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Filtration and qualification for target biomarkers of traditional Chinese medicine formula “fuzi lizhong decoction” acting on stomach ulcer by UPLC/Q-TOF MS

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Abstract: This study aimed to illustrate the curative effect of Fuzi Lizhong Decoction, which is composed of *Aconitum carmichaeli Debeaux*, *Zingiber officinale Roscoe*, *Codonopsis pilosula (Franch.)*, *Atractylodes macrocephala Koidz* and *Glycyrrhiza uralensis Fisch.* and contained components such as benzoyl aconitine, benzoyl aconitine, atractyl lactone I, atractylenolide II, ginsenoside, emodin, glycyrrhizin, glycyrrhizin, 8-gingerol and 10-gingerol, on stomach ulcer based on ultra-performance liquid chromatography/mass spectrometry metabolomics analysis. Rats urine samples of three groups were detected by ultra-performance liquid chromatography/mass spectrometry. Then, stomach ulcer biomarkers were filtered out by EZinfo 2.0 software. After that, analysis of variance was used for picking up significantly retroved biomarkers in treated group. Compounds were identified based on MS/MS spectrum, accurate mass weight and retention time. Twelve metabolites including 2-Methylhippuic acid, Kynurenic acid, 2-Indolecarboxylic acid, Pyrocatechol, Caproic acid, 2,3-Dihydrobenzofuran, 4-Ethylphenol, Lanthionine ketimine, Traumatic acid, Indole-3-carboxilic acid-O-sulphate, Vanillic acid 4-sulfate, 6-Mecaptopurine ribonudeoside 5’-diphosphate were identified as stomach ulcer biomarkers. Among them, ten ones except for 2-Methylhippuic acid and 2-Indolecarboxylic acid retrieved. Pathways involved tryptophan metabolism, benzoate degradation, bisphenol degradation and α-linolenic acid metabolism. In conclusion, Fuzi Lizhong Decoction may cure stomach ulcer by improving tryptophan metabolism, benzoate degradation, bisphenol degradation and α-linolenic acid metabolism.

Keywords: Ultra-performance liquid chromatography coupled with quadrupole-time of flight mass spectrometry, metabolomics, Fuzi Lizhong Decoction, stomah ulcer, biomarkers.

INTRODUCTION

Stomach ulcer occurs when its mucosa is damaged under the influence of various factors such as high pepsin and acid levels, invasion by *Helicobacter pylori*, etc. (Harsha *et al*., 2017). It generally causes severe symptom which requires long-term medical care even surgery, What’s worse, it can lead to death in the condition of perforation or hemorrhage (Theodore *et al*., 2018). So, preventing and remedying stomach ulcer is of importance for being health. Now, there were many reports regarding the preventiong and therapy of botanical medicine on stomach ulcer. Pharmacological experiments conducted by Ilhan Gürbüz, etc.(Gürbüz *et al*., 2005) showed gastric protection against the ethanol-induced gastric ulcer model rats after six plants being given orally. Lakshmanan Hariprasath etc. (Hariprasath *et al*., 2012) found that aqueous leaf extract of *Senecio candicans* possessed curative effect on gastric ulcer and stomach pain in experimental models. It is clearly seen that national medicine is promising for curing stomach ulcer.

FLD (Fuzi Lizhong Decoction) is a traditional Chinese formula which derives from “San Yin Ji Bing Zheng Fang Lun” edited by Wuze Chen (China), contained *Aconitum carmichaeli Debeaux* (ACD), *Zingiber officinale Roscoe* (ZOR), *Codonopsis pilosula (Franch.)* (CPF), *Atractylodes macrocephala Koidz.* (AMK) and *Glycyrrhiza uralensis Fisch.* (GUF). FLD is primarily used to treat digestive diseases caused by spleen deficiency in China (Zheng *et al*., 2014) through several aspects of increasing the activity of super oxide dismutase in the gastric mucosa (Liu *et al*., 2012), improvement of immune function (Wei *et al*., 2013), and mobilizing the inflammatory system (Xie *et al*., 2013).

Our previous study showed that FLD could cure reserpine caused stomach ulcer (Xin *et al*., 2017). But, the action mechanism of FLD treating stomach ulcer has not been ilustrated. Therefore, this study was carried out to elucidate how the metabolic mechanism of FLD acts in treatment of stomach ulcer, treating stomach ulcer.

In view of the overall concept of Chinese medicine curing disease, an systemic study method is preferable. So, various omics technology which characterised by overall

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research strategy was popular in the study on Chinese medicine (Liu *et al*., 2019; Lee *et al*., 2019; Wang *et al*., 2021). Especially, metabolomics combination with HPLC/UPLC-MS technology has been an indispensable method for filtering target biomarkers and metabolic pathways on which Chinese herbal formulars acting on (Wang *et al*., 2018; Zhang *et al*., 2019), which showed that metabolomics is an useful approach for diagnozing disease and clarifying the curative effect Chinese medicine.

Based on the above, urine metabolomics study on the treatment of FLD on stomach ulcer was developed for investigating the rectified effect of FLD on stomach ulcer biomarkers and relative metabolic pathways. Two statistical analysis methods (Li *et al*., 2015; Zhong *et al*., 2016), PLS-DA (partial least squares discriminate analysis) and ANOVA, were applied for demonstrating the overall separation between groups and selecting target biomarkers of FLD.

On the basis of all results, the curative effect as well as the action mechanism of FLD treating stomach ulcer could be evaluated. Moreover, these biomarkers may be used for preventing and diagnosing stomach ulcer.

MATERIALS AND METHODS

Solvent and medicine

Reference standards for 2-Methylhippuic acid, Kynurenic acid, 2-Indolecarboxylic acid, Pyrocatechol, were supplied by Sigma (St. Louis, Missouri, USA) and Avanti (Alabama, USA). All the herbal medicine was bought from Qi Tai Pharmacy (Qiqihar, China). Reserpine was supplied by Rong He company (Shanghai, China). Acetonitrile and methanol were of HPLC grade. Ethanol was of analytical grade. Deionized water was prepared by using a Millipore water purificating equipment.

Animals groups

Thirty of male SD rats (180–220g) were provided by Beijing Laboratory Animal Research Center (Beijing, China) and were acclimated under the condition with temperature of 23±2℃ as well as relative humidity of 50±10% for a week. During this period, rats were freely given water and food. After that, all rats were averaged into three groups (blank group, model group, treated group). The whole experimental cycle experienced the modeling period (one week) and the curing period (two weeks) successively. In the modeling period, the blank group were fed normally, whereas in the model and treated groups, rat were fed every other day and reserpine was intraperitoneally injected with 0.5mg·kg^{-1} every day, (Zavodskaya *et al*., 1964). During the curing period, FLD was only administered intragastically to the treated group with 1.8 g·kg^{-1}. Additionally, body weight and food intake of all rats were supervised once a week, hair status and faeces morphology were monitored every day.

Sample preparation

Reserpine saline solution of 0.125 mg·ml^{-1} was maked up for rats modeling. QC (Quality control) samples were prepared by mixing ten blank urine samples equally, after being dried by nitrogen gas flow, 100μL ethanol was used for dissolving residue, then 2μL filtrate obtained by microporous membrane was to be tested by UPLC-MS equipment. Preparion of FLD was consistent with our published study (Xin *et al*., 2017).

Thirty rats urine were gathered for 24h after the modeling period as well as the curing period respectively. All the sixty urine samples were processed and tested with the same method as QC samples and 2μL of it was injected into the UPLC-MS system.

UPLC-MS procedure

The UPLC-MS (ultra-performance liquid chromatography /mass spectrometry) equipment consist of Acquity Ultra Performance LC and Xevo G2 QTof mass spectrometer (Waters Co., USA).

The UPLC separation condition was performed with QC sample. The CSH C_{18} column (2.1×50mm, 1.7μm) purchased from Waters Co. Was used. The mobile phase was composed of acetonitrile (A) and 0.1% acetic acid aqueous solution (B), which could garantee the high ionization efficacy and good peak shape. The flow rate was 0.3mL·min^{-1}. UPLC resolution was optimized with gradient elution as follows: at 0 min, A was 2%; at 2 min, A was 20%; at 9 min, A was 100% and lasted until 10 min. Column temperature was set at 30℃, which displayed better resolution than other temperature.

The mass spectrometer was equipped with an electrospray source. Mass spectrometry signal was optimal as follows: capillary of (+4.0/-3.5 kV), desolvation gas flow of 600 L·h^{-1}, sampling cone of 30V, extraction cone of 4V, desolvation temperature of 300℃, source temperature of 100°C, scan range between 50~2000 Da, cone gas flow of 50 L·h^{-1}. Mass scan was conducted in positive and negative ion modes simultaneously. Leucine-enkephalin (Waters Co., USA) was used as a reference compound to guarantee the mass accuracy.

The UPLC-MS method was validated by detecting eight endorgenous metabolites in QC samples for observing accuracy and stability. The accuracy was carried out by detecting the same QC sample successively for three times. The stability was implemented through injecting the same QC sample at 0h, 24h and 48h. RSD% (Relative standard deviation percentage) of peak area and retention time below 10% was considered as good detecting method.

Data analysis

Mass lynx software (version 4.1, Waters Co., USA) was used to acquire the UPLC/MS raw data. Then, Marker lynx was used for processing all the data and generated a data set which was composed of peak intensity normalized to the total ion count, *m/z* value and t_R (retention time). After that, with the help of EZ info software (version 2.0), a multivariate statistical analysis was performed. PLS-DA (Partial least-squares discriminate analysis) was applied for data cluster analysis and picking out significantly differential variables which contributed to the groups discrimination on the basis of VIP (variable importance plot) values. Variables of VIP value above 1.5 were considered to significantly affect on the data cluster and picked out as possible biomarkers. Furthermore, these biomarkers were identified through searching references, HMDB (Human Metabolome Database), as well as comparing the mass spectrum to that of standard. Finally, KEGG (Kyoto Encyclopedia of Genes and Genomes) was used to analyze biomarkers which was related to metabolic pathways.

Ethical approval

This study was approved by the Beijing Laboratory Animal Research Center (SYXK (Beijing) 2015-0046) and complied with all national and international guidelines for research involving animals. After the experiments, all rats were euthanized with CO_2 under anesthesia to alleviate suffering.

STATISTICAL ANALYSIS

With the help of IBM SPSS Statistics 19 software, one-way ANOVA (analysis of variance) was performed to evaluate the callback of potential biomarkers in TG.

RESULTS

Evaluation of methodology

Seven compounds in QC sample were selected for inspecting the accuracy and stability of detecting method, which were showed in tables 1-2. The RSD% of them was lower than 10%, which demonstrated that the analytical method was of good accuracy and stability.

Biomarkers of stomach ulcer

Stomach ulcer could be preliminarily judged by abnormal behavior of decreased body weight and food intake, diarrhea, as well as rough hair in model group rats. In addition, there were lots of lymphocyte infiltrating in model rat stomach tissue which could be seen in our previous study (Xin *et al*., 2020). To filter out stomach ulcer biomarkers, simultaneous positive and negative scan mode was used during UPLC/MS detection. Mass accuracy of mass spectrometry was guaranteed by real-time correction through injecting corrected fluid. Reserpine was selected as internal standard for all samples to ascertain precise signal response. Figs. 1-2 showed the BPI (basic peak ion) chromatograms of the urine samples in different groups during modeling period.

With the help of EZinf software, biomarkers filtration was achieved. PLS-DA which was a supervised pattern recognition method was selected for data cluster analysis among groups. At the same time, significantly differential variables with VIP>1.5 were considered to contribute to discriminate model from blank group. They were deemed as potential stomach ulcer biomarkers. Fig. 3 illustrated the separation of the model and treated groups from the blank group (in modeling period, the model and treated groups were received same prescription), which consisted with expected assumption and indicated that stomach ulcer model was successfully constructed (positive ion mode: R^2X [1]=0.3405, $R^2X[2]$=0.08014; negative ion mode: R^2X [1]=0.2397, $R^2X[2]$=0.07881). There were 657 ions (36 ones in positive and 621 ones in negtive) with VIP>1.5, as shown in fig. 4. In this part, identifying these features was pivotal. So, HMDB (Human Metabolome Database) which can provide the comprehensive information on human metabolites was used. Specifically, the matched compounds of each significant feature were searched for in HMDB. Usually, there were multiple compounds corresponding to one feature, but only one which simultaneously meet to origin, biofluid location, as well as fragmentation information is correctly matched. In order to illustrate the identifying process of biomarkers, the positive ion of *m/z* 190.0492, one of the significant different features, was selected as an example. Firstly, the *m/z* was imported in HMDB. Secondly, each appeared compound was screened based on the above conditions and only one named kynurenic acid was eligible. Thirdly, its MS/MS spectrum was compared to that of literature and result showed consistence. At last, structure confirmation was conducted by comparing the precise molecular weight, t_R and MS_2 data with those of kynurenic acid reference substance (fig. 5a~5f).

On the basis of the above analyzing method, twelve compounds were deduced as stomach ulcer biomarkers which were showed in table 3. Among them, four ones were relative to metabolic pathways. From table 3, it could be seen that α-linolenic acid metabolism, tryptophan metabolism, benzoate degradation, bisphenol degradation and were abnormal under the stomach ulcer.

Curative effect of FLD

The curative effect was examined through two aspects. On the one hand, the behavior status of treated group reverted to near normal. On the other hand, stomach from treated group involved less lymphocyte infiltration than the model group without medicine intervening based on our previous study. Namely, the curative effect of FLD on

Table 1 (A): Accuracy for peak area

Compounds name	Kynurenic acid 190.0492	2-Methylhippuic acid 194.0804	2-Indolecarboxylic acid 162.0547	Pyrocatechol 109.0283	Homogentisic acid 167.0338	Phenylpyruric 163.039	Pimelicacid 159.0641
Ion mode	+	+	+	-	-	-	-
Peak area	10733	425	80888	3687	520	3841	10733
	10844	447	80857	3573	527	3590	10844
	10609	419	80609	3851	522	3727	10609
RSD%	1.10%	3.43%	0.19%	3.77%	0.69%	3.38%	1.10%

Table 1 (B): Accuracy for retention time

Compounds name	Kynurenic acid 190.0492	2-Methylhippuic acid 194.0804	2-Indolecarboxylic acid 162.0547	Pyrocatechol 109.0283	Homogentisic acid 167.0338	Phenylpyruric 163.039	Pimelicacid 159.0641
Ion mode	+	+	+	-	-	-	-
Retention time	3.02	3.08	3.00	3.98	3.69	4.06	2.79
	3.02	3.08	3.00	3.98	3.69	4.06	2.79
	3.02	3.08	3.00	3.98	3.69	4.06	2.79
RSD%	0.33%	0.19%	0.19%	0.15%	0.00%	0.14%	0.21%

Table 2 (A): Stability for peak area

Compounds name	Kynurenic acid 190.0492	2-Methylhippuic acid 194.0804	2-Indolecarboxylic acid 162.0547	Pyrocatechol 109.0283	Homogentisic acid 167.0338	Phenylpyruric 163.039	Pimelicacid 159.0641
Ion mode	+	+	+	-	-	-	-
Peak area	11548	473	90961	2675	611	4136	11548
	11007	417	78775	2974	510	4095	11007
	11146	394	87898	2358	568	3899	11146
RSD%	2.50%	9.49%	7.38%	7.49%	9.00%	3.13%	2.50%

Table 2 (B): Stability for retention time

Compounds name	Kynurenic acid 190.0492	2-Methylhippuic acid 194.0804	Indolecarboxylic acid 162.0547	Pyrocatechol 109.0283	Homogentisic acid 167.0338	Phenylpyruric 163.039	Pimelicacid 159.0641
Ion mode	+	+	+	-	-	-	-
Retention time	3.02	3.08	3.00	3.98	3.69	4.06	2.79
	3.02	3.08	3.00	3.98	3.69	4.06	2.79
	3.02	3.08	3.00	3.98	3.69	4.06	2.79
RSD%	0.38%	0.38%	0.19%	0.14%	0.16%	0.14%	0.21%

Table 3: Identification results of stomach ulcer related biomarkers

No.	Deduced metabolites	t_R (min)	Exact mass	Elemental composition	Deviation (ppm)	Pathways	Ion mode
1	2-Methylhippuic acid[a]	3.08	194.0804	$C_{10}H_{11}NO_3$	-1.1	—	Positive
2	Kynurenic acid[a]	3.00	190.0492	$C_{10}H_7NO_3$	-0.9	Tryptophan metabolism	Positive
3	2-Indolecarboxylic acid[a]	3.00	162.0547	$C_9H_7NO_2$	-0.6	—	Positive
4	Pyrocatechol[a]	3.99	109.0283	$C_6H_6O_2$	-0.5	Benzoate degradation	Negative
5	Caproic acid[b]	3.23	115.075	$C_6H_{12}O_2$	-0.8	—	Negative
6	2,3-Dihydrobenzofuran[c]	4.07	119.0489	C_8H_8O	-0.7	—	Negative
7	4-Ethylphenol[c]	5.47	121.0646	$C_8H_{10}O$	-0.4	Bisphenol degradation	Negative
8	Lanthionine ketimine[c]	1.79	187.9997	$C_6H_7NO_4S$	-0.7	—	Negative
9	Traumatic acid[b]	2.91	227.1256	$C_{12}H_{20}O_4$	-0.3	α-linolenic acid metabolism	Negative
10	Indole-3-carboxilic acid-O-sulphate[c]	3.13	239.9962	$C_9H_7NO_5S$	-0.5	—	Negative
11	Vanillic acid 4-sulfate[b]	3.68	246.9908	$C_8H_8O_7S$	-0.4	—	Negative
12	6-Mecaptopurine ribonudeoside 5'-diphosphate[c]	1.31	442.9808	$C_{10}H_{14}N_4O_{10}P_2S$	-2.0	—	Negative

Metabolites with the superscript of "a", "b" and "c" represents the identification of them was conducted by using standards and HMDB, by literatures reports and HMDB, as well as by searching database HMDB, respectively.

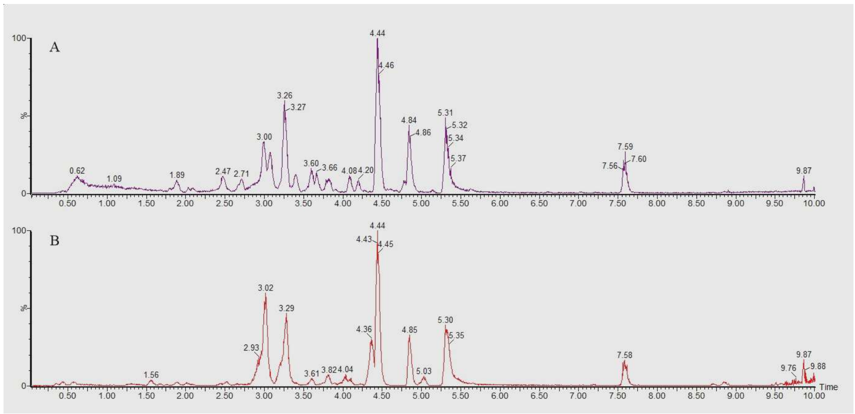

Fig.1. positive basic peak ion chromatograph of urine sample from MG (A) and BG (B) during modeling period

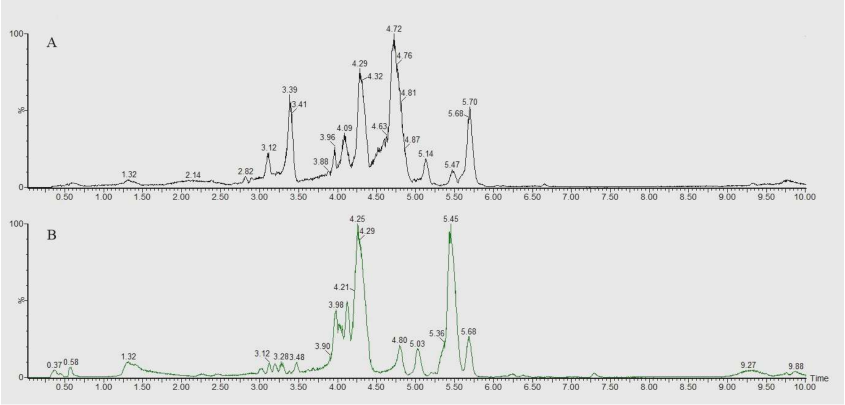

Fig. 2: negative basic peak ion chromatograph of urine sample from MG (A) and BG (B) during modeling period

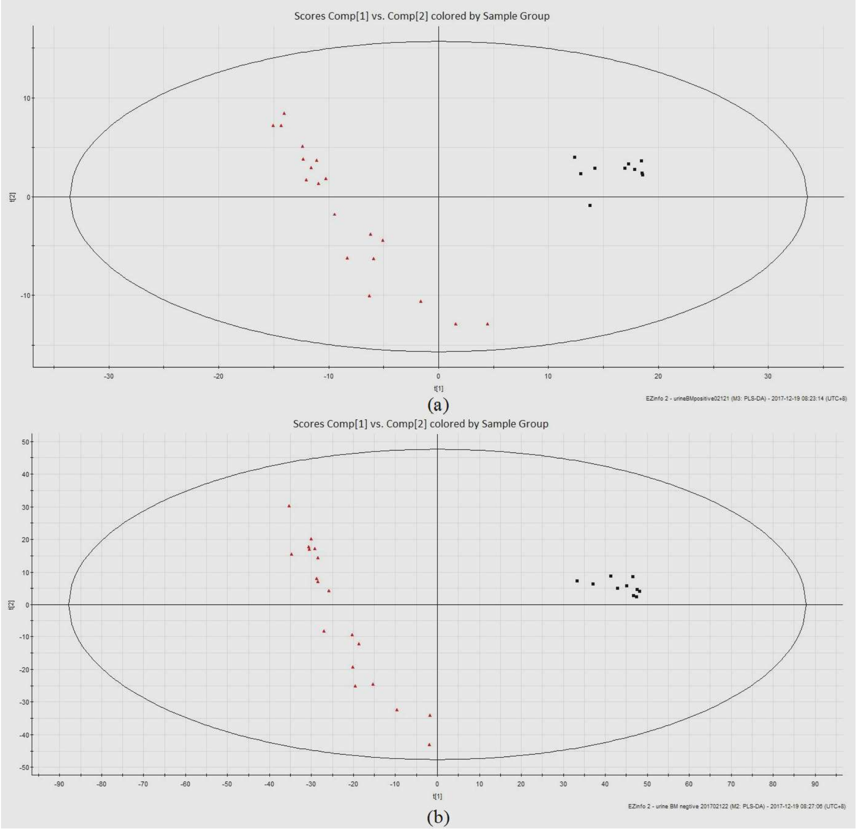

Fig. 3: PLS-DA score plots from different groups during modeling period in (a) positive ion mode and (b) negative ion mode (◆: blank group, ■: model group, ▲: treated group).

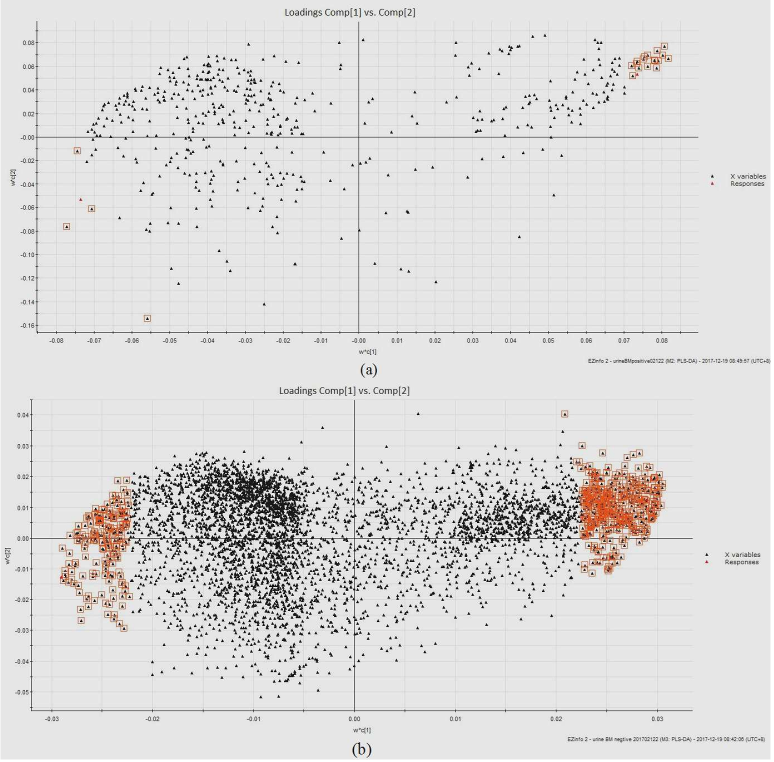

Fig. 4: PLS-DA loading plots from blank group, model group and treated group during modeling period in (a) positive ion mode and (b) negative ion mode (Triangles with square indicate features of which VIP are above one point five)

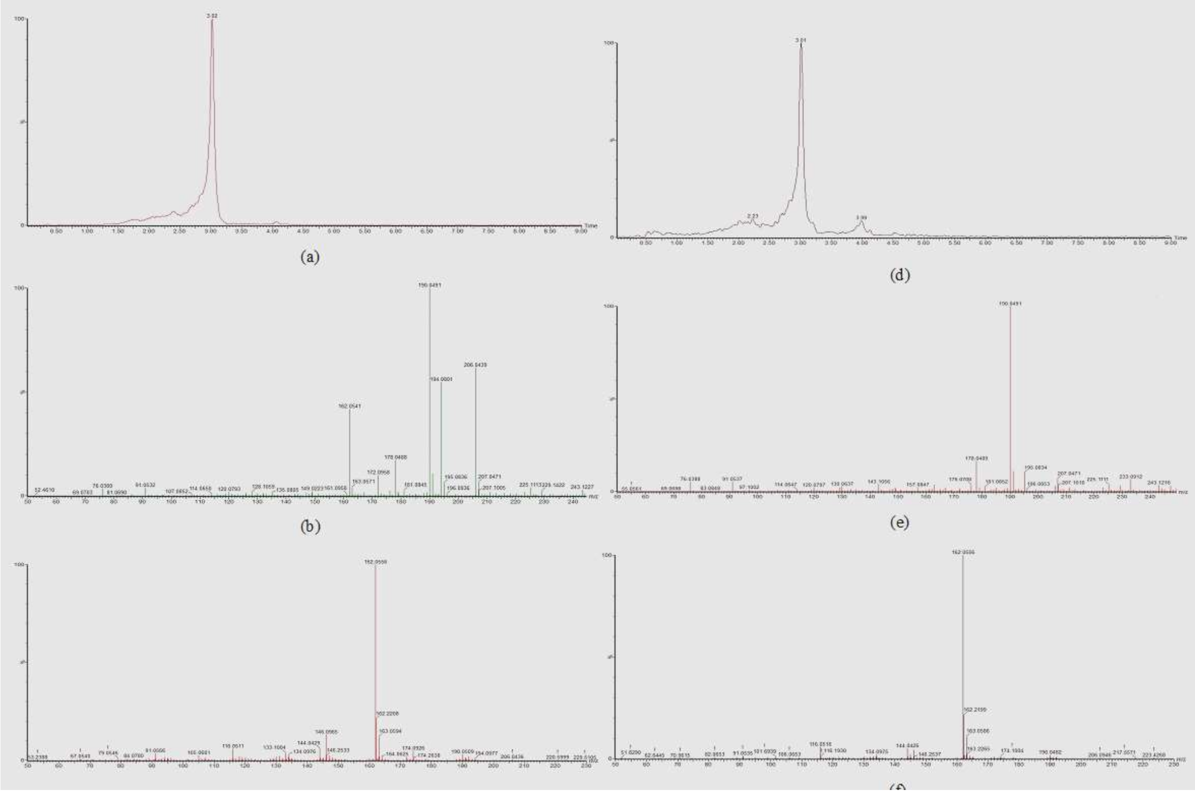

Fig. 5: Identification of kynurenic acid. (a) extracted ion chromatogram of kynurenic acid in urine sample; (b) corresponding mass spectrum to (a); (c) MS/MS spectrum of kynurenic acid in urine sample with collision energy setting at 20 eV; (d) extracted ion chromatogram of kynurenic acid standard added in blank urine; (e) corresponding mass spectrum to (d); (f) MS/MS spectrum of kynurenic acid standard with collision energy setting at 20 eV.

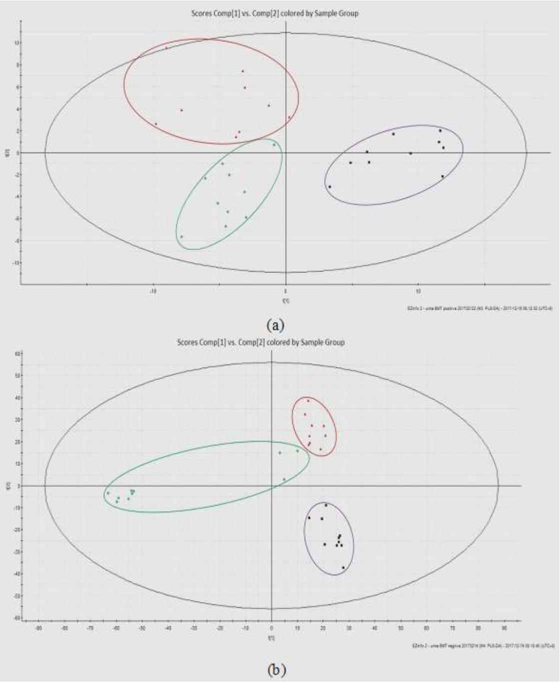

Fig. 6: PLS-DA score plots from different groups during treating period in (a) positive ion mode and (b) negative ion mode (◆: blank group, ■: model group, ▲: treated group).

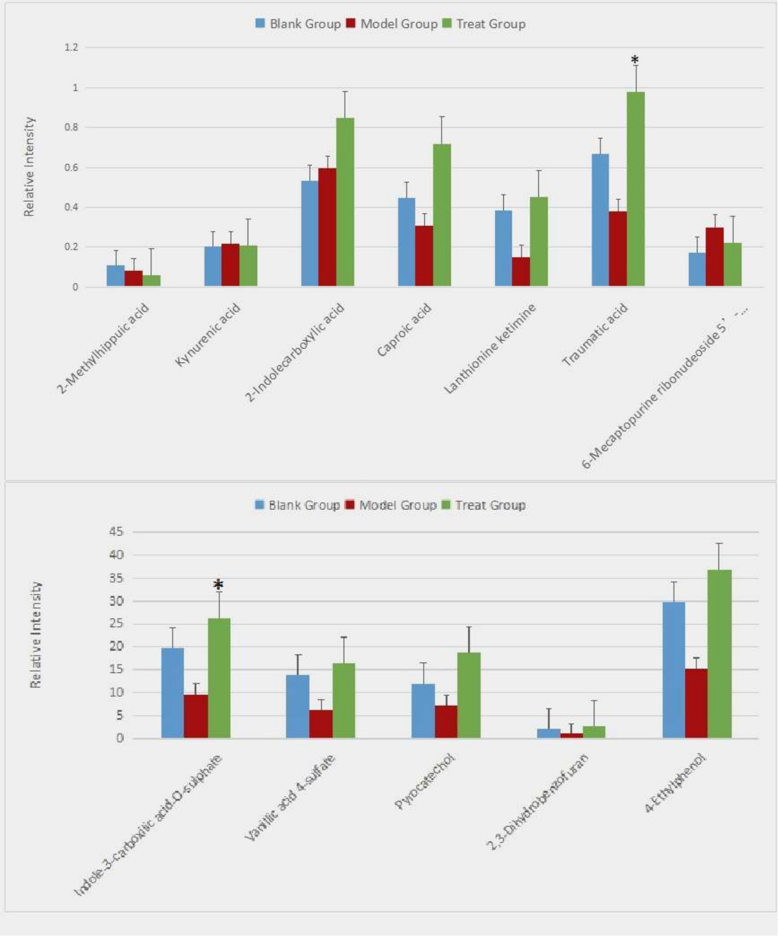

Fig. 7: ANOVA histogram of relative signal intensities for twelve metabolites in BG, MG and TG groups. Data are expressed as mean. Significant differences (*α<0.05) exist between MG and TG for two metabolites.

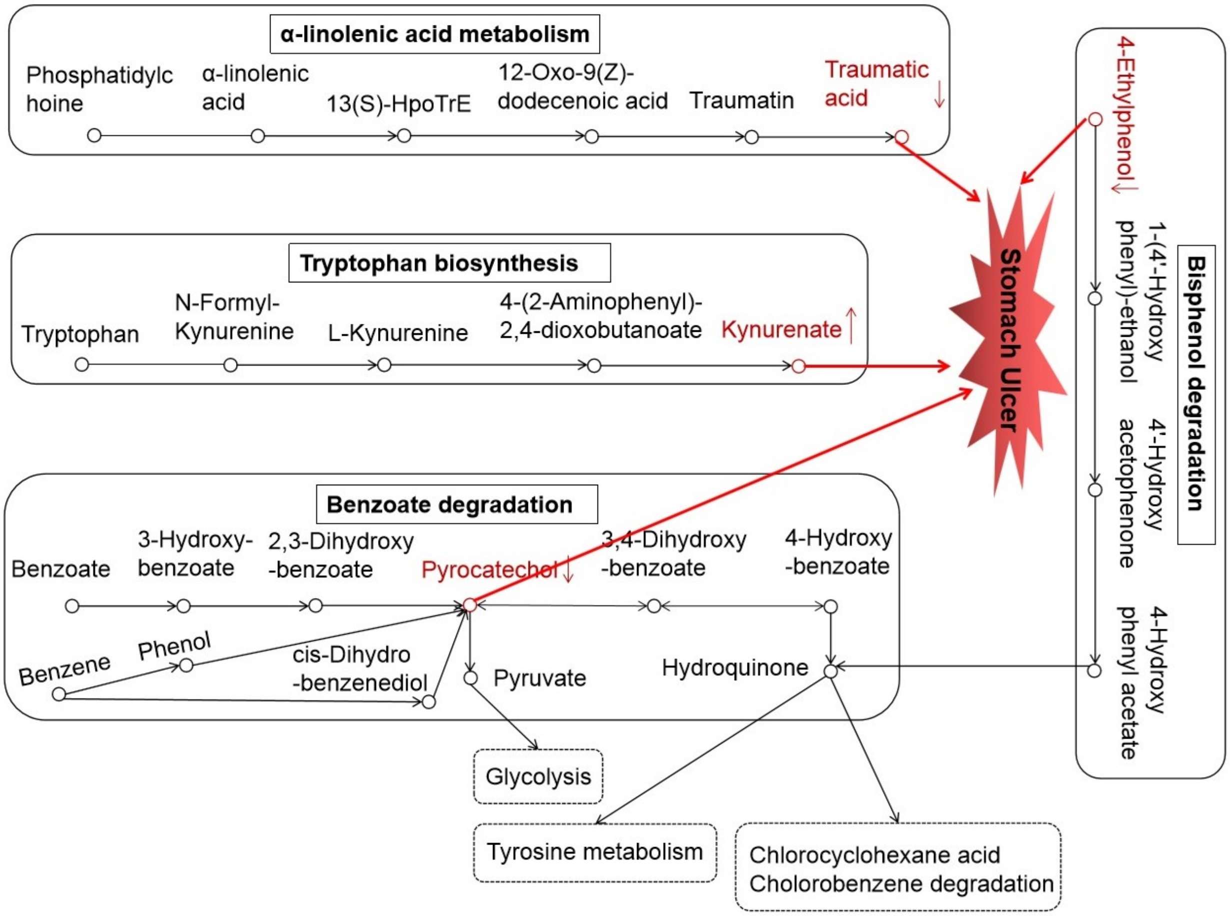

Fig. 8: Deduced metabolic network

stomach ulcer was undoubted, which was intuitively displayed in PLS-DA score plot (fig. 6). Further, mass spectrometry intensity of stomach ulcer biomarkers in three groups were examined by ANOVA to select target biomarkers on which Fuzi Lizhong Decoction act on. Results of ANOVA showed that there were total ten biomarkers including Kynurenic acid, Caproic acid, Lanthionine ketimine, Traumatic acid, 6-Mecaptopurine ribonudeoside 5'-diphosphate, Indole-3-carboxilic acid-O-sulphate, Vanillic acid 4-sulfate, Pyrocatechol, 2,3-Dihydrobenzofuran and 4-Ethylphenol returned, Among them, traumatic acid and Indole-3-carboxilic acid-O-sulphate exhibited significant recovery (fig. 7).

DISCUSSION

Stomach ulcer is a common digestive disease, which may develop into stomach cancer if not being treated in time, so it is important to early diagnose and treat it to prevent the development of cancer. FLD was reported to benefit spleen and stomach. Recently, study on FLD mainly involved network pharmacology (Ding *et al*., 2021; Pan *et al*., 2021) which may predict the functional mechanism. But the predicted analysis can not replace the experimental study. Therefore, this study tried to clarify the mechanism of FLD on stomach ulcer through animal experiment.

Generally, Chinese herbal concoction contains complex components and its efficacy is explained in view of overall concept. It is difficult to determine exact biological target of them. Fortunately, overall research strategy of metabolomics may provide means for assessing the curative effect of Chinese herbal concoction and filtering out the target which they acting on. This study combined the overall concept of Chinese herbal concoction with the overall research strategy of metabolomics for filtering out stomach ulcer-related endorgenous metabolites target as well as FLD acting on ones successfully.

UPLC/Q-TOF MS was the essential technology for the identification of stomach ulcer biomarkers. Among twelve biomarkers, ten ones retrieved after FLD intervening and three ones were reported to related to ulcer indirectly.

Kynurenic acid was reported to reduce basal gastric acid secretion significantly and was protective against ethanol-induced gastric mucosal injury as well as protected gastroduodenal ulcers (Glavin *et al*., 1990; Glavin *et al*., 1989). In this study, we found that the kynurenic acid level was significantly increased in model rats, which was consistent with literature (Jung *et al*., 2011). In the tryptophan metabolism pathway, kynurenic acid was indirectly synthesized by tryptophan, L-tryptophan diet could prevent indomethacin induced ulcer relapse and accelerate the healing process of gastric ulcers induced by acetic acid (Iwona *et al*., 2017; Krzysztof *et al*., 2012), the abnormal level of kynurenic acid indicated that the biotransformation of tryptophan was stimulated. Based on the above, it is speculated that self-protection system might be activated when stomach ulcer happened.

Pyrocatechol is a mid product in benzoate degradation, possessed antiulcer activity (Garro *et al*., 2015), showed decreased level in model group. One of the final products of pyrocatechol is pyruvate which decreased in rat's plasma with gastric ulcer (Yang *et al*., 2017). Therefore, it could be deduced that benzoate degradation was disturbed when gastric ulcer happened.

Traumatic acid is the final product in the pathway of α-linolenic acid metabolism. Although there was no relational report involving traumatic acid connecting to stomach ulcer, the decreased level of traumatic acid in this study could indicate the effect of stomach ulcer on the biotransformation of it.

Traumatic acid and Indole-3-carboxilic acid-O-sulphate which showed significantly callback in TG were considered as target biomarkers upon which FLD acted. Therefore, it was concluded that FLD cured stomach ulcer mainly through intervening in α-linolenic acid metabolism. The metabolic network deduced from this study was illustrated in fig. 8.

The significance of this study depends on it filtering stomach ulcer biomarkers and interpreting the mechanism of FLD. Results may be useful for the diagnosis of stomach ulcer and can provide experimental basis for developing pharmaceuticals treating stomach ulcer.

CONCLUSION

The curative effect of FLD on stomach ulcer was successfully clarified by applying UPLC/MS technology in urine metabolomics. PLS-DA was applied for the data cluster analysis and filtering differential variable. After structural inference, twelve compounds were deduced as biomarkers of stomach ulcer and traumatic acid and indole-3-carboxilic acid-O-sulphate of them were proved as target substances of FLD by ANOVA. The obvious retrieval of biomarkers indicates the intervention of FLD on α-linolenic acid metabolism pathway. In short, This paper reflected the intersecting of pharmacology, metabolomics and medicinal plant, as well as the application of them in the study of pharmaceutical. Results indicated that FLD is expected to be developed as a beneficial pharmaceutical for the stomach in future and this paper would provide the pre-foundation for further filtering out stomach benefited compounds from it. Our further studies will focus on stomach ulcer human urine metabolomics to ascertain the twelve biomarkers of stomach ulcer, which will provide the clinical diagnostic indicators of stomach ulcer.

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