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# The crude extract from the flowers of *Trollius chinensis* Bunge exerts anti-influenza virus effects through modulation of the TLR3 signaling pathway

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ABSTRACT

*Ethnopharmacological relevance:* The flowers of *Trollius chinensis* Bunge (Ranunculaceae) is a traditional Chinese medicine used to treat various inflammatory diseases, including upper respiratory infections, chronic tonsillitis, and pharyngitis. Recently, there has been growing research on the antiviral role of the flowers of *T. chinensis* Bunge. However, little is known about its anti-influenza virus effects and the underlying mechanisms.

*Aim of the study:* This study aims to evaluate the therapeutic effects of the crude extract from the flowers of *T. chinensis* Bunge (CEFTC) on mice infected with influenza virus. We further explored its mechanism by detecting the expression of vital proteins (TLR3, TBK1, TAK1, IKKα, IRF3, and IFN-β) related to TLR3 signaling pathway.

*Materials and methods:* Mice were infected with influenza A virus (H1N1) through the nasal cavity and were intragastrically administered CEFTC at the dose of 0.2 mg/g once daily. The therapeutic effects of CEFTC were evaluated by blood cell count, lung index, spleen index, alveolar lavage fluid testing, and HE staining. Network pharmacology analysis predicted the potential signaling pathway between the flowers of *T. chinensis* Bunge and pneumonia. The expression of TLR3, TBK1, TAK1, IKKα, IRF3, and IFN-β in lung tissues were examined by Western blot assay. In addition, the immunofluorescence assay was applied to assess the effect of CEFTC on the distribution of IRF3 and IFN-β between nuclei and cytoplasm.

*Results:* Compared with the infected group, the lung index was markedly reduced, and the pathological damage of the lungs was also attenuated in the CEFTC treatment group. The network pharmacology analysis indicated that the NF-κB pathway was a potential signaling pathway in the flowers of *T. chinensis* Bunge for the treatment of pneumonia, TLR3, IRF3, and TBK1 were crucial targets associated with pneumonia. Western blot assay demonstrated that in the high-dose virus infected group, CEFTC reduced the expression of TLR3, TAK1, TBK1, and IRF3. Furthermore, CEFTC could increase the nuclear distribution of IRF3 in alveolar epithelial cells after virus infection.

*Conclusions:* These results suggested that different doses of influenza virus could cause varying infection symptoms in mice. Moreover, CEFTC could exert anti-influenza virus effects by regulating the expression of TLR3, IRF3, IFN-β, TAK1, and TBK1 in the TLR3 signaling pathway.

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**Abbreviations**

| | |
|---|---|
| CEFTC | the crude extract from the flowers of *Trollius chinensis* Bunge |
| TLR3 | Toll-like receptor 3 |
| IRF3 | interferon regulatory factor 3 |
| PRRs | pattern recognition receptors |
| PAMPs | pathogen-associated molecular patterns |
| DAPI | 4′,6-diamidino-2-phenylindole |
| TBK1 | TANK binding kinase 1 |
| IFN-β | interferon beta |
| NF-κB | nuclear factor kappa B |
| RIPA | radio immunoprecipitation assay |
| TTD | Therapeutic Target Database |
| TCMSP | Traditional Chinese Medicine System Pharmacology |
| FITC | fluorescein isothiocyanate |
| SIRT1 | NAD-dependent protein deacetylase sirtuin-1 |
| PTGS2 | prostaglandin G/H synthase 2 |
| AKT1 | RAC-alpha serine/threonine-protein kinase |
| MMP9 | matrix metalloproteinase 9 |
| TRIF | Toll-interleukin-1 receptor domain-containing adapter-inducing interferon-β |
| TAK1 | transforming growth factor-β activated kinase 1 |
| IKKα | inhibitor of kappa B kinase alpha |
| $LD_{50}$ | median lethal dose |
| TLRs | Toll-like receptors |
| RIPK1 | receptor-interacting serine/threonine-protein kinase 1 |
| $TCID_{50}$ | Tissue Culture Infective Dose 50% |
| IFNs | interferons |
| TIR | toll-interleukin-1 receptor |
| dsRNA | double-stranded RNA |
| BCA | bicinchoninic acid |
| PVDF | polyvinylidene difluoride |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| ECL | enhanced chemiluminescence |
| HPLC | high-performance liquid chromatography |
| EGFR | epidermal growth factor receptor |
| PPARG | peroxisome proliferator-activated receptor gamma |
| TRAF6 | TNF receptor-associated factor 6 |

## 1. Introduction

Influenza is an acute respiratory infectious disease caused by the influenza virus. Due to its rapid spread and high contagiousness, influenza has become one of the threats to public health. According to data analysis, there are approximately 290,000–650,000 deaths worldwide each year due to seasonal influenza, which is higher than the value estimated by the World Health Organization (Iuliano et al., 2018). At present, vaccination can be used as the primary method to prevent influenza. Together with vaccines, antiviral drugs such as adamantanes and NA inhibitors play a vital role in the prevention and treatment of influenza virus infections (Krammer et al., 2018). However, in recent years, the clinical application of some anti-influenza drugs has been limited due to the drug resistance caused by widespread use (Wu et al., 2017). In addition, influenza viruses are susceptible to mutation, resulting in a reduction in the effectiveness of influenza vaccines, so the vaccine needs to be continually updated to improve its protective effect (Lopez and Legge, 2020; Nuwarda et al., 2021). Therefore, there is an urgent need to research new anti-influenza drugs for clinical application.

The innate immune response is the first defense to prevent viral infection. After influenza virus infection, host pattern recognition receptors (PRRs) can trigger the innate immune response by recognizing pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are common PRRs, which can lead to the activation of innate immune signaling (Chen et al., 2018; Duan et al., 2022; Malik and Zhou, 2020). As an important member of TLR family, TLR3 has been proved to be an essential PRR involved in the regulatory response to viruses (Jung and Lee, 2020). The TLR3 signaling pathway is utterly mediated by the toll-interleukin-1 receptor (TIR) domain-containing adapter-inducing interferon-β (TRIF), which leads to the activation of interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-κB), thus mediating the production of type I interferons (IFNs), proinflammatory cytokines, and chemokines (Chen et al., 2021). Double-stranded RNA (dsRNA) is a molecular feature of most viruses during proliferation and can be produced as an intermediate product of virus replication. TLR3 recognizes dsRNA and activates interferon regulatory factors through a TRIF-dependent pathway, mediating the host antiviral immune response (Biondo et al., 2019). Traditional Chinese medicines have been reported to exert antiviral effects by modulating the TLR3 signaling pathway, such as Huang Lian-Hou Pu Combination (Zhang et al., 2021), Mosla chinensis Maxim (Shi Xiang Ru) (Zhang et al., 2018), and Astragalus Membranaceus (Huang Qi) (Y. Liang et al., 2019).

Compared with chemical drugs, traditional Chinese medicine used for antiviral treatment has the advantages of low drug resistance and fewer adverse reactions, which provides a new direction for developing new antiviral drugs (Yin et al., 2021). The flowers of *T. chinensis* Bunge was a traditional Chinese medicine, first recorded in "Bencao Gangmu Shiyi" as "bitter in taste, cold in nature, non-toxic, mainly used for heat-clearing and detoxicating" (J.W Liang et al., 2019). Studies have shown that total flavonoid from the flowers of *T. chinensis* Bunge has anti-influenza virus effects and can be used as a potential antiviral drug in the clinical treatment of influenza (Liu et al., 2018). Here we observed the anti-influenza virus effects of CEFTC and investigated its mechanism, expecting to provide scientific evidence for the development and application of antiviral drugs in the future.

## 2. Material and methods

### 2.1. Materials and instruments

The mouse influenza A virus strain A/FM/1/47 (H1N1) was provided by the Institute of Virology, Chinese Academy of Preventive Medicine. In our laboratory, Tissue Culture Infective Dose 50% ($TCID_{50}$) of influenza virus was measured by the Reed and Muench method. In this study, the median lethal dose ($LD_{50}$) of influenza virus used to infect mice was $10^{4.5}$ $TCID_{50}$/0.05 ml. The hematoxylin-eosin (HE) staining kit, radio immunoprecipitation assay (RIPA), and bicinchoninic acid (BCA) protein assay kits were purchased from Beijing Solarbio Science & Technology Co. Ltd (Beijing, China). Wright's-Giemsa Staining Solution was produced by Beijing Chemical Works. The primary antibodies used in the experiment included TLR3 (Novus, Littleton, CO, USA), IRF3 (Santa Cruze, Dallas, Texas, USA), transforming growth factor-β activated kinase 1 (TAK1, Bioss, Beijing, China), interferon beta (IFN-β, Bioss, Beijing, China), TANK binding kinase 1 (TBK1, Boster, Wuhan, China), inhibitor of kappa B kinase alpha (IKKα, Boster, Wuhan, China) and β-Tubulin (Biodragon Immunotechnologies, Beijing, China). The secondary antibodies used in this research were supplied by Zhongshan Golden Bridge Bio-technology Co. Ltd (Beijing, China). The laser confocal microscope was produced by Olympus (Nishi-Shinjuku, Shinjuku-Ku, Japan). The Mini PROTEAN® Tetra Cell vertical electrophoresis system and Trans-Biot® SD Cell were all products of Bio-Rad (Hercules, California, USA). The BHC–1300II1/B3 biosafety cabinet produced by SUZHOU ANTAI AIRTECH Co. Ltd (Jiangsu, China) was

also an essential instrument in the research.

### 2.2. Preparation of the CEFTC

The flowers of *T. chinensis* Bunge (lot: 20110901) were purchased from the herbal market in Anguo, Hebei province, also known locally as "Jin Lian Hua." The flowers were collected in July, during which time they bloomed most luxuriantly. A voucher specimen was deposited in the herbarium of the School of Life Sciences, Beijing University of Chinese Medicine. 250 g *T. chinensis* Bunge was extracted twice with 12 times the amount of distilled water for 2 h. The extracted liquid was then filtered, concentrated, and freeze-dried. Finally, a total of 95.5 g CEFTC was obtained (Liu et al., 2020). Each gram of *T. chinensis* Bunge was equivalent to 0.382 g CEFTC. According to the records on the oral dose of *T. chinensis* Bunge in "Zhong Hua Ben Cao," 4 g/d could be used as the standard dose for human oral administration, which was within the range of clinical dose. So, the daily dose of CEFTC should be 1.528 g/d for human. The final dose of CEFTC given to mice was 0.2 mg/g/d, which was the equivalent dose converted according to the dose of CEFTC for humans. CEFTC was dissolved in distilled water to a concentration of 20 mg/ml for intragastric administration.

### 2.3. Animal grouping

Male and female ICR mice (30 in total, 14–16 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd (SCXK (Jing) 2011-0004). The mice were randomly divided into 6 groups: uninfected group, CEFTC group, high-dose infected group, high-dose infected + CEFTC group, low-dose infected group, and low-dose infected + CEFTC group. Virus infection and gavage treatment for each group were as follows: 50 μl physiological saline was given to mice in uninfected and CEFTC groups by nasal drip; 50 μl virus with a titer of $10LD_{50}$ was given to mice in high-dose infected and high-dose infected + CEFTC groups by nasal drip; 50 μl virus with a titer of $LD_{50}$ was given to mice in low-dose infected and low-dose infected + CEFTC groups by nasal drip. 1 h after infection, each group was given intragastric administration for 7 days. Saline was given in the uninfected, high-dose infected, and low-dose infected groups. CEFTC (0.2 mg/g body weight/day) was given in the CEFTC, high-dose infected + CEFTC, and low-dose infected + CEFTC groups. The specific process is shown in Fig. 1. All these experimental procedures were in accordance with the guidelines of the Animal Care and Use Committee.

### 2.4. Blood cell counts and organ index calculation

On the 7th day of infection, the body weight of the mice was weighed and recorded. Mice were sacrificed by exsanguination under anesthesia with sodium pentobarbital. Blood samples were collected from each group, and cell counts of leukocytes, erythrocytes, neutrophils, and lymphocytes were performed. Then the spleen and lung tissues were weighed and recorded from the mice. The index was calculated according to the formula (Cui et al., 2022): lung index = lung weight of mice (mg)/body weight of mice (g) × 100%, spleen index = spleen weight of mice (mg)/body weight of mice (g) × 100%.

### 2.5. Alveolar lavage fluid testing

On the 7th day of infection, alveolar lavage fluid was collected. 1 ml of saline was gently injected into the trachea and pumped back, repeated this step three times to obtain alveolar lavage fluid, stored in Eppendorf tubes, and centrifuged at 3000 r/min for 10 min. The pellets were diluted with normal saline (Lee et al., 2021). The 100 μl dilution was taken to make a smear and stained by immersion in the order of methanol, distilled water, 95% ethanol, ethanol, xylene, Wright-Giemsa staining, and finally placed under the microscope to take photos for observation.

### 2.6. HE staining

The lung tissues were rinsed with physiological saline and then immersed in neutral 4% paraformaldehyde for 24 h. After a series of steps, including rinsing, dehydration, and transparency, the samples

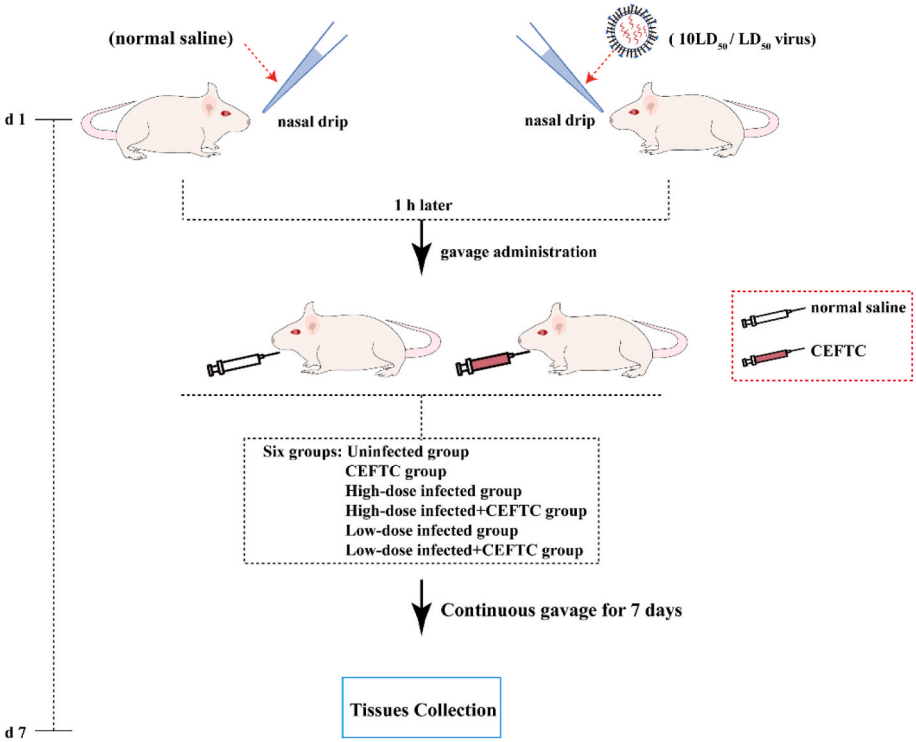


**Fig. 1.** Model-making process of the animal experiment.

were embedded into paraffin. Lung tissues were cut into 4-μm-thick sections, stained with hematoxylin/eosin, and observed under the microscope (Zhang et al., 2018).

### 2.7. Network pharmacology analysis

The active chemical compounds of the flowers of *T. chinensis* Bunge were screened by the Traditional Chinese Medicine System Pharmacology (TCMSP) database. The screening conditions were oral bioavailability (OB) $\geq$ 30% and drug-likeness (DL) $\geq$ 0.18. We converted each compound screened into canonical SMILES via PubChem. Subsequently, these SMILES were imported into SwissTargetPrediction, which could be used to predict potential targets of the compounds. The species were selected as "*Homo sapiens*," and the probability >0 was used as the screening condition.

With "pneumonia" as the keyword, the disease-related targets were retrieved through the Therapeutic Target Database (TTD) (Zhou et al., 2022), Drugbank (Wishart et al., 2018), and GeneCards (Safran et al., 2022). Overlapping targets between potential targets of herb compounds and disease-related targets were considered potential targets for pneumonia. The protein-protein interaction (PPI) network was constructed by the STRING database and visualized by Cytoscape 3.9.1 software. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted by linking targets to the Database for Annotation, Visualization, and Integrated Discovery database (DAVID) (Huang et al., 2009; Sherman et al., 2022). Next, the picture of KEGG pathway enrichment was created using the online tools (https://www.chiplot.online/).

### 2.8. Western blot assay

Lung tissues were lysed with RIPA lysis buffer for 30 min, and then total proteins were obtained by centrifugation at 12,000 g for 5 min. The concentration was measured by the BCA Protein Assay Kit according to the instructions. The proteins were electrophoresed on 8/15% SDS-polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Yu et al., 2018). The membranes were subsequently probed with their antibodies against TLR3, IRF3, TAK1, TBK1, IFN-β, and IKKα, respectively. Then, they were washed and incubated with the corresponding secondary antibodies at 37 °C for 1 h. After adding enhanced chemiluminescence (ECL) solution, the strips were exposed in a dark room.

### 2.9. Immunofluorescence assay

The lung tissue sections were dewaxed with xylene, washed with ethanol (100%, 95%, 90%, 80%, 70%), and water. Then, they were blocked with 5% BSA for 60 min at room temperature and incubated with antibodies against IRF3 and IFNβ, respectively, overnight at 4 °C. Sections were washed three times with phosphate buffer saline tween (PBST) and after that they were incubated using the corresponding secondary antibody labeled with fluorescein isothiocyanate (FITC). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). After the cleaning of sections, they were observed by confocal laser scanning microscopy (Li et al., 2011; Liu et al., 2021).

### 2.10. Statistical analysis

SPSS 20 statistical software was used for data processing, and the results were expressed as mean ± standard deviation. All data between groups were analyzed using analysis of variance (ANOVA), and $P < 0.05$ indicated a statistical difference.

## 3. Results

### 3.1. CEFTC changed the number of inflammatory cells and erythrocytes in mice infected with the influenza virus

On the 7th day of virus infection, blood cell counts results showed that the number of leukocytes, neutrophils, and lymphocytes was reduced due to influenza virus infection. At the same time, erythrocytes were increased (Fig. 2). CEFTC could increase the number of leukocytes, neutrophils, and lymphocytes in mice infected with the low-dose virus. However, in the high-dose infected group, the count of leukocytes, neutrophils, and lymphocytes in mice treated with CEFTC had a decreasing trend. This result indicated that CEFTC could affect the number of inflammatory cells and erythrocytes in mice infected with the influenza virus. In addition, CEFTC had a different effect on blood cell counts due to the distinct doses of the infected virus.

### 3.2. CEFTC affected the spleen index and lung index in mice infected with the influenza virus

We found that CEFTC could change the number of inflammatory cells in mice infected with the influenza virus. To further evaluate the anti-influenza virus effect of CEFTC, spleen index and lung index were analyzed. These results showed that virus infection could increase the spleen index and lung index of mice (Fig. 3), especially the lung index had an noticeable increase ($P < 0.01$). CEFTC could significantly reduce the rising lung index caused by influenza virus infection ($P < 0.01$), indicating that CEFTC had anti-influenza virus activity. Moreover, CEFTC decreased the spleen index in the low-dose infected group, while in the high-dose infected group, CEFTC slightly increased the spleen index. As an important lymphoid organ, spleen played an essential role in immune response (Bégay et al., 2022). Spleen index results indicated that influenza virus infection could activate the immune system. Meanwhile, the effects of CEFTC against infections differed due to the unequal doses of the infected virus.

### 3.3. CEFTC improved lymphocyte infiltration of alveolar lavage fluid in mice infected with the influenza virus

Bronchoalveolar lavage, as a well-established method, was introduced to analyze inflammatory cell infiltration in animal models of respiratory diseases (Van Hoecke et al., 2017). By observing the alveolar lavage fluid, we found varying hemolytic changes in the alveolar lavage fluid of virus-infected groups compared with uninfected groups. To further observe the infiltration of lymphocytes in alveolar lavage fluid, we stained the lymphocytes with Wright's stain. There was prominent lymphocyte infiltration in alveolar lavage fluid of virus-infected groups, and the lymphocyte infiltration induced by the high-dose virus was more severe than that caused by a low-dose virus (Fig. 4). Simultaneously, CEFTC could obviously improve lymphocyte infiltration of alveolar lavage fluid in mice infected with influenza virus. It suggested that CEFTC displayed noticeable effect against influenza virus through reducing the severity of lymphocytic infiltration.

### 3.4. CEFTC affected alveolar structure and inflammatory cell infiltration in mice infected with the influenza virus

We also sought to evaluate the effect of CEFTC on pathology changes of the lungs after influenza virus infection. Thus, the morphological and structural characteristics of the lung tissue were investigated by HE staining. These results showed that whether in the uninfected group or the CEFTC group, the lung tissue structure of mice was normal, and there was no inflammatory cell infiltration (Fig. 5). In contrast, a series of pathological changes in the lungs could be observed in virus-infected groups including intensive infiltration of inflammatory cell and destruction of the alveolar wall. We also discovered that the high-dose

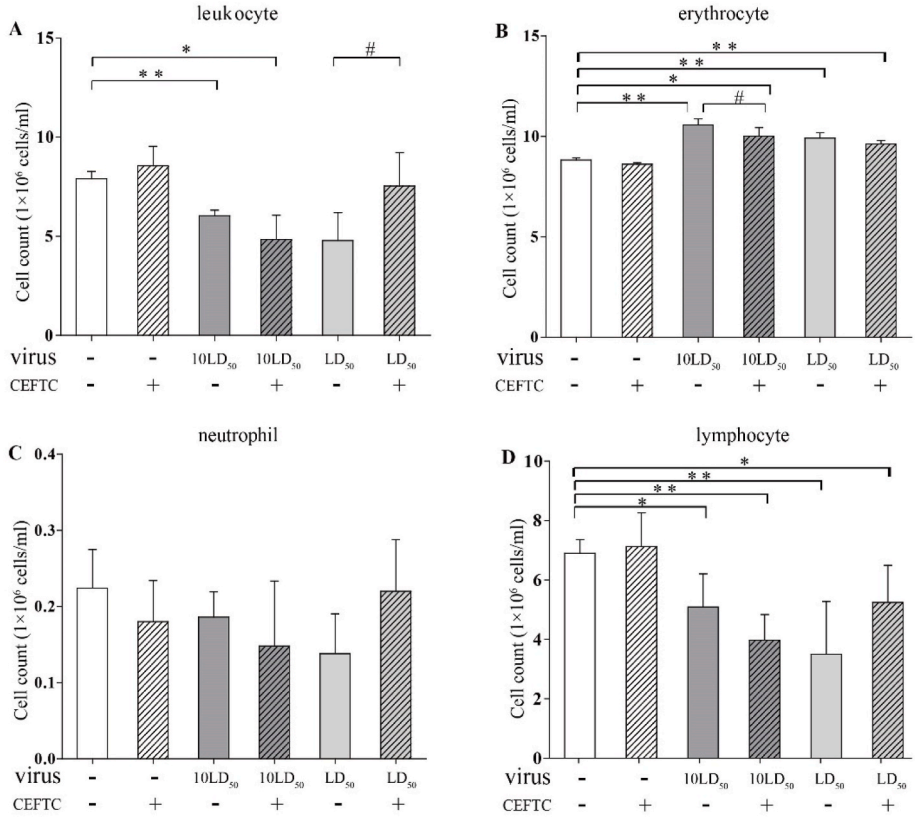


**Fig. 2.** Effects of CEFTC on the blood cell count in mice infected with the influenza virus (n = 5). Compared with uninfected group, *<0.05, **<0.01; Comparison between CEFTC treatment group and corresponding infected group, #<0.05.

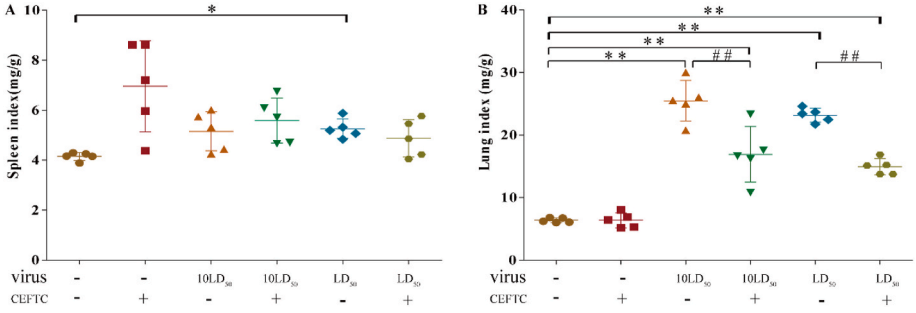


**Fig. 3.** Effects of CEFTC on the organ index in mice infected with the influenza virus (n = 5). Compared with uninfected group, *<0.05, **<0.01; Comparison between CEFTC treatment group and corresponding infected group, ##<0.01.

virus infection caused more serious pathological changes in the lungs, accompanied by the more intensive inflammatory cell infiltration. Compared with the virus-infected groups, CEFTC reduced the inflammatory cell infiltration in the treatment groups. Especially in the low-dose virus infected group, a few normal alveolar structures could be observed after mice were treated with CEFTC.

### 3.5. Potential signaling pathways between the flowers of T. chinensis Bunge and pneumonia were obtained

We confirmed that CEFTC had anti-influenza virus effects, and we next explored the potential mechanism. Network pharmacology was used to predict the possible signaling pathway between the flowers of *T. chinensis* Bunge and pneumonia. Our team had previously analyzed the active compounds of the flowers of *T. chinensis* Bunge using high-performance liquid chromatography (HPLC), finding that flavonoids, phenolic acids, and alkaloids were the main effective ingredients of the flowers of *T. chinensis* Bunge (Guo et al., 2017). Flavonoids were natural phytochemicals found in many plants, which had vigorous antiviral activity against many viruses, including influenza virus, dengue virus, and hepatitis C virus (Badshah et al., 2021). Phenolic acids had been widely used in biomedical fields as antioxidants, antidiabetic agents, and antibacterial agents (Kumar and Goel, 2019). In recent years, the use of alkaloids for antiviral therapy had demonstrated its unique advantages, providing possibilities for the development of new antiviral drugs (Ti et al., 2021). The application of these compounds revealed that they were the essential ingredients related to the antiviral effect of the flowers of *T. chinensis* Bunge.

From the TCMSP database, we retrieved 7 active ingredients of the flowers of *T. chinensis* Bunge, which partially overlapped with those we analyzed using HPLC. And then, we predicted 171 herb targets by SwissTargetPrediction. A total of 1407 potential disease targets were obtained from Genecards, TTD, and Drugbank databases. The results were presented in the Wayne diagram (Fig. 6A) with 50 overlapping

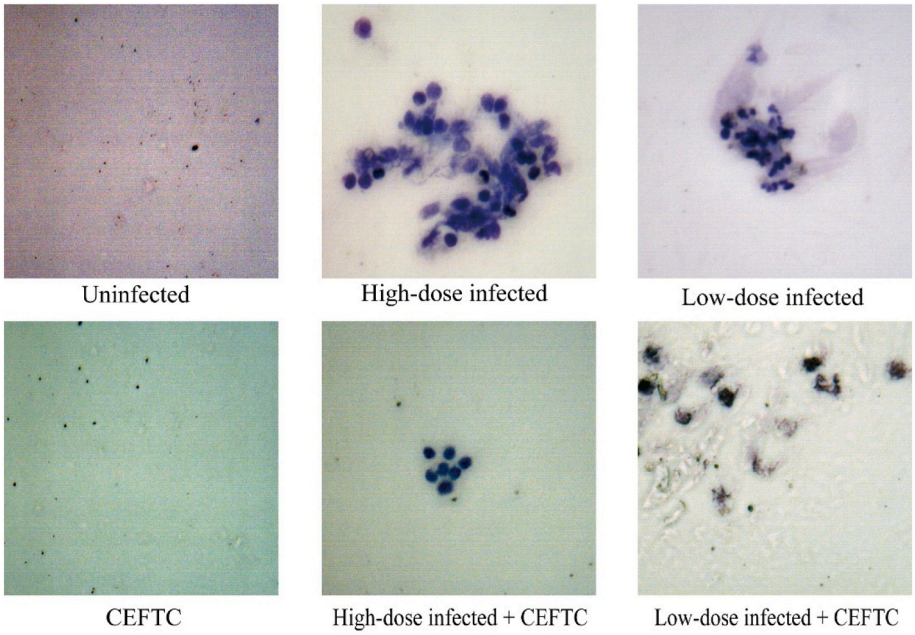


**Fig. 4.** Detection results of alveolar lavage fluid in each group of mice (600 × ).

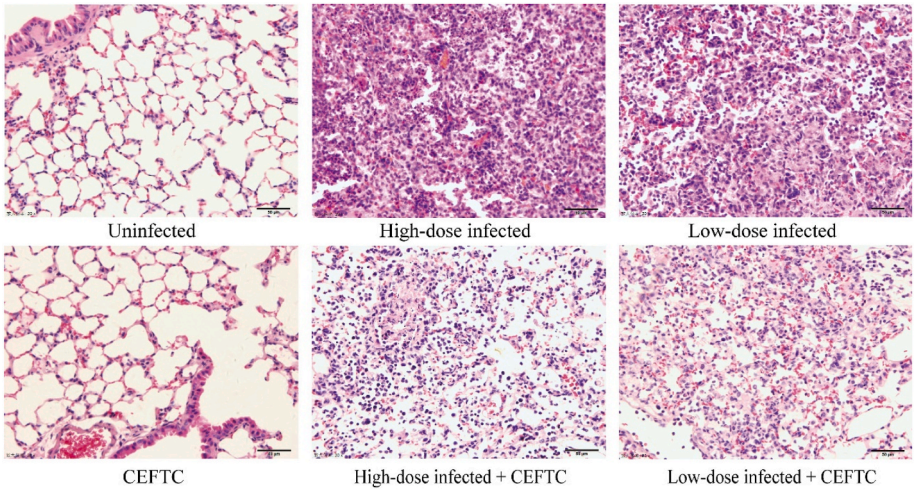


**Fig. 5.** Pictures of HE staining of mice lung tissues (200 × ) in each group.

targets. According to the degree of alignment (Fig. 6B), the essential proteins included RAC-alpha serine/threonine-protein kinase (AKT1), epidermal growth factor receptor (EGFR), prostaglandin G/H synthase 2 (PTGS2), peroxisome proliferator-activated receptor gamma (PPARG), matrix metalloproteinase 9 (MMP9) and NAD-dependent protein deacetylase sirtuin-1 (SIRT1). According to KEGG analysis, 30 signaling pathways were obtained (Fig. 6C), which included the NF-κB pathway.

In inflammation-related responses, AKT1 was a critical upstream kinase related to NF-κB and IRF3 inflammatory signaling pathways (Kan et al., 2021; Kim et al., 2017). Notably, the search results in the Genecards database showed that TLR3, IRF3, and TBK1 were important targets associated with pneumonia (Fig. 6D). In addition, the flowers of *T. chinensis* Bunge could exert anti-influenza virus effects by regulating TLR3 signaling pathway *in vitro* (Liu et al., 2021). Based on literature research and analysis results of network pharmacology, we speculated that the CEFTC could improve the influenza through modulation of the TLR3 signaling pathway.

### *3.6. CEFTC regulated the expression of proteins related to the TLR3 signaling pathway*

To clarify the antiviral mechanism of CEFTC, we further examined the expression of critical proteins in the TLR3 signaling pathway. Compared with the uninfected group, the high-dose virus could increase the expression of TLR3, IRF3, TAK1, TBK1, and IKKα proteins (Fig. 7). In the CEFTC group (uninfected + CEFTC), CEFTC could up-regulate the expression of TLR3, IRF3, TAK1, and TBK1, but it had no significant effect on the expression of IFN-β. In addition, we found that the regulation of CEFTC on protein expression was different between high-dose and low-dose infected groups. In the high-dose infected group, CEFTC reduced the expression of TLR3, IRF3, TAK1, and TBK1, while increasing the expression of IFN-β. However, in the low-dose infected group, CEFTC increased the expression of TLR3, IRF3, IFN-β, and TBK1. Interestingly, compared with the virus-infected group, there were no changes in the expression of IKKα in CEFTC treatment groups. The results suggested that CEFTC exerted the antiviral effect by regulating the protein expression levels of factors related to the TLR3 signaling pathway.

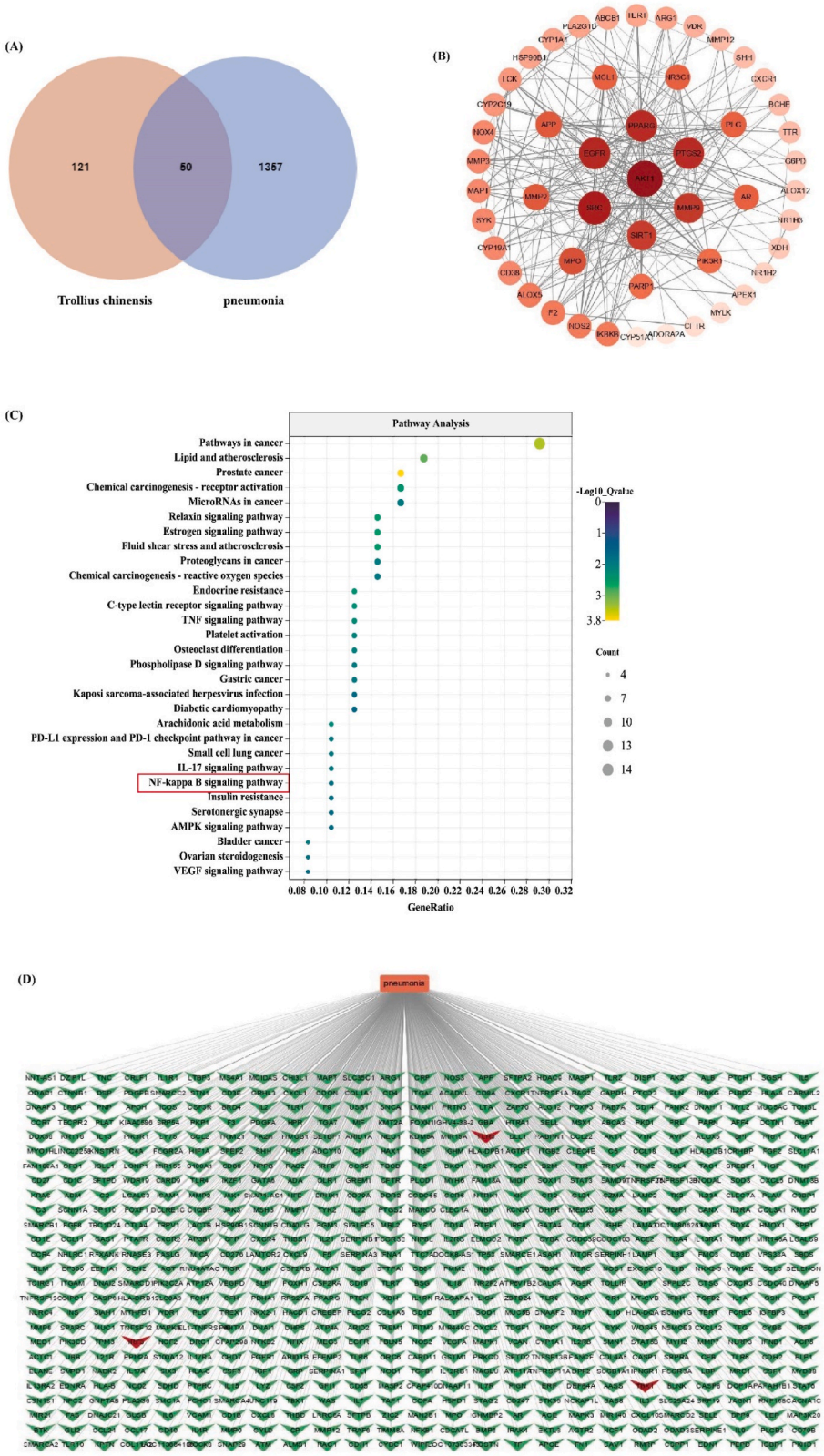


**Fig. 6.** (A) Venn diagram of Trollius chinensis and pneumonia; (B) The PPI network of intersection targets. The circular layout is arranged counterclockwise according to the node from light to dark and small to large. (C) KEGG pathway enrichment analysis. The larger the area of dots, the more the counts; the smaller the P-value, the bluer the dot color. (D) The target of pneumonia. Red nodes represent TLR3, IRF3, and TBK1, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### *3.7. CEFTC altered the distribution of IRF3 and IFN-β between nuclei and cytoplasm*

Finally, to investigate the effect of CEFTC on the distribution of IRF3 and IFN-β between the nucleus and cytoplasm, immunofluorescence analysis was performed using confocal microscopy. IRF3 mainly distributed in cytoplasm in the uninfected group, while in the CEFTC group (uninfected + CEFTC), it distributed in both nuclei and cytoplasm. After mice were infected with the influenza virus, we found IRF3 could translocate into the nuclei (Fig. 8). Furthermore, compared with

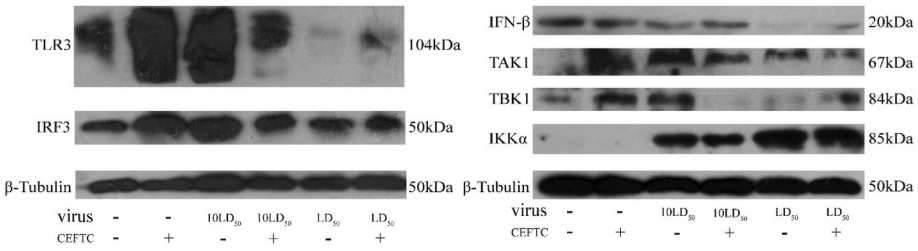


**Fig. 7.** Effects of CEFTC on protein expression of vital genes related to TLR3 signaling pathway in response to influenza virus stimulation.

influenza virus infected groups, the amount of IRF3 in nuclei was markedly increased after CEFTC treatment. IFN-β distributed in the cytoplasm and nuclei of uninfected and CEFTC groups (Fig. 9). Compared with the virus-infected group, the distribution of IFN-β was increased in alveolar epithelial cells after CEFTC treatment. From these results, we observed that in the virus-infected group, CEFTC could promote the translocation of IRF3 into nuclei and increase the distribution of IFN-β in alveolar epithelial cells.

## 4. Discussion

Virus infections, physical and chemical factors, drugs, and some diseases can trigger a decrease in peripheral blood leukocyte count. Leukocytes play an essential role in resisting the invasion of pathogenic microorganisms. Adults infected with severe influenza A (H1N1) virus usually show a decreasing trend in leukocyte count and lymphocyte count in the early stage of the disease (Cheng et al., 2019). The influenza virus can directly suppress the adaptive immune response by infecting and killing activated lymphocytes, and this may explain why peripheral lymphocytes are reduced during influenza virus infection (Bohannon et al., 2021). In our experiments, we found that influenza virus infection could reduce the number of leukocytes, lymphocytes, and neutrophils. However, the number of these cells could be increased in low-dose virus-infected mice after CEFTC treatment. In addition, virus infection could raise the number of erythrocytes, and we speculated that virus infection led to the abnormality of respiratory function, resulting in a compensatory increase in erythrocytes to improve oxygen supply.

H1N1 virus infection can cause pathological changes in the lungs, including the destruction of alveolar structure, inflammatory cell infiltration, and pulmonary congestion (Du et al., 2020; Zhao et al., 2021). The organ index is a basic target for biomedical research. Congestion, edema, and hypertrophy of organs can increase the index, while atrophy or other degenerative changes of organs can decrease the index (Dai et al., 2015). The lung index results of this trial suggested that CEFTC treatment was effective in relieving pulmonary enlargement caused by the influenza virus. Spleen is a vital lymphoid organ, which plays an essential role in the immune response. The size of the spleen correlates with immune competence, and an enlarged spleen may indicate that the immune system is reacting to infection (Bégay et al., 2022). CEFTC had also been found to improve the elevated spleen index induced by an influenza virus infection, thus exerting antiviral effects. Bronchoalveolar lavage is a common and relatively safe diagnostic method for evaluating lung diseases, and it has been introduced for the analysis of inflammatory cell infiltration in animal models of respiratory diseases (Davidson et al., 2020; Van Hoecke et al., 2017). We further demonstrated that CEFTC alleviated inflammatory cell infiltration of alveolar lavage fluid in mice infected with influenza virus. Moreover, we discovered that CEFTC effectively relieved alveolar structure injury and inflammatory cell infiltration of the lungs in influenza virus-infected mice.

As an important activator of type I interferon, TLR3 can interact with the TIR domain-containing bridging protein to activate TBK1, phosphorylate IRF3, and ultimately induce IFN-β expression (Ascough et al., 2018). In this study, CEFTC was found to inhibit the increased expression of TLR3, TBK1, TAK1, and IRF3 induced by the high-dose influenza virus, suggesting that the antiviral mechanism of CEFTC might be related to TLR3-TBK1-IRF3 signaling pathway. Immunofluorescence results revealed that after influenza virus infection, CEFTC promoted the translocation of IRF3 into nuclei and increased the expression of IFN-β in alveolar epithelial cells. Based on these results, we considered that CEFTC could exert the antiviral effect by regulating the TLR3-TBK1-IRF3 signaling pathway. In addition, the TRIF-dependent pathway can directly recruit TNF receptor-associated factor 6 (TRAF6), which can activate receptor-interacting serine/threonine-protein kinase 1 (RIPK1). RIPK1 then triggers TAK1 via phosphorylation and further leads to the activation of NF-κB (Zhao et al., 2015). Numerous studies have shown that drugs can exert antiviral effects by regulating expression of NF-κB (Ding et al., 2017; Wei et al., 2019). Here, we also found that CEFTC reduced the expression of TAK1 after influenza virus infection, suggesting that CEFTC might also exert antiviral effects by regulating the TAK1-NF-κB signaling pathway, and need further verification. Notably, CEFTC had some different effects on the protein expression in TLR3 signaling pathway in the low-dose infected group and the high-dose infected group. We speculated that CEFTC decreased the expression of TLR3、TBK1, and IRF3 to avoid immune storm events caused by high-dose virus infection.

In addition, we also had some interesting discoveries in this study. Following infection with different doses of influenza virus, CEFTC exhibited different regulating effects on the expression of vital factors in TLR3-TBK1-IRF3 signaling pathway to exert its antiviral activity. Infection with different doses of influenza virus led to various degrees of lung injury in mice, and the pathological changes induced by high-dose influenza virus were more serious. After the intervention of CEFTC, we noticed that its therapeutic effects on mice infected with the low-dose virus were better than those infected with the high-dose virus. Therefore, we considered it necessary to double the dose of the drug after a high-dose influenza virus infection, and this might explain why the first oral dose was doubled in clinical treatment.

## 5. Conclusion

The present study showed that different doses of influenza virus could cause varying infection symptoms in mice. CEFTC possessed therapeutic effects on mice infected with influenza virus, and its antiviral mechanism was related to the activation of the TLR3 signaling pathway. In addition, CEFTC displayed different therapeutic effects in treating mice infected with high-dose and low-dose influenza virus, and this discovery might provide drug applications reference in the clinic.

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## CRediT authorship contribution statement

**Yuxi Liang:** Investigation, Data curation, Writing – original draft. **Xiaoli Liu:** Investigation, Data curation, Writing – original draft. **Jingyan Hu:** Investigation. **Songli Huang:** Investigation. **Xin Ma:**

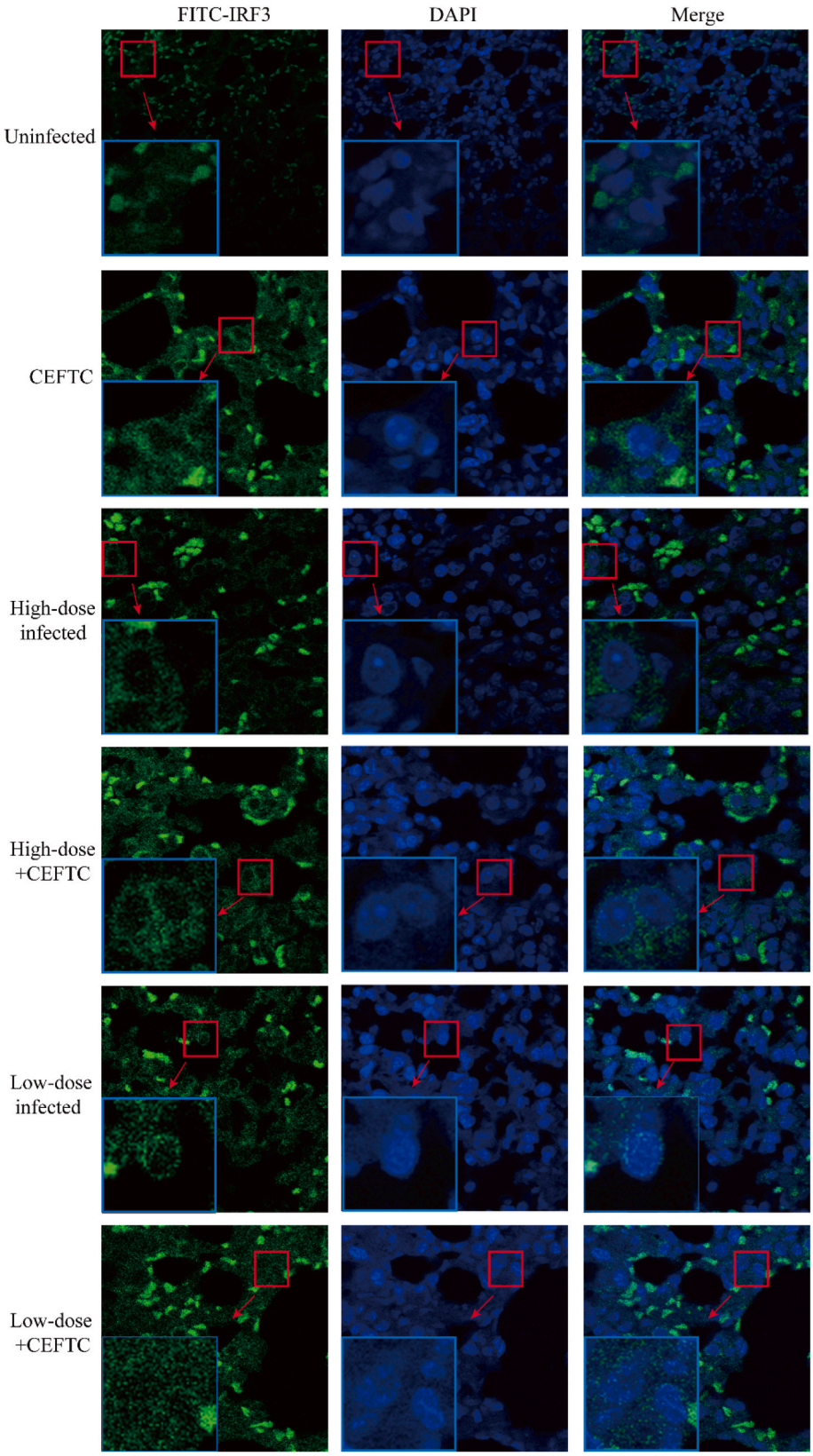


**Fig. 8.** Effects of CEFTC on translocation of IRF3 into the nuclei (600 × ). The blue represents the nuclei, and the green represents IRF3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

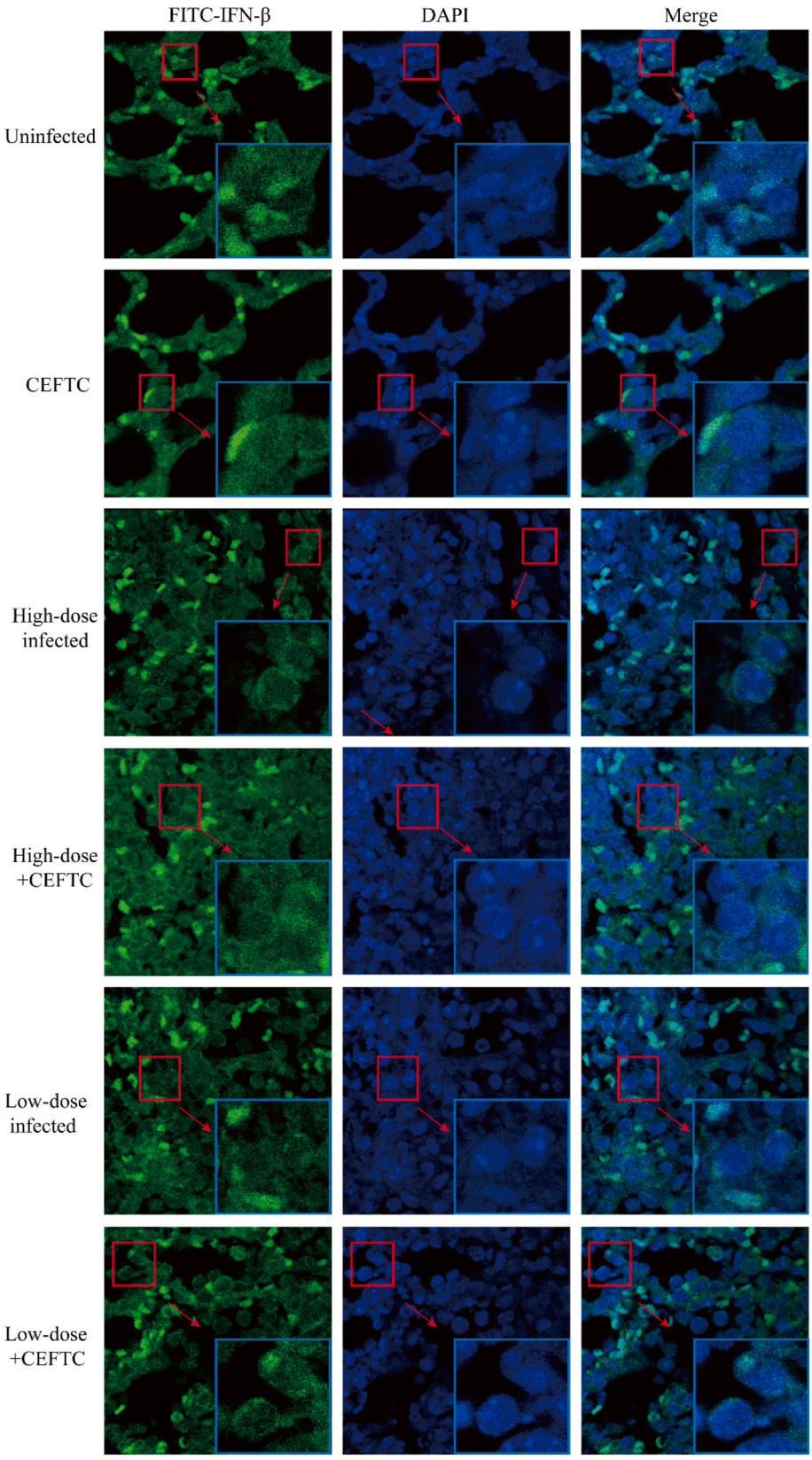


**Fig. 9.** Effects of CEFTC on translocation of IFN-β into the nuclei (600 × ). The blue represents the nuclei, and the green represents IFN-β. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Methodology. **Xiaoyan Liu:** Writing – review & editing, Methodology. **Rufeng Wang:** Resources, Project administration. **Xiuhua Hu:** Writing – review & editing, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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