

# Antidiarrheal Activity of Ethanol Extract of *Ophioglossum Vulgatum* In Mice And Spasmolytic Effect on Smooth Muscle Contraction of Isolated Jejunum In Rabbits

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**Research**

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# Abstract

## Background

*Ophioglossum vulgatum* Linn. (Ophioglossaceae) (OV), which is traditionally used on wounds and burns, enjoys a reputation as the king of medicine in Taiwan. There are few studies on its role in gastrointestinal diseases. Our aim was to assess the antidiarrheal and spasmolytic effect of the ethanol whole plant extract of *Ophioglossum vulgatum* (EWOV).

## Methods

Study was conducted from June 2018 to July 2019. The chemical constituents of EWOV were analyzed by high performance liquid chromatography (HPLC). *In vivo*, the antidiarrheal activity of EWOV (125, 250 and 500 mg/kg; orally) in castor oil-induced Kun Ming mice was evaluated. *In vitro*, the effect of EWOV (0.01-10 mg/mL) on the spontaneous contraction of isolated rabbit jejunum smooth muscle was studied. Verapamil was the positive control group in both vivo and vitro studies. The jejunum stripes were pre-contracted by ACh ( $10^{-5}$  M) and KCl (60 mM) which could induce the jejunum spasm. The possible spasmolytic effect was analyzed in the pretreatment of the jejunum preparations with EWOV (0.3, 1 mg/mL) or verapamil (0.03, 0.1  $\mu$ M) in  $Ca^{2+}$ -free and high- $K^{+}$  (60 mM) solution containing EDTA.

## Results

EWOV (250 and 500 mg/kg) exhibited antidiarrheal effect. EWOV (0.01-10 mg/mL) inhibited the spontaneous and ACh/KCl-induced contraction with an  $EC_{50}$  value of 1.46 (0.89-2.04), 1.06 (0.63-1.48) and 0.48 (0.29-0.67), and it shifted the concentration-response curves of  $CaCl_2$  to right with decreased in max, similar to verapamil.

## Conclusions

EWOV has significant antidiarrheal and spasmolytic effect, possibly by mediating calcium channel blocking activity, this provides the pharmacological basis for use in gastrointestinal disorders.

## Background

Diarrhea is a disease characterized by increased defecation, diluted stool, often associated with the discharge of mucus or undigested food [1]. Diarrhea is one of the main reasons of morbidity and mortality in developing countries, especially, the death of children [2]. In 2016, it accounted that more than 1300 children die per day and approximately 480,000 die per year [3]. In view of the high incidence of diarrhea and the side effects of Western medicine, the World Health Organization (WHO) is encouraging the search for traditional Chinese antidiarrheal herbs. [4]. *Ophioglossum vulgatum* Linn. (OV) (Drug Administration of Guizhou Province, China, 2003) is a little fern and the whole plant has been used as a Chinese herb "Yizhijian" for several hundred years [5]. OV has the functions of clearing heat and

detoxifying toxins, activating blood circulation and dispersing blood stasis, treating hepatitis and pneumonia, etc. [6]. Studies show that the characteristic chemical constituents of OV are flavonoids, glycerides and amino acids [5]. Quercetin is a flavonoid compound, which widely exists in vegetables, fruits and Chinese herbal medicines. It has been shown that quercetin has many biological activities such as antioxidation, anti-inflammation, antibacterial, and antimutation [7, 8, 9, 10]. In addition, quercetin can inhibit the metastasis of gastric, colon and lung cancer [11, 12, 13]. It is extremely important that quercetin also has spasmolytic effect on intestinal smooth muscle, which can delay intestinal transport and inhibit intestinal peristalsis [14], it provides clues for us to assess the antidiarrheal and spasmolytic effect of EWOV.

## Methods

### Chemicals and Drugs

Acetylcholine chloride was from Chengdu Huaxia Chemical Testing Co., Ltd. (Chengdu, China). Sodium bicarbonate, magnesium sulphate, potassium chloride, glucose, sodium dihydrogen phosphate, calcium chloride and sodium chloride were produced by Chengdu Chemicals Co., Ltd. (Chengdu, China). Distilled water was used for the preparation of reference solutions, sample solutions, diluents, and physiological salt solutions (Tyrodé's solutions). Verapamil was from MedChemexpress Co., Ltd. (USA). The castor oil was from Hualong Pharmaceutical Co., Ltd. (Henan, China). The quercetin was from Chengdu Alpha Biotech Co., Ltd. (Chengdu, China). All research-grade chemicals were used in experimental work.

### Plant material and preparation of EWOV

The whole plant of OV was collected from Nanchong (Sichuan, China) in the month of June 2017. In the process of collection, attention should be paid to the protection of ecological environment. On the premise of "keeping roots and preserving species", appropriate amount of plants should be collected for this study. OV was identified by teacher Lan Yang, who was from School of Pharmacy, North Sichuan Medical College and the voucher specimen (CBY-2017-0003) was deposited in the herbarium of the same institution. The whole plant of OV was dried in an electric oven at constant temperature (50 °C) and pulverized into a coarse powder (shredding machine: FW177, Taisite, Tianjin, China). The preparation process of EWOV was as follows: OV powder(100 g) were immersed with 75% ethanol and performed ultrasonic-assist extraction for 3 times (1 h each). All the extracting solution was merged for rotatory evaporation till no ethanol under reduced pressure(50 °C) (Rotary evaporator: RE-52AA, Yarong, Shanghai, China).The extract was further dried thoroughly in a vacuum decompression drying oven (ZK 6050B, Opson, Wuhan, China) and preserved in a vacuum desiccator at 4 °C until further use. Obtained EWOV was dark greenish brown solid and weighed 21.06 g, the percentage yield of OV powder was 21%.

### Animals

Adult male Kun Ming mice weighing 18–22 g and locally bred rabbits weighing 2.0-2.5 kg (License No. : SYXK (Chuan) -2018-076) were provided by the Animal Laboratory Center of North Sichuan Medical

College (Sichuan, China). The 12 h light-dark cycle (temperature 23–26 °C, humidity 70 ± 5%) was maintained for the animals, and the white wood chips were used as bedding while animals were given free access to water, but fasted for 24 h before the experiment.

### Ethics and Consent to Participate

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 and experimental practices.

## Phytochemical study

### Preparation of the reference solution and the sample solution

Quercetin was accurately weighed and with 75% ethanol as solvent, to prepare a reference solution with 8.928 µg/mL quercetin. The solution was passed through a 0.22 µm nylon microporous membrane and kept at 4 °C before use.

Appropriate amount of EWOV (0.2127 g/g DW) was accurately weighed and with 75% ethanol as solvent, to prepare a sample solution with 40.05 mg/mL (Crude drug concentration) EWOV, The solution was filtered by a 0.22 µm nylon microporous membrane, and then used for HPLC analysis.

### Chromatographic conditions

The reference and sample solutions were analyzed by an Agilent-1220 high performance liquid chromatograph system (Agilent, American). The column was Kromasil 100-5-C<sub>18</sub> (250 mm × 4.6 mm, 5 µm). 0.2% acetic acid was used as mobile phase A, chromatographic acetonitrile was used as mobile phase B. Then the mobile phase was filtered by passing through a 0.45 µm filter membrane. The column loaded with the reference solution and the sample solution was run with a mobile phase consisting of acetonitrile and 0.2% acetic acid water (27 : 73, pH = 2–3) for the determination of quercetin from EWOV. The detection wavelength was 360 nm, and the flow rate was 1.0 mL/min. The column temperature was 30 °C, and a sample of 10 µL of the solutions was directly injected.

### Total flavonoid content

The Total flavonoid content of EWOV was determined by a reported method (Shima et al. 2019). Appropriate amount of EWOV in 25 mL brown glass flask volumetric was mixed with 1.0 mL 5% NaNO<sub>2</sub> (w/v), after 6 min interval, 1.0 mL 10% AlCl<sub>3</sub> (w/v) was added, then 10 mL 4% NaOH (w/v) solution was added in order. The reaction mixture was allowed to incubate for 15 min at room temperature before the absorbance was detected at 510 nm. Water (2 mL) was used to substitute aluminum chloride for blank. Rutin was used as a standard for the calibration curve. The result was expressed as rutin mg/g rutin equivalents after triplicate analysis.

# Total polyphenol content

The content of total polyphenols in EWOV was determined by Folin-Ciocalteu (F-C) colorimetry as Gallic acid was used as the reference material. Appropriate amount of EWOV in 25 mL brown glass flask volumetric was mixed with 1.0 mL F-C reagent, after 3 min interval, 1.0 mL 10% Na<sub>2</sub>CO<sub>3</sub> (w/v) was added, ultrapure water for volume fixing and shake well, reaction for 2 h in 25 °C constant temperature water bath, then the absorbance was measured at the wavelength of 765 nm.

## In vivo experiments

### Acute oral toxicity test

Mice were kept on fasting for 24 h before the experiment, and were divided into 6 groups with 10 mice in each group. EWOV was diluted with normal saline to different concentrations. According to the dosage of 500, 1000, 2000, 4000, 8000, 16000 mg/kg body weight of EWOV, each mouse in the experimental group was given 0.2 mL EWOV normal saline diluent orally. Any signs of toxicity and death were strictly documented for 14 days after administration. During these days, the mice were free to access water and food. A dose-response curve for the determination LD<sub>50</sub> was established. The safety of EWOV was assessed with the single maximum dose [15].

### Castor oil-induced diarrhea

Reference to the method of Gong et al. (2017) and Guo et al. (2014), this study first carried out a preliminary experiment. The mice were screened by giving 0.4 mL castor oil and those presenting with diarrhea were randomly divided into 5 groups with 10 mice in each. Random numbers were generated using the standard = RAND() function in Microsoft Excel. The negative control group was given 0.2 mL normal saline (20 mg/kg). While the positive group was given 0.2 mL verapamil (50 mg/kg) and the test groups were administrated orally with EWOV (125, 250 and 500 mg/ kg) respectively. Each mouse was caged individually and blotting paper placed under it. Castor oil (20 mL/kg) was provided orally, the subsequent onset of castor oil-induced diarrhea was observed after half an hour of treatment. The amount of solid feces, semi-solid feces, liquid feces and the time of initial semi-solid appearance was recorded within 4 h after castor oil. The following formula was used to evaluate the severity of diarrhea. Evacuation Index (EI) = solid feces × 1 + semi-solid feces × 2 + liquid feces × 3 (The distribution was as follows: 1 referred to solid feces, 2 referred to semi-solid feces, and 3 referred to liquid feces)

## In vitro experiments

### Preparation and calibration of isolated tissues (Rabbit jejunum)

The local bred rabbits were killed after 24 h of fasting then the tissues (jejunum) were isolated. Isolated rabbit jejunum smooth muscle was used to study the spasmolytic action and likely mechanism of action of EWOV. Rabbits were sacrificed by skull impingement. The isolated jejunum was cut into 2–3 cm and

was flushed in 4 °C Tyrode's solution. Then it was mounted vertically in a tissue bath (20 mL) comprising of Tyrode's solution, which was kept at normal body temperature of  $37 \pm 0.5$  °C with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> aerated. After preloading 1 g, the jejunum was balanced to reach a stable level before the active compounds were added. Jejunum activity was measured with a force sensor and recorded by BL-420F Physiological Signal Collection and Handling System.

## **Effect of EWOV on spontaneous contraction and ACh/KCl-induced contraction of rabbit jejunum**

Rabbit isolated jejunum smooth muscle showed spontaneous contraction under the standard experimental settings. After an equilibrium period of jejunum in Tyrode's solution, cumulative concentrations of EWOV (0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/mL), quercetin (0.3, 1, 3, 10, 30, 100 µM), verapamil (0.01, 0.03, 0.1, 0.3, 1, 3, 10 µM) were added to determine the effect on the spontaneous contraction.

To investigate the spasmolytic activity and mechanism of EWOV, the isolated jejunum smooth muscle was pretreated with ACh ( $10^{-5}$  M) and KCl (60 mM). When the ACh/KCl-induced contraction reached a stable level, EWOV (0.01, 0.1, 1, 3, 10 mg/mL) was added in the test group. While the positive group was treated with verapamil (0.003, 0.03, 0.3, 1, 3 µM).

## **Effect of EWOV on CaCl<sub>2</sub>-induced cumulative contraction**

To determine the effect of EWOV on Ca<sup>2+</sup> influx, the jejunum smooth muscle was stabilized in Tyrode's solution and was then incubated with Ca<sup>2+</sup>-free and high-K<sup>+</sup> (60 mM) solution containing EDTA (0.1 mM) for 30 min in order to eliminate Ca<sup>2+</sup> from tissue, followed by a Ca<sup>2+</sup>-free and high-K<sup>+</sup> (60 mM) for 15 min. The samples were then treated in the absence and presence of EWOV (0.3, 1 mg/mL) and verapamil (0.03, 0.1 mM), Ca<sup>2+</sup> was added in a cumulative manner ( $3 \cdot 10^{-5}$ - $3 \cdot 10^{-2}$  M) to obtain the concentration-response curves of CaCl<sub>2</sub>. The contraction induced by  $3 \cdot 10^{-2}$  M CaCl<sub>2</sub> was regarded as 100% in the absence of EWOV and verapamil.

## **Statistical analysis**

Being expressed as mean  $\pm$  standard error (SEM), all data were analyzed by single-line statistical significance variance analysis (ANOVA) followed by the Dunnett's test. SPSS 19.0 system was used for testing.  $P \leq 0.05$  was considered statistically significant.

## **Results**

### **Chemical content of EWOV**

Under the optimal liquid chromatography conditions, the separation between quercetin and other chemicals peaks were excellent. The reference solution and the sample solution chromatography profiles

were shown in (Figure. 1). The reference solution (Figure. 1A) and the sample solution (Figure. 1B) had corresponding chromatographic peaks at the same retention time. Figure. 1B illustrates the chromatograms obtained from EWOV. The content of quercetin in EWOV was 0.1924%. Meanwhile, the total flavonoids content of EWOV was 2.74% (27.4 mg/g), and the total polyphenols content of EWOV was 1.47% (14.70 mg/g).

## Acute oral toxicity test

In the LD50 test, after EWOV was given in the increasing doses (500, 1000, 2000, 4000, 8000, 16000 mg/kg) of intragastric administration, there were no death or changes in physical behavior during the observation period in the single maximum dose trial. Based on these results, the LD50 value was estimated to be > 16000 mg/kg.

## Castor oil-induced diarrhea

EWOV (125, 250 and 500 mg/kg; orally) exhibited dose-dependent antidiarrheal activity. The negative control group (saline) did not show protection against diarrhea. The onset time of semi-solid feces in negative control group and the positive group were  $57.9 \pm 3.70$  and  $80.80 \pm 3.77$  min, while the EI scores were  $13.00 \pm 1.05$  and  $6.5 \pm 1.08$ . The EI scores in test group were  $12.79 \pm 1.70$ ,  $10.10 \pm 1.37$  and  $8.70 \pm 1.16$ , respectively ( $p > 0.05$ ,  $p < 0.05$ ,  $p < 0.01$ ), while the onset time of semi-solid feces were  $73.00 \pm 3.88$ ,  $77.00 \pm 3.43$ ,  $85.20 \pm 3.99$  min ( $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ). The results showed that the EI scores decreased and the onset time of semi-solid feces prolonged in the test group, same to the positive group ( $p < 0.05$ ). Overall, EWOV showed significant antidiarrheal activity compared with the negative control group (Figure. 2).

## Effect of EWOV on spontaneous or ACh/KCl-induced contraction of rabbit jejunum

Rabbit jejunum was put in Tyrodé's solution. EWOV (0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/mL) inhibited the isolated jejunum in a concentration-dependent manner with an  $EC_{50}$  value of 1.46 mg/mL (95% CI, 0.89–2.04,  $n = 6$ ), similar to verapamil (0.01, 0.03, 0.1, 1, 3, 10  $\mu$ M) with an  $EC_{50}$  value of 0.23  $\mu$ M (95% CI, 0.19–0.27,  $n = 6$ ) (Figure. 3). Furthermore, quercetin (0.3, 1, 3, 10, 30, 100  $\mu$ M) inhibited the isolated jejunum in a concentration-dependent manner with an  $EC_{50}$  value of 7.91  $\mu$ M (95% CI, 7.24–8.63,  $n = 6$ ) (Figure. 4). EWOV also inhibited the contractions induced by ACh ( $10^{-5}$  M) and KCl (60 mM) in a concentration-dependent manner, with respective  $EC_{50}$  values of 1.06 mg/mL (95% CI, 0.63–1.48,  $n = 6$ ) and 0.48 mg/mL (95% CI, 0.29–0.67,  $n = 6$ ), similar to verapamil at 0.001–3  $\mu$ M with an  $EC_{50}$  value of 0.28  $\mu$ M (95% CI, 0.25–0.33,  $n = 6$ ) and 0.12  $\mu$ M (95% CI, 0.08–0.15,  $n = 6$ ). The maximum suppression ratios were 92.76% and 95.50% (Figure. 5).

## Effect of EWOV on $CaCl_2$ -induced cumulative contractions

Our results indicated that EWOV antagonized the contraction of isolated tissue induced by cumulative concentration of  $CaCl_2$ . EWOV (0.3, 1 mg/mL) could move the cumulative  $CaCl_2$  curve to the right with a

decrease in the maximum. Verapamil, the calcium antagonist, also moved the  $\text{CaCl}_2$  curve to the right with a decrease in the maximum. Compared with the negative group, EWOV (0.3, 1 mg/mL) and verapamil (0.03, 0.1  $\mu\text{M}$ ) reduced the maximum contraction induced by 0.3 mM  $\text{CaCl}_2$  to  $48.26 \pm 2.27\%$ ,  $69.53 \pm 1.93\%$ ,  $59.06 \pm 1.11\%$  and  $73.14 \pm 1.63\%$  ( $p < 0.001$ ) respectively (Figure. 6).

## Discussions

As mentioned in the introduction, diarrhea has a high incidence rate and is a worldwide public health problem, we will elaborate further on its classification and pathogenesis. The classification of diarrhea can be roughly divided into osmotic diarrhea, secretory diarrhea, exhaling diarrhea, absorptive disturbance diarrhea and gastrointestinal motility disorder diarrhea [16]. Diarrhea is an intestinal disease caused by multiple pathogens and factors, and its pathological basis is inflammation and edema of mucosa, hyperactivity of intestinal secretion and motor function, the occurrence of loose stools, frequent stools and other symptoms under the stimulation of infection or non-infection factors of the intestinal tract [17]. In recent years, Chinese herbal medicines have been widely used in the medical system [18]. Compared with chemical substances, Chinese herbal medicines are safer [19]. Chinese herbal medicine also has the advantages of rapid curative effect, less toxic and side effects and low cost in the treatment of diarrhea [20]. Therefore, it is necessary to develop natural Chinese herbal medicines for the treatment of diarrhea. OV is one of the commonly used Chinese medicinal materials in clinics, mainly distributed in the provinces of Yunnan, Sichuan, Hubei, Guizhou and Tibet [21], which has anti-inflammatory, antibacterial, antitumor, and other pharmacological effects [22]. It is widely used in clinical practice and has high medicinal value.

In the acute toxicity experiment, the single dose of EWOV was 16000 mg/kg, and the mice did not cause any death or toxic effects during the observation period. According to Lorke [23], any 5 g/kg non-toxic substance can be considered relatively safe. The above conclusions demonstrate the safety of EWOV and provide necessary theoretical basis for subsequent experiments. Castor oil is from castor seed and is a kind of effective laxative, which can reduce the absorption of small intestine and increase the secretion of it [24]. The active ingredient of castor oil inducing diarrhea is ricinoleic acid, which produces irritating and inflammatory actions on the intestinal mucosa and leads to the release of prostaglandins [25]. In addition, it also can promote the release of nitric oxide (NO) and the activation of adenylate cyclase, reduce the activity of ATPase pump, so as to reduce the absorption of potassium sodium ions, which leading to the accumulation of electrolytes and water, then diarrhea phenomenon appears [3]. Therefore, the castor oil-induced diarrheal model including secretory and motility diarrhea [25]. In the castor oil-induced diarrhea model in mice, EWOV can delay the onset time of semi-solid and reduce the diarrhea index, showing significant antidiarrheal effects. Chemical composition studies show that the characteristic chemical composition of EWOV are flavonoids and glycoside compounds, particularly, flavonoids can prevent diarrhea by reducing intestinal peristalsis and hydro-secretion [26] and inhibiting the release of autacoids and prostaglandins [27]. Based on the experimental results, we can infer that

EWOV may inhibit the castor oil-induced diarrhea in mice by inhibiting the release of prostaglandin and reducing gastrointestinal smooth muscle propulsion.

In order to explore the mechanism of EWOV, we extended it to rabbit isolated jejunum smooth muscle experiment. In the experiment of spontaneous contraction of isolated jejunum smooth muscle, both EWOV and quercetin showed concentration-dependent inhibition. Intestinal smooth muscle has low excitability and self-discipline, and its contraction is mainly regulated by intracellular  $\text{Ca}^{2+}$  concentration [28]. Intracellular  $\text{Ca}^{2+}$  is increased mainly through voltage-dependent  $\text{Ca}^{2+}$  channel inflow or release from intracellular storage in the sarcoplasmic reticulum [25]. Therefore, the blocking of  $\text{Ca}^{2+}$  channels or the reduction of  $\text{Ca}^{2+}$  release from intracellular storage will weaken the contraction of intestinal smooth muscle. ACh is one of the most important neurotransmitters in gastrointestinal tract, which can bind to muscarinic M receptor and involve the activation of PLC, leading to the generation of inositol-1, 4, 5-trisphosphate (IP3), which induces  $\text{Ca}^{2+}$  release and leads to small intestine contraction [29, 30]. The experimental results showed that EWOV could significantly relax the contraction of ACh ( $10^{-5}$  M) induced jejunum smooth muscle in vitro. Therefore, we believed that EWOV's antispasmodic effect on the jejunum tract might be related to blocking M receptor and reducing  $\text{Ca}^{2+}$  internal flowing. High concentrations of  $\text{K}^{+}$  can horizontally open voltage-dependent L-type calcium ion channels and allow extracellular  $\text{Ca}^{2+}$  to enter the cell, thereby promoting contraction of intestinal smooth muscle [31, 32]. Therefore, the existence of calcium antagonists is suggested as the substance that relaxing the contraction induced by high- $\text{K}^{+}$ . To verify this hypothesis, as verapamil is an L-type calcium channel blocker [33], we used KCl (60 mM) solution to induce jejunum contraction and used verapamil as the control group. The results showed that EWOV could relax the contraction of jejunum smooth muscle induced by KCl (60 mM) solution, and with the increase of EWOV dose, the concentration-response curves of  $\text{CaCl}_2$  moved to the right and down, which was similar to verapamil, suggesting that EWOV inhibited the contraction of jejunum smooth muscle may be related to blocking calcium channel.

## Conclusion

The results showed that EWOV had significant antidiarrheal effect in castor oil-induced diarrheal mice. In addition, EWOV 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.

## Abbreviations

OV: *Ophioglossum vulgatum* Linn.; EWOV: ethanol whole plant extract of *Ophioglossum vulgatum*; HPLC: high performance liquid chromatography; WHO: the World Health Organization; EI: evacuation index; IP: inositol-1, 4, 5-trisphosphate.

## Declarations

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## Ethics approval and consent to participate

**The statement on ethics approval is provided in the supplementary material.**

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no conflict of interests regarding this work.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

JZ organized all the experiments. LY determined the contents of EWOV. JZ and YF were the major contributors in writing the manuscript. JL and CG studied the contraction of the jejunum smooth muscle. Other authors were involved in the whole experiments. All authors read and approved the final manuscript.

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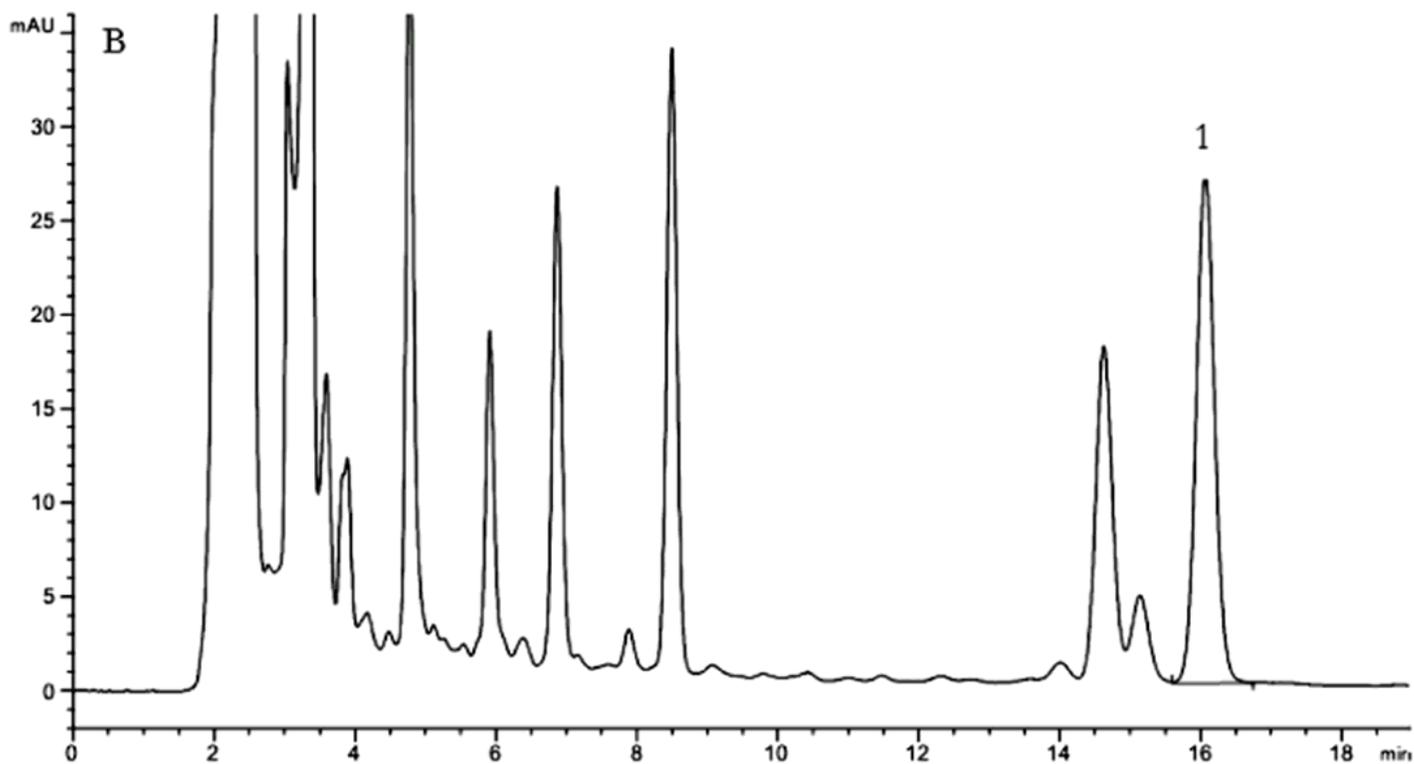
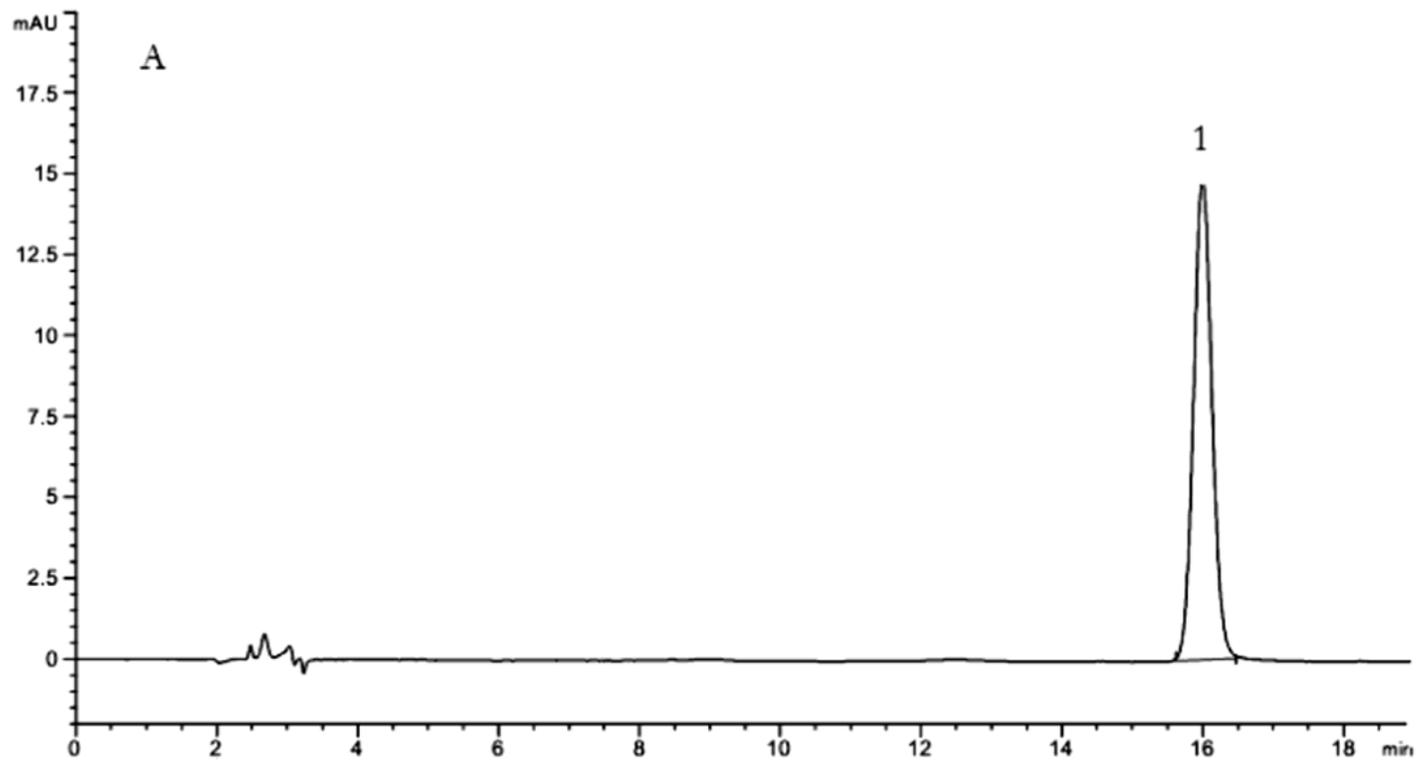
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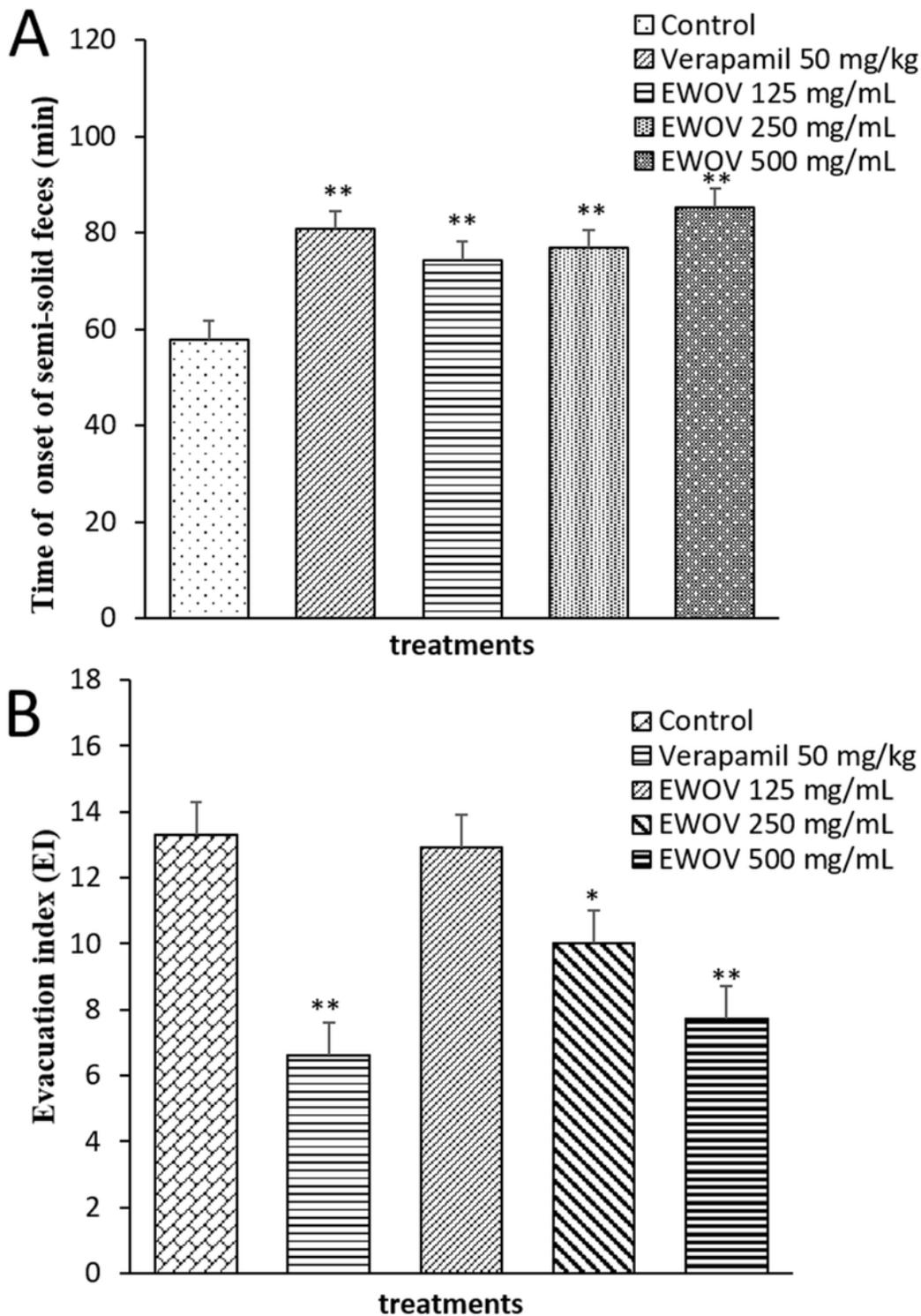
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## Figures



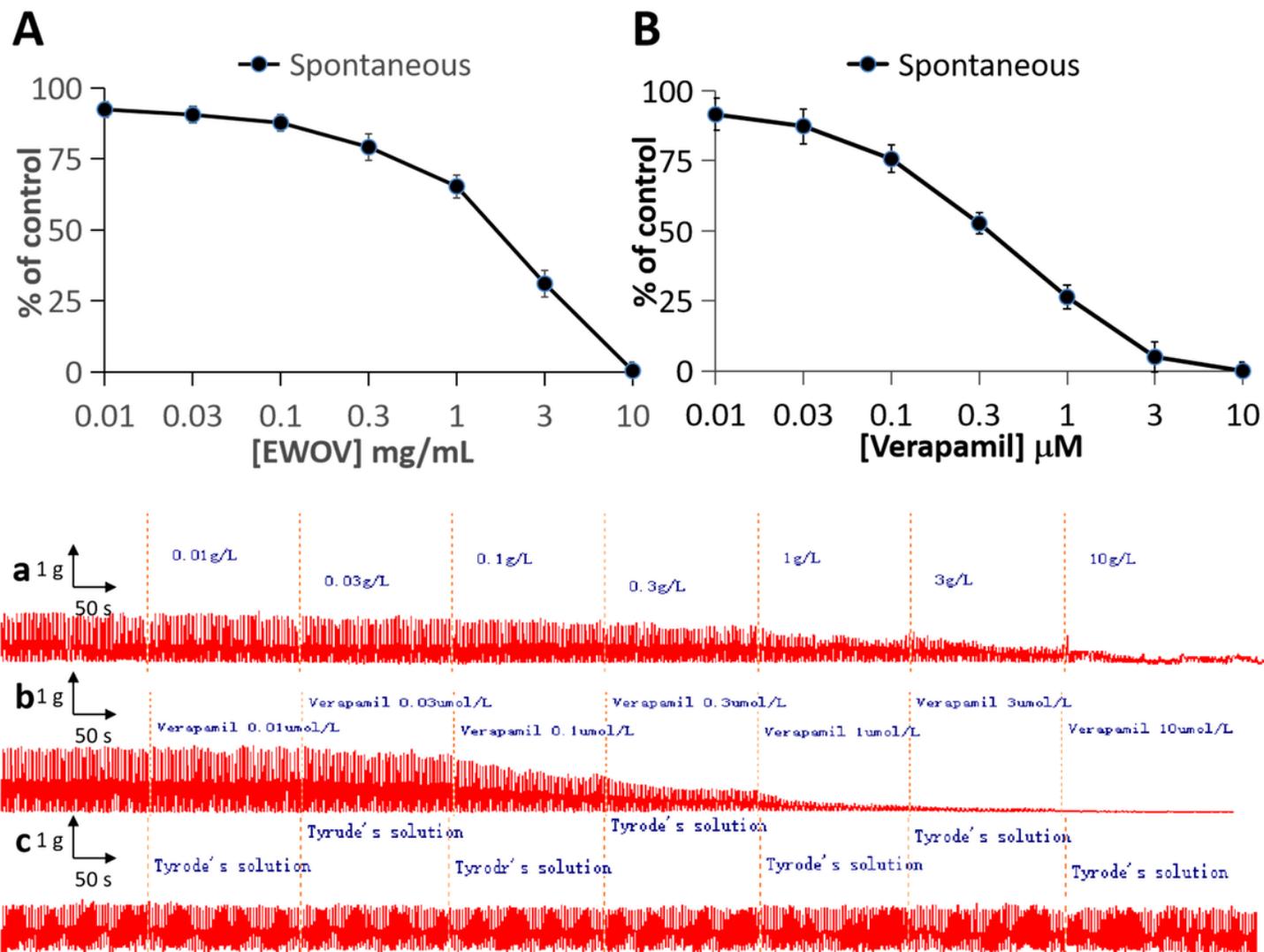
**Figure 1**

HPLC chromatograms of reference substances (A) and EWOV (B) (1 quercetin). A. HPLC chromatogram of the stand ard. B. HPLC chromatogram of EWOV.



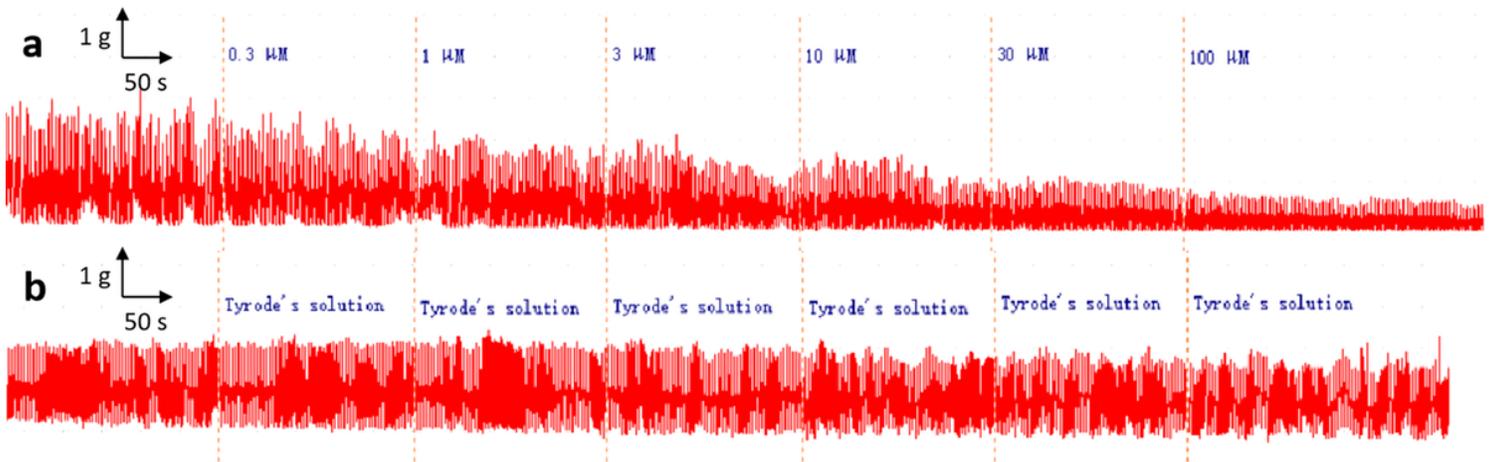
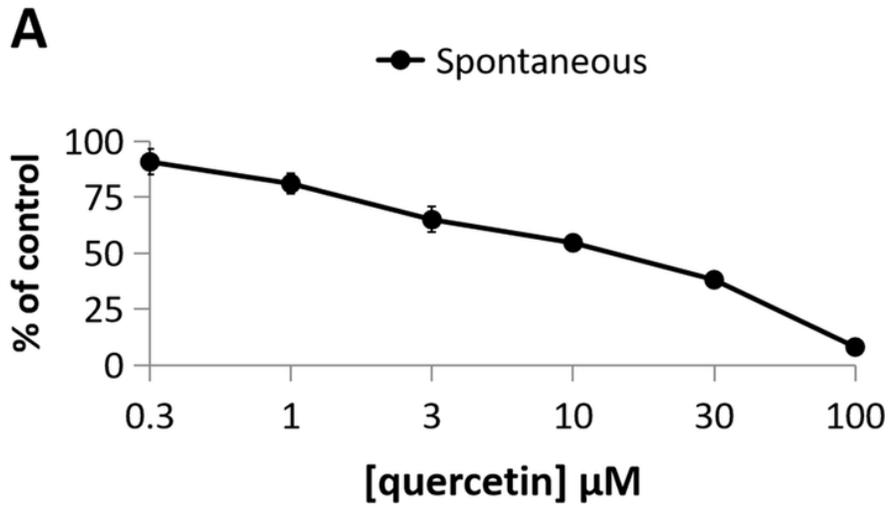
**Figure 2**

Effect of EWOV on castor oil-induced diarrhea in mice. A. Evacuation index (EI); B. The onset of semi-solid feces (min). Results are mean  $\pm$  SEM, n = 10. Results test was performed, \*p < 0.05, \*\*p < 0.01, \*\*\* p < 0.001.



**Figure 3**

Effect of Tyrode's solution on spontaneous contraction of isolated jejunum smooth muscle. Tracings showing A (a) spasmolytic effect of EWOV B (b) spasmolytic effect of verapamil (c) spontaneous contraction of isolated rabbit jejunum. Plant extract was added in cumulative manner and Values listed were the final tissue bath concentrations, Results are mean  $\pm$  SEM, n = 6.



**Figure 4**

Effect of (a) Tyrodé's solution and (b) quercetin on spontaneous contraction of isolated jejunum smooth muscle and the concentration-response curve of quercetin (A). Results are mean  $\pm$  SEM, n = 5.

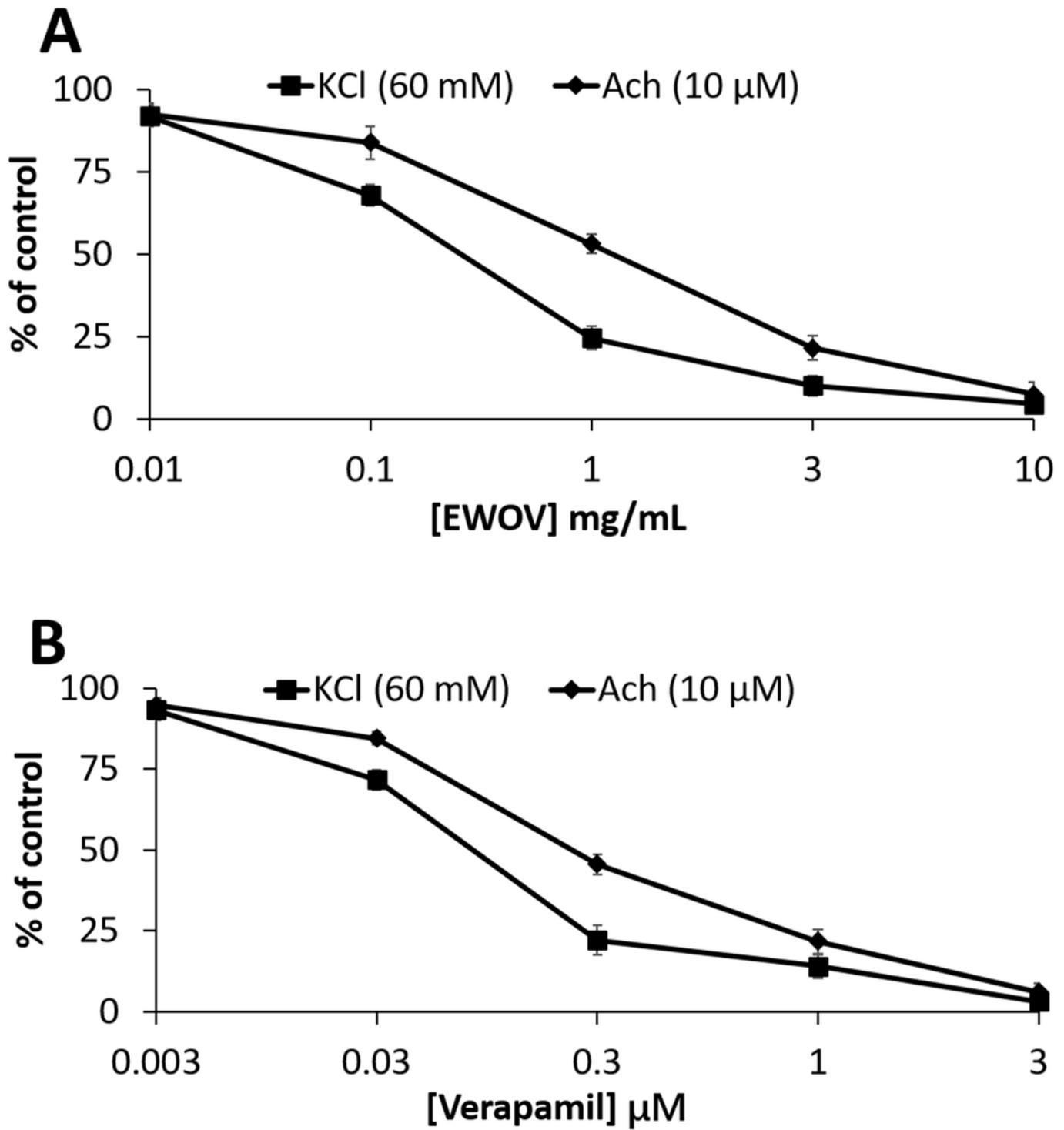
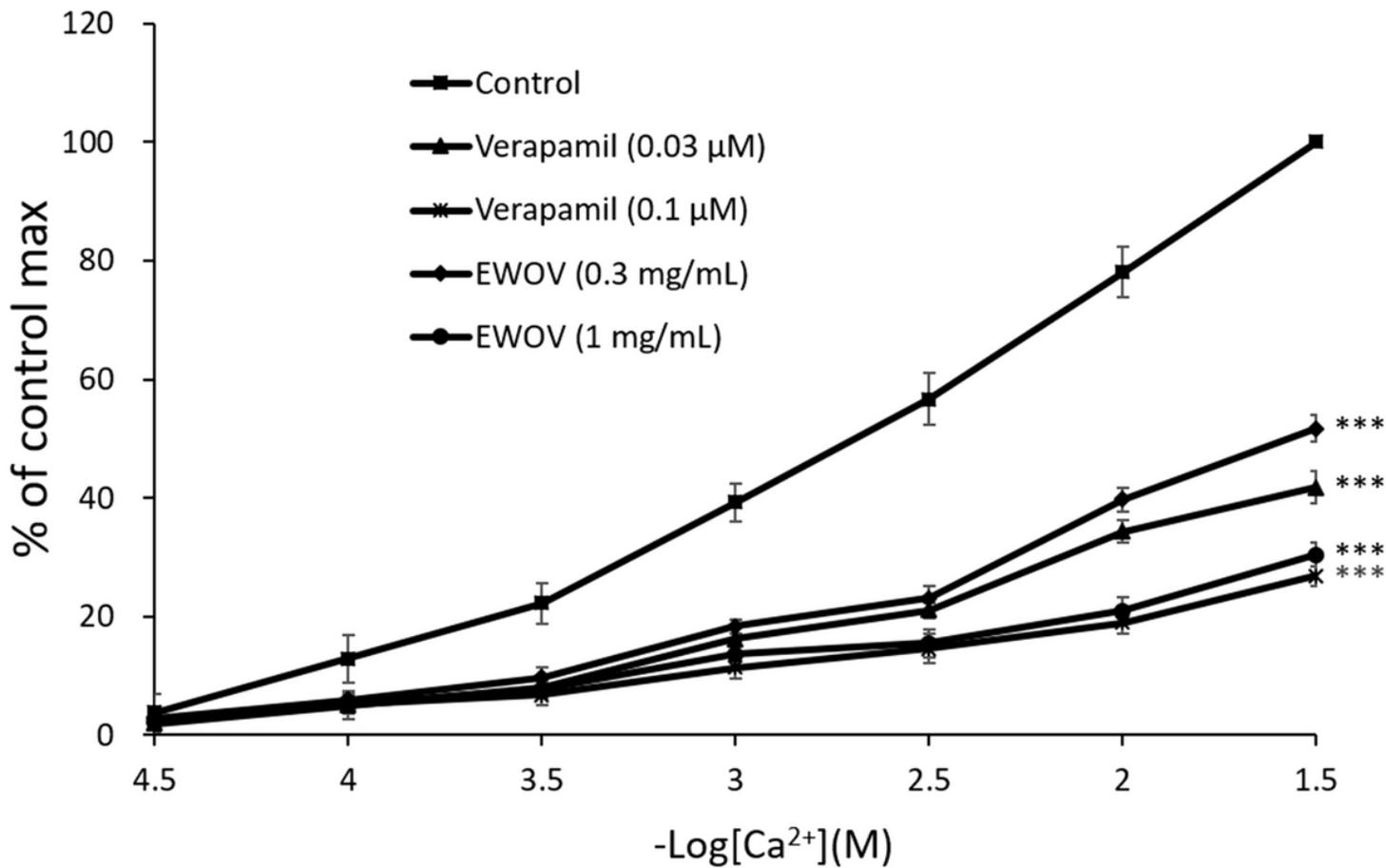


Figure 5

Concentration-dependent inhibitory effect of (A) EWOV and (B) verapamil on ACh (10-5 M) and K+ (60 mM) induced pre-contracted isolated jejunum. Results are mean  $\pm$  SEM, n = 6.



**Figure 6**

Concentration-response curves of CaCl<sub>2</sub> in the presence of verapamil (0.03, 0.1 μM) and EWOV (0.3, 1 mg/mL). Results are mean ± SEM, n = 6. Results test was performed, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.