



Original article

Qingfei oral liquid alleviates airway hyperresponsiveness and mucus hypersecretion via TRPV1 signaling in RSV-infected asthmatic mice

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ABSTRACT

Pediatric asthma is exacerbated by Respiratory Syncytial Virus (RSV) infection, and Transient Receptor Potential Vanilloid 1 (TRPV1) promotes production of inflammatory cytokines and mucus hypersecretion in the pathology of this disease. Our previous research revealed that Qingfei oral liquid (QF) inhibited airway inflammation and mucus hypersecretion in RSV-infected asthmatic mice models and that this may be associated with the TRPV1-regulation of NF- κ B and Mucin 5AC (MUC5AC) expression, but the exact mechanism is unknown. In the present study, LC-MS was used for analyzing the chemicals in QF, ovalbumin (OVA)-induced asthmatic mice inhaled RSV three consecutive times to create an RSV-infected asthmatic model. We found treatment from QF alleviated airway hyperresponsiveness (AHR) and reduced congestion, edema, and infiltration of inflammatory cells into pulmonary tissues. Additionally, QF was found to decrease expression of NF- κ B and its downstream inflammatory cytokines IL-1 β , IL-4, IL-5, and IL-13, as well as a decrease in MUC5AC and pro-inflammatory cytokines in PKC via a reduction in Protein Kinase C-dependent signaling. These findings suggest that QF can alleviate AHR and mucus hypersecretion caused by RSV infection in asthmatic mice, and its mechanism may be associated with the regulation of the TRPV1 signaling pathway.

1. Introduction

Asthma is a complex disease characterized by airway inflammation and remodeling [1]. Respiratory syncytial virus (RSV), an RNA virus that is the most common respiratory pathogen in infants and children [2], exacerbates these symptoms in asthmatic models by inducing the release of cytokines that amplify the inflammatory response, triggering airway hyperresponsiveness (AHR) [3]. Additionally, RSV infection triggers a loss of ciliated cells and metaplasia of goblet cells, which are also associated with enhanced Mucin 5AC (MUC5AC) production and mucus hypersecretion [4–7]. Recent investigations suggest, however, that mucus hypersecretion and airway inflammation may not only be due to MUC5AC stimulation, but also the activation of Transient Receptor Potential Vanilloid 1 (TRPV1) [8]. TRPV1 is expressed in epithelial cells of the respiratory airway, smooth muscle cells, sensory neurons, and dendritic cells in the lungs [9]. It is stimulated by various factors, including viruses, harmful chemicals, low pH, heat, and other endogenous mediators [9,10]. Upregulation of TRPV1 enhances expression of pro-inflammatory neuropeptides such as substance P and Calcitonin Gene-Related Peptide, which contribute to neurogenic inflammation in asthmatic mice [11–13] (Fig. 1 [9–12,14–17]). As such,

it is believed that the severity of asthmatic symptoms in pediatric and adult patients infected with RSV [15] can be minimized when TRPV1 antagonists (e.g., siRNA) are administered, and data show they reduce inflammation of respiratory airways, hypersensitivity and levels of cytokines IL-4, IL-5, IL-13 [16], and IL-1 β [17]. IL-1 β is an inflammatory cytokine that exacerbates symptoms in RSV-asthma [18,19], and as we all known Th2 cytokines play an important role in RSV-asthma [20–22], such as IL-4 is high level to accelerate IgE synthesis, IL-5 recruits and activates eosinophils reproduction, IL-13 increased due to induces of AHR, mucus hyperproduction (i.g. MUC5AC).

At present, it is known that TRPV1 participates in inflammation via NF- κ B [13,23], which triggers expression of cytokines associated with the pathogenesis of asthma, such as IL-4, IL-5, and IL-13 [24]. Additionally, TRPV1 phosphorylation by protein kinase C (PKC) is known to mediate mucin production in asthmatic models through the stimulation of MUC5AC [25,26]. Some scholars suggest that Traditional Chinese Medicine can reduce mucus hypersecretion by modulating TRPV1 [27,28]. Qingfei oral liquid (QF), notably, is a Chinese medicine that has been used to treat patients with viral pneumonia and asthma for decades, we also identified and analyzed bio-active compounds in QF including ephedrine/pseudoephedrine, amygdalin, Mulberroside A,

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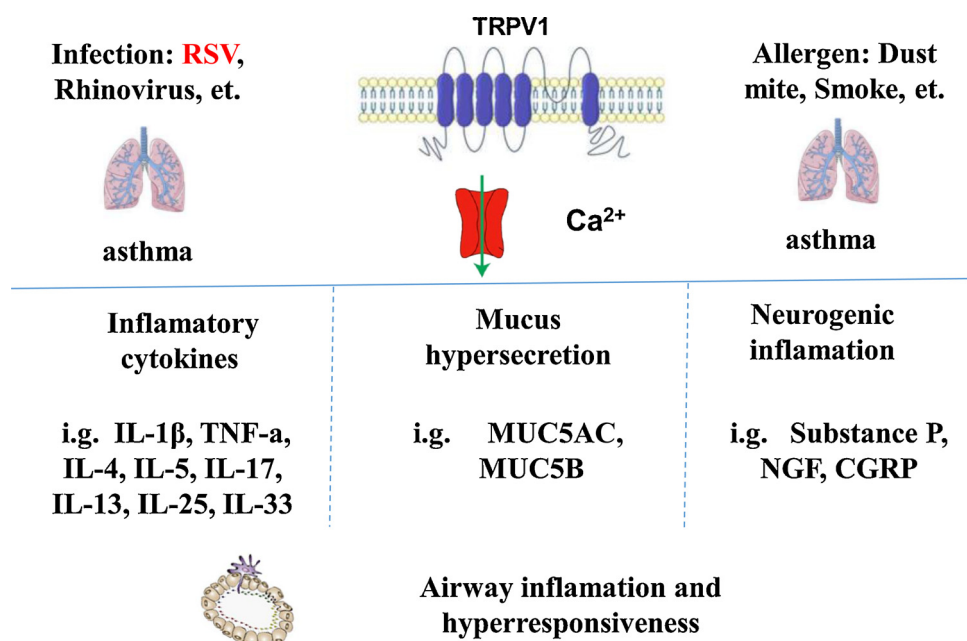


Fig. 1. Roles of TRPV1 in RSV-asthma.

The schematic illustrates how TRPV1 signaling triggers inflammatory pathways that influence airway remodeling and mucus hypersecretion in RSV-asthma. The reference for this figure is Bujak JK et al. Front Oncol. 2019 [9–12,14–17].

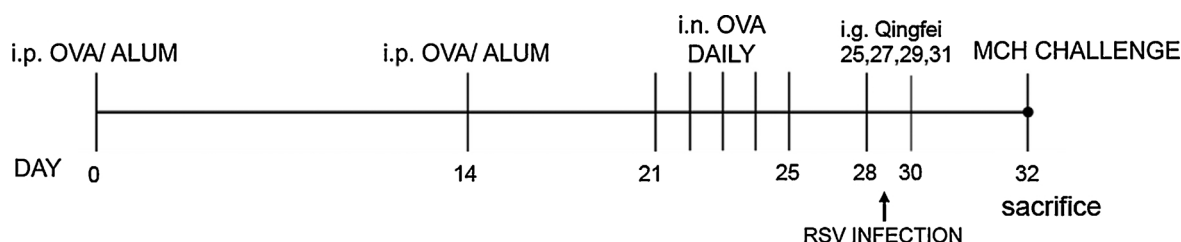


Fig. 5. Timeline of the experiment.

All mice except those in the control group received an IP injection of OVA complexed with Al(OH)₃ on day 0, 14 and 21. They then received a continuous nebulized spray of OVA (1% in PBS) for 30 min using a 402 AI ultrasonic atomizer from days 21–25. They received a nebulized spray of OVA (1% in PBS) for 30 min each morning, every other day from day 27–32. RSV (ATCC) was administered intranasally on day 28, 30 and 32. The QF and Dxms treatment groups were given doses in the afternoon on day 21, 23, 25, 27, 29 and 31.

etc. by using a LC–MS method, and based on accumulating evidence, we hypothesize this may occur via inhibition of the TRPV1 signaling pathway. Our previous research discovered QF decreases airway hyperemia edema, mucus hypersecretions, and hyperplasia of respiratory airways in RSV-infected asthmatic mice [29], though the exact mechanism remains to be elucidated.

In this study, we explore the effect of QF on pulmonary function and TRPV1 signaling in RSV-infected asthmatic mice as a potential treatment for pediatric asthma.

2. Materials and methods

2.1. Drugs and chemicals

QF was purchased from the Chinese Pharmacy at Putuo Hospital, affiliated with the Shanghai University of Traditional Chinese Medicine. A standard dose of QF contains *Herba Ephedrae* (3 g), *Semen Armeniacae Amarum* (10 g), *Gypsum Fibrosum* (20 g), *Scutellariae Radix* (6 g), *Mori Cortex* (10 g), *Descurainiae Semen Lepidii Semen* (10 g), *Peucedani Radix* (10 g), *Polygoni Cuspidati Rhizoma et Radix* (12 g), and *Salviae Miltiorrhizae Radix et Rhizoma* (10 g), totaling 91 g. We weighed out 6 times the prescribed amount of medicine and then performed a water reflux extraction 4 times, one hour each time. Vacuum concentration of the infiltrate created a fluid extract weighing 91 g. The ratio of the

extract to the medicine was 0.3498 g·g⁻¹. For a 25 g mouse, a single dose was 0.1591 g, or 6.365 g·kg⁻¹·d⁻¹, 0.25 mL. The extraction was completed by the Department of Pharmacology at Hangzhou Medical College; all other solvents used in this study were commercially available and of reagent grade.

2.2. LC–MS conditions

QF was dissolved in methanol solution (50 %). The mixture was then centrifuged to get the supernatant. Afterwards, QF solution was made into the concentration of 150 mg/mL. LC was performed on a Waters LC system (Milford MA), consisting of a binary solvent delivery system, an autosampler and PDA detector system. An ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm i.d., 1.7 μm; Waters) was used for all analyses, respectively maintaining at 30 °C and 10 °C. The mobile phase was composed of 0.1 % formic acid (v/v) (A) and acetonitrile (B). The flow rate was 0.4 mL/min with 30 min of gradient elution: 0–1 min, 5–5% B; 1–10 min, 5–20 % B; 10–25 min, 20–50 % B; 25–27 min, 50–95 % B; 27–29 min, 95 % B; 29–30 min, 95–5% B. The MS analysis was performed on a Waters ACQUITY Synapt QTOF mass spectrometer connected to the Waters ACQUITY UPLC system via an electrospray ionization (ESI) interface. High purity nitrogen was chosen to be the nebulizer and auxiliary gas while argon as the collision gas. The Q-TOF MS was operated in the negative ion mode with a capillary voltage of

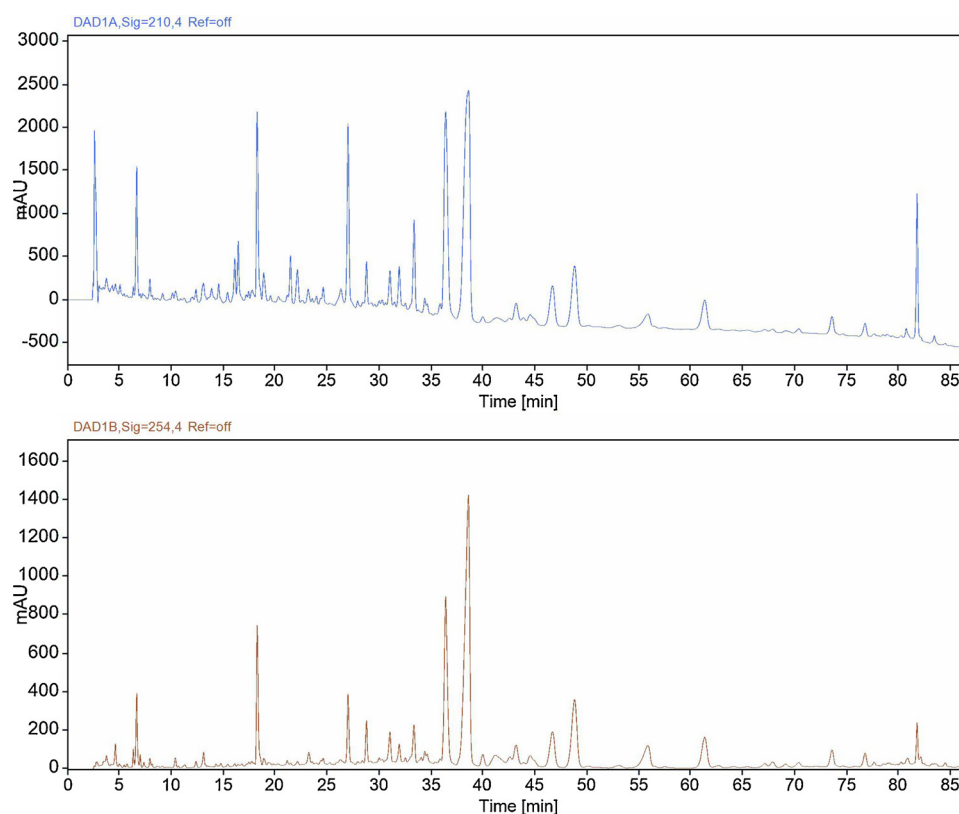


Fig. 2. UV chromatogram of QF (A: 210 nm, B: 254 nm). The extract of QF was analyzed by LC–MS. The LC–MS chromatogram is shown in Fig. 2.

Table 1
main compounds in the aqueous extract of QF by LC–MS.

No.	Source	Retention time(min)	Relative abundance (MS)	<i>m/z</i>	Identified compounds
1	<i>Herba Ephedrae</i>	10.2	0.97 %	166.2	Ephedrine/pseudoephedrine
2	<i>Semen Armeniacae Amarum</i>	16.3	5.86 %	480.3	Amygdalin
3	<i>Cortex Mori</i>	18.5	1.73 %	407.2/569.3	Mulberroside A
4	<i>Rhizoma Bistortae</i>	18.5	1.73 %	355.2	Chlorogenic Acid
5	<i>Rhizoma Polygoni Cuspidati</i>	27.1	1.41 %	229.1	Resveratrol
6		56.5	1.36 %	271.1	Emodin
7	<i>Radix Salviae Miltiorrhizae</i>	36.5	2.59 %	521/741	Salvianolic acid B
8	<i>Radix Scutellariae</i>	38.7	7.89 %	447.3	Baicalin
9	<i>Radix Scutellariae</i>	43.3	1.43 %	447.3	Oroxyloside
10	<i>Radix Scutellariae</i>	61.4	4.37 %	271.2	Baicalein
11	<i>Radix Scutellariae</i>	46.8	4.13 %	461.2	Oroxin
12	<i>Radix Scutellariae</i>	48.9	6.78 %	461.2	Baicalin
13	<i>Radix Scutellariae</i>	73.7	2.43 %	285	Scutellarin
14	<i>Radix Peucedani</i>	81.9	9.33 %	409.3	Nodakenin

3 kV, a sampling cone voltage of 35 V, a desolvation gas flow of 600 L/h, a collision energy of 6 eV, an ion source temperature of 120 °C, and full scan spectra from 100 to 1500 Da. The LockSpray was used to maintain MS accuracy. The $[M - H]^-$ and $[M + H]^+$ ions of leucine-enkephalin at m/z 554.2615 and 556.2771 were used as the lock mass in respectively negative and positive ESI modes, with a concentration of 50 pg/mL and a infusion flow rate of 10 μ L/min.

2.3. Subjects and experimental design

2.3.1. RSV infection of Hep-2 cells

Following Yan Sun's protocol [30], the RSV A2 strain was incubated in Hep-2 cells and cultured in a basal medium with 10 % FBS and 1% Penicillin-Streptomycin at 37°C and 5% CO₂ until it was 80–90 % full. We washed the surface twice with PBS, added RSV and shook the flask gently for 15–30 min. It sat for 2 h and then we added 2% FBS and 1%

Penicillin-Streptomycin. We harvested the cells in a centrifuge tube with appearance of syncytia formation, death, and floating cells after a 3–5 day incubation in same condition. After 3–5 days incubation at 37°C and 5% CO₂, Scrape and harvest the cells and supernatant into centrifuge tube when appeared syncytia formation, death and floating cells in flask. We resuspended the pellet and flash froze the mixture at –80°C, followed by a quick thaw in a 37°C water bath and a vortexing, all repeated 3 times. We centrifuged the liquid for 10 min at 300 × *g* and 4°C, and then filtrated at 0.45 μ m. The liquid was equally divided among test tubes, flash frozen in liquid nitrogen and then stored at –80°C.

2.3.2. Animals

Male BALB/c mice, 3–4 weeks of age and weighing 13 $\pm$ 3 g, were purchased from Slack Animal Company (Shanghai, China) and maintained at a temperature of 24 $\pm$ 2 °C with a relative humidity of

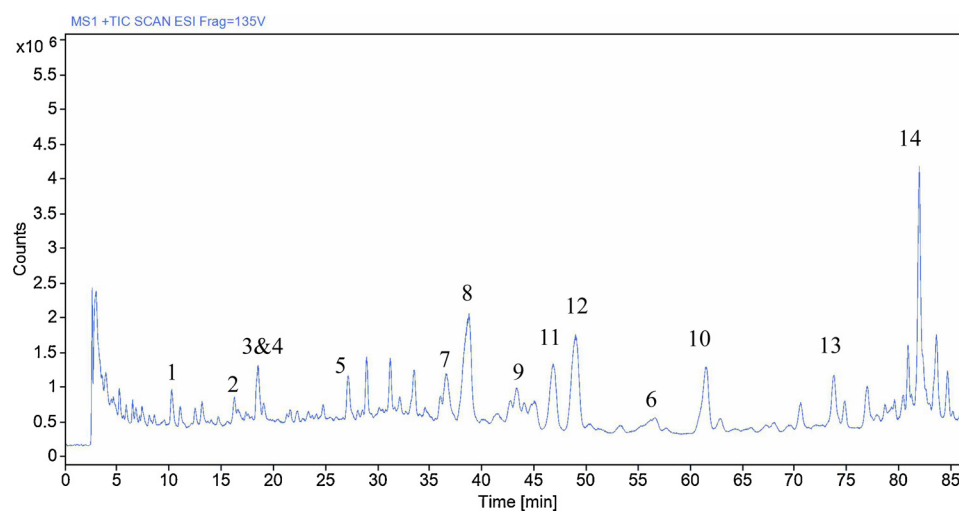


Fig. 3. Total ion flow polarity modes of QF.

After comparing the mass spectrum information in the blank extract and the compound medicine extract, 14 chemical components in the compound were preliminarily identified. The atlas is shown in Fig. 2 and 3.

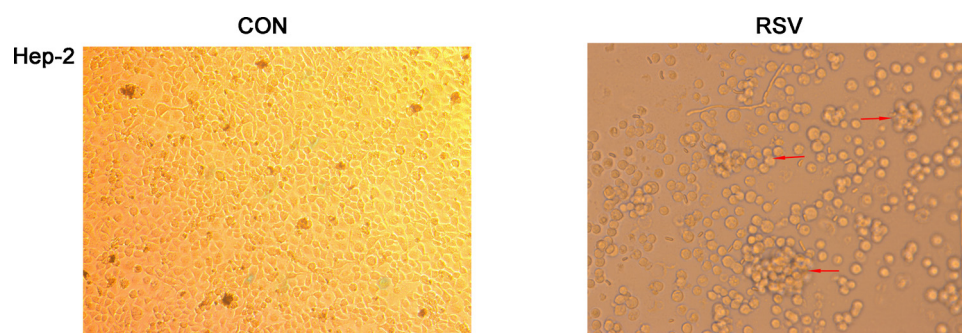


Fig. 4. RSV-infected Hep-2 cells.

Hep-2 cells adhere to the bottom of the culture plate and form the shape of “paving stones”. Following RSV infection, the cells fuse to form a “multinucleated giant cell” syncytium.

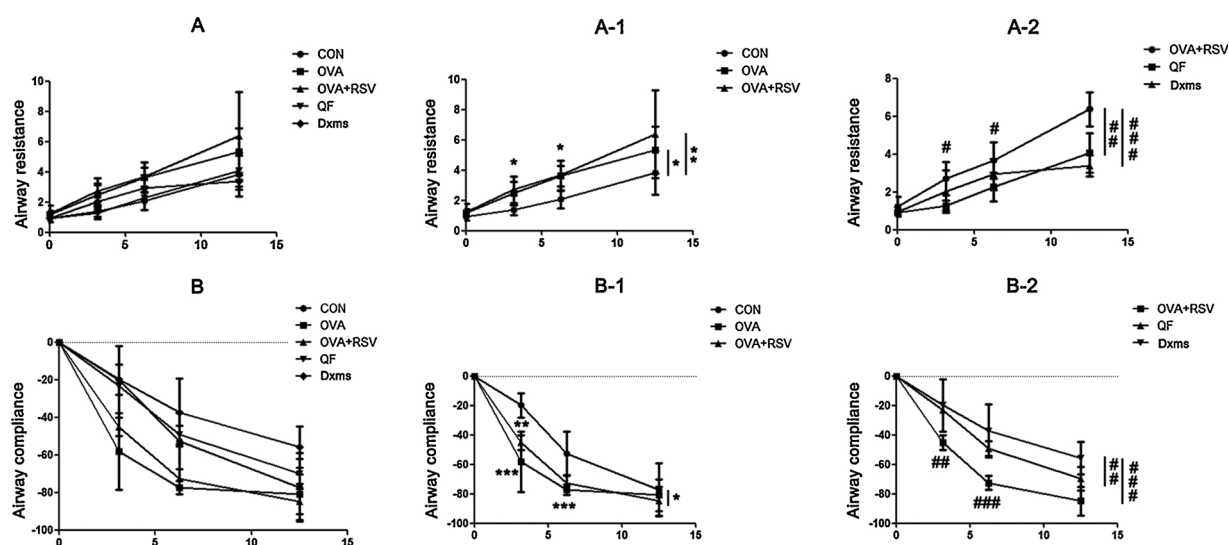


Fig. 6. Effect of QF on AHR and compliance in mice.

Airway responsiveness to aerosolized methacholine was evaluated by Buxco's modular and invasive system (see Materials and methods section). The rats were laid in a supine position inside the plethysmograph chamber and nebulized with PBS, followed by increasing doses of methacholine (3.125–25 mg/ml). The data are expressed as the mean $\pm$ SD. (* $P < 0.05$, ** $P < 0.01$, compared to the control group; and # $P < 0.05$, ## $P < 0.01$; $n = 3$, compared to the OVA + RSV group).

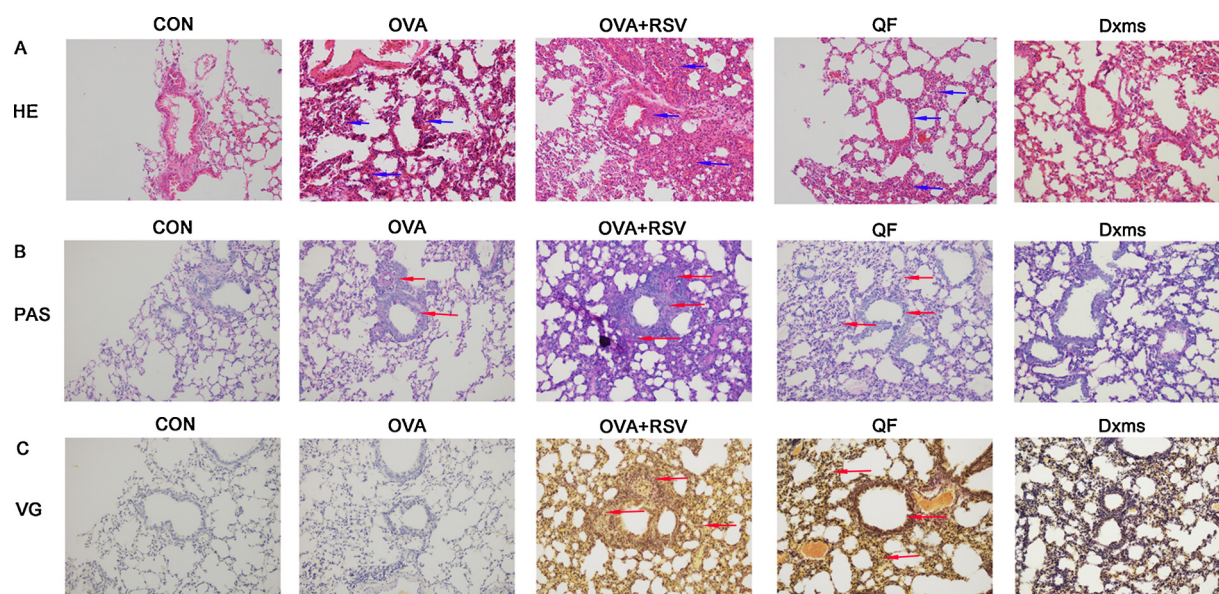


Fig. 7. Effects of QF on pulmonary tissues.

According to the H&E staining, pulmonary tissues from the OVA + RSV group showed interstitial pneumonia, a narrowed alveolus cavity, an infiltration of inflammatory cells, and capillaries that had become dilated and congested, though the QF group had significant improvements for all pathologies. From the PAS staining, the OVA + RSV group experienced mucus hypersecretions, whereas the QF treatment group only experienced minor secretions. From the VG staining, the QF group had the lowest amount of collagen hyperplasia around the respiratory airways and interstitial tissues when compared to the OVA + RSV group.

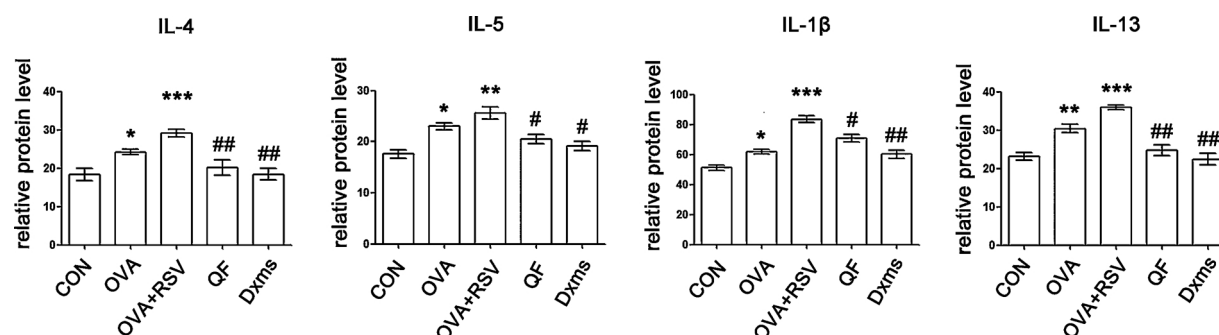


Fig. 8. Effects of QF on inflammation.

Serum inflammation concentrations were evaluated by ELISA. Data are presented as means $\pm$ SD. Serum inflammation levels with a superscripted symbol indicate a significant difference when comparison to the control group (* P < 0.05, ** P < 0.01 and *** P < 0.001) and the OVA + RSV group (# P < 0.05, ## P < 0.01), (n = 3).

46 $\pm$ 5% and a 12 h light/dark schedule. Mice were given free access to food and water and acclimated to their new environment for 5–7 days before the experiments began. Fifty mice were randomly divided into 5 equal groups: control group treated with sodium, OVA group, OVA + RSV group, QF treatment group (based on OVA + RSV group), and Dxms treatment group (based on OVA + RSV group). This study was approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University.

2.3.3. Model and treatment

The OVA sensitization and challenge procedures were performed as indicated previously, with a few modifications [31]. Briefly, the control group received intraperitoneal (IP) injections of PBS containing aluminum hydroxide gel, but were not subjected to intranasal challenge. These mice were sensitized via IP injection of OVA (OVA 20 μ g, V grade, Sigma, plus 2 mg aluminum) on day 0, 14, and 21, and via aerosol nebulization with OVA (1% in PBS) using a 402 AI ultrasonic atomizer (Shanghai Yuyue Medical Equipment Co., Ltd) from days 22–30. For the OVA + RSV group (based on OVA group, RSV1.0 $\times$ 10⁶ PFU·mL⁻¹ in 50 μ L), the procedures for RSV (ATCC) were modified from methods described by Sun [32] and occurred in the morning of

days 28, 30, and 32. The QF group (based on OVA + RSV group, QF 6.365 g·kg⁻¹·d⁻¹) and the Dxms group (based on OVA + RSV group, Dexamethasone 0.2 mg kg⁻¹·d⁻¹) were given doses during the afternoon on days 25, 27, 29, and 31, while OVA and OVA + RSV groups were treated with PBS. At the end of the challenge, all mice were anesthetized with 2.5 % Pentobarbital sodium to test for pulmonary function, and then blood and lungs were collected for further research. The timeline of the experiment showed in (Fig. 5).

2.3.4. Measurement of airway hyperresponsiveness (AHR)

AHR was evaluated by Buxco's modular and invasive system (Buxco Electronics Inc., NY, USA). Changes in airway resistance (R_L) and pulmonary dynamic compliance (C_{dyn}) were measured as described by Amdur and Mead [33]. Briefly, each anesthetized mouse was tracheostomized and intubated with a cannula, and then placed in a supine position inside a plethysmograph chamber connected to a ventilator. After a stable baseline airway pressure (< 5% variation over 2.5 min) was achieved, all mice were given aerosolized PBS or various concentrations of methacholine (3.125, 6.25, 12.5, or 25 mg mL⁻¹) via a jet nebulizer spraying into the head chamber. Minimum values for R_L and C_{dyn} were calculated in response to increasing concentrations of

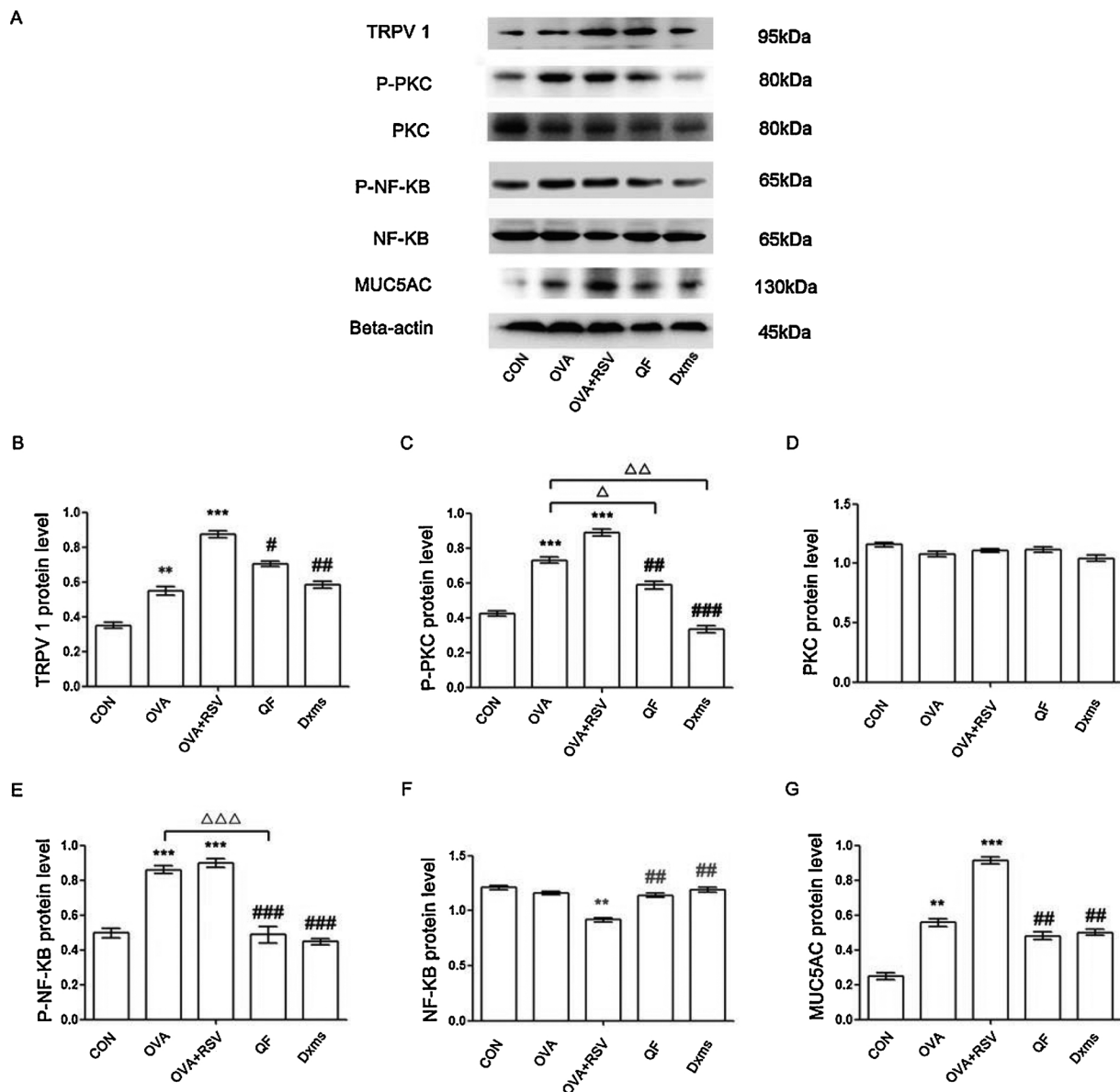


Fig. 9. Effects of QF on levels of TRPV1, P-PKC, PKC, P-NF-κB, NF-κB and MUC5AC in pulmonary tissues. Expression levels of TRPV1, P-PKC, PKC, P-NF-κB, NF-κB and MUC5AC were normalized to the levels of β-actin, and protein levels of the control group were set to 0. Values represent the means ± SD of 3 mice. Levels obtained from the QF and Dxms groups were significantly different when compared to the control group (**P < 0.01, ***P < 0.001) and the OVA + RSV group (##P < 0.01, ###P < 0.001; (n = 3).

inhaled methacholine and expressed as a percentage of change from the baseline value, as in previous studies [34].

2.3.5. Histopathological evaluation

The lungs were fixed overnight in 4% buffered paraformaldehyde and embedded in paraffin. Lung sections (4 μm) were stained with hematoxylin and eosin (H&E), Periodic Acid Schiff (PAS), and Van Gieson (VG), and observed under a light microscope at high magnification (200×) (Leica, German).

2.3.6. ELISA

The levels of inflammatory factors in the serum were analyzed using an ELISA according to the manufacturer's instructions (USCN Business Co., Ltd. China).

2.3.7. Western blotting

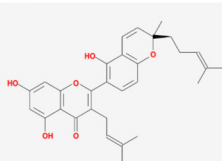
Lung tissues were split by an ultrasound homogenizer in a sample buffer of 80 % RIPA (Beyotime, China, P0013B) and 10 % PMSF

(Beyotime, China, ST506), and then centrifuged at 20,000 × g and 4°C for 15 min. After letting the sediment rest, the lysates were mixed with SDS-PAGE Sample Loading Buffer (Beyotime, China, P0015 L) and heated in a metal bath at 100°C for 10 min, then fractionated by electrophoresis on different concentrations of polyacrylamide-SDS gel, according to their molecular weights, at 80 V for 30 min and then 120 V for an hour. Next, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), incubated in a 5% BSA sealing buffer at room temperature for 2 h, and then placed in a buffer containing rabbit or mouse polyclonal antibodies against TRPV1 (Proteintech, Wuhan, China, 1:1,000 dilution), Phospho-Protein Kinase C (P-PKC) (CST, USA, 1:1,000 dilution), PKC (CST, USA, 1:1,000 dilution), NF-κB (CST, USA, 1:1,000 dilution), and MUC5AC (GeneTex, CA, USA, 1:200 dilution) overnight at 4°C. This was followed by an incubation with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies at room temperature. After washing, proteins were detected by enhanced chemiluminescence (Thermo Scientific, Waltham, MA, USA, and Clarity™ Western

A

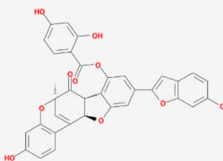
B

<http://tcmspw.com/molecule.php?qn=12757>

Molecule ID	MOL012757								
Molecule name	sanggenone J								
Download structure (click to download)									
Pharmacological and molecular properties data	MW	AlogP	Hdon	H...	OB (%)	Caco-2	BBB	DL	F
	488.62	6.83	3	6	0.99	0.79	-0.08	0.91	0
InChIKey	JGWYWHQILBNHBH-SSEXGKCCSA-N								
Pubchem Cid	N/A								
CAS	♦ 86450-78-4								
Molecule synonyms	Molecule synonyms								
	sanggenone J								
Related diseases	N/A								
Related targets	N/A								
Related Herbs	Chinese name	Latin name							
	桑白皮	Mori Cortex							

MOL012757 sanggenone J

<http://tcmspw.com/molecule.php?qn=12728>

Molecule ID	MOL012728						
Molecule name	mulberrofuran M						
Download structure (click to download)							
Pharmacological and molecular properties data	MW	AlogP	Hdon	H...	OB (%)	Caco-2	BBB
	590.56	5.56	4	10	10.67	0.11	-1.34
InChIKey	YZGCRYQEDYGFIA-JSWXEYGCZSA-N						
Pubchem Cid	N/A						
CAS	♦ 101365-03-1						
Molecule synonyms	Molecule synonyms						
	mulberrofuran M						
Related diseases	N/A						
Related targets	N/A						
Related Herbs	Chinese name	Latin name					
	桑白皮	Mori Cortex					

MOL012728 Mulberrofuran M

MOL012757 sanggenone J

MOL012728 Mulberrofuran M

Fig. 10. Absolute values of the binding constants of Sanggenone J-TRPV1 and Mulberrofuran M-MUC5AC complexes.

(A) The binding constants of the Sanggenone J-TRPV1 complex.

(B) The binding constants of the Mulberrofuran M-MUC5AC complex.

ECL Substrate, Bio-Rad, USA). Of note, β -actin was used in each lane as a control to normalize the expression of the proteins.

2.3.8. 3D modeling

The structure of the ingredients in QF, including *Herba Ephedrae*, *Semen Armeniacae Amarum*, *Gypsum Fibrosum*, *Scutellariae Radix*, *Mori Cortex*, *Descurainiae Semen*, *Lepidii Semen*, *Peucedani Radix*, *Polygoni Cuspidati Rhizoma et Radix*, and *Salviae Miltiorrhizae Radix et Rhizoma*, are available at TCMSP (<http://tcmspw.com/index.php>). The hydrogenation of the ingredients and calculation of their charges were carried out by MGLTools. The structures of TRPV1 (PDB: 3J5R) and MUC5AC (PDB: 5AJN) were reconstructed by single particle cryo-microscopy. These structures were then uploaded to POCASA (<http://altair.sci.hokudai.ac.jp/g6/service/pocasa/>) to predict their ligand binding sites. Here, the grid box coordinates and sizes were entered, and then the docking of receptors by the aforementioned ingredients in QF was obtained using AutoDock Vina 1.1.2. The strongest complexes were chosen according to the docking score, and then drawn and analyzed by Pymol 2.3 and Meastro 11.9.

2.3.9. Statistical analysis

The data were analyzed and presented as the mean $\pm$ standard deviations (SD). Datasets with 3 or more groups were analyzed by one-way analysis of variance (ANOVA) or one-way ANOVA with repeated measures and the least significant difference post hoc test when doing multiple comparisons. Comparisons of 2 groups were analyzed using Student's *t*-test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Compounds of QF

The extract of QF was analyzed by LC-MS. The LC-MS

chromatogram is shown in Fig. 2. The separated compounds were clarified by comparing the *Rt* values and the MS characteristics in total ion flow polarity modes (see Table 1).

After comparing the mass spectrum information in the blank extract and the compound medicine extract, 14 chemical components in the compound were preliminarily identified. The identification results are shown in Table 1, and the atlas is shown in Fig. 2 and 3.

3.2. Appearance of RSV-infected Hep-2 cells

Under the microscope, Hep-2 cells form a single layer at the bottom of the culture plate, packed evenly and closely together, and forming the shape of polygons or "paving stones". After 3–5 days, RSV A2 infects Hep-2 cells, causing them to fuse and form a "multinucleated giant cell" syncytium (Fig. 4).

3.3. Effect of QF on AHR

To evaluate the effect of QF on AHR in the OVA and OVA + RSV groups, airway compliance was assessed 24 h after aerosolized PBS or methacholine (at 3.125, 6.25, and 12.5 mg/mL) were given via a jet nebulizer spraying into the head chamber. Only mild changes in R_L and C_{dyn} were observed in control mice; however, there was a significant enhancement of AHR in the OVA and OVA + RSV groups, with an increase in R_L and decrease in C_{dyn} compared to control mice ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). When subjected to methacholine, administration of QF reduced AHR and enhanced airway compliance in the treatment groups compared to the OVA and OVA + RSV groups ($##P < 0.01$, $###P < 0.001$). Interestingly, the Dxms treatment group also experienced a reduction of AHR and an increase in airway compliance (Fig. 6).

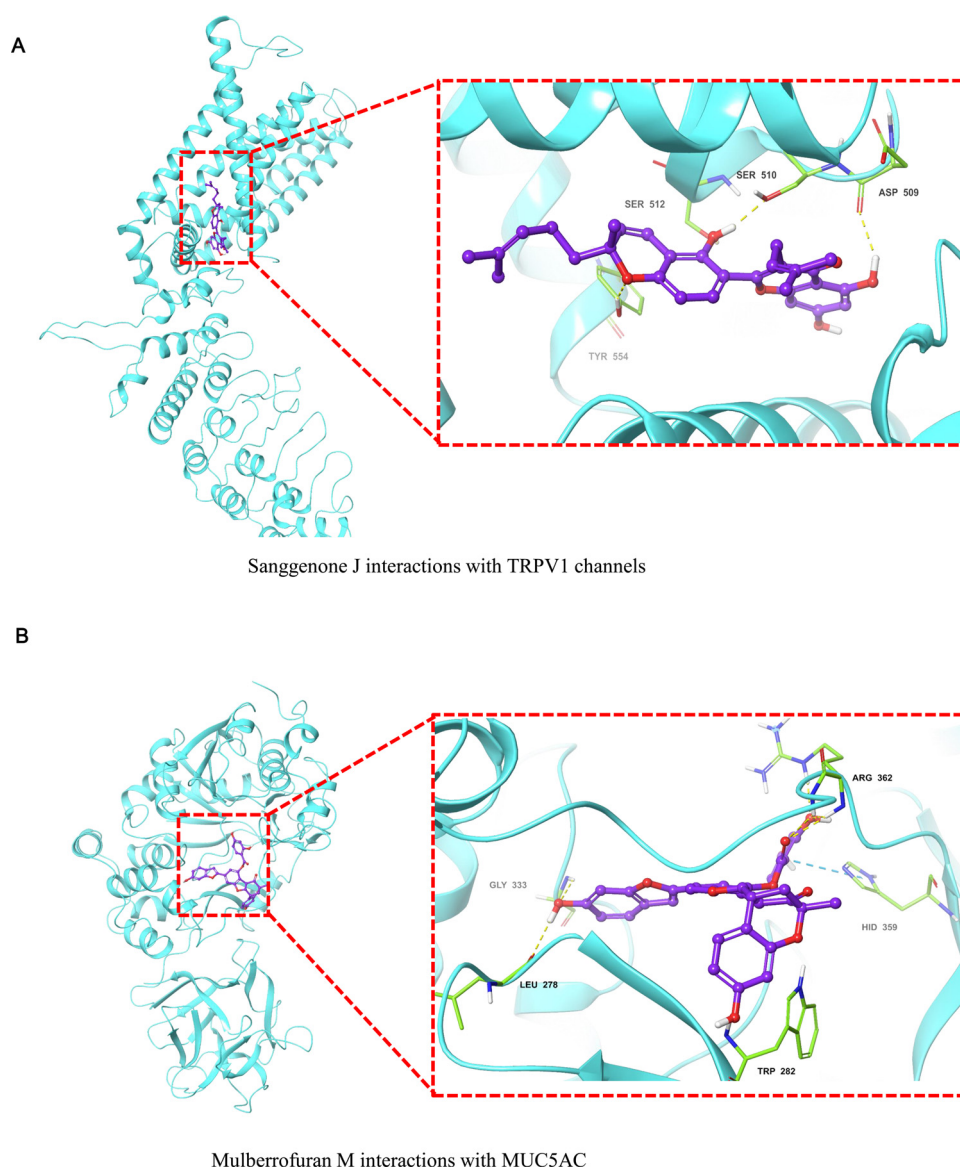


Fig. 11. Modeling of Mulberrofuran M interactions with MUC5AC and Sanggenone J interactions with TRPV1 channels.

(A) Interaction between Sanggenone J and TRPV1. Key functional residues for TRPV1 are marked.

(B) Interaction between Mulberrofuran M and MUC5AC. Key functional residues for MUC5AC are marked.

3.4. Effect of QF on pulmonary inflammation

To evaluate the inhibitory effects of QF on pulmonary inflammation, tissue samples were stained with H&E, PAS, and VG. As shown in Fig. 7, a significant infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues was observed in all OVA + RSV groups; however, treatment by QF attenuated airway inflammation at a dose of $6.365 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. In addition, the OVA + RSV groups experienced a marked increase of peribronchial collagen deposition and mucus hypersecretion within the bronchi of their lungs, though treatment by QF and Dxms reduced both of these pathologies.

3.5. Effect of QF on inflammatory cytokines

Asthmatic airway inflammation is characterized by an imbalance of interleukin cytokines, such as IL-1 β , IL-4, IL-5, and IL-13. Thus, to determine whether QF has the potential to modulate this imbalance, levels of IL-1 β , IL-4, IL-5, and IL-13 were measured by ELISA in serum collected from the mice. As shown in Fig. 8, we found that the serum levels of IL-1 β , IL-4, IL-5, and IL-13 were higher in the OVA and

OVA + RSV groups than in the control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The QF and Dxms treatment groups experienced large decreases in serum levels of these cytokines compared to their respective OVA + RSV groups ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

3.6. Effects of QF on TRPV1, P-PKC, PKC, NF- κ B, and MUC5AC protein levels

It was found that TRPV1 is involved in the regulation of airway inflammation and mucus hypersecretion by TRPV1/PKC/MUC5AC signaling, and our research showed QF could reduce this inflammatory response in mice. As shown in Fig. 9, we found that the protein levels of TRPV1, P-PKC, NF- κ B, and MUC5AC were higher in the OVA and OVA + RSV groups than in the control group (** $P < 0.01$, *** $P < 0.001$). Importantly, mice exposed to QF treatment exhibited significantly reduced levels of TRPV1, P-PKC, NF- κ B, and MUC5AC compared to the OVA and OVA + RSV groups ($^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$), and exposure to Dxms decreased the protein levels of TRPV1, P-PKC, NF- κ B, and MUC5AC compared to the OVA + RSV group ($^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$).

3.7. Identification of the binding sites of TRPV1 and MUC5AC

For MUC5AC, residues LEU278, TRP282, GLY333, HID359, and ARG362 at two adjacent loops and a β -sheet were found to comprise a key binding spot. For TRPV1, residues ASP509, SER510, SER512, and TYR554 at two adjacent α -helices were considered a key binding spot, positioned to potentially coordinate the docking of a ligand. The binding energy required for docking compounds to MUC5AC or TRPV1 is shown in Figs. 8A and B, respectively. Large values for the binding constant indicate less free energy, which reveal a strong affinity between the receptor and its ligand. When the binding constant is greater than 8, the docking of small molecules to receptors is more likely (Figs. 10A and B).

Following screening with multiple ligands, Mulberrofuran M was found to bind most strongly to MUC5AC and Sanggenone J was found to bind most strongly to TRPV1 (Figs. 11A and B, respectively).

4. Discussion

Asthma is a respiratory disease that restricts airflow, characterized by wheezing, shortness of breath, tightness of chest and coughing culminating in chronic airway inflammation [35]. According to the GINA Committee and the ISAAC Phase Three Study Group, 300 million people worldwide are affected - 4.9 % being children and 3.2 % located in Asia-Pacific, specifically [1,35]. RSV infection in asthmatic models triggers the release of inflammatory cytokines, reduces pulmonary function, damages airways, as well as promotes AHR and mucus hypersecretion. In the treatment of pediatric asthma, corticosteroids, theophylline, and β 2 agonists have been successful in improving pulmonary airflow and decreasing inflammation, but controlling airway remodeling events such as AHR and mucus hypersecretion has been limited [36].

In this study, we infected Hep-2 cells with RSV to form syncytia and then introduced them into OVA-induced asthmatic mice. Evaluation of their pulmonary tissues revealed a significant infiltration of inflammatory cells, mucus secretions, and collagen hyperplasia. Treatment with QF, however, significantly minimized these pathologies on all counts.

ELISA tests informed us that QF could dominate RSV-asthma conditions with reducing IL-1 β cytokine levels in the serum of mice. QF inhibited Th2 cells to secrete IL-4, IL-5, and IL-13 in a certain way and delayed the pathological changes of asthma in different aspects.

Most prominently, our results suggest that QF inhibits TRPV1 signaling, thereby downregulating NF- κ B protein expression and helping to balance inflammatory cytokines. Our previous studies found that QF reduced the expression of IL-13, IL-25, IL-33 cytokine levels in RSV-infected asthmatic mice [37], but the mechanism was unclear. A novel finding in the field is that TRPV1 regulates NF- κ B and its downstream cytokines, both Th2 cytokines (e.g., IL-4, IL-5, and IL-13) and epithelial cell-derived cytokines (i.g. IL-25, and IL-33) in an asthmatic attack [38,39], and blocking TRPV1 expression has been shown to decrease these cytokine levels [13,16]. Additional research has shown that TRPV1 activation is promoted by PKC phosphorylation [40], and in our study, we found that the PKC phosphorylation level was significantly increased in OVA + RSV mice and decreased in mice exposed to QF treatment. It is possible that by inhibiting PKC phosphorylation of TRPV1, QF inhibits TRPV1-mediated channel activation, thereby alleviating AHR and mucus hypersecretion. This finding aligns with our previous studies showing QF reduces expression of the TRPV1 and MUC5AC proteins [37]. Finally, we identified the active binding sites of TRPV1 and MUC5AC, respectively, to Sanggenone J and Mulberrofuran M. It is these compounds, specifically, that help to prevent the stimulation of inflammatory pathways and pulmonary damage in RSV-asthma.

In conclusion, RSV-infection in asthmatic models includes the release of inflammatory cytokines triggered by TRPV1 and inflammation

of the respiratory airways [25]. We demonstrate that QF inhibits TRPV1 signaling, resulting in a reduction of asthmatic complications such as AHR and mucus hypersecretion in RSV-infected asthmatic mice. Further studies are examining the anti-asthmatic actions of QF in vitro and in TRPV1^{-/-} mice are needed to sufficiently support our conclusions.

Declaration of Competing Interest

The authors declare no conflicts of interest regarding the study or preparation of the manuscript.

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