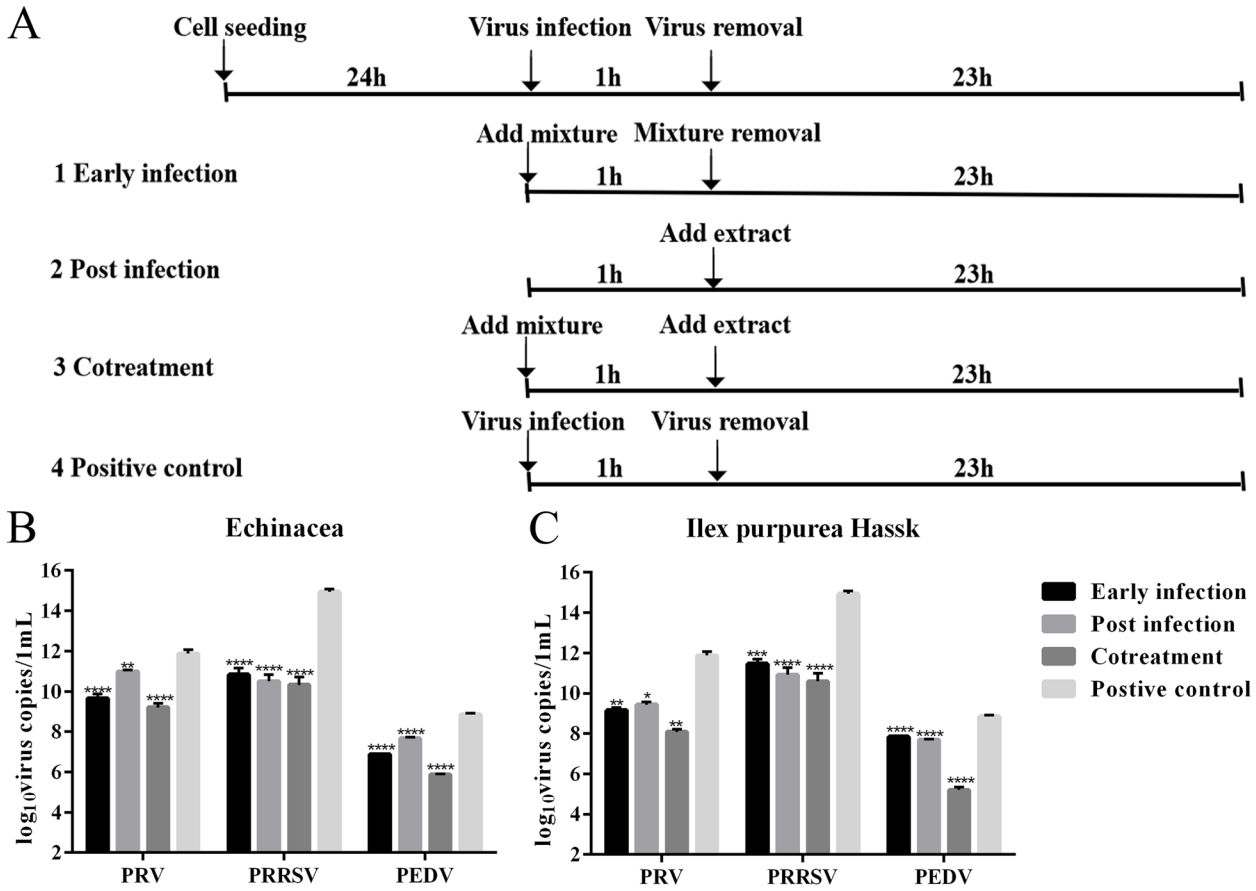


Fig. 5 Time-of-addition experiment. (**A**) Time mark of adding extract. (1) Early infection studies; (2) Post infection studies; (3) Cotreatment; (4) Positive control. The infectious virus released in the infected cell culture supernatant was quantified by qPCR. Time-of-addition assays of Echinacea (**B**) and Ilex purpurea Hassk (**C**) are shown. All data are represented as the mean ± SD, ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *vs* positive control. PRV: porcine pseudorabies virus; PEDV: porcine epidemic diarrhea virus; PRRSV: porcine reproductive and respiratory disorder syndrome

that were previously vaccinated with the pseudorabies vaccine (Leng et al. 2013). These mutant strains are more pathogenic than the nonmutant PRV strains (Zhang et al. 2014). Additionally, persistent PRV infection and the occurrence of other pig viruses, such as PRRSV or PEDV (Chen et al. 2021; Tan et al. 2020), have resulted in significant economic losses and social impacts for the swine industry. Recent research has confirmed that a PRV variant strain can cause acute encephalitis in humans (Liu et al. 2020; Wang et al. 2020a, b). With the global outbreak of the novel coronavirus since December 2019, the infected population has exceeded 15 million as of July 22, 2020 (Parikh et al. 2020). As a result, there is an urgent need for effective research on safe and broad-spectrum antiviral drugs to prevent the spread of the virus, which not only affects human and animal health but also causes economic losses.

The use of traditional Chinese herbal agents for health promotion and adjuvant viral disease therapy is gaining popularity worldwide. Due to the rich resources and complex and diverse ingredients of Chinese herbs, identifying new environmentally friendly antiviral drugs with different mechanisms of action is crucial. In this study, eleven CHEs were screened based on their known pharmacological use, and the pharmacological applications and antiviral activities of the five strongest PRV inhibitors are discussed.

Taraxacum mongolicum, has been shown to protect hepatocytes and contribute to antiviral effects in the replication of HBV or DHBV in vitro (Jia et al. 2014). Ilex rotunda Thunb (Jiubiying) is rich in triterpenoid compounds and has demonstrated strong anti-inflammatory and antioxidant activities (Gang et al. 2019). However, there are no relevant reports about the antiviral function of Ilex rotunda Thunb in cells. Echinacea, is a plant extract that has gained increasing attention from academia and the public. It is reported to be one of the most widely used herbs in Europe and North America for

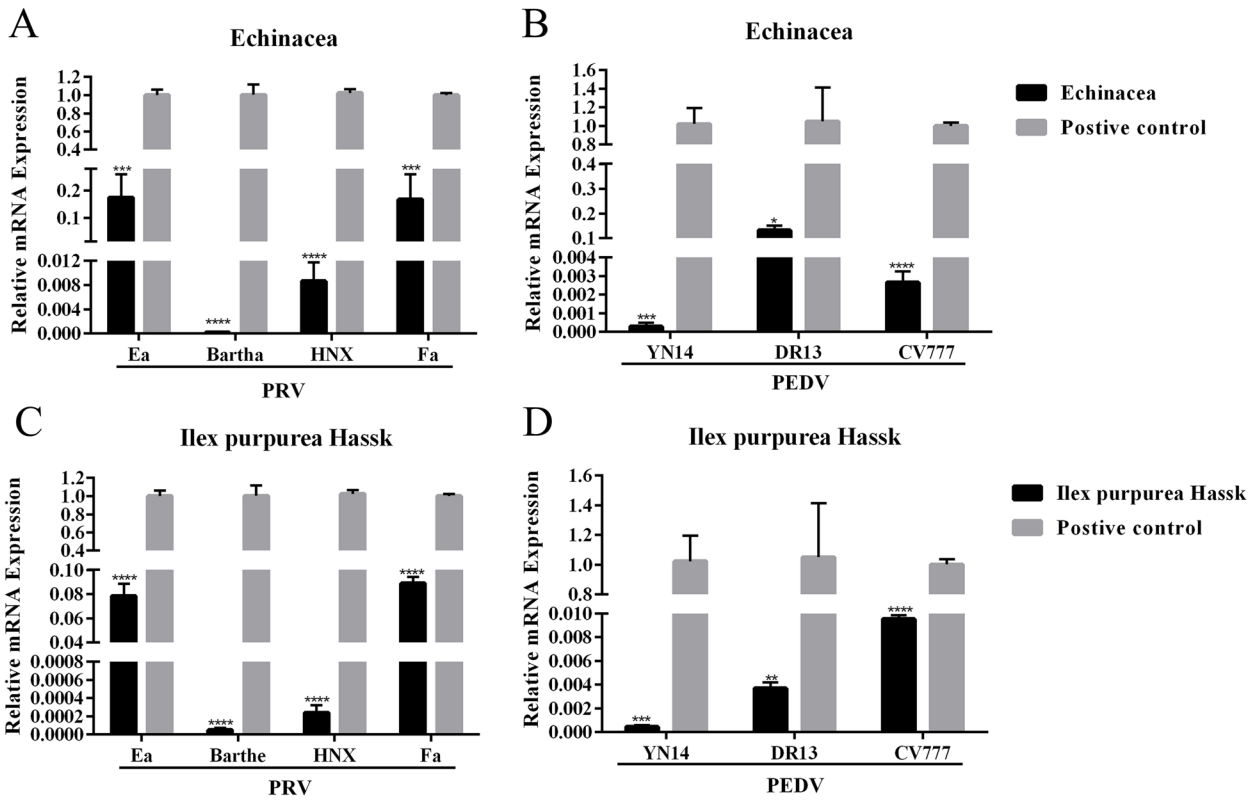


Fig. 6 The inhibitory activity of Ilex purpurea Hassk or Echinacea extracts on different PRV and PEDV subtypes. Vero cells were infected with either PRV or PEDV at an MOI of 0.01 in the presence and absence of CHE. After incubation for 23 h, the viral mRNA expression level was analyzed by RT–qPCR. Echinacea (**A**, **B**); Ilex purpurea Hassk (**C**, **D**). All data are represented as the mean ± SD, ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *vs* the positive control. PRV: porcine pseudorabies virus; PEDV: porcine epidemic diarrhea virus

preventing or treating the common cold, cough, bronchitis, and other upper respiratory infections (Karsch-Völk et al. 2014). Ilex purpurea Hassk has been used for clinical antibacterial, anti-cold, and invasive bleeding purposes, both internally and through injections. However, there is no evidence suggesting that Ilex purpurea Hassk has antiviral properties (Tang et al. 2006). Ganoderma lucidum has been used to treat numerous types of diseases in many countries, including China and Japan (Mishra et al. 2018). Ganoderma lucidum is a mushroom rich in bioactive natural components, which mainly include triterpenes, polysaccharides, proteins, and steroids. According to reports, triterpenoids from Ganoderma lucidum have been shown to exhibit significant antiviral activity against DENV and EV7 (Shiv et al. 2019; Zhang et al. 2014), with the content of Ganoderma triterpene being significantly higher in ethanol extract than in water extract of Ganoderma lucidum (Shiv et al. 2019).

The inhibitory activity of select extracts against PRV infection in vitro was confirmed, with a selectivity index (SI) of approximately > 21 (Taraxacum mongolicum), > 6 (Ilex rotunda Thunb), > 49 (Ilex purpurea Hassk), > 21 (Ganoderma lucidum Kars (ethanol)), and > 144 (Echinacea). These findings suggest that specific extracts may be used for the prevention of PRV illness. Additionally, these extracts were tested for anti-RNA virus (PEDV and PRRSV) activity, with only 4 extracts exhibiting a selectivity index (SI) against PEDV. Among them, the Echinacea extract exhibited the highest selectivity index. In an anti-PRRSV research study, five extracts were found to possess significant antiviral activity. Among these extracts, Echinacea and Ilex purpurea Hassk extracts, known as the strongest PRV PEDV and PPRSV inhibitory extracts, have been tested in an antiviral activity assay. Further research data suggested that these two extracts show antiviral activity during early viral infection and postinfection, and the antiviral activity of the two extracts in vitro is due to the direct impact on the virus and the indirect target cells. Our findings support the potential therapeutic use of Echinacea and Ilex purpurea Hassk extracts against various strains of PRV (Fa, Bartha, Ea) and PEDV (DR13, CV777) infection, which is consistent with previous research.

HPLC analysis of Echinacea (ethanol) showed that it contained a variety of acid derivatives and phenolic

compounds, including chlorogenic acid, cichoric acid, echinacoside, and quercetin (Capek et al. 2015; Brown et al. 2010). The mechanism of action for the Echinacea extract was not determined in this study, but there is evidence that quercetin interacts with the HA2 subunit and inhibits the entry of influenza A virus (Wu et al. 2015). The docked poses of SARS-CoV-2 M^{pro} with echinacoside showed conformational stability through molecular dynamics (Antagonist et al. 2021; Khan et al. 2021); Chicoric acid has been shown to affect the entry of HIV and HIV-1 integrase (Nobela et al. 2018), which is an intracellular mechanism of action; Chlorogenic acid has shown a strong anti-IBV compound that could effectively regulate innate immunity through the MDA5, TLR7 and NF-κB signaling pathways (Abaidullah et al. 2021). It has been shown to have an antiviral effect on the influenza A virus by inhibiting neuraminidase to affect the later stages of the viral infection cycle (Ding et al. 2017). Although there is no relevant report that Ilex purpurea Hassk has antiviral activity, Ilex purpurea Hassk possesses several antiviral active principles including kaempferol, quercetin, and caffeic acid (Tang et al. 2006) Kaempferol and quercetin were reported to bind to the substrate-binding pocket of SARS-CoV-2 $3CL^{pro}$ with high affinity (Khan et al. 2021) and show antiviral activity against PRV in vivo and in vitro (Li et al. 2021; Sun et al. 2021); caffeic acid has been shown to have antiviral effects against various families of viruses, including herpes simplex (HSV), VSV-Ebola pseudotyped, and vaccinia viruses (Jacobs et al. 2018). This is due to its ability to interact with the main protease of the virus, inhibit its activity in the host cell and induce the IFNα antiviral response to inhibit HCV replication through the p62-mediated Keap1/Nrf2 signaling pathway (Shen et al. 2018).

Conclusion

In conclusion, five extracts were screened and identified from eleven commonly used CHEs that displayed broad-spectrum antiviral activity against several viruses (PRV, PEDV, and PRRSV) in vitro. Among them, Echinacea and Ilex purpurea Hassk extract exhibited strong preventive and therapeutic effects against PRV, PEDV, and PRRSV infection, exhibiting direct and indirect in vitro antiviral properties. Therefore, CHE is a potential source of molecules with antiviral activity. This research provides a selection basis for the development and application of antiviral Chinese herbal veterinary drugs.

Methods

The cells, viruses and antibodies

Vero cells were cultured in Dulbecco's modified Eagle's high glucose medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Natocor, Cordoba, ARG) at 37 °C in 5% CO_2. The virulent PEDV YN14 strain (GenBank Accession No. KT02123) and PRV HNX strain (GenBank accession: KM189912) were isolated and preserved by our laboratory. The PEDV (CV777, DR13) strain, PRV (Ea, Fa, Bartha) strain, and highly pathogenic PRRSV WUH3 strain were preserved by our laboratory. All the viruses were titrated using the tissue culture infectious dose 50 ($TCID_{50}$) (Smither et al. 2013). Polyclonal antisera from PRRSV-vaccinated pigs were preserved in our laboratory. The monoclonal PRV-gE antibody was provided by Professor Wu Bin from Huazhong Agricultural University. The monoclonal PEDV-N antibody was made and preserved in our laboratory. Goat anti-mouse secondary antibody and goat anti-pig secondary antibody were purchased from ABclonal (ABclonal, China).

Chinese herbal extract preparation

The Chinese herbal extract and paeonol were obtained from Viand Biotech. The Chinese herbal extract powder was dissolved in DMEM and mixed thoroughly for 1 h at room temperature. This extract solution was filtered through a 0.22 μm filter and then stored at -80°C. The initial concentrations of the 11 dissolved CHEs are shown in Supplementary Table 1.

Cytotoxicity and antiviral activity of Chinese herbal extract

The MTT assay was performed to assess both the cytotoxicity and inhibitory activity of the extract. The cytotoxicity assessment involved culturing Vero cells in 96-well plates and diluting the CHEs 2^0, 2^1, 2^2, 2^3, 2^4, 2^5, 2^6, 2^7, and 2^8 times with DMEM from the original concentration. The diluted extracts were then added to the cell culture. For antiviral activity assessment, Vero cells were seeded in a 96-well plate for 24 h, washed with PBS twice, and inoculated with serially diluted extracts and the virus at 100 $TCID_{50}$ for 1 h. After washing to remove the unabsorbed virus, the cells were sustained in serum-free DMEM with serially diluted extracts. Following 48 h of incubation, the culture medium was removed, and the cells were washed with PBS three times. Then, 20 μL of MTT solution was added to each cell and incubated at 37°C for 4 h. The supernatant was removed and replaced with 150 μL/well of DMSO. To determine cell viability, the plate was shaken at room temperature for 10 min, and the absorbance was measured at 490 nm using a multiwell spectrophotometer. The results were expressed as a percentage of the vehicle control (DMEM). GraphPad Prism software version 7 (GraphPad Software Inc., La Jolla, CA, USA) was used for the cytotoxic concentrations of 50% (CC_{50}) and 90% (CC_{90}) and the inhibitory concentrations of 50% (IC_{50}) and 90% (IC_{90}) values of extracts. The selectivity index (SI) was determined as the ratio of CC_{50} to IC_{50} for each compound.

Indirect Immunofluorescence Analysis (IFA)

Confluent Vero cells in a 24-well plate were incubated with the virus-extract mixture for 1 h at 37°C. The viral inoculum was then removed, and cell monolayers were washed three times with PBS to eliminate any unattached virus particles. To conduct the experiment, a safe concentration of the extract was added to well-grown Vero cells in 12-well plates, followed by additional incubation for 23 h at 37°C. The supernatant was aspirated, and the cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 4 min. The cells were then incubated with the virus monoclonal antibody for 2 h at 37°C and the secondary fluorescent antibodies for 1 h at 37°C. The nucleus was stained with 4',6-Diamidino-2-phenylindole (DAPI) for 20 min. Immunofluorescence was visualized under a fluorescence microscope (Nikon Eclipse Ti microscope, Japan).

Plaque reduction test

Well-grown Vero cells were seeded into 12-well plates at 37°C in 5% CO_2. The virus at an MOI of 0.1 with the safe concentration of extract was added to the cell for 1 h at 37 °C. These cells were washed with PBS and replaced with fresh medium containing 0.8% carboxymethyl cellulose. At 3 d postinfection, these cells were fixed with 10% formaldehyde for 20 min at room temperature. Then, the fixing solution was discarded. Crystal violet staining (0.1%) was carried out to observe plaque formation in the experimental and control groups.

Quantitative Real-time PCR (RT–PCR)

Total RNA was isolated from cells using TRIzol reagent and reverse transcribed utilizing the Primescript RT reagent kit (TaKaRa, China). Relative quantitative real-time PCR (RT–qPCR) was performed to quantify the mRNA levels of M (PEDV), ORF7 (PRRSV), and US6 (PRV), and the mRNA of the β-actin encoding gene served as the internal reference. Total RNA was extracted from cell culture supernatants of PEDV and PRRSV using a Total DNA/RNA/Protein Kit (OMEGA, USA). Total DNA was extracted from cell culture supernatants of PRV using a Total DNA/RNA/Protein Kit (OMEGA, USA). Quantitative real-time PCR (qPCR) was used to quantify PRV DNA, PRRSV RNA, and PEDV RNA. Standard curves were obtained in triplicate using serial dilutions of linearized plasmids containing each target sequence. Data were collected and analyzed using ANOVA.

Different treatment schemes

Cell pretreatment: Vero cells were treated with extracts for 8 h at 37°C, washed with PBS to remove supernatant, and infected with the virus for 1 h. The cells were washed with PBS, and fresh medium was added to continue the culture for 23 h. Virus pretreatment: The virus was preincubated with the extracts for 1 h before infecting the cells, and then the cells were washed three times with PBS and cultured for 23 h. Cotreatment: Vero cells infected with the virus in the presence of extract for 24 h at 37°C. Positive control: Vero cells infected with the virus in the absence of extract for 24 h at 37°C served as a positive control. The infectious virus released in the infected cell culture supernatant was quantified by qPCR.

Time-of-addition experiment

Early infection studies: Vero cells were treated with virus and extracts for 1 h at 37°C and washed with PBS to remove the unattached virus and extract. The infected cells were added to fresh medium and allowed to proceed at 37°C for 23 h. Post infection studies: confluent monolayers of Vero cells were infected with the indicated virus in extract-free medium at 37°C for 1 h, the cells were washed with PBS to remove the unattached virus, and fresh medium containing extract was added. Cotreatment: Vero cells were infected with the virus in the presence of extract for 24 h at 37°C. Positive control: Vero cells were infected with the virus in the absence of extract for 24 h at 37°C. The infectious virus released in the infected cell culture supernatant was quantified by qPCR.

Statistical analysis

The CC_{50} and CC_{90} were performed with SPSS version 6 for Windows (SPSS, Chicago, IL). The same software was used to calculate the IC_{50} and IC_{90} values of the testing compound. The selectivity index (SI) was determined as the ratio of CC_{50} to IC_{50} for each compound. All data were expressed as the mean values ± standard deviations (SD) in triplicate. Statistical analysis of experimental data was performed using one-way analysis of variance (GraphPad Software Inc., USA). $p < 0.05$ was considered statistically significant (*), $p < 0.01$ was regarded as highly significant (**), $p < 0.001$ was regarded as highly significant (***), and $p < 0.0001$ was regarded as highly significant (****).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-023-00091-x.

Additional file 1: Supplementary Table 1. The preparation and source of CHE (Chen et al. 2022; Liang et al. 2019; Liu et al. 2015; He et al. 2011; Shawky et al. 2015; Schoop et al. 2006; Han et al. 2011; Yang et al. 2020; Shang et al. 2011; Jadhav and Karuppayil 2021; Ming et al. 2017; Liu et al. 2019). **Supplementary Table 2.** Primers and probe for quantitative real-time PCR for PRV, PEDV, and PRRSV detection.

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Authors' contributions
Conceived and designed the experiments: Y.S., W.L., Q.H., S.Z. Performed the experiments: Y.S., C.L.,W.L., Analyzed the data: Y.S., C. L., W.Z., Z.L. Drafted the manuscript: Y.S., C.L., M.J.A., M. Z., L. L.,W.L., Q.H. Project administration: QH., W.L. Supervision: QH., W.L. All authors read and approved the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Authors Shujun Zhang and Wentao Li were not involved in the journal's review or decisions related to this manuscript.

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