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Original Research Article

Yiqi Huoxue Huatan formula attenuates inflammation in chronic obstructive pulmonary disease by suppressing the activation of PI3K/AKT and NF-κB signaling pathways

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Abstract

Purpose: *To explore the mechanism with which Yiqi Huoxue Huatan formula regulates inflammation in chronic obstructive pulmonary disease (COPD).*
Methods: *COPD rat models (n=60) were procured and administered with prednisone and Yiqi Huoxue Huatan formula. Histological examination of lung tissues was carried out and bronchoalveolar fluid was collected for further analysis. In addition, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were used to evaluate the expression of different genes and proteins. The study also assessed pulmonary functions in each rat.*
Results: *The results showed that Yiqi Huoxue Huatan formula relieved the lung inflammation and helped to preserve alveolar structure, and its anti-inflammatory efficacy was superior to prednisone. Moreover, it significantly improved pulmonary function in COPD rats. This formula was shown to repress the activity of both PI3K/AKT and NF-κB signaling pathways.*
Conclusion: *Yiqi Huoxue Huatan formula may be effective in treating COPD by attenuating local inflammation and improving pulmonary function.*

Keywords: *Yiqi Huoxue Huatan formula, COPD, PI3K/AKT signaling pathway, NF-κB signaling pathway*

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by airway obstruction, destruction of the extracellular matrix near the alveoli, and persistent inflammation [1]. There are many risk factors associated with this disease, for example, smoking and tobacco use, and asthma [2]. There are many theories attributed to the pathogenesis of COPD such as, oxidant/antioxidant imbalance, the dysregulation of protease and anti-protease, and the activation of NF-κB signaling pathway [1,3,4]. Current treatment options include bronchodilators, corticosteroids and oxygen, and the latest advances into COPD treatment include

antioxidants, IL-1β inhibitor, IL-6 inhibitor and endothelin inhibitors [5]. Suppressing inflammation and preventing the airway structure from damage are the two main approaches to using pharmaceutical options.

Yiqi Huoxue Huatan formula has been proven to be effective in treating many diseases. For example, it improves recovery from cerebral ischemic stroke [6]. In addition, it inhibits the expression levels of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule 1 (ICM-1) in ulcerative colitis [7], indicating its protective effects against inflammation. This formula has been shown to suppress the fibrosis in cirrhosis by inhibiting transforming growth factor-β1 (TGF-β1)/Smads [8]. Also, it can improve the clinical outcome of pneumonia patients [9]. For these reasons, we supposed that *Yiqi Huoxue Huatan* formula might be effective against COPD.

In this research, The COPD rat models were administered with the *Yiqi Huoxue Huatan* formula, and a group of rats received prednisone and were identified as the positive control group, so as to compare the efficacy of *Yiqi Huoxue Huatan* formula with prednisone.

EXPERIMENTAL

Reagents

Yiqi formula is composed of codonopsis, astragalus and licorice; *Huoxue* formula is made up of safflower, peach kernel and chuanxiong; while the components of *Huatan* formula include pinellia, orange peel and poria. Thus, the *Yiqi Huoxue Huatan* formula is a combination of *Yiqi*, *Huoxue* and *Huatan* recipes. These formulas were bought from Sanjiu Pharmaceutical Co Ltd. Prednisone was purchased from MedChemExpress, USA.

Animals and grouping

The animal experiments were approved (approval no. 44007200065273) by the Ethical Committee of Guangzhou University of Chinese Medicine, and experiments were conducted based on the protocols instituted by this Committee All procedures were conducted in accordance with the guidelines of 'Animal Research: Reporting in Vivo Experiments Guidelines 2.0' [10].

Male Wistar rats (60, weight: 200 - 250 g; 8 weeks old) were purchased from the Animal Research Center of Guangzhou University of Chinese Medicine. All the rats were kept in pathogen-free conditions with 50 - 60 % humidity and clean air under 12 h light cycles. After they adapted to the environment, they were randomly divided into 6 groups: the control group (clean air exposed only), COPD grouqc p, COPD + prednisone co-treatment group, COPD + *Yiqi Huoxue Huatan* formula co-treatment group, COPD + Yiqi recipe co-treatment group, COPD + Huoxue recipe co-treatment group and COPD + Huatan recipe co-treatment group. Each group had 10 rats.

Treatments

In order to establish COPD models in rats, the research used a smoke apparatus. The smoke exposure was done as reported, and the smoke concentration was monitored [11]. The rats were first placed in inhalation chambers and smoke was delivered into each chamber using cigarettes purchased for experimental purposes from Beyotechnology, China, with each cigarette stick generating smoke for 15 min. Each cigarette stick contained 25 mg tar and 1.4 mg nicotine. They were exposed to smoke for 1 h in the morning and 1 h in the evening for 7 days a week, and the duration of this treatment was 20 weeks. Rats allocated in the control group did not receive any treatment and were infused with clean air.

In addition, these groups received different treatments. In the COPD + prednisone co-treatment group, 3 mg/kg prednisone was administered to each rat by gastric lavage every day for 20 weeks. In the COPD + Yiqi Huoxue Huatan formula co-treatment group, 14.4 g/kg *Yiqi Huoxue Huatan* formula was given to each rat by gastric lavage every day for 20 weeks. In COPD + Yiqi recipe co-treatment group, 6.6 g/kg Yiqi recipe was administered to each rat via gastric lavage every day for 20 weeks. In COPD + Huoxue recipe co-treatment group, 3.34 g/kg Huoxue recipe was administered to each rat via gastric lavage every day for 20 weeks. In COPD + Huatan recipe co-treatment group, 4.34 g/kg Huatan recipe was administered to each rat via gastric lavage every day for 20 weeks. In the COPD group, equal amounts were given through gastric lavage at the same time.

Pulmonary function assessment

At the end of the 20 weeks of treatment, each rat was anesthetized by injecting pentobarbital (40 ml/kg) intraperitoneally, and then they were tracheostomized and trachea-cannulated. The canula was connected to an animal ventilator (SCIREQ system) and lung function was evaluated by employing the forced oscillation

technique. Through this system, forced vital capacity (FCV), forced expiratory capacity at 0.3 second (FEV 0.3), and peak expiratory flow (PEF) were measured three times for each rat, and the highest values were used for data analysis.

Lung histopathological examination

The lungs were first placed in 10% formalin overnight after they were collected from the rats. Afterwards, they were embedded in paraffin and sectioned at a thickness of 4 mm. They were then stained with hematoxylin and eosin (H & E) (Boster, Wuhan, China). Finally, the sections were mounted and observed under a microscope.

In order to evaluate MMP-9 expression in lung tissues, immunohistochemistry was performed. Slides were then briefly heated and H_2O_2 was used to treat them. Subsequently, 10 % bovine serum albumin was employed to block the slides and then these slides were washed with phosphate buffer saline (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, the slides were incubated with the primary antibody against MMP-9 (dilution: 1:100, Abcam, Cambridge, MA, USA, ab76003), and the secondary antibody was utilized to incubate the slides. Finally, slides were observed under microscope and pictures were taken. The MMP-9 expression was determined using Image Pro-plus 6.0.

Western blotting

Tissues were lysed with radio immune-precipitation assay (RIPA) lysis buffer, and extraction buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) at 4 ^oC. Total protein concentration was calculated using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein samples (25μg) were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Beyotechnology, Shanghai, China) and then separated. Proteins in gels were then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and incubated with 4 % bovine serum albumin, and then treated with the primary antibodies at 4 ^oC overnight. The primary antibodies against PI3K (dilution: 1:1000, Abcam, Cambridge, MA, USA; ab139307), phosphor-PI3K (phosphor-Y607) (dilution: 1:1000, Abcam, Cambridge, MA, USA; ab182651), Akt (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA; 4685), phospho-Akt (Ser473) (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA; 4060), IKKα (dilution: 1:1000, Abcam, Cambridge, MA, USA; ab32041), phospho- IKKα (T23) (dilution: 1:1000, Abcam, Cambridge, MA, USA; ab38515), p65 (dilution: 1:1000, Abcam, Cambridge, MA, USA; ab32536) and phospho-p65 (phosphor-S536) (dilution: 1:1000, Abcam, Cambridge, MA, USA; ab76302) were used in this research, and β-actin was used as the internal control. Twenty-four hours later, these membranes were incubated with HRP-secondary antibodies at room temperature for 1 h. The protein bands were visualized using High-sig ECL Western Blotting Substrate (Tenon, Shanghai, China).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the cells by employing Trizol reagent (TaKaRa, Tokyo, Japan). MicroRNA was extracted using Molpure Cell/Tissue miRNA Kit (Yeasen, Shanghai, China). In addition, mRNA was transcribed using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), and miRNA was transcribed using TaqMan MicroRNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). The mRNA expression levels of related genes were measured using SYBR Premix Ex TaqII kit (Tli RNaseH Plus) (TaKaRa, Tokyo, Japan). β-actin was used as the endogenous control to detect other genes. The primers of the genes used in this study are listed in Table 1.

Table 1: Primers used for qRT-PCR

Gene	Primer 5'→3'
PI3K	F: TATTTGGACTTTGCGACAAGACT
	R: TCGAACGTACTGGTCTGGATAG
AKT	F: AGCGACGTGGCTATTGTGAAG
	R: GCCATCATTCTTGAGGAGGAAGT
IKKα	F: GGCTTCGGGAACGTCTGTC
	R: TTTGGTACTTAGCTCTAGGCGA
NF-κB	F: ATGTGGAGATCATTGAGCAGC
	R: CCTGGTCCTGTGTAGCCATT
GAPDH	F: GGAGCGAGATCCCTCCAAAAT
	R: GGCTGTTGTCATACTTCTCATGG

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for detecting the concentrations of IL-6, IL-8 and TNF-α were bought from Abcam (Cambridge, MA, USA), and the respective catalogue numbers were ab178013, ab214030 and ab181421. The measurement of the concentration of these cytokines was carried out, and IL-6, IL-8 and TNF-α were extracted from the tissue supernatant by centrifugation for 20 min at 1,000g. After the supernatant was collected, ELISA kits were employed to determine the concentration of cytokines, and the absorbance results were read at 450 nm.

Statistical analysis

The data were analyzed using a GraphPad Prism 8.0 (La Jolla, CA, USA) and presented as mean ± standard deviation (SD). All our experiments were performed five times independently. Student's *t*-test and one-way analysis of variance (ANOVA, Bonferroni post hoc test) were adopted during the analysis, depending on the experiment. Two-tailed $p < 0.05$ was considered statistically significant.

RESULTS

Histopathological features of rat lung tissue

Rat lung tissues were stained using H & E. In the COPD model group, there were signs of inflammation and decrease in the number of pulmonary epithelial cells, and the alveoli were partly distended or contracted, compared to the control group (Figure 1). In the COPD + prednisone co-treatment group, inflammation still existed with the aggregation of macrophages, and the structure of the pulmonary alveoli was not organized. In the COPD + *Yiqi Huoxue Huatan* formula co-treatment group, the inflammation seemed to be milder than that of the COPD + prednisone co-treatment group, but the structure of pulmonary alveoli remained blurry. To elaborate on the role of Yiqi Huoxue *Huatan* formula, this formula was divided into three recipes: Yiqi recipe, Huoxue recipe and *Huatan* recipe. In the COPD + Yiqi recipe co-treatment group, there were still signs of inflammation and mucus in the acini. In the COPD + Huoxue recipe co-treatment group, there was obvious inflammation and tissue injury. In the COPD + *Huatan* recipe co-treatment group, the tissue inflammation and injury was not as serious as those in the COPD + prednisone co-treatment group. For these reasons, it is assumed that *Yiqi Huoxue Huatan* formula can treat COPD, and the efficacy might be superior to prednisone.

Levels of MMP-9 and cytokines in lung tissue

The MMP-9 is an important molecule that regulates inflammation, and so its expression was therefore measured in lung tissue. It was discovered that in COPD models, its expression level was upregulated and the respective prednisone and *Yiqi Huoxue Huatan* formula treatment downregulated its expression level (Figure 2 and Table 2). In particular, *Huoxue* recipe and *Huatan* recipe could cause a decrease in MMP-9 expression. Also, the concentration of different cytokines was evaluated, and when it was compared to the COPD group, the expression levels of TNF-α, IL-6 and IL-1 in COPD models were much higher (Table 3). Interestingly, *Yiqi Huoxue Huatan* formula significantly downregulated the concentrations of these cytokines. Thus, *Yiqi Huoxue Huatan* formula reduce inflammation in COPD.

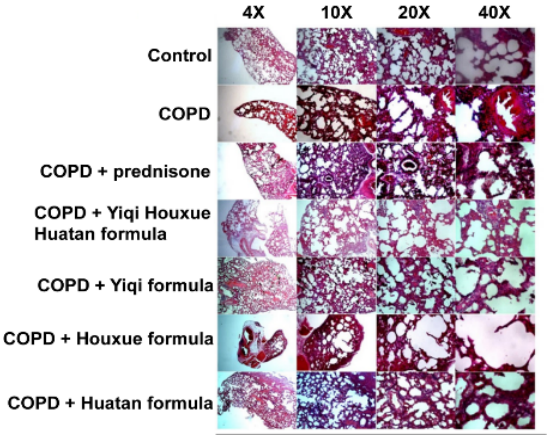

Figure 1: Histopathological features of rat lung tissue. COPD chronic obstructive pulmonary disease

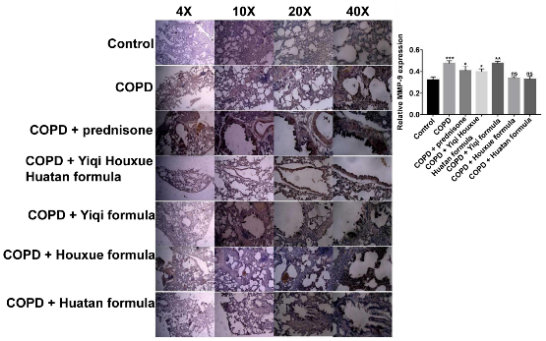

Figure 2: Levels of MMP-9 and cytokines in lung tissue. Immunohistochemistry of lung tissues stained against MMP-9 and relative MMP-9 expression in lung tissues. NS non-significant, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, versus control group

Yiqi Huoxue Huatan formula improves pulmonary function in COPD

Pulmonary function tests were performed on the rats. In COPD models, FVC values decreased remarkably compared to rats in the control group. Moreover, prednisone or Yiqi Huoxue Huatan formula increased FVC values, when compared to the COPD group (Table 4). In addition, FEV0.3/FVC ratio was employed to assess the obstruction of airways in these rats. It was discovered that in COPD models, the FEV0.3/FVC ratio reduced, in contrast to the controls.

Table 2: MMP-9 expression levels in lung tissue (mean ± SD, n = 10)

Group	IOD/Area
Control	0.3240±0.0264
COPD	0.4769±0.0238
COPD + Prednisome	0.4065±0.0469
COPD + Yiqi Huoxue Huatan formula	0.3983±0.0276
COPD + Yiqi formula	0.4758±0.0201
COPD + Huoxue formula	0.3395±0.0137
COPD + Huatan formula	0.3313±0.0246

Table 3: Levels of cytokines in bronchoalveolar fluid (mean ± SD, n = 10)

Group	TNF-a (pg/ml)	IL-6 (pg/ml)	IL-1 (pg/ml)
Control	20.48±4.05	30.05±8.56	34.53±12.51
COPD	130.59±12.27	65.77±8.67	88.67±14.47
COPD + Prednisone	75.42±13.48	55.97±6.56	69.21±12.47
COPD + Yiqi Huoxue Huatan formula	61.76±18.12	42.30±9.18	56.86±11.78
COPD + Yiqi formula	70.48±15.27	50.72±10.02	64.19±14.58
COPD + Huoxue formula	78.74±21.12	55.21±13.58	68.04±12.16
COPD + Huatan formula	56.58±16.78	43.44±12.20	54.64±15.66

Table 4: Pulmonary function results (mean ± SD, n = 10)

Group	FVC (ml)	FEV0.3/FVC	PEF (ml/s)
Control	8.79±1.53	0.8932±0.0585	35.35±1.88
COPD	5.59±1.10^{a}	0.7341±0.0242^{a}	26.24±1.26^{a}
COPD + Prednisone	7.10±1.86^{b}	0.8156±0.0447^{b}	32.23±1.57^{b}
COPD + Yiqi Huoxue Huatan formula	7.13±1.70^{b}	0.7958±0.0239^{b}	31.28±1.49^{b}
COPD + Yiqi formula	6.38±1.94	0.7336±0.0295^{cd}	27.87±1.61^{cd}
COPD + Huoxue formula	5.94±1.00	0.7199±0.0161^{ced}	26.74±1.17^{cd}
COPD + Huatan formula	7.03±0.98^{b}	0.7924±0.0710^{bef}	31.49±1.47^{bef}

It was also discovered that prednisone and Yiqi Huoxue Huatan formula could increase FEV0.3/FVC ratios, thus indicating that prednisone and *Yiqi Huoxue Huatan* formula alleviates airway inflammation and obstruction, yet the Huatan recipe did not change the ratio of FEV0.3/FVC in COPD. Similarly, it was determined that prednisone and Yiqi Huoxue Huatan formula improved PEF values in COPD. Therefore, Yiqi Huoxue Huatan formula improved pulmonary function in COPD.

Yiqi Huoxue Huatan formula mediated anti-inflammatory effects in COPD via PI3K/AKT and NF-κB signaling pathways

To unveil the underlying mechanism behind the regulation of COPD by the *Yiqi Huoxue Huatan* formula, the activity of two main signaling pathways was measured in the lung tissues of these rats. It is noteworthy to state that in COPD rats, the PI3K/AKT signaling pathway was activated, and the activity of this signaling pathway can be suppressed by prednisone and Yiqi Huoxue Huatan formula (Figure 3 A). Yiqi Huoxue Huatan in particular, decreased the activity of PI3K/AKT signaling pathway. The relative expression levels of IKKα and NF-κB in lung tissues were also measured, and it was discovered that NF-κB activity was increased in COPD models (Figure 3 A and B). In contrast with the prednisone treatment, *Yiqi Huoxue Huatan* formula can suppress the activity of NF-κB signaling. In addition to these findings, it was concluded that *Yiqi Huoxue Huatan* formula can repress inflammation in COPD by decreasing the activity of PI3K/AKT and NF-κB signaling pathways.

DISCUSSION

Chronic Obstructive Pulmonary Disease (COPD) affects tens of millions of people worldwide, and the underlying mechanism of COPD is quite complex. Various kinds of immune cells participate in this process, for example, neutrophils, macrophages, T cells and B cells [12]. These cells can secrete cytokines which induce the apoptosis of alveolar cells and cause the destruction of the alveolar wall and the degradation of extracellular matrix. IL-6 is an example, and its concentration is increased in COPD patients with acute exacerbations [13]. IL1-β was reported to lead to persistent inflammation and pulmonary emphysema in lungs [14].

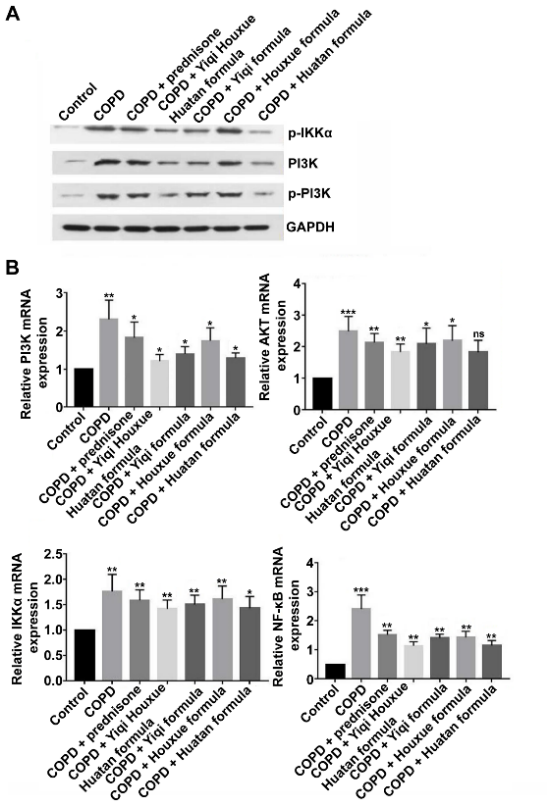

Figure 3: Yiqi Huoxue Huatan formula mediates anti-inflammatory effects in COPD via PI3K/AKT and NF-κB signaling pathways. (A) Western blotting for PI3K/AKT and NF-κB signaling pathways. (B) Relative mRNA expression levels of PI3K, AKT, IKKα and NF-κB. NS: non-significant, ***p < 0.001, **p < 0.01, *p < 0.05, versus control group

TNF-α (Tumor necrosis factor-α) is associated with airway remodeling, and can increase the expression of MMP-2 and MMP-9, both of which can lead to the destruction of alveoli [15]. A clinical study has revealed that persistent systemic inflammation is related to the poor outcome of COPD patients [16]. Therefore, attenuating inflammation systemically and locally in COPD carries clinical significance. In this research, it was discovered that Yiqi Huoxue Huatan formula can improve COPD by reducing inflammation.

It was also discovered that following *Yiqi Huoxue Huatan* formula treatment, the concentrations of key inflammatory mediators in bronchioalveolar fluid, such as IL-6 and TNF-α decreased, and histopathological examination showed that the alveolar structure was partly preserved.

Matrix metallopeptidase 9 (MMP-9) which mediates inflammation and extracellular degradation, is downregulated by *Yiqi Huoxue Huatan* formula [17]. These results indicated that *Yiqi Huoxue Huatan* formula relieves local inflammation. More importantly, the results showed that the efficacy was superior to prednisone. Prednisone is a common treatment option in the acute exacerbations of COPD, but it can result in many side effects such as hyperglycemia and hypertension [18]. To investigate which component plays an important role in treating COPD, we used different recipes to treat COPD rats. It was found that *Yiqi* recipe, *Huoxue* recipe and Huatan recipe hold the same effects, but the combination of these recipes relieves local inflammation to a large extent.

To explore how Yiqi Huoxue Huatan formula modulates local inflammation in COPD, the activity of two main signaling pathways were measured: PI3K/AKT and NF-κB signaling pathways. The results showed that this formula suppresses the activation of both signaling pathways. PI3K/AKT has been shown to increase the release of proinflammatory cytokines in COPD, and regulate the polarization of macrophages [19]. It is noteworthy to state that inhibiting PI3K/AKT signaling pathway is also a promising direction to go in treating COPD. NF-κB signaling is also important, as it functions to manage inflammation in COPD and even induce cell apoptosis [20].

It has been reported that suppressing both NF-κB signaling and COX-2 reduces local inflammation in COPD [21]. In addition, persistent activation of NF-κB signaling in COPD may increase the risk of lung cancer [22]. Hence, the suppression of the activity of NF-κB signaling could decrease local inflammation and improve the clinical outcome in COPD patients. Results showed that Yiqi Huoxue Huatan formula can exert anti-inflammatory effects by downregulating both PI3K/AKT and NF-κB signaling, and thus this formula has potential clinical therapeutic value to treat COPD.

However, there are pitfalls in this research. First, experimental studies in vitro should be performed to further confirm these findings. Second, there might be diverse chemical compounds in Yiqi Huoxue Huatan formula, and further research is required to determine which chemical has the strongest effect. Third, clinical trials may need to be conducted to further confirm the therapeutic effects of *Yiqi Huoxue Huatan* formula.

CONCLUSION

This study has shown that *Yiqi Huoxue Huatan* formula improves COPD by attenuating local inflammation through the suppression of PI3K/AKT and NF-κB activity, thus providing a

new therapeutic strategy for the management of the pulmonary disease.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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