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Antidiarrheal effect of the ethanol extract from *Scutellaria barbata* and its effect on the contraction of jejunum smooth muscles

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Abstract: *Scutellaria barbata* (*S. barbata*), a traditional herbal medicine used in southern China, possesses anti-inflammatory, antitumor, spasmolytic and expectorant effects. However, there are not many recent studies on its gastrointestinal effects. This study aimed to evaluate the antidiarrheal effect of the ethanol extract of *S. barbata* (SBE) and its effect on the isolated jejunum smooth muscle. Methods: The antidiarrheal effect of SBE (doses: 125, 250 and 500 mg/kg) on castor oil-induced diarrhea was investigated *in vivo*. The effect of SBE (0.01-10 mg/mL) on spontaneous or acetylcholine chloride (ACh, 10μM)/KCl (60mM)-induced contraction of isolated rabbit jejunum smooth muscle was examined *in vitro*. The possible spasmolytic mechanism of SBE (1 and 3mg/mL) was analyzed by accumulating $CaCl_2$ in a Ca^{2+}-free high-K^+ (60mM) solution. Results: SBE (125, 250 and 500mg/kg) could delay the initial semi-solid onset time of mice and also reduce the diarrhea index *in vivo*. Furthermore, SBE (0.01-10mg/mL) could alleviate the spontaneous or ACh/KCl-induced contraction *in vitro*. SBE (1 and 3mg/mL) also inhibited the contraction induced by $CaCl_2$, and the concentration–response curves of $CaCl_2$ moved downward and to the right, similar to those of verapamil (0.01 and 0.1μM). Conclusions: SBE exerts antidiarrheal and spasmolytic effects, which provides a pharmacological basis for its use in functional gastrointestinal disorders.

Keywords: *S. barbata* ethanol extract, castor oil-induced diarrhea, Ca^{2+} channel, antidiarrheal, spasmolytic.

INTRODUCTION

Diarrhea is a common pediatric disease with multiple causes and factors (Kotloff *et al*., 2013). Ten percent of children aged 1-59 months die from diarrhea, which ranks second among the diseases causing child mortality (Wang *et al*., 2014). Each year, approximately 7.6 million children under 5 years of age die from diarrhea, with 1.7 billion cases reported globally. Diarrhea is the chief cause of death in children under 5 years of age (Forsberg *et al*., 2017; Aleem *et al*., 2018).

Diarrhea is characterized by sparse stool, watery stool, mucous stool, or pus–bloody stool and is accompanied by fever, abdominal pain, and vomiting. In severe cases, water, electrolyte acid-base balance disorder, toxic symptoms, and systemic manifestations may be seen (Agarwal *et al*., 2018). The pathogenesis of diarrhea is based on dysfunction in gastrointestinal secretion, digestion, absorption, and movement, which results in increased secretion, incomplete digestion, reduced absorption, and accelerated motility. These effects eventually lead to feces thinning and increased frequency (Cavalcanti *et al*., 2019).

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The current treatment for diarrhea mainly involves balancing the electrolytes and restoring the fluid volume by several approaches (Binder, 2020). However, the adverse effects of these treatments have led more and more people to turn to alternative medicine for treating diarrhea (Patel *et al*., 2013). Traditional Chinese medicine has few side effects and can significantly reverse gastrointestinal dysfunction (Lv *et al*., 2019). Therefore, the therapeutic effect of traditional Chinese medicine on digestive system diseases has become the new research trend (Feng *et al*., 2019).

Scutellaria barbata (SB) is the dried whole herb of *Scutellaria barbata* (Labiatae), which is widely known as *hanxin grass* and *head grass.* SB holds a prominent position in the Chinese Pharmacopeia (State Pharmacopoeia Commission, 2015). SB is rich in flavonoids (Lin *et al*., 2015), alkaloids (Ren *et al*., 2014), and polysaccharides (Liu *et al*., 2013); however, its physiologically active substances are mainly flavonoids (Zheng *et al*., 2010). Modern pharmacological studies have demonstrated that the ethanol extract of SB (SBE) possesses many pharmacological activities such as antipyretic, anti-inflammatory, antimutation, antioxidant, antitumor, and immune regulation (Zhang *et al*., 2017; Chen *et al*., 2020). Nonetheless, few studies have so far explored its gastrointestinal effects.

Hence, this study was aimed at evaluating the antidiarrheal and spasmolytic effects of SBE and the possible pharmacological mechanism involved in inhibiting the contraction of isolated jejunum smooth muscle.

MATERIALS AND METHODS

Drugs and reagents

Acetylcholine chloride (ACh) was obtained from Chengdu Hua Xia Chemicals Co., Ltd. (Chengdu, China). Calcium chloride, potassium chloride, magnesium sulfate, sodium chloride, glucose, sodium dihydrogen phosphate, and sodium bicarbonate were purchased from Chengdu Cologne Chemicals Co., Ltd.. Verapamil was sourced from Med Chem Express Co., Ltd. (NJ. USA). Castor oil was sourced from Henan Hua Long Pharmaceutical Co., Ltd. (Henan, China). Distilled water was used for the preparation of stock solutions of all the chemicals.

Animals

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

Drug extraction

The leaves and stems of SB were purchased from Nanchong, Sichuan, China. The voucher samples (CBY-2019-0002) were deposited in the specimen room of the North Sichuan Medical College. SB was dried in the thermostat at 25°C and pulverized into coarse powder with the grinding machine. Next, 50 g of the coarse powder was weighed and placed in a round flask to which 70% ethanol solution (totaling 350 mL) were added at 7 times the volume. The extract was cohobated thrice at 60°C for 50 min each and combined with the filtrate. The extract was dried at 60°C and concentrated into a paste form; the SBE was obtained with the percentage yield of 9.21% and stored at 4°C before use.

Phytochemical analysis

The standard solution was made up to 0.24mg/mL of eleutheroside B and 0.08mg/mL of scutellarin in methanol, and the sample solution was made up to 447 mg/mL of SBE (11.13 g/g DW) in methanol. Both the standard and sample solutions were sieved through a 0.22-μm nylon microporous membrane. The column was tested as the Aglient-ZORBAX SB-C18 (4.6 × 250 mm, 5 μm). The mobile phase was passed through a 0.45-μm filter membrane. The mobile phase consisting of 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) using a linear gradient (table 1) was used to determine the eleutheroside B and scutellarin from SBE. The detection wavelength was changed from 335 nm to 260 nm at 10 min and the flow rate was set to 0.6 mL/min. The solution was maintained in a constant temperature water bath at 28°C.

Study designs

This study involved two phases: the antidiarrheal effect of SBE in mice was evaluated *in vivo*. The spasmolytic effect and the possible mechanism of SBE on the contraction of the isolated jejunum smooth muscles in rabbits were investigated i*n vitro*.

In vivo studies

Acute toxicity of SBE in mice

The experimental mice were categorized into 6 random groups (n = 6). The mice were provided SBE at doses of 500, 1000, 2000, 4000, 8000 and 16000 mg/kg orally. The toxicity signs and death of mice were recorded for 14 days. The safety of SBE was evaluated at the highest dose ((Li *et al*., 2019).

Effect of SBE on castor oil-induced diarrhea in mice

The experimental mice were categorized into 5 random groups (n = 10). The test groups were provided SBE (125, 250, 500 mg/kg); the positive control group was provided verapamil (50 mg/kg); the negative control group was provided normal saline (20 mL/kg). After 30 min, 0.4 mL of castor oil was administered orally and each mouse was placed into a separate cage, and an ink-absorbing paper was placed 3-cm below each cage. Then, the initial semi-solid onset time and the amount of solid feces, semi-solid feces and liquid feces collected within 4 h were determined. The diarrhea index (EI) was used to evaluate the severity of diarrhea (Tadesse *et al*., 2014).

EI = Number of Liquid Feces × 3 + Number of Semi-Solid Feces × 2 + Number of Solid Feces

In vitro study

Preparation of isolated jejunum smooth muscles in rabbits

The healthy locally bred rabbits were selected, and the mice were euthanized by cervical dislocation, after which the jejunum was removed and cut into 1.5-2.0-cm section. The intestinal contents were washed with the Tyrode's solution and the jejunum was stored in the Tyrode's solution at a constant temperature of 37±0.5°C. A section of jejunum was then collected and suspended vertically in an organ bath containing 18mL of the Tyrode's solution (95% O_2 and 5% CO_2 were mixed in the organ bath, and the bubble velocity was set to 1-2/s). The pre-load pressure was set to 1 g. The jejunal activity was recorded with the FT-100 biosensor connected to the BL-420F biological function experimental system.

Effect of SBE on spontaneous contraction of the isolated jejunum smooth muscles

After the jejunum was stabilized in the Tyrode's solution, different concentrations of SBE (0.01, 0.03, 0.1, 0.3, 1, 3,

and 10mg/mL) were added to determine the effect on spontaneous contraction of the jejunum smooth muscles. The contractibility of the jejunum smooth muscles was set at 100% before administration. SBE was replaced with verapamil (0.01, 0.03, 0.1, 0.3, 1, 3, 10μM) as the positive control. The concentration-response curves and the EC_{50} of SBE and verapamil were evaluated.

Effect of SBE on Ach- or KCl-induced contraction of the isolated jejunum smooth muscles

To investigate the spasmolytic activity of SBE, ACh (10 μM) or KCl (60 mM) were added into the Tyrode's solution to make the isolated jejunum continuously spasmodic. After the jejunum was stabilized, SBE (0.01, 0.03, 0.1, 0.3, 1, 3, 10mg/mL) or verapamil (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3μM) were added. The concentration-response curves and the EC_{50} of SBE and verapamil were evaluated.

Effect of SBE on $CaCl_2$-induced contraction of the isolated jejunum smooth muscles

We stabilized the jejunum smooth muscle in Ca^{2+}-free high-K^+ (60 mM) solution and incubated it with EDTA (0.1 mM) for 30 min to eliminate Ca^{2+} from the tissues, followed by incubation without EDTA for 15 min. The experiment was divided into 5 phases. To the first and second groups, SBE (1 and 3 mg/mL) were added and the third and fourth groups received verapamil (0.01 and 0.1 μM). In comparison, the fifth group received the 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%.

STATISTICAL ANALYSES

The results were expressed as mean ±S.E.M and the difference between several groups and the negative control group was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's method. The SPSS 19.0 system was used for analysis. $p<0.05$ was considered to indicate statistical significance.

RESULTS

Chemical compounds identified in SBE with HPLC

Under optimal liquid chromatography conditions, although the scutellarin content in the extract was low, the separation between eleutheroside B and scutellarin was good. The chromatography profiles of the reference solution and the test sample solution are shown in fig. 1. fig. 1 (A) is the blank solution, fig. 1 (B) is the reference solution and fig. 1 (C) is the sample solution from SBE (fig. 1).

Acute toxicity of SBE in mice

After SBE was intragastrically administrated in increasing doses of 500, 1000, 2000, 4000, 8000 and 16000 mg/kg, neither mortality nor signs of toxicity were observed in 14 days. The safe dose of SBE was, hence, established to be >16000 mg/kg.

Table 1: Liquid Chromatography Conditions

Time (min)	Phase A (%)	Phase B (%)
0-10	80	20
10-20	80→60	20→40

Effect of SBE on castor oil-induced diarrhea in mice

After 4 h, the mice in the negative control group (normal saline, 20mL/kg) experienced acute diarrhea. The EI was 13.46±1.13 and the initial semi-solid onset time was 40.23±3.98 min. The EI of the positive control group (verapamil, 50mg/kg) was 7.30±0.82 ($p<0.001$) and the initial semi-solid onset time was 80.40±3.84min ($p<0.001$). The EIs of the test group for 125, 250 and 500 mg/kg of SBE were 12.80±1.14 ($p<0.05$), 11.90±0.99 ($p<0.1$) and 9.40±0.97 ($p<0.001$), and the initial semi-solid onset times were 51.90 ± 3.93 ($p<0.05$), 57.80±4.21 ($p<0.01$), and 64.50±3.75 min ($p<0.01$), respectively (fig. 2).

Effect of SBE on spontaneous contraction of the isolated jejunum smooth muscle

SBE (0.01, 0.03, 0.1, 0.3, 1, 3 and 10mg/mL) inhibited the spontaneous contraction of the isolated jejunal smooth muscle in a concentration-dependent manner, with an EC_{50} value of 0.84mg/mL (95% confidence interval [CI], 0.96–1.11, n = 6). In the positive control group, the EC_{50} value of verapamil (0.01-10μM) was 0.42mg/mL (95% CI, 0.40–0.47, n = 6) (fig. 3).

Effect of SBE on ACh- or KCl-induced contraction of the isolated jejunum smooth muscle

SBE (0.01, 0.03, 0.1, 0.3, 1, 3 and 10mg/mL) relieved the intestinal contraction induced by ACh (10 μM) or KCl (60 mM) in a concentration-dependent manner. The EC_{50} values of SBE were 0.81 mg/mL (95% CI, 0.74-1.06, n = 6) and 0.51mg/mL (95% CI, 0.48-0.57, n = 6), and their maximum inhibition rates were 89.41% and 84.61% for ACh- and KCl-induced contractions, respectively. The results were similar for verapamil (0.003-3μM), with EC_{50} values of 0.21μM (95% CI, 0.14-0.32, n = 6) and 0.05μM (95% CI, 0.04-0.07, n = 6), and with maximum inhibition rates of 92.74% and 88.02% for ACh- and KCl-induced contractions, respectively (fig. 4).

Effect of SBE on $CaCl_2$-induced contraction of the isolated jejunum smooth muscle

SBE inhibited the contraction of jejunum smooth muscle induced by the cumulative concentration of $CaCl_2$ (3×10^{-5}–3×10^{-2} M) in a concentration-dependent manner. The maximum inhibition rate was 100%. Further studies showed that 1 and 3mg/mL of SBE moved the $CaCl_2$ concentration–response curves to the lower right and that the maximum inhibition rates were 50.62±4.07% and 29.31±1.88% ($p<0.001$), respectively.

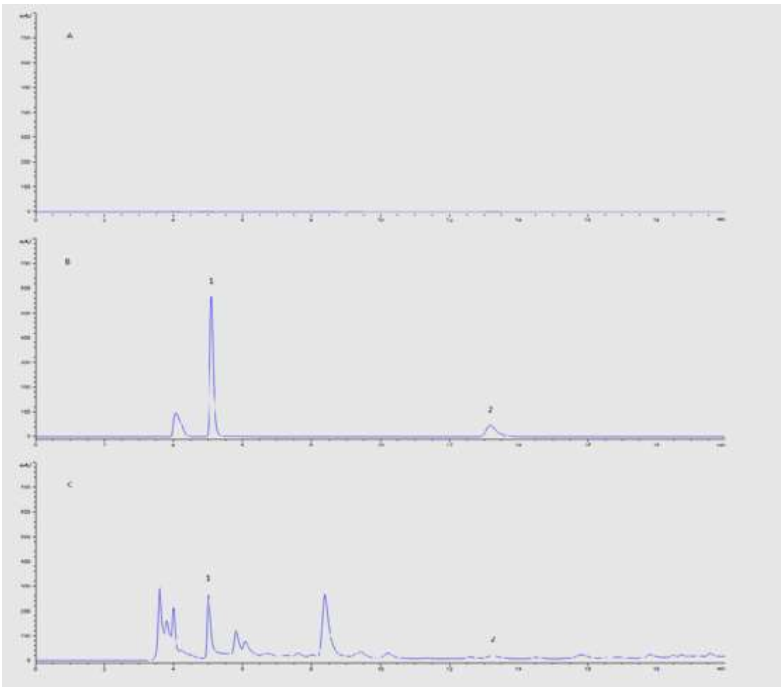

Fig. 1: HPLC chromatograms of the blank (A), reference substances (B) and SBE (C) (1 eleutheroside B, 2 scutellarin).

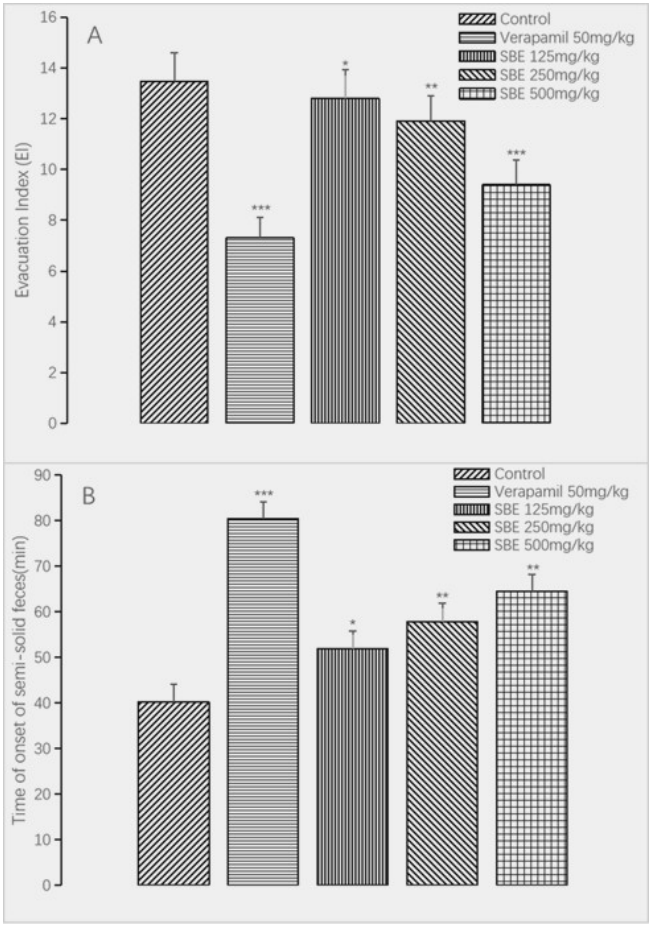

Fig. 2: The effect of SBE or verapamil on castor oil-induced diarrhea in mice. Evacuation Index (EI) (A) and the initial semi-solid onset time of mice (B) are recorded. The results are presented as mean ±SEM, n = 10. **p*<0.05, ***p*<0.01, ****p*<0.001.

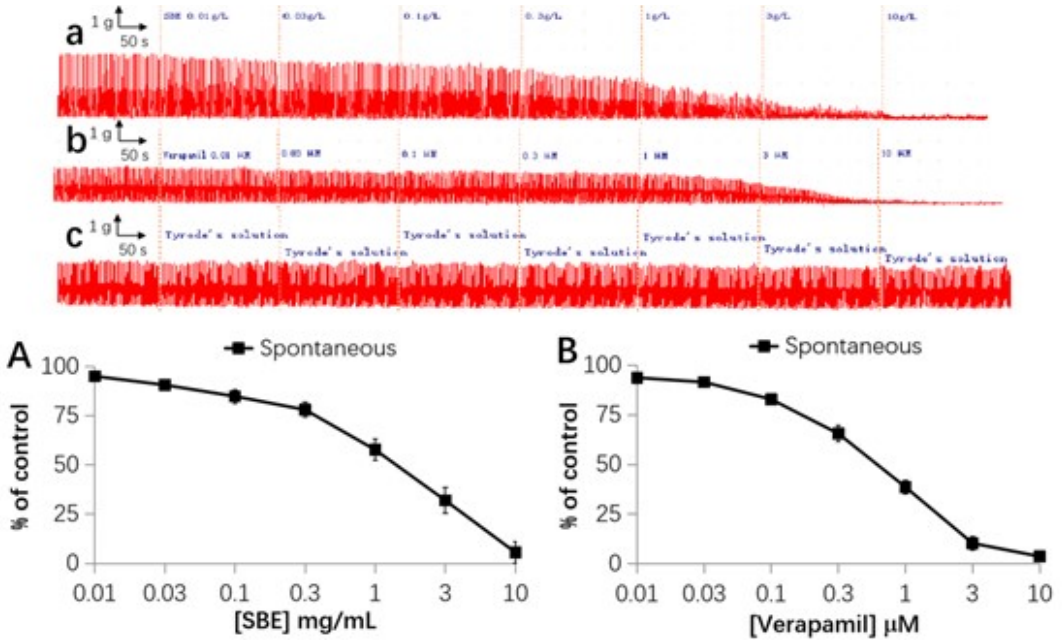

Fig. 3: Concentration-response curves of SBE (A) or verapamil (B) on the spontaneous contraction of isolated jejunum smooth muscles. Tracing demonstrating SBE (a), verapamil (b) and Tyrode's solution (c) of isolated rabbit jejunum. The results are expressed as mean ±SEM, n = 6.

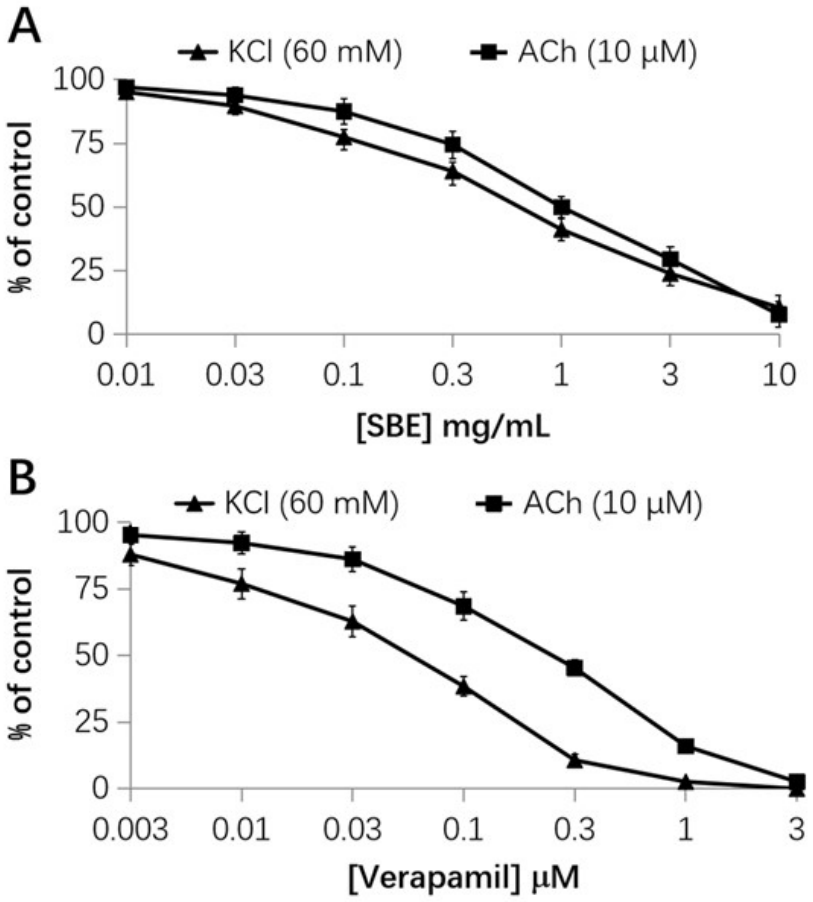

Fig. 4: Concentration-response curves of SBE (A) or verapamil (B) on ACh (10μM)/KCl (60mM)-induced contraction of isolated jejunum smooth muscles. The results are expressed as mean ±SEM, n = 6.

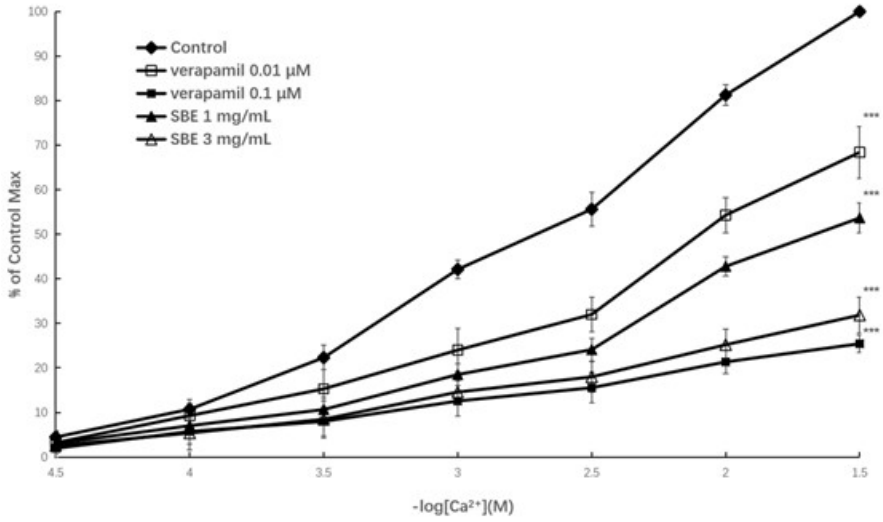

Fig. 5: Concentration-response curves of $CaCl_2$ on isolated jejunum smooth muscles in the presence of SBE (1mg/mL; 3mg/mL) or verapamil (0.01μM; 0.1μM). The results are presented as mean ±SEM, n = 6. **p*<0.05, ***p*<0.01, ****p*<0.001.

The maximum inhibition rates of 0.01 and 0.1μM verapamil were 65.18%±5.80% and 23.49%±3.35% (p<0.001), respectively. Hence, both SBE and verapamil showed similar trends in the $CaCl_2$ concentration–response curves (fig. 5).

DISCUSSION

Castor oil is considered as an "irritant" laxative (Vani *et al*., 2018). The active ingredient of castor oil inducing diarrhea is ricinoleic acid, which has irritating and inflammatory actions on the intestinal mucosa and leads to the release of prostaglandins (Mahmood *et al*., 2017). Ricinoleic acid binds to the prostanoid receptor E3; the concentration of cytosolic cyclic adenosine phosphate (cAMP) decreases, which causes Ca^{2+} desensitization (Bos *et al*., 2003; Fukata *et al*., 2001). The trigger for the contraction of smooth muscle cells is Ca^{2+}; thus, its reduction inhibits the contraction of the intestinal smooth muscle. In addition, ricinoleic acid promotes the release of nitric oxide (NO) and the activation of adenylate cyclase, while reducing the activity of ATPase pump. This effect lowers the absorption of potassium and sodium ions, thereby leading to the accumulation of electrolytes and water and triggering diarrhea (Dos Santos Negreiros *et al*., 2019).

Our study evaluated the potential antidiarrheal effect of SBE. The extract acted by reducing the urgency and frequency of defecation of mice in the castor oil-induced diarrhea model. It was found that SBE could delay the initial semi-solid onset time and reduce the EI.

One of the mechanisms of antidiarrheal effect is to reduce the motility of the gastrointestinal tract (Russell *et al*., 2019). The isolated rabbit jejunum smooth muscles were used to study the dose-dependent spasmolysis as well as the putative mechanisms of the spasmolytic effect of SBE.

The results revealed that SBE could inhibit the spontaneous contraction of the jejunum smooth muscle in a concentration-dependent manner. ACh is one of the principal neurotransmitters in the enteric nervous system (Huang *et al*., 2019). By activating the M receptors on the membrane of the intestinal smooth muscle cells, ACh couples G protein with adenylate cyclase, decreases the concentration of intracellular cAMP and increases the intracellular Ca^{2+} concentration, thereby increasing the contraction, tension, and peristalsis of the intestinal tract (Guo *et al*., 2014; Delvalle *et al*., 2018). It was found that SBE could antagonize the contraction of jejunum induced by ACh. Therefore, the antispasmodic effect of SBE on isolated smooth muscle may be regulated by blocking the M receptors.

Smooth muscle contraction is triggered by free Ca^{2+} in the cytoplasm (Li *et al*., 2019). Extra cellular Ca^{2+} enters the cells via voltage-gated L-type Ca^{2+} channels present on the cell surface; on the other hand, intracellular sarcoplasmic reticulum releases Ca^{2+}, which increases the intracellular free Ca^{2+} concentration (Abdur *et al*., 2017). Therefore, whether SBE blocks the Ca^{2+} channel or decreases the release of Ca^{2+} stored in the cells, the contraction of intestinal smooth muscle will be weakened (Li *et al*., 2018). The high concentration of K^+ induces the depolarization of muscle cells and activates the voltage-dependent L-type Ca^{2+} channel on the cell membrane, thus allowing the extra cellular Ca^{2+} to enter the cells and promoting the contraction of the intestinal smooth muscle (Türck and Leonhard, 2010; Ventura *et al*., 2011).

Therefore, the presence of Ca^{2+} channel antagonists may alleviate the contraction induced by the high concentration of K^+ in intestinal smooth muscle (Khan *et al*., 2016). To verify whether the mechanism is related to voltage-dependent L-type Ca^{2+} channel, we used a high K^+ solution to induce smooth muscle contraction and added the cumulative external Ca^{2+} to the Tyrode's solutions containing SBE or verapamil. Verapamil is a Ca^{2+} channel antagonist (Awe *et al*., 2011). With the increase in $CaCl_2$ concentration, the concentration–response curves shifted downward and to the right, which was similar to that of verapamil. Therefore, the relaxing effect of SBE on the jejunal smooth muscle might be regulated by blocking the voltage-gated L-type Ca^{2+} channels.

CONCLUSION

The results demonstrate that SBE exerts significant antidiarrheal effect in castor oil-induced diarrheal mice. In addition, SBE could relax the spontaneous contraction of the jejunum as well as the contraction induced by ACh (10^{-5} M) and KCl (60 mM) in rabbits. The mechanism may be related to the inhibition of the Ca^{2+} channel, which provides a pharmacological basis for the use of SBE in gastrointestinal disorders.

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