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# Serum metabonomics study on antidiabetic effects of fenugreek flavonoids in streptozotocin-induced rats

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ABSTRACT

Fenugreek is a well-known medicinal plant used for treatment of diabetes. In this study, the antidiabetic effect of fenugreek flavonoids was investigated by metabonomics based on ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Fenugreek flavonoids were purified using polyamide resin and D101 macroporous adsorption resin, characterized by UPLC-Q-TOF-MS, and administered to streptozotocin (STZ)-induced diabetic rats for 28 days. Pharmacological study results indicated that fenugreek flavonoids exerted a strong antidiabetic effect characterized by significant reduction of fasting blood glucose ($P < 0.01$), increase in serum insulin level ($P < 0.01$) and liver glycogen content ($P < 0.01$), attenuation of weight loss, and improvement of pancreatic islet and kidney conditions. The antidiabetic effect of fenugreek flavonoids was further analyzed by metabonomics. Serum samples of health and diabetic rats treated or not with fenugreek flavonoids were evaluated by UPLC-Q-TOF-MS, followed by principal component analysis (PCA) and orthogonal projection to latent structures squares-discriminant analysis (OPLS-DA). The PCA model revealed significant differences among the animal groups, and OPLS-DA identified fenugreek flavonoids-induced changes of 11 potential biomarkers involved in lipid metabolism (docosahexaenoic acid, arachidonic acid, sphinganine, sphingosine-1-phosphate, and lysophosphatidylcholines 20:4, 18:2, 16:0, and 20:2), amino acid metabolism (hippuric acid and tryptophan), and kidney function-related metabolism (2-phenylethanol glucuronide). Our study demonstrates that flavonoids are bioactive components of fenugreek with potent antidiabetic activity, which exert their therapeutic effects by multiple mechanisms, including reducing insulin resistance, improving gluconeogenesis, and protecting islet cells and kidneys from damage.

## 1. Introduction

Fenugreek (*Trigonella foenum-graecum* Linn.) is a medicinal plant cultivated in southwest Asia, Africa, and the Mediterranean region as a food crop [1,2]. Recent pharmacological studies found that dried ripe fenugreek seeds exert significant medicinal effects beyond traditional nutritional values, including antidiabetic, antihyperlipidemic, anti-obesity, antioxidant, and immunomodulating properties [3–8]. The interest in the antidiabetic activity of fenugreek has increased because of high incidence of diabetes worldwide and the established safety of fenugreek as a food crop [9,10]. Currently, a number of research institutions and pharmaceutical companies are working with fenugreek seeds as raw material for developing new drugs to treat diabetes.

Identification of biologically effective components is important for developing new drugs from fenugreek. Fenugreek mainly contains polysaccharides, saponins, flavonoids, alkaloids, and proteins. Previous studies have clarified antidiabetic effect of proteins and amino acids (4-hydroxyisoleucine), alkaloids (trigonelline), saponins, and polysaccharides from fenugreek [11–23]. However, there is a lack of information on such therapeutic activity of flavonoids. A previous study has evaluated the hypoglycemic effect of a single fenugreek flavonoid derivative in alloxan-induced diabetic mice [24]; however, the antidiabetic activity of total fenugreek flavonoids has not been yet investigated. Fenugreek contains 1–2% flavonoids which include apigenin and luteolin as the main aglycones, and xylose, arabinose, glucose, galactose or rhamnose as C-glycosides [25–27]. Given that flavonoids are considered as potential antidiabetic components of fenugreek seeds, their effect should be confirmed experimentally.

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In this study, we explored the antidiabetic effect of fenugreek flavonoids in diabetic rats by measuring fasting blood glucose (FBG), serum insulin, liver glycogen, and evaluating pancreatic and kidney histopathology. In addition, the metabolic response underlying fenugreek flavonoids effects in diabetes was elucidated by metabonomics. The biomarkers identified by metabonomics could be used to assess the antidiabetic effect of flavonoids from fenugreek seeds and to develop flavonoid-based drugs.

## 2. Materials and methods

### 2.1. Materials

Fenugreek seeds were produced in Sichuan Province, China, 2013. The seeds were authenticated by Professor Shumin Wang (Changchun University of Chinese Medicine, Changchun, China), an expert in Chinese medicine. Streptozotocin (STZ), hippuric acid, tryptophan and rutin were obtained from Sigma-Aldrich (St. Louis, MO, USA). D101 macroporous adsorption resin and polyamide resin (30–60 mesh) were obtained from Tianjin Agricultural Chemical Company (Tianjin, China) and China Pharmaceutical Group, Shanghai Chemical Reagent Company (Shanghai, China), respectively. Leucine encephalin (LE) was obtained from Waters Corporation (Milford, MA, USA). HPLC-grade acetonitrile, acetic acid, and formic acid were purchased from Tedia Company (Fairfield, OH, USA). Ultrapure water was produced in a Milli-Q plus water purification system (Millipore Corporation, Billerica, MA, USA). Kits for liver glycogen assay and rat insulin (INS) ELISA were obtained from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China).

### 2.2. Preparation of fenugreek flavonoids

Fenugreek seeds were ground to 60–80 mesh and defatted in petroleum ether. The residues were dried under vacuum and then refluxed in 70% ethanol for 1.5 h three times. The extracts were filtered, concentrated at reduced pressure, dissolved in water, and loaded into a polyamide resin column. After washing with water to remove carbohydrates and proteins, the adsorbed flavonoids were eluted with 70% ethanol, further purified on D101 macroporous adsorption resin, and eluted with 30% ethanol to obtain fenugreek flavonoids.

### 2.3. Identification of fenugreek flavonoids

The total flavonoid content of fenugreek flavonoids was determined by colorimetry using rutin as a reference. Chemical composition of fenugreek flavonoids was analyzed by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). UPLC analysis was performed using the Acquity UPLC system (Waters Corporation) equipped with an Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, Ø = 3 μm). The injection volume was 5 μL, and mobile phases A and B were acetonitrile and 0.1% formic acid solution (v/v), respectively, applied at a flow rate of 0.5 mL/min; column temperature was maintained at 30 °C. Gradient elution was performed as follows: 0 min, 15% A; 0–25 min, 15%–20% A; 25–28 min, 20%–25% A; and 28–40 min, 25%–100% A. The MS analysis was carried out using the ESI source in the $MS^E$ mode, and full-scan mass range was *m/z* 50–1600 Da. The source temperature was 120 °C, and the desolvation gas temperature was 350 °C. The cone and solvent removal gas was nitrogen used at the flow rates of 50 L/h and 700 L/h, respectively. The voltages of capillary, cone, and extraction cone in negative ion mode were set at 2.5 kV, 30 V, and 5.0 V, respectively. $MS^E$ was applied for MS/MS analysis with low collision energy of 6 eV and high collision energy of 20–40 eV. Argon was used as collision gas. Sodium formate was used to construct a standard curve for quality control, and LE was used as mass reference.

### 2.4. Induction of diabetes and experimental design

#### 2.4.1. Ethics statement

The care and use of animals, and the experimental protocol were in accordance with the Guidelines for the Care and Use of Experimental Animals of Jilin University, and were approved by the Animal Experiment Ethics Committee of Jilin University.

#### 2.4.2. Induction of diabetes

Male Sprague-Dawley rats weighing 220 ± 20 g were obtained from the Experimental Animal Center of Jilin University (Changchun, China). Rats were housed in a climate-controlled room (temperature, 20 ± 2 °C; humidity, 60 ± 10%; 12 h/12 h dark/light cycle) with free access to food and water. After one week of acclimatization, the rats were subjected to 16-h fast; then, diabetes was induced with a single intraperitoneal injection of STZ (65 mg/kg body weight) freshly dissolved in 0.1 M citrate buffer (pH 4.2). After 7 days, blood glucose levels were measured by tail vein puncture using glucose meters (Beijing Yicheng Electronic Technology Company, Beijing, China); animals with glucose level over 16.7 mM were classified as diabetic.

#### 2.4.3. Experimental design

Diabetic rats were randomly divided into two groups: the diabetic untreated group was administered water intragastrically (10 mL/kg/day) and the diabetic/fenugreek flavonoids-treated group was administered 0.5 g fenugreek flavonoids in 10 mL of water intragastrically (10 mL/kg/day). Healthy control rats were administered water as described. The experiments were terminated after 28 days.

### 2.5. Antidiabetic effect of fenugreek flavonoids

At day 28, rats were fasted for 16 h. After fasting blood glucose (FBG) levels were determined using glucose meters, rats were euthanized by exsanguination under anesthesia and blood was collected. Serum was isolated by centrifuging blood at 3000 × *g* for 10 min at 4 °C, immediately aliquoted, and frozen at −80 °C until analysis. To determine serum insulin levels, serum samples were thawed at room temperature and analyzed using the INS ELISA kit. Liver glycogen content was determined using the liver glycogen assay kit. The pancreas and kidney were fixed in 10% neutral buffered formalin and used for histological examination after hematoxylin-eosin (H&E) staining. Unpaired two-sided Student's *t*-test (SPSS software, version 19.0) with subsequent Bonferroni correction was used to evaluate significant differences between the groups. The level of significance was set at $P < 0.01$ (Bonferroni's correction for multiple comparisons).

### 2.6. Metabonomics

#### 2.6.1. Preparation of serum samples

Prior to UPLC-Q-TOF-MS analysis, serum samples were thawed at room temperature and deproteinized by the addition of 200 μL acetonitrile to 50 μL serum. The mixture was vortexed for 60 s, centrifuged at 12,000 × *g* for 10 min, and supernatants were filtered through a 0.22-μm membrane. A serum QC sample was prepared by mixing 20 μL of each serum sample, treated as above, and used to monitor UPLC-Q-TOF-MS performance.

#### 2.6.2. UPLC-Q-TOF-MS conditions

UPLC-Q-TOF-MS conditions for serum analysis were the same as described in our previous study [28]. UPLC was performed using the Acquity UPLC system (Waters Corporation) equipped with an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, Ø = 1.7 μm) operated at 40 °C. During analysis, serum samples were kept at 4 °C. The injection volume was 5 μL, and mobile phases A and B were acetonitrile and 0.1% aqueous formic acid, respectively, applied at the flow rate of 0.5 mL/min. MS was performed in a QTOF SYNAPT G2 High Definition

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