Xie S, Shu X, Wu X et al. Comparison of different extraction procedures for isolating bacteriostatic and anti-pseudorabies virus components from *Rodgersia Sambucifolia* (Hemsl). Am J Trad Chin Vet Med 2023; 18(1):3-11.

Basic Science Studies

Comparison of Different Extraction Procedures for Isolating Bacteriostatic and Anti-pseudorabies Virus Components from *Rodgersia Sambucifolia* (Hemsl)

Shuimei Xie, Xianghua Shu, Xin Wu, Ying Zhang, Yulei Wang, Xin Huang, Zhihui Zhang, Yalun Zhang, Qiong Pan, Junyi Lin, Wangzhi Zhang, Zhicheng Geng, Hong Yuan, Yonghui Li, Chunlian Song

ABSTRACT

Medicinal plants are considered a repository of bioactive ingredients. *Rodgersia sambucifolia* (Hemsl), a herbaceous perennial originating from East Asia, has demonstrated bacteriostatic and antiviral properties. The aim of this study was to identify an effective extraction procedure for isolating potential bacteriostatic and antiviral compounds from the plant. The crude extracts were obtained using ethanol, methanol or acetone, and stepwise extracted with ethyl acetate, n-butanol or water. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and diameters of the inhibition zone were evaluated for bacteriostatic efficacy. These tests were carried out with *Enterococcus faecalis*, *Enterococcus faecium*, *Shigella dysenteriae*, *Streptococcus lactis*, *Staphylococcus aureus*, and *Streptococcus lactis*. The antiviral activities of prevention, treatment, and neutralization, utilizing pseudorabies virus, were evaluated using swine testicular cell viability. The active ingredients from the best bioactivity extraction procedure were analyzed using liquid chromatography-mass spectrometry (LC-MS). When ranked highest to lowest, the bacteriostatic and antiviral effects were obtained in the order of the crude extracts prepared from ethanol, methanol, and acetone. Bacteriostatic and antiviral effects were highest in the stepwise extraction of ethyl acetate, followed by the extraction of n-butanol and water. The main compounds identified by LC-MS in the ethyl acetate phase in descending order of content were: bergenin, palmitic acid, baicalein, linoleic acid, chrysin, γ-linolenic acid, catechin gallate, catechin, ursolic acid, and baicalin. This study provides a research basis for the development of bacteriostatic and antiviral drugs from traditional Chinese herbal medicines.

Keywords: active ingredients, antiviral, anti-pseudorabies virus, bacteriostasis, Chinese herbal medicine, extraction procedures, pseudorabies virus, *Rodgersia sambucifolia*, stomach cell line, swine

ABBREVIATIONS

AC: Ethyl acetate phase; **AQ:** Aqueous phase; **AT:** Acetone; **AT-AC:** Acetone extract of ethyl acetate phase; **AT-AQ:** Acetone extract of aqueous phase; **CCK-8:** Cell counting kit; **ET:** Ethanol; **ET-AC:** Ethyl acetate phase of ethanol extract; **LC-MS:** Liquid chromatography-mass spectrometry; **MBC:** Minimum bactericidal concentration; **MIC:** Minimum inhibitory concentration; **MT:** Methanol; **MT-AC:** Methanol extract of ethyl acetate phase; **MT-AQ:** Methanol aqueous phase; **MT-NBA:** Methanol extract n-butanol phase; **NBA:** N-butanol phase; **PRV:** Pseudorabies virus; **RSHE:** *Rodgersia sambucifolia* (Hemsl) extract; **ST:** Porcine testicular cells; **$TCID_{50}$:** Median Tissue Culture Infectious Dose

The excessive use of antimicrobials in the clinic has rendered antimicrobial resistance one of the most severe global public health problems. Additionally, viral pathogens are highly harmful and have proven difficult to impossible to treat with therapeutic medicines available at the current time. The pseudorabies virus (PRV) is one of the most common viral pathogens threatening the global swine industry.[1] It has been detected in at least 44 countries since its discovery in the early 20th century. The virus causes reduced fertility in sows, respiratory disease in adult pigs, nervous system disorders and high piglet mortality.[2] According to reports, the estimated losses due to PRV infection in the United States swine industry ranged from $21 million to nearly $33 million per year.[3]

Medicinal plants are considered a repository of bioactive ingredients possessing varied therapeutic features. A unique approach to both the overuse of antibiotics and identification of effective antiviral agents is investigation of Chinese herbal medicines (CHM) that have been associated with antibacterial and antiviral effects.[4] For example, glycyrrhizin, a bioactive compound of licorice root, has been used as a traditional CHM for a variety of

Author Professional Degrees and Certifications: MS of Veterinary Medicine (Xie); DVM (Shu); **From:** College of Veterinary Medicine, Yunnan Agricultural University, Kunming, China; ***Address correspondence to** Dr. Chunlian Song (Chunlian-Song@outlook.com).

beneficial clinical effects including anti-inflammatory properties. Diammonium glycyrrhizate, derived from glycyrrhizin, is more stable, soluble and has significant antiviral activity. In particular, it inhibits cellular infection by PRV and reduces apoptosis during PRV infection.[5]

Rodgersia sambucifolia (Hemsl) (RSHE) is a herbaceous perennial originating from East Asia (i.e. Rodgers flower, rhizome of featherleaf). In China, known as *Hong Jiang* or *Jin Yue Tuo*, it has been used as a traditional Chinese medicine, exhibiting suitable pharmacological activities and little toxicity or side effects.[6,7] The plant is known for its active component, bergenin, which has antinociceptive and anti-inflammatory properties; however, other active components are less explored. In particular, the components associated with antibacterial and antiviral properties are especially compelling.[8]

Different extraction procedures can influence bacteriostatic and antiviral effects of natural products.[9] Ethanol (ET) and ethyl acetate extracts of *Rodgersia aesculifolia* (Batal) exhibited the best antiproliferative effects on *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Bordetella bronchiseptica*.[10,11] The antiviral activity of the n-butanol and ethyl acetate extracts was better than that of the acetone extract against Coxsackie B group I-VI viruses. The bioactivities of these extracts, however, were the same for the Herpes simplex type I virus.[12]

The aim of this study was to compare different extraction methods in order to identify an efficient extraction procedure for isolating bacteriostatic and antiviral components from *Rodgersia sambucifolia* (Hemsl). The effect of the extraction methods on potential bacteriostatic and antiviral activity was evaluated on six bacteria and PRV under *in vitro* conditions. Additionally, liquid chromatography-mass spectrometry (LC-MS) was used to investigate and identify active ingredients in the *R. sambucifolia* extracts. The objectives of this study, therefore, were to identify an effective extraction procedure that optimized bacteriostatic and antiviral efficacy and to identify the main ingredients in the extracts that might contribute to the plant extract's biological activity. Study findings provide a basis for the development and use of a natural antibacterial and antiviral drug.

MATERIALS AND METHODS

Preparation of *Rodgersia sambucifolia* (Hemsl) extracts (RSHE)

Rodgersia sambucifolia (Hemsl) was purchased from a *R. sambucifolia* plantation in China[a]. Its crude extracts were obtained as follows: the plant was dried and granulated with a 60 mesh sieve and mixed with ET, methanol (MT) or acetone[b] (AT). The powder was further dispersed by ultrasonication for 15 minutes and overnight magnetic rotation at room temperature. The yield rate of the crude extracts was calculated after filtration, rotary steaming, concentration and freeze-drying.

The extraction phase of *R. sambucifolia* was obtained as follows: the crude extracts of ET, MT and AT were concentrated and extracted 3 times with ethyl acetate, n-butanol or distilled water. The extraction liquids from these 3 steps were combined, and yield rate of crude extract was calculated after rotary steaming, concentration and freeze-drying.

In vitro bacteriostatic test

The bacteriostatic efficacy test was performed by measuring the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and bacterial growth inhibition zones. These tests were conducted against *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus lactis*, *Shigella dysenteriae*, *Klebsiella pneumonia*, and *Staphylococcus aureus*[c].

The broth dilution assay determined the MIC and MBC values of RSHE extracts using two-fold serial dilutions (100 to 0.195 mg/ml). The bacterial solution (100 µL of 10^6 CFU/ml) was incubated for 18 hours. Iodonitrotetrazolium chloride blue was added as an indicator of microbial growth. The MIC value was visually determined when the solution turned colorless. The minimum bactericidal concentration without any growth was considered the MBC. The inhibition zone was determined by the Oxford cup assay.[13] Three concentrations of RSHE (100 µL each of 100 mg/ml, 50 mg/ml, and 25 mg/ml) were added into Oxford cups in triplicates. The blank control contained distilled water. The evaluation criteria for the bacteriostatic zone followed a previous study.[14]

In vitro anti-pseudorabies virus (anti-PRV) test

Swine testicular (ST) cells were obtained from a standard supplier[d]. Following a previous report, the cells were revived and cultured.[15] The procedure was initiated by thawing cells in a 37°C water bath and collected after centrifugation at 1000 rpm for 4 minutes.[16] Culture medium (2 ml) was added and the cells were incubated (37°C, 5% CO_2) and grown to a 90% confluence. The culture medium was collected, twice washed with 1ml of phosphate buffered saline and digested by 0.25% trypsin. The collected cells were gently blown into suspension and divided into 2-3 culture flasks which were placed in a cell incubator (37°C, 5% CO_2). Cell concentration and survival rate were calculated.

The ST cells were then mixed with lyophilization solution and divided into sterile lyophilization tubes which were frozen overnight at -80°C with gradient cooling. The tubes were transferred to a liquid nitrogen tank for storage.

To assess a safe RSHE concentration, the ST cells were incubated for 24 hours with 10 RSHE doses (200, 100, 50, 25, 12.5, ..., 0.78 µg/ml; 6 replicates of each dose). The CCK-8 (cell counting kit) test determined the safe concentrations according to cell viability. The dose for PRV infection of ST cells was calculated by using the Reed-Muench assay [50% tissue culture infectious dose/ $TCID_{50}$ (Median Tissue Culture Infectious Dose)].

The ST cells (10^4 cells) were cultured on a 96-well culture plate and infected with 10^{-1} to 10^{-8} doses of the PRV solution into eight replicates. The PRV was isolated at the Preventive Veterinary Laboratory of the College of Veterinary Medicine, Yunnan Agricultural University (virus titer = $10^{5.6}$ $TCID_{50}$/ml).[17] The $TCID_{50}$ was calculated from the cytopathic effect.

The antiviral effect of RSHE was evaluated according to cell viability using the CCK-8 kit for prevention, treatment and neutralization of PRV[e]. The prevention efficacy was evaluated as follows: a safe concentration of RSHE was first added to 10^4 ST cells, and then the cells were infected with 100 µL of PRV (100 $TCID_{50}$). The treatment efficacy was assessed as follows: ST cells (10^4) were first infected with 100 µL of PRV (100 $TCID_{50}$); the safe concentration of RSHE was then added. The neutralization efficacy of PRV was evaluated by mixed liquid incubation 100 µL of PRV (100 $TCID_{50}$) + safe concentration RSHE. The PRV control group was infected for 4 hours and then incubated until 80% of the cells showed pathology (around 2 hours).

Screening of the potential active components from RSHE

The best biological extraction phase (with maximum antibacterial and antiviral properties) was selected for active ingredients screening. The potential active components in RSHE were analyzed by LC-MS. The parameter settings were: column temperature 40°C, flow rate 400 µL/min, sample loading 2 µL. For positive ion mode: Phase A was 0.1% formic acid in water; Phase B was 0.1% formic acid in acetonitrile. For anion mode: Phase A was water (with 2 mM ammonium acetate); Phase B was acetonitrile. The flow elution gradient was: 0-1.5 min, 95% A, 5% B; 2.5 min, 90% A, 10% B; 14 min, 60% A, 40% B; 22-25 min, 5% A, 95% B; and 26-30 min, 95% A, 5% B. For electrospray ionization mass spectrometry: the ion source was the atomizing pressure of 60 PSI (pounds/square inch); auxiliary pressure was 60 PSI; air curtain pressure was 35 PSI; temperature was 650°C; spray voltage was 5000 V (positive ion mode) or -4000 V (negative ion mode).

Statistical analysis

The extraction rate of RSHE = (W/50)×100% with "W" as the weight of the extract, that is, the extraction rate of RSHE is the ratio of the weight of the extract to 50 g RSHE. The data from the bacteriostatic and anti-PRV tests were statistically analyzed by commercial software[f] using a one-way ANOVA test. Statistical significance was $p < 0.05$.

RESULTS

Extraction rates of different phases of RSHE

The extraction rates of ET, MT and AT phases were 33.2%, 29.8% and 14.02%, respectively. The extraction yields of ethanol with ethyl acetate (ET-AC), n-butanol (ET-NBA) and water (ET-AQ) were 8.80%, 15.28% and 11.98%, respectively. The extraction yields of methanol solvent with ethyl acetate (MT-AC), n-butanol (MT-NBA) and water (MT-AQ) were 8.08%, 14.64% and 1.90%, respectively. The extraction yields of acetone with ethyl acetate (AT-AC), n-butanol (AT-NBA) and water (AT-AQ) were 6.74%, 9.88% and 1.90%, respectively. The highest to the lowest extraction rate was obtained for the ET, MT, AT solvents, and the n-butanol phase (NBA), ethyl acetate phase (AC), and aqueous phase (AQ) (Figure 1).

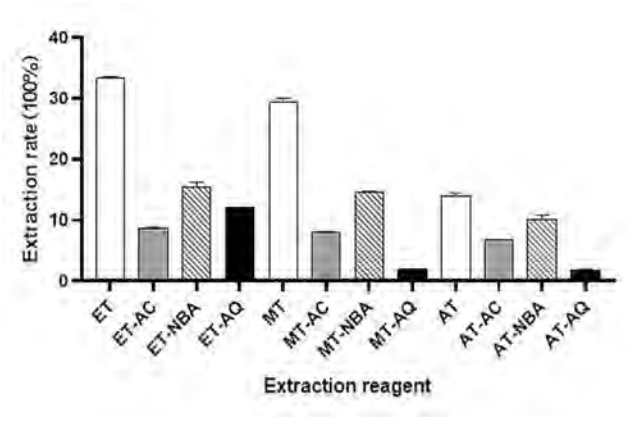


Figure 1: The highest to the lowest extraction rate was obtained for the ET, MT, AT solvents, and the n-butanol phase (NBA), ethyl acetate phase (AC), and aqueous phase (AQ).

***In vitro* bacteriostatic tests (MIC, MBC, Oxford cup with bacteriostatic zone tests)**

The ET-AC extract achieved the most effective inhibition based on MIC results against *Enterococcus faecalis* and *Streptococcus lactis* (MICs = 0.39 mg/ml). The ET-AC extract against *Shigella dysenteriae*, the MT-NBA extract against *Enterococcus faecalis* and *Streptococcus lactis* and the AT-AC against *Staphylococcus aureus* and *Streptococcus lactis* had an MIC value of 0.78 mg/ml. The MBC results (0.78 mg/ml) for the best inhibition were consistent with the MIC results. Study findings, therefore, demonstrated RSHE inhibited the proliferation of six bacteria and the highest inhibition was observed in the ET-AC extract (Table 1). The zone of inhibition of bacterial growth resulted in 27 combinations of bacteria tested being sensitive to RSHE, 21 of which were associated with ethyl acetate (Table 2). The RSHE, therefore, inhibited bacterial proliferation, and the AC extract displayed a notable antibacterial effect.

***In vitro* evaluation of the anti-PRV effect ($TCID_{50}$ determination of the PRV)**

A $TCID_{50}$ of $10^{-5.39}$/0.1 ml of PRV was obtained from the Reed-Muench method. That is, inoculating 100 µL of $10^{5.39}$ virus dilution caused 50% of ST cells to become cytopathic (Table 3).

Effect of RSHE on the ST cells and determination of safe concentration

The viability of ST cells treated with 200~6.25 µg/ml of ET, 100 and 50 µg/ml of ET-AC, 100~12.5 µg/ml of ET-NBC, and 200 µg/ml of ET-AQ were significantly lower

than the control group. Cell viabilities of 200~100 µg/ml of MT, MT-AC, MT-NBC and MT-AQ were substantially lower than those of the control group. The cell viability of 200~100 µg/ml of AT, 200~50 µg/ml of AT-AC, 20~50 µg/ml of AT-NBC, and 200 µg/ml of the AT-AQ extracts were significantly lower than that of the control group. These specific concentrations of RSHE, therefore, were toxic to ST cells, while the other concentrations were safe. According to the cell viability of these 3 types of RSHE, the safe concentration of RSHE ranged from 0.78 to 12.5 µg/ml (Figure 2).

In vitro anti-pseudorabies virus (anti-PRV) test

The cell viability of the PRV groups was significantly lower than the control groups ($p<0.05$), indicating the successful infection of the ST cells by PRV. The viability of ST cells in the groups treated with 12.5 µg/ml of ethanol extract of n-butanol phase, 12.5 µg/ml of MT-AC, and 3.13 µg/ml of AT-AC was significantly higher than that of the PRV groups. The viability of these cells increased by 30.54% ($p<0.001$), 35.66% ($p<0.001$), and 22.52% ($p<0.01$), respectively. These results revealed the prevention efficacy of RSHE (Figure 3).

The ST cell viability of the groups treated with 1.56 µg/ml of ET, MT, and AT was significantly higher than that of the PRV groups. The cell viability increased by 12.26% ($p<0.01$), 12.34% ($p<0.01$) and 22.48% ($p<0.01$), respectively. These results revealed the treatment efficacy of RSHE (Figure 4).

The ST cell viability of the groups treated with 3.13 µg/ml of ET-AC and 12.5 µg/ml of the MT-AC groups were significantly higher than those of the PRV groups. There was an increase of 17.27% ($p<0.01$) and 25.17% ($p<0.01$), respectively. These results revealed the neutralization efficacy of the RSHE (Figure 5).

Table 1: Mean inhibitory concentration (MIC) and mean bactericidal concentration (MBC) values of *Rodgersia sambucifolia* (Hemsl) extracts (RSHE, mg/ml); table findings demonstrate RSHE inhibited the proliferation of six bacteria and the highest inhibition was observed in the ET-AC extract (ethyl acetate phase of ethanol extract)

Group		EFA	EFE	SLA	SDY	KPN	SAU
ET	MIC	3.125	3.1250	3.125	6.250	12.50	6.250
	MBC	6.250	6.250	6.250	100.00	50.00	25.00
ET-AC	MIC	0.390	1.560	0.390	0.780	3.125	1.560
	MBC	0.780	3.130	1.560	25.00	6.250	12.50
ET-NBA	MIC	1.560	1.560	1.560	3.125	12.50	3.125
	MBC	12.50	12.50	6.250	12.50	12.50	12.50
ET-AQ	MIC	6.250	12.50	3.125	12.50	25.00	6.250
	MBC	12.50	100.0	6.150	100.0	50.00	100.0
MT	MIC	6.250	1.560	1.560	3.125	6.250	3.125
	MBC	25.00	3.125	3.125	12.50	25.00	6.250
MT-AC	MIC	3.125	3.125	1.560	1.560	1.560	1.560
	MBC	12.50	12.50	3.125	12.50	6.250	6.250
MT-NBA	MIC	3.125	0.780	0.780	6.250	3.125	3.125
	MBC	12.50	1.560	1.560	12.50	100.0	6.260
MT-AQ	MIC	25.00	1.560	1.560	12.50	12.50	6.250
	MBC	100.0	3.125	6.250	50.00	200.0	12.50
AT	MIC	6.250	6.250	3.125	3.125	1.560	1.56
	MBC	12.50	50.00	6.250	12.50	25.00	25.00
AT-AC	MIC	3.125	3.125	1.560	1.560	1.560	0.780
	MBC	12.50	25.00	3.125	6.250	25.00	12.50
AT-NBA	MIC	12.50	6.250	6.250	6.250	3.125	1.560
	MBC	25.00	100.0	12.50	50.00	12.50	25.00
AT-AQ	MIC	12.50	12.50	0.780	6.250	6.250	6.250
	MBC	25.00	25.00	6.250	100.0	12.50	50.00

EFA = *Enterococcus faecalis*, EFE = *Enterococcus faecium*, SLA = *Streptococcus lactis*, SDY = *Shigella dysenteriae*, KPN = *Klebsiella pneumonia*, SAU = *Staphylococcus aureus*

Table 2: Bacteriostatic determination (zone of inhibition*, mm) of *Rodgersia sambucifolia* (Hemsl) extracts **(**RSHE)

Concentration/ (mg/ml)	EFA	EFE	SLA	SDY	KPN	SAU
ET100	16.00 ± 0.00(I)	0.00 ± 0.00(-)	11.00 ± 0.00(R)	15.00 ± 0.00(I)	16.00 ± 0.00(I)	15.00 ± 0.00(I)
ET-AC100	20.17 ± 0.29(S)	15.67 ± 0.58(I)	15.00 ± 0.00(I)	20.00 ± 0.00(S)	20.00 ± 0.00(S)	13.00 ± 0.00(R)
ET-AC50	17.00 ± 0.00(S)	15.00 ± 0.00(I)	12.00 ± 0.00(R)	18.00 ± 0.00(S)	18.00 ± 0.00(S)	11.33 ± 0.58(R)
ET-AC25	15.00 ± 0.00(I)	12.00 ± 0.00(R)	12.00 ± 0.00(R)	15.00 ± 0.00(I)	15.00 ± 0.00(I)	0.00 ± 0.00(-)
ET-NBA100	15.67 ± 0.58(I)	12.67 ± 0.58(R)	0.00 ± 0.00(-)	15.67 ± 0.58(I)	16.00 ± 0.00(I)	21.00 ± 0.00(S)
ET-NBA50	13.00 ± 0.00(R)	10.00 ± 0.00(R)	0.00 ± 0.00(-)	13.00 ± 0.00(R)	13.50 ± 0.00(R)	17.00 ± 0.00(S)
ET-NBA25	10.00 ± 0.00(R)	0.00 ± 0.00(-)	0.00 ± 0.00(-)	11.33 ± 0.58(R)	12.00 ± 0.00(R)	14.00 ± 0.00(I)
MT100	16.00 ± 0.00(I)	13.00 ± 0.00(R)	14.00 ± 0.00(I)	17.00 ± 0.00(S)	16.34 ± 0.58(I)	13.00 ± 0.00(R)
MT50	12.00 ± 0.00(R)	10.00 ± 0.00(R)	10.00 ± 0.00(-)	14.00 ± 0.00(I)	14.00 ± 0.00(I)	11.00 ± 0.00(R)
MT-AC100	20.00 ± 0.00(S)	18.00 ± 0.00(S)	19.00 ± 0.00(S)	20.00 ± 0.00(S)	19.00 ± 0.00(S)	19.00 ± 0.00(S)
MT-AC50	17.00 ± 0.00(S)	14.00 ± 0.00(I)	15.00 ± 0.00(I)	18.67 ± 0.58(S)	17.00 ± 0.00(S)	15.00 ± 0.00(I)
MT-AC25	14.00 ± 0.00(I)	12.00 ± 0.00(R)	14.00 ± 0.00(I)	15.00 ± 0.00(I)	16.00 ± 0.00(I)	13.00 ± 0.00(R)
MT-NBA100	15.33 ± 0.58(I)	14.00 ± 0.00(I)	15.00 ± 0.00(I)	16.00 ± 0.00(I)	14.67 ± 0.58(I)	11.00 ± 0.00(R)
MT-NBA50	12.17 ± 0.29(R)	11.00 ± 0.00(R)	12.00 ± 0.00(R)	14.00 ± 0.00(I)	14.00 ± 0.00(I)	11.00 ± 0.00(R)
AT100	19.00 ± 0.00(S)	12.00 ± 0.00(R)	15.33 ± 0.58(I)	16.33 ± 0.29(I)	17.67 ± 0.58(S)	14.00 ± 0.00(I)
AT50	16.00 ± 0.00(I)	10.67 ± 0.58(R)	11.67 ± 0.58(R)	15.00 ± 0.00	16.00 ± 0.00(I)	12.50 ± 0.00(R)
AT25	14.00 ± 0.00(I)	10.00 ± 0.00(R)	10.00 ± 0.00(R)	13.00 ± 0.00(R)	13.67 ± 0.58(R)	0.00 ± 0.00(-)
AT-AC100	19.00 ± 0.00(S)	13.00 ± 0.00(R)	18.00 ± 0.00(S)	17.17 ± 0.29(S)	17.67 ± 0.58(S)	18.00 ± 0.00(S)
AT-AC50	18.00 ± 0.00(S)	11.00 ± 0.00(R)	15.00 ± 0.00(I)	16.00 ± 0.00(I)	16.67 ± 0.58(I)	15.00 ± 0.00(I)
AT-AC25	15.00 ± 0.00(I)	10.00 ± 0.00(R)	11.33 ± 0.58(R)	14.00 ± 0.00(I)	15.00 ± 0.00(I)	11.00 ± 0.00(R)
AT-NBA100	18.00 ± 0.00(S)	13.00 ± 0.00(R)	14.00 ± 0.00(I)	16.00 ± 0.00(I)	12.67 ± 0.58(R)	14.00 ± 0.00(I)
AT-NBA50	15.00 ± 0.00(I)	12.00 ± 0.00(R)	11.00 ± 0.00(R)	14.00 ± 0.00(I)	14.00 ± 0.50(I)	12.00 ± 0.00(R)
AT-NBA25	12.00 ± 0.00(R)	0.00 ± 0.00(-)	9.00 ± 0.00(R)	14.00 ± 0.00(I)	13.00 ± 0.00(R)	0.00 ± 0.00(-)
AT-AQ100	15.00 ± 0.00(I)	11.00 ± 0.00(R)	10.83 ± 0.29(R)	15.00 ± 0.00(I)	14.00 ± 0.00(I)	11.00 ± 0.00(R)

All bacteria tested were resistant to: ET-AQ100&50&25, MT-25, MT-NBA25, MT-AQ-100&50&25, AT-AQ50&25 extracts; therefore, removed from table.

* The inhibition zone criteria: diameter ⩽13 mm depicts resistance (R) to inhibition; 14 mm to 16 mm represents intermediate (I) inhibition, and ⩾ 17 mm depicts sensitivity (S) to inhibition; 0 mm shown as "-"; *Enterococcus faecalis* (EFA), *Enterococcus faecium* (EFE), *Streptococcus lactis* (SLA), *Shigella dysenteriae* (SDY), *Klebsiella pneumonia* (KPN), *Staphylococcus aureus* (SAU).

Table 3: Determination of pseudorabies virus (PRV) in ST cell $TCID_{50}$; inoculating 100 µL of $10^{-5.39}$ virus dilution caused 50% of ST cells to become cytopathic

Virus dilution	With CPE	Without CPE	Cumulative		Percentage of holes with CPE (%)
			With CPE hole number	Without CPE hole number	
10^{-1}	8	0	39	0	(39/39)100
10^{-2}	8	0	31	0	(31/31)100
10^{-3}	8	0	23	0	(23/23)100
10^{-4}	8	0	15	0	(15/15)100
10^{-5}	5	3	7	3	(7/10)70
10^{-6}	2	6	2	9	(2/11)18.2
10^{-7}	0	8	0	15	0
10^{-8}	0	8	0	23	0
10^{-9}	0	8	0	31	0
10^{-10}	0	8	0	39	0

CPE= cytopathic effect

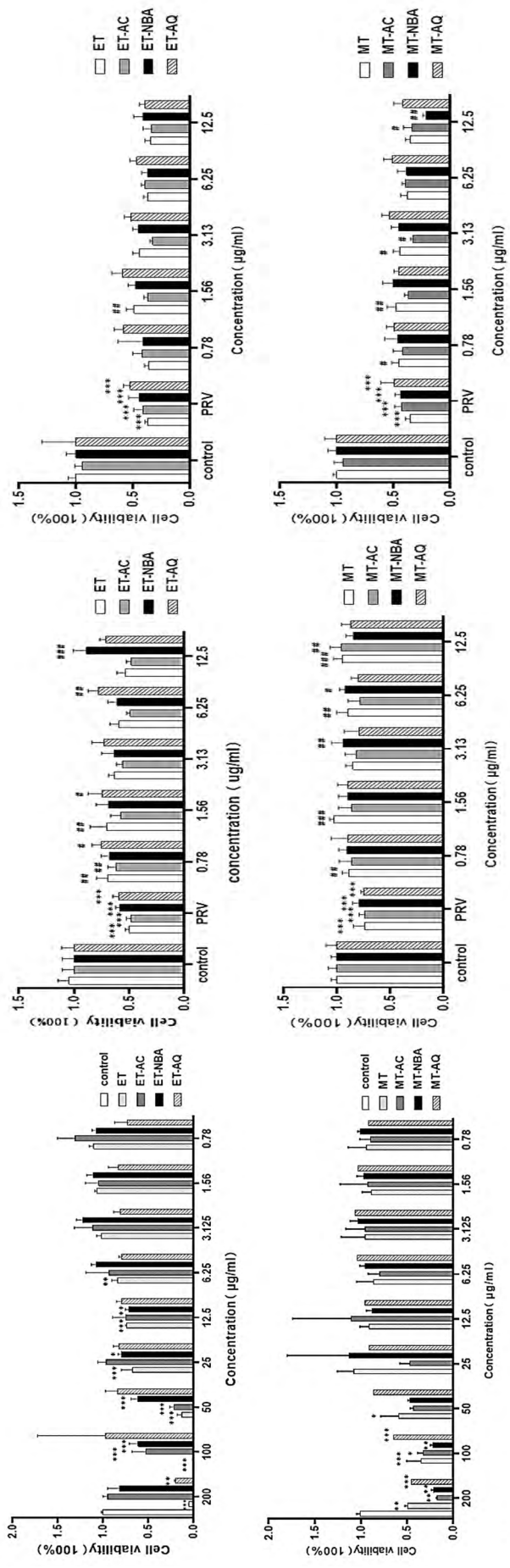


Figure 2: The effect of *R. sambucifolia* extracts on ST cell viability. The bar graphs demonstrate treatment with different doses of ethanol (top), methanol (middle), and acetone (bottom) extracts. The extracts with cell viability significantly lower than the controls were considered toxic to ST cells.

Figure 3: ST cell viability assessment for PRV infection prevention of RSHE.

Figure 4: ST cell viability assessment depicting treatment efficacy of RSHE for PRV infection.

Figure 5: ST cell viability assessment of PRV neutralization efficacy of RSHE; extracts - left bar graph (ethanol), middle (methanol), right (acetone).

Different extraction procedures influence the prevention, treatment and neutralization of PRV activities. The ET-AC, MT-AC, and AT-AC extracts of RSHE exhibited the most effective prevention, treatment and PRV neutralization efficacy. Consistent with *in vitro* bacteriostatic results, the AC extraction phase of *R. sambucifolia* exhibited the most effective bacteriostatic and antiviral properties. Subsequently, the main active ingredients were analyzed by LC-MS from the AC extract.

Determination and analysis of the active components in the AC extract of RSHE

A total of 9 flavonoids (baicalein, baicalin, isoquercitrin, quercetin, daidzein, 3-hydroxyflavone, emodin, geranylin, puerarin); 8 polyphenols (catechin gallate, bergenin, ellagic acid, caffeic acid, gallic acid, catechin gallate, resveratrol, epigallocatechin); and 3 fatty acids (palmitic acid, γ-linolenic acid, linoleic acid) were identified in the ET, MT and AT extracts, respectively. In descending order of content, the main compounds were: bergenin, palmitic acid, baicalein, linoleic acid, chrysin, γ-linolenic acid, catechin gallate, catechin, ursolic acid, and baicalin (Figures 6 and 7).

DISCUSSION

In this study, different extraction procedures were used to evaluate the bacteriostatic and antiviral efficacies of the Chinese herbal medicine, *Rodgersia sambucifolia* (Hemsl). The order of stepwise extraction for the 3 solvents was AC, NBA and AQ, which was consistent with previous reports describing higher bacteriostatic effect in the AC extract than other organic phases.[12,18] Study findings then demonstrated that the ethyl acetate extraction of AC yielded the most active bacteriostatic/antiviral ingredients, which were further analyzed by LC-MS. The main compounds identified in this extract included: bergenin, palmitic acid, baicalein, linoleic acid, chrysin, γ-linolenic acid, catechin gallate, catechin, ursolic acid, and baicalin. Findings from this study provide a basis for the use and development of natural antibacterial and antiviral drugs from *Rodgersia sambucifolia* (Hemsl).

Study findings demonstrated RSHE had a notable antibacterial effect. The ET-AC extract achieved the most effective inhibition based on MIC results against *Enterococcus faecalis* and *Streptococcus lactis* and the AT-AC against *Staphylococcus aureus* and *Streptococcus lactis.* The RSHE demonstrated inhibition of 6 bacteria (ET-AC greatest effect) and the zone of inhibition of bacterial growth resulted in 27 combinations of bacteria demonstrating sensitivity to RSHE (21 of these with ethyl acetate).

Specific antiviral assessment demonstrated 1.56 μg/ml concentration of the ET, MT, and AT-AC extracts of *R. sambucifolia* exhibited positive treatment efficacy in PRV-infected ST cells. The prevention efficacies of the MT and ET-AC groups were better than those of other extracts. The MT-NBA extract had the most effective PRV neutralization action. These results indicated that the dose might impact antiviral activities. The bacteriostatic and antiviral mechanisms of RSHE could be related to direct killing, inhibition of virus replication or adsorption into cells.

Similar to the flavonoid containing extracts of *R. sambucifolia*, studies have shown other flavonoids such as myricetin, and flavonoid containing herbs such as *Panax notoginseng*, and *Radix isatidis* have an antiviral effect on PRV. Myricetin can effectively inhibit PRV infection and prevent PRV from entering cells early in infection. It can regulate NF-κB and mitogen-activated protein kinase pathways to inhibit viral replication and control the inflammatory response and apoptosis caused by viral infection.[19] *Panax notoginseng* polysaccharides inhibit PRV infection by interfering with PRV adsorption and replication in vitro.[20] *Radix isatidis* polysaccharide can inhibit PRV replication, prevent infection, and kill the virus.[21]

LC-MS results of the RSHE in the present study indicate the presence of a variety of flavonoids with antibacterial, antitumor, antiviral and immune function-enhancing properties. Components such as baicalein, gallic acid, catechin, and quercetin (flavonoid components) have been associated with bacteriostatic and antiviral properties.[22-26] The flavonoid components baicalein, baicalin and catechin are used for their antibacterial, anti-PRV, anti-influenza effects and the treatment of viral pneumonia.[27,28]

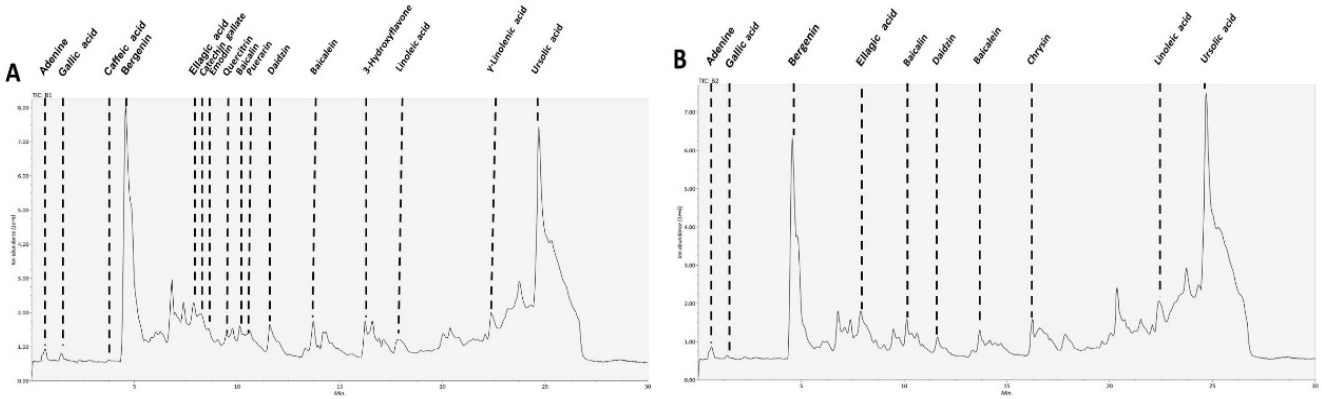
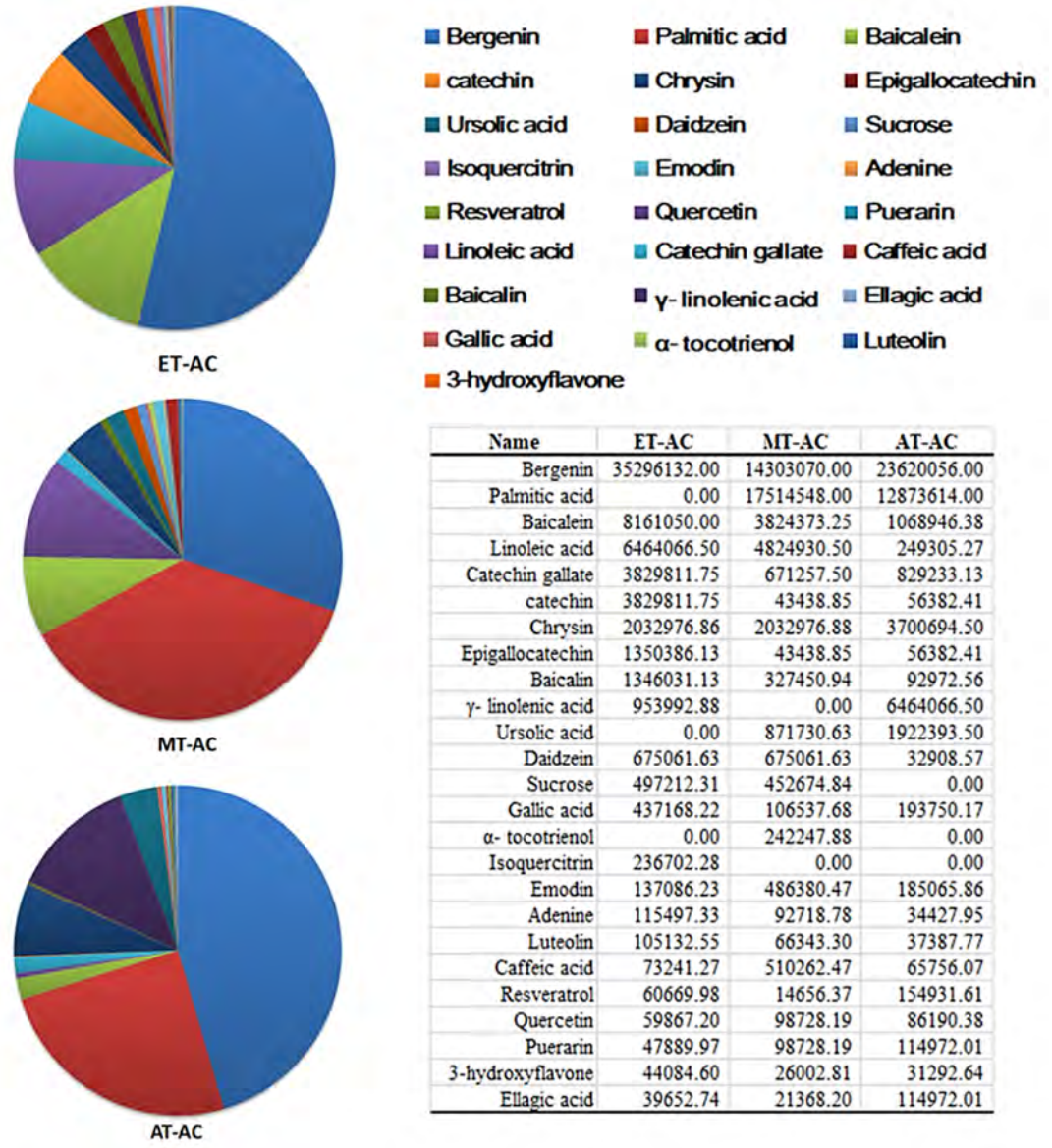


Figure 6: The active ingredients from the best bioactivity extraction procedure (AC) were analyzed using liquid chromatography-mass spectrometry (LC-MS). (A) the total ion flow diagram of the ET-AC extract in the positive ion detection mode, (B) the total ion flow diagram of the ET-AC extract in the negative ion detection mode.



Name	ET-AC	MT-AC	AT-AC
Bergenin	35296132.00	14303070.00	23620056.00
Palmitic acid	0.00	17514548.00	12873614.00
Baicalein	8161050.00	3824373.25	1068946.38
Linoleic acid	6464066.50	4824930.50	249305.27
Catechin gallate	3829811.75	671257.50	829233.13
catechin	3829811.75	43438.85	56382.41
Chrysin	2032976.86	2032976.88	3700694.50
Epigallocatechin	1350386.13	43438.85	56382.41
Baicalin	1346031.13	327450.94	92972.56
γ- linolenic acid	953992.88	0.00	6464066.50
Ursolic acid	0.00	871730.63	1922393.50
Daidzein	675061.63	675061.63	32908.57
Sucrose	497212.31	452674.84	0.00
Gallic acid	437168.22	106537.68	193750.17
α- tocotrienol	0.00	242247.88	0.00
Isoquercitrin	236702.28	0.00	0.00
Emodin	137086.23	486380.47	185065.86
Adenine	115497.33	92718.78	34427.95
Luteolin	105132.55	66343.30	37387.77
Caffeic acid	73241.27	510262.47	65756.07
Resveratrol	60669.98	14656.37	154931.61
Quercetin	59867.20	98728.19	86190.38
Puerarin	47889.97	98728.19	114972.01
3-hydroxyflavone	44084.60	26002.81	31292.64
Ellagic acid	39652.74	21368.20	114972.01

Figure 7: Compositional ratio of the active ingredients in 3 extracts from *R. sambucifolia*

The present study sought to define an optimal procedure for extraction of antibacterial and antiviral biocompounds from *Rodgersia sambucifolia* Hemsl. The antibacterial and antiviral effects of ethanolic, methanolic, acetonic extracts and their isolates after sequential extraction with ethyl acetate, n-butanol and water were determined. The ethyl acetate phase displayed the best bacteriostatic and anti-PRV properties, therefore, was considered the best extraction procedure and was subjected to LC-MS analysis. The main active ingredients determined by LC-MS included the flavonoid components of baicalin, baicalein, chrysin, quercetin, and polyphenol components: bergenin, gallic acid, catechin. These isolates may be related to *R. sambucifolia*'s bacteriostatic and anti-PRV effects but await further mechanistic study for proof of this hypothesis. In conclusion, *Rodgersia sambucifolia* (Hemsl) with its variety of active ingredients has broad application potential and this study provides a basis for developing bacteriostatic and antiviral agents from this traditional Chinese medicine.

ACKNOWLEDGMENTS

The authors thank the Yunnan Provincial Major Science and Technology Special Project for financial help and Dr. CS, XS, and XW for their helpful suggestions.

Declaration of Interest and Funding

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest. This study was supported by the major science and technology project in Yunnan Province Construction and Application of a Technical System for Prevention and Control of Important Pig Diseases in Yunnan Province (202102AE090007).

Author Contributions

SX, YW, and SX conceived and designed the experiments. SX, HY, LYZ, XH, YZ, XL, and ZZ performed the experiments. SX, QP, ZG, JL, YL, BL, and WZ analyzed the data. SX, XW, and CS contributed toward the reagents and materials and completed the paper. All authors have read and approved the final manuscript.

Data availability statement

The original contributions presented in the study are included in the author contributions section of the article, and further inquiries can be directed to the corresponding authors.

FOOTNOTES

[a] *R. sambucifolia* plantation, Dali City, Yunnan Province, China
[b] Tianjin Zhiyuan Chemical Reagent Co., Ltd. Tianjin, China
[c] Beijing Bei Chuang Na Institute of Biotechnology, Beijing, China
[d] Hunan Fenghui Biotechnology Co., LTD., China
[e] Shenzhen Branch Biological Technology Co., LTD., Guangdong, China
[f] GraphPad Prism 8 ANOVA test, manufacturer name and city/ country here

REFERENCES

1. Li J, Li X, Hao G et al. Fusion of pseudorabies virus glycoproteins to IgG Fc enhances protective immunity against pseudorabies virus. Virol J 2019; 536:49-57. doi.org/10.1016/j.virol.2019.07.027
2. He W, Auclert L, Zhai X et al. Interspecies transmission, genetic diversity, and evolutionary dynamics of pseudorabies virus. J Infect Dis 2019; 219(11):1705-1715. doi: 10.1093/infdis/jiy731
3. EFSA Panel On Animal Health And Welfare, Mors S, Bøtner A et al. Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): Aujeszky's disease. EFSA J 2017; 15(7):4888. doi.org/10.2903/j.efsa.2017.4888
4. Zhang B, Ku X, Yu X et al. Prevalence and antimicrobial susceptibilities of bacterial pathogens in Chinese pig farms from 2013 to 2017. Sci Rep 2019; 9, 9908. doi.org/10.1038/s41598-019-45482-8
5. Sui X, Yin J, Ren X. Antiviral effect of diammonium glycyrrhizinate and lithium chloride on cell infection by pseudorabies herpesvirus. Antiviral Res 2010; 85(2):346-53. doi: 10.1016/j.antiviral.2009.10.014
6. Song M, Zhang L, Zhang Y et al. Effects of genetic variation and environmental factors on bergenin in *Rodgersia sambucifolia* Hemsl. J Ethnopharmacol 2020; 247:112201. doi.org/10.1016/j.jep.2019.112201
7. Wann G, Sunilson A, Samuel J et al. In vitro cytotoxic effect of *Rodgersia Sambucifolia* (Hemsl) in the treatment of leukemia. IJNTR 2017; 3(4):30-33.
8. Barai P, Raval N, Acharya S et al. Neuroprotective effects of bergenin in Alzheimer's disease: Investigation through molecular docking, in vitro and in vivo studies. Behav Brain Res 2019; 356:18-40. doi: 10.1016/j.bbr.2018.08.010
9. Deng J, Xiao X, Tong X et al. Preparation of bergenin from Ardisia crenata sims and *Rodgersia sambucifolia* hemsl based on microwave-assisted extraction/high-speed counter-current chromatography. Sep Purify Techno 2010; 74(2):155-159. doi:10.1016/j.seppur.2010.05.018
10. Zhai W, Xu Q. Research on the divided extraction of Rodgersia aesculifolia-batal and the compound formation. J Shaanxi Xueqian Norm Univ 2007; 2:62-64. doi:10.3969/j.issn.1008-598X.2007.02.017
11. Zhang D J, Yang Y J, Shi Y B, et al. In vitro antibacterial and acute toxicity test of Rodgersiaaesculifolia. Batal Chin Trad Patent Med 2005; 5:604-605. doi: 10.3969/j.issn.1001-1528.2005.05.040
12. Hu Z, Shi Y, Luo Y, et al. Experimental study on inhibiting bacteria in vitro and acute toxicity of Rodgersia aesculifolia batal. Prog Vet Med 2005, 26(10):86-88. doi: CNKI:SUN:DYJZ.0.2005-10-019
13. Ye C, Dai H, Hu W. Antimicrobial and antioxidant activities of the essential oil from onion (Allium cepa L.). Food Contr 2013; 30:48-53. doi.org/10.1016/j.foodcont.2012.07.033
14. Zhang S Q, Wang M, Lei N, et al. Experimental study on bacteriostasis of Escherichia coli O_8 from pig by traditional Chinese medicine in vitro. J Domestic Anim Ecol 2015; 36(8):62-65. doi: CNKI:SUN:JCST.0.2015-08-013
15. Lin J, Li Z, Feng Z et al. Pseudorabies virus (PRV) strain with defects in gE, gC, and TK genes protects piglets against an emerging PRV variant. J Vet Med Sci 2020; 82(6):846-55. doi: 10.1292/jvms.20-0176
16. Saganuwan A. Application of median lethal concentration (LC50) of pathogenic microorganisms and their antigens in vaccine development. BMC Res Notes 2020; 13:-6. doi.org/10.1186/s13104-020-05126-x
17. Zhu L Y, Zhang Z X, Zang L L, et al. Isolation and identification of goat-derived Pseudorabies virus. Prog Vet Med 2016; 12:113-117. doi: CNKI:SUN:DYJZ.0.2016-12-025
18. Dan F J. Antibacterial action in vitro of Rodgersiaaesculifoliabatal. J Anhui Agr Sci, 2009; 10:4491-2, 502. doi:10.13989/j.cnki.0517–6611.2009.10.081.
19. Song X, Hu H, Hu Z et al. Myricetin inhibits pseudorabies virus infection through direct inactivation and activating host antiviral defense. Front Microbiol 2022; 3529. doi.org/10.3389/fmicb.2022.985108
20. Huan C, Zhou Z, Yao J et al. The antiviral effect of panax notoginseng polysaccharides by inhibiting PRV adsorption and replication in vitro Molecules 2022; 27(4):1254. doi: 10.3390/molecules27041254
21. Tong C, Chen Z, Liu F, et al. Antiviral activities of Radix isatidis polysaccharide against pseudorabies virus in swine testicle cells. BMC Complement Med Ther 2020; 20:48. doi.org/10.1186/s12906-020-2838-4
22. Choubey Y, Goyal S, Varughese L et al. Probing gallic acid for its broad spectrum applications. Mini Rev Med Chem 2018; 18(15):1283-1293. doi: 10.2174/1389557518666180330114010
23. Wu W, Li R, Li X et al. Quercetin as an antiviral agent inhibits influenza A virus (IAV) entry. Viruses 2015; 8(1):6. doi: 10.3390/v8010006
24. Yang F, Feng C, Yao Y et al. Antiviral effect of baicalin on Marek's disease virus in CEF cells. BMC Vet Res 2020; 16(1):371. doi: 10.1186/s12917-020-02595-x
25. Zhang Y, Yu J, Dong X et al. Research on characteristics, antioxidant and antitumor activities of dihydroquercetin and its complexes. Molecules 2017; 23(1):20. doi: 10.3390/molecules23010020
26. Zhao T, Tang H, Xie L et al. Scutellaria baicalensis Georgi.(Lamiaceae): A review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. J Pharm Pharmacol 2019; 71(9):1353-1369. doi: 10.1111/jphp.1312
27. Huang H, Zhou W, Zhu H et al. Baicalin benefits the anti-HBV therapy via inhibiting HBV viral RNAs. Toxicol Appl Pharmacol 2017; 323:36-43. doi: 10.1016/j.taap.2017.03.016
28. Xu G, Dou J, Zhang L et al. Inhibitory effects of baicalein on the influenza virus in vivo is determined by baicalin in the serum. Biol Pharm Bull 2010; 33(2):238-243. doi: 10.1248/bpb.33.238