Analytical Biochemistry 631 (2021) 114310

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Using UPLC-QTOF/MS and multivariate analysis to explore the mechanism of Bletilla Striata improving $PM_{2.5}$-induced lung impairment

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A R T I C L E I N F O

Keywords:
$PM_{2.5}$
Bletilla striata
Lung impairment
UPLC-QTOF/MS
Metabolomics

A B S T R A C T

Exposure to fine particulate matter ($PM_{2.5}$) is closely related to lung diseases and has become more and more harmful to public health. The traditional Chinese medicine of Bletilla Striata has the effect of clearing and nourishing the lungs in clinics. The purpose of the study is using metabolomics methods to explore the mechanism of $PM_{2.5}$-induced lung injury and Bletilla Striata's therapeutic effect. In this article, we used an Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-QTOF/MS) method to identify the potential biomarkers. The results showed that there were 18 differential metabolites in the plasma and urine of rats with $PM_{2.5}$-induced lung injury, involving the glycerophospholipid metabolism pathway, the tryptophan metabolism pathway, and the purine metabolism pathway, etc. After the administration, Bletilla Striata changed the levels of 21 metabolites, and partly corrected the changes in the level of metabolites caused by $PM_{2.5}$. The results indicated that Bletilla Striata could exert a good therapeutic effect by reversing the levels of some biomarkers in the rats with $PM_{2.5}$-induced lung impairment.

1. Introduction

Numerous epidemiological studies have certified that the Particulate Matter (PM) exposure causes impairment effects to public health [1–3]. Especially, the fine particulate matter ($PM_{2.5}$), defined as particles with aerodynamic diameter of less than 2.5 μm, has been associated with a series of diseases such as asthma, lung cancer and cardiovascular disease [4–7]. According to recent estimates, the $PM_{2.5}$ air pollutant is widespread in many countries and is considered a health risk worldwide. Therefore, it is critical to investigate the toxicological mechanisms of $PM_{2.5}$-induced adverse health effects for more efficient clinical treatment.

Bletilla Striata is a dry tuber of the orchid family Bletilla Striata (Thunb.) Reichb.f., its nature and taste are bitter, sweet, astringent, slightly cold, and enter the lung meridian. Its effects included of invigorating lung, stopping bleeding, reducing swelling, growing muscles, and restraining sores. Modern pharmacological studies have shown that Bletilla Striata has antibacterial function such as clearing the lung, anti-tumor, anti-ulcer, regulating immune system, and promoting wound healin [8,9]. Current toxicological mechanistic studies of $PM_{2.5}$ include DNA damage, inflammation, reactive oxygen species (ROS), and oxidative stress [10–13]. A previous study from our group showed that $PM_{2.5}$ induces several gene expression alterations in rat lung tissues [14]. In our study, the rats were exposed to $PM_{2.5}$ for different periods of time, and the lung impairment of the rat was also increased. In recent years, many studies have demonstrated that the lung impairment could induce a change in the endogenous metabolites in body fluids and tissues, including changes in phospholipid, glycerophospholipid, sphingolipid and purine metabolism and DNA damage in lung [15,16]. Therefore, the determination of the changes in the endogenous substances in $PM_{2.5}$-induced rats plasma and urine samples could identify potential markers for lung impairment.

Metabolomics is an emerging field that involves the analysis of low weight molecules or metabolites in biological samples. Dynamic multi-parameter quantitative measurement of the metabolites of the pathophysiological stimulation of the life system helps to discover the relationship between the disease and metabolic characteristics [17–19]. As an emerging technology, metabolomics can be used to search for possible biomarkers with including GC/LC-mass spectrometry and NMR-spectroscopy, which can provide molecular insights into the entire

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https://doi.org/10.1016/j.ab.2021.114310
Received 26 May 2021; Received in revised form 12 July 2021; Accepted 16 July 2021
Available online 17 July 2021

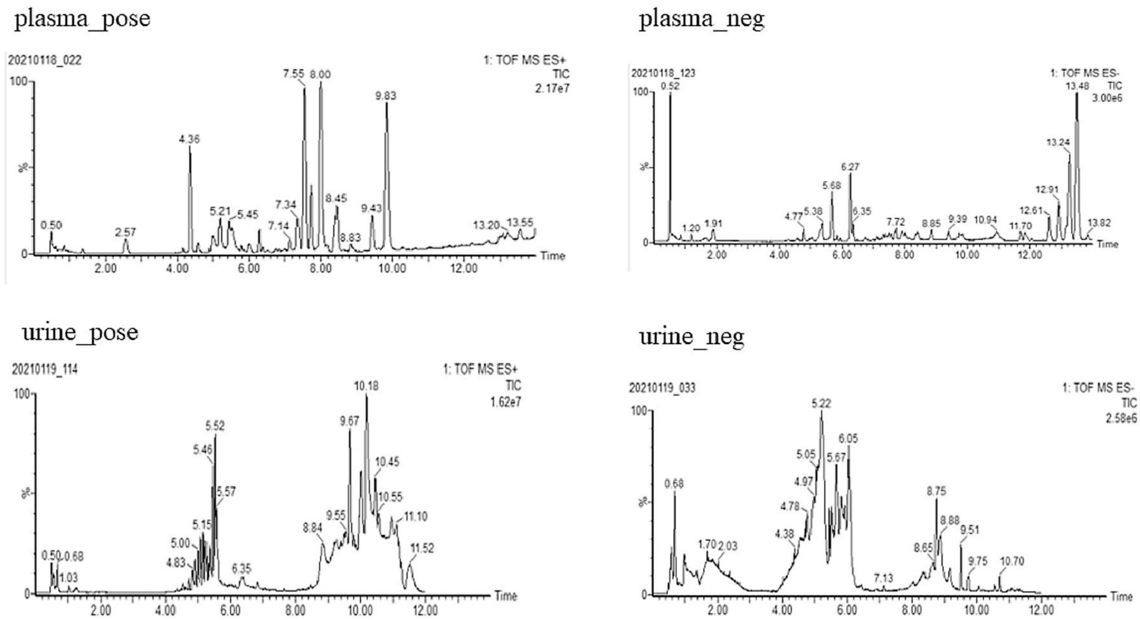

Fig. 1. Base peak ion current (BPI) chromatograms obtained from plasma sample and urine sample (a. plasma sample in positive mode, b. plasma sample in negative mode, c. urine sample in positive mode, d. urine sample in negative mode.).

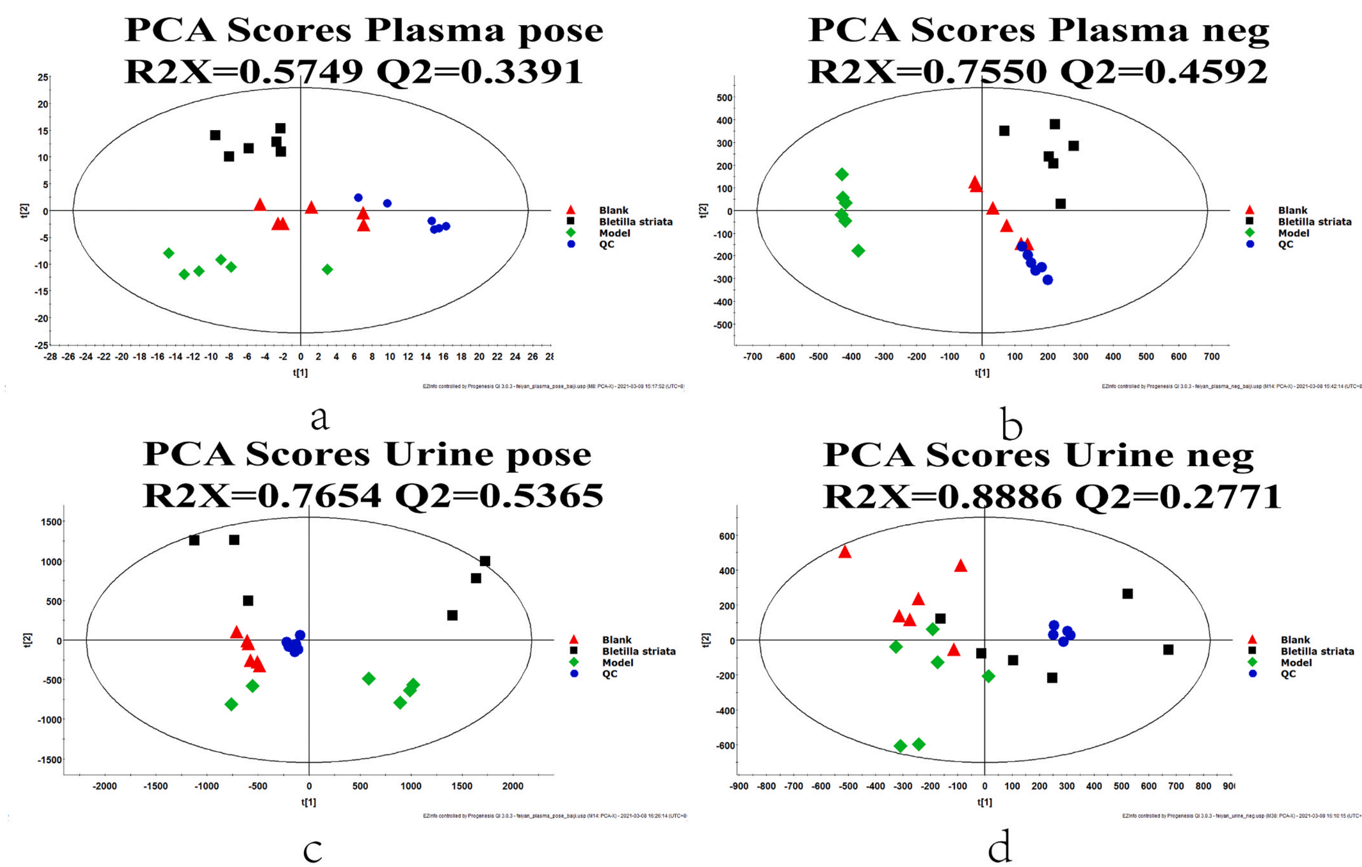

Fig. 2. PCA score plots of plasma and urine metabolic profiles. a. in plasma in ESI (+); b.in plasma in ESI (−); c.in urine in ESI (+), d. in urine in ESI (−), respectively.

metabolic process of the diseases. It is an ideal tool for identifying physiological or pathophysiological conditions in the field of drug research and health care. In this study, we used UPLC-QTOF/MS to analyze the plasma and urine of rats in the control group and the $PM_{2.5}$-exposed model group. In addition, metabolomics was used for the first time to study the mechanism of $PM_{2.5}$-induced lung impairment and the therapeutic effect of Bletilla Striata. This study may provide a basis for a better understanding of the metabolic spectrum of $PM_{2.5}$-infected pneumonia and new insights into the clinical application of Bletilla Striata.

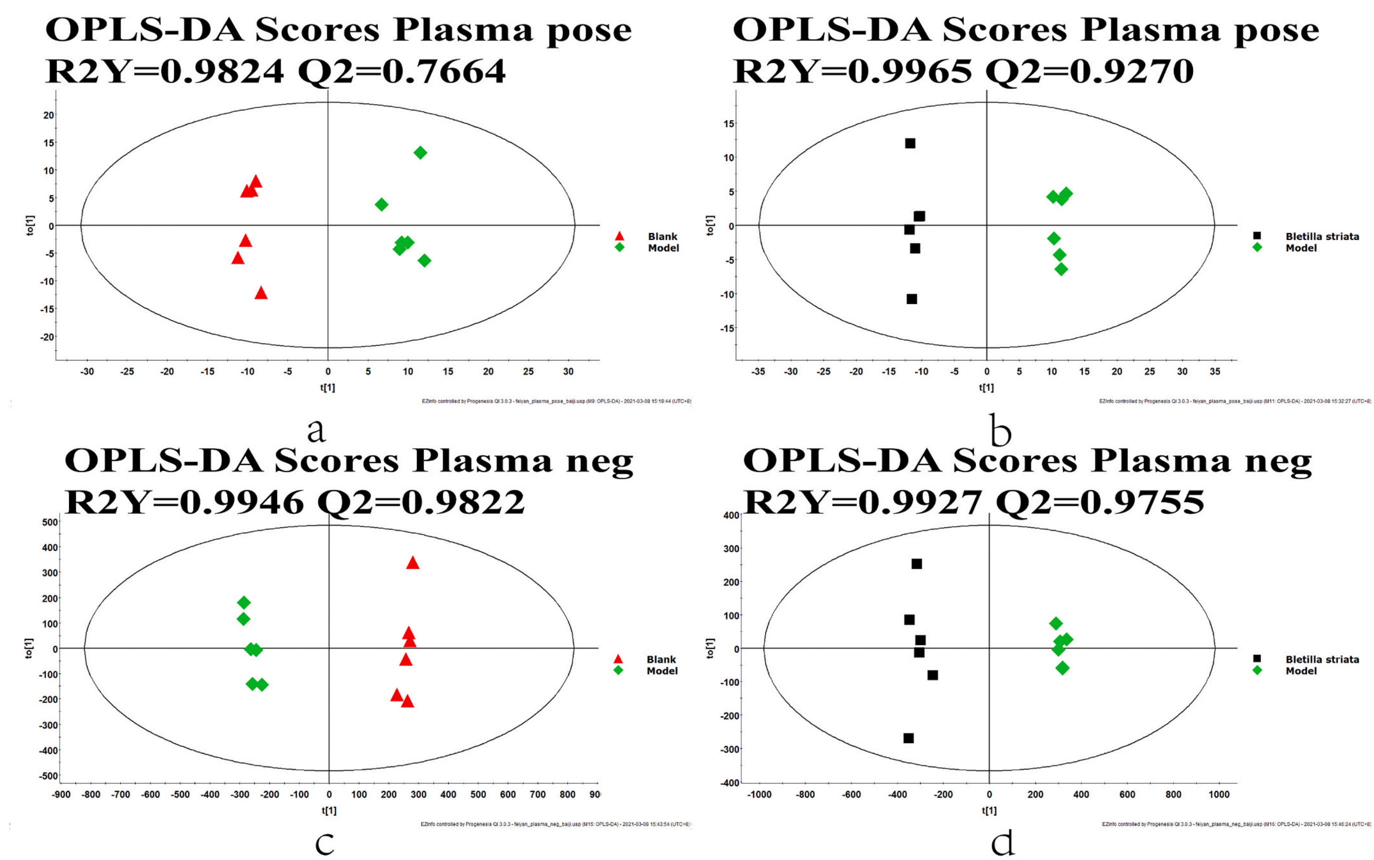

Fig. 3. OPLS_DA score plots between each two groups in plasma. a. OPLS-DA scores plots of blank and model group (ESI (+)), b. OPLS-DA scores plots of Bletilla striata and model group (ESI (+)), c. OPLS-DA scores plots of blank and model group (ESI (−)), d. OPLS-DA scores plots of Bletilla striata and model group (ESI (−)).

2. Materials and methods

2.1. Materials and reagents

Bletilla Striata was purchased from Tianyitang TCM store (Shenyang, China). Acetonitrile, methanol and formic acid of LC/MS class were purchased from Fisher Scientific (Company Inc, USA). Distilled water prepared with Watson demineralized water was employed throughout this experiment.

2.1.1. Preparation for Bletilla Striata decoction

The Bletilla Striata was pulverized to fine powder. And then powders were extracted three times by refluxing in water (1:10 w/v) for 2 h. The extracted solutions were concentrated under reduced pressure to 1.0 g mL^{-1}.The decoction was stored in the refrigerator at 4 °C.

2.2. Animals

18 adult female wistar rats (age: 8-week old, weight: 200–300 g) were obtained from the Vital River Laboratories, Beijing, China. The animal study was carried out in accordance with the Guideline for Animal Experimentation of Shenyang Medical College and the protocol was approved by the Animal Ethics Committee of the institution.

2.3. p.m.$_{2.5}$ collection and exposure protocol

$PM_{2.5}$ samples were prepared according to the method used in the previous studies of our group [20]. $PM_{2.5}$ samples were prepared in distilled water at 10 mg/mL prior to use. 18 Wistar rats were randomly assigned to 3 groups of 6 control、6 model and 6 Bletilla Striata rats each. The model and Bletilla Striata group rats were exposed to $PM_{2.5}$ (0.4 mg/mL/rat) by intratracheal instillation for 30 days, while the Bletilla Striata group rats were treated with Bletilla Striata. And the 6 control group rats were treated with 1 mL normal saline by the same route of administration. After the treatment times, the 3 group rats were sacrificed, the plasma and urine samples were collected for the determination of endogenous metabolites.

2.4. UPLC-Q-TOF/MS metabolomics analysis

2.4.1. Plasma sample preparation and LC-MS condition

We add 450 μL prechilled methanol to 150 μL plasma sample, and the mixture was vortexed for 15 s and let stand at −20 °C for 20min, then centrifuged at 12,000 rpm for 10 min under 4 °C. The supernatant was transferred and evaporated to dryness under nitrogen. The residue was dissolved in 150 μL of methanol-water (7:3, v/v). After vortexing centrifugation again at 12,000 rpm for 10 min at 4 °C, the supernatant was transferred to a sample vial for UPLC/Q-TOF-MS analysis.

The analysis was done using an Acquity UPLC system (Waters, Milford, MA, USA) coupled with Q-TOF mass spectrometer. Chromatographic separation was achieved using an Acquity UPLC T3 C18 column (100 mm × 2.1 mm, 1.7 μm; Waters) at 40 °C. The mobile phase consisted of aqueous 0.1% formic acid (solvent A) and acetonitrile (solvent B) in the positive mode, and consisted of water (solvent A) and acetonitrile (solvent B) in the negative mode, which all were delivered at a flow rate of 0.4 mL min^{-1}. For plasma metabolomic using a gradient elution of 5% B at 0–2 min, 5%–60% B at 2–5 min, 60%–80% B at 5–13 min , 80%–95% B at 13–14 min and 95% B at 14–16 min. The sample injection volume was 3 μL, and the sample room temperature was 4 °C.

Mass spectrometry was performed at 1.5 kV (ESI^+) and at 2.0 kV (ESI^-) for plasma samples. The flow rate of desolvation gas was 500 L/h. The source temperature was 120 °C and the desolvation temperature

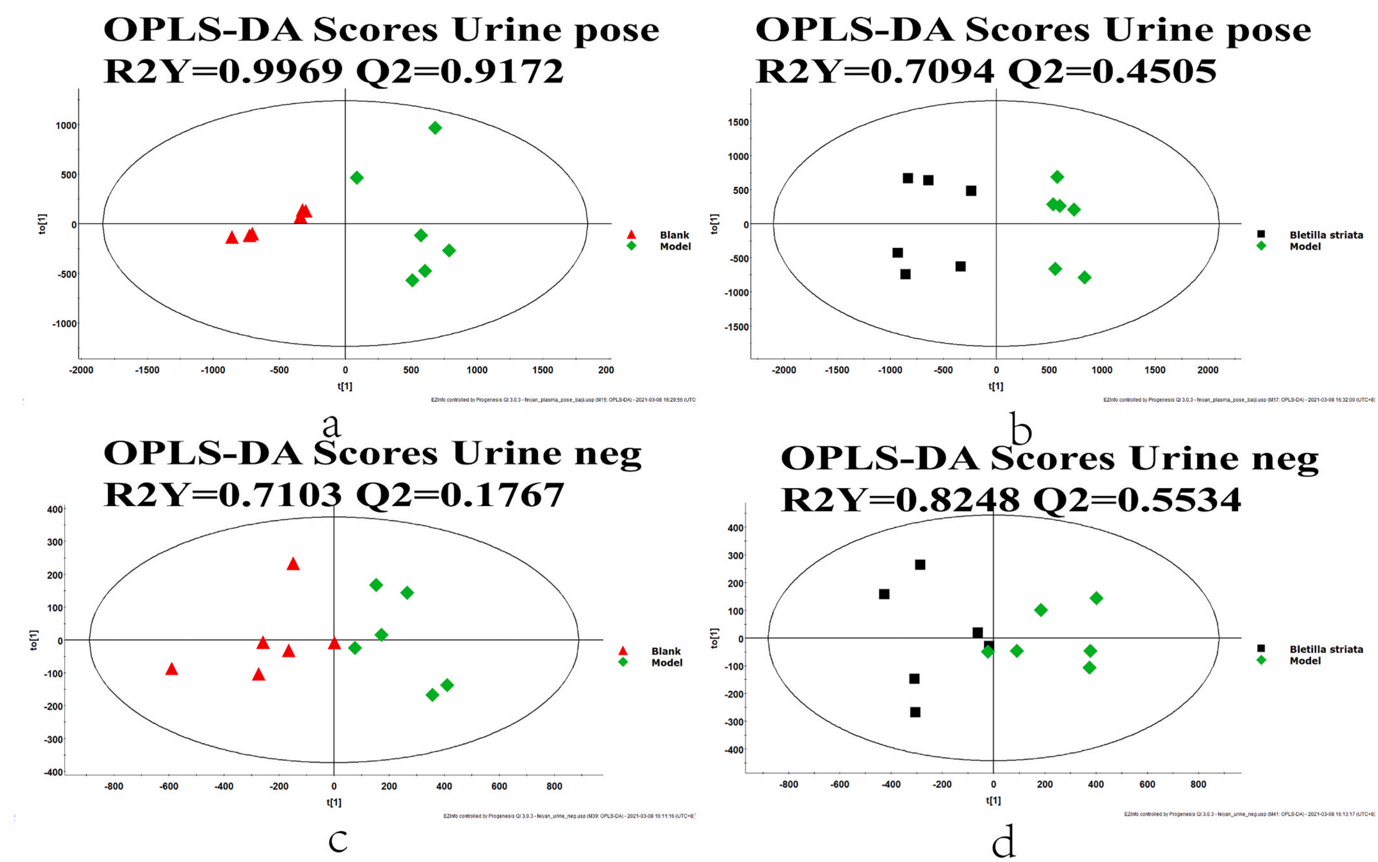

Fig. 4. OPLS_DA score plots between each two groups in urine. a. OPLS-DA scores plots of blank and model group (ESI (+)), b. OPLS-DA scores plots of Bletilla striata and model group (ESI (+)), c. OPLS-DA scores plots of blank and model group (ESI (−)), d. OPLS-DA scores plots of Bletilla striata and model group (ESI (−)).

was 500 °C. The TOF acquisition rate was 0.2 s/scan. MS data were acquired in continuum mode. Leucineen kephalin (250 pg/uL) was used as a lock mass reference (*m/z* 556.2771) with the Lock Spray interface to ensure mass accuracy and reproducibility.

2.4.2. *Urine sample preparation and LC-MS condition*

The urine sample (150 μL) was added to 300 μL of prechilled methanol, and the mixture was vortexed for 15 s and let stand at −20 °C for 20min, then centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred through 0.22 μm filter and then to a sample vial for UPLC/Q-TOF-MS analysis.

The analysis was performed using an Acquity UPLC system (Waters, Milford, MA, USA) coupled with Q-TOF mass spectrometer. Chromatographic separation was achieved using an Acquity UPLC T3 C18 column (150 mm × 2.1 mm, 1.7 μm; Waters) at 40 °C. The mobile phase consisted of aqueous 0.1% formic acid (solvent A) and acetonitrile (solvent B) in the positive mode, and consisted of water (solvent A) and acetonitrile (solvent B) in the negative mode, which all were and acetonitrile (solvent B), which was delivered at a flow rate of 0.4 mL min^{-1}. For urinary metabolomic with a gradient elution of 2% B at 0–2 min, 2%–60% B at 2–8 min, 60%–95% B at 8–10 min, and 95% B at 10–12 min.

Mass spectrometry was performed at 1.5 kV (ESI+) and at 2.0 kV (ESI−) for urine samples. The flow rate of desolvation gas was 500 L/h. The source temperature and desolvation temperature were 120 °C and 500 °C, respectively.

2.5. *Data processing and statistical analysis*

All of the original raw files were imported into the Progensis QI software for analysis and processing. Peak alignment, extraction and peak reduction were performed for each peak. The quality, retention time and relative intensity of the detected peaks were listed, and metabolites molecules among them were selected as the next analysis targets. In order to process more diversified data, we used Ezinfo3.0 software, including the principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The variable importance for the projection (VIP) value > 1 in OPLS-DA and $P < 0.05$ for Students t-test were choosed as potential biomarkers for further analysis. The screened differential metabolies were considered as possible potential biomarkers and were searched using HMDB, KEGG and other databases.

Significantly altered metabolite data were imported into MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) to investigate the therapeutic mechanisms related to Bletilla Striata treatment. The p value < 0.05 was regarded as significant pathways.

3. Results

3.1. *UPLC-MS results*

The UPLC/MS Base peak ion current chromatogram (BPI) of plasma samples and urine samples were shown in positive ionization modes (ESI+) and negative ionization modes (ESI-) in Fig. 1. Under the optimized gradient elution program and the metabolomics map of each sample, the BPI showed ideal separation results. In this study, a multivariate statistical analysis method was established to identify biomarkers related to the treatment of Bletilla Striata on $PM_{2.5}$-induced lung injury.

3.2. *Metabonomic profiling*

To assure the reliability of the data, QC samples were injected before

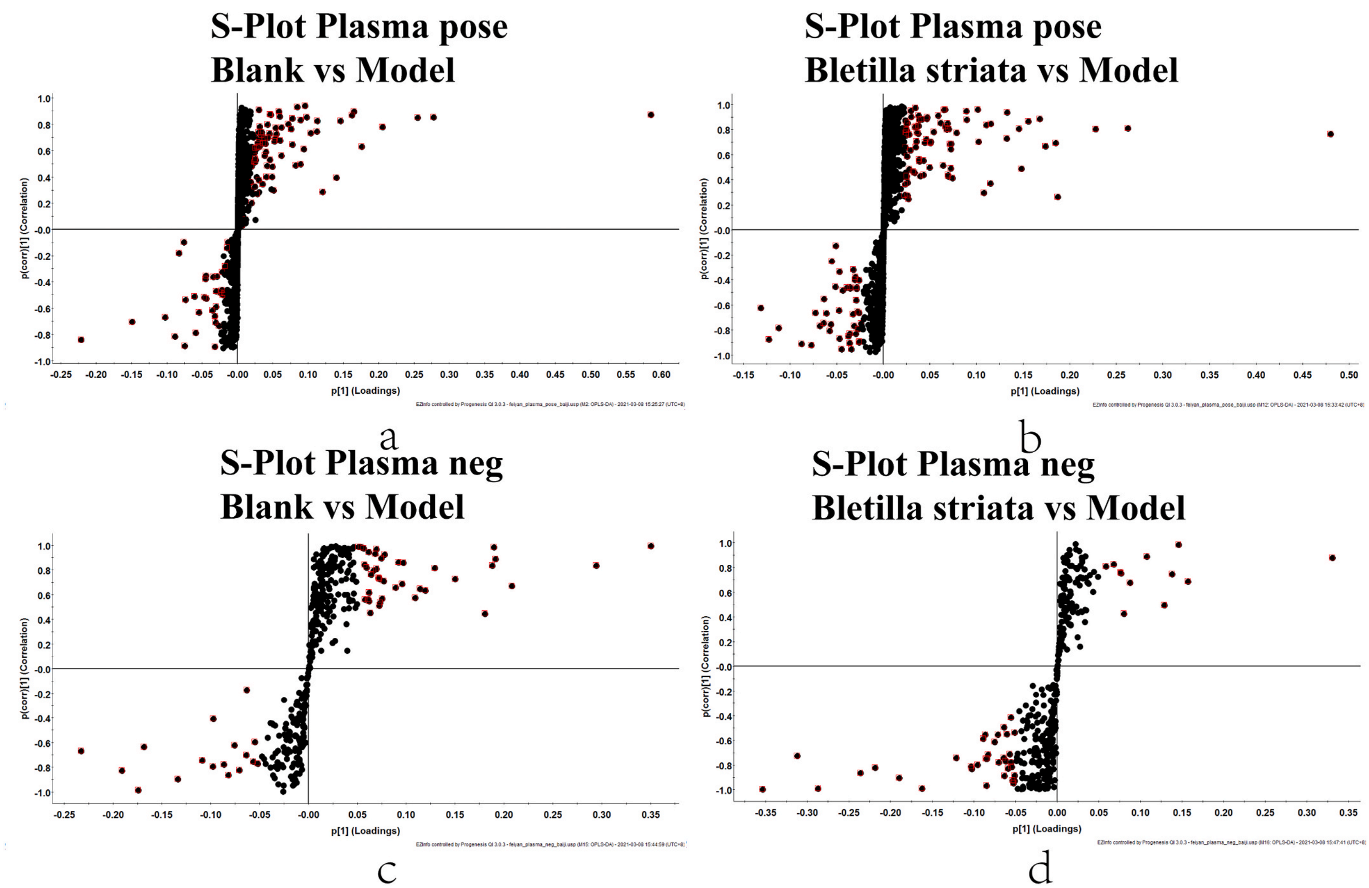

Fig. 5. OPLS–DA S-plot between each two groups in plasma. a. S-plot of blank and model group (ESI (+)), b. S-plot of Bletilla striata and model group (ESI (+)), c. S-plot of blank and model group (ESI (−)), d. S-plot of Bletilla striata and model group (ESI (−)).

and during the analysis process. PCA is an unsupervised multivariate analysis method which has been widely used to preliminary analyze MS data and is often performed to view grouping trends. Fig. 2 showed the score plot made by PCA in positive and negative ion mode. The x-axes and y-axes in Fig. 2 represented the different component after dimension reduction by PCA algorithm, respectively. The icons of different colors represented Bletilla Striata group, model group, QC samples and control group, respectively. Among them, the box in black represent the six samples of Bletilla Striata group, the triangle in red represent the six samples of control group, the diamond with green color represent the six samples of exposure group, the dots in blue represent the six samples of QC samples. After PCA algorithm calculation, the sample points of different groups had distinct aggregation. It is indicated that there were significant differences between the three groups. In addition, an obvious aggregation state was observed among QC samples in PCA, indicating that the experimental conditions of sampling from the first to the last were all in stable state.

The supervised OPLS-DA has the function of prediction, and can screen out clearly the difference variables between groups. Fig. 3 showed the score plot and the result of permutation test made by OPLS-DA in positive and negative ion mode of plasma samples. Fig. 4 showed the score plot and the result of permutation test made by OPLS-DA in positive and negative ion mode of urine samples The x- and y-axes in Figs. 3 and 4 represented the algorithm is used to fit the first principal component and orthogonal principal component of the data. After OPLS-DA algorithm calculation, the sample points of different groups had distinct aggregation, indicating that there were significant differences between exposure group and control group and exposure group and Bletilla Striata group.

The permutation test was used to assess the model fitting ability and prediction ability of the model established by OPLS-DA. The principle of permutation test was to rearrange the samples in and establish a new model again. Each permutation test presented a set of model related parameters R2 and Q2. The R2 represented the fitting ability of the model and the Q2 evaluate the predictive power of the model. The closer the R^2 and Q^2 value is to 1, the better the predictive ability. In our study, both values are greater than 0.5 which indicates that the fitting ability and prediction ability of the model are in good agreement.

The variables with a VIP value of >1.0 were selected as potential biomarkers shown in Figs. 5 and 6. These differential compounds (CV $\leq$ 30% and P $\leq$ 0.05) were identified in the online database HMDB by matching the MS raw data. As a result, total 18 endogenous metabolites in exposure group and control group and 21 endogenous metabolites in exposure group and Bletilla Striata group were selected as potential biomarkers for further study(Table 1 , Fig. 7). In Table 1, Fold Change (FC) value is to evaluate the change and trends between blank and model group, model and Bletilla group. There are significant differences between the two groups of metabolites shown in Fig. 8a. MetaboAnalyst 5.0 was used to evaluate a comprehensive view of the metabolic network and reveal the most relevant pathways affected by PM2.5 and the therapeutic effect of Bletilla shown in Fig. 8b. Among them, metabolism of glycerophospholipid, sphingomyelin, purine and tryptophan metabolism were the most significant pathways compared exposure and control group. Meanwhile, we found that the relative concentration of these different metabolites could be reversed after taking Bletilla Striata. Compared with the alterations of PM2.5-related metabolites, most of them were reset to a normal level after Bletilla Striata administration. By integrating related metabolic pathways, the metabolic network of the potential biomarkers are established and shown in Fig. 9.

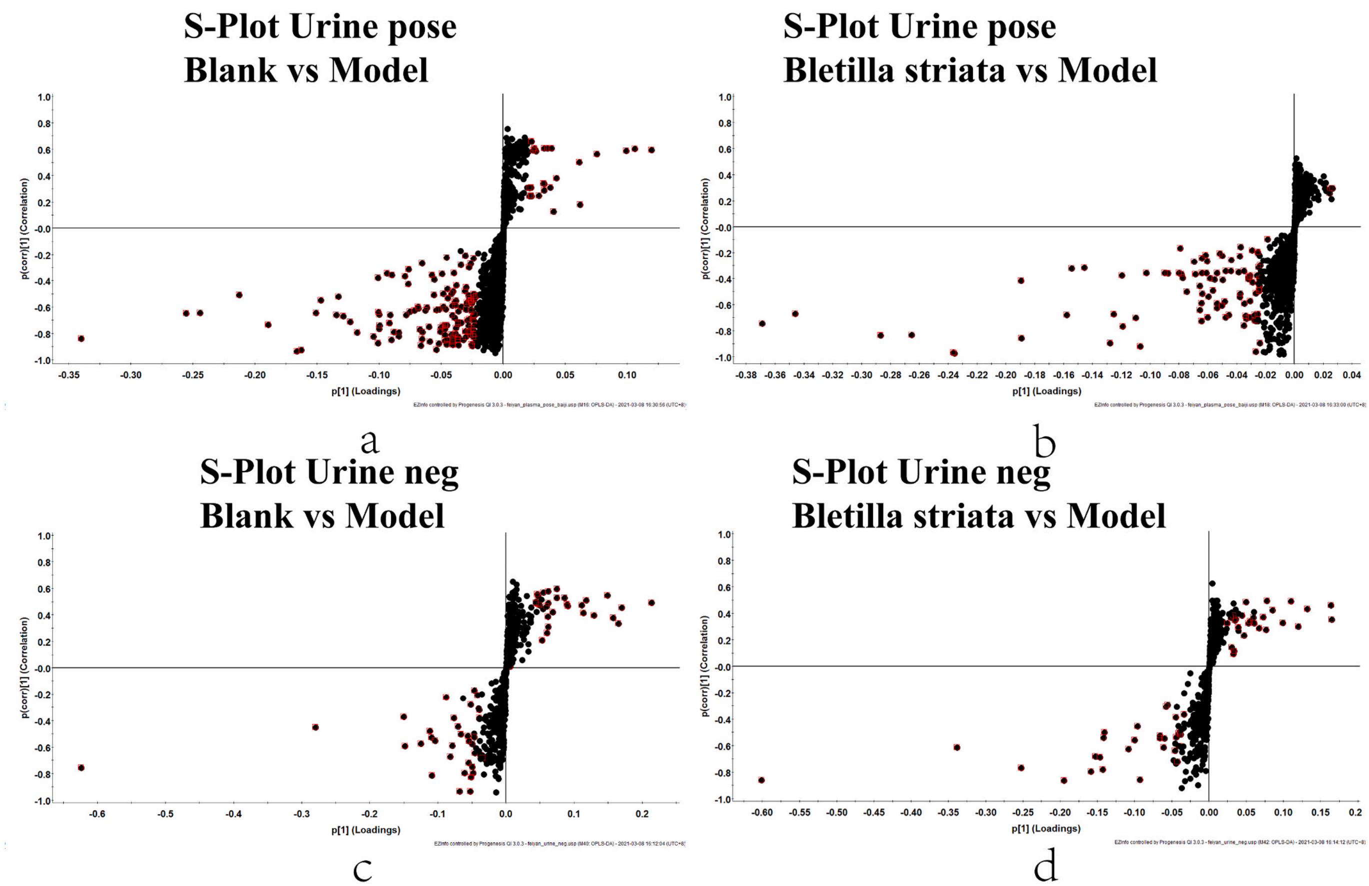

Fig. 6. OPLS–DA S-plot between each two groups in urine. a. S-plot of blank and model group (ESI (+)), b. S-plot of Bletilla striata and model group (ESI (+)), c. S-plot of blank and model group (ESI (−)), d. S-plot of Bletilla striata and model group (ESI (−)).

Table 1
Metabolic pathway classification, fold change (FC) and p value for identified biomarkers.

Compound ID	Adducts	Formula	Mass Error (ppm)	Description	*m/z*	Anova (p)	q Value	FC_B vs M	FC_M vs Bletilla Striata
plasma									
HMDB0000207	M + H–H2O, M + H	C18H34O2	−0.7557	Oleic acid	265.2524	0.0017	0.0005	1.0324	0.9083
HMDB0032033	M + H	C22H39NO	−1.8985	2,4,12-Octadecatrienoic acid isobutylamide	334.3098	0.0000	0.0000	1.0254	0.8400
HMDB0002212	M + NH4	C20H40O2	−3.9759	Arachidic acid	330.3354	0.0000	0.0000	0.9556	6.3198
HMDB0240262	M + H–H2O, M + H	C24H50NO7P	−1.0999	LysoPC(0:0/16:0)	496.3375	0.0031	0.0008	0.8582	/
HMDB0002815	M + H, M + Na	C26H52NO7P	−4.7489	LysoPC(18:1(9Z))	522.3529	0.0002	0.0001	0.8871	1.2757
HMDB0011511	M + H	C25H52NO7P	−2.2583	LysoPE(20:0/0:0)	510.3543	0.0004	0.0002	0.9091	1.3714
HMDB0031923	M + Na	C20H40O4	4.8259	10,20-Dihydroxyeicosanoic acid	367.2835	0.0294	0.0045	/	0.8768
HMDB0008590	M + H, M + Na, M + H-H2O	C44H84NO8P	−3.8665	PC(22:2(13Z,16Z)/14:0)	786.5977	0.0019	0.0006	/	0.9499
HMDB0000467	M + H–H2O, M + H	C24H38O4	−2.2461	Nutriacholic acid	391.2829	0.0001	0.0001	/	0.9818
HMDB0000269	M + H–H2O	C18H39NO2	−0.8289	Sphinganine	284.2945	0.0000	0.0000	/	3.2845
urine									
HMDB0002285	M + H–H2O, M + H	C9H7NO2	1.7473	2-Indolecarboxylic acid	162.0533	0.0027	0.0018	0.5189	1.2821
HMDB0003320	M + H–H2O, M + H	C9H7NO2	1.7473	Indole-3-carboxylic acid	162.0533	0.0027	0.0018	0.5189	1.2821
HMDB0004077	M + H–H2O, M + H	C9H7NO2	1.7473	4,6-Dihydroxyquinoline	162.0533	0.0027	0.0018	0.5189	1.2821
HMDB0031172	M + H–H2O, M + H	C9H7NO2	1.7473	3-Formyl-6-hydroxyindole	162.0533	0.0027	0.0018	0.5189	1.2821
HMDB0011174	M + H	C11H20N2O3	−2.0259	Isoleucylproline	229.1542	0.0015	0.0012	0.5762	0.8206
HMDB0011175	M + H	C11H20N2O3	−2.0259	Leucylproline	229.1542	0.0015	0.0012	0.5762	0.8206
HMDB0014954	M + NH4	C11H17NO3	−2.1893	Orciprenaline	229.1542	0.0015	0.0012	0.5762	0.8206
HMDB0014861	M + NH4	C11H17NO3	−2.1893	Methoxamine	229.1542	0.0015	0.0012	0.5762	0.8206
HMDB0000292	M + Cl	C5H4N4O2	0.7854	Xanthine	187.0029	0.0020	0.0007	0.8397	2.1606
HMDB0000786	M + Cl	C5H4N4O2	0.7854	Oxypurinol	187.0029	0.0020	0.0007	0.8397	2.1606
HMDB0001182	M + Cl	C5H4N4O2	0.7854	6,8-Dihydroxypurine	187.0029	0.0020	0.0007	0.8397	2.1606
HMDB0035002	M-H2O–H	C21H22O5	4.3595	Xanthogalenol	335.1304	0.0076	0.0023	0.7134	0.6451

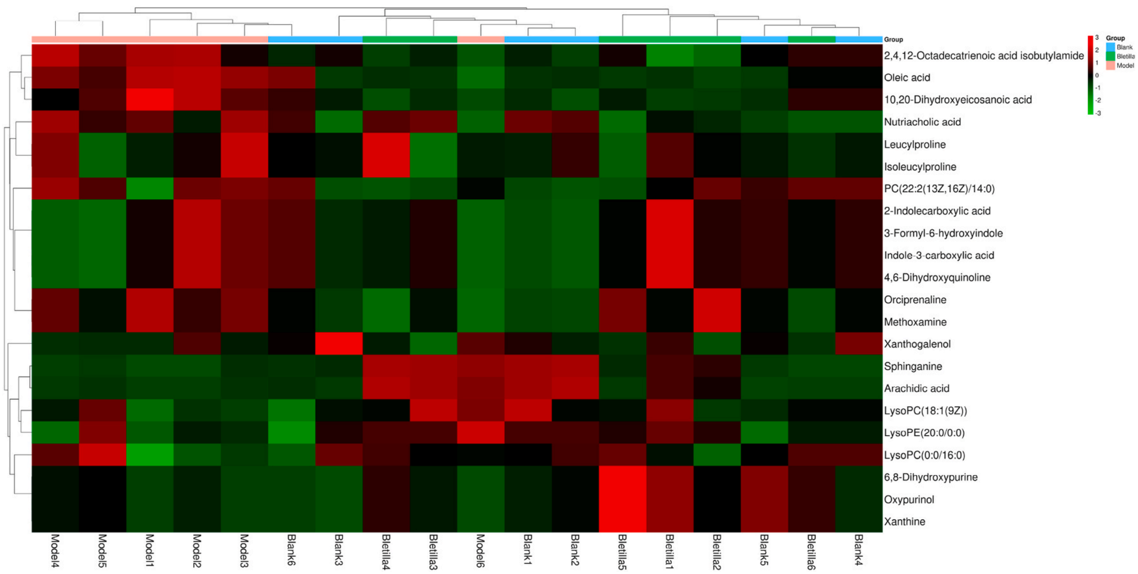

Fig. 7. Heatmap of metabolics.

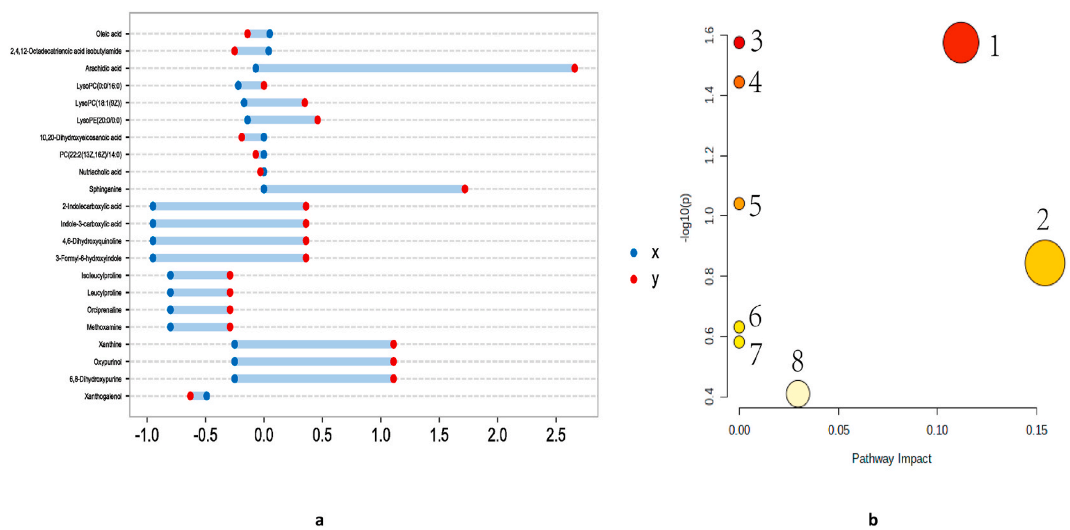

Fig. 8. FC Dumbbell Figure ((a), x represent Bletilla striata vs model, y represent model bs blank) and pathway bubble Figure (b)
*1 Glycerophospholipid metabolism 2 Sphingolipid metabolism 3 Synthesis of unsaturated fatty acids 4 Linoleic acid metabolism 5 alpha-Linoleic acid metabolism 6 Arachidonic acid metabolism 7 Tryptophan metabolism 8 Purine metabolism.

4. Discussion

Currently, metabolomics is an excellent and advanced discipline which assesses comprehensive endogenous metabolites of a biological system. Metabolomics has a global metabolic profiles analysis matching tightly with the holistic view of TCM. The use of omics techniques is crucial for understanding and interpreting TCM's efficacy and toxicity [21]. Bletilla Striata is the first classic and famous formula recorded in the Compendium of Materia Medica. Its function is to clear the lungs and stop bleeding. In China, Bletilla Striata has been used clinically for hundreds of years to be effective in curing pneumonia and lung cancer [22]. However, although this information is essential for our further exploration, the treatment mechanism is still unclear.

The lungs are the main organs of the body and are rich in lipids, amino acids and nucleotides. In this study, there are lipid (phospholipids, sphingolipids, unsaturated fatty acids), purines, tryptophan and other metabolic disturbance in the blood and urine of $PM_{2.5}$-induced rats, which can be improved by Bletilla Striata. Abnormal lipid metabolism is related to the activation of oxidative and inflammatory pathways [23]. According to reports, the use of PM10 can stimulate neutrophils to flow into the respiratory tract, increase TF-α and IL-6 levels, and change the related genes expression of inflammation, cholesterol and lipid metabolism [24]. In our research, 10 different lipid compounds were found, accounting for nearly 50% of the total identified biomarkers. The metabolic pathways of unsaturated fatty acids, glycerophospholipids and sphingomyelin may be the potential mechanism of $PM_{2.5}$-induced toxicity.

The interaction between $PM_{2.5}$ and cell membrane is the first step to induce cytotoxicity. Surface-active phospholipids (PC, PG, PE) are the main lipid components of cell membranes, and are important structure that maintains an independent intracellular environment and regulates the exchange of substances inside and outside the cell. In inflamed lungs, phospholipids hydrolyzed into lysophospholipids and caused surface active substance dysfunction. In addition, the phospholipids and lipoprotein produced in the lungs affect the respiratory function by reducing the surface tension of the alveoli during breathing [25,26]. $PM_{2.5}$

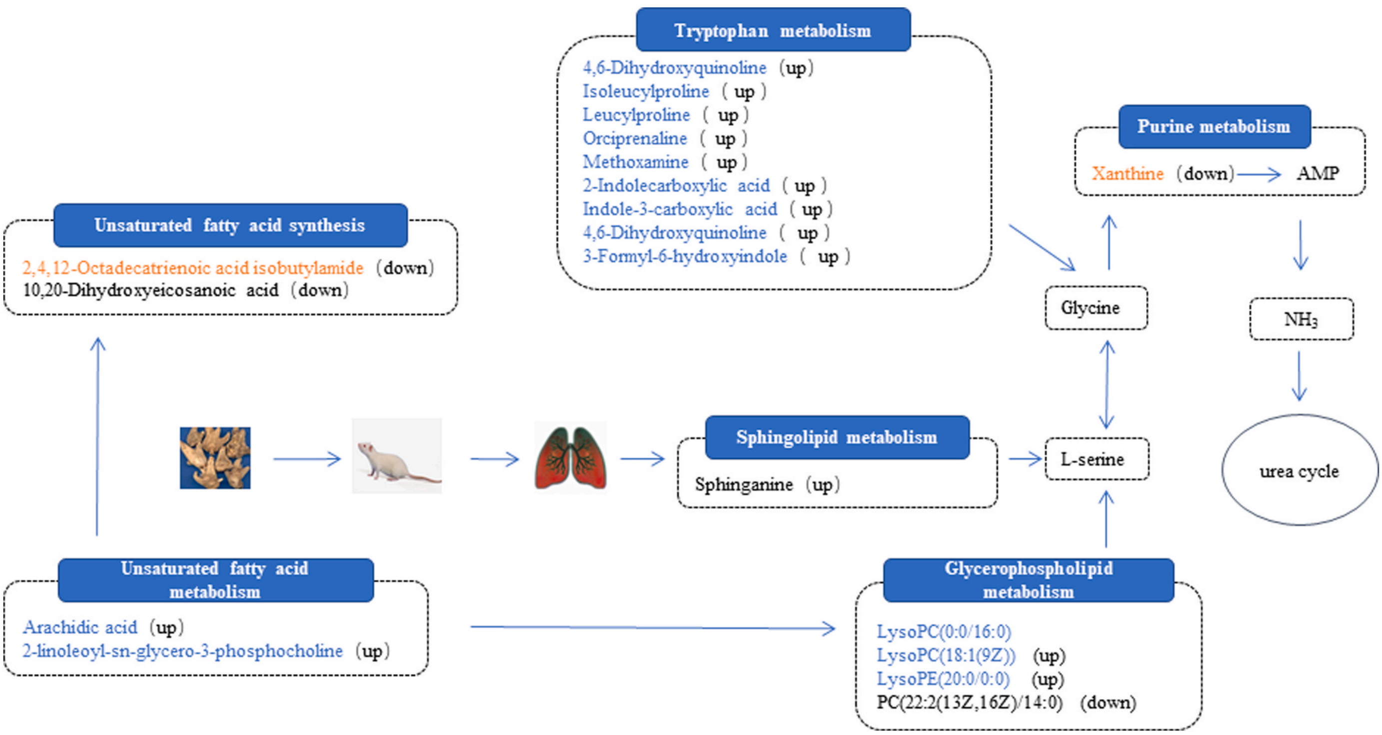

Fig. 9. Total pathway map (The yellow word means that the metabolic level of the model group increased compared with the blank group, the blue word means that the metabolic level of the model group decreased compared with the blank group; up means that compared with the model group, the metabolic level of the Bletilla striata group increased, down means that compared with the model group, the metabolic level of the Bletilla striata group decreased). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

exposure can trigger an increase in oxidized phospholipids, and then mediate systemic inflammation through a TLR4/NADPH oxidase-dependent mechanism. The changes in lung lipids are closely related to the occurrence and development of asthma [27,28]. However, after the administration of Bletilla Striata, the differential metabolites showed a significant correction, which proves that Bletilla Striata is effective.

Sphingomyelin is another important component of cell membranes, accounting for 10–15% of total membrane lipids. Sphingosine is the main component of sphingomyelin and plays a key role in the response of cells to oxidative stress. We observed that the levels of sphingosine and sphingomyelin in the model group were significantly increased, but effectively decreased after Bletilla Striata administration, suggesting that there is a potential correlation between $PM_{2.5}$ exposure and the sphingomyelin pathway and Bletilla has a therapeutic effect.

Regarding amino acid biomarkers, we found that the concentration of tryptophan of model group in the urine of rats was significantly lower than that of the control group, while the concentration of tryptophan and its metabolites in the Bletilla Striata group was significantly increased. This result indicated that the change of tryptophan concentration was related to various types of systemic inflammation [29–31]. It has been found that patients with IBD have significantly lower serum tryptophan level, the increased tryptophan metabolism is related to IBD activity [32].

Purines and pyrimidines have been reported to play a regulatory role in signal transmission through purinergic receptors, which are related to lung injury [33]. Purine metabolism disorder is associated with decreased lung function [34]. Purines and their metabolites are highly active biochemical substances that occur in the organism. The significant upregulation of some purines and their metabolites indicates that PM2.5 stimulates the degradation process of purines in the rat lung. Purine metabolism generates oxygen free radicals through the xanthine oxidase pathway [35]. Hypoxanthine and adenosine have also been found to be associated with neutrophil inflammation in chronic obstructive pulmonary disease [36]. In this experiment, the purine concentration of rats in the treatment group was significantly increased by more than 2 times, further confirming that $PM_{2.5}$ exposure can significantly disrupt nucleotide metabolism.

5. Conclusions

In this study, an UPLC-QTOF/MS metabolomics method was successfully developed to clarify the metabolic characteristics of $PM_{2.5}$-induced lung impairment and the therapeutic effect of Bletilla Striata. We tested 22 potential biomarkers and predicted the main metabolite network of $PM_{2.5}$-induced lung impairment. We concluded that Bletilla Striata can reverse the pathological process by regulating the disordered metabolic pathway, phospholipids, glycerophospholipid metabolism, sphingolipid metabolism, tryptophan metabolism, purine metabolism, etc. This study will help to better understand the mechanism of $PM_{2.5}$-induced lung impairment and the effect of Bletilla Striata.

Ethical approval and consent to participate

The experimental protocol was approved by the Animal Ethics Committee of Shenyang Medical College (Permit number: 2016–0151). Written informed consent was obtained from individual or guardian participants.

Consent for publish

All the authors agreed to publish the article in the journal.

Authors contributions

Ronghua Fan: conceived and designed the experiments. Ronghua Fan, Xinyue Wang and Meiqi Xing: performed the experiments. Ze Zhang, Chen wang and Lili Deng: analyzed the data. Ronghua Fan: wrote the paper. Yumo Han and Xinyue Wang: reviewed the paper. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by the National Natural Science Foundation of China. (No. 81903793), Scientific Project of Liaoning Province (No. SYYX202013), Shenyang Scientific Project (No. RC190372) and Shenyang Medical College student scientific research Project (No.20198010 and 20219015).

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