Effects of imperatorin on airway remodeling in bronchial asthma through S1PR2/STAT3 signaling pathway

Dandan Wang[1], Yunmei Cui[1], Fan Gao[1], Weiwei Zheng[1], Jinzi Li[1*], Zhemin Xian[2*]

[1] Department of Ultrasound Medicine, Affiliated Hospital of Yanbian University, Yanji, Jilin,133000, China
[2] Department of Pediatrics, Affiliated Hospital of Yanbian University, Yanji, Jilin,133000, China

ARTICLE INFO

ABSTRACT

In this study, we analyzed the effect of Imperatorin (IMP) on airway remodeling in bronchial asthma (BA) through the S1PR2/STAT3 signaling pathway. First, 30 BALB/c mice were randomized into control, model, and intervention groups. The control group was left untreated; the model and intervention groups were BA modeled and; the intervention group was further intraperitoneally injected with IMP following modeling. Lung tissue pathological changes, inflammatory cell deposition in bronchoalveolar lavage fluid (BALF), expression of inflammatory factors, and oxidative stress (OS) were detected in three groups of mice. We found that the intervention group had reduced macrophage and lymphocyte counts in BALF and ameliorated pathological damage of lung tissue than the control group after intervention. In addition, the post-interventional inflammatory factors and malonaldehyde (MDA) in the intervention group were elevated compared with the control group but reduced versus the model group, while the superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were lower than those in the control group and higher compared with the model group ($P<0.05$). In addition, the expression of S1PR2/STAT3 pathway in three groups of mice showed that S1PR2/STAT3 signaling was activated in the model group, while the expression of S1PR2 and STAT3 in the intervention group was lower than that in the model group ($P<0.05$). These results demonstrate that IMP reverses pathological injury in BA and alleviates airway remodeling by inhibiting the S1PR2/STAT3 axis.

Introduction

Bronchial asthma (BA), one of the most common respiratory diseases in clinical practice, is characterized by hyperresponsiveness, airway inflammation and airway remodeling (1). BA occurs in all age groups, with epidemiological statistics suggesting a global incidence of approximately 1 to 3 percent in 2020 (2). Clinically, BA is believed to be caused by a family history of asthma, allergic diseases (rhinitis, conjunctivitis, etc.), obesity, smoking, etc. (3). During the episode, patients will be accompanied by obvious dyspnea and tachypnea, which if not intervened in time, is highly likely to cause respiratory failure and death (4). According to the World Health Organization (WHO) statistics, about 400,000-600,000 people die of BA every year on average (5). At present, there is no complete clinical cure for BA, and patients need lifelong remission treatment once they develop the disease (6). Therefore, finding a new cure for BA is a hotspot and difficulty in modern clinical research.

Imperatorin (IMP) is a natural 6,7-furanocoumarin compound, which is present in Umbelliferae plants such as Radix Angelicae Pubescentis, Radix Angelicae Sinensis, Masterwort and other commonly used traditional Chinese medicines, and has been proved to have anti-inflammatory, anti-tumor, anti-convulsant and other pharmacological effects (7, 8). In a recent study, Wang N et al. found that IMP inhibits mast cell-mediated allergic airway inflammation (9). With the deepening of research, IMP has also shown excellent effects in anti-oxidative stress (OS) and anti-allergy (10, 11). These studies undoubtedly suggest that IMP may also have the potential to be a therapeutic drug for BA, but its application needs further study and confirmation.

Sphingosine-1-phosphate (S1P) is a biologically active lipid mediator that plays an important role in regulating inflammatory responses and abnormal cell biological behaviors (12). Existing clinical evidence has confirmed that S1P can accelerate the occurrence of airway inflammation by binding with its receptor S1PR2 and activating the downstream signal-transducing activator of transcription 3 (STAT3) (13). The anti-inflammatory effect of IMP on alveolar macrophages is related to the S1P receptor 2 (S1PR2)/STAT3 axis (14). Therefore, we speculate that the anti-BA effect of IMP may also be related to the S1PR2/STAT3 signaling pathway.

Therefore, this study will analyze the influence of IMP on airway remodeling through the S1PR2/STAT3 axis to provide reliable reference and guidance for future clinical applications of IMP in treating BA.

Materials and Methods

Animal data

Thirty BALB/c mice (6-8 weeks old, 16-20g) of specific pathogen-free (SPF) grade were purchased from

---

Ketongnuobei Biomedical Technology (Beijing) Co., Ltd. (certificate number: SYXK (Beijing) 2023-0044). The animals were housed 5 per cage under constant environmental conditions (12/12h day/night, 20-26℃, and 40-70% humidity). The Animal Ethics Committee at our hospital approved this study, and all experiments strictly followed the Regulations for the Administration of Affairs Concerning Experimental Animals.

Reagent information

The sensitizing solution was prepared by mixing 10 μg of ovalbumin (OVA), 100 mg of aluminum hydroxide powder and 200 μL of normal saline (NS). The trigger solution was made by mixing 0.1 g of OVA and 10 mL of NS. All the above materials were ordered from Abcam, USA. IMP, supplied by Beijing Solarbio Science & Technology, was dissolved in 200 μL of dimethyl sulfoxide (DMSO). Masson and Hematoxylin-eosin(HE) Staining Kits: Shanghai Gefan Biotech; Enzyme-linked Immunosorbent Assay (ELISA) kit: Beijing TransGen Biotech; Wright's Stain: Merck, Germany; Bicinchoninic Acid (BCA) Kit: Shanghai Yisheng Biotech; Western Blot Antibodies: Abcam, USA; Polymerase Chain Reaction (PCR) Kit: Thermo Fisher Scientific, USA.

Grouping and treatment

All mice were acclimated for one week and then were randomized into control, model, and intervention groups (n=10 for each). Mice in model and intervention groups were treated with an intraperitoneal injection of 200 μL of sensitizing solution on the 1st, 7th, and 14th day; on the 17th day, 10 mL of trigger solution was inhaled by atomization (completed within 30 min). The control group was given intraperitoneal injection and atomized inhalation of the same amount of NS. Atomization was performed 3 times a week for 8 weeks. In the intervention group, 30 mg/kg IMP was injected intraperitoneally 1 hour before each atomization.

BA scoring

After the intervention, the behaviors of mice in each group such as nose scratching and sneezing were observed within 3 minutes, and BA scoring was performed (15): 0 points for non-sneezing, 1 point for 1-3 sneezes, 2 points for 4-10 sneezes, and 3 points for more than 10 sneezes; 0 points for no nose scratching, 1 point for mild nose scratching, 2 points for frequent nose scratching, and 3 points for continuous nose scratching; 0 points for no wheezing, 1 point for shortness of breath, 2 points for obvious wheezing, and 3 points for death from wheezing. The total score was 9 points.

Sample collection

All mice were killed under anesthesia, and the trachea was exposed by a longitudinal incision along the neck. After a small cut, a hose was inserted, through which 1 mL of NS was injected and then pumped back (three times in a row). The bronchoalveolar lavage fluid (BALF) was collected and divided into two parts: one was centrifugalized to collect supernatant for storage in a refrigerator at -80℃, and the other part was subpackaged into Eppendorf (EP) tubes, stained with Wright's stain, and then placed under a light microscope for macrophages and lymphocyte counting. In addition, intact lung tissue was taken and fixed in 4% formaldehyde for testing.

Lung histological examination

After dehydration, waxing, embedding and slicing (thickness 4 μm), the mouse lung tissue sections were stained with Masson and HE strictly following the kit instructions. After completion, the collagen deposition in lung tissue was observed microscopically. Image J software was used to analyze HE-stained lung tissue sections. The circumferences of luminal and abluminal membranes, the total area of the tracheal wall, and the area of airway smooth muscle were measured to calculate tracheal wall thickness (total area of the tracheal wall/circumferences of luminal and abluminal membranes), and airway smooth muscle thickness (area of the airway smooth muscle/circumferences of luminal and abluminal membranes).

Detection of inflammatory factors and OS markers

Tumor necrosis factor (TNF)-α, interleukin (IL)-1β/6, transforming growth factor (TGF)-β, superoxide dismutase (SOD), malonaldehyde (MDA) and glutathione peroxide (GSH-PX) concentrations were measured strictly according to the kit instructions.

Protein detection

Total protein was extracted from mouse lung tissues after lysis with a BCA kit. Following purity verification, 20 μg of the protein was taken for gel electrophoresis, membrane transfer, one hour of sealing with 5% skim milk, and immersion in α-SMA, MMP-9, S1PR2, p-STAT3 primary antibodies (1:2,000) for a night-long incubation at 4℃. A second antibody (1:5,000) was added to the membrane the next day for 4 hours of room-temperature incubation, followed by exposure and development using ECL and calculation of gray values of the bands using ImageJ software.

PCR

TRIzol extracted total RNA from lung tissue and reverse transcribed it into cDNA for PCR reaction. Reaction conditions (40 cycles): 95℃ for 30s, 95℃ for 30s, and 60℃ for 2min. α-SMA, MMP-9, S1PR2, and STAT3 mRNA levels normalized against GAPDH were calculated by $2^{-\Delta\Delta CT}$. The primer sequence was commissioned to be designed and constructed by Thermo Fisher Scientific, USA.

Statistical analyses

All experiments were done in triplicates, and the results were expressed in the form of mean standard deviation ($\bar{\chi}\pm s$). The variance analysis and Bonferroni post-hoc test were used for comparison among groups, and the difference was considered statistically significant when P<0.05.

Results

Effect of IMP on pathological symptoms of BA

First, the multi-group comparison of BA scores revealed higher BA scores in the model and intervention groups compared with the control group, and lower scores in the intervention group as compared to the model group (*P*<0.05). According to Masson staining, the lung tissue of control mice was clearly reticulated, the bronchial lumen was normal and the lung was not damaged. The model group showed significantly increased collagen deposi-
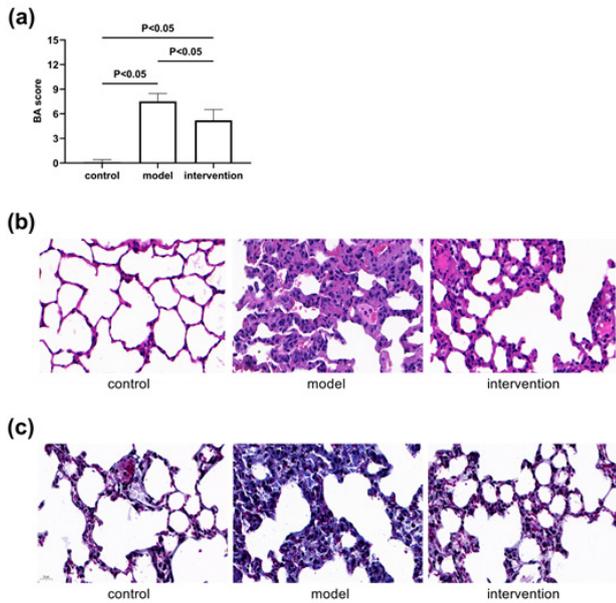
Figure 1. Effect of IMP on pathological symptoms of BA. a) BA scores, b) HE staining, c) Masson staining.

Figure 2. Impact of IMP on airway remodeling. a) thicknesses of the tracheal wall, b) thicknesses of airway smooth muscle.

Figure 3. Influence of IMP on pulmonary inflammation. a) number of inflammatory cells (macrophages and lymphocytes), b) concentration of inflammatory factors (TNF-α, IL-1β/6, and TGF-β).

Figure 4. Impact of IMP on lung OS. a) SOD, b) GSH-Px, c) MDA.

Figure 5. Influence of IMP on pulmonary inflammation. a) number of inflammatory cells (macrophages and lymphocytes), b) concentration of inflammatory factors (TNF-α, IL-1β/6, and TGF-β).

tion in lung tissue than control mice, with airway smooth muscle hypertrophy and hyperplasia, obvious airway wall thickening, mucosal epithelium hyperplasia, lower layer widening, and mucus-blocking mucosal lumen. In contrast, the mouse pulmonary fibrosis in the intervention group was markedly reduced compared with the model group, and the pathological condition was significantly ameliorated. These results preliminarily suggest that IMP can reverse the pathological injury process of BA. Fig 1

Impact of IMP on airway remodeling

Through calculation, it was found that the thicknesses of the tracheal wall and airway smooth muscle in the model group were (18.70±1.64)μm and (10.50±1.18)μm, respectively, which were the highest among the three groups, while those in the intervention group was (11.90±3.84)μm and (7.20±1.93)μm, respectively, lower than compared with the model group and higher than those in the control group ($P<0.05$). It indicates that IMP can also alleviate airway remodeling in BA mice. Fig 2

Influence of IMP on pulmonary inflammation

After cell counting, it was found that the BALF macrophage and lymphocyte counts were notably higher in the model group than in the control group, while those in the intervention group were lower compared with the model group and higher versus the control group ($P<0.05$). Subsequently, the detection results of inflammatory factors showed that TNF-α, IL-1β/6, and TGF-β were the highest in the model group among the three groups, while those in the intervention group were higher than the control groups and lower compared with the model group ($P<0.05$). It can be seen that IMP can inhibit the release of pulmonary inflammatory factors. Fig 3

Impact of IMP on lung OS

In the detection of OS markers, SOD and GSH-Px were found to be higher in the model and intervention groups compared with the control group and were higher in the intervention group as compared to the model group. While
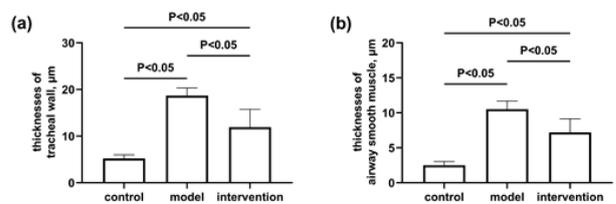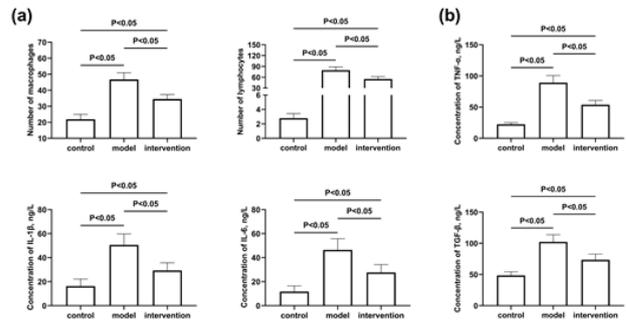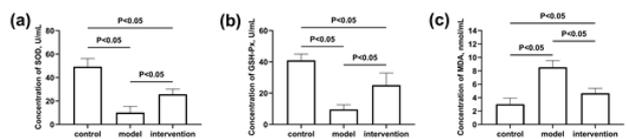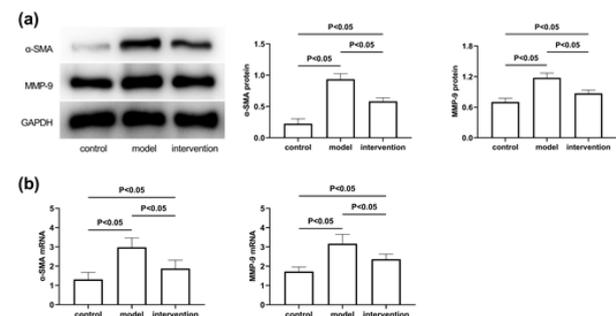
model and intervention groups had higher MDA levels than the control group, and the intervention group showed lower MDA than the model group ($P<0.05$). These results suggest that IMP can also inhibit OS progression. Fig 4

Effect of IMP on pulmonary fibrosis

After testing, it can be seen that α-SMA and MMP-9 protein levels were higher in the model group compared with the control group, while those in the intervention group were lower compared with the model group and higher versus the control group ($P<0.05$). Consistent findings were observed in the PCR assay, that is, the α-SMA and MMP-9 mRNA in the intervention group were lower

compared with the model group and higher versus the control group ($P<0.05$). In other words, IMP can effectively inhibit the fibrosis process of lung tissue in BA mice. Fig 5

Influence of IMP on S1PR2/STAT3 axis

Finally, the detection of S1PR2/STAT3 axis-associated gene expression identified the highest S1PR2 and p-STAT3 protein expression in the model group among the three groups, while S1PR2 and p-STAT3 protein levels in the intervention group were higher compared with the control group but lower versus the model group ($P<0.05$). According to PCR analysis, S1PR2 and STAT3 mRNA levels were the highest in the model group, followed in descending order by the intervention group and control group ($P<0.05$). This confirms that the S1P/S1PR2/STAT3 axis is active in BA and that IMP can inhibit this pathway. Fig 6

Discussion

The potential threat of BA, a global health problem, must attract our sufficient attention, and finding a cure for BA is also the focus of modern clinical research (16). In this study, we found that IMP effectively reversed the pathological damage of BA, which has important reference significance for the future clinical treatment of BA.

First of all, after establishing the BA mouse model, we found that the BA score of the model group was significantly higher than that of the control group, and the pathological damage of lung tissue was aggravated, with obvious inflammatory infiltration and collagen deposition, which is in line with the pathological manifestations of BA (17), confirming the successful modeling. In contrast, the intervention group showed reduced BA scores and obviously ameliorated pathological damage of lung tissue, suggesting that IMP can reverse the pathological progression of BA, which is also consistent with the study of Xian Z et al. on the effect of IMP on the improvement of airway inflammation (18). Besides, airway remodeling is known to be one of the most important pathological features in BA, and reversing airway remodeling in BA patients has direct implications for alleviating BA (19). In this experiment, we also found that the thicknesses of the tracheal wall and airway smooth muscle in the model group were significantly increased compared with the control group, confirming the existence of significant airway remodeling in BA mice. After IMP treatment, airway remodeling was significantly improved in the intervention group, which further confirms the above inference, indicating that IMP has excellent therapeutic potential for BA. Similarly, IMP is shown to reduce extracellular matrix deposition, inhibit hepatic stellate cell activation and reverse liver fibrosis progression by up-regulating HNF-4α (20), which also supports our experimental results. In addition, α-SMA plays a key role in airway smooth muscle contraction and is closely related to airway remodeling. Increasing the level of α-SMA can promote smooth muscle cell proliferation and migration (21). MMP-9, on the other hand, is associated with collagen deposition in the tracheal wall, which may cause airway stenosis (22). In the intervention group, the expression of α-SMA and MMP-9 was also significantly inhibited, which fully demonstrates the ameliorating effect of IMP on airway remodeling.
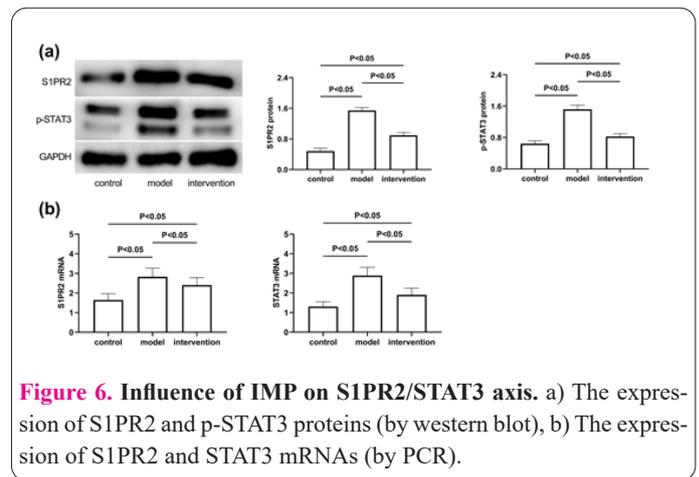
As we all know, OS and inflammatory infiltration of

Figure 6. Influence of IMP on S1PR2/STAT3 axis. a) The expression of S1PR2 and p-STAT3 proteins (by western blot), b) The expression of S1PR2 and STAT3 mRNAs (by PCR).

lung tissue are the key to the destruction of patients' lung function during the onset of BA, which is also an important reason for the eventual respiratory failure and repeated attacks of BA (23). In this study, we can also see that the level of inflammatory factors in the model group increased and the OS response intensified. While IMP intervention contributed to obviously suppressed inflammatory factors in mice and significantly reversed OS responses, indicating that the improvement mechanism of IMP on BA might be related to its anti-inflammatory and anti-OS actions. In previous studies, IMP has also shown excellent anti-inflammatory and anti-OS effects on diseases such as arthritis and colitis (24, 25), which can support our view.

Finally, in order to confirm that the influence of IMP on BA is related to the S1PR2/STAT3 axis, we detected the expression of this pathway in BA. The results showed significantly higher S1PR2 and p-STAT3 protein levels in the model group compared with the control group, confirming the activated S1PR2/STAT3 pathway in BA. In previous studies, the abnormal activation of the S1PR2/STAT3 axis has been repeatedly confirmed to accelerate the release of downstream inflammatory mediators and promote the intensification of the body's inflammatory response (26, 27), which is also in line with the pathological manifestations of BA. Similarly, the expressions of S1PR2 and p-STAT3 in the intervention group decreased significantly, which confirms that IMP can inhibit the S1PR2/STAT3 axis and eventually reverse the pathological changes of BA, consistent with the research results of Chen L et al. (14).

However, more experiments are needed to further confirm the influence of IMP on BA and its mechanism of action and to analyze and compare the effects of different doses of IMP. Finally, before IMP is put into clinical use, a large number of clinical trials are still needed to confirm its efficacy and safety, which will also be the focus of subsequent studies.

IMP can effectively reverse the pathological injury of BA and ameliorate airway remodeling, and its mechanism is related to the suppression of S1PR2/STAT3 axis-associated gene expression and the alleviation of inflammatory responses and OS. IMP is expected to be a new treatment choice for BA in the future, providing a more reliable guarantee for patients' health and life safety.

Ethical approval
Not applicable.

Conflicts of interest

The authors report no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

Not applicable.

References

1. Kunc P, Fabry J, Lucanska M, Pecova R. Biomarkers of Bronchial Asthma. Physiol Res. 2020;69(Suppl 1):S29-S34.

2. Hammad H, Lambrecht BN. The basic immunology of asthma. Cell. 2021;184(6):1469-85.

3. Schoettler N, Strek ME. Recent Advances in Severe Asthma: From Phenotypes to Personalized Medicine. Chest. 2020;157(3):516-28.

4. Nakamura Y, Tamaoki J, Nagase H, Yamaguchi M, Horiguchi T, Hozawa S, et al. Japanese guidelines for adult asthma 2020. Allergol Int. 2020;69(4):519-48.

5. Liu F, Liu YR, Liu L. Effect of exercise rehabilitation on exercise capacity and quality of life in children with bronchial asthma: a systematic review. Zhongguo Dang Dai Er Ke Za Zhi. 2021;23(10):1050-7.

6. Minagawa S, Araya J, Watanabe N, Fujimoto S, Watanabe J, Hara H, et al. Real-life effectiveness of dupilumab in patients with mild to moderate bronchial asthma comorbid with CRSwNP. BMC Pulm Med. 2022;22(1):258.

7. Deng M, Xie L, Zhong L, Liao Y, Liu L, Li X. Imperatorin: A review of its pharmacology, toxicity and pharmacokinetics. Eur J Pharmacol. 2020;879:173124.

8. Ma T, Liu P, Wei J, Zhao M, Yao X, Luo X, et al. Imperatorin alleviated endometriosis by inhibiting the activation of PI3K/Akt/NF-kappaB pathway in rats. Life Sci. 2021;274:119291.

9. Wang N, Wang J, Zhang Y, Zeng Y, Hu S, Bai H, et al. Imperatorin ameliorates mast cell-mediated allergic airway inflammation by inhibiting MRGPRX2 and CamKII/ERK signaling pathway. Biochem Pharmacol. 2021;184:114401.

10. Huang Y, Liao X, Wang H, Luo J, Zhong S, Zhang Z, et al. Effects of imperatorin on apoptosis and synaptic plasticity in vascular dementia rats. Sci Rep. 2021;11(1):8590.

11. Xu WW, Huang ZH, Liao L, Zhang QH, Li JQ, Zheng CC, et al. Direct Targeting of CREB1 with Imperatorin Inhibits TGFbeta2-ERK Signaling to Suppress Esophageal Cancer Metastasis. Adv Sci (Weinh). 2020;7(16):2000925.

12. Loh KC, Leong WI, Carlson ME, Oskouian B, Kumar A, Fyrst H, et al. Sphingosine-1-phosphate enhances satellite cell activation in dystrophic muscles through a S1PR2/STAT3 signaling pathway. PLoS One. 2012;7(5):e37218.

13. Oskeritzian CA, Hait NC, Wedman P, Chumanevich A, Kolawole EM, Price MM, et al. The sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 axis regulates early airway T-cell infiltration in murine mast cell-dependent acute allergic responses. J Allergy Clin Immunol. 2015;135(4):1008-18 e1.

14. Chen L, Xu W, Yang Q, Zhang H, Wan L, Xin B, et al. Imperatorin alleviates cancer cachexia and prevents muscle wasting via directly inhibiting STAT3. Pharmacol Res. 2020;158:104871.

15. Shi ZG, Sun Y, Wang KS, Jia JD, Yang J, Li YN. Effects of miR-26a/miR-146a/miR-31 on airway inflammation of asthma mice and asthma children. Eur Rev Med Pharmacol Sci. 2019;23(12):5432-40.

16. Mandlik DS, Mandlik SK. New perspectives in bronchial asthma: pathological, immunological alterations, biological targets, and pharmacotherapy. Immunopharmacol Immunotoxicol. 2020;42(6):521-44.

17. Ravi A, Goorsenberg AWM, Dijkhuis A, Dierdorp BS, Dekker T, van Weeghel M, et al. Metabolic differences between bronchial epithelium from healthy individuals and patients with asthma and the effect of bronchial thermoplasty. J Allergy Clin Immunol. 2021;148(5):1236-48.

18. Xian Z, Choi YH, Zheng M, Jiang J, Zhao Y, Wang C, et al. Imperatorin alleviates ROS-mediated airway remodeling by targeting the Nrf2/HO-1 signaling pathway. Biosci Biotechnol Biochem. 2020;84(5):898-910.

19. Li H, Duan C, Zhou L, Wang M. [Application progress of metabonomics evaluation methods in bronchial asthma]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue. 2021;33(8):1021-4.

20. Ge JW, Deng SJ, Xue ZW, Liu PY, Yu LJ, Li JN, et al. Imperatorin inhibits mitogen-activated protein kinase and nuclear factor kappa-B signaling pathways and alleviates neuroinflammation in ischemic stroke. CNS Neurosci Ther. 2022;28(1):116-25.

21. Dong L, Wang Y, Zheng T, Pu Y, Ma Y, Qi X, et al. Hypoxic hUCMSC-derived extracellular vesicles attenuate allergic airway inflammation and airway remodeling in chronic asthma mice. Stem Cell Res Ther. 2021;12(1):4.

22. Zou F, Zhang J, Xiang G, Jiao H, Gao H. Association of Matrix Metalloproteinase 9 (MMP-9) Polymorphisms with Asthma Risk: A Meta-Analysis. Can Respir J. 2019;2019:9260495.

23. Crimi C, Ferri S, Campisi R, Crimi N. The Link between Asthma and Bronchiectasis: State of the Art. Respiration. 2020;99(6):463-76.

24. Ahmad N, Ansari MY, Bano S, Haqqi TM. Imperatorin suppresses IL-1beta-induced iNOS expression via inhibiting ERK-MAPK/AP1 signaling in primary human OA chondrocytes. Int Immunopharmacol. 2020;85:106612.

25. Luo M, Luo Y. Imperatorin Relieved Ulcerative Colitis by Regulating the Nrf-2/ARE/HO-1 Pathway in Rats. Inflammation. 2021;44(2):558-69.

26. Terashita T, Kobayashi K, Nagano T, Kawa Y, Tamura D, Nakata K, et al. Administration of JTE013 abrogates experimental asthma by regulating proinflammatory cytokine production from bronchial epithelial cells. Respir Res. 2016;17(1):146.

27. Nikolskii AA, Shilovskiy IP, Barvinskaia ED, Korneev AV, Sundukova MS, Khaitov MR. Role of STAT3 Transcription Factor in Pathogenesis of Bronchial Asthma. Biochemistry (Mosc). 2021;86(11):1489-501.