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Original Research Article

Effective-constituent compatibility-based analysis of Bufei Yishen formula, a traditional herbal compound as an effective treatment for chronic obstructive pulmonary disease

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ABSTRACT

Objective: Critical effective constituents were identified from Bufei Yishen formula (BYF), a traditional herbal compound and combined as effective-constituent compatibility (ECC) of BYF I, which may have potential bioactive equivalence to BYF.
Methods: The active constituents of BYF were identified using four cellular models and categorised into Groups 1 (Bufeiqi), 2 (Bushen), 3 (Huatan) and 4 (Huoxue) according to Chinese medicinal theory. An orthogonal design and a combination method were used to determine the optimal ratios of effective constituents in each group and the ratios of "Groups 1 to 4" according to their pharmacological activity. We also comprehensively assessed bioactive equivalence between the BYF and the ECC of BYF I in a rat model of chronic obstructive pulmonary disease (COPD).
Results: We identified 12 active constituents in BYF. The numbers of constituents in Groups 1 to 4 were 3, 2, 5 and 2, respectively. We identified the optimal ratios of effective constituents within each group. In Group 1, total ginsenosides:*Astragalus* polysaccharide:astragaloside IV ratio was 9:5:2. In Group 2, icariin:schisandrin B ratio was 100:12.5. In Group 3, nobiletin:hesperidin:peimine:peiminine:kaempferol ratio was 4:30:6.25:0:0. In Group 4, paeoniflorin:paeonol ratio was 4:1. An orthogonal design was then used to establish the optimal ratios of Group 1, Group 2, Group 3 and Group 4 in ECC of BYF I. The ratio for total ginsenosides:*Astragalus* polysaccharide:astragaloside IV:icariin:schisandrin B:nobiletin:hesperidin:peimine:paeoniflorin:paeonol was determined to be 22.5:12.5:5:100:12.5:4:30:6.25:25:6.25. A comprehensive evaluation confirmed that ECC of BYF I presented with bioactive equivalence to the original BYF.
Conclusion: Based on the ECC of traditional Chinese medicine formula method, the effective constituents of BYF were identified and combined in a fixed ratio as ECC of BYF I that was as effective as BYF itself in treating rats with COPD.

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1. Introduction

Traditional Chinese medicine (TCM) is a unique system originally developed to help the ancient Chinese population contend with various diseases. It is still regarded as an important part of the current medical system [1,2]. TCM treats disease mainly with herbal formulae based on the holistic theory and combinatorial principles such as tonifying qi (Buqi), tonifying kidney (Bushen),

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resolving phlegm (Huatan), and activating blood (Huoxue), according to each syndrome ("Zheng" in Chinese) [2]. Herbal medicines are mixtures of numerous active ingredients. Further, most of the active constituents in the herbs act on multiple targets and exert different biological functions [3,4]. Consequently, identifying the effective constituents and elucidating their modes of action are essential for understanding the scientific basis of TCM formulae. To this end, a constituent-based Chinese medicine was proposed. It comprises novel TCM formulae consisting of effective constituents of Chinese medicine [5]. As a rule, the first step is to identify the constituents of the medicines in the formulae [3]. Next, cellular or animal models of the target disease or its pathological stages are established, the effective constituents of the Chinese medicine are identified, and a candidate prescription is constructed and optimised according to TCM compatibility theory and principles, experimental studies, and clinical trials. In this way, constituent-based Chinese medicines containing stable, effective substances are prepared [5]. Identification of the effective constituents in constituent-based Chinese medicine facilitates clarification of their modes of actions.

In TCM, chronic obstructive pulmonary disease (COPD) is classified as a lung distention (Feizhang disease) [6] whose typical syndrome is a lung-kidney qi deficiency characterised by panting, shortness of breath, lassitude, spontaneous sweating, weakness in the lower back and knees, tinnitus, vertigo, frequent urination at night, and soreness and weakness of the waist and knees [7]. The Bufei Yishen formula (BYF; Pat. No. ZL.201110117578.1) comprises twelve medicinal herbs and is prescribed for COPD patients with the aforementioned syndrome. BYF is composed of twelve medicinal herbs belonging mainly to one of the four efficacy-oriented groups (Group 1, Group 2, Group 3 and Group 4). BYF treatment alleviates clinical symptoms, reduces exacerbation frequency, delays acute exacerbation, and improves pulmonary function and exercise capacity in COPD [8]. Moreover, a system pharmacology-based analysis of BYF showed that the twelve medicinal herbs in BYF contained 216 active compounds and hit 195 potential targets [9]. A comprehensive analysis of system pharmacology, transcriptomics, proteomics, and metabolomics data for BYF-based COPD treatment showed that BYF had long-term therapeutic efficacy against COPD by regulating lipid metabolism, oxidative stress, cell junction pathways, and inflammatory responses at the system level [10]. However, the complexity of the effective substance makes it difficult to standardise quality and stability, validate efficacy, safety and dosage, and understand the therapeutic mechanisms of BYF.

Here, we established four cellular models by subjecting alveolar epithelial cells (A549) to tumor necrosis factor-α (TNF-α), exposing macrophages (THP-1) to lipopolysaccharide (LPS), treating A549/THP-1 co-cultures with LPS, and challenging endothelial cells with hypoxia and LPS. The aforementioned treatments characterised the critical pathological changes associated with COPD. Cellular models were then applied to identify the active constituents in each group of candidate constituents and evaluate their pharmacological activity in combination. An orthogonal design was used to establish the optimal ratios of effective constituents within each group and the ratios of Groups 1–4. A combination of effective constituents in four groups with optimal ratios was designated as having effective-constituent compatibility (ECC) of BYF I. We also investigated the therapeutic effects of BYF and ECC of BYF I on a laboratory-induced rat COPD model and comprehensively assessed bioactive equivalence between BYF and ECC of BYF I. Finally, we constructed the effective-constituent compatibility of BYF by identifying active constituents, optimizing their proportion, and evaluating therapeutic effect on COPD rats.

2. Materials and methods

2.1. Reagents and chemicals

Ginsenosides Rb1, Re, Rg1 and Rg3, icariin, betaine, and synephrine were purchased from the National Institutes for Food and Drug Control (Beijing, China). Ginsenosides Rh1 and Rh2, protopanoxadiol, astragaloside IV, schisandrin B, peimine, peiminine, hesperidin, nobiletin, bergenin, quercetin, kaempferol, paeoniflorin, and paeonol were obtained from Chengdu Must Bio-Tech Co. Ltd. (Chengdu, China). *Astragalus* polysaccharide, *Lycium barbarrum* polysaccharide, and total ginsenoside were prepared by Dr. Feng Suxiang of the Henan University of Chinese Medicine, Henan, China. LPS from *Escherichia coli* 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF-α was obtained from PeproTech (Rocky Hill, NJ, USA). Human interleukin (IL)-8, IL-6, IL-1β, IL-23, matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1, endothelin, tissue factor, tissue plasminogen activator (tPA), and tPA inhibitor-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster (Wuhan, China). Thrombomodulin and endothelial nitric oxide synthase ELISA kits were obtained from Elabscience Biotechnology (Wuhan, China).

2.2. Cell culture, treatment, and cell viability assay

A549 and THP-1 cell lines, and human umbilical vein endothelial cells (HUVECs) were obtained from the Chinese Academy of Science Type Culture Collection (Shanghai, China). A549 and THP-1 cells were cultured in RPMI 1640 medium (Beijing Solarbio Science & Technology, Beijing, China) containing 10% foetal bovine serum (FBS) (Sijiqin, Tianhang Biotechnology, Hangzhou, China). HUVECs were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified 5% CO_2 atmosphere.

A549, THP-1 and HUVEC cells were seeded in six-well plates. The A549 cells were stimulated with TNF-α (10 ng/mL) and the THP-1 cells were stimulated with LPS (2 μg/mL). Both were subjected to various concentrations of the constituents for 48 h. The HUVECs were incubated at 37 °C in 5% CO_2 and 95% N_2, stimulated with LPS (2 μg/mL), and exposed to different constituent concentrations for 48 h. In the co-culture system, A549 cells were cultured in 6-well plates and THP-1 cells were seeded in a Transwell insert to prevent direct contact between the two cell types. The cells in the co-culture system were treated with LPS (2 μg/mL) and subjected to various concentrations of constituents for 48 h. The cells and supernatants were separately harvested and their protein expression levels were measured.

2.3. Preparing effective constituents

In a previous study, a systems pharmacology model derived 216 active compounds from BYF [9]. Earlier studies on the pharmacological activity of these compounds identified 24 constituents in nine herbal medicines as candidate constituents exerting pharmacological activity against pulmonary diseases. BYF was assigned to four groups in accordance with the theory of Chinese herbal medicine (Table 1).

2.3.1. Preparation of total ginsenosides

Dried Ginseng Radix et Rhizoma powder was extracted twice by boiling with 70% (v/v) ethanol for 90 min each time. The extracts were combined, filtered, concentrated under reduced pressure, added to water, absorbed by a D101 macroporous resin column, and eluted with water, 30% (v/v) ethanol, and 70% (v/v) ethanol

Table 1
Bufei Yishen formula and their candidate constituents were divided into four groups.

Efficacy-oriented group	Herbal medicine	Candidate compounds
Buqi	Ginseng Radix et Rhizoma	Ginsenosides Rb1, Re, Rg1, Rg3, Rh1 and Rh2, protopanoxadiol, and total ginsenosides
	Astragali Radix	Astragaloside IV and *Astragalus* polysaccharide
Bushen	Epimedii Folium	Icariin
	Lycii Fructus	*Lycium barbarum* polysaccharide and betaine
	Schisandrae Chinensis Fructus	Schisandrin B
Huatan	Fritillariae Thunbergii Bulbus	Peimine and peiminine
	Citri Reticulatae Pericarpium	Hesperidin, nobiletin and synephrine
	Ardisiae Japonicae Herba	Bergenin, quercetin and kaempferol
Huoxue	Paeoniaeradix Rubra	Paeoniflorin and paeonol

to yield major fractions that were later concentrated under reduced pressure. The extracts were exsiccated and pulverised. The total ginsenosides content was determined to be 58.21%. Finally, per gram dry extract obtained is equivalent to 25 g raw medical herbs.

2.3.2. Preparation of Astragalus polysaccharide and Lycium barbarum polysaccharide

Dried Astragali Radix or Lycii Fructus powder was extracted twice with boiling water for 1 h each time. The extracts were combined, filtered, concentrated under reduced pressure, and mixed with ethanol to a final 85% concentration. Overnight, the Lycii Fructus extract was filtered and powdered by freeze-drying. The Astragali Radix extract was isolated by centrifugation at 3500 r/min for 5 min, washed with ethanol, acetone, and diethyl ether, and freeze-dried. The polysaccharide content was determined by the phenol–sulfuric acid method. The Astragalus and Lycium barbarum polysaccharide concentrations were 60.18% and 56.93%, respectively. Total ginsenosides, *Astragalus* polysaccharide, and Lycium barbarum polysaccharide were analysed by ultraviolet Vis spectroscopy (supplementary Fig. 1S). Finally, per gram dry extract of *Astragalus* polysaccharide and Lycium barbarum polysaccharide obtained is equivalent to 18 and 14 g raw medical herbs, respectively.

2.4. Optimising the proportions of effective constituents

We performed a two-step orthogonal study to establish the optimal proportion for each effective constituent. First, we determined the optimal proportions of the effective constituents in each group. Second, we performed orthogonal experiments to optimise the proportions of Group 1, Group 2, Group 3 and Group 4.

In Step one, we ran orthogonal experiments to optimise the proportions of effective constituents in Group 1 and Group 3 and identified two effective constituents in Group 2 and two in Group 4. Combinations of the effective constituents and their 4 different concentrations were used to optimise their proportions. Sixteen proportions of effective constituents per group were obtained using various factors (Table 2) in accordance with an orthogonal design (4 levels for each factor; $L_{16}(4^n)$). The effects of each combination (16 proportions per group) on the inflammatory response (IL-1β, IL-6, IL-8, IL-23 and MMP-9) were evaluated in the A549/THP-1 co-culture model and the optimal proportions of the effective constituents in each group were identified according to their weighted averages.

In Step two, effective constituents in each group were subjected to orthogonal experiments ($L_{16}(4^4)$) to form the ECC of BYF I (Table 3). Ratio of each constituent, which was set in 4 dose levels, was based on the results of the first-step selection.

2.5. COPD rats and drug administration

Sprague-Dawley rats ([200 ± 20] g) and twenty C57BL/6 mice were obtained from Biotechnology Co., Ltd. (Beijing, China). Animals were maintained in specific pathogen-free facilities and housed in filter-top cages with free access to food and water under a 12 h light:12 h dark cycle in plastic cages at (25 ± 2) °C with a relative humidity of 50% ± 10%. The animal experiments were approved by Experimental Animal Care and Ethics Committee of the First Affiliated Hospital, Henan University of Chinese Medicine. COPD rats and BYF were prepared as described in a previous study [11]. Briefly, the rats were placed in a closed chamber and exposed to tobacco from weeks 1 to 12 and recurring *K. pneumoniae* infections from weeks 1 to 8. Each group has 12 Sprague-Dawley rats. The COPD rats were orally treated with normal saline, BYF (3.7 g/kg), ECC of BYF I (19.74, 9.87 and 4.94 mg/kg), and aminophylline (APL, 54 mg/kg) daily from weeks 9 to 20. Normal saline was orally administered to the control rats. All rats were anaesthetised and sacrificed at week 20 and the lung tissues, blood, and bronchoalveolar lavage fluid (BALF) were harvested.

Table 2
Concentration of constituents in Buqi, Hutan, Bushen and Huoxue groups for Step one.

Group	Constituent	Level 1 (μg/mL)	Level 2 (μg/mL)	Level 3 (μg/mL)	Level 4 (μg/mL)
Buqi	Total ginsenosides (A)	11.25	22.5	45	90
	Astragalus polysaccharide (B)	0	25	50	100
	Astragaloside IV (C)	0	10	20	40
Huatan	Nobiletin (A)	0	2	4	8
	Hesperidin (B)	0	7.5	15	30
	Peimine (C)	0	6.25	12.5	25
	Peiminine (D)	0	6.25	12.5	25
	Kaempferol (E)	0	10	20	40
Bushen	Icariin (A)	12.5	25	50	100
	Schisandrin B (B)	12.5	25	50	100
Huoxue	Paeoniflorin (A)	12.5	25	50	100
	Paeonol (B)	3.13	6.25	12.5	25

Table 3
Concentration of effective constituents in Buqi, Bushen, Huatan and Huoxue groups.

Group	Constituent	Level 1 (μg/mL)	Level 2 (μg/mL)	Level 3 (μg/mL)	Level 4 (μg/mL)
Buqi	Total ginsenosides (A)	11.25	22.5	45	90
	Astragalus polysaccharide (B)	6.25	12.5	25	50
	Astragaloside IV (C)	2.5	5	10	20
Bushen	Icariin (A)	12.5	25	50	100
	Schisandrin B (B)	1.5625	3.125	6.25	12.5
Huatan	Nobiletin (A)	1	2	4	8
	Hesperidin (B)	7.5	15	30	60
	Peimine (C)	1.56	3.13	6.25	12.5
Huoxue	Paeoniflorin (A)	12.5	25	50	100
	Paeonol (B)	3.13	6.25	12.5	25

The herbal drugs in BYF were identified and prepared as fluid extracts. BYF fingerprints for 10 batches and 20 chemical constituents of BYF were identified in a previous study [11]. All experiments were conducted in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the First Affiliated Hospital, Henan University of Chinese Medicine, Henan, China.

2.6. Pulmonary function and histological analyses

Pulmonary function was evaluated every 4 weeks by unrestrained pulmonary function testing plethysmography from week 0 to week 20. At week 20, all rats were anaesthetised and their tidal volume (TV), minute volume (MV), peak expiratory flow (PEF), forced vital capacity (FVC), forced expiratory volume at 0.1 s (FEV0.1 s), functional residual capacity (FRC), and FEV0.1 s/FVC were measured.

Lung tissues were fixed with 10% (v/v) formalin neutral buffer solution, embedded in paraffin, cut into sections, and stained with Mayer's haematoxylin and 1% eosin alcohol solution.

2.7. Kit analysis

The IL-1β level in BALF and IL-6 and MMP-9 levels in serum were measured with ELISA kits according to the manufacturer's instructions. Total antioxidant capacity (T-AOC) and lipid peroxidation (LPO) in the serum were detected with kits (Jiancheng, Nanjing, China). All determinations were performed in triplicate.

2.8. Comprehensive R-value evaluation

The *R*-value was calculated according to formulae (1)–(5).
Model effect:

$$dM = \frac{\bar{x}_2 - \bar{x}_1}{s} s = \frac{s_2 + s_1}{2} \quad (1)$$

Treatment effects:

$$\text{APL effect}: dMe_1 = \frac{\bar{x}_3 - \bar{x}_2}{z_1} z_1 = \frac{s_2 + s_3}{2} \quad (2)$$

$$\text{BYF effect}: dMe_2 = \frac{\bar{x}_4 - \bar{x}_2}{z_2}, z_2 = \frac{s_2 + s_4}{2} \quad (3)$$

$$\text{ECC of BYF I effect}: dMe_3 = \frac{\bar{x}_5 - \bar{x}_2}{z_3}, z_3 = \frac{s_2 + s_5}{2} \quad (4)$$

$$R\text{-value}: R_i = \frac{dMe_i}{dM}, i = 1, \ldots 4 \quad (5)$$

Here, $\bar{x}$ and s represent the mean and standard deviation of the outcomes (lung function: TV, MV, PEF, FVC, FEV0.1s, FEV0.1s/FVC and FRC; histological changes: MLI and MAN; biomarkers: IL-1β, IL-6, MMP-9, T-AOC and LPO), respectively. The groups were: normal $\bar{x}_1 \pm s_1$, model $\bar{x}_2 \pm s_2$, APL $\bar{x}_3 \pm s_3$, BYF $\bar{x}_4 \pm s_4$, ECC of BYF I (high dose) $\bar{x}_5 \pm s_5$, ECC of BYF I (mid-dose) $\bar{x}_6 \pm s_6$, and ECC of BYF I (low dose) $\bar{x}_7 \pm s_7$.

For convenience, the *R*-values were transformed into *D*-values:

$$D = R - (-1) \quad (6)$$

2.9. Statistical analysis

The datasets were subjected to normality and homogeneity of variance tests. The least significant difference test and one-way analysis of variance in SPSS v. 22.0 (IBM Corp., Armonk, NY, USA) were applied to normally distributed data. Dunnett's T3 was used for all other data. Data are expressed as mean ± standard error of mean. $P < 0.05$ indicated significant difference.

3. Results

3.1. Candidate compounds identified

Here, we established an inflammatory model using co-cultured A549 and THP-1 cells in filter-separated mode. Paeoniflorin, paeonol, nobiletin, hesperidin/nobiletin/synephrine, hesperidin/nobiletin, ginsenosides Rg3 and Rh1, and total ginsenosides downregulated IL-1β, IL-6, IL-8, IL-23 and MMP-9. Hesperidin alone downregulated IL-1β, IL-8 and IL-23. Bergenin alone downregulated IL-6, IL-23 and MMP-9. Quercetin alone downregulated IL-6 and MMP-9. Kaempferol alone downregulated IL-23 and MMP-9 levels (supplementary Tables S1–S5).

Airway inflammation is a symptom of COPD. Hypoxia and inflammation often occur simultaneously as gas exchange is inadequate in this state. We exposed airway epithelial-like A549 cells to TNF-α and established an epithelial cell inflammatory response model. We also subjected the monocyte/macrophage cell line THP-1 to LPS and challenged endothelial cells with hypoxia and LPS. Using these models, we evaluated the activity of the candidate constituents.

Total ginsenosides, icariin, schisandrin B, peimine, peiminine, hesperidin, nobiletin, kaempferol, paeoniflorin and paeonol exerted substantial biological activity and were considered effective constituents.

3.2. Optimising the proportions of effective constituents

Based on the aforementioned candidate compound classifications, we identified 12 effective constituents among the main herbals in BYF and selected those whose pharmacological activity was typical for the groups to which they were assigned (Table 4). As Astragali Radix predominated in BYF, astragaloside IV and *Astragalus* polysaccharide were also treated as effective constituents.

Table 4
Candidate effective constituents of Bufei Yishen formula.

Efficacy-oriented group	Herbal medicine	Candidate compounds
Buqi	Ginseng Radix et Rhizoma	Total ginsenosides
	Astragali Radix	Astragaloside IV and *Astragalus* polysaccharide
Bushen	Epimedii Folium	Icariin
	Schisandrae chinensis Fructus	Schisandrin B
Huatan	Fritillariae Thunbergii Bulbus	Peimine and peiminine
	Citri Reticulatae Pericarpium	Hesperidin and nobiletin
	Ardisiae Japonicae Herba	Kaempferol
Huoxue	Paeoniaeradix Rubra	Paeoniflorin and paeonol

3.2.1. Optimising the proportions of effective constituents in various groups

In "Group 1", different ratios of constituents had different effects on the IL-1β, IL-6, IL-8, IL-23 and MMP-9 levels (Table 5). We then used the orthogonal design and weighted average method and established that the optimal ratio of total ginsenosides, *Astragalus* polysaccharide and astragaloside IV was 9:5:2 (A4B3C3, Table 6). The optimal ratio of the effective constituents in "Group 3" (nobiletin, hesperidin, peimine, peiminine and kaempferol) was 4:30:6.25:0:0 (Tables 7 and 8, A3B4C2D1E1). The optimal ratios for the effective constituents in "Group 2" and "Group 4" were identified by a combination method. For icariin and schisan-

Table 5
Effects of Buqi group constituents with different proportion on co-cultured A549 and THP-1 cells induced by lipopolysaccharide.

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (μg/mL)	IL-23 (pg/mL)	MMP-9 (μg/mL)
Normal	1.83 ± 0.74	14.15 ± 1.89	2.48 ± 0.21	418.23 ± 26.92	18.35 ± 1.51
Model	7.31 ± 1.20$^{\#\#}$	27.68 ± 2.13$^{\#\#}$	4.83 ± 0.15$^{\#\#}$	808.35 ± 43.34$^{\#\#}$	170.60 ± 8.73$^{\#\#}$
1	7.55 ± 0.26	28.33 ± 0.88	4.93 ± 0.15	885.21 ± 68.72	170.64 ± 1.24
2	6.39 ± 1.01	21.63 ± 3.34*	5.32 ± 0.49	641.33 ± 61.75*	93.67 ± 7.07**
3	5.37 ± 1.09	20.29 ± 1.34**	3.35 ± 0.29**	710.81 ± 46.82	72.18 ± 1.74**
4	15.14 ± 0.72	29.75 ± 2.01	4.98 ± 0.24	557.29 ± 25.92**	110.69 ± 4.01**
5	8.86 ± 5.34	22.13 ± 3.19	3.42 ± 0.23**	670.93 ± 50.32*	137.19 ± 12.59*
6	26.65 ± 0.77	31.31 ± 2.93	4.66 ± 1.01	759.51 ± 41.20	93.53 ± 11.46**
7	10.54 ± 1.10	16.47 ± 0.96**	5.36 ± 0.22	474.32 ± 62.93**	119.18 ± 3.09**
8	6.37 ± 0.05	16.97 ± 1.11**	4.65 ± 0.18	474.41 ± 49.11**	141.51 ± 12.93*
9	15.92 ± 0.89	34.07 ± 4.38	4.39 ± 1.72	877.21 ± 16.44	93.71 ± 4.10**
10	5.75 ± 0.91	14.63 ± 1.84**	4.24 ± 0.88	507.43 ± 40.13**	134.72 ± 3.86**
11	8.69 ± 0.65	15.93 ± 0.95**	2.02 ± 0.21**	517.42 ± 23.72**	98.34 ± 7.65**
12	13.84 ± 1.58	14.78 ± 0.65**	5.85 ± 1.48	464.23 ± 10.23**	110.86 ± 1.81**
13	12.19 ± 0.84	21.51 ± 0.74**	5.19 ± 0.11	529.72 ± 29.53**	73.67 ± 7.98**
14	7.28 ± 1.78	14.62 ± 0.73**	2.43 ± 0.26**	471.31 ± 38.22**	86.18 ± 4.69**
15	10.71 ± 0.56	17.86 ± 1.23**	3.70 ± 0.23**	516.84 ± 10.21**	82.40 ± 3.37**
16	6.55 ± 0.92	16.23 ± 0.38**	3.00 ± 0.13**	447.90 ± 24.39**	51.58 ± 1.96**

Data are expressed as mean ± standard error of mean, with n = 3. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model. IL: interleukin; MMP-9: matrix metalloproteinase-9.

Table 6
Results of $L_{16}(4^3)$ orthogonal experiment of Buqi group.

Group	Total ginsenosides (A)	*Astragalus* polysaccharide (B)	Astragaloside IV (C)	D	E	Weighted score
1	1	1	1	1	1	48.65
2	1	2	2	2	2	63.58
3	1	3	3	3	3	74.65
4	1	4	4	4	4	44.78
5	2	1	2	3	4	60.20
6	2	2	1	4	3	41.34
7	2	3	4	1	2	61.86
8	2	4	3	2	1	65.83
9	3	1	3	4	2	45.40
10	3	2	4	3	1	75.87
11	3	3	1	2	4	78.57
12	3	4	2	1	3	62.36
13	4	1	4	2	3	57.48
14	4	2	3	1	4	83.09
15	4	3	2	4	1	66.18
16	4	4	1	3	2	86.30
K1	57.92	52.93	63.72	63.99	64.13	
K2	57.31	65.97	63.08	66.37	64.29	
K3	65.55	70.32	67.24	74.26	58.96	
K4	73.26	64.82	60.00	49.43	66.66	
Range *R*	15.96	17.38	7.25	24.83	7.70	

Table 7
Effects of Huatan group constituents with different proportion on co-cultured A549 and THP-1 cells induced by lipopolysaccharide.

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-23 (pg/mL)	MMP-9 (μg/mL)
Normal	1.90 ± 0.16	0.35 ± 0.11	136.81 ± 18.64	9.51 ± 1.48	5.16 ± 1.32
Model	3.32 ± 0.28$^{\#\#}$	1.33 ± 0.01$^{\#\#}$	613.23 ± 5.8$^{\#\#}$	24.76 ± 1.25$^{\#\#}$	30.53 ± 1.21$^{\#\#}$
1	2.62 ± 0.07*	1.06 ± 0.01**	493.87 ± 8.71**	19.54 ± 0.44**	30.54 ± 1.40
2	5.05 ± 1.31	1.71 ± 0.07	710.26 ± 24.52	27.00 ± 0.78	27.72 ± 0.56*
3	3.98 ± 0.40	1.67 ± 0.06	714.25 ± 21.22	29.82 ± 1.38	32.00 ± 0.44
4	3.90 ± 0.38	1.51 ± 0.06	637.14 ± 19.90	23.61 ± 0.64	32.52 ± 1.91
5	3.58 ± 0.20	1.38 ± 0.06	546.34 ± 20.12**	20.60 ± 0.64**	26.15 ± 0.56**
6	3.64 ± 0.04	1.14 ± 0.06**	465.31 ± 20.23**	16.87 ± 0.64**	20.39 ± 0.54**
7	3.26 ± 0.10	1.03 ± 0.11**	394.60 ± 19**	15.91 ± 0.61**	16.91 ± 0.44**
8	2.52 ± 0.44	0.68 ± 0.10**	234.40 ± 16.8**	11.05 ± 0.54**	24.32 ± 3.25*
9	2.72 ± 0.22*	0.80 ± 0.10**	271.90 ± 17.5**	12.27 ± 0.56**	12.04 ± 0.26**
10	2.44 ± 0.13**	0.59 ± 0.10**	207.60 ± 17.00**	10.36 ± 0.54**	9.55 ± 0.25**
11	2.72 ± 0.14*	0.61 ± 0.12**	221.00 ± 20.90**	11.65 ± 0.67**	14.45 ± 4.02**
12	1.71 ± 0.49**	0.48 ± 0.12**	179.90 ± 20.00**	10.10 ± 0.64**	9.27 ± 0.34**
13	3.73 ± 0.15	1.09 ± 0.06**	461.10 ± 20.80**	18.59 ± 0.66**	18.58 ± 0.41**
14	3.74 ± 0.07	1.10 ± 0.13*	384.30 ± 21.80**	17.02 ± 0.70**	15.25 ± 0.54**
15	2.96 ± 0.29	0.79 ± 0.10**	267.60 ± 17.40**	12.41 ± 0.56**	13.59 ± 0.40**
16	3.55 ± 0.25	1.24 ± 0.06*	497.60 ± 19.40**	18.70 ± 0.62**	23.27 ± 0.64**

Data are expressed as mean ± standard error of mean, with n = 3. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model. IL: interleukin; MMP-9: matrix metalloproteinase-9.

Table 8
Results of $L_{16}(4^5)$ orthogonal experiment of Huatan group.

Group	Nobiletin (A)	Hesperidin (B)	Peimine (C)	Peiminine (D)	Kaempferol (E)	Weighted score
1	1	1	1	1	1	46.58
2	1	2	2	2	2	29.70
3	1	3	3	3	3	30.93
4	1	4	4	4	4	34.36
5	2	1	2	3	4	39.09
6	2	2	1	4	3	45.45
7	2	3	4	1	2	51.72
8	2	4	3	2	1	69.18
9	3	1	3	4	2	69.74
10	3	2	4	3	1	87.12
11	3	3	1	2	4	72.60
12	3	4	2	1	3	97.50
13	4	1	4	2	3	45.01
14	4	2	3	1	4	48.84
15	4	3	2	4	1	67.15
16	4	4	1	3	2	42.59
K1	35.39	50.11	51.81	61.16	67.51	
K2	51.36	52.78	58.36	54.123	48.44	
K3	81.74	55.6	54.67	49.93	54.72	
K4	50.90	60.90	54.55	54.18	48.72	
Range *R*	46.35	10.80	6.56	11.23	19.07	

drin B, it was 100:12.5 (Group 13, Table 9); for paeoniflorin and paeonol, it was 4:1 (Group 16, Table 10).

3.2.2. Optimising the proportions of groups 1–4

Sixteen proportions among four groups were obtained with an orthogonal design. We tested the anti-inflammatory effect of 16 ratios of four groups on the A549/THP-1 co-culture model, and then identified the optimal ratios of the four groups based on the best efficacy determined from the weighted average. Most of the combinations in the four-group combinations significantly downregulated IL-1β, IL-6, IL-8, IL-23 and MMP-9 levels (Table 11). Using the orthogonal design and weighted average, we identified 10 effective constituents in the four groups as candidate ECC of BYF I and the optimal ratios for them as total ginsenosides:*Astragalus* polysaccharide:astragaloside IV:icariin:schisandrin B:nobiletin:hesperidin:peimine:paeoniflorin:paeonol = 22.5:12.5:5:100:12.5:4:30:6.25:25:6.25 (A2B4C3D2, Table 12).

3.3. Therapeutic effects of the ECC of BYF I on COPD rats

To investigate the therapeutic effects of the ECC of BYF I and compare its efficacy with that of BYF, ECC of BYF I and BYF were orally administered to COPD rats and their effects on lung function, pathological changes, inflammatory response and oxidative stress were tested.

3.3.1. BYF and the ECC of BYF I ameliorated COPD induced by cigarette smoke and bacterial infection

BYF and ECC of BYF I were orally administered to rats from weeks 13 to 20. Body weight, lung mechanics, and pulmonary histopathology were analysed. Fig. 1 shows that cigarette smoke and bacterial infection exposure lowered body weight and growth rate but BYF suppressed these reductions.

Fig. 2A–C show that TV, MV and PEF decreased in the model rats relative to the control from weeks 4 to 20. In contrast, compare to the model group, BYF, medium-dose ECC of BYF I, and the bronchodilator APL significantly inhibited the aforementioned

Table 9
Effects of Bushen group constituents with different proportion on co-cultured A549 and THP-1 cells induced by lipopolysaccharide.

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-23 (pg/mL)	MMP-9 (μg/mL)	Weighted score
Normal	0.76 ± 0.13	4.80 ± 0.23	0.31 ± 0.04	23.42 ± 2.46	2.98 ± 0.45	
Model	1.55 ± 0.07$^{\#\#}$	8.45 ± 0.90$^{\#\#}$	0.79 ± 0.03$^{\#\#}$	46.83 ± 4.40$^{\#\#}$	12.42 ± 0.28$^{\#\#}$	
1	0.92 ± 0.18**	6.01 ± 0.41*	1.20 ± 0.27	42.44 ± 2.14	13.93 ± 1.09	56.20
2	0.53 ± 0.13**	5.91 ± 0.41*	0.88 ± 0.03*	33.80 ± 3.22*	6.71 ± 0.28*	74.70
3	0.98 ± 0.20**	9.42 ± 0.50	1.43 ± 0.27*	91.04 ± 6.80**	3.34 ± 0.16**	46.30
4	2.79 ± 0.59*	13.80 ± 1.10**	2.76 ± 0.47**	125.00 ± 31.90*	24.29 ± 6.89	22.10
5	0.68 ± 0.19**	5.48 ± 0.50**	1.16 ± 0.21*	32.64 ± 5.83*	11.21 ± 2.42	66.50
6	0.99 ± 0.15**	6.89 ± 0.47	0.99 ± 0.08*	36.61 ± 5.38	4.49 ± 0.64**	63.10
7	1.71 ± 0.41	13.64 ± 2.80*	0.15 ± 0.10**	93.00 ± 13.66**	3.01 ± 0.53**	39.80
8	1.92 ± 0.19*	18.59 ± 3.90*	3.52 ± 0.78**	140.60 ± 35.30*	21.00 ± 1.89*	20.30
9	0.57 ± 0.22**	6.01 ± 1.48	1.10 ± 0.19*	63.30 ± 4.21**	8.08 ± 0.68	60.10
10	0.46 ± 0.15**	6.73 ± 0.65	1.04 ± 0.08**	72.72 ± 15.92	5.49 ± 1.03*	63.50
11	0.87 ± 0.10**	11.14 ± 0.90*	1.40 ± 0.27*	153.00 ± 10.02**	2.81 ± 0.91**	45.10
12	1.98 ± 0.48	11.72 ± 1.30*	2.99 ± 0.64**	110.00 ± 18.83**	14.09 ± 1.81	25.80
13	0.78 ± 0.08**	4.61 ± 0.30**	0.83 ± 0.15	31.40 ± 2.40**	9.55 ± 1.48	74.80
14	0.79 ± 0.45*	7.78 ± 0.70	0.93 ± 0.11	61.40 ± 4.78*	3.39 ± 0.11**	60.00
15	0.54 ± 0.23**	10.24 ± 2.50	0.89 ± 0.04*	74.00 ± 11.49*	1.45 ± 0.19**	73.10
16	0.87 ± 0.12**	18.20 ± 2.90**	1.78 ± 0.18**	129.00 ± 11.40**	4.79 ± 0.16*	35.80

Data are expressed as mean ± standard error of mean, with n = 3. $^{\#\#}P < 0.01$, *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model. IL: interleukin; MMP-9: matrix metalloproteinase-9.

Table 10
Effects of Huoxue group constituents with different proportion on co-cultured A549 and THP-1 cells induced by lipopolysaccharide.

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-23 (pg/mL)	MMP-9 (pg/mL)	Weighted score
Normal	0.57 ± 0.35	1.61 ± 0.02	219.68 ± 12.30	11.04 ± 2.02	1.92 ± 0.22	
Model	3.36 ± 1.02$^{\#}$	4.96 ± 0.60$^{\#\#}$	993.00 ± 87.8$^{\#\#}$	32.10 ± 4.17$^{\#\#}$	62.57 ± 4.00$^{\#\#}$	
1	1.99 ± 0.56	4.94 ± 2.20	799.05 ± 128.00	34.69 ± 7.51	29.53 ± 3.13**	47.44
2	1.42 ± 0.16*	5.85 ± 1.61	764.36 ± 47.00*	36.04 ± 7.60	30.32 ± 11.82*	46.73
3	1.75 ± 0.12	4.59 ± 1.29	576.90 ± 17.00**	23.27 ± 4.16	23.34 ± 4.52**	61.08
4	0.62 ± 0.32*	3.89 ± 0.76	677.50 ± 31.70*	19.53 ± 4.04*	20.60 ± 0.96**	71.26
5	1.69 ± 0.53	4.37 ± 0.42	651.30 ± 122.00*	25.35 ± 2.91	23.11 ± 3.02**	58.88
6	1.64 ± 0.57	6.53 ± 1.96	533.60 ± 24.00**	23.32 ± 2.73*	22.58 ± 4.51**	59.57
7	0.46 ± 0.20**	4.54 ± 0.60	592.00 ± 58.40**	20.3 ± 2.56**	21.44 ± 1.19**	73.19
8	0.32 ± 0.16**	5.80 ± 0.87	590.00 ± 27.50**	21.74 ± 3.41*	20.19 ± 5.94**	76.11
9	1.31 ± 0.95	3.71 ± 0.75	664.90 ± 31.00**	28.13 ± 1.21	27.29 ± 0.82**	58.31
10	1.03 ± 0.24*	4.44 ± 1.29	640.90 ± 125.00*	21.77 ± 2.76*	32.37 ± 0.85**	58.93
11	0.62 ± 0.29*	3.24 ± 0.21**	523.30 ± 10.00**	19.20 ± 1.38**	21.98 ± 3.85**	77.64
12	1.57 ± 0.15*	3.09 ± 0.37*	678.00 ± 108.00**	19.00 ± 2.45**	18.33 ± 2.15**	71.86
13	1.23 ± 0.35*	4.06 ± 0.38	497.00 ± 12.00**	19.90 ± 1.33**	27.96 ± 9.59**	66.63
14	1.50 ± 0.39*	2.96 ± 0.26**	497.00 ± 32.90**	16.00 ± 0.63**	18.81 ± 1.52**	80.84
15	0.32 ± 0.10**	3.75 ± 0.44*	550 ± 21.60**	23.39 ± 2.77*	17.14 ± 0.62**	84.53
16	0.28 ± 0.02	2.79 ± 0.74*	628 ± 93.50**	16.89 ± 1.60**	22.20 ± 3.94**	90.27

Data are expressed as mean ± standard error of mean, with n = 3. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model. IL: interleukin; MMP-9: matrix metalloproteinase-9.

Table 11
Effects of four-group combinations with different proportion on co-cultured A549 and THP-1 cells induced by lipopolysaccharide.

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (μg/mL)	IL-23 (pg/mL)	MMP-9 (μg/mL)
Normal	3.16 ± 0.88	0.81 ± 0.23	0.57 ± 0.02	7.80 ± 1.50	15.77 ± 0.72
Model	13.10 ± 1.25$^{\#\#}$	3.93 ± 0.32$^{\#\#}$	4.01 ± 0.27$^{\#\#}$	180.10 ± 26.50$^{\#\#}$	75.21 ± 2.13$^{\#\#}$
1	12.58 ± 0.92	3.70 ± 0.45	3.75 ± 0.09	164.00 ± 3.4.	58.80 ± 4.90*
2	5.56 ± 0.36**	2.95 ± 0.12*	2.34 ± 0.16**	144.60 ± 4.10	49.47 ± 5.39**
3	4.47 ± 0.65**	2.22 ± 0.22**	1.92 ± 0.06**	121.40 ± 2.30	30.08 ± 2.53**
4	3.60 ± 0.11**	1.13 ± 0.09**	1.07 ± 0.05**	22.00 ± 2.50**	14.58 ± 0.55**
5	14.52 ± 1.01	4.73 ± 0.17	1.76 ± 0.34**	180.80 ± 5.80	62.09 ± 2.84*
6	10.79 ± 1.12	4.24 ± 0.09	4.21 ± 0.12	166.80 ± 2.90	58.01 ± 0.48**
7	3.19 ± 0.09**	1.75 ± 0.16**	1.37 ± 0.16**	76.50 ± 3.70**	38.31 ± 0.39**
8	2.87 ± 0.07**	0.76 ± 0.12**	0.69 ± 0.06**	14.20 ± 2.10**	24.35 ± 2.08**
9	13.37 ± 1.16	4.15 ± 0.04	4.24 ± 0.12	169.80 ± 5.70	57.18 ± 2.30**
10	8.08 ± 1.05**	3.01 ± 0.87	2.60 ± 0.08**	145.00 ± 2.00	42.55 ± 0.25**
11	4.38 ± 0.33**	2.08 ± 0.18**	1.85 ± 0.21**	97.50 ± 4.00*	43.64 ± 1.94**
12	3.75 ± 0.27**	1.59 ± 0.05**	1.73 ± 0.15**	71.20 ± 4.90**	21.81 ± 0.18**
13	10.80 ± 0.16*	3.58 ± 0.35	3.09 ± 0.07**	162.00 ± 2.20	43.53 ± 3.29**
14	5.26 ± 1.30**	1.98 ± 0.36*	2.01 ± 0.06**	137.70 ± 3.10*	59.01 ± 0.61**
15	3.94 ± 0.27**	1.52 ± 0.06**	1.32 ± 0.12**	62.40 ± 1.70**	40.48 ± 0.38**
16	3.29 ± 0.06**	1.08 ± 0.04**	1.09 ± 0.04**	33.80 ± 4.20**	22.44 ± 1.08**

Data are expressed as mean ± standard error of mean, with n = 3. $^{\#\#}P < 0.01$, *vs* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model. IL: interleukin; MMP-9: matrix metalloproteinase-9.

Table 12
Results of $L_{16}(4^4)$ orthogonal experiment of Huoxue, Bushen, Huatan and Buqi groups.

Group	Huoxue (A)	Bushen (B)	Huatan (C)	Buqi (D)	E	Weighted score
1	1	1	1	1	1	19.05
2	1	2	2	2	2	29.23
3	1	3	3	3	3	38.94
4	1	4	4	4	4	75.20
5	2	1	2	3	4	21.28
6	2	2	1	4	3	18.92
7	2	3	4	1	2	48.12
8	2	4	3	2	1	91.98
9	3	1	3	4	2	17.99
10	3	2	4	3	1	26.27
11	3	3	1	2	4	37.49
12	3	4	2	1	3	50.23
13	4	1	4	2	3	22.48
14	4	2	3	1	4	32.49
15	4	3	2	4	1	46.76
16	4	4	1	3	2	65.63
K1	40.61	20.20	35.27	37.47	46.02	
K2	45.08	26.73	36.88	45.30	40.24	
K3	33.00	42.83	45.35	38.03	32.64	
K4	41.84	70.76	43.02	39.72	41.62	
Range *R*	12.08	50.56	10.08	7.82	13.37	

decreases by week 20. We tested the effects of BYF and ECC of BYF I on changes in FVC, FEV0.1, FEV0.1s/FVC and FRC. BYF, ECC of BYF I and APL inhibited changes in these factors induced by cigarette smoke and bacterial infection (Fig. 3).

A histopathological analysis of pulmonary tissue revealed that bronchiole wall thickness, alveolar diameter, and inflammatory cell infiltration increased whilst alveolar number decreased in the model rats and these changes were suppressed in the BYF, high-dose ECC of BYF I, medium-dose ECC of BYF I, and APL groups (Fig. 4).

3.3.2. Effects of BYF and ECC of BYF I on inflammatory response, protease expression, and oxidative-antioxidative status in COPD rats

We monitored pulmonary inflammatory responses by measuring proinflammatory cytokines in BALF and serum. IL-1β and IL-6 were markedly upregulated in COPD rats but these increases were suppressed by BYF and ECC of BYF I treatment (Fig. 5A and B).

Protease-mediated connective tissue breakdown is a critical mechanism of COPD pathogenesis. Serum MMP-9 was distinctly lower in the ECC of BYF I-treated group than the model rats (Fig. 5C). Several oxidants and antioxidants may participate in the pathogenesis of the inflammatory process in COPD. Thus, we investigated their effects on LPO and T-AOC. BYF and ECC of BYF I significantly decreased LPO and increased T-AOC, whereas APL only decreased LPO (Fig. 5D and E).

3.3.3. Bioactive equivalence between BYF and ECC of BYF I

ECC of BYF I and BYF showed high protective efficacy in COPD rats. They suppressed changes in lung function and histopathology, downregulated IL-1β, IL-6 and LPO, and upregulated TAC. However, it was uncertain whether ECC of BYF I had bioactive equivalence to BYF. We implemented the *R*-value comprehensive evaluation assay to establish their bioactive equivalence based on the lung function and/or inflammatory response, protease expression, and oxidative-antioxidative status results. Comprehensive evaluation of lung function measurements showed that the order of improvement from high to low was high-dose ECC-BYF I, medium-dose ECC-BYF I, BYF, low-dose ECC-BYF I, and APL. There were no significant differences among high- and medium-dose ECC-BYF I, and BYF (Fig. 6A). Moreover, a comprehensive evaluation of all outcomes also indicated no significant differences among ECC-BYF I and BYF (Fig. 6B). Thus, ECC of BYF I had bioactive equivalence to BYF.

4. Discussion

TCM formulae have been clinically tested on a large scale for a very long time. They are promising for the holistic treatment of complex diseases [4]. TCM formulae consist of various medicinal herbs containing thousands of constituents. The efficacy of TCM formulae is determined by their combinatorial effective constituents rather than single compounds [12]. However, the com-

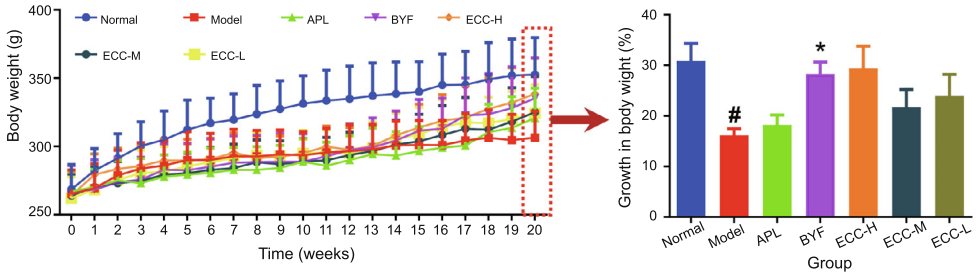

Fig. 1. Changes in body weight of rats from weeks 0 to 20. Data are expressed as mean ± standard error of mean (n = 8–12). $^{\#}P < 0.01$, *vs* normal group; $^{*}P < 0.05$, *vs* model group. APL: aminophylline; BYF: Bufei Yishen formula; ECC-H: high-dose effective-constituent compatibility of BYF I; ECC-M: medium-dose effective-constituent compatibility of BYF I; ECC-L: low-dose effective-constituent compatibility of BYF I.

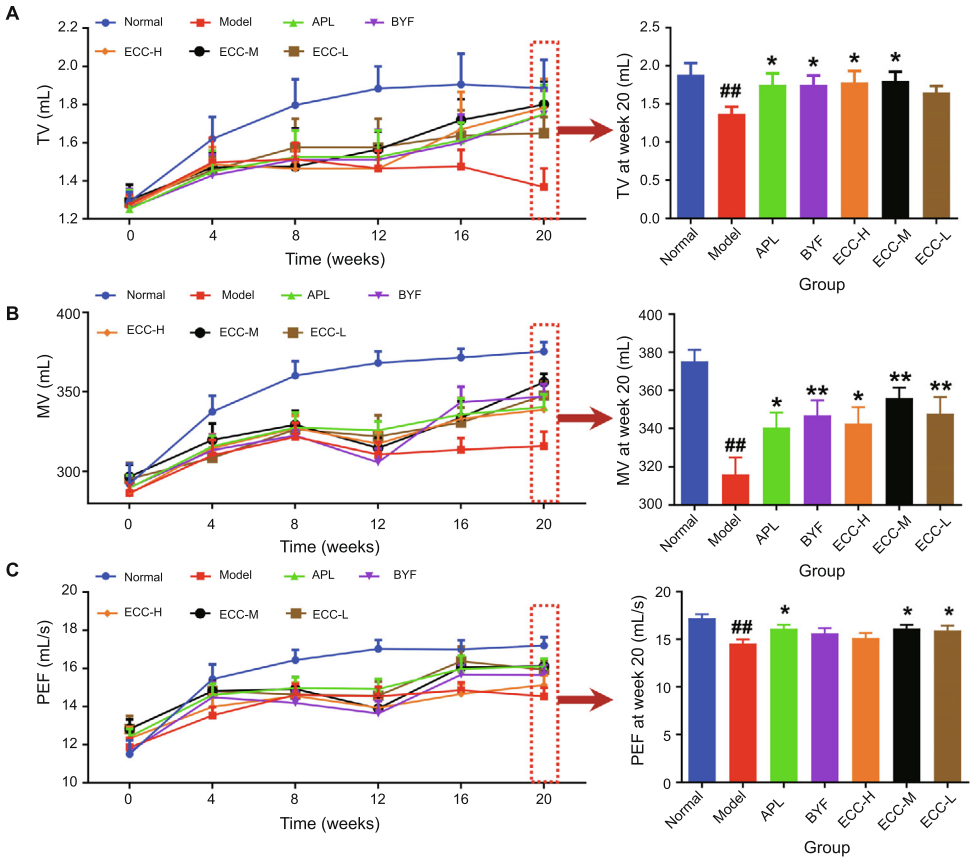

Fig. 2. Changes in pulmonary function of rats from weeks 0 to 20. Data are expressed as mean ± standard error of mean (n = 8–12). $^{\#\#}P < 0.01$ *vs* normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model group. APL: aminophylline; BYF: Bufei Yishen formula; ECC-H: high-dose effective-constituent compatibility of BYF I; ECC-M: medium-dose effective-constituent compatibility of BYF I; ECC-L: low-dose effective-constituent compatibility of BYF I; MV: minute volume; PEF: peak expiratory flow; TV: tidal volume.

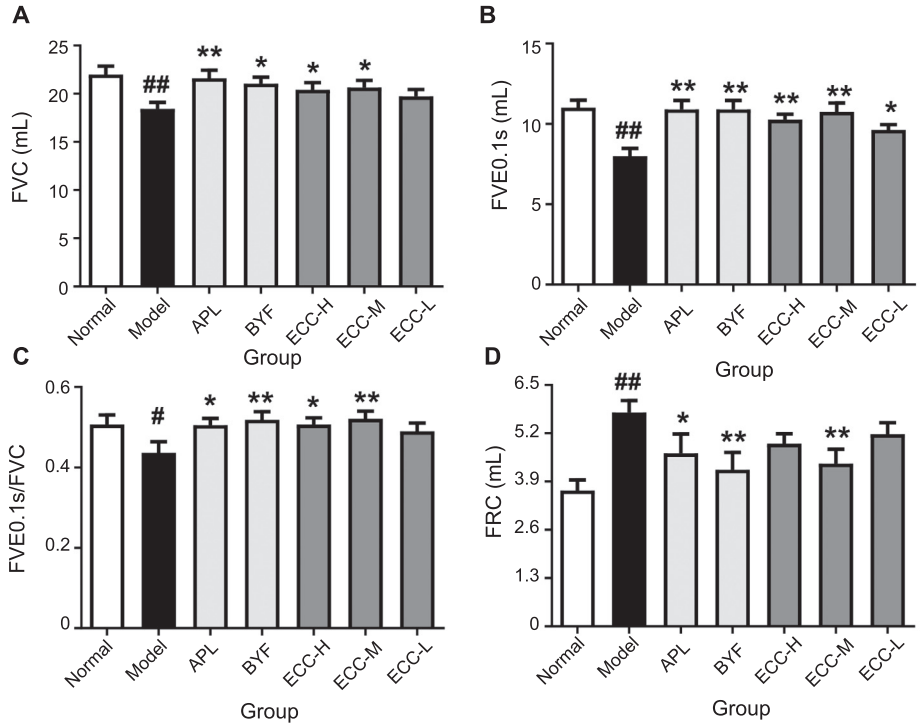

Fig. 3. Changes in pulmonary function of rats at week 20. Data are expressed as mean ± standard error of mean (n = 8–12). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs* normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model group. APL: aminophylline; BYF: Bufei Yishen formula; ECC-H: high-dose effective-constituent compatibility of BYF I; ECC-M: medium-dose effective-constituent compatibility of BYF I; ECC-L: low-dose effective-constituent compatibility of BYF I; FEV0.1s: forced expiratory volume at 0.1s; FRC: functional residual capacity; FVC: forced vital capacity.

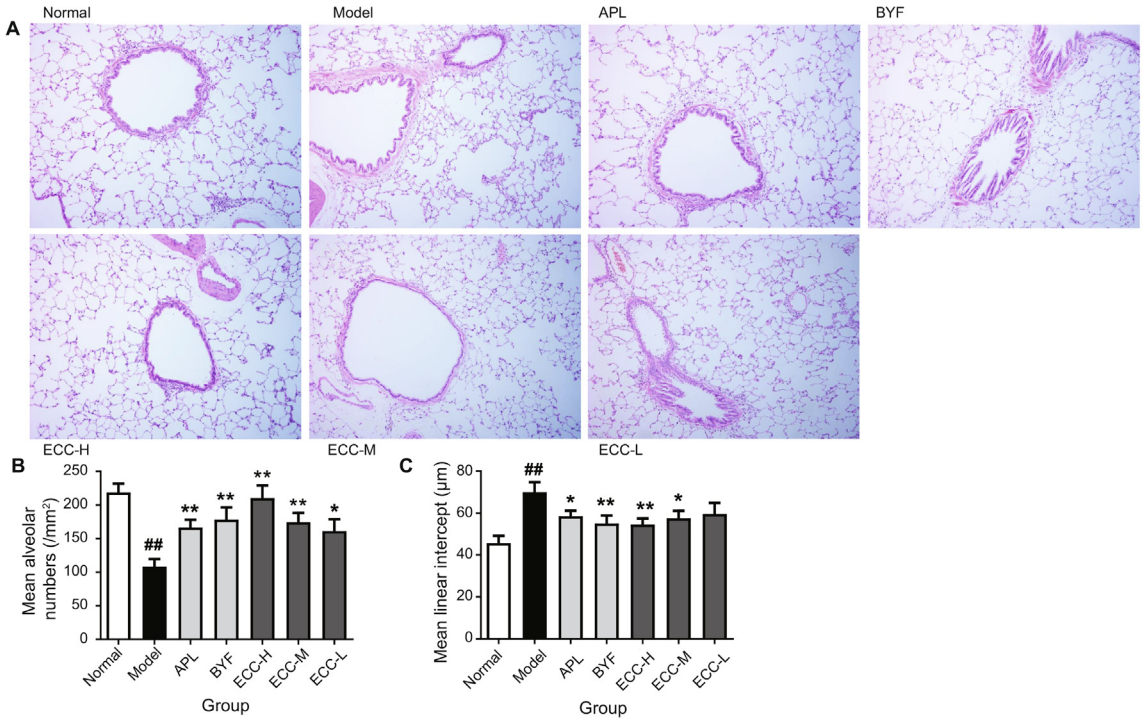

Fig. 4. Changes in lung tissue morphology. (A) Pathological changes in lung tissues (haematoxylin and eosin staining; ×100); (B) mean alveolar number; (C) mean linear intercept. Values are expressed as mean ± standard error of mean (n = 6). $^{\#\#}P < 0.01$, *vs* normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model group. APL: aminophylline; BYF: Bufei Yishen formula; ECC-H: high-dose effective-constituent compatibility of BYF I; ECC-M: medium-dose effective-constituent compatibility of BYF I; ECC-L: low-dose effective-constituent compatibility of BYF I.

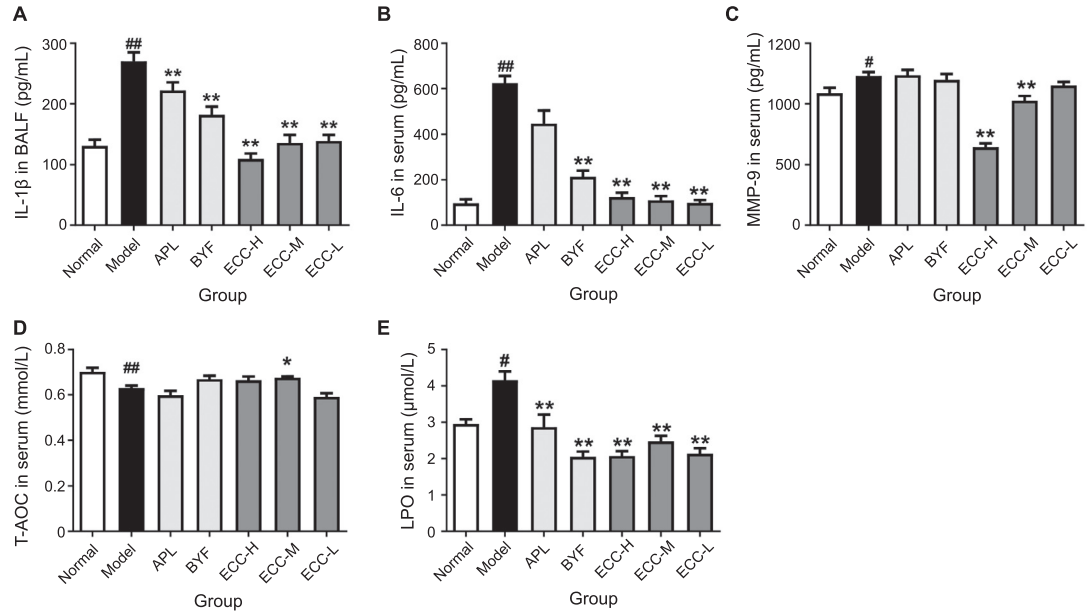

Fig. 5. IL-1β in BALF and IL-6, MMP-9, T-AOC and LPO levels in serum. Values are expressed as mean ± standard error of mean (n = 6). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs* normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model group. APL: aminophylline; BALF: bronchoalveolar lavage fluid; BYF: Bufei Yishen formula; ECC-H: high-dose effective-constituent compatibility of BYF I; ECC-M: medium-dose effective-constituent compatibility of BYF I; ECC-L: low-dose effective-constituent compatibility of BYF I; IL: interleukin; LPO: lipid peroxidation; MMP-9: matrix metalloproteinase-9; T-AOC: total antioxidant capacity.

plexity of effective constituents makes it difficult to enhance and standardise their quality, stability, and efficacy. For these reasons, appropriate methodologies and strategies are urgently needed. Here, we applied the method of effective-constituent compatibility to identify the effective constituents in BYF and determined the primary effects of BYF [5].

Thousands of constituents are contained in the BYF herbal medicines. Nevertheless, only some of them are primary effective substances of BYF for COPD treatment [9,13]. Thus, we implemented cellular models characterising the critical pathological changes that occur in COPD to identify the primary effective substances of BYF. The symptoms of COPD include airflow obstruction, chronic

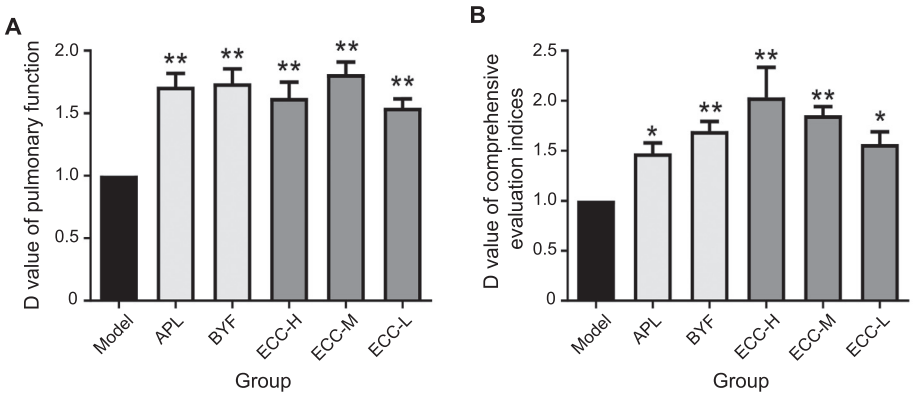

Fig. 6. *D* values of pulmonary function and comprehensive evaluation indices. Values are expressed as mean ± standard error of mean (n = 7 for A and n = 15 for B). $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* model group. APL: aminophylline; BYF: Bufei Yishen formula; ECC-H: high-dose effective-constituent compatibility of BYF I; ECC-M: medium-dose effective-constituent compatibility of BYF I; ECC-L: low-dose effective-constituent compatibility of BYF I.

bronchitis, and systemic inflammation. Airway epithelial cells and macrophages release proinflammatory cytokines in response to pulmonary inflammatory stimuli. These substances may play important roles in COPD development and progression [14,15]. Epithelial cells stimulated by cigarette smoke and other inflammatory mediators such as TNF-α, release large amounts of proinflammatory cytokines such as IL-1β, IL-6 and IL-8, and secrete excess mucus. Macrophages are stimulated by cigarette smoke and bacterial infection and produce inflammatory mediators such as TNF-α, IL-6 and IL-8 and secrete elastolytic enzymes including MMP-2, -9 and -12 which orchestrate chronic inflammation in COPD [16]. Hence, we established cellular models by exposing alveolar epithelial cells (A549) to TNF-α, subjecting macrophages (THP-1) to LPS, and challenging A549/THP-1 co-cultures with LPS.

In BYF, Ginseng Radix et Rhizoma, Astragali Radix, Epimedii Folium, Lycii Fructus, Fritillariae Thunbergii Bulbus, Citri Reticulatae Pericarpium, and Paeoniaeradix Rubra play the major roles [17]. Ginseng Radix et Rhizoma and Astragali Radix belong to TCM Group 1 and Lycii Fructus belongs to TCM Group 2. Fritillariae Thunbergii Bulbus and Citri Reticulatae Pericarpium belong to TCM Group 3. Paeoniaeradix Rubra belongs to TCM Group 4. Here, we selected 24 candidate active constituents among the four herbal medicine groups and applied the aforementioned cellular models to identify 12 effective constituents among the 24 candidates.

The next step was to determine the proportions of these effective constituents. Based on the principles of TCM, the 12 effective constituents were divided into Group 1, Group 2, Group 3 and Group 4, with 3, 2, 5 and 2 effective constituents, respectively. An orthogonal design was applied to identify the optimal ratios of the constituents in Group 1 and Group 3 and the permutation for Group 2 and Group 4. The ratios of the four groups were detected by orthogonal design. Ten compounds in four groups were identified as candidate ECC of BYF I. Candidate ECC is considered bioactive equivalence with the original herbal formula if the ratios of their anti-COPD efficacy fall within an acceptable range. We evaluated the therapeutic effects of ECC of BYF I on COPD rats [18] and comprehensively assessed bioactive equivalence between BYF and ECC of BYF I using the *R*-value comprehensive evaluation method. We found that ECC of BYF I had bioactive equivalence with the original BYF.

Here, we evaluated the efficacy of the candidate constituents in the four groups of BYF herbal medicines on various cell models. Based on our efficacy assessment, we identified optimal ratios for the effective constituents within each group and among the four groups to recommend candidate ECC of BYF I. We also demonstrated that the ECC of BYF I was as effective as BYF in a rat COPD model. Successful identification of the effective constituents and ECC from the original formula indicated that the effective-constituent compatibility method could be used to develop modern Chinese medicines based on efficacious traditional formulae.

5. Conclusion

Here, we applied the method of effective-constituent compatibility to identify 10 effective constituents that were combined as candidate ECC of BYF I. We demonstrated that the ECC of BYF I was as effective as BYF in treating rats with COPD. Therefore, ECC is a type of modern Chinese medicine developed from effective constituents according to the compatibility theory and principle in TCM. ECC has similar advantages to TCM itself and has stable, effective constituents.

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Author contributions

JSL designed the outline of the study. XFL, HRD, WCZ, JDM and ZXR performed experiments, conceived the study, draft and revised the manuscript. SXF and YGT were involved in performing experiments, acquisition of data and statistical analysis. PZ and YX contributed to the data analysis and interpretation. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Conflicts of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joim.2020.04.004.

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