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Prevention of secretory diarrhea by ethanol extract of *Bistortae rhizoma* through inhibition of chloride channel

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Abstract

Inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) and Ca²⁺-activated Cl⁻ channel (CaCC) represents an attractive approach for the treatment of secretory diarrhea. The aim of the study is to investigate the molecular basis of the anti-diarrheal effect of traditional Chinese herbal antidiarrheal medicine *Bistortae rhizoma*. Fluorescence quenching assay indicated that the 40% methanol /water fraction (D5) dose-dependently inhibited both CFTR and CaCC function in transfected Fischer rat thyroid (FRT) cells. *Ex vivo* studies indicated that D5 inhibited both forskolin (FSK)-activated CFTR current and CCh-induced CaCC current in rat colonic mucosa. In the mouse closed-loop model, intraluminal application of D5 (200 µg/mL) significantly reduced cholera toxin-stimulated fluid secretion. In the intestinal motility model, D5 significantly delayed intestinal peristalsis in mice. Our research suggests that CFTR and CaCC-mediated intestinal epithelial Cl⁻ secretion inhibiting and gastrointestinal motility delaying may account for the anti-diarrheal activity of *B. rhizoma*.

Introduction

Intestinal fluid and electrolyte secretion is pivotal for digestion, absorption and stool movement. The process is elaborately regulated by coordinated activities of enterocyte ion channels. Direction and rate of fluid movement in intestine are decided by activities of chloride channels at the apical membrane. Chloride-dependent intestinal secretion is regulated by cyclic nucleotide and/or Ca²⁺ signaling pathways. Cystic fibrosis transmembrane conductance regulator (CFTR), cloned and characterized by Riordan and colleagues in 1989 (Riordan et al., 1989), has been associated with cAMP-dependent Cl⁻ secretion. Although Ca²⁺-activated Cl⁻ channel (CaCC) was speculated to exist for quite some time, the first CaCC, anoctamin 1 (ANO1 or TMEM-16A), was unveiled by three independent groups until

2008 (Caputo et al., 2008; Schroeder et al., 2008). Because CFTR and CaCC involve in major enterotoxin-induced fluid secretion in the intestine, they represent attractive targets for diarrhea therapy.

CFTR is localized predominantly in the intestinal crypt cells, and it is the Cl⁻ pathway in enterotoxin-induced secretory diarrheas caused by cholera toxin and *E. coli* heat-stable enterotoxin (Thiagarajah et al., 2004; Sonawane et al., 2007; Chao et al., 1994; Grubb, 1995; Berschneider et al., 1988; O'Loughlin et al., 1991). ANO1 is expressed in a variety of cells including the intestinal epithelial and pacemaker cells. There is strong evidence that ANO1 is involved in rotavirus and drug-related diarrheas (Morris et al., 1999). Inhibition of CFTR and CaCC has been considered an effective strategy of treating secretory diarrhea.



Traditional Chinese medicine (TCM) has been considered to be effective among herb users and patients over a long time. Although studies confirmed the efficacies of TCM (Jiang et al., 2010; Li and Peng, 2013), its pharmacological basis remains largely under investigation. Uncovering the relationships between the active ingredients and molecular targets is the principal and basic issue for transforming TCM into evidence-based medicine. Because CFTR and ANO1 are validated molecular targets for the therapy of secretory diarrheas, it is reasonable to find ingredients having CFTR and/or CaCC inhibitory activities from anti-diarrheal TCM herbs.

Bistortae rhizoma, the dry rhizome of *B. bistorta* is widely used in TCM for the treatment of a variety of diseases including secretory diarrhea. Aim of the present study is to investigate the molecular basis of the anti-diarrheal effect of *B. rhizoma*.

Materials and Methods

Plant acquisition and extraction: Chinese herb *B. rhizoma* was purchased from Dalian Chinese Medicine decoction pieces trade CO., Ltd (Dalian, China) authenticated macroscopically and microscopically according to the descriptions found in the Pharmacopoeia (National Pharmacopoeia Committee, 2010). A voucher sample (DS00346), was deposited at Liaoning Provincial Key Laboratory of Biotechnology and Drug Discovery, Liaoning Normal University. *B. rhizoma* was crushed and passed through 100 mesh screen to get 1.0 kg powder. Dry powder was extracted with 95% ethanol for 12 hours. After cooling to room temperature, the solution was filtered, collected and then further concentrated at reduced pressure to a 215 g extractum. The extractum was separated using D101 macroporous resin with a step gradient of methanol/water (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%) to get 10 fractions (D1~D10).

Cell lines, animals and compounds: Fischer rat thyroid (FRT) epithelial cells cotransfected with human wild type CFTR (Clarke et al., 2001; Harmon et al., 2010) or ANO1 (Hao et al., 2011) and YFP-H148Q/I152L fluorescence protein were cultured in Nutrient F12 coon's medium (Sigma Chemical Co., USA.) supplemented with 10% fetal bovine serum (HyClone company, USA), 2 mM L-glutamine, 100 u/mL penicillin and 100 µg/mL streptomycin in a 37°C incubator with 5% CO₂ and 95% humidity. Forskolin (FSK), genistein (Gen), IBMX, cholera toxin, indomethacin and amiloride were all purchased from Sigma Chemical Co, USA. ATP and NaI were purchased from Sangon Biotech (Shanghai) Co., Ltd. CFTR_{inh}-172 was synthesized in our own lab as described (He et al., 2004). Carbachol (CCh) was purchased from EDM Chemicals, Inc. (San Diego, CA).

Wistar rats (180-220 g) and male C57BL/6 mice (8-10 weeks) were fed a chow diet and kept under specific pathogen-free conditions at Dalian Medical University (Permit Number: SCXK liao 2008-0002, 2013-0003). The research was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Liaoning Normal University Committee on Animal Research.

Iodide influx fluorescence assay: For the measurement of fractions on CFTR inhibition, FRT cells transfected with CFTR were plated in a black wall clear bottom 96 well plate (Corning Inc. Corning, NY, U.S.A) at a density of 20,000 per well and incubated until confluence. Cells were washed three times with 200 µL PBS before 50 µL cocktail (5 µM FSK, 25 µM Gen and 100 µM IBMX) plus test compounds was added into the wells. Cocktail only was set as negative control and cocktail plus CFTR_{inh}-172 was set as positive control. After 10 min incubation, fluorescence data were recorded with FLUOstar Galaxy microplate reader (BMG Lab Technologies, Inc.) equipped with HQ500/20X (500 ± 10 nm) excitation and HQ 535/30M (535 ± 15 nm) emission filters (Chroma Technology Corp.) and syringe pumps. Iodide fluorescence was recorded continuously for 14 sec (2 sec before and 12 sec after iodide addition).

For the measurement of fractions on ANO1 inhibition, FRT cells transfected with ANO1 were plated as mentioned above until confluence. Cells were washed three times before test compounds addition into each well and incubated for 10 min. T16A_{inh}-A01 was set as positive control. Fluorescence data were recorded with the same equipment mentioned above, at 2 sec ATP was pumped into the system along with iodide.

Rat colonic short-circuit current measurement: Wistar rats were sacrificed with overdose of intraperitoneal sodium pentobarbital (100 mg/kg) and the colon was quickly removed, cut open along the mesentery and washed with ice cold Krebs-Henseleit buffer containing (in mM): 117 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 11.1 D-glucose, 1.2 KH₂PO₄, 24.8 NaHCO₃, pH 7.4. After blunt dissection of the muscularis, tissue was mounted in an Ussing Chamber system (Physiological Instruments) with each hemi-chamber filled with 5 mL KH buffer. Then the chambers were connected to a VCC MC 6 multichannel voltage-current clamp via silver/AgCl electrodes and 3 M KCl agar bridges. The solutions were continuously bubbled at 37°C with 95% O₂ and 5% CO₂. Indomethacin (10 µM) was added into both hemi-chambers to prevent the impact of prostaglandin synthesis, amiloride (10 µM) was added into mucosal side of the tissue to prevent epithelial Na⁺ transport. Transepithelial short-circuit current was recorded using Acquire and Analyze 2.3 software, voltage was clamped at 0 mV during the whole experiment.

Intestinal fluid secretion measurement: Closed loop mice model were used to evaluate the efficacy of D5 on cholera toxin-induced intestinal fluid secretion. Briefly, C57BL/6 mice were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg) after depriving of food and free of water for 24 hours, a small abdominal incision was made to expose the small intestine, four closed ileal loops (10 ~ 15 mm length) were produced proximal to the cecum with sutures. Each loop was injected with 50 μ L saline containing 0.5 μ g cholera toxin plus 10 μ g D5, 0.5 μ g cholera toxin as negative control, 0.5 μ g cholera toxin plus CFTR_{inh}-172 as positive control and saline as blank. Then the abdominal incision was closed with suture and mice were allowed to recover. Body temperature was maintained at 37°C during experiments. After 6 hours, the mice were again anesthetized and the loops were removed and measured their weight and length. Intestinal fluid secretion was measured as loop weight/length (g/cm).

Intestinal motility measurement: C57BL/6 mice were deprived of food for 24 hours, fraction D5 (20 μ g) was administered intraperitoneally or orally, after 30 min, 10% activated charcoal diluted in 5% gum arabic were administered orally for another 30 min. Mice were sacrificed and the small intestines were removed. The peristaltic index was calculated as the ratio of the length that activated charcoal traveled to the total length of the small intestine.

High performance liquid chromatographic (HPLC) analysis of active fractions: Fraction D5, (-)-epicatechin-3-gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG) were analyzed on Waters alliance HPLC system (Waters 2695) with a diode assay detector (Waters 2996), using C₁₈ column reversed-phase column (Venusil ASB, 10 × 250 mm, 5 μ m particle size, China) at 30°C. The samples were eluted at a flow rate of 1 mL/min using 2 solvent systems: (A) 0.2% acetic acid in water; (B) 0.2% acetic acid in acetonitrile. The solvent A was 5% at 0 min and 50% at 35 min, using linear gradient elution.

Statistical analysis: All data are expressed as mean \pm SE or as representative traces. Intestinal motility data were entered into and the results were analyzed using the SPSS software (Version 16). Student's t test was used to compare the mean values of continuous variables for the test and control groups, p values less than 0.05 were considered statistically significant.

Results

Fluorescence assay models of CFTR and ANO1 chloride channel inhibition: Figure 1A shows the fluorescence assay model of CFTR inhibitors. FRT cells expressing CFTR were pretreated with cocktail, fluorescence data were recorded for 14 sec, at 2 sec iodide was pumped into each well, inactive compounds led to the

decrease of fluorescence while incubation of active compounds made CFTR chloride channel blockage producing no fluorescence quenching.

As shown in Figure 1A, an assay was developed to identify ANO1 inhibitors. FRT cells transfected with ANO1 and I⁻ sensitive fluorescent protein H148Q/I152L were used in the experiments. Fluorescence was measured in response to the addition of I⁻ and ATP at 2 sec. Because of the presence of ANO1 inhibitors, there was little fluorescence quenching.

Inhibitory effect of components from *B. rhizoma* on CFTR and ANO1 chloride channel inhibitory activities: The inhibitory activity of CFTR by fractions D1~D10 was evaluated using cell-based fluorescence quenching test assay. After incubation with cocktail to produce the maximal activation of CFTR, 100 μ g/mL test compounds were added into wells and fluorescence data were recorded, I⁻ was pumped into the wells at 2 sec. As shown in Figure 1B (left panel), D1~D8 differed in degree in the inhibitory effect on CFTR in which D4 and D5 produced the maximal inhibitory activity.

ANO1 inhibition was also investigated using the fluorescence assay mentioned above. Cells were washed three times with PBS, 100 μ g/mL test compounds were added into each well and were incubated for 10 min. I⁻ and ATP were both pumped into the system at 2 sec. Similar to that of CFTR, D2~D10 acted as ANO1 inhibitors as well with D5 showing the highest potency of ANO1 inhibition (Figure 1B right panel).

CFTR and ANO1 inhibition by active components from *B. rhizoma*: FRT cells transfected with wild type CFTR and EYFP were incubated with indicated concentrations of active fraction D5. Figure 1C (left panel) shows the inhibitory effect of D5 on wild type CFTR. Results indicated that D5 inhibited CFTR chloride channel in a dose-dependent way (IC₅₀=42.4 μ g/mL).

In the meanwhile, ANO1 inhibition by D5 was also tested using FRT cells stably cotransfected with ANO1 and H148Q/I152L EYFP protein. Indicated concentrations of D5 were added into each well and cells were incubated for 10 min, ATP and iodide were concurrently pumped into the wells. Figure 1C (right panel) shows that D5 also exhibited ANO1 inhibitory effect dose-dependently (IC₅₀ = 32.2 μ g/mL).

Inhibition of CFTR and CaCC chloride channel activities in rat colonic epithelia: It has been well documented that CFTR expresses in rat colonic epithelia, our study used isolated rat colonic mucosa to investigate the effect of D5 on epithelial Cl⁻ secretion. Indomethacin and amiloride were present to prevent prostaglandin and Na⁺ current before CFTR activation by FSK (cAMP agonist). As shown in Figure 2A, mucosal application of indicated concentrations of D5 dose-dependently inhibited FSK-induced short-circuit current, CFTR_{inh}-

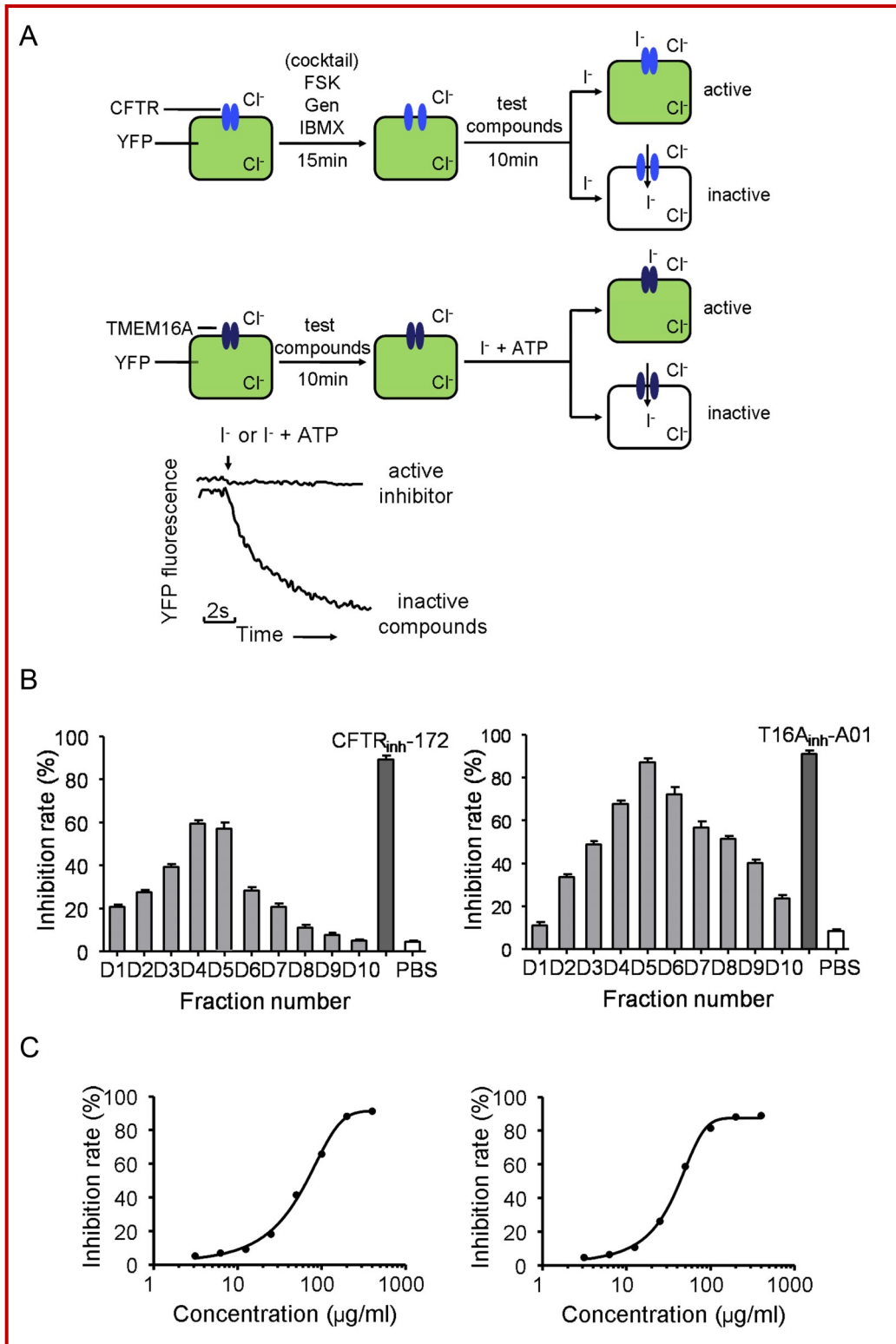


Figure 1: Inhibitory effects of *B. rhizoma* fractions on CFTR and ANO1 chloride channels

(A) Fluorescence assay models used in the identification of chloride channel inhibitors. FRT cells cotransfected with CFTR were pretreated with cocktail for 15 min and then 100 μg/mL test compounds were added for another 10 min (upper panel). FRT cells cotransfected with ANO1 were pretreated with test compounds for 10 min. Fluorescence was monitored in response to I⁻ or I⁻ plus 300 μM ATP addition (middle panel). The examples of putative traces of active inhibitors and inactive wells with test compounds (down panel) on CFTR and ANO1 inhibition. (B) Summary of inhibition rate on CFTR (left panel) and ANO1 (right panel) by fractions D1~D10. CFTR_{inh}-172 and T16A_{inh}-A01: positive control. Data were expressed as mean ± SE of 5 independent experiments. (C) CFTR (left panel) and ANO1 (right panel) chloride channel inhibitory effects by active component D5

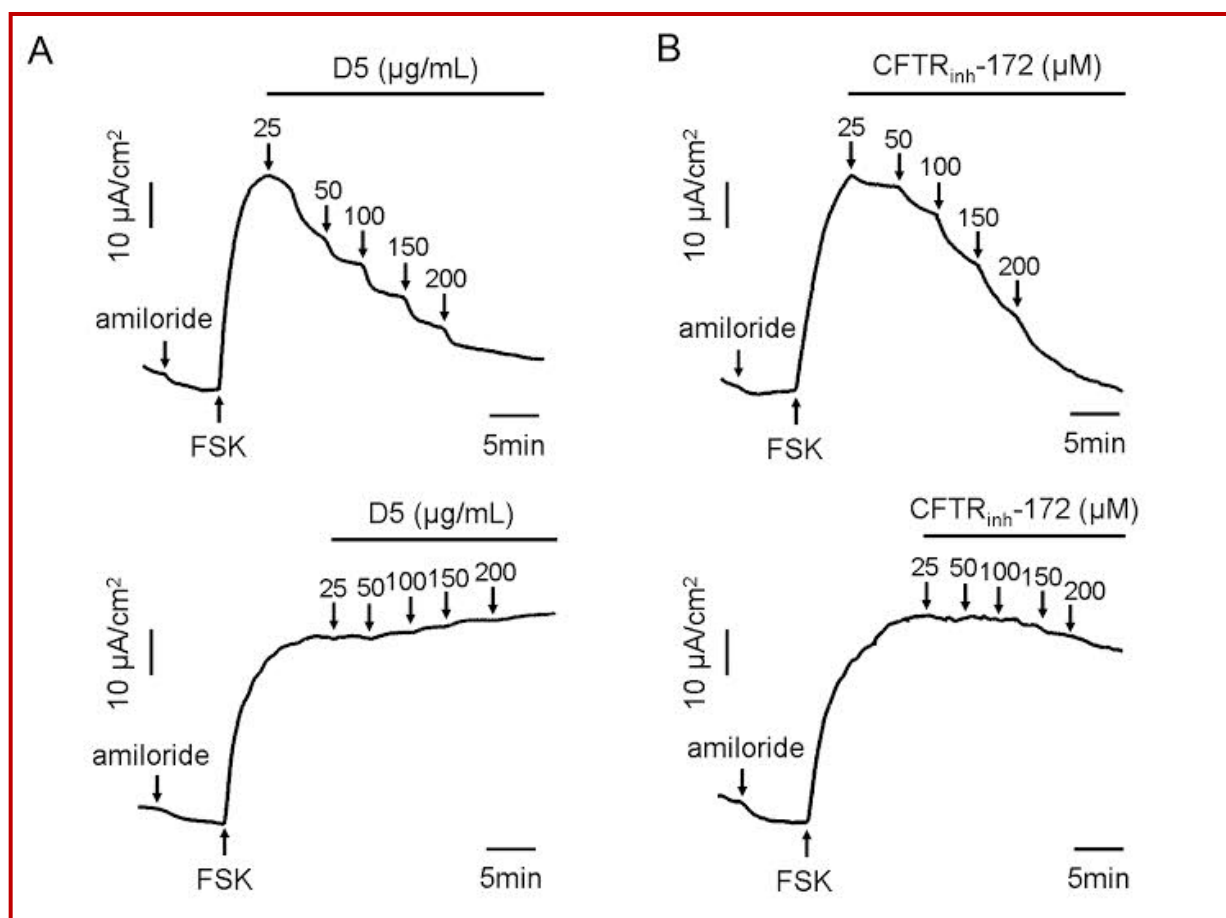


Figure 2: D5 inhibits CFTR Cl⁻ currents in rat colonic epithelia

(A) Inhibitory effect on CFTR chloride channel in rat colonic epithelia by mucosal (upper panel) and serosal (down panel) application of indicated concentrations (25, 50, 100, 150, 200 µg/mL) of D5. (B) Inhibitory effect of CFTR_{inh}-172 on mucosal (upper panel) side and serosal (down panel) side of rat colonic epithelia on CFTR chloride channel. FSK (20 µM) was used to induce a CFTR-mediated Cl⁻ current

172 was set as positive control. Tests were further done to evaluate the inhibitory effect of D5 on the serosal side, results showed that D5 didn't affect Cl⁻ current on the serosal side.

Studies were further done to investigate the role of CaCC in colonic epithelial chloride transport by Ussing Chamber. Different concentrations of D5 were added into the mucosal sides of the tissues following 15 min incubation and addition of 500 µM CCh (muscarinic receptor agonist). Results indicated that D5 inhibited CCh-induced transepithelial short-circuit current (Figure 3A). Effects of 50 µM and 200 µM D5 on serosal sides were also tested, Figure 3B shows that D5 didn't act as an inhibitor on the serosal side of rat colonic epithelia.

Inhibition of intestinal fluid secretion by fraction D5 in mouse ileum: Next, we investigated *in vivo* efficacies of D5 on cholera toxin-induced intestinal fluid secretion using live male mice. Results and summarized data are shown in Figure 4. Large amount of fluid was accumulated in the loop where cholera toxin was applied

compared with saline control. Intraluminal injection of active fraction D5 significantly reduced the cholera toxin-induced fluid secretion, although no more than the application of CFTR_{inh}-172 used as positive control.

Inhibition of intestinal motility by fraction D5: Intestinal motility measurements were performed using mice as intestinal motility disorder is one of the main causes in the pathophysiology of diarrhea. Figure 5 shows that both intraperitoneally and orally administration of fraction D5 prevented intestinal peristalsis as in activated charcoal movement delayed in these groups with peristaltic indexes of 38.3% and 29.3%, respectively, compared to that of control (50% and 49.3%).

HPLC analysis of active fraction characteristics: HPLC analysis of fraction D5 revealed the presence of 8 major constituents (representing >4% of the %area) labeled as a~h with retention time 13.2, 13.7, 16.9, 17.5, 18.3, 19.5, 20.6 and 21.7 min, respectively (Figure 6A). The analytical chromatogram shows that compounds ECG and EGCG had different retention time (21.4 and 16.6 min) from major components of fraction D5 (Figure 6B

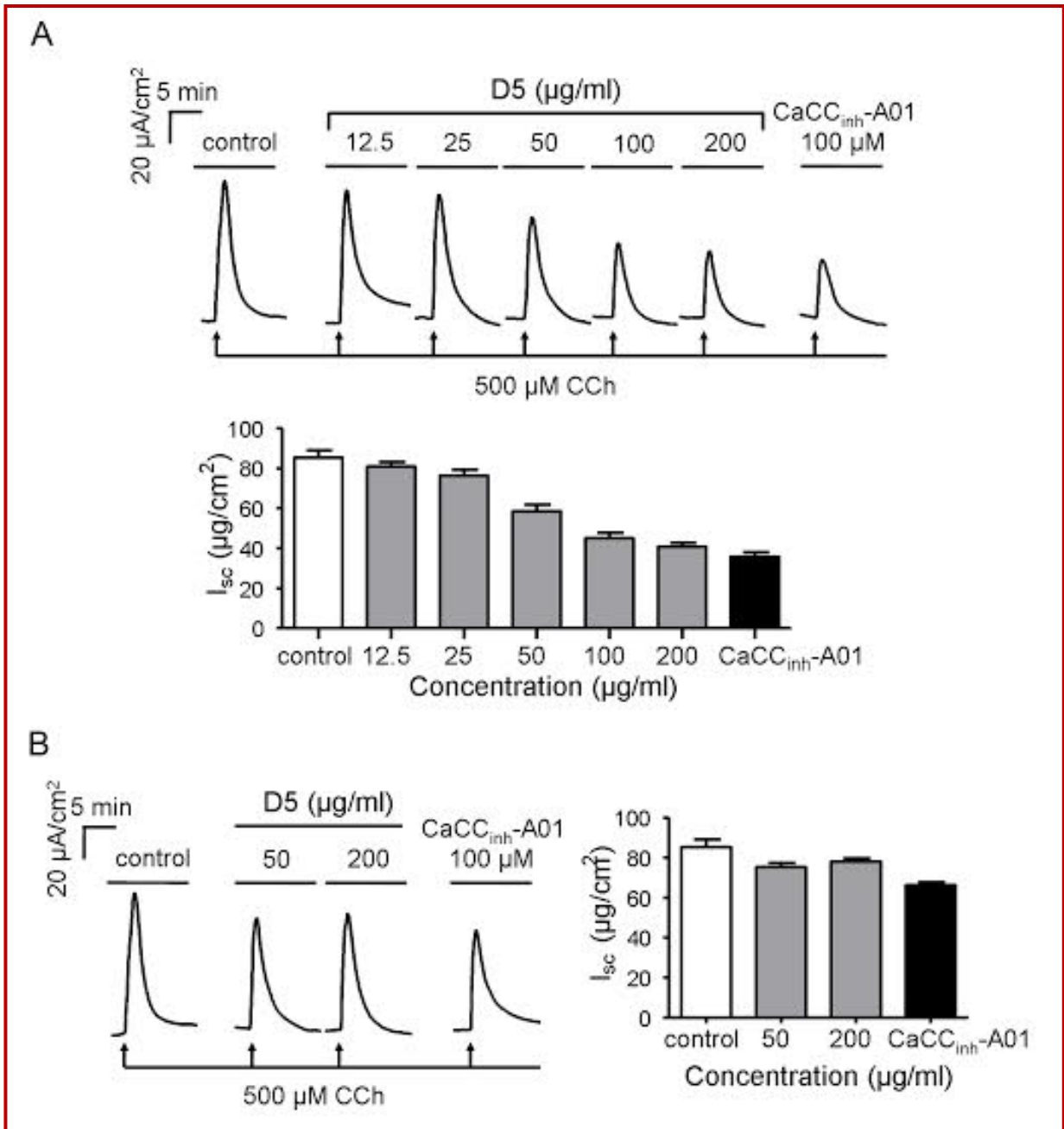


Figure 3: CCh-induced Cl^- currents were inhibited by incubation of indicated concentrations of D5 (12.5, 25, 50, 100, 200 $\mu\text{g}/\text{mL}$)

(A) CCh-induced Cl^- currents were inhibited after mucosal application of indicated concentrations of D5 in a dose-dependent way ($n=5$). (B) Serosal application of D5 affected little on the CCh-induced Cl^- currents ($n=5$). CaCC_{inh}-A01 was used as positive control

and C). Our future work is focusing on isolating, testing, and structurally elucidating compounds a~h using preparative HPLC.

Discussion

The purpose of the present study is to investigate the pharmacological basis of the anti-diarrheal effect of *B. rhizoma*. A 40% methanol /water fraction (D5) from

D101 macroporous resin was found to have CFTR and CaCC inhibitory activities in FRT cells and isolated rat colonic mucosa. *In vivo*, D5 significantly reduced cholera toxin-stimulated fluid secretion in mouse closed-loop model. In addition, D5 remarkably delayed intestinal peristalsis in mice. The inhibitory effects on CFTR and CaCC-mediated Cl^- secretion and gastrointestinal motility may provide a molecular basis for the anti-diarrheal properties of *B. rhizoma*.

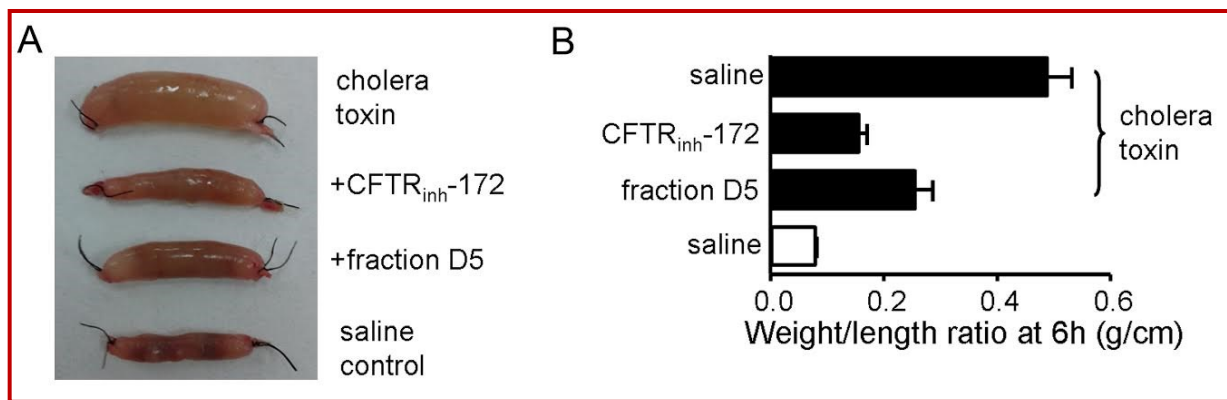


Figure 4: Fraction D5 inhibits cholera toxin-induced intestinal fluid secretion. (A) Photograph of isolated mouse ileal loops at 6 hours after injection with cholera toxin (0.5 µg), cholera toxin plus CFTR_{inh}-172 or D5 and saline control. Ratio of ileal loop weight/length (g/cm) at 6 hours (n=6)

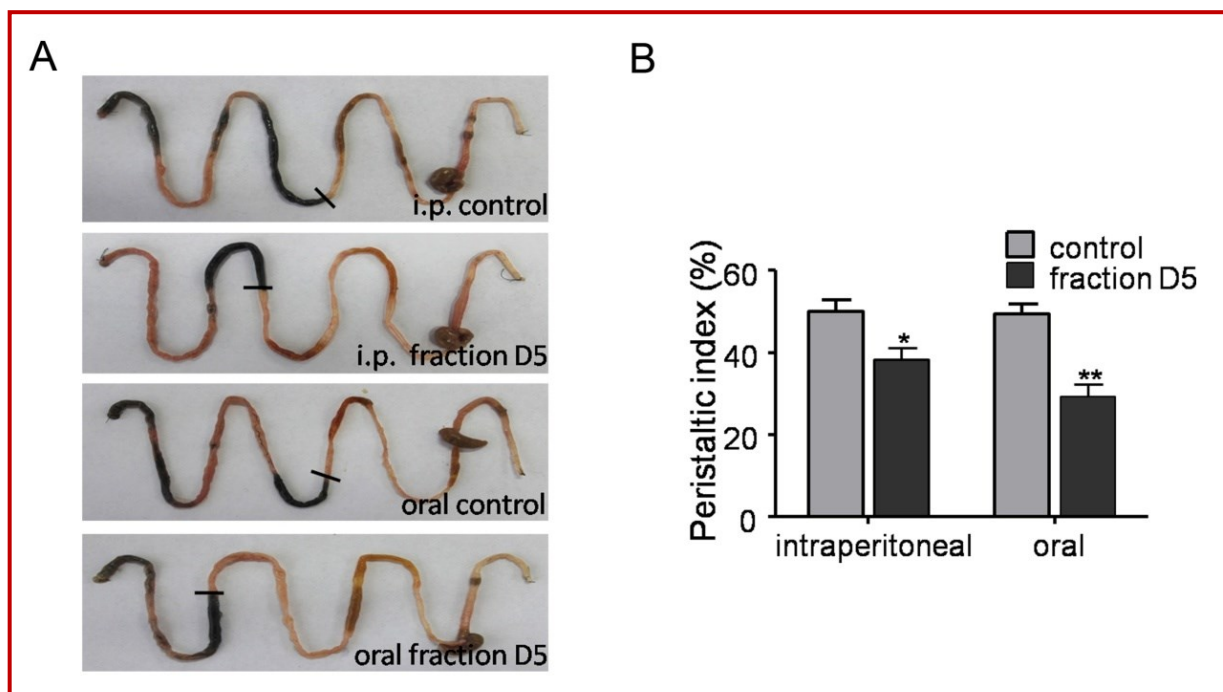


Figure 5: Intestinal motility inhibition by fraction D5. (A) Photographs of isolated mouse intestinal tracts after intraperitoneal injection and oral gavage of PBS and fraction D5, showing travelled distance of activated charcoal. (B) Summary of peristaltic indexes. Data were expressed as mean ± SE of six independent experiments. *p<0.05, **p<0.01

Leading causes of secretory diarrhea include both increasing in net intestinal fluid secretion and reduction in intestinal fluid absorption. Increased net fluid secretion results from activation of CFTR and CaCC-mediated ion secretion, and reduced fluid absorption results from increased movement of intestine. Our result indicated that D5 inhibited both CFTR and CaCC-mediated Cl⁻ transport, as well as intestinal motility. Synergic effect of these activities may result in a significant inhibition of intestinal salt and water secretion. These results support the theory of multi-component and multi-target therapy in TCM.

Previous studies indicated that ANO1 was expressed in interstitial cells of Cajal (ICC) where generated slow-wave pacemaker activity in the gastrointestinal (GI) tract (Ferrera et al., 2010; Huang et al., 2009; Hwang et al., 2009). Inhibition of ANO1 on ICC may reduce the motility of intestine, and thus increase intestinal fluid absorption. In the present study, we found that D5 could significantly inhibit ANO1-mediated iodide transport in transfected FRT cells and intestine motility in mice, these results correlates well with the notion that GI motility can be inhibited by pharmacological inhibition of ANO1. ANO1 may be a therapeutic target

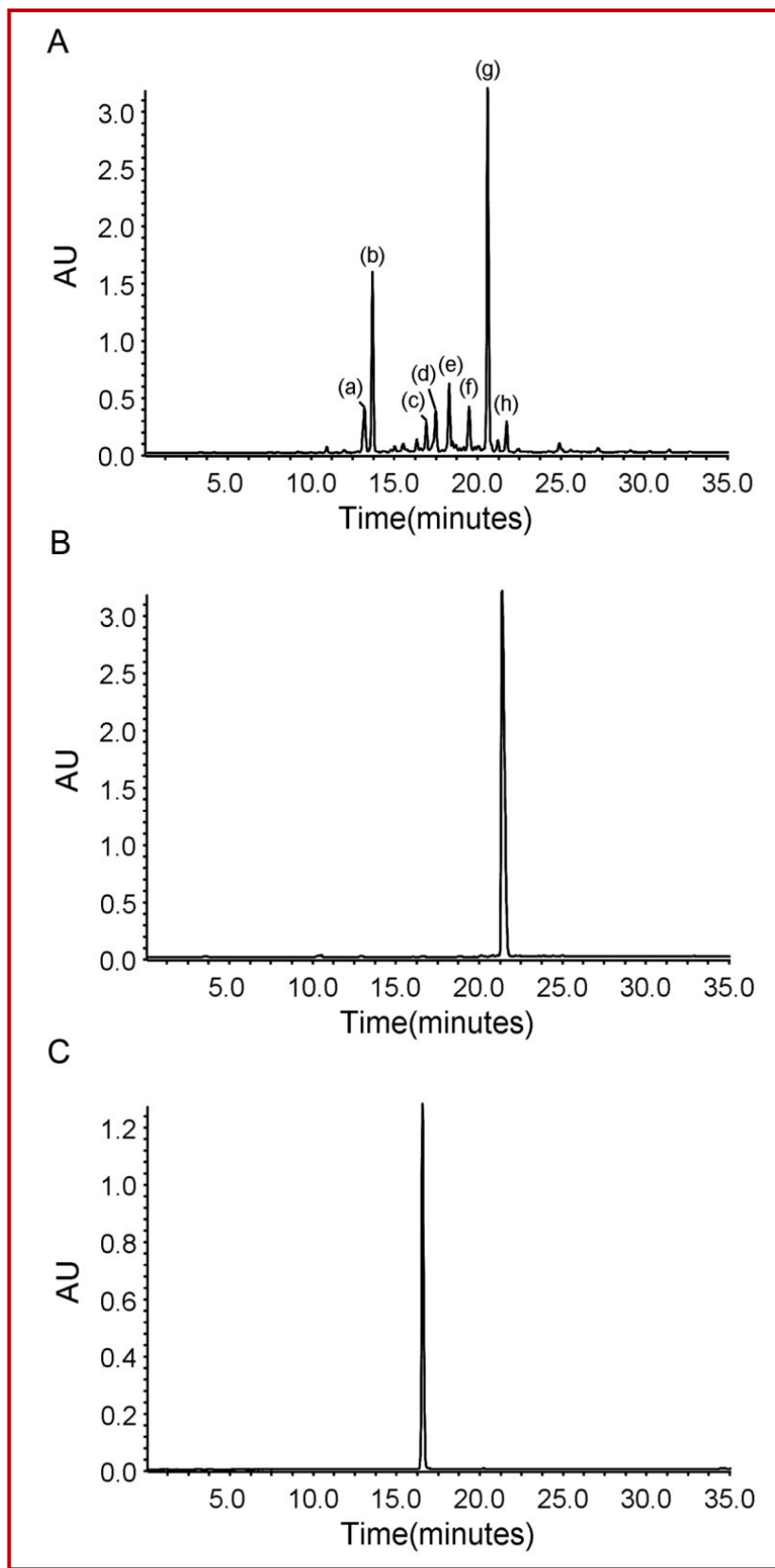


Figure 6: Chromatograms of fraction D5, ECG and EGCG. (A) Chromatograms of fraction D5 with the greatest CFTR and CaCC inhibitory effect. (B) and (C) show chromatograms of two known CFTR and CaCC inhibitors ECG and EGCG

for GI hypermotility-related disease such as secretory diarrhea.

Our efforts were vain in isolation of active compounds from D5. Previous studies by Chen et al indicated that ECG and EGCG inhibited CFTR-mediated Cl⁻ current in rat colonic epithelia (with IC₅₀ values both around 400 μM) and cholera toxin-stimulated diarrhea in mice (Chen et al., 2015). Namkung et al. reported that ECG and EGCG (>300 μM) inhibited CaCC-mediated Cl⁻ current in T84 cells (Namkung et al., 2010). Because ECG and EGCG exist in a wide range of natural products and extracts including ethanol extract of *B. rhizoma*, we analyzed contents of these two compounds using HPLC. It turned out that there were low contents of ECG and EGCG in D5. These results suggest that CFTR and CaCC inhibitory activities by EGCG and ECG may account in part for the anti-diarrheal effect of *B. rhizoma*.

Conclusion

The present study revealed that CFTR and CaCC-mediated intestinal epithelial Cl⁻ secretion inhibiting and gastrointestinal motility delaying may account for the anti-diarrheal activity of *B. rhizoma*. Our results support the proposition that TCM are multi-component and multitarget agents.

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Conflict of Interest

Authors declare no conflict of interest

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