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Original Article

Broad antiviral and anti-inflammatory activity of Qingwenjiere mixture against SARS-CoV-2 and other human coronavirus infections

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ABSTRACT

Background: Qingwenjiere Mixture (QJM) is a traditional Chinese medicine (TCM) that has been shown to have remarkable clinical efficacy against COVID-19. However, little is known about the antiviral and anti-inflammatory activities of QJM against a wider range of human coronavirus (HCoV) strains.

Purpose: The study aims to investigate the antiviral and anti-inflammatory activities of QJM, as well as the underlying mechanisms against HCoV infections.

Methods: The chemical compositions from QJM were analyzed by LC-MS. The inhibitory effect of QJM on infections of HCoV-OC43, HCoV-229E, HCoV-NL63, and SARS-CoV-2 was evaluated in HRT-18 cells, Huh7 cells, LLC-MK2 cells, and Vero-E6 cells, respectively, by using cytopathic effect (CPE) inhibition assay or RT-qPCR detection of viral \(n_s\) or \(RdRp/Hel\) genes. The expression of pro-inflammatory cytokines induced by HCoV-OC43, HCoV-229E, and SARS-CoV-2, as well as the host \(ace2\) gene was also determined by RT-qPCR assay. Furthermore, the expression of key molecules in the NF-\(\kappa B/\)MAPKs signaling pathways was determined by western blot.

Results: In alcohol-extraction groups of QJM and reference decoction pieces, 53 similar ion peaks were identified, the majority of which were phenylpropanoids, iridoids, and flavonoids. In addition, QJM reduced CPE caused by HCoVs and the expression of viral \(n_s\) genes or N protein. Pretreatment with QJM also exerted inhibitory effect on viral \(n_s\) gene expression. QJM also inhibited the expression of \(RdRp/Hel\) and \(s\) genes of SARS-CoV-2, as well as the host \(ace2\) gene. Besides, QJM markedly reduced virus-induced mRNA expression of a panel of pro-inflammatory cytokines, such as IL-6, CXCL-8/IL-8, CXCL-10/RANTES, TNF-\(\alpha\), IFN-\(\alpha\), CCL-2/MCP-1, CXCL-9/MIG, and IL-1-\(\alpha\). We further showed that QJM inhibited the phosphorylation of NF-\(\kappa B\) p65, and JNK, ERK 1/2, and p38 MAPKs in HCoV-OC43-infected HRT-18 cells.

Conclusions: QJM has broad antiviral and anti-inflammatory activity against both common and newly emerged HCoVs possibly by inhibiting the activation of key components in NF-\(\kappa B/\)MAPKs signaling pathway. QJM also has a prevention effect against HCoV infections and inhibits the host receptor required for virus entry. These results indicate that QJM may have the therapeutic potential in the treatment of diseases caused by a broad range of HCoVs.

Abbreviations: ARB, Arbidol; ARDS, acute respiratory distress syndrome; CC\textsubscript{50}, the 50% cytotoxic concentration; COVID-19, coronavirus disease 2019; CPE, cytopathic effect; DMSO, dimethyl sulfoxide; HCoVs, human coronaviruses; hpi, hours post infection; IC\textsubscript{50}, the 50% inhibition concentration; LC-MS, liquid chromatograph-mass spectrometer; LH, Lianhuaqingwen capsule; MOI, multiplicity of infection; MTT, methyl thiazolyl tetrazolium; N protein, Nucleocapsid protein; QJM, Qingwenjiere Mixture; \(RdRp/Hel\), \(RdRp/Helicase\); RDV, Remdesivir; S protein, Spike glycoprotein; TCM, traditional Chinese medicine; vRNP, viral ribonucleoprotein.

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Introduction

Human coronaviruses (HCoVs) are single-stranded, positive-sense, enveloped RNA viruses that belong to the family Coronaviridae. There are seven strains of coronaviruses (HCoVs) that are known to infect humans. Among them, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and currently circulating SARS-CoV-2 that caused coronavirus disease 2019 (COVID-19) are highly pathogenic Betacoronaviruses and can cause acute respiratory distress syndrome (ARDS), cytokine storms, and even death worldwide. The other common HCoVs which include two Betacoronaviruses (HCoV-OC43 and HCoV-HKU1) and two Alphacoronaviruses (HCoV-229E and HCoV-NL63), do not frequently cause severe disease, but they are related to high rate of morbidity and mortality in young children and elderly people (Brown et al., 2019; Hui et al., 2014; Tian et al., 2020; Yao et al., 2020). The common HCoVs and newly emerging HCoVs share a similar life cycle, but their genomic sequences and pathogenicity are different. Unfortunately, vaccines and pharmacological treatment for HCoV infections are still limited, and symptomatic relief and complication prevention remain the most important management strategy (Zumla et al., 2016). Given the threats posed by circulating and emerging viruses, it is urgent to explore medicines with a broad spectrum of antiviral drugs that target a range of HCoVs.

Antiviral agents, particularly those that target RNA viruses, have been repurposed for antiviral therapy against COVID-19. Several drugs, such as remdesivir (RDV), favipiravir, chloroquine, and arbidol (ARB), efficiently inhibited viral replication in SARS-CoV-2-infected Vero E6 cells and RDV was even considered as the antiviral hope against SARS-CoV-2, however, clinical trials was required to further evaluate the efficacy and safety of these drugs (Lam et al., 2020). Traditional Chinese medicine (TCM) has a long history of clinical use in the treatment of infections caused by respiratory viruses. During the last year, TCM has been widely used (almost 85%) for the treatment of COVID-19 patients in China (Yang et al., 2020). For example, a Chinese patent medicine, Lianhuaqingwen capsule (LH), has been shown to reduce the time to recovery of fever and cough in patients infected with SARS-CoV-2 (Hu et al., 2021). Qingfei paidu decoction (QPD) is another TCM prescription that has been demonstrated to significantly shorten symptom recovery time and increase cure rates without causing adverse effects in over 100 patients infected with SARS-CoV-2 (Chen et al., 2020a).

Qingwenjieri Mixture TQM (QJM) is a TCM formula that is developed based on the theory of prevention and cure of ‘‘plague’’. The main ingredients of this formula are usually prescribed for the treatment of flu-like symptoms, asthma, inflammation, tonsillitis, and sore throat (Lin et al., 2020). Indeed, QJM is effective in reducing fever, sore throat, and cough, as well as decreasing the levels of C-reactive protein (CRP) and hypersecretive C-reactive protein in patient with flu-like symptom (unpublished). During the COVID-19 outbreak, QJM was used in designated hospitals in Yunnan and Hubei Provinces, as well as neighboring countries such as Laos and Myanmar, and demonstrated remarkable clinical efficacy in SARS-CoV-2-infected patients, including reducing CRP level in serum, improving the absorption of pulmonary inflammation, and shortening the length of hospital stay (unpublished). A network pharmacological analysis of QJM has identified 32 putative targets against COVID-19, which were retrieved from both the OMIM and Gene cards data C databases (Zhu et al., 2020). These findings indicated that QJM may be effective against HCoV by alleviating the inflammatory responses.

Infections with HCoVs may result in inflammatory responses that promote viral replication and cause respiratory tissue injury. For example, HCoV infections activated NF-κB signaling pathway by depleting IkBα and promoting p65 chromatin recruitment, and thereby allowed the synthesis of A20 protein (TNFAIP3) essential for effective viral replication (Poppe et al., 2017). Additionally, the generation of dsRNA activated the NF-κB/MAPK pathways, resulting in the production of substantial amounts of pro-inflammatory cytokines, such as CCL-2/MCP-1, IL-10, and CXCL-10/IP-10, which may cause lung tissue damage, edema, and even ARDS (Kono et al., 2008). Furthermore, it has been demonstrated that the MAPK signaling pathway is involved in the development of SARS-CoV-related lung fibrosis (Li et al., 2016). It remains unclear whether QJM could interfere with these pathways, hence inhibiting HCoV replication and excessive pro-inflammatory responses. In this study, we aimed to explore the antiviral and anti-inflammatory effects of QJM against common and newly emerging HCoVs. We also investigated the possible mechanism for the anti-viral and anti-cytokine activities of QJM.

Materials and methods

Drugs

The effects of QJM on various HCoVs were compared with LH, ARB, and RDV. The ingredients of QJM were shown in Table 1. The powdered raw materials of LH was provided by the Institute of Chinese Materia Medica, China Academy of Chinese medical sciences. ARB tablets (0.1 g per tablet) were purchased from CSPC Ouyi Pharmaceutical Co., Ltd, and RDV was provided by Prof. Zifeng Yang from Guangzhou Medical University. For the preparation of drug stock solution, QJM was dissolved in phosphate-buffered saline (PBS) to a concentration of 600 mg/ml, and RDV, LH and ARB were dissolved in dimethyl sulfoxide (DMSO).

Table 1

| Herbs (Chinese Pinyin) | Herbs (Latin name) | Herbs (English name) | Function |
|-----------------------|------------------|---------------------|----------|
| Huoxiang              | Pogostemon cablin | Pogostemonis Herba  | Aromatherapy, resolve damp (Zheng et al., 2013) |
| Chaihu                | Bupleuran chinense DC. | Bupleuri Radix | disperse heat and lift up yang qi (Cheng et al., 2013) |
| Huangqin              | Scutellaria baicalensis Georgi. | Scutellariae Radix | clear heat, relieve toxicity (Chen et al., 2020b) |
| Lianqiao              | Forsythia suspensa (Thunb.) Vahl. | Forsythiae Fructus | clear heat, relieve toxicity (Chen et al., 2020b) |
| Fabansxia             | the processed products of the stem tubers of Pinellia ternata (Thunb.) Breit. | Pinelliae Rhizoma | transform phlegm, descend counterflow (Chen et al., 2020b) |
| Caoguo                | Amomum tsao-ko | Tsakoo Fructus | resolve damp, eliminating phlegm (Li W., 2020) |
| Yinchen               | Artemisia capillaris | Artemisiae Herba | clear damp-heat, treat yang type jaundice (Li, 2020) |
| Matixiang             | Valeriana jatamansi Jones | Valerianae Jatamansi Rhizoma et Radix Talcum | relieve heat, promote urination (Li W., 2020) |
| Huashi                | Glycyrrhiza inflata Bat. | Glycyrrhizeae Radix et Rhizoma | clear heat-protect intestine (Li, 2020) |
| Gancao                | -- | -- | tonifying spleen, clear heat, reconcile all drug (Chen et al., 2020b) |
| Baiwei                | Cynanchum atratum Bunge. | Cynanchi AtratiRadix et Rhizoma | clear heat, cool blood, promote urination (Li, 2020) |
| Chaoshenqu            | -- | Massa Medicata Fermentata | improve digestion (Li, 2020) |
| Chaohoupi             | the processed bark of Magnolia officinalis Rehd. et Wils. var. biloba Rehd. et Wils. | Magnoliae Officinalis Cortex | dispelling phlegm, stop asthma (Chen et al., 2020b) |
to concentrations of 100 mM, 300 and 100 mg/ml, respectively. These solutions were stored at −20 °C before use.

**LC-MS analysis of QJM**

The black powder of QJM and the reference decoction pieces of chaishu (*Bupleurum chinense* DC.), huangquin (*Scutellaria baicalensis* Georgij.), huxiao (*Pogostemon cablin* (Blanco) Bent.), liqiao (*Forsythia suspensa* (Thunb.) Vahl.), matixiang (*Valeriana jatamansi* Jones), baizhi (*Cynanchum atratum* Bunge.), yinchen (*Artemisia capillaris* Thunb.), and chaohoupu (*Magnolia officinalis* Rehd. et Wils. var. *biliba* Rehd. et Wils.) were provided by Chinese medicine hospitals in Yunnan Province, and were respectively extracted with 95% EtOH (3 times) at room temperature for 24 h each time. The filtrate was subsequently concentrated under vacuum to yield a crude extract. An appropriate amount of crude extract was dissolved in water or MeOH and filtered before being analyzed by using a liquid chromatograph-mass spectrometer (LC-MS). The ion peaks (or quasi-molecular ion peaks) with the same retention time (tR) and mass-to-charge ratio (m/z) in QJM and a certain decoction piece were identified. Then, the possible chemical composition of the ion peaks was determined by reviewing the literature and compared with the reference decoction pieces.

**Cell lines and virus**

The human rectal carcinoma cell line HRT-18 (ATCC CCL-244) was cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, HyClone, USA). The rhesus monkey kidney epithelial cell line LLC-MK2 (ATCC CCL-7), the African green monkey kidney epithelial cell line Vero E6 (ATCC CCL-81), and human hepatocellular carcinoma cell line Huh-7 (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA). All cells were cultured in medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, USA). HCoV-OC43 (VR-1558), HCoV-229E, and HCoV-NL63 were gifted from Prof. Jincun Zhao (Guangzhou Medical University). HCoV-OC43, HCoV-229E, and HCoV-NL63 or SARS-CoV-2 at a multiplicity of infection (MOI) of 1. After adsorption at 34 °C for 2 h, the cells were rinsed twice with PBS and incubated with drug-containing culture medium. The supernatants of Huh7 and HRT-18 cells were collected at 72 and 120 h post infection (hpi), respectively, and the copy numbers of viral genes were determined by RT-qPCR assay.

Pretreatment and treatment experiments

For pretreatment experiment, confluent monolayer of Huh7 and HRT-18 cells grown in 12-well plates was incubated with the medium containing QJM at 2000, 1000, 500, and 250 μg/ml for 0, 2, and 6 h, which was followed by the infection with HCoV-229E and HCoV-OC43, respectively, at a multiplicity of infection (MOI) of 1. After adsorption at 34 °C for 2 h, the cells were rinsed twice with PBS and incubated with drug-containing culture medium. The supernatants of Huh7 and HRT-18 cells were collected at 72 and 120 h post infection (hpi), respectively, and the copy numbers of viral genes were determined by RT-qPCR assay.

**Cytotoxicity assay**

The cytotoxic effects of drugs on HRT-18, Huh7, LLC-MK2, and Vero E6 cells were measured by methyl thiazolyl tetrazolium (MTT) assay. Briefly, cell monolayer grown in 96-well plates was incubated with indicated concentrations of drugs. After 48 h, the cells were stained with MTT solution at 1 mg/ml at 37 °C for 4 h. The supernatants were then discarded, and 150 μl dimethyl sulfoxide (DMSO) was added to the each well to dissolve formed formazan crystals in cells. The absorbance at 490 nm was measured by using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher, USA). The 50% cytotoxic concentration (IC50) of drugs to cells was calculated by the Reed-Muench method.

**Western blot assay**

HRT-18 cells were seeded into 6-well plates at a density of 2 × 10^5 cell/well. After growing into monolayer, the cells were infected with HCoV-OC43 (MOI = 1) for 2 h. The inoculums were removed, and cells were treated with different concentrations of QJM. At 96 hpi, total protein was extracted for western blot analysis as mentioned in our previous study with the modification of using 10% SDS-PAGE and PVDF membranes (0.2 μm, Bio-Rad, USA) (Dong et al., 2019). Membranes were probed with the antibodies against n, RdRp/Hel genes and cytokines/chemokines were shown in supplementary Table 1.
were incubated overnight at 4 °C with antibodies against P-Ser536-NF-kB p65, p65, P-ERK1/2, ERK1/2, P-p38, p38, P-SAPK/JNK, SAPK/JNK, GAPDH (Cell Signaling, USA), HCoV-OC43 (Millipore, USA, MAB9012), followed by an HRP-conjugated secondary antibody. The immunocomplexes were detected using enhanced chemiluminescence (ECL, Amersham, USA) and quantified by Image J software.

Data analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) (IBM SPSS statistics 21.0, USA). All results were the representative or the average data from at least 3 independent experiments. All the numerical results were presented as mean ± standard error of the mean (SEM). Significance was considered when p < 0.05.

Results

LC-MS analysis of alcohol-extraction group of QJM

To better understand the chemical composition of QJM, samples of QJM were analyzed by UPLC fingerprints. The water- and alcohol-extraction groups of the powdered QJM and 8 compatible decoction pieces were analyzed by LC-MS. When the retention time and ion flow data were combined, few ion peaks were yielded from the water-extraction group, while 53 common ion peaks were identified in the alcohol-extraction group of QJM powder and the reference decoction pieces (Fig. 1 and Supplementary Figures). The possible chemical constituents of these peaks were further determined, and 44 phenylpropanoids, 36 iridoids, 13 flavonoids, 8 steroids, 6 sesquiterpenoids, 5 triterpenoids, 4 furanones, 4 aromatic derivatives, and 12 additional chemical compounds were identified (Supplementary Table 2). The majority of compounds were derived mostly from cycloditerpenes and their degradation products, as well as from biphenyleolignans of matixiang (Valeriana jatamansi Jones) and chaohoupu (the processed bark of Magnolia officinalis Rehd. et Wils. var. biloba Rehd. et Wils) (Supplementary Figs. 1, 2 and Supplementary Table 2). Flavonoid triterpenes and triterpene saponins were the main chemical constituents from chainhu (Bupleurum chinense DC.) (Supplementary Fig. 3 and Supplementary Table 2). Flavonoids and Guaiacane sesquiterpenes were the main chemical constituents from huangqin (Scutellariae baicalensis Georgi.) (Supplementary Fig. 4 and Supplementary Table 2) and huoxiang (Pogostemon cablin (Blanco) Bent.) (Supplementary Fig. 5 and Supplementary Table 2), respectively. The components from lianqiao (Forsythia suspensa (Thunb.) Vahl.) included cyclohexanol glycoside, lignans, and triterpenes (Supplementary Fig. 6 and Supplementary Table 2). The components from lianqiao (Forsythia suspensa (Thunb.) Vahl.) included cyclohexanol glycoside, lignans, and triterpenes (Supplementary Fig. 6 and Supplementary Table 2). The C21 steroidal saponins and aromatic compounds were main constituents from baiwei (Cynanchum atratum Bunge.) (Supplementary Fig. 7 and Supplementary Table 2). Few components from yinchen (Artemisia capillaris Thunb.) were found (Supplementary Fig. 8 and Supplementary Table 2).

QJM inhibits the infection of common HCoVs at nucleotide or protein levels

The antiviral effects of QJM against HCoVs were investigated and
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Table 2
Antiviral activity of drugs against HCoVs in vitro.

| Medicine | Cell strain/ Virus type | CC<sub>50</sub> ± SEM (μg/ml) | IC<sub>50</sub> ± SEM (μg/ml) | SI ± SEM |
|----------|-------------------------|-----------------------------|-----------------------------|---------|
| QJM (μg/ml) | HRT-18/ OC43-1558 | 12,179.12 ± 74.17 | 298.34 ± 8.69 | 46.88 ± 1.07 |
| Huh7/ HCoV-229E | 6534.38 ± 74.04 | 54.04 ± 4.23 | 120.66 ± 4.80 |
| LLC-MK2/ NL63 | 11,617.78 ± 43.39 | 118.07 ± 3.60 | 93.68 ± 1.03 |
| Vero/E6/ SARS-CoV-2 | 22,411.36 ± 21.81 | 388.27 ± 4.72 | 57.72 ± 0.34 |
| Arbidol (μg/ml) | HRT-18/ OC43-1558 | 11.57 ± 0.33 | 2.86 ± 0.18 | 4.06 ± 0.15 |
| Huh7/ HCoV-229E | 9.68 ± 0.14 | – | – |
| LLC-MK2/ NL63 | 34.57 ± 0.50 | – | – |
| Vero/E6/ SARS-CoV-2 | 17.44 ± 0.40 | 1.33 ± 0.08 | 14.27 ± 1.42 |
| LH (μg/ml) | HRT-18/ OC43-1558 | 1033.59 ± 11.75 | 366.05 ± 11.75 | 2.83 ± 0.06 |
| Huh7/ HCoV-229E | 1330.84 ± 21.28 | 349.94 ± 19.50 | 3.82 ± 0.20 |
| LLC-MK2/ NL63 | 4167.53 ± 17.68 | 354.37 ± 29.20 | 11.92 ± 0.97 |
| Vero/E6/ SARS-CoV-2 | 2215.00 ± 14.53 | 163.81 ± 14.53 | 13.84 ± 1.45 |

† The CC<sub>50</sub> (50% cytotoxic concentration) of drugs to cells was determined by MTT assay.

§ The IC<sub>50</sub> (50% inhibitory concentration) of drugs on HCoV-NL63 was evaluated using RT-qPCR detection of viral gene from the supernatant, while the IC<sub>50</sub> for other HCoVs was determined by CPE assay.

‡ The SI (selectivity index) was calculated as the ratio of CC<sub>50</sub> to IC<sub>50</sub>.

Compared to that of LH and ARB, LH and ARB were both effective against influenza virus infection, and recent studies showed that these two drugs could inhibit SARS-CoV-2 replication (Nojomi et al., 2020; Yang et al., 2020). In this study, the cytotoxicity of these drugs was firstly determined by MTT assay, and the CC<sub>50</sub> value of QJM in HRT-18, Huh7, and LLC-MK2 was 12,179.12 ± 74.17, 6534.38 ± 74.04, and 11,617.78 ± 43.39 μg/ml, respectively (Table 2). The infections of HCoV-OC43 and HCoV-229E induced obvious CPE, which was inhibited by QJM treatment with IC<sub>50</sub> values of 298.34 ± 8.69 and 54.04 ± 4.23 μg/ml, respectively. QJM appeared to be more effective than LH in inhibiting these two viruses, as evidenced by lower IC<sub>50</sub> values (Table 2).

In addition, ARB was effective against HCoV-OC43 but not against HCoV-229E (Table 2). The IC<sub>50</sub> values of QJM and LH against HCoV-NL63 at 7 dpi were 118.07 ± 3.60 and 354.37 ± 29.20 μg/ml, respectively (Table 2). However, ARB was ineffective in inhibiting the n gene of this virus. Furthermore, QJM showed higher SI values than LH and ARB, which suggests that QJM may be more effective and safer in the treatment of HCoVs (Table 2).

We further investigated the influence of QJM on the expression of mRNA or protein of an Alphacoronavirus, HCoV-229E, and a Betacoronavirus, HCoV-OC43. Briefly, viral RNA was extracted from the supernatant of virus-infected cells treated with or without drugs, and the expression of n gene was determined by RT-qPCR assay. We demonstrated that LH, ARB, and all concentrations of QJM effectively inhibited the n gene expression of HCoV-229E at 48 hpi and HCoV-OC43 at 72 hpi, and the inhibition decreased at the following time points (Fig. 2 Aa, b). Furthermore, QJM, LH, and ARB significantly reduced the expression level of N protein of HCoV-OC43 detected by western blot assay (Fig. 2 Ca, h). These results indicated that QJM inhibited common HCoV replication at nucleic acid or protein level.

Given that many Chinese medicines were more effective when used prophylactically (Xu et al., 2012), we next evaluated the inhibitory effect of QJM pretreatment on the viral n gene expression. The drugs were added to cells at 6, 2, and 0 h prior to infection, as well as 2 h following virus incubation, and the viral n gene was detected at 120 hpi (HCoV-OC43) or 72 hpi (HCoV-229E). The results showed that LH, ARB, and QJM effectively inhibited the viral gene expression when added to cells at all time points before viral infection (Fig. 2 B). Interestingly, the inhibition rate of QJM was significantly higher in the pretreatment assay than in the treatment assay (Fig. 2 B).

**Antiviral effect of QJM against SARS-CoV-2**

Besides common HCoVs, the effects of QJM against the newly emerging SARS-CoV-2 were further investigated and compared to those of LH, ARB, and RDV (Nojomi et al., 2020; Sreekanth Reddy and Lai, 2021; Yang et al., 2020). Despite less effective in inhibiting SARS-CoV-2-induced CPE, QJM had a higher SI value than LH and ARB (Table 2). QJM could also significantly inhibit the expression of viral RdRp/Helicase gene in supernatant of infected cells (Fig. 2 Ac). It was reported that the entry process of SARS-CoV-2 was mediated by the binding of envelope-embedded surface-located spike (S) glycoprotein to the entry receptor ACE2 from humans and other species (Liu et al., 2015). We therefore investigated the effect of QJM on both viral s and host ace2 genes by using RT-qPCR assay. The results showed that QJM was more potent than RDV, LH, and ARB in inhibiting the mRNA expression levels of these two genes (p < 0.05, p < 0.01, p < 0.001) (Fig. 2b, c). These results indicated that QJM was effective in suppressing not only the viral gene but also the host factor associated with SARS-CoV-2 infection.

QJM inhibits the expression of cytokine/chemokine induced by HCoV infections

Aberrant immune responses characterized by excessive production of pro-inflammatory mediators and increased recruitment of immunocytes could accelerate the disease process of HCoV infections (Tortura and Baric, 2012; Yamaya et al., 2020). Therefore, we investigated whether QJM has the inhibitory effect on the expression of pro-inflammatory mediators in HCoV-infected cells. As shown in Fig. 3 A and B, infection with HCoV-OC43 and HCoV-229E dramatically increased the mRNA levels of IL-6, TNF-α, CXCL-8/IL-8, IFN-α, CCL-2/MCP-1, and CXCL-9/MIG, while QJM treatment exhibited different degree of inhibition on the expression of these cytokines and chemokines in a dose-dependent manner (p < 0.001). Furthermore, 500 μg/ml of QJM was more effective than ARB in reducing levels of CXCL-8/IL-8 and CXCL-9/MIG and was also superior to LH in suppressing the level of CXCL-8/IL-8 (p < 0.001) in HCoV-OC43-infected cells (Fig. 3 Ac, h). In HCoV-229E-infected cells, QJM at high concentration (1000 μg/ml) had the anti-inflammatory effect comparable to LH, while QJM at low concentration (250 μg/ml) was generally less effective than LH but better than ARB (Fig. 3 B).

Moreover, the mRNA levels of IL-1α and CCL-5/RANTES were significantly upregulated at 48 hpi in Vero E6 cells infected with SARS-CoV-2 (p < 0.001), which was remarkably reduced to varying degrees by the treatment of QJM, LH, ARB, or RDV (p < 0.001) (Fig. 3 C). Furthermore, QJM at 500 or 250 μg/ml was more effective than RDV and LH in reducing the level of IL-1α (p < 0.05, p < 0.001) (Fig. 3 Ca), and QJM at 250 μg/ml also showed superior inhibition on the expression of CCL-5/RANTES as compared with LH (p < 0.001) (Fig. 3 C). These results indicate that QJM could have the potential to attenuate HCoV-induced pro-inflammatory responses.

QJM inhibits the activation of NF-κB p65 and p38/ERK/JNK MAPKs stimulated by HCoVs

HCoV infections can trigger the activation of diverse cellular signaling pathways, such as the canonical NF-κB pathway and MAPK pathway, which regulates the downstream production of pro-inflammatory cytokines (De Wilde et al., 2018). Given that QJM potentially inhibited the production of multiple cytokines, we next...
examined whether the anti-cytokine activity of QJM was related to its impact on the expression of key components in cellular pathways. As expected, infection with HCoV-OC43 for 96 h resulted in significant phosphorylation of p38, NF-κB p65, SAPK/JNK, and ERK 1/2 in HRT-18 cells, which was markedly inhibited by the treatment of QJM, LH, or ARB (p < 0.05, p < 0.001) (Fig. 4A, B). In addition, QJM was more effective or had a broader range of effective concentrations in inhibiting P-p38 and P-p65 than P-SAPK/JNK and P-ERK1/2 (Fig. 4B). These results indicate that QJM inhibits the cytokine production possibly by interrupting with the activation of NF-κB p65 and p38/ERK/JNK MAPK signaling pathways induced by HCoV-OC43.
Discussion

The ongoing outbreak of COVID-19 has made clear that efficient remedies for life-threatening coronavirus infections are definitely lacking. TCM has been proved to be effective against newly emerged HCoV outbreaks. However, previous studies mainly focused on the effect of TCM in treating specific HCoV strain, and our understanding of the antiviral mechanism remains limited (Abd El-Aziz and Stockand, 2020; Cai and Gu, 2020). In this study, we showed that QJM treatment inhibited the viral load of various HCoV strain in vitro. Interestingly, pretreating cells with QJM resulted in higher inhibition rates on virus replication than treating cells after virus incubation. These results partially support a role for QJM in preventing the entry of HCoVs into the host. It has been shown that the entry of HCoVs was facilitated by the binding of S protein to host receptors, which determined the virus tissue tropism (Petersen et al., 2014; De Wilde et al., 2018). The inhibition of SARS-CoV-2 s gene and host ace2 gene suggests that QJM may target not only the viral gene but also target the host factor required for HCoV entry.

In previous studies, TCMs such as LH, QPD, Xuebijing injection, Jinhua Qinggan granules presented remarkable effects in clinical treatment of COVID-19, and network pharmacology analysis revealed that the active ingredients of these drugs could directly act on viral proteins and exhibit anti-inflammatory and immunoregulatory effects against HCoVs infection (Huang et al., 2020b). QJM is a formula composed of 13 decoction pieces with intricate chemical composition. According to the TCM theory, the benefits of the combination of these herbs include clearing damp-heat, lifting up yang qi, reducing toxicity, eliminating...
In our study, flavonoids were the main chemical compounds in QJM and 8 reference decoction pieces by using LC-MS analysis, with phenylpropanoids, iridoids, and flavonoids being the 3 most common constituents. Chlorogenic acid is a phenylpropanoid that has been identified in Chaihu (*Bupleurum chinense* DC) and Yinchen (*Artemisia capillaris* Thum). Chlorogenic acid has been reported to be the main active ingredient in Xuebijing injection and was believed to have an anti-inflammatory effect against SARS-CoV-2 (He et al., 2020). However, the effects of iridoids on HCoV infection were barely reported (Gong et al., 2008). In addition, flavonoids extracted from many herbs were capable of inhibiting the replication of HCoVs (Gong et al., 2008). In general, the antiviral and anti-inflammatory activities of QJM were in line with the pharmacological activities of these chemical constituents identified in decoction pieces, which may provide a better understanding of the effect and mechanism of QJM in the treatment of HCoV infection.

The infection with HCoV could result in overproduction of cytokines and chemokines, which contributes to the disease severity. Patients with SARS-CoV-2 infection who required ICU admission had higher concentrations of CSF3/G-CSF, CXCL-10/IP-10, CCL-2/MCP-1, CCL-3/MIP-1α, and TNF-α than non-ICU patients (Huang et al., 2020a). In a previous study, network pharmacological analysis identified 32 potential targets between QJM and COVID-19, including IL-6, TNF, and CCL-2/MCP-1, which was consistent with our finding that QJM suppressed the mRNA expression of these cytokines (Zhu et al., 2020). It has also been reported that disruption of the CCL5-CCR5 axis could have the potential to reduce inflammation and viral load in terminally-ill, critical COVID-19 patients (Patterson et al., 2020). In the present study, QJM significantly reduced the mRNA expression of CCL-5/RANTES in SARS-CoV-2-infected Vero E6 cells, indicating that it may restore the immunologic dysfunction and reduce viral burden in COVID-19 patients. Besides, suppressing IL-1 released by damaged epithelial and endothelial cells in response to SARS-CoV-2 infection has been suggested another host-directed therapy for COVID-19 (Van de Veerendonk and Neetea, 2020). The reduction of IL-1α expression by QJM may add to our understanding of its anti-inflammatory activity in the treatment of COVID-19.

Cellular signaling cascades are involved in the network of virus-host interaction and act as a double-edged sword. The activation of NF-κB and/or MAPKs signaling pathways could result in the production of pro-inflammatory cytokines such as CCL-2/MCP-1, CXCL-10/IP-10, IFN-α and CXCL-8/IL-8, which further recruited white blood cells to defeat viral infection (Bonizzi and Karin, 2004; Mubarak and Alturaiki, 2019). However, continued activation of the signaling pathway by pro-inflammatory cytokines, as well as signaling pathway-regulated cytokine production, could intensify the feed forward loop (Bonizzi and Karin, 2004), resulting in cytokine storm and the resultant severe tissue injury in HCoV-infected patients (Wang et al., 2020). Apart from their role in inflammation, prior research established that the recruitment of p65 chromatin was required for a step after N protein production, as well as the development of host A20 protein, both of which were required for efficient virus replication (Poppe et al., 2017). Additionally, increased expression of MAPKs pathways family members, such as p38, ERK1/2 and JNK, would promote the synthesis of viral nonstructural proteins (NSPs) protease, which could bind to N protein, tether the viral genome to the replicase-transcriptase complex (RTC), and further package the encapsidated genome into viral particles (Li et al., 2016; Mubarak and Alturaiki, 2019; Varshney and Lal, 2011; Wang et al., 2020). It has also been demonstrated that p38 kinase facilitated the replication of HCoV-229E, Murine Coronavirus (BHV), and MERS-CoV...
(Hemmat et al., 2021). Therefore, it is possible that QJM treatment may help control of cytokine storm associated with HCoV infections by inhibiting the activation of the signal cascades. On the other hand, the suppression of p38, JNK and ERK MAPKs by QJM may alter the intracellular environment, making it less favorable to viral multiplication. However, the limitation of our study is that we did not evaluate whether these putative targets contribute to the pharmacological activities of QJM. It is important to use agonists of P-p65, P-p38, P-ERK, and P-SAPK/JNK following QJM treatment to determine the antagonistic effects on the antiviral and/or anti-cytokine activity of QJM, which will allow for the validation of the molecules that QJM utilizes to inhibit viral replication or cytokine production. In general, these results provide a primary understanding of the possible mechanism of QJM against innate immune response in HCoV infections.

Conclusion

In conclusion, the current study demonstrates that QJM exerts a broad spectrum of antiviral and anti-inflammatory activities against both common and newly emerged HCoVs by interfering with the activation of NF-κB/MAPKs pathways. In addition, QJM has a prophylactic effect against HCoVs and inhibits the host receptor required for virus entry. All these findings may contribute to a better understanding of the clinical application of QJM.

Ethic statement

All experiments and protocols in this study were reviewed and approved by the Institutional Ethical Committee of Kunming University of Science and Technology, and the Institutional Ethical Committee of Guangzhou Medical University.

CRediT authorship contribution statement

Peifang Xie: Writing – original draft, Investigation, Data curation. Yue Fang: Investigation, Validation, Writing – review & editing. Zhi Chen: Data curation. Yulan Shao: Data curation. Qinhai Ma: Investigation, Validation. Zifeng Yang: Resources, Funding acquisition. Jinzuo Zhao: Resources. Hongmei Li: Investigation. Rongtao Li: Investigation. Shuwei Dong: Conceptualization, Methodology, Writing – review & editing. Weibo Wen: Conceptualization, Resources, Supervision. Xueshan Xia: Conceptualization, Methodology, Project administration, Funding acquisition, Supervision.

Declaration of Compering Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153808.

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