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Preliminary screening of traditional medicine against porcine reproductive and respiratory syndrome virus

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Summary

Objective: Due to the lack of specific anti-PRRSV drugs and limited cross-protection of the vaccine, PRRSV outbreaks cause substantial economic and social losses. This study aimed to identify anti-PRRSV agents from traditional Chinese medicines and predict their antiviral mechanisms. Methods: Screen anti-PRRSV agents by CCK-8/RT-qPCR assays and predict their mechanisms using network pharmacology. Results: Resveratrol, berberine, puerarin, *Portulaca oleracea* water extract, and vanillic acid exhibited anti-PRRSV activity. Their potential molecular targets/mechanisms were predicted via network pharmacology. Conclusion: Five anti-PRRSV agents were screened, their targets and mechanisms elucidated, providing a theoretical basis for the development of PRRSV drugs and further studies.

Keywords: porcine reproductive and respiratory syndrome virus, antiviral drugs, network pharmacology

The porcine reproductive and Respiratory Syndrome Virus (PRRSV) threatens the global swine industry. Typical symptoms occur in the reproductive and respiratory systems, with high morbidity and mortality during outbreaks. Currently, there are no specific anti-PRRSV drugs or treatments. Although vaccines are the main preventive measure, inactivated ones have weak immunogenicity, while attenuated live vaccines risk virulence reversion. In addition, antibody-dependent enhancement and new strains – both due to the high genetic variability – further reduce the efficacy of the vaccine (15). These challenges hinder effective PRRS control and severely constrain swine industry development. Thus, novel antiviral agents are urgently needed to reduce PRRSV transmission and mitigate its impact.

Traditional Chinese medicine (TCM) has a long history of prevention and treatment of diseases. Investigating the antiviral mechanisms and therapeutic potential of TCMs is a hot research focus. Through a literature review, this study selected 8 agents: Xiaochaihu Granules (XCHG), Qingkailing Granules (QKLG), Lianhuaqingwen Capsules (LHQWC), resveratrol, *Portulaca oleracea*, berberine, puerarin and vanillic acid. These agents have antiviral/immunomodulatory properties, but no reports exist on their anti-PRRSV effects. In this study, we identified anti-PRRSV agents *in vitro*, predicted their antiviral mechanisms via network pharmacology, and laid a foundation for further research.

Material and methods

Cells, viruses, and drugs. Marc-145 cells were cultured in a humidified 37°C incubator with 5% CO_2, using DMEM (Gibco, China) supplemented with 10% (v/v) fetal bovine serum (FBS; Biosharp, China) and 1% (v/v) penicillin-streptomycin (Seven, China). The HP-PRRSV strain (GenBank: PQ699728) was propagated in Marc-145 cells and stored at –80°C in our laboratory. *Portulaca oleracea* powder (Hao Yuan Tang, China), Xiaochaihu Granules (XCHG; Baiyunshan, China) and Qingkailing Granules (QKLG; Yuanda, China) were each dissolved in purified water to prepare 20% (w/v) aqueous solutions. Lianhuaqingwen capsules (LHQWC; Yiling, China) were solubilized in DMSO (Biosharp, China) to a 60% (w/v) solution. Berberine (Huilin Bio, China), resveratrol (MACKLIN, China), puerarin (YUZHOU BIOLOGICAL, China) and vanillic acid (MACKLIN, China) were individually dissolved in DMSO to final concentrations of 800 mM, 800 mM, 800 mM and 100 mM, respectively. All solutions were aliquoted and stored at –20°C for subsequent use.

Cell viability measurement. Marc-145 cells were seeded in 96-well plates to 80% confluency. Test agents were diluted in maintenance solution (DMEM with 1% (v/v) penicillin streptomycin and 2% (v/v) FBS). Next, diluted drugs (various concentrations), solvent control, and cell

control were added to pre-seeded wells. After 24 h incubation, CCK-8 was added, incubated at 37°C for 30 min, and absorbance was measured at 450 nm via a microplate reader.

Determination of anti-PRRSV cell viability. Marc-145 cells were seeded in 96-well plates to 80% confluency, incubated with test agents at safe concentrations for 24 h, and all groups except the cell control were infected with PRRSV (MOI = 1). CPE was monitored and imaged by inverted microscope upon appearance, after which the CCK-8 assay was performed.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Marc-145 cells were inoculated in 6-well plates to 80% confluency. Except for the PRRSV control cells, cells were treated with test agents at safe concentrations for 24 h, and then infected with PRRSV (MOI = 1). At 48 h post-infection, cells were harvested for RNA extraction and reverse transcription. RT-qPCR was performed using the LineGene 9600 Plus Detection System (Bioer, China) and SYBR Green PCR Master Mix (Takara, Japan), with PRRSV nucleocapsid (N) gene- and β-actin gene-specific primer pairs synthesized by Kumei Biotechnology (Tab. 1). The reaction protocol was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s (14).

Tab. 1. List of the primers used in this study

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
PRRSV N	AAACCAGTCCAGAGGCAAGG	TCAGTCGCAAGAGGGAAATG
β-Actin	CTCCATCATGAAGTGTGACGT	GTGATCTCCTTCTGCATCCTGTC

Acquisition and analysis of agent and disease targets. TCMSP retrieved the chemical components and protein targets of each agent; UniProt mapped these to the *Sus scrofa* gene targets. PRRS-related genes from OMIM and GeneCards (correlation scores ≥ median) were selected. BioLadder visualized the agent-PRRS intersection targets. PPI analysis using STRING (medium confidence > 0.4 as threshold). PPI network was visualized using Cytoscape 3.10.2; agent-PRRS intersection targets were imported into DAVID for GO/KEGG enrichment analyses, with results visualized via https://www.bioinformatics.com.cn.

Data analysis. Data were analyzed with GraphPad Prism 9.5.1: t-tests for comparisons between groups, one-way ANOVA for ≥.

Results and discussion

To verify agent safety, their cytotoxicity in Marc-145 cells was assessed via CCK-8 assay (Fig. 1A). Safe concentrations for subsequent experiments: XCHG 0.1% (w/v), QKLG 0.02% (w/v), LHQWC 0.01% (w/v), WEPO 3.75% (w/v), resveratrol 100 μM, puerarin 100 μM, berberine 100 μM, vanillic acid 20 μM. For anti-PRRSV screening, cells were pretreated with each agent at safe concentrations for 24 h, infected with PRRSV, and cultured until cytopathic effect (CPE) emerged (Fig. 1B). Marked CPE was observed in XCHG, QKLG and LHQWC groups. CCK-8 viability assays (Fig. 1C) showed resveratrol, puerarin, berberine, WEPO and vanillic acid exerted the most significant antiviral effects relative to the PRRSV group ($P < 0.0001$), so these five agents were selected. RT-qPCR quantified PRRSV N gene mRNA expression in agent-treated cells (Fig. 1D). All five agents significantly downregulated PRRSV N gene mRNA levels, confirming inhibition of PRRSV replication.

Pharmacological analysis of the network. Potential agent targets were identified through TCMSP: resveratrol (151 targets), *Portulaca oleracea* (92 targets), puerarin (55 targets), berberine (19 targets), and vanillic acid (8 targets). A total of PRRS-related targets were retrieved from OMIM and GeneCards. Aent-PRRS overlapping targets were identified (Fig. 2 A-E): resveratrol (89 targets), *Portulaca oleracea* (59 targets), puerarin (40 targets), berberine (12 targets), vanillic acid (5 targets).

Data were imported into STRING to construct PPI networks (Fig. 2 F-J): resveratrol-PRRS (84 nodes, 661 edges), *Portulaca oleracea*-PRRS (54 nodes, 239 edges), berberine-PRRS (11 nodes, 15 edges), puerarin-PRRS (38 nodes, 139 edges), and vanillic acid-PRRS (3 nodes, 2 edges).

PPI network data were input into Cytoscape 3.10.2 for analysis, with network average degree as the screening criterion (Fig. 2 K-O). Resveratrol (top 5): *IL6*, *IL1B*, *STAT3*, *TP53*, *CASP3*; *Portulaca oleracea* (top 3): *PTGS2*, *PPARG*, *ALB*; berberine (top 3): *PRKACA*, *NOS3*, *PTGS2*; puerarin (top 3): *CASP3*, *STAT3*, *MMP9*; vanillic acid: *NOS3*, *PTGS2*, *PTGS1*.

The intersecting targets of Agent-diseases were analyzed via DAVID to obtain GO enrichment results. Functional clusters with P-values < 0.05 and the top 10 enriched genes were selected for visualization (Fig. 2 P-T). For resveratrol, BP: Positive regulation of angiogenesis, miRNA transcription, and apoptotic process (negative); CC: Cytoplasm, extracellular space; MF: Identical protein binding, cytokine activity, protein homodimerization activity. For *Portulaca oleracea*, BP: Positive regulation of the MAPK cascade, gene expression, and RNA polymerase II-mediated transcription; CC: Membrane raft, perinuclear region of the cytoplasm; MF: Protein homodimerization activity, scaffold protein binding, heme binding. For berberine, BP: Cyclooxygenase pathway, sodium ion transport regulation, nitric oxide biosynthetic process; CC: Nucleus, Golgi apparatus; MF: Scaffold protein binding, heme binding, ubiquitin protein ligase binding. For puerarin, BP: Positive regulation of miRNA transcription, extrinsic apoptotic signalling (through death domain receptors), apoptotic process; CC: Extracellular space, cell surface; MF: Ubiquitin protein ligase binding, protease binding, transcription cis-regulatory region binding. For vanillic acid: BP: Cyclooxygenase pathway, calcium ion transport (negative regulation), prostaglandin biosynthetic process; CC: Neuron projection; MF: Heme binding, prostaglandin-endoperoxide synthase activity.

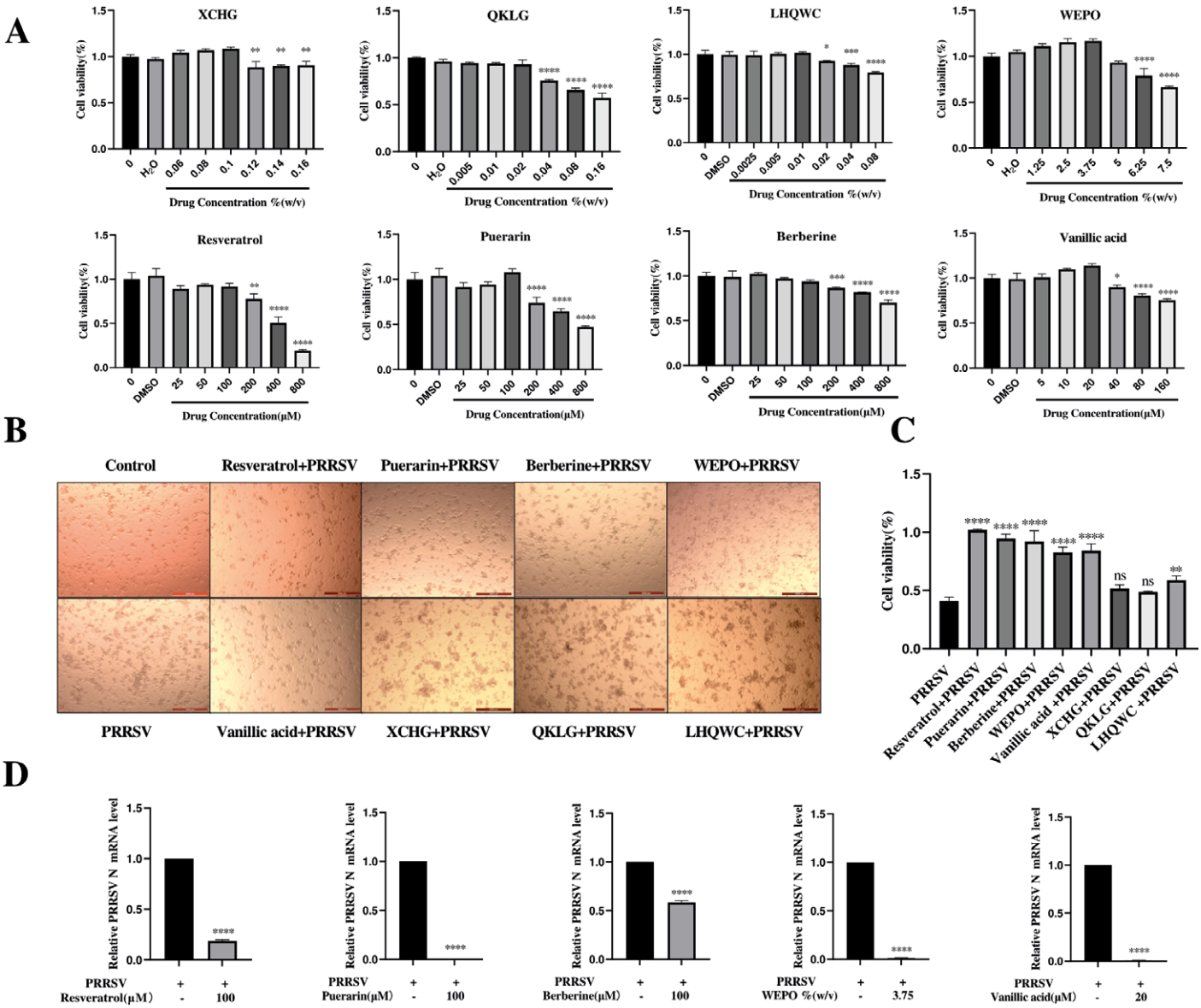

Fig. 1. Screening for anti-PRRSV drugs. (A) Cytotoxicity of different concentrations of agents in Marc-145 cells at 24 h. (B) CPE after PRRSV infection. (C) Detection of cell viability post-PRRSV infection via CCK-8 assay. (D) Verification by RT-qPCR of the anti-PRRSV effect of the screened agents at 48 h after PRRSV infection

KEGG pathways were analyzed, sorted by P-value, and the top 20 were selected for visualization (Fig. 2 U-Y). Resveratrol: Lipid and Atherosclerosis signaling pathway, AGE-RAGE signaling pathway in Diabetic Complications, MAPK signaling pathway, Apoptosis, PI3K-Akt signaling pathway, Non-alcoholic Fatty Liver Disease (NAFLD), TNF signaling pathway. *Portulaca oleracea*: Lipid and Atherosclerosis signaling pathway, AGE-RAGE signaling pathway in Diabetic Complications, MAPK signaling pathway, PI3K-Akt signaling pathway, NAFLD, TNF signaling pathway. Berberine: Calcium signaling pathway, Regulation of Lipolysis in Adipocytes, Lipid and Atherosclerosis signaling pathway, Platelet Activation, cAMP signaling pathway, cGMP-PKG signaling pathway, Arginine Biosynthesis. Puerarin: Lipid and Atherosclerosis signaling pathway, AGE-RAGE signaling pathway in Diabetic Complications, NAFLD, TNF signaling pathway, Apoptosis, IL-17 signaling pathway, HIF-1 signaling pathway. Vanillic acid: Arachidonic Acid Metabolism, Regulation of Lipolysis in Adipocytes, VEGF signaling pathway. Other pathways (e.g., cancer, parasite, nervous/secretory system-related) had limited relevance to PRRSV mechanisms, and their correlations require further validation.

XCHG is recommended for the prevention and treatment of viruses such as severe acute respiratory syndrome (SARS), influenza A virus (IAV), dengue virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It inhibits inflammatory storms, regulates immunity, and acts against hepatitis B virus (HBV), herpes viruses, and influenza viruses (8). QKLG is the first choice for pharyngitis, tonsillitis, and other virus-linked diseases. It works against IAV, parainfluenza virus type B (PIV-B), and respiratory syncytial virus (RSV), and is also commonly used for

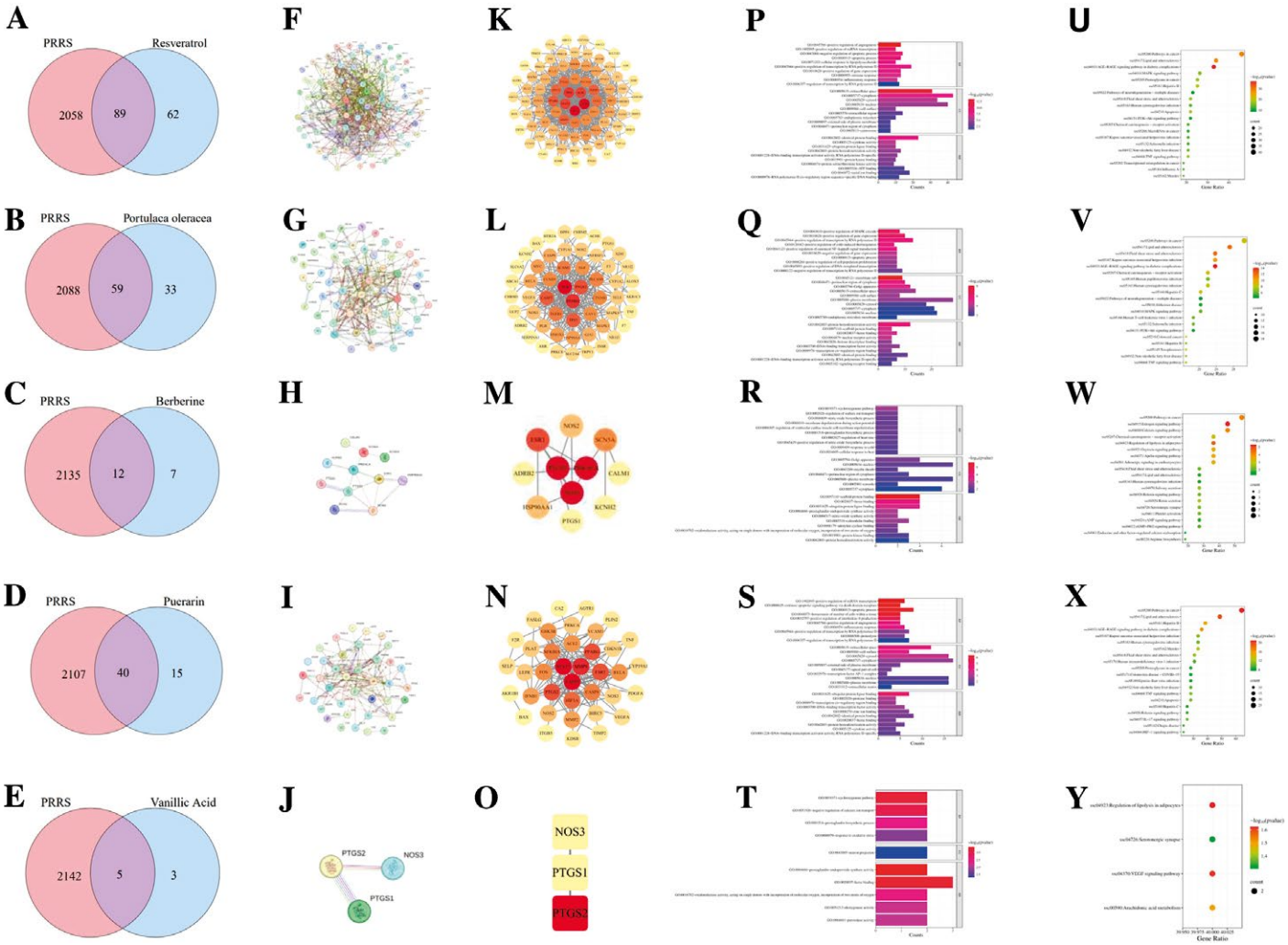

Fig. 2. Network pharmacology analysis of 5 specific agents against PRRS. (A-E) Target interaction analysis between PRRS and the 5 specific agents. (F-J) PPI network diagram of intersecting targets. (K-O) Topology analysis via Cytoscape 3.10.2. (P-T) KEGG pathway analysis of intersecting targets. (U-Y) GO enrichment analysis of intersecting targets

herpes zoster caused by varicella-zoster virus (VZV) (18). LHQWC has broad-spectrum antiviral effects targeting SARS-CoV-2, influenza viruses, and SARS (13). Though these three compound drugs (XCHG, QKLG, LHQWC) are confirmed to have antiviral properties, this conflicts with our findings – they showed no protective effect on PRRSV-infected cells. This may be due to their complex composition and insufficient proportion of active ingredients for therapeutic efficacy. Optimizing their component ratios is needed to explore their potential against PRRSV.

Resveratrol acts against herpes simplex virus (HSV), VZV, pseudorabies virus (PRV), RSV, Zika virus (ZIKV), SARS-CoV-2, enterovirus 71 (EV71), dengue virus and influenza virus (4). Berberine inhibits human immunodeficiency virus (HIV), RSV, hepatitis C virus (HCV), human papillomavirus (HPV), human cytomegalovirus (HCMV) and influenza virus (16). *Portulaca oleracea* is a nutrient-rich medicinal plant with diverse biological activities and complex phytochemicals. Studies show its antiviral mechanisms: regulating the PI3K/AKT/mTOR pathway to suppress porcine rotavirus (PoRV) replication; its aqueous extract preventing H1N1/H3N2 attachment to cells in the early stage of infection; and reducing porcine epidemic diarrhea virus (PEDV) adsorption efficiency (21). Puerarin acts against HBV, HIV-1, RSV (17), PEDV, rotavirus (RV), and influenza virus (3). Vanillic acid has anti-inflammatory effects, and emerging evidence highlights its regulatory role at the crossroads of inflammation, immunity, and metabolic pathways (10). Current research shows that the antiviral properties of five phytochemicals – resveratrol, berberine, vanillic acid, puerarin, and WEPO – align with our experimental confirmation of their anti-PRRSV activity.

The network pharmacology analysis shows that the main shared targets of 5 drugs and PRRSV are involved in regulating inflammation, apoptosis, immunity, and metabolism. Activated by host PRRs, PRRSV activates NF-κB/NLRP3 inflammasomes, induces cytokine secretion (IL-10, IL-12, IL-15, IL-17, TNF-α); triggers mitochondrial apoptosis through Bax-mediated release of cytochrome C and caspase-9 activation, with MAPK, PI3K/Akt and p53-dependent

pathways linked to PRRSV-regulated apoptosis (1). Resveratrol exerts anti-inflammatory effects via multiple pathways: via the p38 MAPK/SIRT1 pathway to alleviate inflammation and decrease TNF-α, IL-6, and IL-1β secretion; via significantly downregulating the MyD88-dependent TLR4 and NF-κB pathway activity; and via regulating the PI3K/Akt/mTOR pathway to reduce inflammatory cytokine levels (4). *Portulaca oleracea* extract blocks MAPK, synergistically suppresses NF-κB (11); its active ingredients inhibit PI3K phosphorylation, modulate mTOR/NF-κB, and exert anti-inflammation (20). Berberine regulates the MEK-ERK, AMPK/mTOR, and NF-κB signaling pathways, as well as reducing inflammatory cytokine secretion (16). Puerarin activates PI3K-Akt-mTOR, upregulates anti-apoptotic proteins, inhibits caspase cascade and PTEN-ROS-p53, suppresses TNF-α-induced apoptosis (9). Vanillic acid inhibits IL-1β-induced MAPK and PI3K/AKT/NF-κB pathways induced by IL-1β (6).

PRRSV infection is associated with host metabolism, as it promotes glycerophospholipid hydrolysis in cell membranes, releases arachidonic acid to activate its metabolic cascade, and generates inflammatory mediators. Activated NF-κB further enhances the transcription of the ALOX, COX, and PLA2 genes, ultimately forming a pathogenic positive feedback loop between inflammation and lipid metabolism (12). Resveratrol promotes fatty acid oxidation via upregulation mediated by AMPK of PPARα, and regulates cholesterol transport by enhancing apolipoprotein A-I and low-density lipoprotein receptor expression (19). *Portulaca oleracea* activates the AMPK pathway, inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase to reduce cholesterol synthesis, modulates miR-33 and miR-34a expression to limit cholesterol uptake, and upregulates PPARα to enhance cholesterol catabolism (7). Berberine regulates blood glucose, lipids, and inflammatory responses in metabolic syndrome patients (2). Puerarin inhibits TGF-β/Smad signaling to downregulate lipogenic genes, reduces hepatic lipid accumulation, and ameliorates insulin resistance through AMPK activation, indirectly suppressing lipolysis and free fatty acid release (5). Vanillic acid exerts anti-inflammatory effects by directly inhibiting the COX-2 pathway and blocking the formation of PGE2 (10).

In summary, this study systematically evaluated 8 antiviral agents, identifying 5 agents with potent anti-PRRSV activity via in vitro screening. Integrating network pharmacology, we elucidated their molecular targets and related signaling pathways, providing a reference for the screening of anti-PRRSV drugs and an in-depth study of their mechanisms of action.

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