

# Antibacterial, antidiarrheal, anti-inflammatory and analgesic activities of compound Shikuqin powder

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**Abstract:** The MIC and MBC values of Shikuqin (SKQ) against 5 bacteria that readily cause diarrhea were measured by the broth micro dilution method. The castor oil-induced diarrhea method was used to evaluate the antidiarrheal activity. Intestinal transit and gastric emptying were also evaluated with normal and neostigmine-induced intestinal transit in rodents. In addition, the antidiarrheal activity of SKQ was assessed *in vivo* with isolated rabbit ileum. Xylene-induced ear edema was used to evaluate the anti-inflammatory activities in mice, while hot plate and writhing tests were performed to assess the analgesic effects. Senna decoction (0.3g/mL) was administered intragastrically to induce a rat model of diarrhea. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to detect AQP4 mRNA, and Western blot was performed to quantify the protein level of AQP4 in the colon. SKQ exhibits remarkable antidiarrheal, anti-inflammatory and analgesic effects in the gastrointestinal tract disorders, and can therefore be developed as a promising antidiarrheal agent.

**Keywords:** Analgesic, antibacterial, antidiarrheal, anti-inflammatory, compound Shikuqin powder.

## INTRODUCTION

Diarrhea represents a common gastrointestinal issue with potentially fatal implications and poses substantial challenges for public health (Anne-Laure *et al.*, 2011). Antibiotic therapy for diarrhea is controversial, as the etiological agents often exhibit resistance to multiple broad spectrum antibiotics (Casburn-Jones and Farthing 2004). At present, traditional Chinese medicines have been attracting increasing attention due to their few side-effects, abundant resources, absence of drug resistance, and long history of safe application in human. Therefore, the development of traditional Chinese medicine preparations is an alternative approach for the treatment and prevention of infectious intestinal diseases in farm animals and humans.

Shikuqin (SKQ) powder is a new compound Chinese medicine preparation developed from Chinese herbal medicines including *Granati Pericarpium*, *Sophorae Flavescentis Radix* and *Fraxini Cortex*. As a traditional Chinese medicine, *Granati Pericarpium* is the dried peel of *Punica granatum L*, *Pomegranate* branch and has intestinal astringent, hemostasis and insecticidal effects. Modern pharmacological studies have shown that it is effective for the treatment of bacillary dysentery, amebic dysentery and a variety of infectious diseases (Devatkal *et al.*, 2010, Viana *et al.*, 2010, Sreekumar *et al.*, 2014). *Sophorae Flavescentis Radix* is a traditional Chinese medicine with a long history, and is derived from the

dried root of *Sophora flavescens* Ait. Its traditional pharmacological effects have been mainly demonstrated in the antibacterial and antiarrhythmic treatment. Modern pharmacological studies show that it has anti-tumor, anti-virus, anti-allergy and anti-liver fibrosis activities (Kong *et al.*, 2010, Han and Wang 2012). *Fraxini Cortex* is one of the most commonly used Chinese medicines, and is derived from the dried bark of *Fraxinus rhynchophylla* Hance, *Fraxinus szaboana* Lingelsh, *Fraxinus chinensis* Roxb. or *Fraxinus stylosa* Lingelsh, *Oleaceae*. Based on herbology, it has the functions of clearing heat, drying dampness, and treating diarrhea caused by damp-heat or dysentery. It is also effective in treating the damp-heat syndrome. Modern studies indicate that it has anti-inflammatory, analgesic and urinary acid-reducing effects, and is therefore a promising treatment option for gout (Fang *et al.*, 2008).

In order to elucidate the therapeutic effects of SKQ powder on diarrhea, we carried out relevant pharmacodynamics studies and thus provide reference for future clinical applications. In this study, we aim to assess the antibacterial, antidiarrheal, anti-inflammatory and analgesic effects of SKQ.

## MATERIALS AND METHODS

### *Drug preparation*

In the present study, the SKQ samples (No. 20160501) were prepared in the Institute of Animal Husbandry and Veterinary Medicine of GAAS (Guizhou, China). The samples consisted of three Chinese herbs including

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*Sophorae Flavescentis Radix* (Batch number: 20160302), *Fraxini Cortex* (Batch number: 20160313) and *Granati Pericarpium* (Batch No. 20160325). The ratio of each component in the mixture was 3:3:2.

#### **Bacterial strains and growth conditions**

Freeze-dried *E. coli* powder (CVCC3419, CVCC1555) was purchased from the China Veterinary Microbial Culture Collection (Beijing, China). The other two bacterial strains used in this antibacterial study were *E. coli* clinical isolates EZYP09-47 and *E. coli* ATCC25922, which were obtained from the Natural Medicine Research Center, Sichuan Agricultural University, Chengdu, China.

#### **Animal preparation**

Young adult female and male (average weight: 18-20g) Kunming (KM) mice were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (License No. SCXK [Sichuan] 2015-30). Female (average weight: 180-260 g) and male (average weight: 180-260g) Juvenile Sprague Dawley (SD) rats were bought from Chengdu Dossy Experimental Animals Co., Ltd. (License No. SCXK [Sichuan] 2015-028). New Zealand rabbits (1.5-3.0 kg) were obtained from Chengdu Dossy Experimental Animals Co, Ltd. [License No. SCXK (Sichuan) 2013-17]. The animals were separated according to gender and were housed in well-ventilated sterile polypropylene cages. Based on the Guidelines of the International Committee on Laboratory Animals, the animals were acclimatized to housing conditions under standardized conditions (25±3°C, 35-60% humidity, 12-hr light/12-hr day cycle) for a period of one week before the commencement of the experiment. Free access to normal diet and water were allowed during the adaptation period.

#### **Ethics statement**

All procedures that involved animals and the care of animals in the present study were approved by the Ethics Committee of Sichuan Agricultural University, according to the Regulation of Experimental Animal Management (State Scientific and Technological Commission of the People's Republic of China, No.2, 1988) and The Interim Measures of Sichuan Province Experimental Animal Management (Science and Technology Bureau of Sichuan, China, No.25, 2013).

#### **Evaluation of antibacterial activity of SKQ**

The minimum inhibitory concentration (MIC) was determined with the broth microdilution method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (Wikler 2012). SKQ was dissolved with a mixture of Mueller-Hinton broth and DMSO (9:1) to achieve a final concentration of 250 mg/ml, followed by testing in a range of 250 mg/ml to 1.95mg/ml.

After incubation for 24h at 37°C, the optical density was

recorded at 630 nm with a Biotek ELX 808 plate spectrophotometer to evaluate bacterial proliferation. The results were further confirmed with macroscopic observation.

The MIC values were measured as the lowest concentration at which no bacterial growth was observed. All measurements were repeated in at least three independent experiments.

The minimum bactericidal concentration (MBC) was recorded based on a modified CLSI recommendation. A loop full of broth was sampled from the MIC wells followed by spreading on Mueller-Hinton agar plates. After 24 hr of incubation, the plates were examined for bacterial growth, and MBC was determined as the lowest concentration at which the colony number was lower than three (Karadal and Yildirim 2014).

#### **Evaluation of antidiarrheal activity of SKQ**

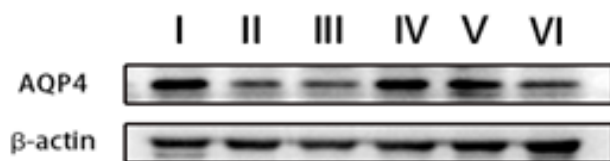
##### *Castor oil-induced diarrhea in mice*

120 mice were allocated randomly into six groups, including the normal group, positive control group, negative control group and three SKQ-treated groups (with equal numbers of male and female mice). The procedures developed by Sunil *et al.* (Bajad *et al.*, 2001) were adopted in the construction of this model. The mice were subjected to overnight fasting (provide with water, but no food) for 18 hr before the start of the experiment. Animals in the first (normal group), second (control group), third, fourth, fifth and sixth groups were orally administered physiological saline solution (the vehicle in which SKQ was dissolved), Loperamide hydrochloride capsules (0.032 g/kg), 1, 2 and 4 g/kg body weight of SKQ, respectively. Each mouse was kept in a separate cage, and the floor was lined with blotting paper, which was replaced hourly. At 30 min after the treatment, the mice were orally administered 0.3 ml castor oil. Except for the mice in the normal group, the time between the appearance of the first diarrheal drop and the administration of castor oil was recorded. The severity of diarrhea was determined each hr over a duration of 6 hr by documenting the total number of diarrheic stools excreted.

##### *Normal gastrointestinal motility*

The methods developed by Dicarolo *et al.* and Gerald *et al.* (Carlo *et al.*, 1994, Teke *et al.*, 2007) were used to assess the effect of SKQ on the gastrointestinal transit in mice. The mice were randomly divided into 5 groups with 20 mice in each group (equal male and female numbers). The mice in group 1 (normal group) were each administered by gavage 10ml/kg (p.o.) of physiological saline solution (vehicle for solubilization of SKQ). Mice in groups 2-4 were administered by gavage 10 ml/kg (p.o.) of SKQ equivalent to doses of 1, 2 and 3 g/kg, respectively. The mice in group 5 were each administered

0.032 g/kg (p.o.) Loperamide hydrochloride capsules. The mice were orally treated once a day at 8:00 in the morning for 5 days. It is noteworthy that the mice in this study underwent fasting for 18 hr (in this period they had access to drinking tap water *ad libitum*) before the last administration on the fifth day. Forty minutes after oral administration of the mice with the corresponding drugs on the fifth day, each animal in the five groups received 0.8 ml (0.8 g) of the test meal (semi-solid nutrient paste). Twenty minutes after the test meal, the mice were anaesthetized by inhalation of ether and then sacrificed by cervical dislocation. The abdomen of each mouse was cut open, and the upper and lower sides of the stomach and the entire length of intestine from the pylorus to the caecum were ligated, dissected and carefully removed. An analytical balance was used to determine the weight of the full stomach. The stomach was opened, and its contents were collected and rinsed. After removal of excess moisture with cotton wool, the empty stomach was weighed. To determine the quantity emptied from the stomach during the experiment period, the difference between empty and full stomach was subtracted from the weight of the test meal (0.8 g). The residual index (RI) is defined as the ratio of the quantity emptied from the stomach in the experiment to the weight of the test meal ingested. The peristaltic index (PI), which describes the distance traveled by the test meal relative to the entire length of the small intestine, was calculated and expressed as a percentage of the full length of the small intestine.



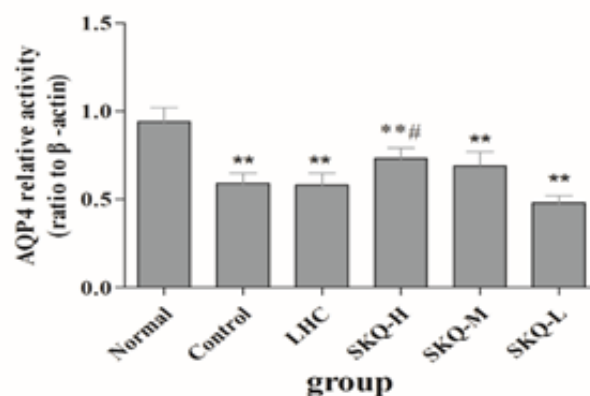
Note: I, II, III, IV, V and VI represent the groups of normal control, control, LHC-treated, high dose, medium dose and low dose of SKQ treatment, respectively.

**Fig. 1:** Effects of SKQ on AQP4 protein expression in rats with diarrhea

#### Neostigmine sulfate-induced gastrointestinal motility

The mice were allocated into six groups of twenty animals each. Three groups were orally administered SKQ (1, 2 and 4 g/kg). Another group was treated with Loperamide hydrochloride capsules (0.032 g/kg) orally, and the other groups (normal group and control group) were given physiological saline solution (10 ml/kg) orally. The mice received oral administration once per day at 8:00 in the morning for 5 days. It is noteworthy that the mice underwent fasting for 18 hr (in this period they had free access to tap water) before the last administration on the fifth day. To probe the mechanism of action, after twenty minutes of the last administration, all groups of mice, except the normal group, were treated with neostigmine sulfate (0.2 mg/kg) by means of

subcutaneous injection of the neck. At the same time, the mice in the normal group were injected with an equal volume of physiological saline solution. Twenty minutes after the treatments, the mice were fed 0.8 ml (0.8 g) of the test meal (semi-solid nutrient paste). All mice were sacrificed twenty minutes following administration of the test meal. Subsequently, repeated the experimental procedures on the measurements of residual index (RI) and peristaltic index (PI) described in detail above.



Note: \* P<0.05, \*\* P<0.01 vs. Normal group; # P < 0.05, ## P < 0.01 vs. Control group.

**Fig. 2:** Densitometric analysis of AQP4 proteins in rats with diarrhea ( $x \pm s$ ,  $n = 6$ )

#### Intestinal peristalsis on isolated rabbit ileum

Antidiarrheal activity was evaluated on isolated rabbit ileum using a modified protocol of Mehjabeen *et al.* (2014). The rabbits were sacrificed by a blow on the back of neck. The abdomen was immediately opened, and the caecum was pulled forward to reveal the length of small intestine. Subsequently, the intestine was removed from the rabbit and placed in a beaker or Petri-dish containing Tyrode's solution (Muto *et al.*, 2005). Next, ileum segments (about 3-4 cm long) were immediately dissected from the isolated intestine, and then placed in a beaker or Petri-dish containing Tyrode's solution. For the following experiments, a piece of isolated smooth muscle was mounted on an organ bath with 70 ml capacity, followed by filling with Tyrode's solution. Throughout the experiment, the circulating water temperature of the organ bath was maintained at 37°C. The perfusion solution was bubbled with a mixture of 5% carbon dioxide and 95% oxygen. The spontaneous ileum movements were recorded on a polygraph or oscillograph using an isotonic transducer (Mehjabeen *et al.*, 2014). To assess the effects of SKQ on spontaneous intestine movement, SKQ was dissolved in physiological saline solution and then added to the organ bath following an equilibration period (Ahmad *et al.*, 2012). The effects of SKQ on the tension and frequency of the contraction and relaxation patterns of isolated rabbit ileum (smooth muscles) are listed in the tables. The SKQ doses used for the observation of responses were 1.48, 2.96 and 5.91 mg/ml.

### **Evaluation of anti-inflammatory activity of SKQ**

The animals were randomly allocated into six groups, each including 10 males and 10 females. The mice in different groups were administered physiological saline solution (normal and control groups), indomethacin (0.04 g/kg) and SKQ (4, 2 and 1g/kg), respectively. The animals were subjected to oral administration at 8:00 once per day for a duration of 5 days. One hour after the administration on the 5th day, the mice in all groups, except those in the normal group, were daubed with xylene (0.03 ml) on both sides of the right ear, while the left ear was observed as control (Ibrahim *et al.*, 2012). One hour after xylene application, all animals were sacrificed with ether anesthesia, and both ears were removed along the auricle baseline. Round pieces of the ears were prepared by a punch (6 mm in diameter) and weighed by an electronic balance.

The ear edema degree (A) = weight of right piece - weight of left piece.

Inhibition rate% = (Difference of ear weight (control) - Difference of ear weight (treated))/Difference of ear weight (control)×100%

### **Evaluation of analgesic activity of SKQ**

#### *Acetic acid-induced abdominal writhing test*

Animals were randomly allocated into 5 groups with equal numbers of males and females. The animals were administered physiological saline solution, indomethacin (0.04 g/kg), high-dose SKQ (4 g/kg), medium-dose SKQ (2 g/kg) and low-dose SKQ (1 g/kg), respectively. The animals received oral administration once per day over 5 days. One hour after the administration on the 5th day, all animals were subjected to intraperitoneal injection of 0.7% acetic acid (10 ml/kg) (Koster *et al.*, 1959). Afterwards, the number of writhes within 20 min (the writhing reactions include stretching of hind paws, abdominal contractions, upward movement of hips and writhing of abdominal muscles) was documented (Decigan *et al.*, 2005). The analgesic percentage was determined as follows:

Inhibition rate% = (number of writhes (control) - number of writhes (treated))/number of writhes (control)×100%

#### **Hot plate test**

The female animals were kept on a hot plate at a constant temperature of 55.5±0.5°C (Lanhers *et al.*, 1991). Multiple activities, including jumping and lifting or licking of the hind paws, were considered as antinociceptive indicators. The female animals exhibiting a pain threshold (the time elapsed on the hot plate until the animal first exhibited an antinociceptive indicator) in a range of 5~30 sec were qualified for subsequent testing (Asongalem *et al.*, 2004). Fifty qualified animals were randomly allocated into 5 groups, followed by treatment with physiological saline solution, indomethacin (0.04g/kg) and SKQ (4, 2 and 1g/kg), respectively. The

animals received oral administration once per day over 5 days. After the orally administration on the 5th day, the pain threshold of each group was recorded at 30, 60 and 120 min. If the animal showed no antinociceptive indicator within 60 sec during the test, it was immediately removed, and the pain threshold was recorded as 60 sec.

### **Effects on transcriptional and translational levels of aquaporin AQP4**

#### *Animal model*

*Folium sennae*, a well-known laxative, has been widely utilized for the induction of diarrhea in rats, mice and dogs (Kunzelmann and Mall 2002, Viana *et al.*, 2010, Silva *et al.*, 2017). The rats were fasted for 10 hr before the administration of Senna decoction extract (water was provided ad libitum). The rats were repeatedly given 15 ml/kg *Senna* decoction (0.3 g/ml) with intragastrical feeding needles twice a day for six consecutive days in the diarrhea group, and the animals with profuse liquid stool were noted as diarrhea-positive. After successful establishment of the diarrhea model, 72 rats were randomly allocated into six groups with equal numbers of males and females. The rats were treated once per day with physiological saline (normal group and control group), indomethacin (0.016g/kg), high-dose SKQ (2 g/kg), medium-dose SKQ (1 g/kg) and low-dose SKQ (0.5 g/kg), respectively. After the treatment, the animals were immediately subjected to autopsy under ether anesthesia, and the colons were removed 48 hr after administration (Zhang *et al.*, 2012). The colons were washed with phosphate buffered saline (PBS: 140 mM NaCl, 32 mM KCl, 20 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.5mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.4), frozen with liquid nitrogen, and stored at -80°C.

#### **Real-time polymerase chain reaction**

Approximately 15 mg frozen colon was used for RNA extraction. To reveal the underlying molecular pathways, it was necessary to determine the mRNA levels of the relevant genes. First, total RNA was extracted from the colon using TRIzol reagent (No. 15596018, Invitrogen) according to the manufacturer's instructions. Next, the quality of extracted RNA was assessed by determining the A260/A280 ratio (>2.0). For each colon sample, the RNA (1µg) was reverse-transcribed by the RevertAid First Strand cDNA Synthesis Kit (No. K1621, Thermo Scientific). For each tissue sample, 1µL of cDNA sample was then used for RT-PCR with the SYBR Green Supermix Kit (No. 1708882, Bio-Rad). Based on previous reports (Imam *et al.*, 2015, Wang, Hu *et al.* 2017), the mRNA levels, expressed as the relative mRNA levels in comparison with the control group, were determined in three repeats after normalization using β-actin. The Oligo 7 software was used for the design of primer sequences (table 9). The primers were synthesized by Liuhe Genomics Technology Co., Ltd. (Beijing, China).

### Western blot

Total protein in the colon was extracted for evaluation in this test. The colon samples were collected, washed with cold PBS, and then lysed with lysis buffer containing 1 mM protease inhibitor PMSF (No.P0100, Solarbio). The total protein was extracted using a total protein extraction reagent for mammalian tissue (No.AR0101-10, Boster). The concentrations of extracted proteins were quantified using the BCA Protein Assay Kit (No. AR0146, Boster). Next, the samples were denatured with a 4× dual-color protein loading buffer for 5 min at 100 °C (No. AR1142, Boster). An equal amount of protein (30µg) in each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Skim milk (5%) or BSA (5%) was used to block the protein blots for 1 h at room temperature. The membrane was next incubated with primary antibodies against β-actin or AQP4 at 4 °C overnight. After being washed three times with TBST buffer [20 mM Tris-HCl (pH 7.4), 100 mM NaCl and 0.1% (w/v) Tween 20], the protein blots were incubated with peroxidase-conjugated secondary antibody (goat anti-rabbit, CST, USA) at room temperature for 1 h. The immunoreactive bands were visualized with chemiluminescence (ECL Prime, Pierce Chemical, USA) using the ChemiDoc MP Imaging System (Bio-Rad) and quantified relative to β-actin (CST, USA) with the ImageJ software (National Institutes of Health) (Zhi *et al.*, 2014, Mei *et al.*, 2016).

## STATISTICAL ANALYSIS

The data were expressed as mean ± SEM of 10 determinations, and statistically analyzed using Duncan Multiple Range Test (DMRT) and One-way Analysis of Variance (ANOVA). The differences were considered statistically significant when  $P < 0.05$  or  $P < 0.01$ .

## RESULTS

### Evaluation of antibacterial activity of SKQ

The MIC and MBC values of SKQ are shown in table 1. The results showed that SKQ had inhibitory effects on the tested bacteria strains. SKQ exhibited the strongest antibacterial effect on *E. coli* clinical isolates O78 (EZYP09-47) and *E. coli* O101 (CVCC3749), showing MIC and MBC values of 7.81 and 15.63 mg/ml, respectively. In addition, both the MIC and MBC values against *E. coli* O6 (ATCC25922) were 15.63 mg/ml. In contrast, the antibacterial effect of SKQ on *E. coli* O78 (CVCC1555) was relatively weak, and the MIC and MBC values were 15.63 mg/ml and 31.25 mg/ml, respectively.

### Evaluation of antidiarrheal activity of SKQ

#### Castor oil-induced diarrhea in mice

One hour after the administration of castor oil by gavage,

except for the normal group, the mice gradually showed different levels of diarrhea, which was aggravated over time (table 2). Frequent passage, increased fecal water and loose stool that was poorly formed were observed. In comparison with the control group, the diarrhea index of the treatment groups significantly decreased ( $P < 0.05$  or  $P < 0.01$ ), except for the low-dose SKQ group. In comparison with the positive control group, all other groups showed significant differences except for the normal group ( $P < 0.05$  or  $P < 0.01$ ). Therefore, SKQ induced dose-dependent reduction of the diarrhea index.

### Normal Gastrointestinal motility

The results are shown in table 3. Compared with the normal group, the residual rate inside the stomach of the LHC group and all SKQ-treated groups increased significantly ( $P < 0.01$ ). Except for the low-dose SKQ group, the intestinal propulsive rates all increased significantly ( $P < 0.01$ ). Compared with the LHC group, the residual rate inside the stomach of the middle and high SKQ dosage groups increased significantly ( $P < 0.01$ ), while the intestinal propulsive rate of all the SKQ-treated groups was elevated significantly ( $P < 0.01$ ). The residual rate inside the stomach and the intestinal propulsive rate were both dose-independent in the SKQ treatment groups.

### Neostigmine sulfate-induced gastrointestinal motility

In comparison with the animals in the normal group used for the study of normal intestinal transit, the animals in the control group showed faster travelling of test meals ( $P < 0.01$ ). In addition, no significant difference was observed when comparing the residual index and peristaltic index of SKQ at all doses compared with that of the control group. However, LHC (0.032 g/kg, p.o.) induced greater antimotility effect than SKQ at high dosage (4 g/kg, p. o.) (table 4).

### Intestinal peristalsis on isolated rabbit ileum

As shown in table 5, the tonic contraction of smooth muscle in each group decreased significantly in comparison with the normal group ( $P < 0.01$ ). The tonic contraction of smooth muscle in the high-dose SKQ group significantly decreased in comparison with the loperamide hydrochloride group ( $P < 0.01$ ). The effect of SKQ was enhanced with increased drug concentration.

### Evaluation of anti-inflammatory activity of SKQ

In our study, significant swelling was observed after xylene was applied on both sides of the right ear (table 6). In comparison with the control group, the degree of ear edema in the indomethacin- and SKQ-treated groups significantly declined ( $P < 0.01$ ). The inhibition rate in the indomethacin-treated group was 78.81%, whereas the rates in the groups treated with high, medium and low doses of SKQ were 74.83%, 72.85% and 53.64%, respectively. The results indicated that both SKQ and indomethacin possessed anti-inflammatory activities, and

**Table 1:** MIC and MBC values of SKQ against four bacteria strains

Bacteria	MIC	MBC
O78 (CVCC1555)	15.63	31.25
O6 (ATCC25922)	15.63	15.63
O78 (EZYP09-47)	7.81	15.63
O101 (CVCC3749)	7.81	15.63

**Table 2:** Effects of SKQ against castor oil-induced diarrhea in mice

Groups	Dosage/(g/kg)	Diarrhea index
Normal	/	0
Control	/	1.01±0.04**
LHC	0.032	0.06±0.09##
SKQ-L	2.3	0.97±0.21**
SKQ-M	4.5	0.74±0.22**##
SKQ-H	9	0.35±0.27**##

**Table 3:** Effect of SKQ against normal peristaltic index and residual index in mice

Groups	Dosage/(g/kg)	Peristaltic index%	Residual index%
Normal	/	54.67±7.30	66.44±8.10
LHC	0.032	95.33±18.49**	38.41±1.90**
SKQ-L	2.3	103.33±16.51**	64.78±8.91**
SKQ-M	4.5	142.80±18.88**	52.91±3.30**
SKQ-H	9	152.74±12.71**	52.22±5.44**

**Table 4:** Effect of SKQ against on neostigmine sulfate-induced peristaltic index and residual index in mice

Groups	Dosage/(g/kg)	Peristaltic index%	Residual index%
Normal	/	55.69±5.04	62.44±5.78
Control	/	24.61±7.10**	79.35±6.10**
LHC	0.032	104.34±10.20**##	55.55±6.25##
SKQ-L	2.3	60.17±10.37**##	70.10±8.14**
SKQ-M	4.5	100.52±10.58##	68.10±7.29##
SKQ-H	9	143.95±8.89**##	61.62±10.03##

**Table 5:** Inhibitory effect of SKQ on the spontaneous contraction of rabbit ileum.

Groups	Dosage (mg/mL)	Average tension/g
Normal	/	4.67±0.04
LHC	/	3.46±0.06**
SKQ-L	1.48	3.70±0.14**
SKQ-M	2.96	3.37±0.07**
SKQ-H	5.91	2.43±0.24**

Note: \* P<0.05, \*\* P<0.01 vs. Normal group; # P<0.05, ## P<0.01 vs. Control group.

indomethacin had stronger activity than SKQ. Regarding the doses of SKQ, the anti-inflammatory effect of the middle-dose group was the most significant, the low-dose group showed moderate effect, and the high-dose group exhibited promotion of swelling (P>0.05).

**Evaluation of analgesic activity of SKQ**

*Acetic acid-induced abdominal writhing test*

As shown in table 7, In comparison with the control

group, the number of writhes in indomethacin- and SKQ-treated groups was significantly reduced (P<0.05), and the inhibition rates in the groups treated with low, medium and high doses of SKQ (55.30%, 53.92% and 12.46%, respectively) were all lower than that in the indomethacin-treated group (64.07%).

**Hot plate test**

The results are shown in table 8. To varying degrees, both

**Table 6:** Effects of SKQ against xylene-induced ear edema in mice

Groups	Dosage/(g/kg)	Ear oedema degree (mg)	Inhibition rate%
Normal	/	0.16±0.11 <sup>a</sup>	/
Control	/	1.51±0.53 <sup>*</sup>	/
Indomethacin	0.04	0.32±0.20	78.81
SKQ-L	1	0.70±0.51	53.64
SKQ-M	2	0.41±0.35	72.85
SKQ-H	4	2.64±1.86 <sup>*</sup>	-74.83

**Table 7:** Effects of SKQ against acetic acid-induced writhing test in mice

Groups	Dosage/(g/kg)	Number of writhes	Inhibition rate%
Control	/	67.71±12.59	/
Indomethacin	0.04	24.33±6.89 <sup>*</sup>	64.07
SKQ-L	1	59.27±10.88	12.46
SKQ-M	2	31.20±8.68 <sup>*</sup>	53.92
SKQ-H	4	30.82±13.38 <sup>*</sup>	55.30

**Table 8:** Analgesic effects of SKQ in the hot plate test

Groups	Dosage/(g/kg)	Pain threshold on different time points		
		30 min	60 min	120 min
Normal	/	11.98±1.90	14.23±1.46	13.88±1.77
Indomethacin	0.04	18.92±1.70 <sup>*</sup>	23.51±0.95 <sup>*</sup>	43.47±12.20 <sup>*</sup>
SKQ-L	1	19.53±5.27 <sup>*</sup>	27.34±1.71 <sup>*</sup>	35.62±9.92 <sup>*</sup>
SKQ-M	2	21.92±3.23 <sup>*</sup>	29.38±6.41 <sup>*</sup>	41.37±8.83 <sup>*</sup>
SKQ-H	4	18.92±2.05 <sup>*</sup>	25.34±2.72 <sup>*</sup>	32.13±2.67 <sup>*</sup>

Note: \* P < 0.05, \*\* P < 0.01 vs. Normal group; # P < 0.05, ## P < 0.01 vs. Control group.

**Table 9:** Primer sequences used for RT-PCR

Primer	Primer sequences (5'-3') (forward/reversed)	Fragment length (bp)
AQP4	F-5'AGGGAAGGCATGAGTGACG3'	250
	R-5'TGCTGAGTCCAAAGCAGAGG3'	
$\beta$ -actin	F-5'GGAGATTACTGCCCTGGCTCCTAGC3'	155
	R-5'GGCCGGACTCATCGTACTCCTGCTT3'	

indomethacin and SKQ elevated the pain thresholds at 30, 60 and 90 min. Compared with the normal saline group, the drug treatment groups showed significant differences at 30, 60 and 90min (P<0.05). Nevertheless, no significant difference was observed among the treatment groups (SKQ and indomethacin; P>0.05).

### Effects on transcriptional and translational levels of aquaporin AQP4

#### Real-time polymerase chain reaction assay

Under the influence of SKQ, the mRNA expression level of AQP4 in diarrhea model rats declined significantly (table 9; P<0.01). Meanwhile, the expression levels of AQP4 mRNA with medium and high doses of SKQ significantly increased in comparison with the control group (P<0.01). No significant difference was observed in the groups with medium and high doses of SKQ in comparison with that of the LHC group (P>0.05). Therefore, SKQ treatment up-regulated the expression of

AQP4, which was at a low level in the control group, to reach the normal expression level.

#### Western blot assay

As shown in fig. 1 and fig. 2, the AQP4 protein level in the control group was significantly lower than that in the normal group (P<0.01), consistent with previous results. The AQP4 levels in the groups with high and middle SKQ doses were up-regulated in comparison with that of the control group (P<0.05). The results indicated that SKQ achieved antidiarrheal effect by up-regulating the expression of intestinal aquaporin and promoting intestinal mucosal water reabsorption.

## DISCUSSION

Diarrhea is a common symptom of gastrointestinal dysfunction. This study used a castor oil-induced diarrhea model to evaluate the anti-diarrhea function of SKQ. It is

reported that castor oil causes diarrhea within 1-2 hr in mice after administration of 0.1-0.2 ml (Rouf *et al.*, 2003). Owing to the high dose of castor oil (0.3 ml/mice) in our study, diarrhea responses were detected within 1 h. Only the animals that exhibited diarrheal response were selected for subsequent studies. In this study, SKQ could markedly reduce the diarrhea in a dose-dependent manner, indicating that SKQ was effective for the treatment of castor oil-induced diarrhea. The diarrhea index used in the present study can objectively reflect the quantity and quality changes of diarrhea, as well as the severity of diarrhea (Teng *et al.*, 2007, Hui *et al.*, 2013).

Using the residual rate of the semisolid paste inside the stomach as an indicator, the effect of medication on gastric emptying was observed (Kong *et al.*, 2010). Carbon ink was added to the semisolid nutrient paste as an indicator to allow simultaneous observation of the effect of medication on intestinal propulsive movement (Liang *et al.*, 2016). The results show that SKQ can significantly decrease the frequency of intestinal propulsive movements under normal status and hyper status. It can also increase gastric residues and inhibit gastrointestinal movement. It must be noted that SKQ can stop diarrhea by directly inhibiting excessive intestinal peristalsis and opposing acetylcholine secretion.

In addition, SKQ was observed to relax isolated rabbit ileum in a concentration-dependent manner. The findings imply that the anti-motility activity of SKQ can be partially achieved via blockade of the histaminic receptors and/or muscarinic choline receptors in mammalian intestine. It is also possible that muscarinic choline receptor antagonists (for example atropine) can synergistically stimulate the antidiarrheal effect of SKQ by strengthening the effects of the extract on intestinal motility and secretion.

The anti-inflammatory and analgesic effects can not only improve the clinical symptoms associated with diarrhea, but are also important for the treatment of diarrhea. Inflammation is a defense reaction of the body tissues in response to damaging factors, and is categorized into acute and chronic inflammations (Scalia 2013). The results revealed that indomethacin and SKQ could inhibit ear swelling, indicating that they could reduce inflammation by inhibiting capillary exudation and edema.

Having confirmed the potent anti-inflammatory activity of SKQ, we next studied its analgesic effects. Pain is a pathological and physiological process that occurs when the organism responds to environmental stimuli, and is not only a defensive reaction, but also a symptom of many diseases (Saltykov 1900). The results showed that the inhibition rates of indomethacin and SKQ on writhing response reached 64.07% and 55.30%, respectively. Therefore, indomethacin and SKQ could significantly

inhibit the acetic acid-mediated capillary permeability in mice (table 3). As the writhing response test lacks specificity, the observed analgesic effects could occur through different mechanisms. Accordingly, we established a hot plate model to test whether central analgesic or peripheral analgesic effects were involved. Under thermal stimulation conditions of the hot plate test, the nociceptors of mice produce pain signals and transmit them to the central nervous system, and subsequently generate a series of anti-pain reactions, such as head torsion, foot licking (the feet are highly sensitive to heat stimulation), and even leaping action (Hiruma-Lima *et al.*, 2000). The results of the hot plate test showed that both indomethacin and SKQ could significantly improve the pain threshold of mice, suggesting that they may inhibit or decrease the afferent pain through the integration of pain sensory center in mice.

In this work, the unimpaired fluid secretion in AQP4 null mice is in accordance with an exclusively cryptal site for the secretion of colonic fluids. As indicated by the fecal water content, the active fluid absorption was minimally affected by the deletion of AQP4 (Verkman *et al.*, 1996). Kon *et al.* (2014) reported a similar finding that sennoside A, a main rhubarb extract component, exhibits laxative activity by inhibiting water transport from the intestinal tract to the vascular side through down-regulation of AQP3 expression in the colon. The findings are in accordance with the lower rate of active fluid transport in the colon relative to that of the salivary gland and kidney, and with the crypt being a major site for colonic fluid absorption. The identification of specific water channels (aquaporins) on the gastrointestinal tract epithelial cells may provide a reasonable explanation. Aquaporins are a family of highly conserved transmembrane channel proteins that are chiefly involved in rapid water transport (Zhang *et al.*, 2012).

As the most abundantly expressed AQP in the colon, AQP4 plays important roles in water absorption. Based on the pathogenesis-related literature in the past decade, AQP4 is closely associated with the underlying mechanism of diarrhea (Wang 2007, Yamamoto *et al.*, 2007, Zhang *et al.*, 2012, Zhang, Wang *et al.*, 2013, Li *et al.*, 2014). According to Wang *et al.* (2000), the knockout mice deficient in AQP4, which is predominantly expressed in the mucosal epithelial cells of the mouse colon, have higher fecal water content than that of the wild type mice, even under normal physiological conditions. Zhang *et al.* discovered that AQP4 was rarely expressed on the surface of proximal colons in mice with sennoside A-induced diarrhea, whereas treatment with berberine significantly up-regulated AQP4 expression (Zhang, Wang *et al.* 2012). Therefore, the fecal water content increases with decreasing AQPs levels, validating the findings of the current study (figs. 1 and 2).

This work adopted the method of administering *Folium Sennae* extract by gavage to construct a rat diarrhea model, as this method has been shown to excel in studying the pathogenesis and pharmacological treatment of acute diarrhea (Ikarashi *et al.*, 2012). In this study, the results of Western blot and RT-PCR both indicated that during diarrhea, the colonic expression level of AQP4 was on average lower than that in the normal group, leading to decreased water reabsorption in the gastrovascular cavity. Thus, the feces could not be concentrated, creating a large amount of loose stools. From the perspective of pathology, this counterevidence suggests that under normal conditions, AQP4 has the physiological effect on water reabsorption by the colon, and the effect may be attributed to common pathophysiological factors of certain diarrhea-related diseases. The results show that SKQ can up-regulate the expression of AQP4, contributing to one of the key mechanisms for AQP4 to inhibit diarrhea (figs. 1 and 2).

## CONCLUSION

Taken together, the experimental data obtained in this laboratory animal model study indicate that SKQ possesses *in vitro* antimicrobial activities and *in vivo* antidiarrheal, anti-inflammatory and analgesic activities. Therefore, these findings establish the pharmacological foundation for the use of SKQ, a traditional Chinese medicine preparation, for the treatment and/or management of diarrhea. In addition, our conclusion validates the claims made by traditional medicine practitioners on the antibacterial, antidiarrheal, anti-inflammatory and analgesic activities of SKQ.

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