

RESEARCH ARTICLE

A metabolomics research based on UHPLC-ESI-Q-TOF-MS coupled with metabolic pathway analysis: Treatment effects of stir-frying *Xanthii Fructus* on allergic rhinitis in mice model

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Abstract

Xanthii Fructus (XF), a well-known herb in traditional Chinese medicine, has been frequently used for the treatment of allergic rhinitis in the clinic. Its therapeutic metabolic mechanism, however, remains undetermined. In this work, a metabolomics research coupled with metabolic pathway analysis has been employed to screen out the potential mechanism in its effects on allergic rhinitis. Specifically, mouse serum samples containing XF were analyzed based on ultra-high performance liquid chromatography equipped with electrospray ionization quadruple time-of-flight mass spectrometry detection (UHPLC-ESI-Q-TOF-MS) in both positive and negative polarity. In addition, the raw data gained from UHPLC-ESI-Q-TOF-MS were processed by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) in order to discover remarkable metabolites. Twenty-seven potential biomarkers in mouse serum were filtered from free databases like HMDB. Interestingly, this study filtered the potential metabolic pathways including glycerophospholipid metabolism and branch-chain amino acid metabolism. We hope that this paper will provide a feasible strategy for revealing the therapeutic mechanism of XF in allergic rhinitis mice model.

KEYWORDS

allergic rhinitis, metabolomics, stir-frying *Xanthii Fructus*, therapeutic mechanism, UHPLC-ESI-Q-TOF-MS

1 | INTRODUCTION

Allergic rhinitis (AR) is a chronic nasal mucosal inflammatory disease manifested by frequent symptoms such as sneezing, nasal itching, nasal congestion and runny nose and eyes and can occur seasonally or throughout the year. In recent years, we have witnessed a growing number of reports showing the prevalence and severity of AR and the substantial burden it places on patients' quality of life and work

productivity, which makes the management and treatment of AR an important issue.

Xanthii Fructus (XF), the ripe fruits of *Xanthium sibiricum* Patr. XF, was documented to be able to soothe nasal orifices and eliminate wind-dampness in *Sheng Nong's Herbal Classic*. Therefore it has been employed for conditions such as cramping and numbness of the limbs, ulcer, sinusitis, catarrhs and pruritus (Chinese Pharmacopoeia Commission, 2015). XF always need frying into stir-fried *Xanthii Fructus* (SXF) before application. Nowadays, SXF is frequently used for the treatment of rhinitis in the clinic. However, its composition of is very complex, and its clinical effect for the treatment of AR is the result of various components targeting multiple metabolic pathways.

Abbreviations: AR, Allergic rhinitis; BCAA, branch-chain amino acid; ESI, electrospray ionization; OVA, ovalbumin; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; RT, retention time; SXF, stir-fried *Xanthii Fructus*; TCM, traditional Chinese medicine; VIP, variable importance in projection; XF, *Xanthii Fructus*.

To uncover those active components within SXF and possible metabolic pathways, we used ovalbumin (OVA) to duplicate AR in a mouse model and employed metabolomics for further investigation after SXF intervention (Cui et al., 2012; Hu, 2012).

Metabolomics applies large-scale information extraction technology and multiple-variable processing technology to analyze the metabolites of animals. Its research mode is similar to the mechanism of traditional Chinese medicine (TCM). Thus metabolomics can provide new ideas to uncover the complex mechanisms in TCM and is widely applied in TCM research areas such as toxicity mechanism studies, compatibility mechanism exploration of active constituents in Chinese medicine and determination of potential processing mechanisms of Chinese medicine (Guo, Cui, Wang, & Kuang, 2017; Qin et al., 2014; Sun et al., 2017).

Ultra-high-performance liquid chromatography equipped with electrospray ionization quadruple time-of-flight mass spectrometry detection (UHPLC-ESI-Q-TOF-MS) technology is valuable for characterizing the structure of metabolites and isomers since can not only effectively reduce the interference, such as the base quality effect, ionization inhibition and ionization competition, but also increase metabolite identification quantity and accuracy. Thus this article explores the mechanism of effect of SXF on AR mice models using UHPLC-ESI-Q-TOF-MS technology.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

The HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). HPLC-grade formic acid was purchased from Tedia (Fairfield, OH, USA). Ultrapure water was prepared from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2 | Sample preparation

Xanthii Fructus was purchased from Foshan Chinese Medicine Group Co. (Guangzhou, China) and Professor Hao Cai (Nanjing University of Chinese Medicine) authenticated its species. The voucher specimens were stored in the Herbarium of School of Pharmacy, Nanjing University of Chinese Medicine. The herbal (2 kg) was stir-fried according to the standard of the *Chinese Pharmacopoeia* (2015 edition) and then extracted by refluxing with 70% ethanol (1:50, $W_{\text{herbal}}/V_{\text{solvent}}$) for 1 h. The solution obtained was concentrated under reduced pressure to generate dry extract, and then stored at 4°C before administration.

2.3 | Animal experiment

This study was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine. The animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals. Male BALB/C mice ($n = 30$), weighing 10–20 g, were obtained by the Slaccas Experiment Animal Company (Shanghai, China). Temperature, humidity and light were maintained constant, while food and water were provided freely. All of the mice were

acclimated in the laboratory for at least 1 week prior to the experiment. The 30 BALB/C mice were grouped randomly, including (1) the normal control group ($n = 10$), (2) the disease model group ($n = 10$) and (3) the treatment group ($n = 10$). After the mice had been fed for 1 week in the laboratory, they were OVA-sensitized by intraperitoneal injection of 50 μg OVA (chicken egg albumin; Sigma) in 200 μL of normal saline containing 2 mg aluminum hydroxide (Alum; Sigma) on days 0, 4, 7, 11 and 14. Then the mice were challenged with 20 μL normal saline containing 50 $\mu\text{g}/\text{mL}$ OVA into the nasal cavities on both sides every day until day 27. From days 28 to 42, mice in the normal control and disease model groups were given normal saline by perioral administration, and those in the treatment group were orally administered 1 g/kg SXF and sensitized with an intranasal challenge of OVA.

2.4 | Symptom evaluation of rhinitis in mouse

Firstly, OVA was nasal dripped into BALB/C mice. Subsequently, number of times of nasal rubbing and sneezing, as well as a condition of a runny nose were observed 15 min after sensitization with OVA.

2.5 | Mouse serum samples

One hour after the last observation, the mice were anesthetized with 10% chloral hydrate. The blood was collected overnight in 4°C, and then centrifuged to obtain serum frozen at -80°C until UHPLC-ESI-Q-TOF-MS analysis. Each 100 μL of serum sample was mixed with 1 mL methanol and vortexed for 5 min to precipitate the proteins. After centrifuged at 12,000 rpm for 10 min, the supernatant was transferred into another tube and evaporated to dryness under nitrogen. The residue was then reconstituted with 100 μL of methanol, vortexed for 30 s and centrifuged at 12,000 rpm for 10 min to obtain the supernatant fluid for analysis.

2.6 | Metabolic profiling

2.6.1 | Instruments

In order to analyze the endogenous composition of mouse serum, this study developed a strategy using an Ekspt™ ultra LC100-XL system coupled to hybrid quadruple time-of-flight tandem mass spectrometry (LCMS-Triple TOF™5600, AB SCIEX, Foster City, CA, USA) with an electrospray ionization (ESI) interface. The data was processed by Analyst 1.6 software.

2.6.2 | UHPLC-ESI-Q-TOF-MS conditions

2.6.3 | Chromatographic

The UHPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-30AD binary pump, an autosampler (model SIL-30SD) and an online degasser (DGU-20A5R). The chromatographic separation was carried out using a C_{18} reversed-phase column ($2.1 \times 100 \text{ mm}$, $1.8 \mu\text{m}$, Agilent). The column temperature was kept at 30°C . The flow rate was 0.3 mL/min and the sample injection volume was 3 μL . The mobile

phase consisted of (A) 0.1% formic acid in water and (B) methanol. The optimized gradient elution was as follows: 0–5 min, 20–30% B; 5–7 min, 30–35% B; 7–12 min, 35–80% B; 12–17 min, 80–100% B; 17–20 min, 100–20% B.

TABLE 1 Results of mouse behaviors

Group	No.	Score ($\bar{x} \pm SD$)
Normal control group	6	2.17 $\pm$ 0.75
Disease model group**	6	5.67 $\pm$ 0.52
Treatment group (before)**	6	5.50 $\pm$ 0.55
Treatment group (after) ^{††}	6	3.50 $\pm$ 0.55

*Disease model group and treatment group (before) compared with normal control group;

** $p < 0.001$;

[†]treatment group (after) compared with disease model group;

^{††} $p < 0.001$.

2.6.4 | Mass spectrometry

The mass spectrometer was operated with an electrospray ionization in both positive (ESI⁺) and negative (ESI⁻) ion mode. The parameters were set as follows: ion spray voltage, 7 kV; turbo spray temperature, 550°C; declustering potential, 70 V; collision energy, 30; nebulizer gas, 55 psi; heater gas, 55 psi; curtain gas, 35. The nebulizer and auxiliary gas were nitrogen. TOF MS and TOF MS/MS were m/z 100–1200 and 50–1200, respectively. A 200 ms accumulation time and an 80 ms accumulation time were operated for TOF MS and TOF MS/MS, respectively. Instrument adjustment was brought into operation in every 12 samples.

2.7 | Data processing and pattern recognition

The data collected from UHPLC-ESI-Q-TOF-MS were handled by Marker View software and changed to.xlsx format. Parameters were set as follows: minimum retention time, 2.00 min; minimum

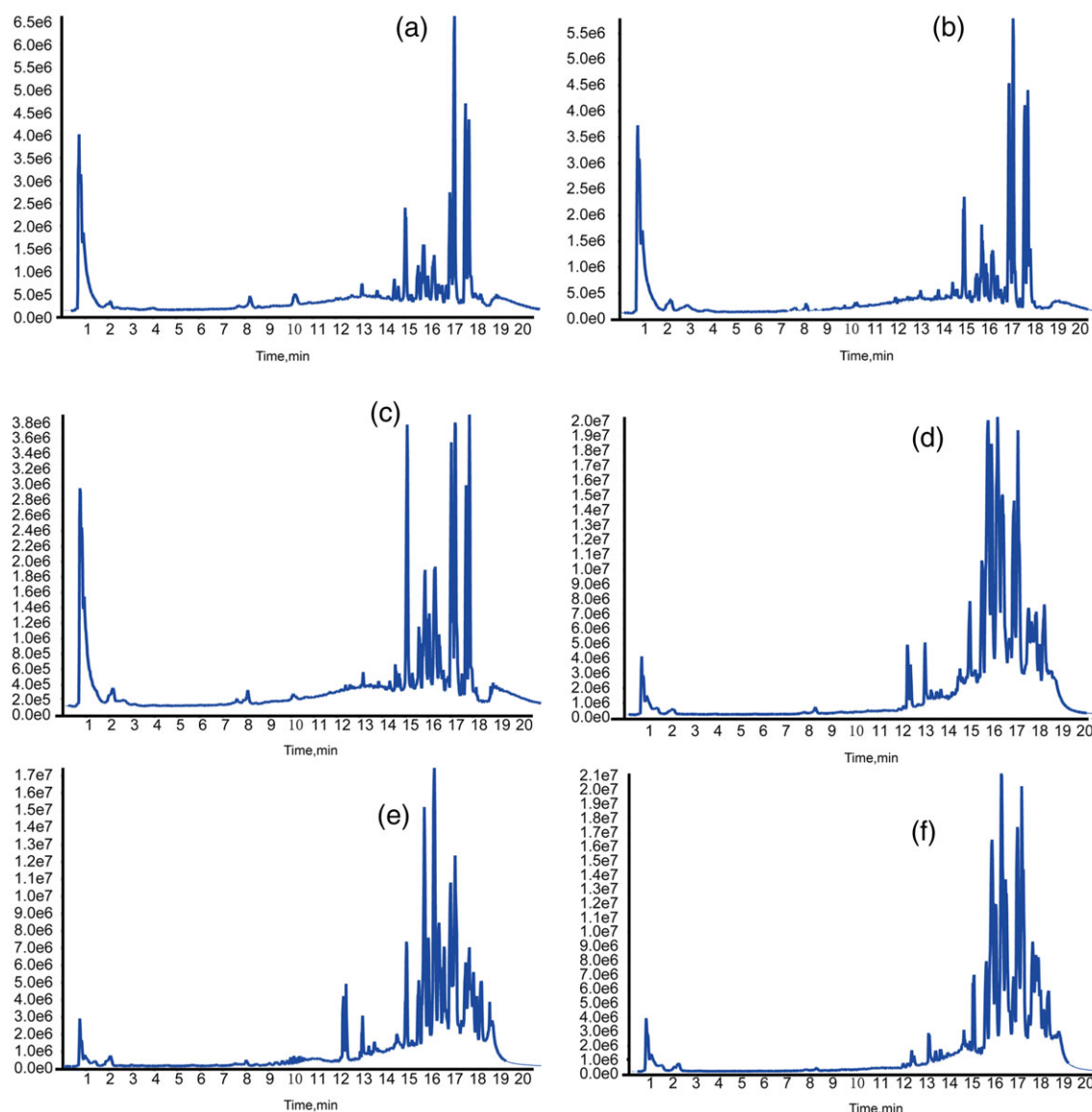


FIGURE 1 UHPLC-MS total chromatograms: (a–c) in positive mode; (d–f) in negative mode normal control group (a and d); disease model group (b and e); treatment group (c and f)

spectral peak width, 25 ppm; minimum RT (retention time) peak width, six scans; noise threshold, 100; retention time tolerance, 0.50 min; mass tolerance, 50.0 ppm; removal of peaks in <15 samples; maximum number of peaks, 8000. The data was imported to SIMCA software for principal component analysis (PCA) and partial least-square-discriminant analysis (PLS-DA), and then some data obtained including molecular weight, RT and VIP (variable importance in projection). The data whose VIP is bigger than 1.0 was finally handled in HMDB and Metaboanalyst.

2.8 | Identification of biomarkers and metabolic pathway analysis

The accurate MS fragments with metabolites were sought in on-line free databases like HMDB to identify potential biomarkers. The metaboanalyst database was used to seek pathway analysis.

3 | RESULTS AND DISCUSSION

3.1 | Behaviors in AR mice models

The frequencies of rubbing, sneezing and runny nose were counted every day after intranasal OVA administration. The frequency was expressed by $\bar{x} \pm SD$, and analyzed by one-way ANOVA using SPSS 20.0. As shown in Table 1, the levels of symptom score in the treatment group (before) and disease model group were much higher than those in normal control group before treatment. The level of symptom score in the treatment group (after) was decreased ($p < 0.001$), compared with the disease model group after 14 days.

3.2 | Data analysis and pattern recognition

The sera were analyzed by UHPLC-ESI-Q-TOF-MS, and the total ion chromatography (TIC) are shown in Figure 1. From Figure 2, there was an obvious separation in the PCA score plot between treatment group, disease model group and normal control group, and these three groups may have had different metabolites. This study distinguished the normal control group, disease model group and treatment group using PLS-DA to identify potential biomarkers. The serum samples were separated into three blocks by PLS-DA score plots and this phenomenon may result from some metabolic patterns. The PLS-DA score plots are shown in Figure 2a and b (Figure 2a, PCA in negative mode and Figure 2b, PCA in positive mode).

3.3 | Identification of potential endogenous biomarkers

The VIP value of metabolites was used to detect ions that contributed significantly to the different groups. In this study, VIP value was set as exceeding 1. Finally, nine significantly different endogenous metabolites and 18 significantly differential endogenous metabolites were selected for further study in both negative and positive modes respectively. By comparing the accurate MS fragments with the metabolites found above, this study inferred the structure information. The signal was measured by UHPLC-ESI-Q-TOF-MS in the form of intensity.

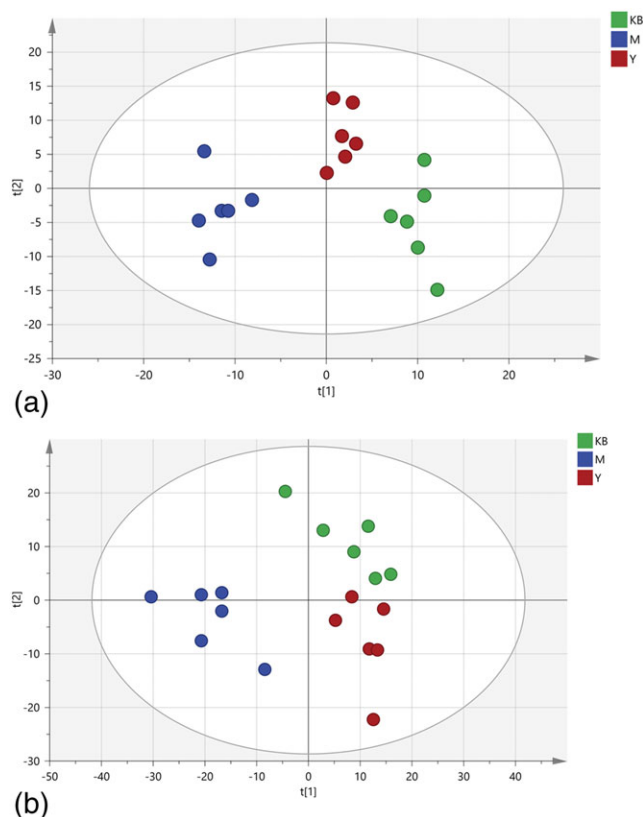


FIGURE 2 Partial least squares discriminant analysis (PLS-DA) score plot. Normal control group, green; disease model group, blue; treatment group, red (a, principal component analysis (PCA) in negative mode; b, PCA in positive mode)

Compared with the normal control group, some endogenous substances like L-valine, 2-methylbutyrylcarnitine, neurine, PC(40:8), heptadecanoic acid, (E)-1-[4-hydroxy-3-(3-methyl-1,3-butadienyl)phenyl]-2-(3,5-dihydroxyphenyl) ethylene, LysoPC(14:0), LysoPC(20:1), neocasomorphin (1–5), 3b,17a,21-trihydroxypregnenone, L-acetylcarnitine, enflurane, eicosenoic acid, L-γ-glutamyl-L-leucine and methyl 5-(hydroxymethyl)pyrrolidine-3-carboxylate were down-regulated in the disease control group, while these endogenous substances were up-regulated to normal levels after treatment. Compared with the normal control group, some endogenous substances like deoxynivalenol 3-glucoside, 3-keto-digoxigenin, 6,7-dimethoxy-7-epirosmanol, LysoPE(20:1), armillary, scutigeral, stearic acid, LysoPE(20:2), LysoPE(24:6), MG(18:4), sphingosine 1-phosphate and (2E,4E)-icosa-2,4-dienoic acid were up-regulated, while these endogenous substances were down-regulated to normal levels after treatment. The corresponding results were shown in Table 2.

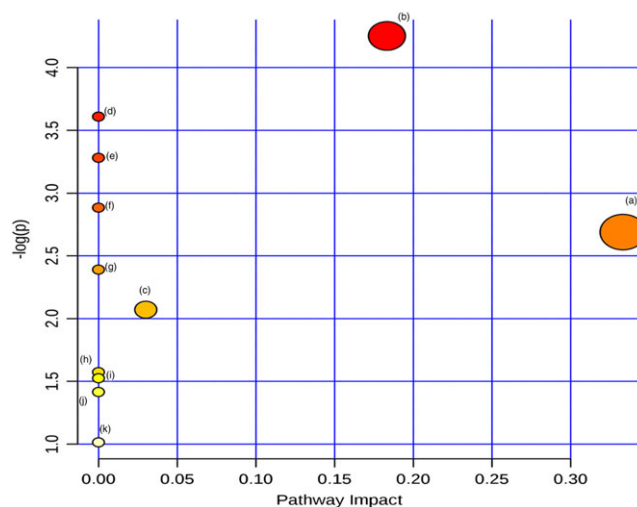
3.4 | Biological pathway analysis

The identification endogenous metabolites were imported into the Metaboanalyst database to discover the potential pathways that contributed significantly to SXF. Eleven pathways—glycerophospholipid metabolism, valine, leucine and isoleucine biosynthesis (branch-chain amino acid, BCAA, metabolism), sphingolipid metabolism, biosynthesis of unsaturated fatty acids, linoleic acid metabolism, α-linolenic acid

TABLE 2 Twenty-seven potential biomarkers between the three groups: ↑, up-regulated; ↓, down-regulated

No.	RT (min)	Formula	Exact mass	Observed mass	Identification	Ion mode	Disease control group/Treatment group	HMDB
1	20.93	C ₅ H ₁₁ NO ₂	118.0866	119.0657	L-Valine		↑↓	HMDB00883
2	2.02	C ₁₂ H ₂₃ NO ₄	246.1701	247.1835	2-Methylbutyrylcarnitine	+	↑↓	HMDB00378
3	15.82	C ₅ H ₁₃ NO	104.1054	105.1213	Neurine	+	↑↓	HMDB31259
4	17.2	C ₄₈ H ₈₀ NO ₈ P	830.5581	831.5453	PC(40:8)	+	↓↑	HMDB08156
5	20.89	C ₁₇ H ₃₄ O ₂	271.2618	272.2492	Heptadecanoic acid	+	↓↑	HMDB02259
6	3.37	C ₁₉ H ₁₈ O ₃	295.1285	296.1135	(E)-1-[4-Hydroxy-3-(3-methyl-1,3-butadienyl)phenyl]-2-(3,5-dihydroxyphenyl)ethylene	+	↓↑	HMDB33331
7	15.25	C ₂₂ H ₄₆ NO ₇ P	468.3085	469.3011	LysoPC(14:0)	+	↑↓	HMDB10379
8	17.4	C ₂₈ H ₅₆ NO ₇ P	550.3865	551.3746	LysoPC(20:1)	+	↑↓	HMDB10391
9	15.71	C ₂₉ H ₄₁ N ₅ O ₉	604.2911	605.2894	Neocasomorphin (1–5)	+	↑↓	HMDB60144
10	17.18	C ₂₁ H ₃₂ O ₄	347.2198	346.2024	3b,17a,21-Trihydroxy pregnenone	–	↑↓	HMDB00382
11	16.88	C ₂₁ H ₃₀ O ₁₁	457.1800	456.1749	Deoxynivalenol 3-glucoside	–	↓↑	HMDB39852
12	15.05	C ₂₃ H ₃₂ O ₅	387.2152	386.2037	3-keto-Digoxigenin	–	↓↑	HMDB60746
13	16.89	C ₂₂ H ₃₀ O ₆	389.1909	388.1827	6,7-Dimethoxy-7-epirosmanol	–	↓↑	HMDB30844
14	16.5	C ₂₅ H ₅₀ NO ₇ P	506.3266	505.3182	LysoPE(20:1)	–	↓↑	HMDB11512
15	16.68	C ₂₄ H ₃₀ O ₆	413.1925	412.1901	Armillarin	–	↓↑	HMDB39380
16	17.11	C ₂₃ H ₃₂ O ₄	371.2201	370.2155	Scutigeral	–	↓↑	HMDB30012
17	18.8	C ₁₈ H ₃₆ O ₂	283.2638	282.2579	Stearic acid	–	↓↑	HMDB00827
18	7.43	C ₉ H ₁₇ NO ₄	202.1085	201.1034	L-Acetylcarnitine	–	↑↓	HMDB00201
19	15.07	C ₃ H ₂ ClF ₅ O	182.9647	181.9668	Enflurane	–	↑↓	HMDB14373
20	15.89	C ₂₅ H ₄₈ NO ₇ P	504.3099	503.3031	LysoPE(20:2)	–	↓↑	HMDB11483
21	15.76	C ₂₉ H ₄₈ NO ₇ P	552.3098	551.3034	LysoPE(24:6)	–	↓↑	HMDB11499
22	17.83	C ₂₁ H ₃₄ O ₄	349.2357	348.2303	MG(18:4)	–	↓↑	HMDB11541
23	15.01	C ₂₀ H ₃₈ O ₂	309.2799	308.2645	Eicosenoic acid	–	↑↓	HMDB02231
24	15.42	C ₁₈ H ₃₈ NO ₅ P	378.2413	377.2326	Sphingosine 1-phosphate	–	↓↑	HMDB00277
25	2.87	C ₁₁ H ₂₀ N ₂ O ₅	259.1296	258.1207	L-γ-Glutamyl-L-leucine	–	↑↓	HMDB11170
26	2.57	C ₇ H ₁₃ NO ₃	158.0827	157.0811	Methyl 5-(Hydroxymethyl)pyrrolidine-3-carboxylate	–	↑↓	HMDB61678
27	18.05	C ₂₀ H ₃₆ O ₂	307.2642	306.2533	(2E,4E)-icosa-2,4-dienoic acid	–	↓↑	HMDB61864

metabolism, pantothenate and CoA biosynthesis, arachidonic acid metabolism, valine, leucine and isoleucine degradation, fatty acid biosynthesis and aminoacyl-tRNA biosynthesis—were found to be the most important answer to SXF. Then glycerophospholipid metabolism, and valine, leucine and isoleucine biosynthesis (BCAA metabolism) were selected for the most important metabolic pathways whose pathway impact is >0.1. The participation of some identified endogenous substances in the pathway was as follows: PC(40:8) participates in glycerophospholipid metabolism, linoleic acid metabolism, α-linolenic acid metabolism and arachidonic acid metabolism; L-valine participates in valine, leucine and isoleucine biosynthesis BCAA metabolism, pantothenate and CoA biosynthesis, and valine, leucine and isoleucine degradation; stearic acid participates in biosynthesis of unsaturated fatty acids and fatty acid biosynthesis; sphingosine 1-phosphate participates sphingolipid metabolism. The remainder do not match any pathway. The results are shown in Figure 3.

**FIGURE 3** Potential pathway

3.5 | Biological pathway functional explanation

This study developed a UHPLC-ESI-Q-TOF-MS method with multivariate data analysis to analyze the metabolomics of mouse serum after administration of SXF. Thirty-one potential biomarkers were found as shown in Table 2, which indicated that SXF could regulate glycerophospholipid metabolism and valine, leucine and isoleucine biosynthesis (BCAA metabolism). As the impact of last metabolism was 0.1, this paper discussed only the first two metabolisms. The potential biomarker changes are shown in Figure 4a–e.

3.5.1 | Glycerophospholipid metabolism

Glycerophospholipid plays a key role in the process of inflammation and immune reaction. Lipid can provide energy for organisms and participates in many life activities. Therefore, it has important physiological functions including maintenance of cell membrane structure, energy storage and signal transduction (Bou et al., 2010). Lipids can be divided into eight categories including glycerides, glycerol phospholipid fatty acids, sphingolipid, sterol ester, pregnenolone lipid sugar, lipid and PE (Fahy et al., 2009). Glycerophospholipid molecules have many biological actions, such as causing metabolic network disorder and bringing about the occurrence of various diseases, including coronary heart disease (Attia et al., 2009), atherosclerosis (Krauss, 2010), diabetes (Zhu, Liang, Hu, Wang, & Luo, 2011), adiposis (Vries, Kappelle, Dallinga, & Dullaart, 2011), cancer (Jeong, Lim, Kim, & Moon, 2011), brain damage (Pasvogel, Miketova, & Moore, 2010), inflammation (Boggara & Krishnamoorti, 2010) and Alzheimer's disease (Lgarashi, Ma, Gao, Kim, & Rao, 2011). Inflammation generated in

any part of the body is one of the most common human disease pathological processes. Most human disease is closely related to the inflammatory process; meanwhile inflammation may result in lipid metabolism disorders. immunoglobulin-mediated reaction being its basis, AR is a disease strongly related to inflammatory. From the above, we may predict that SXF's effect on AR is related to glycerophospholipid metabolism. PC(40:8) is the material composition of glycerophospholipid metabolism. The content of PC(40:8) was reduced in the disease model group, but raised to normal levels after treatment with SXF extract. One of the mechanisms of SXF in treatment of AR is via adjusting the PC(40:8) content and further affecting glycerophospholipid metabolism.

3.5.2 | BCAA metabolism

BCAA is the main nitrogen source of skeletal glutamine and alanine synthesis. There is a definite relationship between BCAAs and glutamine (Holecek, 2005). Glutamine is considered as a conditional essential amino acid, whose regulation role in protein metabolism is associated with the metabolism condition of BCAAs (Holecek, 2005). BCAAs, especially for leucine, can stimulate the synthesis of protein, inhibit the decomposition of protein and promote the synthesis of glutamine. BCAAs can affect the lymphocyte synthetic of proteins, DNA and RNA, and stimulate the differentiation; they can also maintain the function of immune cells (Calder, 2006). L-Valine is an important raw material of BCAA metabolism. The content of L-valine was reduced in the disease model group. When treated with SXF extract, it increased to normal levels. One of the mechanisms of SXF in

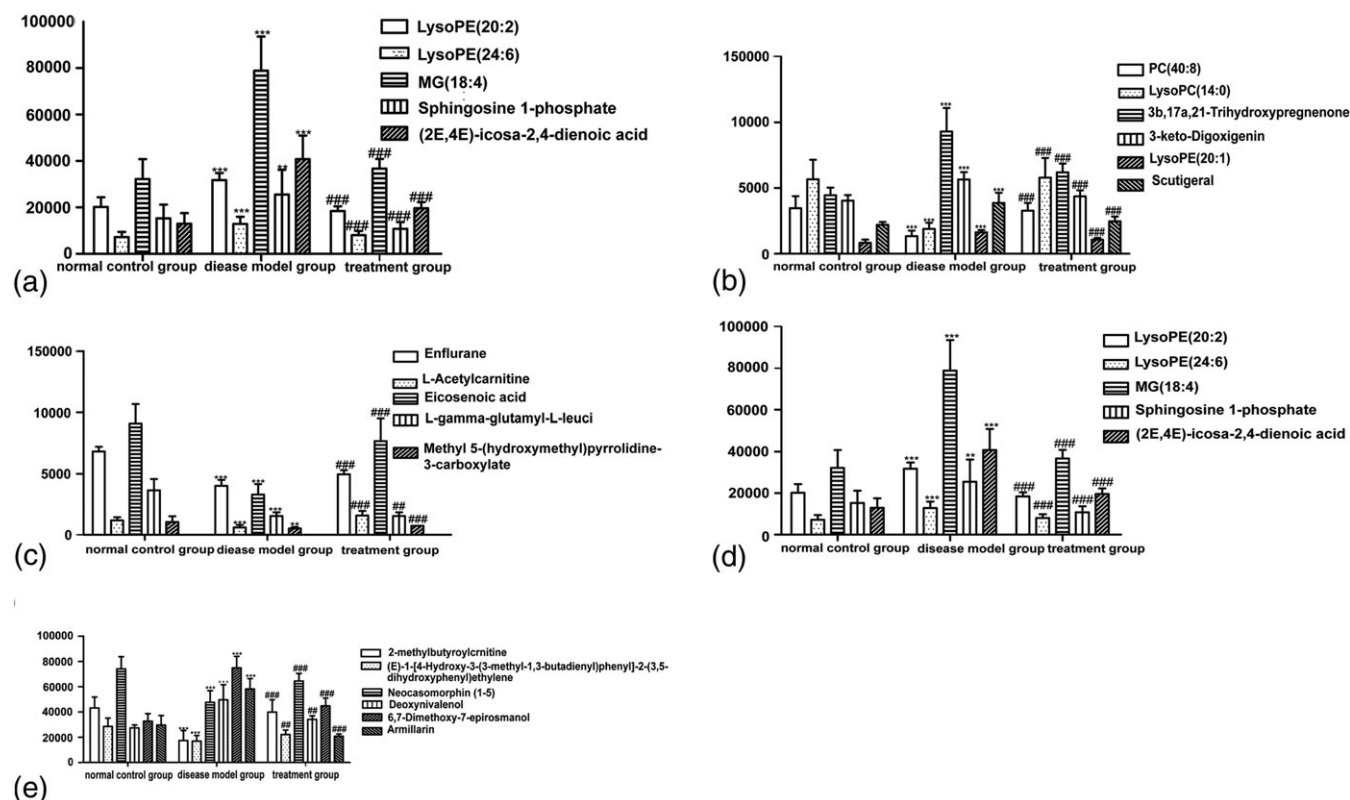


FIGURE 4 Comparison of potential biomarkers in three groups after treatment. ** $p < 0.05$, *** $p < 0.01$ comparing normal control group with disease model group; ## $p < 0.05$, ### $p < 0.01$ comparing disease model group with treatment group

treatment of AR is via adjusting the L-valine content and further affecting BCAAs metabolism.

4 | CONCLUSION

Based on immunoglobulin-mediated type I allergy via releasing histamine, AR is a kind of nasal mucosa chronic inflammation disease that brings inconvenience to people's life and work. Although there has been some development regarding treating AR with TCM, the mechanisms remain unclear. This paper established a stagey of using UHPLC-ESI-Q-TOF-MS coupled with multivariate data analysis method to explore the biomarkers and metabolic pathways for SXF's treatment effect on AR. Finally, this paper determines that both glycerophospholipid metabolism and BCAA metabolism are closely related with SXF's treatment effect on AR.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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