Kangfuxin Liquid Ameliorates Dextran Sulfate Sodium (DSS)-Induced Acute Ulcerative Colitis in Mice by Modulating Immune Response and Suppressing Inflammation

Background: The aim of this study was to determine the effect of kangfuxin liquid (KFXL) on inflammatory response, and its underlying mechanism in treating acute ulcerative colitis (UC) in mice induced by dextran sulfate sodium (DSS).

Material/Methods: Mice were provided drinking water containing DSS (3%) for 7 days to induce acute enteritis. The mice were divided into 6 groups: a control group, a DSS-induced (vehicle) group, a sulfasalazine (SASP) group, and low-, medium-, and high-dose kangfuxin liquid groups. Disease activity index (DAI), colon mucosa damage index (CMDI), histopathological score (HS), and organ index were monitored daily. The levels of interleukin-1β (IL-1β), interleukin-10 (IL-10) in serum and interleukin-17 (IL-17) and epidermal growth factor (EGF) in colon tissue were assessed by enzyme-linked immunosorbent assay (ELISA). Flow cytometry was used to assess the changes of T lymphocyte subsets in spleens of mice to evaluate the therapeutic effect of drugs on acute UC in mice.

Results: Different doses of kangfuxin liquid reduced the DAI, CMDI, and HS scores (P<0.01 or P<0.05) of acute UC mice, reduced the level of IL-1β and IL-17 in serum, increased the expression of IL-10 in serum and EGF in colon tissue, increased the number of CD3+ T cells, and decreased the level of CD4+ T cells and the ratio of CD4+/CD8+.

Conclusions: Kangfuxin liquid has a therapeutic effect on DSS-induced acute UC in mice, and its mechanism of action may be associated with regulating immune function and reducing intestinal inflammatory response.

Keywords: Anti-Inflammatory Agents • Antigens, Differentiation, T-Lymphocyte • Colitis, Ulcerative
Background

Inflammatory bowel diseases (IBDs) are chronic inflammatory conditions, including Crohn’s disease (CD) and ulcerative colitis (UC), involving recurrent inflammation of the intestine [1,2]. The etiology and pathogenesis of IBDs have not been fully elucidated. According to reports in the literature, they may be associated with genetics [3], environment [4], microbiota [5], and intestinal immune dysfunction [6]. At the beginning of the 20th century, there were millions of people suffering from IBDs. IBDs are most prevalent in the Western world and it was estimated that 0.5% of the total world population had an IBD in 2015. This would be equivalent to 2.2 million Americans suffering from IBDs in 2025 [7].

Acute ulcerative colitis is difficult to control and develops into chronic colitis, which can lead to death. The lesions of acute ulcerative colitis are more common in the sigmoid colon and rectum [8]. Recently, the incidence rate of UC has been steadily rising worldwide, which has brought enormous burdens to the medical system [7]. Currently, UC is treated with a variety of drugs, including aminosalicylic acid preparations, steroids, immunosuppressants, and biological agents such as monoclonal antibodies [9,10]. These medicines have limited therapeutic effects in achieving remission UC via different mechanisms. Patients usually develop resistance, resulting in variations in patient outcomes and higher treatment costs, which makes them medically unsuitable for long-term and extensive applications [11]. Therefore, it is important to seek effective and economic remedies that have fewer adverse effects.

Kangfuxin liquid (KFXL) is a type of traditional Chinese medicine, consisting of ethanol extract of the insect Periplaneta americana (Family: Blattidae). The ancient book “Shen Nong’s Materia Medica” in the Han Dynasty reported that Periplaneta americana was used against various ailments such as, burns, cough, asthma, inflammation, and blood disorders [12]. The main components of KFXL are polyphenols derivatives, coumarin, cyclic dipeptides, dopamine derivatives mucin, amino acids, and periplanetols [13]. In China, KFXL has been clinically utilized widely to treat ulcers, fistulas, burns, scalds, and bedsores, manifesting a significant healing effect [14,15]. Moreover, KFXL is used to treat chronic UC in clinical practice [16]. A study found that KFXL had a positive effect on ulcerative colitis induced by trinitrobenzene sulfonic acid in rats [17]. Further mechanism studies showed that KFXL can reduce the expressions of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), and increase the level of hematopoietic growth factor (HGF) to cure UC [18].

Some reports showed the increase of CD4+ T counts and CD4+/CD8+ ratio indicates the aggravation of UC [19]. Meanwhile, the downregulation of accumulation of CD4+ and CD8+ can ameliorate the inflammation and colonic tissue damage [20]. Hence, T cells play a crucial role in UC and help reduce the progression of UC. However, the underlying mechanism and whether KFXL can regulate immune response and the secretions of other inflammatory cytokines remain unclear. Therefore, we evaluated the effect and mechanism of KFXL on acute UC induced by dextran sulfate sodium (DSS) in mice.

Material and Methods

Animals

Male Balb/c mice (18–22 g, 6–8 weeks old, NO. SCXK-2013-0004) were purchased from Hunan Provincial Silaikejingda Experimental Animal Co. Ltd. All mice were housed 5 per cage and fed standard laboratory chow in the animal room with 12-h dark/light cycles, 30-70% humidity, and a constant temperature of 24±1°C. All animal experimental procedures were approved by the Institutional Ethics Committee for Animal Care and Use.

Drugs and Reagents

KFXL was provided by Haoyisheng Pharmaceutical Company Limited (Sichuan, China). DSS was purchased from Shenzhen Lijing Biological Technology Co., Ltd (Shenzhen, China). SASP was obtained from Shanghai Xinyitiangping Pharmaceutical Company, Ltd (Shanghai, China). Interleukin-1β (IL-1β), interleukin-10 (IL-10), interleukin-17 (IL-17), and epidermal growth factor (EGF) kits were from Nanjing Jiancheng Bioengineering Institute. Rat anti-mouse CD3 FITC, Rat anti-mouse CD4 PE, Rat anti-mouse CD8 APC, and Rat anti-mouse CD25 Perp-cy5.5 were acquired from BD Biosciences Co., Ltd.

Animal Treatment

Seventy-two healthy Balb/c mice were adaptively kept for 1 week, and then the mice were randomly divided into 6 groups [2]. According to the clinical dosage conversion of KFXL [21], the concentrations of KFXL were determined. Group A was the normal control group (drinking water and the intragastric administration of saline (15 mL/kg) daily); Group B was the DSS-induced (Vehicle) group (drinking 3% DSS solution); Group C was the positive group (drinking 3% DSS solution and the intragastric administration of Salazosulfapyridine (SASP, 0.6 g/kg) once daily); Group D was the low-dose KFXL group (12 mice) (drinking 3% DSS solution and the intragastric administration of KFXL (0.625 g/kg) solution once daily); Group E was the mid-dose KFXL group (12 mice) (drinking 3% DSS solution and the intragastric administration of KFXL (1.25 g/kg) solution once daily); Group F was the high-dose KFXL group (12 mice) (drinking 3% DSS solution and the intragastric administration of KFXL (2.5 g/kg) solution once daily). Except for the normal control...
group, the rest of the mice were given 3% (w/v) DSS for 7 days, as previously described [22]. After modeling, the whole drug therapy period was 1 week. During the treatment, we checked and recorded the weight, physical condition, stool consistency, and occult blood in feces on the 1st, 4th, and 7th days. After 7 days, blood samples were collected, centrifugated, and stored at -80°C. Mice were euthanized by carbon dioxide asphyxia. All of the animal experiments in this study were carried out with the approval of the Experimental Animal Research Center, and the Institutional Ethics Committee for Animal Care and Use, Chinese Academy of Medical Science (CAMS).

**Evaluation of DAI Score**

The disease activity index (DAI) is recognized as one of the typical indicators for evaluating the pathological degrees of colitis [23]. The DAI scores are based on weight loss, stool consistency, and rectal bleed. DAI was calculated at the beginning (day 1), in the middle (day 4), and at the end of the KFXL and SASP treatment (day 7). According to Table 1 [24], the average of the 3 values constituted the DAI and the formula was as follows:

$$\text{DAI} = \frac{\text{weight loss percentage score} + \text{stool score} + \text{rectal bleed score}}{3}$$

**Measurement of Organ Index, Colon Length, and CMDI**

The liver, spleen, thymus, and colon were dissected to remove fat and fascia. The colon was cut along the mesenteric margin, and the feces were washed away with normal saline. The wet weight of each organ was determined and the organ index was calculated using the formula:

$$\text{Organ index} = \frac{\text{organ weight (mg)}}{\text{mouse weight (g)}}$$

The colon length was measured by using a ruler. The colon was observed and the colonic mucosal damage index (CMDI) was scored [25]. The scoring method was as shown in Table 2.

**Histological Analysis**

Colonic tissues, obtained from the visible ulcer site by unaided vision, were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. The colon was rolled into a Swiss roll, sectioned at 5 μm, and stained with hematoxylin and eosin. Finally, the colon damage and inflammation were observed under a light microscopic. Patel et al described a method for estimating the colon inflammatory scores [26], and these parameters were used to determine the histological score. Each of the individual parameters was graded from 0 to 4 depending on the severity of changes in the colon, as shown in Table 3.

**Assessment of IL-1β and IL-10 Levels in Serum and EGF and IL-17 Levels in Colon Mucosa by ELISA Assay**

Blood samples were obtained from the eyeball. The blood was centrifuged and the supernatant was analyzed according to the instructions of the ELISA kit to detect cytokines, including IL-1β and IL-10. To assess the levels of EGF and IL-17, we took a piece of colon tissue, added phosphate buffer, prepared the tissue homogenate, centrifuged it, and detected the supernatant according to the instructions of the ELISA kit [27].

### Table 1. Disease activity index scoring criteria.

| Score | Weight loss (%) | Stool consistency | Rectal bleed |
|-------|-----------------|-------------------|--------------|
| 0     | <1              | Well-formed pellets | No blood     |
| 1     | 1-5             | Loose stools      | Slight redness in stools |
| 2     | 5-10            | Pasty and semi-formed stools | Clear redness in stools |
| 3     | 10-20           | Diarrhea          | Severe redness in stools |
| 4     | >20             | Liquid stools     | Rectal prolapse |

### Table 2. Colonic mucosal damage index scoring criteria.

| Score | Colon characteristics |
|-------|-----------------------|
| 0     | No damage             |
| 1     | Mucosal congestion, edema, mucosal erosion, or ulceration |
| 2     | Mucosal congestion, edema, mucous membrane rough, mild erosion, or intestinal adhesion |
| 3     | Mucosal congestion, edema, moderate erosion and ulcer formation, but the ulcer diameter was <1 cm |
| 4     | Mucosal congestion, edema, moderate erosion and ulcer formation, but the ulcer diameter was >1 cm |

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Flow Cytometry of UC Spleens

The suspensions of single cells prepared from the mouse spleens were passed through a 200-mesh nylon sieve and then filtered. The single-cell suspensions were added to red blood cell lysate to split erythrocytes for 5 min, then added to 3% FBS-PBS cell-washing liquid, followed by centrifugation at 400×g for 5 min. The cell concentration was adjusted to 1×10^6/mL, and the antibodies (CD3-FITC, CD8-APC, CD4-PE, and CD25-Percp-cyt5.5) were added to the cell suspensions with a staining kit (BD Biosciences) for 30 min, according to manufacturer’s instructions. Then, the supernatant was discarded twice to elute out the superfluous monoclonal antibodies. Finally, the experimental cells were mixed with 500 μl FBS-PBS buffer. Finally, the stained cells were used for analysis with a FACS CantoII flow cytometer (BD, USA).

Statistical Analysis

All tests were performed using SPSS 21.0 software. Results are described as mean±standard deviation (SD). The data with normal distribution were analyzed by one-way ANOVA, and the data with non-normal distribution were tested by the rank-sum test. The least significant difference (LSD) test was used for pairwise comparison between groups, and P<0.05 was used as the standard of statistical significance.

Results

Effects of KFXL on DSS-induced Colitis

The bodyweight of mice who received DSS was significantly lower in comparison with those that were not given DSS. The DAI scores of mice in the DSS-induced group increased significantly compared to the control group. On the 4th and 7th days, the DAI scores of mice in the DSS-induced group were higher than that of mice in the other group. Moreover, the DAI scores in each KFXL-treated group decreased, showing a considerable dose-dependent effect of KFXL (Figure 1).

Effects of KFXL on the CMDI and Colon Length

As shown in Figure 1B, in the control group, the general colon morphology was normal, the mucosa was smooth, and no mucosal congestion, edema, erosion, or ulcers were found. The colon mucosa damage index (CMDI) score in the DSS-induced group increased significantly more than that in the control group (P<0.01). After KFXL and SASP treatment, the CMDI score of intestinal mucosae was lower than that of the DSS-induced group, in a dose-dependent manner. Also, high-dose and low-dose KFXL had a significant difference from the SASP group (P<0.05).

The colon lengths of mice are shown in Figure 1C. The colon length of the DSS-induced group was significantly shorter than that in the control group (P<0.01); however, the colon lengths of the SASP group, KFXL high-dose group (2.5 g/kg), and KFXL middle-dose group (1.25 g/kg) were dramatically greater compared to the DSS-induced group (P<0.01). However, there was no difference between the KFXL and SASP groups.

Effects of KFXL on the Organ Index of Mice

After 7 days of treatment, no obvious changes in the liver index and thymus index were found in any of the groups. Compared with the control group, the spleen index and colon index of the DSS-induced group increased markedly (P<0.01). Whereas, in contrast with the DSS-induced group, the colon index and colonic mucosal edema were significantly reduced in each KFXL-treatment group. However, the spleen index for all the KFXL-treatment groups was not significantly different from the model group, showing a downward trend (Figure 2).

Effects of KFXL on Histopathological Evaluation

A small number of goblet cells and inflammatory cells were found in the colons of the control group, while the loss of

Table 3. Histological scoring criteria.

| Score | Histological feature |
|-------|----------------------|
| 0     | Normal colon mucosa  |
| 1     | Basal crypts shortened with mild edema and inflammatory cell infiltration in the histological score |
| 2     | Basal crypts disappeared with moderate inflammation in the lamina propria |
| 3     | Basal crypts disappeared with severe inflammation of lamina propria but surface epithelium remained |
| 4     | Basal crypts and surface epithelium all disappeared with severe inflammation of mucosa, muscularis propria and submucosa of lamina propria |

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The suspensions of single cells prepared from the mouse spleens were passed through a 200-mesh nylon sieve and then filtered. The single-cell suspensions were added to red blood cell lysate to split erythrocytes for 5 min, then we added it to 3% FBS-PBS cell-washing liquid, followed by centrifugation at 400×g for 5 min. The cell concentration was adjusted to 1×10^6/mL, and the antibodies (CD3-FITC, CD8-APC, CD4-PE, and CD25-Percp-cyt5.5) were added to the cell suspensions with a staining kit (BD Biosciences) for 30 min, according to manufacturer’s instructions. Then, the supernatant was discarded twice to elute out the superfluous monoclonal antibodies. Finally, the experimental cells were mixed with 500 μl FBS-PBS buffer. Finally, the stained cells were used for analysis with a FACS CantoII flow cytometer (BD, USA).
goblet cells and crypt cells was observed in the colons of mice that received DSS, and a large number of inflammatory cells infiltrated into the mucosal muscle layer. Each administration group repaired the lost goblet cells and crypt cells in varying degrees and reduced the infiltration of inflammatory cells. The colonic histopathological score (HS) in the DSS-induced group was significantly higher than that in the control group ($P<0.01$). The HS in the SASP group and administration groups were evidently lower compared with the DSS-induced group ($P<0.01$ or $P<0.05$). The low-dose KFXL group was higher compared to the SASP group ($P<0.05$) (Figure 3).

**Effects of KFXL on the Expression of IL-1$\beta$, IL-10, IL-17, and EGF**

As shown in Figure 4, compared with the control group, the expressions of serum IL-1$\beta$ and tissue IL-17 in the DSS-induced group increased significantly, while the expressions of serum IL-10 and tissue EGF decreased significantly ($P<0.01$). Compared with the DSS-induced group, the expression of serum IL-1$\beta$ in the KFXL high-dose (2.5 g/kg) and middle-dose groups (1.25 g/kg) decreased significantly, and the serum IL-10 expression in each dose administration group increased in a dose-dependent manner, especially in the high-dose KFXL group (2.5 g/kg). The data showed the expression of IL-10 in the high-dose KFXL group had a notable difference with SASP group ($P<0.01$). KFXL could significantly increase the expression of EGF in colon tissue and decrease the expression of IL-17 ($P<0.05$ or $P<0.01$). This result demonstrated that KFXL effectively decreased DSS-induced inflammation and repaired the mucous membranes in the experimental mice.

**Effects of KFXL on T cell Subsets in the Spleens of DSS-induced Ulcerative Colitis Mice**

Splenic T lymphocyte subsets were detected and analyzed by flow cytometry. The results showed that, compared with the control group, the level of CD3$^+$ T cells and the proportion of CD25$^+$/CD4$^+$ in the DSS-induced group decreased significantly, and the level of CD8$^+$ T cells decreased, while the level of
CD4+ T cells and the ratio of CD4+/CD8+ increased significantly (P<0.01). Compared with the DSS-induced group, the KFXL high-dose (2.5 g/kg) and middle-dose groups (1.25 g/kg) had significantly increased level of CD3+ T cells, while the middle-dose (1.25 g/kg) and low-dose groups (0.625 g/kg) had decreased levels of CD4+ T cells, and tended to have a decreased ratio of CD4+/CD8+. There was a significant difference between KFXL and SASP groups in the ratio of CD4+/CD8+ (P<0.01 or P<0.05), and high (2.5 g/kg) and mediate doses (1.25 g/kg) of KFXL were obviously lower in the expression of CD8+ T cells comparing with SASP (Figure 5).

Discussion

Ulcerative colitis (UC) is a chronic and complex autoimmune inflammatory disease, which is linked to a variety of functional disorders, resulting to excessive secretion of inflammatory cells and cytokines [28]. Specially, the continuous progression of UC elevates the risk of colorectal cancer (CRC) [29]. Immune modulators, such as sulfasalazine and glucocorticosteroids, have been broadly used to treat UC [30,31], but they can cause adverse effects like vomiting, anemia, and generalized edema. Thus, interest has grown in the use of traditional/alternative medicines for inflammatory chronic diseases [32].

The DSS-induced colitis mice model is widely used because it resembles features of human UC [2]. There are over 20 animal models of colitis. DSS-induced colitis is one of the most suitable experimental models [33]. The present study is the first to assess and compare the pharmacological effects of sulfasalazine and KFXL in a mice model of UC.

Using a DSS-induced mice colitis model, we evaluated the therapeutic efficacy of KFXL against UC in the present study. We found that KFXL ameliorated DSS-induced colitis symptoms. The DAI was reduced in the treated group compared to the untreated model group. KFXL effectively decreased the disease activity index and organ index, and it markedly increased colon length compared to the DSS-induced group. The results prove that KFXL ameliorated the extent of UC.

IL-17-secreting T cells can stimulate the expressions of IL-1β and tumor necrosis factor (TNF)-α and inhibit the level of
IL-10 [34]. Previous studies confirmed KFXL can reduce the expression of TNF-α [35]. Hence, this study intended to further investigate the effect of KFXL on inflammatory indicators of cellular immune imbalance. IL-1β is an inflammatory cytokine secreted by monocytes, mast cells, smooth muscle cells, and endothelial cells, and it is an important pro-inflammatory factor in the initiation of immunity and inflammation. It has been reported that IL-17 has a strong ability to activate neutrophils and T cells, stimulate macrophages, fibroblasts, and epithelial cells to produce a variety of inflammatory factors, promote the cascade of inflammatory factors, and eventually lead to inflammation and tissue damage [36]. IL-10 is currently recognized as an anti-inflammatory factor that plays a major role in inhibiting the release of inflammatory factors and inflammatory response, regulating the differentiation of diverse immune cells. Clinical reports show that KFXL promotes mucosal repair. Epidermal growth factor (EGF) plays a crucial role in promoting the expression of differentiation genes, inducing cellular growth and migration, and maintaining the normal metabolism of epithelial cells. EGF is very

Figure 3. The effect of KFXL on histopathology of acute UC mice. (A) Effect of KFXL on histopathology colitis score for all groups. (B) Histopathology colitis of DSS-induced acute ulcerative colitis in mice treated with different doses of KFXL and SASP and normal mice by H&E staining. Original magnification ×200. a, goblet cells; b, inflammatory cells; c, crypt cells. * P<0.05, ** P<0.01 compared with the normal control group; a, P<0.05, ** P<0.01 compared with the DSS-induced group (Vehicle); & & P<0.01 compared with the SASP group.
**Figure 4.** The effect of KFXL on IL-1β, IL-10 of serum and IL-17, EGF of colon in acute UC mice. (A) IL-1β, (B) IL-10, (C) IL-17, (D) EGF. * P<0.05, ** P<0.01 compared with the normal control group; # P<0.05, ## P<0.01 compared with the DSS-induced group (Vehicle); & P<0.05, && P<0.01 compared with the SASP group.
**Figure 5.** Effects of KFXL on splenic T lymphocyte subsets in acute UC mice were screened by flow cytometry for the presence of CD4 and CD8 molecules. (A) CD3+ Cell (%), (B) CD3+CD4+ Cell (%), (C) CD3+CD8+ Cell (%), (D) CD4+/CD8+, (E) CD25+/CD4+. (F) FACS Staining. FACS staining is shown different groups, including control group (F1), DSS control group (F2), SASP group (F3), KFXL 0.625 g/kg (F4), KFXL 1.25 g/kg (F5) and KFXL 2.5 g/kg (F6). *P<0.05, **P<0.01 compared with the normal control group; #P<0.05, ##P<0.01 compared with the DSS-induced group (Vehicle); &P<0.05, &&P<0.01 compared with the SASP group.

important for the recovery and integrity of intestinal mucosal barrier function [37].

Our results showed that the expressions of IL-10 and EGF in the high-, middle-, and low-dose KFXL groups were higher than those in the DSS-induced group, while the expression of IL-1β and IL-17 were lower. The data suggest that KFXL can regulate the balance of pro-inflammatory and anti-inflammatory cytokines and promote tissue repair, which is consistent with the previous reports [17,38].

Histopathological changes in the colon tissue extracts from mice showed that the DSS-induced group exhibited mucosal inflammation extending to all layers of the bowel wall, with increased thickness of the muscle layer, crypt distortion, and crypt loss. The growth of monocytes and lymphocytes, as well
as goblet cell depletion, was visible in the mucosa of untreated mice. However, KFXL and sulfasalazine treatment partially improved mucosal injury and inflammatory cell infiltration.

The immune response of the body is mainly divided into cellular immunity and humoral immunity. T lymphocytes mediate cellular immunity; they directly kill target cells, and have an auxiliary and regulatory function on humoral immunity mediated by B lymphocytes. T cells can also be divided into different subsets on the basis of their functions and surface markers. CD3 protein is the surface marker of mature T cells, and CD4 is the main surface marker of helper T cells, which mainly regulate or assist other lymphocytes to exert their functions and have immunomodulatory effects. There is also a group of CD4 regulatory/suppressor T cells, which usually play an important role in maintaining self-tolerance and avoiding excessive immune response damage to the body; its surface marker is CD25. The main surface marker of cytotoxic T cell is CD8, which can kill exogenous infected cells and can kill cells through direct contact and secretion of perforin [39]. The increase of CD4+/CD8+ ratio usually means that the body’s immune function is hyperactive [40].

CD4+ and CD4+/CD8+ were obviously higher than in healthy colons, as reported by Gavidel et al [41]. Our results also showed that CD3+ cells decreased significantly, CD4+ and CD4+/CD8+ cells increased significantly, and CD25+/CD4+ cells decreased in mice with UC induced by DSS, suggesting that T cell subsets were out of balance, immune function was hyperactive, and T cell negative regulation and immune tolerance were weakened. After treatment with KFXL, the level of CD3+ T cells increased significantly, while the level of CD4+ T cells decreased, and the ratio of CD4+/CD8+ cells decreased, suggesting that KFXL can downregulate the activation of CD4+ T cells, reduce the ratio of CD4+/CD8+ cells, and regulate the differentiation of T cell subsets in thymus of mice.

Conclusions

To sum up, KFXL had a therapeutic effect on DSS-induced UC mice, which can downregulate the activation of CD4+ T cells in the thymus, reduce the ratio of CD4+/CD8+, regulate the balance of pro-inflammatory and anti-inflammatory factors in colon and serum, inhibit inflammation, improve the pathological changes of the colon, and promote the repair of intestinal mucosa. However, KFXL regulates the related targeted proteins of immune response and inflammation, which needs to further exploration in the future.

Acknowledgements

The authors are grateful to Yunnan Provincial 2011 Collaborative Innovation Center for Entomoeutics, Yunnan Provincial Key Laboratory of Entomological Biopharmaceutical R&D, National-Local Joint Engineering Research Center of Entomoeutics, Wenzhou Medical University, and Shanghai Jiao Tong University and Nanjing University of Chinese Medicine.

Conflict of Interest

None.
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