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Antidiarrheal effect of ethanol extract from *Lophatheri Herba* and its effect on isolated jejunal smooth muscle in rabbits

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Abstract: *Lophatheri Herba* is a traditional Chinese medicine, which is commonly used in the treatment of fever, stomatitis, urodynia. The aim of the study is to evaluate the antidiarrheal activity of the ethanol extract of *Lophatheri Herba* (Gramineae, ELH) and observe its effect on isolated jejunum smooth muscle in rabbits, so that we can provide a possible pharmacological basis for its clinical use. Methods: In vivo, the antidiarrheal activity of ELH (250, 500 and 1000 mg/kg; orally) in castor oil-induced Kun Ming mice was evaluated. In vitro, the effect of ELH (0.01-10 mg/mL) on the spontaneous and ACh (10μM)/K^+ (60mM)-induced contraction of isolated rabbit jejunum smooth muscle was studied. The possible mechanism of spasmolytic effect of ELH (1, 3mg/mL) was explored by pretreatment of intestinal tract with $CaCl_2$. Results: ELH (500 and 1000mg/kg) exhibited antidiarrheal effect and it (0.01-10 mg/mL) inhibited the spontaneous and ACh/K^+-induced contraction with an EC_{50} value of 1.27 (0.89-1.34), 0.76 (0.54-1.02) and 0.34 (0.27-0.53), it also shifted the concentration-response curves of $CaCl_2$ to right with decreased in max, similar to verapamil. Conclusions: ELH has significant antidiarrheal and spasmolytic effect, this provides the pharmacological basis for use in gastrointestinal disorders.

Keywords: *Lophatheri Herba,* antidiarrhea, spasmolytic, gastrointestinal disorders.

INTRODUCTION

Since ancient times, diarrhea has been recognized as a difficult miscellaneous clinical problem. Diarrhea associated with irritable bowel syndrome (IBS) affects mainly socio-economically challenged populations (Sousa *et al*., 2016), and it becomes the main cause of death to children under 5 in developing countries (Cavalcanti *et al*., 2019). The development of oral rehydration has provided an easy method for patients with acute watery diarrhea to replenish and maintain body fluids, however, rehydration does not treat the diarrhea itself (Kauna *et al*., 2019). Antidiarrheal drugs such as loperamide or codeine phosphate are widely used to treat diarrhea in adults and have shown efficacy, but they are prohibited for use in infants and children (Rehman *et al*., 2020). Abuse of antibiotics to treat diarrhea appears drug-fast, with mixed results, limits the use of this therapy in diarrhea (Tribble, 2017). To combat the problem of diarrhea, the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), has initiated a diarrhea disease control program (Araújo *et al*., 2015), which aims to promote the development of new treatments and encourage research on natural products (Costa *et al*., 2016). *Lophatheri Herba* (LH) is called "Dan-Zhu-Ye" in Chinese, which is the dry leaf and stem of *Lophatherum gracile* Brogn (Chen *et al*., 2019). LH is a traditional Chinese medicine (TCM) (Ge *et al*., 2014), it is first published in Compendium of Materia, then has been published in Chinese Pharmacopoeia since 1977 (Liu *et al*., 2009). LH is widely distributed in East and Southwest China and is commonly used in the treatment of fever, stomatitis, urodynia, etc (Wang *et al*., 2014). In addition, LH is also as an edible plant in China (He *et al*., 2016). Recent studies have shown that LH has antioxidant (Ge *et al*., 2013), antibacterial (Fan, *et al*., 2015), antiviral, anticancer, diuretic and hyperglycemic properties (Kim *et al*., 2016). LH contains many active components, including flavonoids, triterpenoids, polysaccharides, alkaloids, and several kinds of amino acid and mineral elements (Wang *et al*., 2019). Among them, studies have shown that Luteolin, one of the flavonoids in LH, exhibited antispasmodic activities (Lemmens-gruber *et al*., 2006). However, little research has been done on antispasmodic and antidiarrheal effects of LH. The main purpose of this research is to study the antidiarrheal activity of ethanol extract of *Lophatheri Herba* (ELH).

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MATERIALS AND METHODS

Drugs and reagents

Acanthopanthin B: CAS: 551-15-5, Lot: AF8111195, purity: 98%, Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China); Verapamil: Med Chem Express Co., Ltd. (New Jersey, USA); Sodium chloride, calcium chloride, potassium chlorides, odium bicarbonate,, magnesium sulfate, glucose, sodium dihydrogen phosphate: Chengdu Cologne Chemicals Co., Ltd. (Chengdu, China); Acetylcholine chloride: Chengdu Huaxia Chemical Co., Ltd. (Chengdu, China); Castor oil: Henan Hualong Pharmaceutical Co., Ltd. (Henan, China); Distilled water was used for the preparation of stock solutions of all the chemicals.

Instrument

BL-420 Biological function experiment system, FT-100 Biological tension sensor and HW-400E Constant temperature smooth muscle groove: Chengdu Techman Soft Co., Ltd. (Chengdu, China); High performance liquid chromatograph (HPLC) system: Agilent-1220 (California, USA); Ultrasonic machine: KQ-300DE, Dongguan Keqiao Ultrasonic Instrument Co., Ltd. (Guangzhou, China); Electronic balance: Discovery PV215CD, Switzerland OHAUS Co., Ltd. (Switzerland); Pure water manufacturing system: Sichuan ulupure Technology Co., Ltd. (Chengdu, China); Rotary evaporator: RE-52AA, YaRong (Shanghai, China); Constant temperature water bath pot: Yuhua Instrument Co., Ltd. (Gongyi, China).

Plant material and preparation

LH was provided by Sichuan Yuding Tang Traditional Chinese Medicine Pieces Co., Ltd (Sichuan, China) and was identified by professor Yang Lan, the voucher specimen (CBY-2019-0007) was deposited in the specimen room of North Sichuan Medical College. LH was dried in the thermostat at 25°C and was pulverized into coarse powder by the grinding machine. Most of the active components of LH can be extracted by 70% ethanol under reflux (Wang *et al*., 2015). 50 g of LH coarse powder was weighed, and it was placed in a round flask and added 10 times 70% ethanol solution (In total 500 mL). The extract was cohobated for 3 times at 50°C for 30 min each, then combinated the filtrate. At last, the filtrate was Dried in the constant temperature water bath pot (60°C) untill it was concentrated into paste extract, so the ELH was obtained and the percentage yield was 12.01%

Animals

Adult male Kun Ming mice (18-22 g) (SYXK-(chuan)-2019-076) and locally bred rabbits (2.0-2.5kg) were supplied by the Animal Laboratory Center, North Sichuan Medical College, Sichuan, China. All animals were kept under standard environmental condition: Mean temperature of 24±5°C, mean humidity of 45±5% and light-dark cycles for 24 h. Animals had free access to water, but fasted 24 h before the experiments.

Ethical approval

The animal research conformed to the requirements of Ethical Review Committee of SLAS (Sichuan association for Laboratory Animal Sciences) Assessment agencies and followed the requirements of animal welfare.

Phytochemical study

Chromatographic conditions

The column was tested as Aglient-ZORBAX SB-C18 (4.6 × 250 mm, 5μm). The mobile phase consisting of 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) was 80: 20. The mobile phase was passed through a 0.45μm filter membrane. Detection wavelength: 265 nm. Flow rate: 0.6mL/min. Column temperature: 27°C,

Preparation of the standard and the sample solution

The standard solution was made up to Acanthopanthin B with the concentration of 1.1mg/mL in methanol and the sample solution was made up to 0.029mg/mL of ELH (0.1201g/g DW) in 70% methanol. The solution was passed through a 0.22 μm nylon microporous membrane and kept at 4°C before use.

Acute oral toxicity test

Seventy mice were randomly divided into seven groups with ten mice each group. Sixty mice among them were used to evaluate the safety of ELH, which were administered orally at different doses of ELH (500, 1000, 2000, 4000, 8000, 16000mg/kg) in 14 days. The mice of negative control group were administered saline solution orally. Mice were weighed and observed situation about the hair, mental and death every day.

Effect on castor oil-induced diarrhea

The method of castor oil-induced diarrhea in mice was from Palla (2015). Fifty healthy mice were selected and randomly divided into 5 groups (n=10) and kept in individual cages. All drugs were administered through the lavage needles. The negative control group received normal saline (20mg/kg). The positive control group received verapamil (50mg/kg). The test groups received ELH (250, 500, 1000mg/kg). After 30 min, mice of all groups received 0.4mL castor oil (20mL/kg) orally. Blotting papers were placed under the cages. The initial time of semi-solid feces, and the amount of solid feces, semi-solid feces, liquid feces were recorded in 4 h. Evacuation index (EI) is used to evaluate the severity of diarrhea.

EI = Number of Liquid Feces × 3 + Number of Semi-Solid Feces × 2 + Number of Solid Feces (Tadesse *et al*., 2014)

Tissue preparation

The healthy locally bred rabbits were selected. they were euthanized by cervical dislocation. The jejunum was took out, rinsed and stored in the Tyrode's solution at 37± 0.5°C. A section of jejunum (1.5-2 cm) was taken and suspended vertically in an organ bath containing 18mL Tyrode's solution (95% O_2 and 5% CO_2 were mixed in the organ bath, and the bubble velocity was 1-2 per second) (Ali *et al*., 2016). The pre-load pressure was 1 g. Jejunal activity was recorded by the FT-100 biosensor connected to BL-420F biological function experimental system.

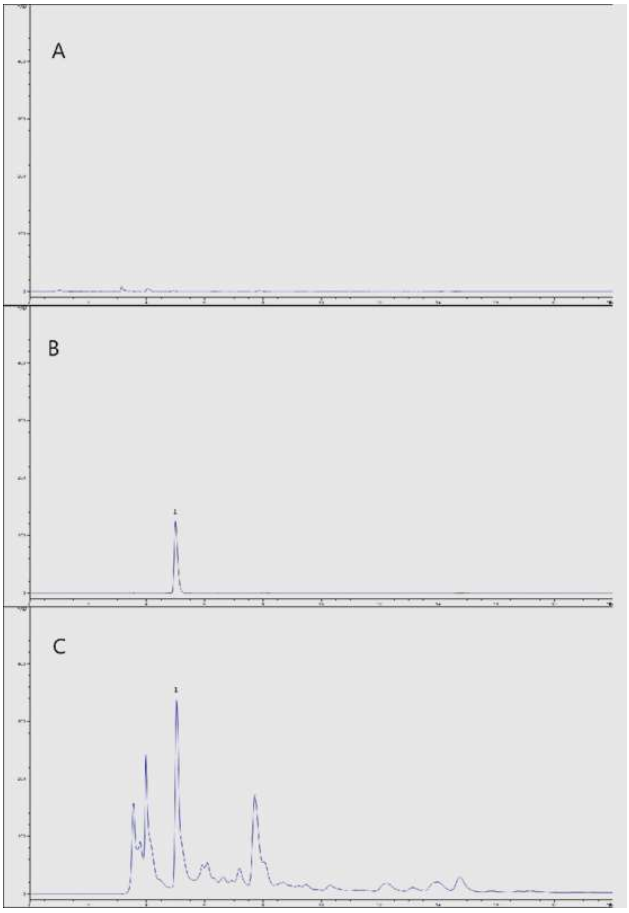

Fig. 1: HPLC chromatograms of blank (A), reference substances (B) and ELH (C) (Acanthopanthin B).

Effect of ELH on spontaneous contraction of rabbit jejunum

Rabbit jejunum tissue showed spontaneous contraction under the standard experimental settings (Chaudhary *et al*., 2012). After the tissue was stabilized, ELH (0.01, 0.03, 0.1, 0.3, 1, 3, 10mg/mL) was added.

Effect of ELH on ACh/K^+-induced contraction

After jejunum smooth muscle contracted stably, ACh (10 μM) or high-K^+ (60mM) was added into in Tyrode's solution to make the isolated jejunum continuously spasticity. When the ACh or high-K^+-induced contractions reached a stable level for about 15 min, ELH (0.01, 0.03, 0.1, 0.3, 1, 3, 10mg/mL) was accumulated as test group. Positive group was treated with verapamil (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3uM).

Effects of ELH on $CaCl_2$-induced contraction

Substances that inhibiting high-K^+ contraction are considered as Ca^{2+} entry blockers. To determine the connection between the inhibition of intestinal contraction by ELH and Ca^{2+} channel, the jejunum mooth muscle was originally equilibrated in Tyrode's solution, then we stabilized it in Ca^{2+}-free high-K^+ (60mM) solution and incubated it with EDTA (0.1mM) for 30 min to eliminate Ca^{2+} from the tissues, then incubated without EDTA for 15 min. The experiment was divided into 5 groups. The first and second groups were added with ELH (1 and 3 mg/mL), and the third and fourth groups were added with verapamil (0.03 and 0.1μM). As a comparision, the fifth group was added with Tyrode's solution. $CaCl_2$ was added cumulatively (3×10^{-5}-3×10^{-2} M) and the $CaCl_2$ concentration-response curves were drawn. Contraction at the concentration of 3×10^{-2} M was set at 100%.

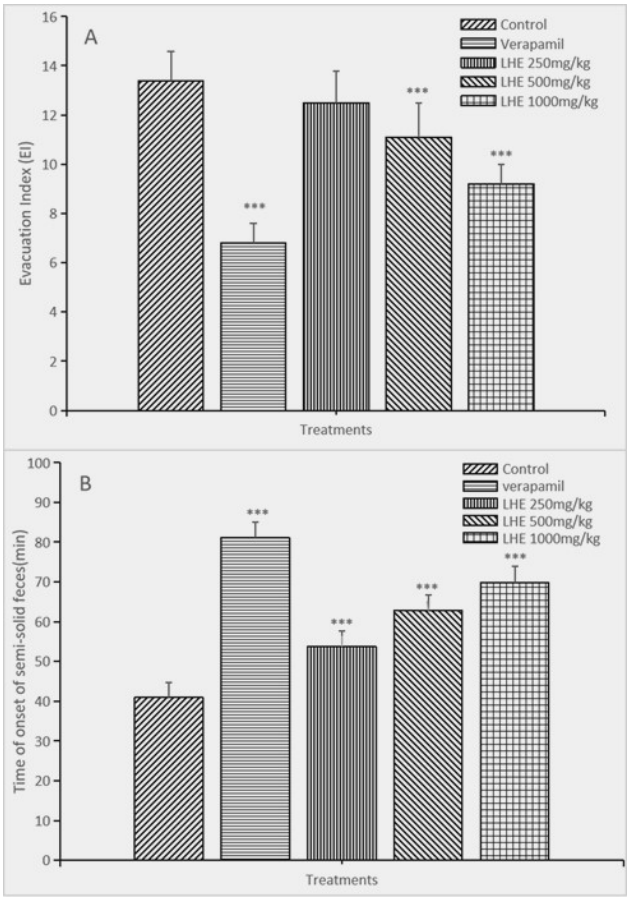

Fig. 2: Effect of ELH on castor oil-induced diarrhea in mice. A. Evacuation Index (EI), B. Time of onset of semi-solid feces (min). *p<0.05, **p<0.01, ***p<0.001. compared with the negative control group; volumes presented as the mean ± SEM, n = 10.

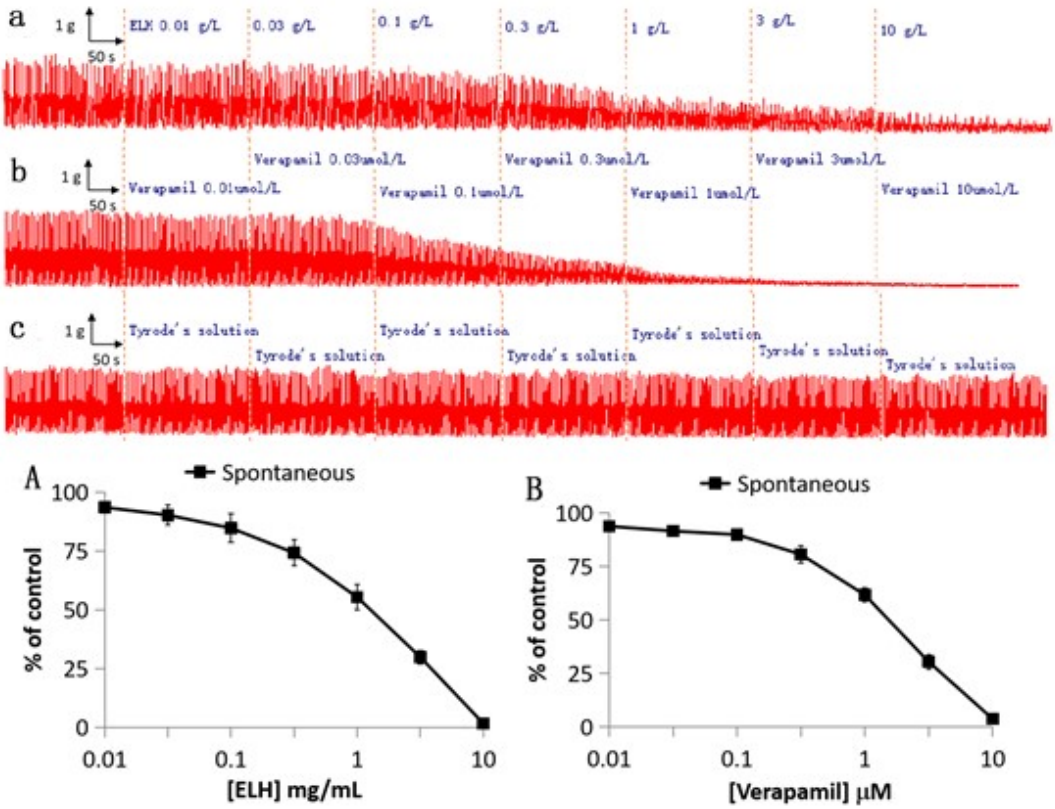

Fig. 3: Concentration-dependent inhibitory effect of (A(a)) crude extract of ELH and (B(b)) verapamil, on spontaneously contracting isolated jejunum. Tracing showing (c) spontaneous contraction of isolation rabbit jejunum. Results are expressed as mean ± SEM, n = 6.

STATISTICAL ANALYSIS

Being represented as mean ± standard error (SEM), all data were analyzed by one-way analysis of variance followed by the Dunnett's test. SPSS 19.0 system was used for testing. $P \leq 0.05$ was considered as statistically significant.

RESULTS

Phytochemical study

The separation of Acanthopanthin B was good. The chromatography profiles of the reference solution and the test sample solution were shown in fig. 1. fig. 1 (A) of blank solution, fig. 1 (B) of reference solution and fig. 1 (C) of sample solution from ELH were as bellow.

Acute oral toxicity test

After ELH was intragastrically administrated in increasing doses of 500, 1000, 2000, 4000, 8000 and 16000 mg/kg, we observed no mortality or signs of toxicity in 14 days. As a results, the safety of ELH was assessed to be greater than16000 mg/kg.

Effect on castor oil-induced diarrhea

After 4 h of castor oil, mice in the negative control group (normal saline, 20mL/kg) showed acute diarrhea. The ELH (500 and 1000mg/kg) significantly inhibited diarrhea by increasing the onset time of semi-solid feces (63.4±4.01 and 69.9±3.9 min) and decreasing the EI (11.1 ±1.37 and 9.2±0.79) compared with the negative control group (40.9±3.78 min; 13.25±1.25) (p<0.001). The EI and the onset time of semi-solid feces of verapamil were 6.8±0.79 and 80.4±3.84 min (p< 0.001) (fig. 2).

Effect of ELH on spontaneous contraction of rabbit jejunum

As shown in fig. 3 (c), there was no significant effect of Tyrode's solution on the spontaneous contraction of rabbit jejunum (p>0.05). The naturally contracting isolated rabbit jejunum was significantly relaxed by ELH at 0.01-10 mg/mL, EC_{50} =1.27 mg/mL (0.89-1.34 mg/mL, n = 6) (fig. 3A (a)), similar to verapamil (0.01-10 μM) with an EC_{50} value of 2.03 uM (2.01-2.43, n = 6) (fig. 3B (b)).

Effect of ELH on ACh/K^+-induced contraction

ELH (0.01, 0.03, 0.1, 0.3, 1, 3, 10mg/mL) relaxed both the ACh (10μM) and K^+(60mM)-induced contraction with the EC_{50} value of 0.76mg/mL (0.54-1.02mg/mL, n = 6) and 0.34 mg/mL (0.27-0.53mg/mL, n = 6) (fig. 4A). It is similar to verapamil at (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 μM) with an EC_{50} value of 0.33 μM (0.21-0.49, n = 6) and 0.15 μM (0.10-0.27, n = 6) (fig. 4B).

Effect of ELH on $CaCl_2$-induced cumulative contractions

ELH inhibited the contraction induced by cumulative concentration of $CaCl_2$ (3×10^{-5}-3×10^{-2} M), As the effect of verapamil, the ELH shifted the $CaCl_2$ curves to the lower right and down with the decrease in maximum contractions. Compared with the negative group, ELH (0.3, 1mg/mL) and verapamil (0.03, 0.1 μM) reduced the maximum contraction induced by 0.3mM $CaCl_2$ to 39.37 ±0.42%, 71.98±0.72%, 56.56±0.83% and 79.06±0.56% (fig. 5).

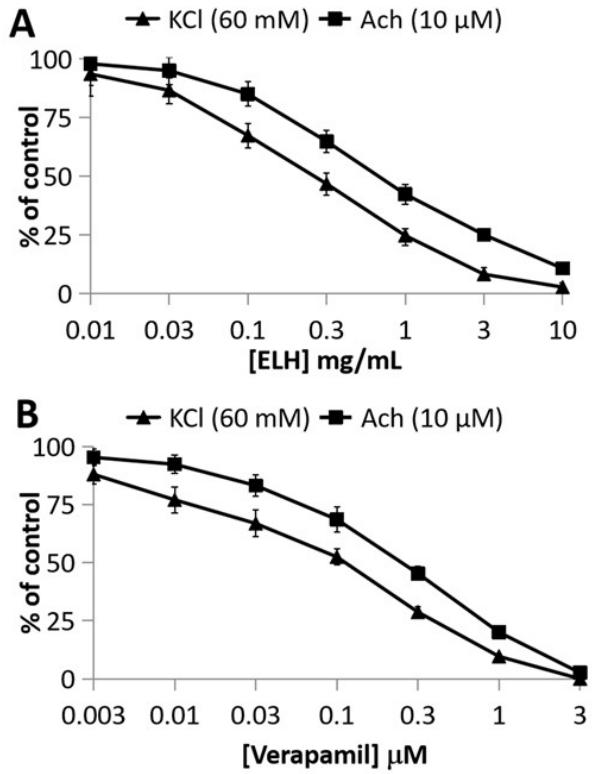

Fig. 4: Concentration-dependent inhibitory effect of (A) crude extract of ELH and (B) verapamil on Ach (10 μM) or high K^+ (60 mM) induced pre-contracted isolated jejunum. Results are expressed as mean ± SEM, n = 6.

DISCUSSION

IBS is a frequent gastrointestinal disease (Forte *et al*., 2012), characterized by abdominal pain, constipation or diarrhea and flatulence. At present, conventional drugs are not always safe or effective especially in extended use (Aleem and Janbaz, 2018). Abnormal contraction of intestinal smooth muscle is onn of the main symptoms of IBS, so modifying the contractility of smooth muscle is often the main treatment of IBS (Wei *et al*., 2013). Therefore, it is imperative to find a natural antidiarrheal drug that is resistant to bowel movements and has few side effects.

In this study, castor oil-induced accelerated intestinal motility in mice was used as a diarrhea model. Reducing the fecal output and inhibiting the experimental diarrhea are the basis for the pharmacological evaluation of a potential antidiarrheal agents (Ching *et al*., 2013). Castor oil is derived from the seeds of Ricinus communis (Guo *et al*., 2014a), ricinoleic acid is an active hydrolytic metabolite of castor oil followed by alterations in ion transport and water flux in the intestine (Fujita *et al*., 2014). Moreover, it can stimulate and inflame intestinal mucosa, leading to the release of several mediators such as prostaglandins (Sisay *et al*., 2017), cAMP, nitric oxide (NO) and tachykinin (Costa *et al*., 2016). ELH reduced the EI score of castor oil-induced diarrheal mice and prolonged the onset time of semi-solid feces, which indicated antidiarrheal effect. Research has attributed the antidiarrheal activity of many plants to the presence of tannins, alkaloids, saponins, flavonoids, sterols and terpenes (Yakubu and Salimon, 2014) and coumaric acids and flavonoids are the major constituents of ELH (Tang *et al*., 2015). The antidiarrheal activity of flavonoids has been demonstrated and attributed to inhibit intestinal motility and electrolytic secretion and also reduce the release of autacoids and prostaglandins, inhibiting the motility and secretion induced by castor oil (Dosso *et al*., 2012). In the experiment, as a reference drug for this experiment, the pharmacological actions of verapamil can be related to its specific blocking effect on the transmembrane transport of Ca^{2+} (Ansari *et al*., 2020). In other words, the contraction of intestinal depends on the mediation of Ca^{2+}, verapamil can reduce intestinal peristalsis to inhibit diarrhea by blocking Ca^{2+} channels and reducing the entry of Ca^{2+}. We hypothesize that the antidiarrheal effect of ELH is related to Ca^{2+}, which is similar to verapamil.

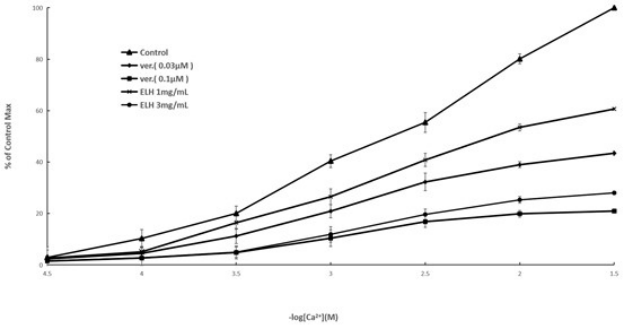

Fig. 5: Concentration-response curves of $CaCl_2$ on rabbit-isolated jejunum in the presence of ELH (1, 3 mg/mL) and verapamil (0.03, 0.1 μM). Results are mean ± SEM, n = 6.

In vitro study, ELH inhibited intestinal contraction in a concentration-dependent manner, which is the reason for the antidiarrheal effect of ELH confirmed above. Gastrointestinal contraction is regulated by many physiological mediators, such as histamine, ACh,

prostaglandin and Serotonin (5-HT) (Zhang *et al*., 2013). ACh is a neurotransmitter in the gastrointestinal tract, which is important in the contraction of intestine and colon (Huang *et al*., 2019). ACh stimulates muscarinic receptor, which couples G protein (GP) and then increases the concentration of inositol triphosphate (IP_3) and diacylglycerol (DG), it causes Ca^{2+} release and protein phosphorylation to stimulate smooth muscle contraction (Jia *et al*., 2014). ELH inhibited ACh-induced contraction in a concentration-dependent manner, so we hypothesize the ELH has the function of relaxing jejunal smooth muscle might be regulated by blocking muscarinic receptor. In addition, the contraction of jejunum is mediated through Ca^{2+} inside the cytoplasm, mainly by Ca^{2+} influx from extra-cells or Ca^{2+} release from sarcoplasmic reticulum (Hussain *et al*., 2016). Much of the Ca^{2+} needed for the contraction of intestinal smooth muscle enters the myocytes from outside the cells when action potential occurs, and a small part comes from the sarcoplasmic reticulum. When the intracellular Ca^{2+} concentration rises to a certain level, the contraction of smooth muscle is triggered (Li *et al*., 2019). It has been shown that the concentration of K^+ above 30 mM (what is high K^+) can contract smooth muscle by opening voltage-dependent L-type Ca^{2+} channels that allows Ca^{2+} influx (Türck and Leonhard-Marek, 2010). Substances that cause high potassium-induced contractile inhibition are thought to be calcium influx blockers (Guo *et al*., 2014b). ELH can inhibit the high K^+-induced contraction in a concentration-dependent manner. Therefore, ELH may have the function of blocking L-type Ca^{2+} channels. To verify this inference, the cumulative external Ca^{2+} was added into Tyrode's solutions and set verapamil, the L-type Ca^{2+} channel blocker, as the control group (Li *et al*., 2019). From results, with the increase of ELH concentration, the dose-response curve of $CaCl_2$ shifted to the right and down, similar to that caused by verapamil. Therefore, we also hypothesize ELH has the function of relaxing jejunal smooth muscle might be regulated by blocking L-type Ca^{2+} channels. This study provides a pharmacological basis for the clinical application of ELH in diarrhea, the efficacy of ELH in the treatment of diarrhea should be further evaluated in the future, more data should be provided for clinical studies from the cellular and molecular levels, and we believe that ELH is promising in the treatment of diarrhea.

CONCLUSION

The results showed that ELH had significant antidiarrheal effect in castor oil-induced diarrheal mice. In addition, ELH could relax the spontaneous contraction of jejunum and the contraction induced by ACh (10^{-5} M) and KCl (60 mM) in rabbits. The mechanism may be related to inhibiting the calcium ion channel, this provided the pharmacological basis for use in gastrointestinal disorders.

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