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ORIGINAL RESEARCH

Exploration of the in vitro Antiviral Effects and the Active Components of Changyanning Tablets Against Enterovirus 71

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Purpose: This study aims to investigate the in vitro antiviral effects of the aqueous solution of Changyanning (CYN) tablets on Enterovirus 71 (EV71), and to analyze its active components.

Methods: The in vitro anti-EV71 effects of CYN solution and its herbal ingredients were assessed by testing the relative viral RNA (vRNA) expression level and the cell viability rates. Material basis analysis was performed using HPLC-Q-TOF-MS/MS detection. Potential targets and active components were identified by network pharmacology and molecular docking. The screened components were verified by in vitro antiviral experiments.

Results: CYN solution exerted anti-EV71 activities as the vRNA is markedly reduced after treatment, with a half maximal inhibitory concentration (IC_{50}) of 996.85 μg/mL. Of its five herbal ingredients, aqueous extract of *Mosla chinensis* (AEMC) and leaves of *Liquidambar formosana* Hance (AELLF) significantly inhibited the intracellular replication of EV71, and the IC_{50} was tested as 202.57 μg/mL and 174.77 μg/mL, respectively. Based on HPLC-Q-TOF-MS/MS results, as well as the comparison with the material basis of CYN solution, a total of 44 components were identified from AEMC and AELLF. Through network pharmacology, AKT1, ALB, and SRC were identified as core targets. Molecular docking performed between core targets and the components indicated that 21 components may have anti-EV71 effects. Of these, nine were selected for in vitro pharmacodynamic verification, and only rosmarinic acid manifested in vitro anti-EV71 activity, with an IC_{50} of 11.90 μg/mL. Moreover, rosmarinic acid can stably bind with three core targets by forming hydrogen bonds.

Conclusion: CYN solution has inhibitory effects on EV71 replication in vitro, and its active component was identified as rosmarinic acid. Our study provides a new approach for screening and confirmation of the effective components in Chinese herbal preparation.

Keywords: antiviral effects, material basis analysis, component-target-pathway-disease network, protein–protein interaction network, core targets, rosmarinic acid

Introduction

Enterovirus 71 (EV71) is the main pathogen causing hand, foot, and mouth disease (HFMD).[1] Since 1997, HFMD has emerged as a serious childhood infection in the Asia-Pacific region.[2] From 2008 to July 2022, up to 26.1 million cases of HFMD and 3727 deaths had been reported in China. The disease became a serious public health problem.[3] Although the main manifestations of HFMD are low-grade fever, maculopapular or papular blistering rash on the hands, feet, and mouth ulcers,[4] there were growing evidences that a large number of patients with HFMD have atypical presentations, especially those infected with the EV71, and may rapidly develop serious complications such as encephalitis, pulmonary

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edema, and myocarditis.[5,6] With the use of EV71 vaccine,[1,7] severe HFMD cases had significantly decreased in China.[8] However, vaccination does not completely prevent viral infection, and some children who were vaccinated may still be infected and develop complications such as encephalitis.[9] At present, no antiviral drug was approved for the treatment of EV71 infection. Moreover, as enterovirus belongs to a non-envelope virus, it is difficult to be killed in the host environment.[10] Therefore, the constant search for effective medicine is essential for the control of the infection caused by EV71.

The therapeutic effects of Traditional Chinese medicine (TCM) on infectious diseases, such as influenza, dengue, and SARS-CoV, have been proven by long-term historical practice.[11] Generally, the treatment of HFMD with TCM is based on clearing heat and relieving dampness and detoxification. The TCM preparations which had been clinically used for the treatment of HFMD included heat poisoning injection, Xiyanping injection, Pudilan anti-inflammatory oral solution, Shuanghuanglian oral solution, and anti-viral oral solution.[12,13]

Changyanning (CYN) tablets are composed of five herbal ingredients: *Mosla chinensis* (Chinese name: Jiangxiangru, MC), leaves of *Liquidambar formosana* (Chinese name: Fengxiangshuye, LLF), *Euphorbia humifusa* (Chinese name: Dijincao, EH), roots of *Cinnamomum camphora* (Chinese name: Zhangshugen, RCC), and *Hedyotis chrysotricha* (Chinese name: Jingmaoercao, HC). Clinically, CYN tablets have been proven effective for the treatment of diarrhea, acute gastroenteritis, and ulcerative colitis.[14–16] When we screened the anti-EV71 medicines, we found by chance that the aqueous solution of CYN tablets has in vitro anti-EV71 effects. The 2020 edition of the Chinese Pharmacopoeia records that CYN tablets have the effects of clearing heat and relieving dampness, which is consistent with the therapeutic principle of HFMD. Therefore, whether CYN tablets could be used for the treatment of EV71 infection needs further study.

Although TCM has been used to treat infectious diseases for many years,[17] the complexity of the components led to the ambiguity of its mechanism of action, therefore hindering the research and application of TCM.[18] Identification and search for active components are crucial for clarifying the mechanism of TCM. With the advantages of high resolution, high efficiency and sensitivity, Liquid Chromatography-Mass Spectrometry (LC-MS) has become one of the most powerful tools for the analysis of chemical components of TCM.[19] Hence, the HPLC-Q-TOF-MS/MS method was used to identify the active components after confirming the in vitro antiviral activities of CYN and its herbal ingredients. Network pharmacology uses a variety of cutting-edge technologies such as omics, high-throughput screening, and network analysis to reveal the complex network relationship between "component-gene-target-disease", which helps to understand the molecular basis from multiple dimensions and predict the potential pharmacological mechanism of drugs.[20–22] Molecular docking is a method for studying the interaction and recognition between protein receptors and small-molecule ligands[22]. It predicts the binding ability between different molecules and receptors when two molecules bind to each other to form a stable complex based on the 3D structure of the molecules.[23] Its operating procedures include the search for receptor and ligand structures, the search for binding sites, the selection of computational tools and algorithms, and the selection of scoring methods.[24] Because it relies heavily on computer operations, it can save a lot of experimental materials and reduce the time and energy of repeated experiments.[25] Therefore, molecular docking has become an important tool for drug discovery and molecular simulation.[26–28]

CYN tablet has been approved for the treatment of bacillary dysentery by the Chinese Pharmacopoeia Commission since 2015, but its antiviral activities, especially if it could be used for the treatment of infection caused by enterovirus, are unclear. In addition, as CYN is a Chinese herbal prescription and its compositions are very complicated, the effective components that could play an essential role in resisting viruses or bacteria have not been well studied. Therefore, this study aims to verify whether CYN tablets have antiviral effects on EV71 by in vitro pharmacodynamic experiments and also to find its active herbal ingredients. Furthermore, the antiviral mechanism and the effective components with anti-EV71 activity were analyzed by integrating network pharmacology with HPLC-Q-TOF-MS/MS and molecular docking techniques.

Materials and Methods

Ethics Statement

This study was exempted from ethics review and approval by the Ethics committee of Zhejiang provincial Center for Disease Control and Prevention (Zhejiang CDC) as it does not involve the use of any clinical samples, human or animals.

The EV71 strain used was a virus stock in laboratory of Zhejiang CDC, hence there was no operation of sampling from patients as well.

Medicines and Chemical Components

CYN tablets (Lot number: N211010), provided by Zhejiang CONBA Pharmaceutical Co., Ltd., were prepared into a solution by dissolving it with pure water, making the initial concentration of 100 mg/mL. Five herbal ingredients (MC, LLF, EH, RCC, and HC) that constitute CYN tablets were also provided by Zhejiang CONBA Pharmaceutical Co., Ltd. and were authenticated by Dr. Jianbiao Yao, Zhejiang CONBA Pharmaceutical Co., Ltd. The herbal ingredients were stored at the Zhejiang Provincial Key Laboratory of Traditional Chinese Medicine Pharmaceutical Technology. The numbers of MC, LLF, EH, RCC, and HC were YZ004-200,801, YZ005-190,601, YZ001-191,001, YZ003-200,904, and YZ002-200,814, respectively. To maintain consistency, all of the crude slices of these medicines conformed to quality standards in Chinese Pharmacopeia (2020 edition) or the Standards of Chinese Medicinal Materials in Jiangxi Province (2014 edition). The aqueous extracts of five herbal ingredients were prepared with the same method. Briefly, the herb was boiled and refluxed in 30 volumes of water for 1h. The extraction process was repeated twice followed by filtration. The aqueous extracts were concentrated under reduced pressure to 50 mL and were determined to contain 1 g/mL of dried herbs.

Astragalin (CAS: 480–10-4), gallic acid (CAS: 149–91-7), cryptochlorogenic acid (CAS: 905–99-7), neochlorogenic acid (CAS: 906–33-2), rosmarinic acid (CAS: 20283–92-5), chlorogenic acid (CAS: 327–97-9) standards were purchased from Chengdu Desite Biotech Co., Ltd., China. Hyperoside (CAS: 482–36-0) standard was purchased from Shanghai High-Tech Chemical Technology Co. Caffeic acid (CAS: 331–39-5) and rutin (CAS: 153–18-4) standard were purchased from National Institutes for Food and Drug Control. These standards were dissolved with dimethyl sulfoxide, making the initial concentration 10 mg/mL and stored at −20°C. Ribavirin (Lot number: 181202) purchased from Biokin Pharmaceutical Co. Ltd. and used as a positive control, was dissolved in phosphate buffer saline (PBS) at a concentration of 5 mg/mL and stored at −20°C.

Virus and Cells

EV71 strain EV71/Zhejiang. CHN/195/2016 (GenBank accession no. OR198186) was an isolate stored at −80°C in the Zhejiang CDC. The human rhabdomyosarcoma (RD) cell line was provided by the Poliomyelitis Laboratory of the Chinese Center for Disease Control and Prevention. RD cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin in an incubator at 37°C and 5% CO_2.

Cytotoxicity of Medicines

Cytotoxicity of medicines was determined using Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China). RD cells seeded in 96-well plates were cultivated at 37°C for 48 h. Subsequently, they were divided into blank group, cell control group, and different concentrations of medicine-treated groups (n=5). The growth medium was discarded and replaced with different concentrations of medicines diluted in maintenance medium (MM) (100 μL/well). After incubation at 37°C for 48 h, the MM-containing medicines were removed and the cells were washed twice with PBS. Then, 100 μL MM containing 10 μL CCK-8 was added to the cells. Cell plates were reincubated for 1.5 h at 37°C, and the optical density of each well was measured at 450 nm. The maximum concentration with a cell survival rate ≥ 90% was recognized as the maximum non-toxic concentration (MNTC) of the medicines. And the 50% cytotoxic concentration (CC_{50}) was calculated according to the equation.[29]

Antiviral Activity Evaluation of Medicines

RD cells were seeded in 24-well plates. After the monolayer was formed, the cells were inoculated with 0.01 MOI of EV71 (100 μL/well), followed by incubation at 37°C for 1 h. The virus was discarded, and the cells were treated with different concentrations of medicines (1 mL/well). Cells were divided into base group, virus control (VC) group, cell control group, and different concentrations of medicine-treated groups (n=6). For VC, 1 mL/well MM was added to cells after infection. Cell control was cells without infection. After 24 h incubation, the culture plates were freeze-thawed

twice, and the anti-EV71 activity was assessed by comparing the viral RNA (vRNA) levels between the VC and medicine-treated groups. Ribavirin (1 mg/mL) was used as a positive control in the same way as for the medicine groups.

To further investigate the intracellular inhibitory effect of medicine on EV71, the half maximal inhibitory concentration (IC_{50}) of medicine was determined by testing cell viability.[30] RD cells were infected with 0.01 MOI of EV71 in 96-well plates for 1 h at 37°C. After infection, the virus was discarded, and 100 μL MM containing various concentrations of medicine was added to the cells. For the VC group, the cells were covered with 100 μL MM without medicine after infection. Cells were divided into cell control group, VC group, and different concentrations of medicine-treated groups (n=5). After 48 h of incubation, the cell viability of each well was measured by CCK-8, and the IC_{50} of medicine was calculated.[29]

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR targeting the VP1 gene of EV71 was performed to test vRNA level. Total RNA was extracted from 300 μL mixture of cells and culture fluid and then eluted into 60 μL RNase-free water using Nucleic Acid (DNA/RNA) Extraction or Purification Kit (SANSURE BIOTECH INC, China). The extracted RNA was detected by One-Step TB Green™ PrimeScript PLUS RT-PCR Kit (Perfect Real-time) (TaKaRa, Kyoto, Japan). The relative RNA expression level was calculated using a classical $2^{-\Delta\Delta Ct}$ method after normalizing against the expression level of β-actin. Sequences of primer pairs are shown in Table 1.

Material Basis Analyzed by HPLC-Q-TOF-MS/MS

The HPLC was carried out using an Agilent 1260 Infinity liquid chromatography system. Chromatographic separation was performed on an Agilent ZORBAX Eclipse XDB-C18 column (4.6×250 mm, 5 μm) at a column temperature of 30°C. The mobile phase flow rate was kept constant at 1.0 mL/min. The mobile phase consisted of methanol (solvent A) and water containing 0.05% acetic acid (v/v) (solvent B) with elution by a linear gradient at a flow rate of 1.0 mL/min. A simple gradient elution system is established as shown in Table 2. Detection was 320 nm wavelength, and the injection volume was 10 μL. The MS was carried out using Agilent 6530 Accurate-Mass Q-TOF LC/MS with an ESI source operated in the negative ion mode. The source parameters were set as follows: capillary voltage, 3500 V; nebulizer, 35 psig; fragmentor, 65 V; skimmer, 65 V; dring gas, 10 L/min; collision energy, 20 V. Mass spectra were recorded across the range m/z 120–1000.

Network Pharmacology

Firstly, the 44 components identified by HPLC-Q-TOF-MS/MS were imported to PubChem (https://pubchem.ncbi.nlm.nih.gov/), and only 40 can be retrieved. Then, the 40 components were downloaded in SDF format in PubChem and

Table 1 Primer Pairs Used for Detection of EV71 and Host Cells

Target	Primer Name	Sequence of Primers
EV71	EV71-F	5'-GAGCATGATTGAGACACGCTGTGT- 3'
	EV71-R	5'-CCCGCCCTRCTGAAGAAACT- 3'
Host gene (RD)	β-actin-F	5'-GAGCTACGAGCTGCCTGACG-3'
	β-actin-R	5'-CCTAGAAGCATTTGCGGTGG-3'

Table 2 Details of HPLC Assay

Elution Time (min)	Mobile Phase A Concentration (%)
0–15	Linearly from 5 to 10
15–20	Maintained at 10
20–50	Linearly from 10 to 30
50–80	Linearly from 30 to 50

imported into Pharmmapper (http://www.lilab-ecust.cn/pharmmapper/) for the prediction of the related targets of each component. Based on the criteria with Norm Fit > 0.80, only 34 components could be used for target prediction. Subsequently, the EV71-related targets were obtained through retrieving three databases, including GeneCards (https://www.genecards.org/), DisGeNET (https://www.disgenet.org/), and OMIM (https://www.omim.org) using keywords EV71, EV-A7, or Human enterovirus 71. The component-related targets and the EV71-related targets were imported into R 4.2.0 software to obtain the intersection targets and plot Venn diagram. The component-gene-target-disease network diagram was drawn using Cytoscape 3.9.1 software. Finally, the intersection targets were imported into the String database (http://string.embl.de/), and the free targets were removed. The PPI network diagram was drawn using Cytoscape 3.9.1 software.

Molecular Docking

Three core targets obtained in PPI were used for molecular docking with the 34 components. The PDB structures of AKT1 (PDB ID: 3mvh), ALB (PDB ID: 6yg9), and SRC (PDB ID: 1o4i) were downloaded from the RSCB PDB database (http://www.rcsb.org/). All proteins were processed by pymol 2.3 software, including the removal of ligands and water, the addition of hydrogen, and the setting of active centers. Then, 34 components identified from HPLC-Q-TOF-MS/MS were imported into the ChemDraw 3D 20.0 for energy minimum processing and saved in pdb format. The docking scores are shown in images using the OmicStudio tools at https://www.omicstudio.cn/tool. The proteins and components were converted to pdbqt format by AutodockTool 1.5.6. Docking was performed with AutoDock vina software and visualized with Pymol 2.5.4 and LigPlot 2.2.

Statistical Analysis

Experimental results were expressed as mean ± standard deviation. Data were tested for normality and homogeneity of variance using the one-way ANOVA test. If the variances were homogeneous, the least significant difference test was selected. If the variances were not uniform, the Games-Howell test was selected. $P < 0.05$ indicated that the difference was significant.

Results

In vitro Anti-EV71 Activity of CYN Solution

To avoid cytotoxicity, the MNTC of CYN solution was first determined on RD cells. Cell viability was tested as 103.39% when the cells were treated with 5 mg/mL of CYN solution, while it dropped to 23.90% when the concentration of CYN solution increased to 10 mg/mL (Supplementary Table S1). Hence, the MNTC of CYN solution was identified as 5 mg/mL and the CC_{50} was calculated as 7.96 mg/mL (Figure 1A). The MNTC and CC_{50} of the positive control ribavirin were 1 mg/mL and 2.43 mg/mL, respectively (Supplementary Table S2). To investigate the antiviral effects, RD cells were treated with various concentrations (5, 2.5, 1.25, 0.63 mg/mL) of CYN solution after EV71 infection. Figure 1B shows that CYN solution treatments efficiently inhibited the replication of EV71 as the vRNA levels of CYN treatment groups were significantly reduced compared to the vRNA of VC ($P<0.01$) (Supplementary Table S3). Also, CYN solution presented dose-dependent inhibitory activities on cell death caused by EV71 infection and the IC_{50} was tested as 996.85 μg/mL (Figure 1C and Supplementary Table S4). The IC_{50} of ribavirin was tested as 289.68 μg/mL (Figure 1D and Supplementary Table S5).

In vitro Anti-EV71 Activity of Five Herbal Medicines Which Compose CYN Tablet

To clarify the active herbal ingredients, we tested the in vitro anti-EV71 effects of the aqueous extracts of five herbal ingredients that compose CYN tablet by comparing the relative vRNA expression level between VC and medicine-treated groups. The MNTC of aqueous extract of MC (AEMC), aqueous extract of LLF (AELLF), aqueous extract of EH (AEEH), aqueous extract of RCC (AERCC), and aqueous extract of HC (AEHC) were determined as 10, 2.5, 1.25, 80, and 20 mg/mL, respectively (Supplementary Table S6–S10). In order to compare the antiviral effects at the same concentration, the minimum MNTC among the five ingredients (1.25 mg/mL) was used in the subsequent experiments.

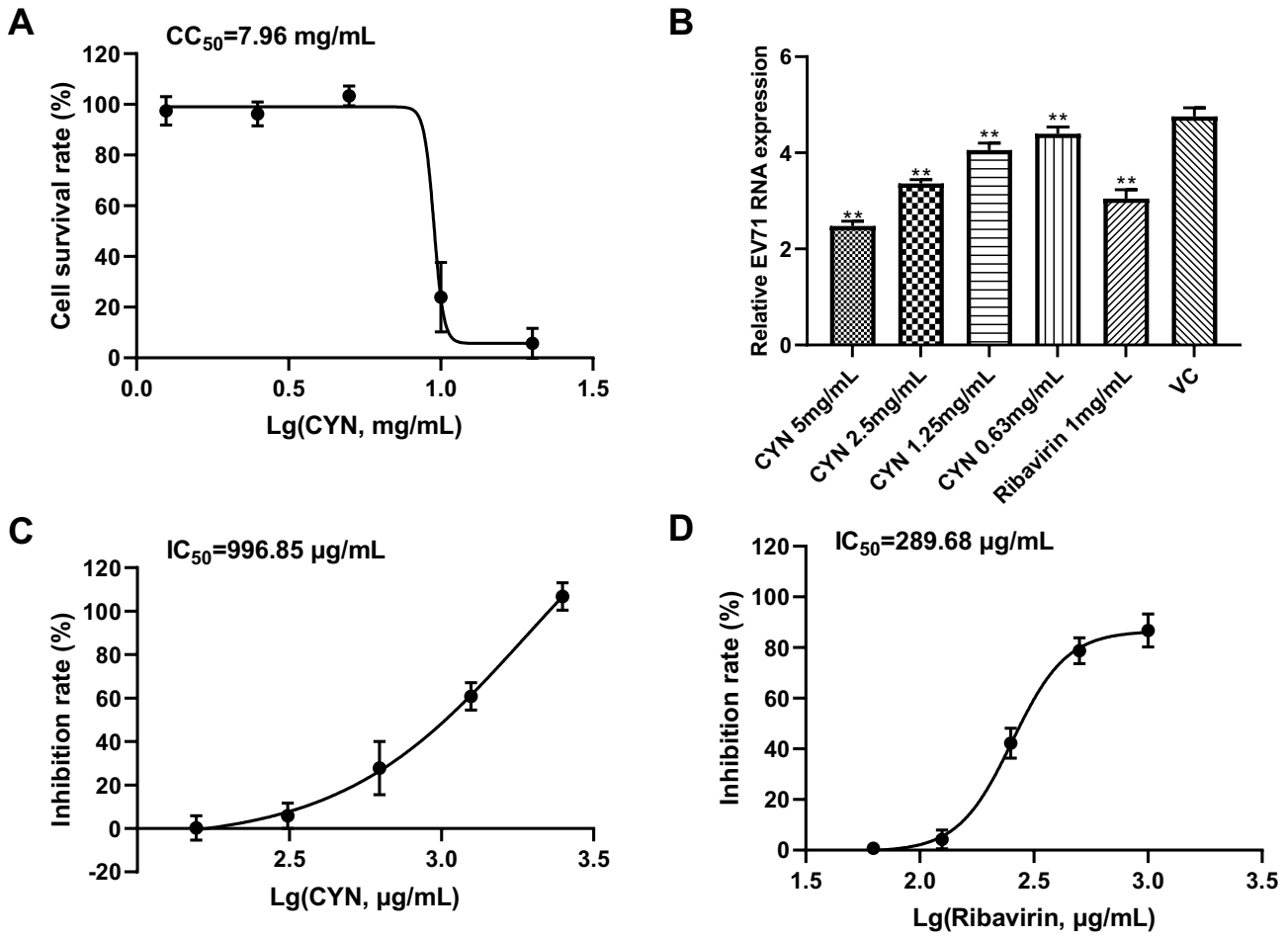

Figure 1 Cytotoxicity (**A**), EV71 RNA expression level (**B**), and anti-EV71 effect (**C**) of aqueous solution of Changyanning (CYN) tablets. (**D**) Anti-EV71 effect of positive control ribavirin. (**A**) Cytotoxicity induced by the aqueous solution of CYN tablets on RD cells. (**B**) RD cells were infected with 0.01 MOI EV71 and treated with different concentrations of CYN solution or ribavirin (1 mg/mL) for 24 h. Quantitative real-time PCR (qRT-PCR) was performed to test EV71 VP1 RNA level. The expression of VP1 transcripts was calculated with the expression level of β-actin mRNA. **significant with $P < 0.01$ compared to VC. (**C**) CYN solution inhibited cell deaths induced by 0.01 MOI EV71. (**D**) Ribavirin inhibited cell deaths induced by 0.01 MOI EV71.

When the infected cells were treated with five herbal ingredients, a marked drop in EV71 RNA level was only observed in AEMC and AELLF treated groups ($P < 0.01$). AEMC and AELLF at a concentration of 1.25 mg/mL resulted in >99% reduction in the expression of EV71 VP1 transcripts, indicating that AEMC and AELLF inhibited EV71 replication intracellularly (Figure 2A and Supplementary Table S11). However, the EV71 RNA levels between other three medicine-treated (AEEH, AEHC, and AERCC) groups and the VC group were not different significantly, indicating that the EV71 replication was not inhibited by treatments with these herbal ingredients. Furthermore, to clarify the inhibitory effects of AEMC and AELLF on EV71, the infected cells were treated with various concentrations of the two herbs. Cell viability rates proved the anti-EV71 activities of AEMC and AELLF. Also, the inhibitory rates showed a dose–effect relationship with the concentration of the medicines. The calculated IC_{50} of AEMC and AELLF were 202.57 μg/mL and 174.77 μg/mL, respectively (Figure 2B and C and Supplementary Table S12 and S13).

Material Basis of AEMC and AELLF

To explore the active components of CYN solution, the material basis of two active herbal ingredients (AEMC and AELLF) which possess in vitro anti-EV71 activities were analyzed by HPLC-Q-TOF-MS/MS. Total ion chromatograms are shown in Figure 3A and B. Through comparison with the material basis of CYN solution,[31] a total of 44 components were identified. Of these, 23 were contained in both AEMC and CYN solutions, while 32 were contained in both AELLF and CYN solutions (Supplementary Table S14). Eleven components were identified in all three. Seven of the 11 are

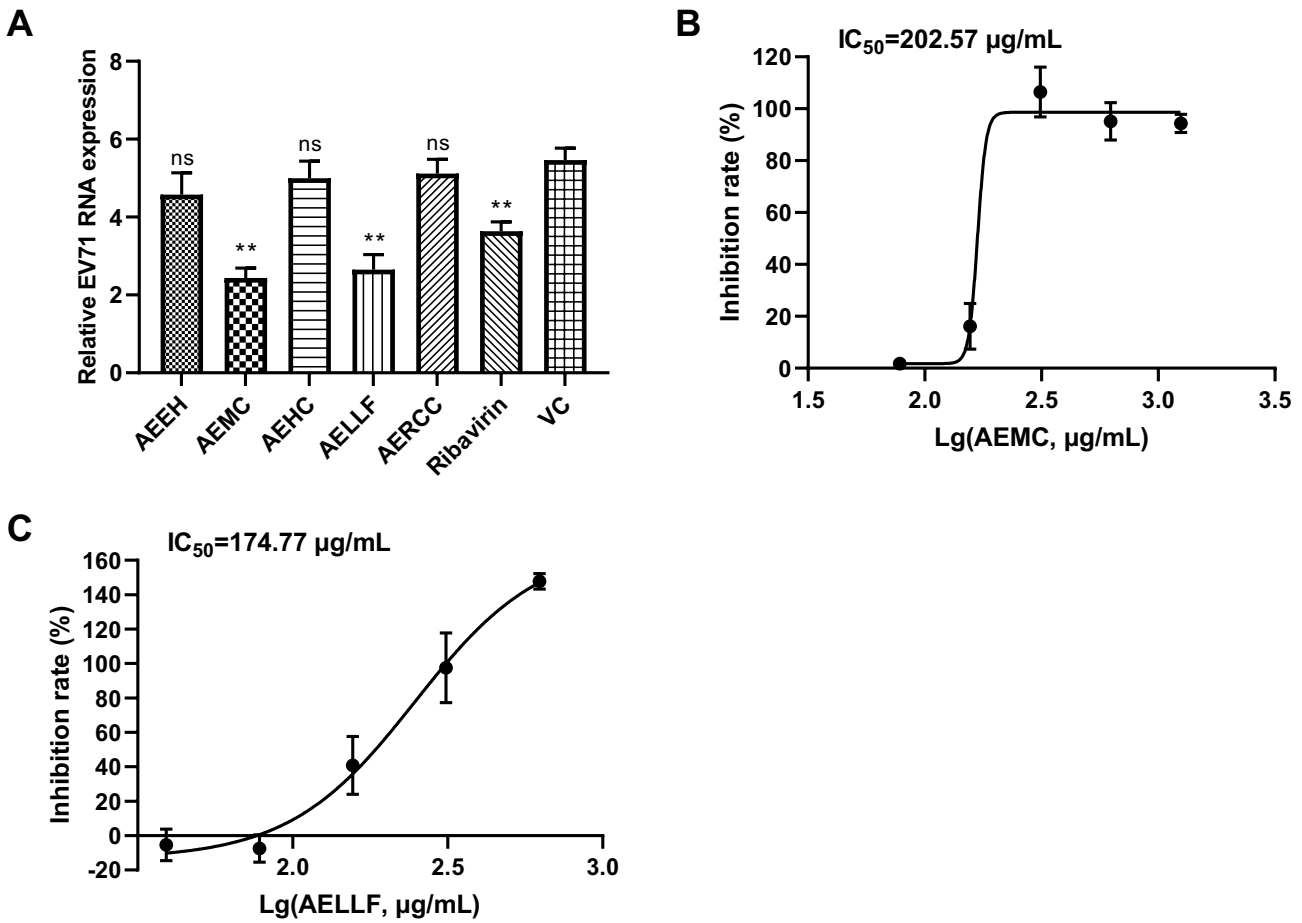

Figure 2 Effects of aqueous extract of five herbal ingredients and ribavirin on the RNA expression of EV71(**A**), and anti-EV71 effect of AEMC (**B**) and AELLF (**C**). (**A**) RD cells were infected with 0.01 MOI EV71 and treated with five herbal ingredients or ribavirin (1 mg/mL) for 24 h. qRT-PCR was performed to test EV71 VP1 RNA level. The expression of VP1 transcripts was calculated with the expression level of β-actin mRNA. ns, not significant with $P > 0.05$; **, significant with $P < 0.01$ compared to VC. (**B**) Aqueous extract of MC inhibited cell deaths induced by 0.01 MOI EV71. (**C**) Aqueous extract of LLF inhibited cell deaths induced by 0.01 MOI EV71.

organic acids, including quinic acid, malic acid, citric acid, gallic acid, hydroxybenzoic acid-glucoside, protocatechuic acid, and vanillic acid. Their structural formulas are shown in Figure 3C. Two of the 11 belong to flavonoids. They are rutin and astragalin (Figure 3D). The remaining two are amino acids N-acetyl-DL-glutamic acid and tannins ellagic acid (Figure 3E and F).

Network Pharmacology

Although we obtained 44 components by material basis analysis, there are only 40 which could be successfully retrieved from PubChem. After screening with the criteria, Norm Fit > 0.80, 34 components could be used for target prediction. A total of 349 component-related targets were screened. Of these targets, 298 were related to MC and 304 were related to LLF. Then, EV71-related targets were retrieved from three databases. Figure 4 shows that 473 targets were screened from OMIM, 298 were screened from Genecards and 21 were screened from DisGeNET database. The component-related targets and the EV71-related targets were imported to plot Venn diagram using R 4.2.0 software, and 29 targets were obtained in the intersection.

The component-target-pathway-disease network was composed of two herbal ingredients, 34 components (used for target prediction), 29 intersection targets, and 20 signaling pathways, showing the potential relationship between herbal ingredients and EV71 infection (Figure 5). The 29 intersection targets were imported into the String platform to construct a PPI network, and the results were imported into Cytoscape 3.9.1 software for visualization. Figure 6 shows there are 25 associated target proteins and 214 edges obtained. The top three targets associated with other proteins were RAC-alpha serine/threonine-protein kinase (AKT1), albumin (ALB), and proto-oncogene tyrosine-protein kinase Src (SRC). Hence, they were regarded as core targets.

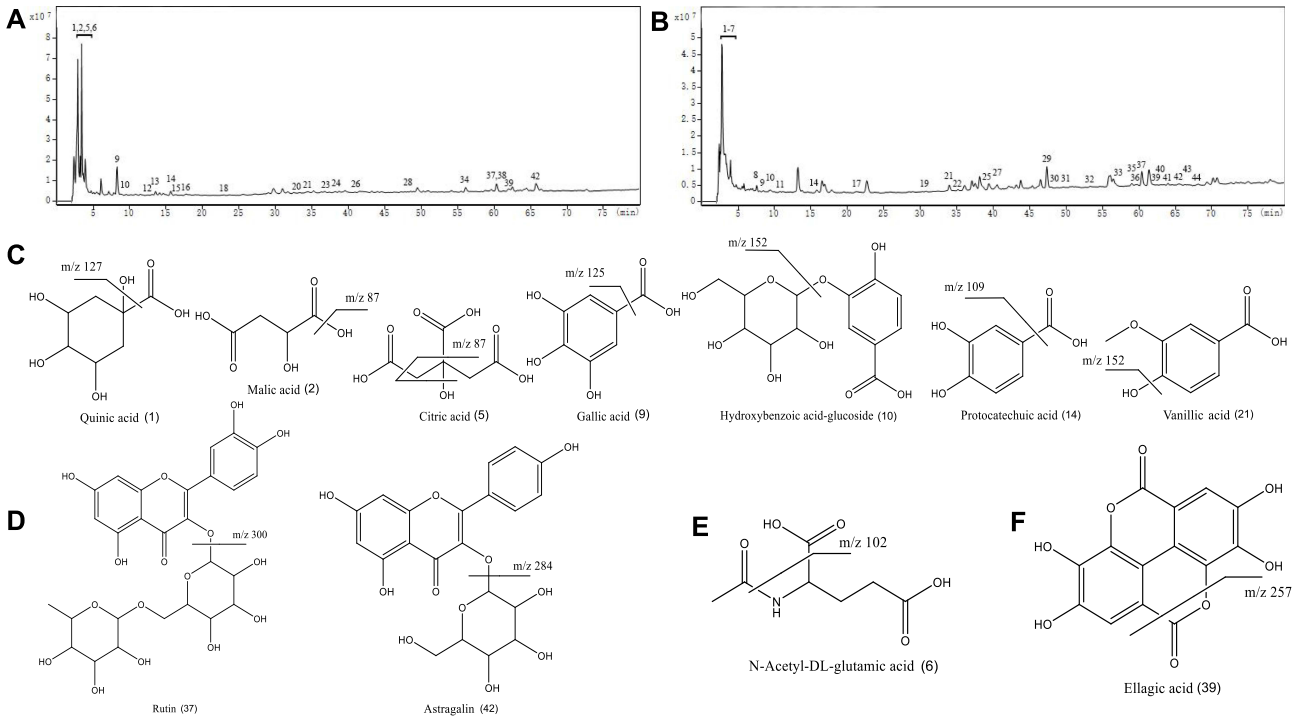

Figure 3 Ion chromatogram of AEMC (**A**) and AELLF (**B**) analyzed by HPLC-Q-TOF-MS/MS, and the structural formula of the 11 common components (**C**–**F**). (**A**) The total ion chromatogram of AEMC. (**B**) The total ion chromatogram of AELLF. (**C**) The structural formulas of seven organic acids. (**D**) The structural formulas of two flavonoids. (**E**) The structural formulas of amino acid. (**F**) The structural formulas of tannin. 9, gallic acid; 17, neochlorogenic acid; 19, chlorogenic acid; 22, cryptochlorogenic acid; 23, caffeic acid; 35, hyperoside; 37, rutin; 38, rosmarinic acid; 42, astragalin.

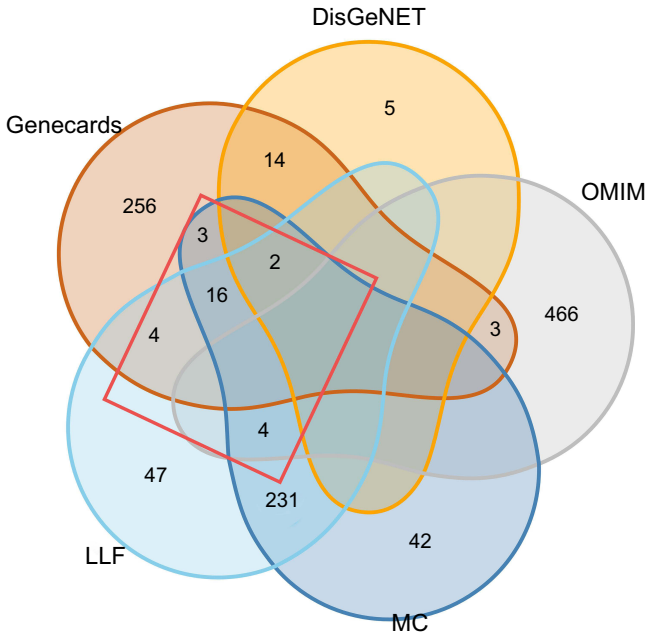

Figure 4 Venn plot. Venn plot was constructed based on the related targets of 34 identified components and the EV71-related targets retrieved from three databases. The 29 intersection targets were marked with the red box.

Molecular Docking and Verification of in vitro Anti-EV71 Activity

For seeking the active components of CYN solution, the 34 components were docked with three core target proteins, respectively. The binding energies between components and proteins are shown in Figure 7. The binding energy ≤-7.0 kcal/mol indicates the stable binding between components and targets.[32] Hence, 21 out of the 34 components may bind tightly with

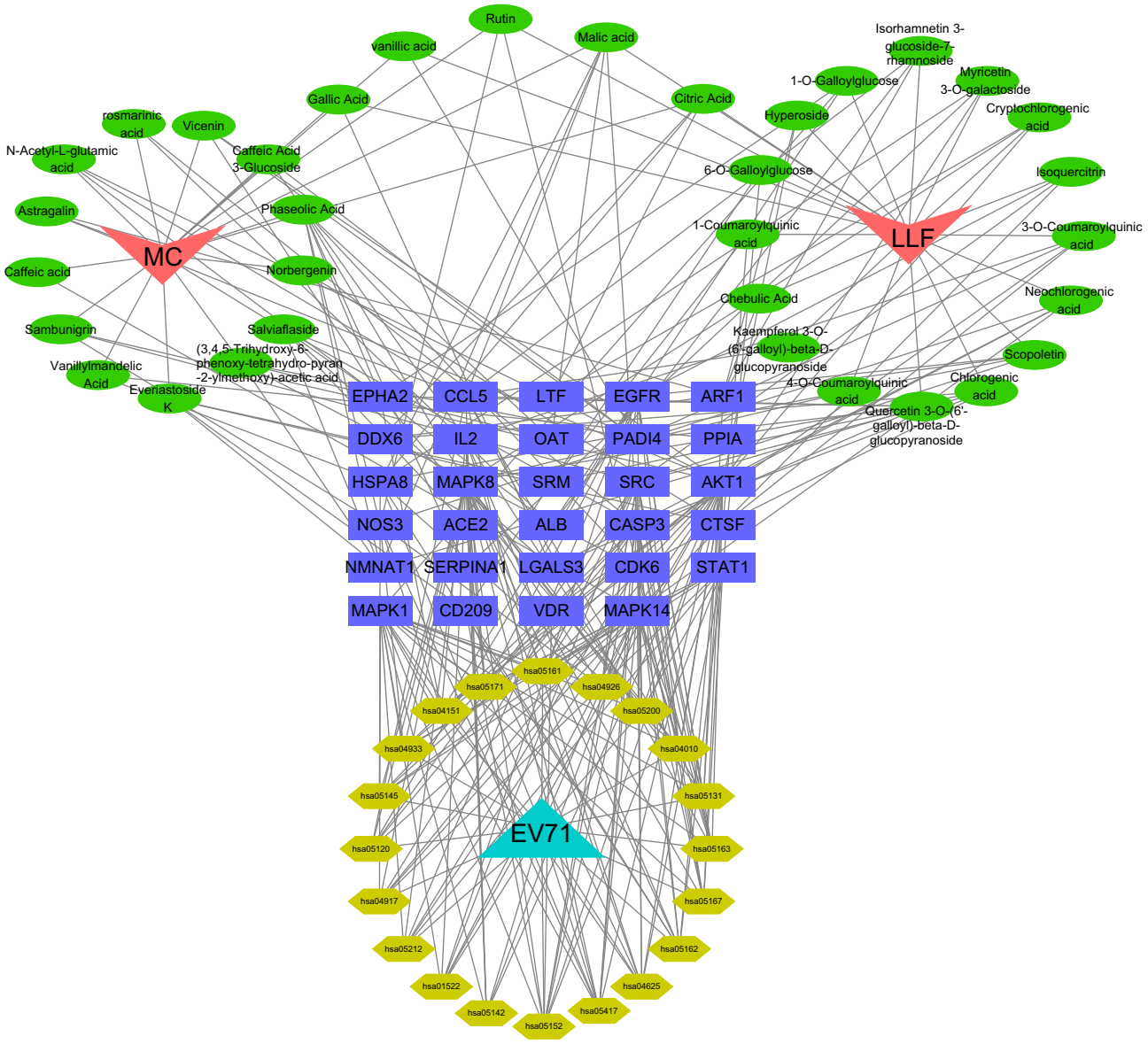

Figure 5 Component-target-pathway-disease network. The component-target-pathway-disease network diagram. Red represents the herbal ingredients MC and LLF. Green represents the 34 identified components. Blue represents EV71. Purple represents the common targets in three. And yellow represents the related pathway.

the core targets and play an anti-EV71 role as the average binding energy with the three targets was ≤ −7.0 kcal/mol. As not all the components were available, only nine including rutin, gallic acid, astragalin, rosmarinic acid, caffeic acid, neochlorogenic acid, cryptochlorogenic acid, chlorogenic acid, and hyperoside were selected for pharmacodynamic verification in vitro. The MNTC of the nine components are shown in Table 3 (Supplementary Table S15–S23). After EV71 infection, cells were treated with each component of the MNTC. Compared to VC, the relative vRNA level markedly dropped only when the cells were treated with 50 μg/mL rosmarinic acid after infection (P<0.01) (Figure 8A, Supplementary Table S24). The results experimentally verified that rosmarinic acid had anti-EV71 activities in vitro. The inhibitory activity of the rosmarinic acid is dose-dependent, with an IC_{50} of 11.90 μg/mL (Figure 8B, Supplementary Table S25).

To further understand the possible mechanism of rosmarinic acid, we analyzed the interaction between rosmarinic acid and three core targets. The docking score of rosmarinic acid was −9.2 kcal/mol with AKT1, −9.9 kcal/mol with ALB, and −5.5 kcal/mol with SRC (Figure 7). Figure 9 shows that rosmarinic acid could form hydrogen bonds with residues ALA-230, GLY-159, GLY-162, and ASP-292 in AKT1 (PDB: ID 3mvh), and residues ARG-10, LEU-66, HIS-

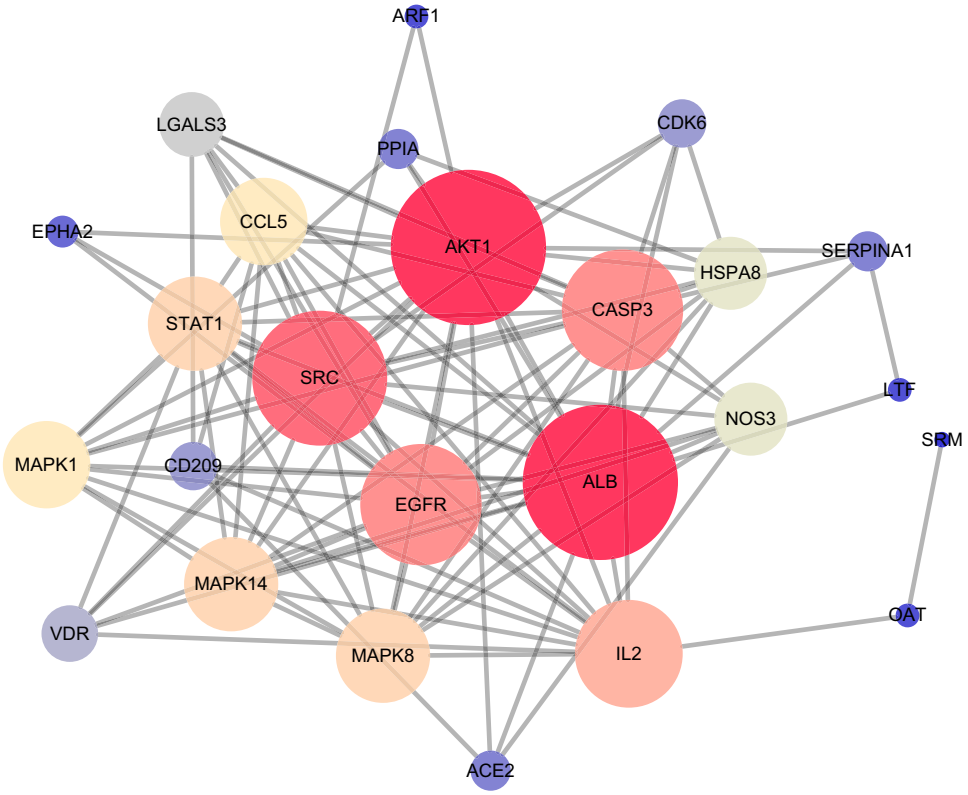

Figure 6 Protein–Protein Interaction (PPI) network diagram. The redder the color, the larger the circle, indicating that the target correlation is stronger.

67, ASN-99, and TYR-30 in ALB (PDB: 6yg9), as well as residues HIS-60, SER-36, THR-38, and GLU-37 in SRC (PDB: ID 1o4i).

Discussion

CYN tablet, a Chinese proprietary medicine used for the treatment of enteritis and diarrhea, was found to have in vitro antiviral effects against EV71 in this study. As CYN tablets consist of five herbal ingredients, the anti-EV71 activities of the aqueous extracts of the five herbal ingredients were also analyzed. Of the five, only AEMC and AELLF exerted inhibitory effects on the replication of EV71 intracellularly.

Due to the ingredients of TCM being complex, we first analyzed the material basis of the two herbal ingredients (AEMC and AELLF) which have in vitro anti-EV71 activities by HPLC-Q-TOF-MS/MS and then compared the results with the material basis of CYN solution reported previously.[31] In total of 11 components were identified in all three medicines. Two of them, namely, rutin and the intestinal metabolites of ellagic acid, were considered to have inhibitory effects on the replication of EV71.[33,34] Among the components shared by AEMC and CYN solution, AELLF and CYN solution, rosmarinic acid, chlorogenic acid, caffeic acid, and hyperoside were reported to have antiviral effects,[35–38] especially rosmarinic acid, which was proved to inhibit the proliferation of EV71 virus.[39]

In order to know which components played an essential role in in vitro anti-EV71 process, we explored the anti-EV71 mechanism of CYN using network pharmacology and molecular docking.

Firstly, the component-related targets and the disease-related targets were screened. Based on Venn diagram, we obtained 29 targets of components and EV71 in the intersection. Then, the 29 intersection targets were used to construct a PPI network. AKT1, ALB, and SRC were considered core targets as they are closely related to other proteins. In terms of AKT1, the expression of AKT1 was significantly down-regulated in the early stage of EV71 virus infection, which indicates the correlation between AKT1 and EV71 infection.[40] SRC is a typical member of the non-receptor protein tyrosine kinase family, which is involved in a variety of basic functions to maintain cell homeostasis, including regulating

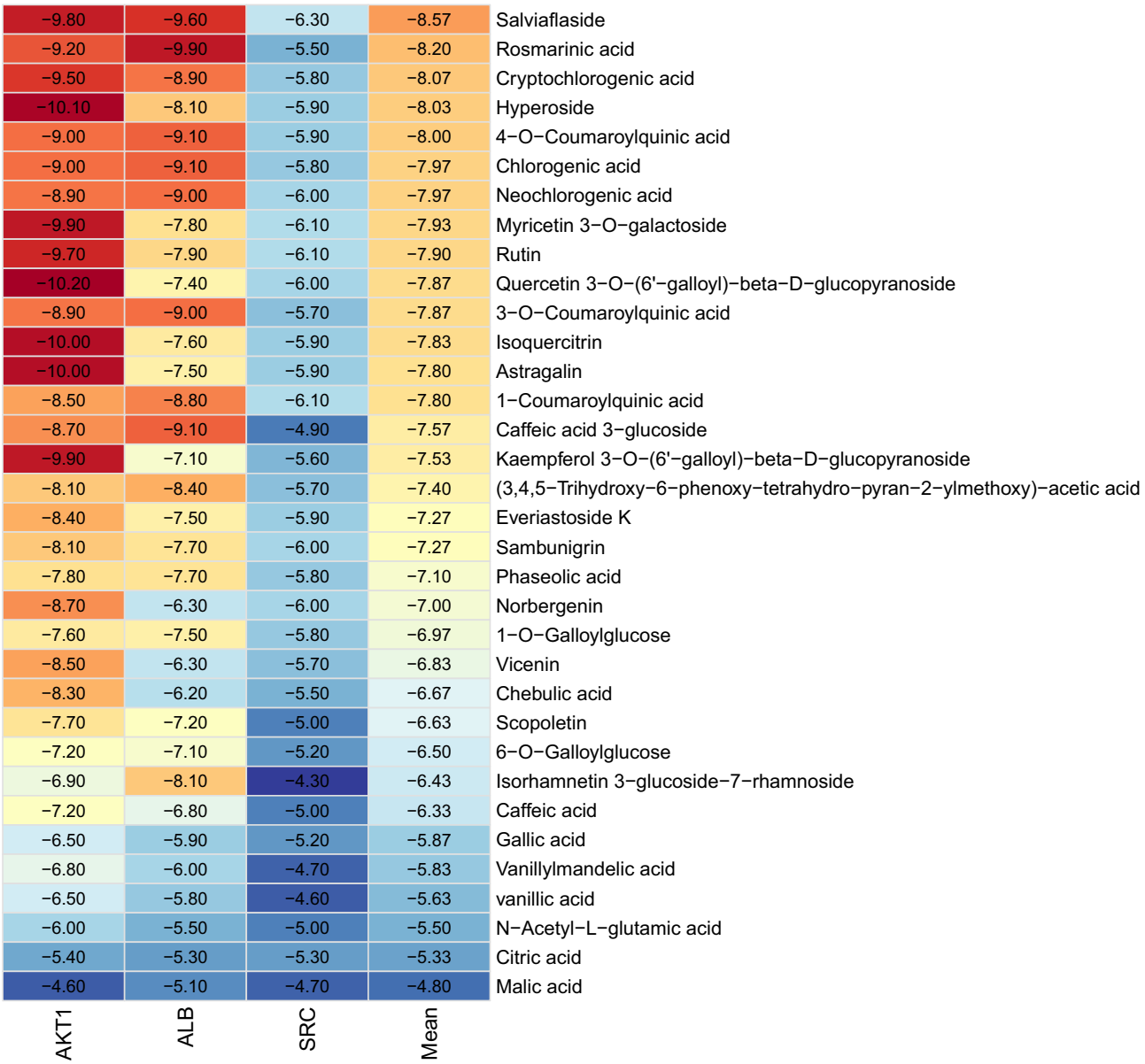

Figure 7 The docking scores of components and core targets. The redder the color, the lower the binding energy between the component and the target, and the component may bind tightly to the target.

cell cycle progression, proliferation, differentiation, survival, and other cellular processes. Studies have found that Src tyrosine kinase inhibitors (STKIs) can be used for the treatment of HIV-1.[41] ALB is produced in the liver and is associated with cell death in the liver.[42] After the identification of core targets, molecular docking was performed to seek the active components of CYN. Combining previous reports and results of molecular docking, nine components were selected for in vitro pharmacodynamic validation. It was found that only rosmarinic acid showed a significant inhibitory effect on EV71 proliferation, which indicated that rosmarinic acid might be the chemical compound mainly responsible for the anti-EV71 activity of CYN tablet.

Rosmarinic acid is an esterification product of caffeic acid and 3.4-dihydroxyphenyl lactic acid, which is widely distributed in the Compositae and Labiatae families. Rosmarinic acid has a variety of biological activities such as antioxidant, anti-inflammatory, antibacterial, antiviral, antitumor, antidiabetic, and neuroprotective.[43] The anti-EV71 effects of rosmarinic acid were recognized in RD cells in previous reports.[44,45] Chung et al put forward that rosmarinic acid could reduce EV71 viral particle production and viral RNA expression in RD cells,[46] which is similar to the results

Table 3 The MNTC of the Nine Components Selected for Pharmacodynamic Verification

No.	Name of Components	MNTC (μg/mL)
1	Neochlorogenic acid	100.00
2	Rutin	200.00
3	Astragalin	200.00
4	Rosmarinic acid	50.00
5	Caffeic acid	50.00
6	Cryptochlorogenic acid	100.00
7	Chlorogenic acid	100.00
8	Hyperoside	200.00
9	Gallic acid	6.25

in this study. Apart from suppressing EV71 replication, rosmarinic acid was considered to inhibit EV71 infection through reducing EV71 viral attachment and entry during the viral absorption stage.[46] In vitro and in vivo studies showed that rosmarinic acid has a protective effect against cytopathic and apoptotic cell death in the early stages of EV71 virus infection.[39] Our results demonstrate that rosmarinic acid could efficiently decrease cell death caused by EV71 infection. After experimental verification, the anti-EV71 effect of rosmarinic acid has been proven by molecular docking. It is generally considered that binding energy less than −7.0 kcal/mol indicates a strong binding activity between the ligand and the receptor.[32] The binding energy between rosmarinic acid and two core targets (AKT1 and ALB) was much lower than −7.0 kcal/mol, indicating it could bind tightly to the target proteins, thereby proving the anti-EV71 activity of rosmarinic acid from the molecular point of view. Besides the anti-EV71 activity, rosmarinic acid was reported to be efficient against a variety of other viruses, including severe acute respiratory syndrome coronavirus, herpes simplex virus, dengue virus, influenza A virus, and hepatitis B virus.[36,47–50] Therefore, rosmarinic acid might be a broad-spectrum antiviral compound.

Another component we are concerned with is rutin, which was identified from both AEMC and AELLF by HPLC-Q-TOF-MS/MS, and was also demonstrated to bind tightly with two of the three core targets (AKT1 and ALB) by

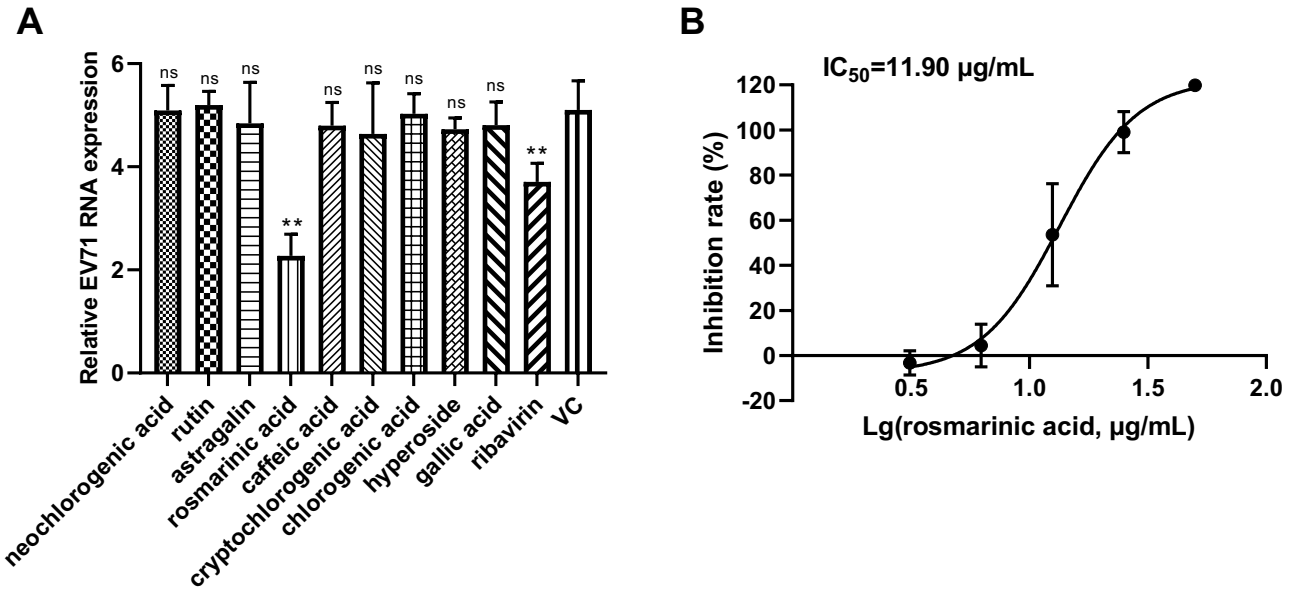

Figure 8 Effects of nine components and ribavirin on the RNA expression of EV71 (**A**), and anti-EV71 effect of rosmarinic acid (**B**). (**A**) RD cells were infected with 0.01 MOI EV71 and treated with nine components or ribavirin (1 mg/mL) for 24 h. qRT-PCR was performed to test EV71 VP1 RNA level. The expression of VP1 transcripts was calculated in relation to the expression level of β-actin mRNA. ns, not significant with $P > 0.05$; **, significant with $P < 0.01$ compared to VC. (**B**) Rosmarinic acid inhibited cell deaths induced by 0.01 MOI EV71.

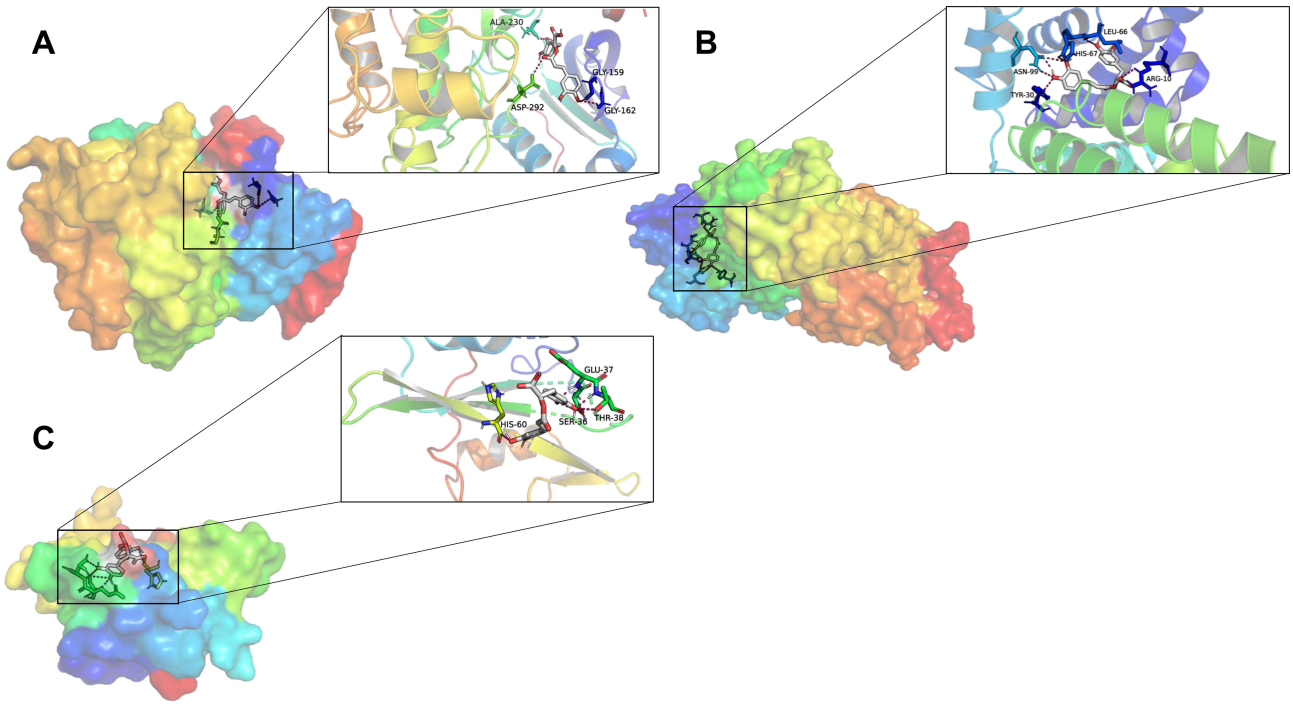

Figure 9 Binding pattern of rosmarinic acid to AKT1 protease(**A**), ALB protease(**B**), and SRC protease(**C**). (**A**) Binding pattern of rosmarinic acid to AKT1. (**B**) Binding pattern of rosmarinic acid to ALB. (**C**) Binding pattern of rosmarinic acid to SRC.

molecular docking. It gave negative results when we verified the anti-EV71 effects through comparing the vRNA level between VC and rutin treated cells, which indicated that rutin could not inhibit EV71 replication by post-infection treatment. However, rutin has been reported to significantly suppress EV71-induced CPE and viral replication on Vero cells when the cells are pretreated with 200 μM of rutin before infection.[33] It could be inferred, therefore, that rutin may exert antiviral effects by reducing viral attachment or entry to cells, but not inhibiting intracellular viral replication.

The limitation of the study is that in vivo anti-EV71 activities of CYN tablets and rosmarinic acid were not assessed because of no animal bio-safety level-2 (ABSL-2) laboratory, which needs to be further studied.

Conclusion

In summary, CYN tablets exerted an in vitro anti-EV71 effect through inhibiting viral replication. Combining the HPLC-Q-TOF-MS/MS with network pharmacology, rosmarinic acid was identified as the effective component with anti-EV71 activities. Our study could provide a reference for the screening and confirmation of antiviral components in Chinese herbal preparation.

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Disclosure

The authors report no conflicts of interest in this work.

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