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# Mechanistic evaluation of gastroprotective effects of Kangfuxin on ethanol-induced gastric ulcer in mice

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ABSTRACT

This study was designed to evaluate the gastroprotective effect of Kangfuxin (KFX), a Chinese patent medicine constituent isolated from American cockroach, on ethanol-induced gastric ulcer in mice and to elucidate the potential mechanisms of the effect involved. According to the results, mice treated with alcohol appeared obvious gastric mucosal injury, while treatment with Cimetidine (a positive control) and KFX significantly relieved the damage, along with decreased oxidative stress and apoptosis indexes. Subsequently, we conducted a label-free quantitative proteomic (LFQ) and found that NF-κB and PI3K/AKT signaling pathway participated in gastroprotective effect of KFX. Furthermore, Western blot analysis revealed that KFX treatment inhibited the expression of TNF-α, IL-1β, greatly reduced the phosphorylation level of IκB and repressed the nuclear translocation of NF-κB p65, which demonstrated that KFX inhibited the activation of NF-κB pathway. Meanwhile, the PI3K/AKT pathway was also involved in regulating the anti-inflammation effect. These findings define for the first time that the gastroprotective effects of KFX against gastric ulcer can be attributed to its role in NF-κB inhibition.



## 1. Introduction

Gastric ulcer is one of the major gastrointestinal disorders with increasing incidence and prevalence globally. Exogenous damaging factors such as excessive drinking habits, poor diets, stress, smoking and prolonged ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) are all relevant to the formation of gastric ulcer [1]. This disease is caused by an imbalance of aggressive factors and defensive factors, which can in turn regulate the inflammatory processes involving the roles of neutrophils, eosinophils, and mast cells [2,3]. Excessive intake of alcohol can cause gastric mucosa injury. Ethanol damages the gastric mucosa vascular endothelia cells, distorts microcirculation and induces hypoxia due to the increase production of reactive oxygen radicals and inflammatory cytokines [4]. Therefore, drugs that have the ability to efficiency protect the gastric mucosa form gastric ulcer should have the capacity to reduce oxidative and inflammation stress. Currently, the treatment options available for gastric ulcer is based on the use of proton pump inhibitor and H2 receptor antagonists; however, the problem of reoccurrence of gastric ulcer after treatment as well as undesirable side effects have been associated with the use of these drug [5,6]. Hence, the search for an ideal antiulcer drug that decreases the incidence of relapse and affords better protection is still needed. Recently, there is a growing attention on the use of traditional medicine and their products as treatment options for different ailments, and natural medical materials are increasingly becoming important sources of new, more effective and safer therapeutic options including for the treatment of gastric ulcer. American cockroach (*Periplaneta americana*), the largest species of pest insect in family Blattidae, is a well-known domestic pest native to Africa and has spread throughout world especially in the tropical and subtropical regions [7]. In China, the ethanol extract of the dried whole body of *Periplaneta americana* has been used as traditional Chinese medicine for the treatment of blood stasis syndrome, abdominal mass and gastric ulcer for hundred years [8]. The previously study showed that *Periplaneta americana* extract can increase the levels of gastric mucosal hexosamine and prostaglandin E2, which contribute to the repair of gastrointestinal

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mucosa [9,10]. Due to its potent gastroprotective activity, several new drugs like KFX, Xinmailong injection, cockroach oil have been developed using *Periplaneta americana* as the raw material, and related pharmacological activity and clinical efficacy have been recognized. KFX is a kind of pure biologic medicine extracted from *Periplaneta americana* that contains multiple constituents [11]. It was reported that KFX can promote the cell increment, granulation tissue growth, and angiogenesis, enhance immune function, anti-inflammation, induce cell apoptosis, treat burn wounds, and promote ulcer healing etc [12]. However, the underlying mechanism of promoting ulcer healing is still unclear. Recently, Chen P et al. [13] studied a prophylactic effect of Kangfuxin and revealed that KFX has the effect of preventing ethanol-induced gastric ulcer via attenuating oxidative stress and ER stress in Mice. Based on the above description, the present study was designed to investigate the anti-ulcer effect of the *Periplaneta americana* extracts against ethanol-induced gastric ulcer in mice, as well as exploring the underlying mechanism.

## 2. Materials and methods

### 2.1. Kangfuxin oral liquid

KFX was gift from GoodDoctor Pharmaceutical Group, which contents 1 g of the dried whole body of *Periplaneta americana* per milliliter.

### 2.2. Animals

Twenty-four, 8-week-old male FVB mice weighing between 22 and 25 g were used in this study. The animals were housed in the animal facility at Wenzhou Medical University, under a 12 h light/dark cycle at a temperature of 25 °C and relative humidity ranging from 60 to 70% through-out the experiment. The use of experimental animals was conducted in strict compliance with the rules and regulations established by the Wenzhou Medical University after obtaining their ethical approval to pursue this study [the ethical approval date and the protocol number: wydw2015-0080]. The animals had free access to diet and water ad libitum. Prior to the induction of gastric ulcer, animals were fasted for 24 h to ensure an empty stomach (water was allowed). The animals were individually housed in wire mesh cages to avoid coprophagy. The induction of gastric ulcer was achieved by intragastric gavage of absolute ethanol at a dosage of 0.15 mL/20 g body weight [14]. Animals were randomly divided into 5 groups (10 mice in each group). Groups 1 and 2 served as the normal and ulcer controls, and then they were given the vehicle (normal saline). Groups 3, 4 were then treated with KFX (50 and 100 mg/kg, respectively), and Group 5 were treated with positive control agent Cimetidine (800 mg/kg). Agents (saline, KFX and Cimetidine) were administered by oral probe once daily for 5 days. The stomachs were then dissected severally at 1, 3, 5 days for further study.

### 2.3. Gross examination of gastric mucosa

After sacrificing the animals, the stomachs were removed and washed with 0.9% saline solution to clean away the waste. This was followed by macroscopic examination of the stomach for the detection of any hemorrhagic lesions on the gastric mucosa. Besides, the stomachs of the mice were opened along the greater curvature for Ulcer Index (UI) scoring [15]. An observer who was blind to the identity of the samples examined the gastric tissues for gastric mucosal lesions, which were expressed in UI scores as follows: 0 = no lesions (normal stomach), 0.5–1 = hyperaemia, 1–2 = haemorrhagic spots, 2–3 = 1–5 small ulcers, 3–4 = several small ulcers, 4–5 = 1–5 small and 1–3 large ulcers, 5–6 = several small and large ulcers, 6 = stomach full of ulcers or perforations. The percentage of inhibition was calculated with the following formula:

$$[(\text{UI model group} - \text{UI treated group})/\text{UI model group}] \times 100\%.$$

### 2.4. LC-MS analysis

The stomachs of the mice were minced and lysed in buffer consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 40 mM Trizma base, 40 mM dithiothreitol (DTT), 1% protease inhibitor cocktail (Sigma). After centrifugation at 15,000 g for 15 min (4 °C), the total protein concentration in the supernatant were measured using the Bradfold protein assay (Bio-Rad, Richmond, CA, USA). Lysates were then subjected to protein digestion according to the filter-aided sample preparation (FASP) protocol with an enzyme to protein ratio 1:50 [16]. The digested peptides were submitted to on-line nanoflow liquid chromatography using the EASY-nLC system (Thermo Fisher Scientific) with 10 cm capillary columns of an internal diameter of 75 μm filled with 3 μm Reprosil-Pur $C_{18}$-A2 resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The elute was electrosprayed through a Proxeon nanoelectrospray ion source for electrospray ionization (ESI)-MS/MS analysis on a Thermo Fisher LTQ Velos Pro (Thermo Fisher Scientific, Bremen, Germany) using full scan mode over the $m/z$ range 200–1800. Identification and LFQ of peptides were done with Masscot Daemon, KEGG pathway and molecular function analysis were performed with DAVID Bioinformatics Resources (https://david.ncifcrf.gov/home.jsp) and STRING analysis (http://string.embl.de). All annotations were extracted from UniProt database.

### 2.5. Measurement of malonaldehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) level

Stomach tissues stored at −80 °C were homogenized in homogenization Tris-buffer (20 mM, pH 7.5) on ice using Ultra Turraks Homogenizer (IKA, Germany) and then were centrifuged at 14,000 g at 4 °C for 15 min. The supernatants were used to determine the activities of SOD, and levels of GSH and MDA. The concentration of protein in the supernatants was determined by the Bradford method using bovine serum albumin (BSA) as a standard. The activities of SOD and the levels of GSH and MDA were determined using commercial assay kits according to the manufacturer's instructions (Beyotime, Shanghai, China).

### 2.6. TUNEL staining

Apoptotic nuclei in the stomach were examined by transferase mediated dUTP nick-end labeling (TUNEL) staining using a cell death detection assay kit (Promega, American, US). Stomach tissue was fixed in buffered neutral 10% formalin, dehydrated in graded alcohol series, embedded in paraffin, and sectioned at 4–6 μm. Hydrated slides were used for TUNEL staining according to the manufacturer's instructions. Nuclei were stained with DAPI. TUNEL positive nuclei within stomach tissue were counted in ten random fields for each of five slides from one mouse with at least six mice (as indicated) each group, and the data were calculated as the percentage of apoptotic myocyte nuclei/total number of nuclei.