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Comparative metabolomics analysis for the compatibility and incompatibility of kansui and licorice with different ratios by UHPLC-QTOF/MS and multivariate data analysis

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ABSTRACT

Kansui, the root of *Euphorbia kansui* T.N. Liou ex T.P. Wang (Euphorbiaceae), is a well-known poisonous traditional Chinese medicine (TCM). However, many monographs of TCM indicated that it cannot be co-used with licorice, as kansui-licorice is a typical "eighteen incompatible" medicaments. Our previous studies have indicated that kansui was effective in treating malignant pleural effusion (MPE), and the efficacy could be weakened by the co-use of licorice, even causing serious toxicity at the given ratio. Nevertheless, the actual mechanisms of their dosage-toxicity-efficacy relationship need to be well clarified. The present study aimed to investigate the effect of individual and combined use of kansui and licorice on MPE rats, and explain the underlying mechanisms from a metabolomic perspective. Urine samples were analyzed by ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS). Partial least-squares discriminate analysis (PLS-DA) models were built to evaluate the interaction between kansui and licorice. Seven potential biomarkers contribute to the separation of model group and control group were tentatively identified. And selenoamino acid metabolism and nicotinate and nicotinamide metabolism with the impact-value 0.31 and 0.24, respectively, were filtered out as the most important metabolic pathways. Kansui and kansui-licorice at a ratio of 4:1 can treat MPE rats by adjusting abnormal metabolic pathways to the normal state, while it may have opposite result with kansui-licorice 1:4. The different influences to the two metabolic pathways may partially explain the dosage-toxicity-efficacy relationship of kansui-licorice with different ratios. The results could offer valuable insights into the compatibility property changes for the two herbs.

1. Introduction

Kansui, the root of *Euphorbia kansui* T.N. Liou ex T.P. Wang, is a well-known traditional Chinese medicine (TCM), and was recorded in *Sheng Nungs Herbal* as a low-grade drug. It possesses a variety of pharmacological activities, including diuretic, purgation, and so on. And it has been widely used for centuries in China as a drastic hydragogue for edema, ascites, and asthma [1,2]. Licorice, is a popular Chinese herbal medicine derived from the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Bat., and *Glycyrrhiza glabra* L. It is one of the oldest and most popular herbal medicines in the world for various biological activities, including antiulcer, anti-inflammatory, and so on [3]. As a tonifying herbal medicine, licorice is extensively used in TCM, and appears as a component herb in about 60% of all TCM prescriptions, but it cannot be co-used with kansui, which is recorded in "eighteen incompatible" medicaments in many monographs of TCM. The toxicity of kansui might be enhanced by licorice, and on the other side the effect of kansui might be decreased by licorice [4,5].

Our previous study [6,7] investigated the dosage-toxicity-efficacy

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relationship of the co-use of kansui and licorice. And the results showed that kansui had certain efficacy of treating malignant pleural effusion (MPE), and the efficacy can be weakened by the co-use of licorice, even to cause serious toxicity on the given ratio. The ratio between 2:1 and 1:1 (kansui:licorice) was considered as the flex point of the dosage-toxicity-efficacy of kansui and licorice. In contrast, the results reminded that kansui and licorice can be co-used in given ratios and dosages. It is meaningfully that the property of the compatibility of kansui and licorice was changed with the different ratios. However the potential mechanism of this interesting phenomenon has not been well clarified.

Metabolomics as a branch of system biology was first put forward by professor Nicholson in 1999, and is defined as systematically qualitative and quantitative analysis of metabolites in a given organism or biological sample and then quantificationally describes changes of endogenous metabolites before and after stimulations or disturbances [8,9]. Metabolomics is to regard organisms as a whole, and the physiological and pathological process to be dynamic. It's consistent with "wholeness", "dynamic concept" and "dialectics" of the TCM theory [10]. In recent years, metabolomics has been widely used in many fields, especially to be used to evaluate the curative effects and mechanism of action of Chinese herbs [11]. With continuous advancement of technologies, many techniques have been used in metabolomics, and different metabolomic techniques have been applied to different herbal medicine research. Among them, UHPLC–MS is recognized as one of the best analytical techniques in selectivity, sensitivity, and reproducibility [12,13].

In this study, two different ratios of kansui and licorice (4:1 and 1:4), which represent their compatibility and incompatibility respectively, were designed to investigate the effect of individual and combined use of kansui and licorice on MPE rats. And metabolomics study was employed to explore the possible mechanisms. UHPLC-QTOF-MS technology was used to analyze the metabolites of rat's urine before and after the drug therapy, then the multivariate statistical were utilized for the metabolomic analysis.

2. Experimental

2.1. Drugs and reagents

Acetonitrile (HPLC grade), methanol (HPLC grade) and formic acid were purchased from Merck KGaA (Darmstadt, Germany); ultra-pure water was purified by an EPED super purification system (Nanjing, China). Other reagents and chemicals were of analytical grade.

The roots of *Euphorbia kansui* T.N. Liou ex T.P. Wang (No. NJUTCM-20100520) were collected from Honghegu, Shanxi Province, China, and the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch. (No. NJUTCM-20101215) were collected from Lingwu City, Ningxia Province, China. The two herbs were identified by Professor Chungen Wang (Department of Pharmacognosy, Nanjing University of Chinese Medicine, Nanjing, China), and the voucher specimens of them have been deposited in the Herbarium of Nanjing University of Chinese Medicine. In our previous study [14], the chemical analyses of kansui and licorice were performed by UHPLC-PDA-TQ-MS, and the major peaks of each herb found in the total ion chromatograms were identified and quantified. The extraction and preparation of drug solution were as same as our previous study [7].

2.2. Animals and treatment

SPF male Wistar rats were provided by Beijing Vital River Company (Beijing, China). All of the rats were kept in Drug Safety Evaluation Center of Nanjing University of Chinese Medicine, Nan-jing, China. And the protocol of the study was approved by the Ethics Committee of the Nanjing University of Chinese Medicine.

After a week of adaptive feeding, fifty-six rats weighing 250–280 g were randomly divided equally into seven groups: normal control group, model group, kansui group, kansui-licorice 4:1 group, kansui-licorice 1:4 group and licorice groups 1 and 2 of corresponding dose. The models were developed by once intrapleural injection with Walker 256 cells (1×10^7/mL, 0.3 mL per rat). After intrapleural injection, the rats of the different groups were given different drugs by gavage for 7 days. The normal control group and model group were given 0.9% saline. The ratios and doses are listed in Table 1.

Table 1
The ratios and dose of different groups.

Groups	*n*	Kansui (g/kg)	Licorice (g/kg)	Ratio (kansui:licorice)
Control	8	–	–	–
Model	8	–	–	–
Kansui	8	0.15	–	–
Licorice 1	8	–	0.04	–
Licorice 2	8	–	0.60	–
4:1	8	0.15	0.04	4:1
1:4	8	0.15	0.60	1:4

2.3. Sample collection and preparation

On days 7 after the administration, all animals were housed in metabolic cages (1 per cage) to collect the 24 h urine samples with the freely diet and drink. Then, all of the rats were weighed and anesthetized with 10% chloral hydrate by intraperitoneal injection before the pleural fluid was collected. The rats were fixed in the supine position, the thorax was opened carefully, and the pleural fluid was removed using an injector and drinking paper. Additionally, the rats were weighed again, and the difference in the weight was regarded as the pleural fluid volume.

All urine samples were immediately centrifuged at 3000 rpm for 10 min, and the supernatants were separated and stored at −80 °C until analysis. Methanol (800 μL) was added into 800 μL of all the urine samples, the mixture was vortexed for 30 s and centrifuged at 13,000 rpm for 10 min. Then, 1400 μL supernatants of the urine samples were transferred into new tubes and evaporated to dryness under vacuum with the Labconco CentriVap concentrator (Kansas City, MO, USA). The residues of urine samples were dissolved in 200 μL of 70% methanol solution, and the mixtures were vortexed for 1 min, and centrifuged at 13,000 rpm for 10 min at 4 °C. Finally, a 2 μL aliquot of supernatant was injected for UHPLC-QTOF/MS analysis.

In addition, the urine samples from each group were mixed together as the quality control (QC) samples. This pooled sample was globally representative of the whole sample set. The QC samples were injected three times at the beginning of the run in order to condition or equilibrate the system and then every ten samples throughout the analytical run to further monitor the stability of the analysis [15,16].

2.4. UHPLC-QTOF/MS conditions

UHPLC was performed using a Waters ACQUITY UPLC system (Waters, Milford, MA, USA) that was equipped with a binary solvent delivery system and an auto-sampler. Chromatographic separation was performed on a Thermo Syncronis C18 column (2.1 mm i.d. × 100 mm, 1.7 μm). The mobile phase was composed of A (water and 0.1% formic acid) and B (acetonitrile) under gradient elution conditions: 5–30% B from 0 to 8 min, 30–70% B from 8 to 11 min, 70–95% B from 11 to 13 min, 95% B from 13 to 14 min, 95–5% B from 14 to 15 min. The flow rate of the mobile phase was 0.4 mL/min, and the column and auto-sampler were maintained at 35 and 4 °C, respectively. Two cycles of weak and strong solvent washing of the injection system were performed between injections. The injection volume was 2 μL.

Mass spectrometry was performed on a Synaptt Q-TOF (Waters, Manchester, UK) using an ESI operated in positive and negative ion mode. The parameters in the source were set as follows: capillary