Effects of Alpinia Officinarum and Euodia Rutaecarpa on the expression of TRPA1 and TRPM8 in rats with irritable bowel syndrome

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[Abstract]  Objective  Alpinia Officinarum and Euodia Rutaecarpa are two traditional Chinese herbs with warm nature, usually called interior-warming medicinal and widely used to treat cold syndrome for a long history. The purpose of this study is to explore the effects of Alpinia Officinarum and Euodia Rutaecarpa on irritable bowel syndrome (IBS) rats with spleen-stomach deficiency cold syndrome, and to clarify the link between “warming the middle to alleviate pain” and transient receptor potential channel A1 (TRPA1), M8 (TRPM8). Methods  IBS rat model with spleen-stomach deficiency cold syndrome was established by cold diarrhea and restraint stress. Rats were randomly divided into the normal group, model group, positive group (pinaverium bromide), Alpinia Officinarum group and Euodia Rutaecarpa group. After continuous modeling and administration for seven days, body weight, loose stool grades, activity of ATP enzyme, pathological morphology of gastrointestinal tissue were evaluated. The expression of TRPA1 and TRPM8 in spinal ganglion, stomach and colon tissue of rats were detected by RT-PCR and immunohistochemistry. Results  The IBS model with spleen-stomach deficiency cold syndrome caused increase in the number of diarrhea and loss in the body weight in rats. Compared with the normal group, the loose stool grades of model group was significantly increased (P<0.01), Na⁺-K⁺-ATP and Ca²⁺-Mg²⁺-ATP activity of erythrocyte membrane were significantly decreased (P<0.01). However, compared with the model group, Alpinia Officinarum and Euodia Rutaecarpa obviously reduced the loose stool grades (P<0.05 or P<0.01), and obviously increased the activity of Na⁺-K⁺-ATP and Ca²⁺-Mg²⁺-ATP (P<0.05). The expression of TRPA1 and TRPM8 in spinal ganglia and colon tissue of the model group was markedly enhanced (P<0.01), but Alpinia Officinarum and Euodia Rutaecarpa significantly inhibited these overexpression. Conclusion  The effect of interior-warming and alleviating pain of Alpinia Officinarum and Euodia Rutaecarpa are traditionally used to cure spleen-stomach deficiency cold syndrome.
1 Introduction

Alpinia Officinarum (Gāo liáng jiāng) is mainly distributed in Guangdong Province and Hainan Province in China [1], while Euodia Rutaecarpa (Wú zhū yú) is mainly distributed in the south of Qinling Mountains [2]. These two traditional Chinese herbs are called interior-warming medicinal with warm nature and interior-warming effect, which are widely used to treat interior-cold syndrome such as abdominal cold and pain, vomiting, diarrhea and other digestive system diseases [3-4]. Pharmacological studies have proved that interior-warming medicinal has many pharmacological effects such as anti-ulcer, anti-diarrhea, anti-inflammatory and analgesic effect [1,5], but the relationship between its warm nature and pharmacological activities is still unclear. Transient receptor potential (TRP) channels are universal biological sensors that detect changes in the environment [6], seven classes of them have been classified: TRPA, TRPC, TRPM, TRPML, TRPP, TRPV and TRPN subfamilies. TRP channels can accept various stimuli in the external environment, such as changes in temperature, pH or osmotic pressure, pressure, shear stress, mechanical stretch, oxidative stress, phospholipids and phospholipid metabolites. In addition, they can respond to endogenous substances and messengers produced during tissue damage and inflammation. Recently, some thermo TRP channels were found associated with traditional Chinese medicine [7-8]. TRPA1, also named ANKTM1, is activated by noxious cold stimulation for human, can also be activated by chemical and mechanical stimuli [9-11]. With further study, a great deal of evidence has shown that TRPA1 plays an important role in the production of pain and the enhancement of pain sensitivity [12].

TRPM8, is activated by cold stimuli, changes in voltage, pH, chemicals, and regulates a series of physiological and pathological processes in the organism. Two of these channels are involved in the generation of cold sensation. For the TRPA1 channel overexpression of people, it can trigger a series of Neuroendocrine immunity (NTM) responses in the corresponding parts such as skin, intestinal, respiratory, which are shown as skin rashes, irritable bowel syndrome and asthma [13]. The previous studies of interior-warming medicinal showed that the Alpinia Officinarum and Euodia Rutaecarpa had obvious effects of alleviating pain and anti-diarrhea in mice or rats [14-17]. However, whether the effect of warming the middle and dispelling cold of the two warm-nature herbs is associated with the TRP adjustments in pathological condition is still not clear.

Irritable bowel syndrome (IBS) is an epidemic disease that endangers human health all over the world. The morbidity of IBS was 7%~21% [18] in the world and 6.5% in China [19]. It is a functional gastrointestinal disorder characterized by recurrent abdominal pain or discomfort that is associated with changes in defecation frequency and stool consistency, but occur in the absence of other organic gastrointestinal disease [20-21]. Because of its complex pathogenesis, current drug effect is not satisfactory. The disease still seriously affects the quality of life and the work efficiency of patients, which cause remarkable increases of health care and costs [21]. Therefore, complementary and alternative therapies are receiving more and more attention from both patients and clinicians, and the therapeutic effects of Chinese medicine [22-23] and even acupuncture [24] are obvious. Specific subtypes of IBS often classify patient symptoms into constipation predominant (IBS-C),...
diarrhea predominant (IBS-D), mixed (IBS-M), indeterminate (IBS-U). Among them, IBS-D is classified as having more than 25% of stools categorized as Bristol Stool Form Scale (BSFS) 6 or 7, less than 25% as BSFS 1 or 2. IBS-D disease is often treated with preprandial loperamide or pinaverium, female patients who fail other therapy for severe IBS-D symptoms may be treated with 5-hydroxytryptamine (5-HT) type 3 antagonists such as alosetron, or ondansetron. Pain and enterospasm can be treated with antispasmodics and tricyclic antidepressants, counseling, probiotics, or a trial of rifaximin\[^{[21]}\]. Although many treatments have been proposed, but the efficacy is still unsatisfactory. Warming the middle energizer to stop diarrhea is an important therapeutic principle for treating IBS-D with traditional Chinese medicine, there are commonly used interior-warming medicinal in clinic, such as Alpinia Officinarum, Euodia Rutaecarpa and their compound prescriptions (Liangfu pill, Sishen pill). Therefore, in this research, IBS rat model with spleen-stomach deficiency cold which induced by cold diarrhea and restraint stress was used to study the effect of Alpinia Officinarum and Euodia Rutaecarpa on IBS disease, we aim to investigate the regulatory effect on the expression of TRPA1 and TRPM8 in interior cold syndrome, then provide scientific evidence for the molecular mechanism of interior-warming medicinal.

## 2 Materials and methods

### 2.1 Herbal material and preparation

Alpinia Officinarum and Euodia Rutaecarpa, Folium Sennae (Fān xiè yè) were purchased from Kangmei Pharmaceutical company limited in Guangdong province (Guangzhou, China). The medicinal materials were identified by Professor LI Shuyuan (School of Traditional Chinese Medicine, Guangdong Pharmaceutical University). Each herb was extracted twice with water (30 min per time), then filtrate was combined and froze in the refrigerator.

### 2.2 Chemical and reagents

Pinaverium bromide tablets (Pinaverium) were purchased from Abbott Healthcare SAS (France). ATP enzyme test box (A016-1-1) and BCA protein assay kit (A045-2-2) were obtained from Nanjing Jiancheng Bioengineering Institute. TRPA1 (ab68847) and TRPM8 (ab104569) antibodies were purchased from Abcam company (UK), SABC-POD (Boster Bioengineering Co., Ltd., Wuhan). Trizol (Invitrogen Biotechnology Co., Ltd.); Reverse transcription system kit (Promega Biotechnology Co., Ltd., Beijing, China); D2000 DNA maker, Premix ExPCR kit (Takara biological engineering Co., Ltd., Dalian, China); trichloromethane (Qiangsheng Chemical Co., Ltd., Jiangsu, China), absolute ethanol (Yongda Chemical Reagent Co., Ltd., Tianjin, China), iso-propyl alcohol (Damao Chemical Reagent Co., Ltd., Tianjin, China).

### 2.3 Experimental animals

Female Sprague-Dawley rats (180–220 g) were obtained from Experimental Animal Center of Southern Medical University (China). They were housed in plastic cages with free access to food and water at (22±2) °C. Rats were acclimatized to the environment for 1 week before the experiment. The experiment procedures were conducted in compliance with the National Institutes of Health Guide for care and use of the laboratory animals, and were approved by the Institutional Animal Ethics Committee of Guangdong Pharmaceutical University (gdpulacSPF2012271).

### 2.4 Groups and detection items

Fifty rats were randomly divided into five groups (ten animals in each group): normal group, model group, positive group (pinaverium bromide), Alpinia Officinarum group (Alpinia group, 2 g/kg), Euodia Rutaecarpa group (Euodia group, 2 g/kg).
The spleen-stomach deficiency cold IBS model was established by cold diarrhea and restraint stress according to previously described method\(^{19,25}\). Rats (except normal group) were given 20% Folium Sennae decoction (2 mL/100 g) by gavage once a day; five minutes later, rats (except normal group) were fixed on the board with their limbs to restrict their movements, and one hour later rats were released. Such practices continued for 7 days, and during this period each group was given corresponding drug treatment every day, the normal group and the model group were given distilled water. During the experiments, all rats were weighed periodically to reflect weight changes.

### 2.4.1 The degree of diarrhea

On the seventh day, 1 h after administration, all rats were kept in separate cages with filter paper underneath to count the loose stools and loose stool grades, which can indicate the degree of diarrhea in each rat. Loose stool grade means the extent of watery stools, it refers to the diameter of stains formed by loose stool on the filter paper. The loose stools grades were divided into 4 grades according to Zhou method\(^{25-26}\). The stain diameter which is less than 1 cm belongs to 1 grade; the stain diameter which is between 1 cm and 1.9 cm belongs to 2 grade; the stain diameter which is between 2 cm and 3 cm belongs to 3 grade; the stain diameter which is more than 3 cm belongs to 4 grade. We kept on observing rats for 6 h after giving Folium Sennae, changed the filter paper every hour, at last recorded loose stools of each rat. The loose stool grade of each rat was calculated according to the following formula: average loose stool grades=the sum of loose stool grades/the total of loose stool numbers.

### 2.4.2 ATP enzyme activity

One hour after the last administration, rats were anesthetized and sacrificed, blood was taken from abdominal aorta and separated into erythrocyte membrane according to kit description method. Then we respectively detected activities of Na\(^+-\)K\(^+-\)ATP enzyme and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP enzyme in erythrocyte membrane according to kit operating procedures.

### 2.4.3 Gastrointestinal tissue morphology

The stomach and colon tissues were clipped and washed, then put into 4% polyformaldehyde, embedded with paraffin, processed with histological section and HE (hematoxylin and eosin) staining techniques. At last, we observed the pathological changes and photographed them by optical microscopy.

### 2.4.4 Gene expression of TRPA1 and TRPM8

RT-PCR analysis was applied to detect gene expression. The stomach (STO) and distal colon (COL) of rats were aseptically removed, the small roots and connective tissue were carefully cleaned after dorsal root ganglion (DRG) removal, all these tissues were put into RNA-free enzyme frozen tube, put in liquid nitrogen immediately, and later preserved at \(-80\) °C refrigerator. The total RNA extraction and the reverse transcription were finished according to the kit instruction (GoScriptTM Reverse Transcription System kit and Premix TaqTM kit). The primers of \(\beta\)-Actin, TRPA1 and TRPM8 were synthesized by Shanghai sangon biological engineering Co., Ltd. \(\beta\)-Actin (sense: 5'-GACCCAGATCATGTTTGAGACC-3', antisense: 5'-GCAGTAATCTCCTTCTGCATCC-3', 600bp), TRPA1 (sense: 5'-AACCGCATAGAGCTCCTCAA-3', 399bp), TRPM8 (sense: 5'-CGTCGTCTTCGCTTACTTCTAC-3', antisense: 5'-TTTGTGTCAGTTTCTGTAACC-3', 219bp).

PCR reaction conditions of \(\beta\)-action: pre-denaturation at 94 °C for 3 min, denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, 30 cycles, extension at 72 °C for 5 min. PCR reaction conditions of TRPA1
and TRPM8: pre-denaturation at 94 °C for 3 min, denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, 35 cycles, extension at 72 °C for 5 min. PCR products were detected by gel electrophoresis (1.5%) and quantified by gray scale scanning software. The relative expression level results were presented as the ratio of target gene and β-Actin reference gene.

2.4.5 Protein expression of TRPA1 and TRPM8

We applied immunohistochemical analysis method to detect TRPA1 and TRPM8 protein expression. Rats were anesthetized and transcardially infused with 0.9% saline and 4% polyformaldehyde in 0.01 M phosphate buffer solution. The tissue samples of corresponding segment DRG, STO and COL were fixed in 4% polyformaldehyde solution, dehydrated and embedded in paraffin following routine methods. Then paraffin blocks were cut into 5 μm-thick sections, dewaxed with xylene and hydrated with ethanol. The sections were blocked endogenous peroxidase with 3% peroxide-methanol for 10 min, and rinsed with phosphate buffer solution (PBS, 0.01 M, pH 7.2). Then sections were immersed in EDTA antigen retrieval solution for microwave antigen retrieval (8 min, 92-98 °C). Afterwards, sections were incubated for 8 hours at 4°C with different primary antibodies: TRPA1 (1:40, Abcam, ab68847), TRPM8 (1:40, Abcam, ab104569), then sections were incubated again with secondary antibody for 20 min and SABC for 20 min. Stained with 3,3-diaminobenzidine (DAB), kept at room temperature without light for 3-5 min. Finally, sections were counterstained with hematoxylin for 30 sec, dehydrated with ethanol and mounted with neutral gums. The negative control group that set separately was carried out with the same steps as described above, but PBS replaced the antibody (TRPA1 or TRPM8). Image-Pro Plus 6.0 system (IPP) image analysis system was used for quantitative analysis. The positive index was calculated as positive area integral optical density (IOD).

2.5 Statistical methods

All data were presented as mean±SD and analyzed by SPSS statistics software (one-way ANOVA). P values of less than 0.05 (P<0.05) were defined as the significant level.

3 Result

3.1 Body weight changes of rats

All rats were acclimated for 1 week before the experiment. During this period, rats had flexible movement and clean fur, the body weight increased steadily (the weight of 1th, 4th, 8th day, Fig. 1). There was no significant difference in rat weight between each group. A week later, except the normal group, other groups were given Folium Sennae and restraint stress, meanwhile all the drug groups were given corresponding treatment by intragastric administration. At day 3, 5 and 7 after establishing models (the 10th, 12th, 14th day during the experimental period, Fig. 1), the result showed that body weight in the model group was significantly lower than the normal group (P<0.05 or P<0.01), which indicating that the model has some influence on body weight of rats, and it may be relative to diarrhea. Although the weight of the
pinaverium, Alpinia and Euodia groups were slightly higher than that of the model group, except Euodia group, there was no significant difference between other treatment groups and the model group.

3.2 Effect of Alpinia Officinarum and Euodia Rutaecarpa on diarrhea degree

One of the most important clinical symptoms of diarrhea type irritable bowel syndrome (IBS-D) is persistent or intermittent diarrhea\cite{20,27-28}. In animal experiments, we can use loose stool grades or diarrhea index to represent the intensity of diarrhea. Before giving Folium Sennae, rats all had normal stools without stains of loose stools. A week later, except the normal group, other groups were given Folium Sennae and restraint stress to establish IBS rats model. Therefore, the stool conditions of these groups changed gradually, especially the model group, a lot of loose or watery stool could be seen in these rats. The results showed that the defecation of normal rats always kept normal stools (0 grade). However, the degree of diarrhea between model group and normal group were significantly different from the second hour to the sixth hour ($P<0.01$), which indicated that irritable bowel syndrome rat model with spleen-stomach deficiency was successfully established. Compared with the model group, the diarrhea degrees of positive group (pinaverium) was significantly decreased at 2 h, 3 h ($P<0.01$) and 4 h ($P<0.05$), the loose stool grades of Alpinia group was significant decreased at 5, 6 h ($P<0.05$), and that of Euodia group was significant decreased at 4, 5, 6 h ($P<0.05$ or $P<0.01$). Pinaverium is a calcium antagonist which acts on the gastrointestinal tract as an antispasmodic agent, and it plays a role in inhibiting calcium influx into smooth muscle cells of the intestine. Therefore, it can be used to treat IBS, as well as for symptomatic treatment of pain related to intestinal dysfunction, abnormal defecation, enterospasm and gastrointestinal discomfort\cite{29-31}. The results showed that this IBS model had obvious symptoms of diarrhea, pinaverium, Alpinia and Euodia significantly improved this symptom (Fig. 2).

![Fig. 2 Effect of Alpinia Officinarum and Euodia Rutaecarpa on loose stool grades in IBS rats. Values were expressed as mean±SD in each group ($n=10$). ▲ $P<0.05$, ▲▲ $P<0.01$, compared with normal group; * $P<0.05$, ** $P<0.01$, compared with model group.](image)

3.3 Effect on activity of Na\(^+\)-K\(^+\)-ATP enzyme and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP enzyme

ATP is a protease on the biomembrane, which exists in tissues, membranes and mitochondria, and can reflect the level of cellular energy metabolism\cite{32}. The cold and heat changes of human body are closely related to energy metabolism. Numerous studies have shown that Na\(^+\)-K\(^+\)-ATP enzyme and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP enzyme have a certain relationship with cold or heat syndrome in traditional Chinese medicine\cite{33-35}. Traditional Chinese medicine with cold and hot attribute may regulate the heat and cold syndromes by affecting some links of energy metabolism such as Na\(^+\)-K\(^+\)-ATP enzyme and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP enzyme. There are no organelles in the red blood cells, so there will be no interference during the determination, so it is sensitive to detect the ATPase activity of red blood cells. Therefore, the activity of Na\(^-\)-K\(^+\)-ATP and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP in red blood cells was used as an indicator of IBS with spleen-stomach deficiency cold syndrome. Fig. 3 showed that there were significant differences in the activities of Na\(^-\)-K\(^+\)-ATP enzyme and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP enzyme of erythrocyte membrane between the model group and the normal group ($P<0.01$), this might be related with spleen-stomach deficiency cold caused by Folium Sennae administration. Comparing with the model group, the activities of
Na\(^+\)-K\(^+\)-ATP enzyme and Ca\(^{2+}\)-Mg\(^{2+}\)-ATP enzyme of Alpinia group and Euodia group were significantly increased, which suggested that Alpinia officinarum and Euodia rutaecarpa could enhance the activity of ATP enzymes to improve symptoms of spleen-stomach deficiency cold.

### 3.4 Effect of Alpinia Officinarum and Euodia Rutaecarpa on gastrointestinal pathological changes

IBS is a functional gastrointestinal disorder characterized by abdominal pain or discomfort that is associated with changes in defecation frequency and stool consistency, generally the patients have no organic disease\(^{[20,27,31]}\). In this study, except the lymphocytes of the colonic tissue increased in some model rats, there were no significant pathological changes in the colon and stomach of Alpinia group and Euodia group, which indicated that by given Folium Sennae administration and restraint stress, the model of irritable bowel syndrome with spleen-stomach deficiency cold only showed local mild inflammation. After treatment with Alpinia Officinarum and Euodia Rutaecarpa, local inflammatory cell infiltration was reduced (Fig. 4).

### 3.5 Effect of Alpinia Officinarum and Euodia Rutaecarpa on the expression of TRPA1 mRNA and protein

According to earlier literature report\(^{[36-37]}\), TRPA1 is mainly distributed in the primary sensory neurons of DRG, trigeminal ganglia (TG) and vagus (VG). In recent years, studies have shown that in some non-neuronal cells there are expressions of TRPA1, such as hair cells of inner ear, vascular endothelial cells, dental pulp fibroblasts, keratinocytes and islet cells, et al\(^{[38-41]}\). However, there are few reports on gastrointestinal tissue. In order to study the relationship between the expression of TRPA1 and IBS with spleen-stomach deficiency cold syndrome, also to clarify the expression sites of TRPA1 in rats, we selected three sites (DRG, COL and STO) for this study. We found that TRPA1 was expressed at all the three sites. The mRNA expression of TRPA1 in DRG tissue of normal rat is very low. The results showed that when compared with the normal group, the IBS model group with spleen-

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**Fig. 3** Effect of Alpinia Officinarum and Euodia Rutaecarpa on ATP enzymes in IBS rats.
Values are expressed as mean±SD in each group (n=10). \(^*^P<0.05, \(^*^*^P<0.01\), compared with normal group; \(^\dagger^\dagger^P<0.05, \(^\dagger^\dagger^*^P<0.01\), compared with model group.

**Fig. 4** Effect of Alpinia Officinarum and Euodia Rutaecarpa on gastrointestinal pathological morphology (HE staining, 100×).
COL: colon tissues; STO: stomach tissues.
stomach deficiency cold significantly increased the expression of TRPA1 mRNA in DRG \((P<0.01)\). However, compared with the model group, Alpinia group and Euodia group significantly inhibited this overexpression \((P<0.05)\). The results also showed the expression of TRPA1 mRNA in the colon of model group significantly increased when compared to the normal group \((P<0.01)\). This is consistent with the clinical research results that the expression level of Alpinia group declined in comparison with the model group although the differences of the statistical analysis was not obvious.

Immunohistochemistry detection further confirmed the expression of TRPA1 in these sites. The results showed the expression levels of TRPA1 protein in the DRG and COL tissue of the model group were significantly higher than the normal group, and the expression levels of TRPA1 protein in the drug groups were also regulated to varying degrees. However, the expression differences in the STO of each group were not significant (Fig. 5B, Fig. 6).

**Fig. 5** Effect of Alpinia Officinarum and Euodia Rutaecarpa on TRPA1 mRNA and protein expression.
A. mRNA expression result of TRPA1; B. Protein expression result of TRPA1. DRG: dorsal root ganglion; COL: colon; STO: stomach; IOD: integral optical density. Values were expressed as mean±SD. *\(P<0.05\), **\(P<0.01\), compared with normal group; ^\(P<0.05\), ^\(^*\)\(P<0.01\), compared with model group.

**Fig. 6** Immunohistochemical detection of TRPA1 protein expression in DRG, COL and STO (400×). DRG: dorsal root ganglion; COL: colon; STO: stomach.
3.6 Effect of Alpinia Officinarum and Euodia Rutaecarpa on the expression of TRPM8 mRNA and protein

TRPM8 was identified as an outwardly rectifying Ca$^{2+}$ permeable non-selective cation channel, which is activated by cold temperatures below 28 °C$^{[43]}$. The localization of this channel in cold responsive small diameter neurons of DRG and trigeminal ganglion (TG) is consistent with a role in cold sensation. TRPM8 can be activated by natural compounds such as eucalyptol and menthol as well as by the synthetic super cooling agent icilin$^{[43]}$. All TRPM8 agonists induce a cooling sensation, suggesting that TRPM8 is a cold receptor. TRPM8 also shows that it is related to pain sensation, bladder function and the occurrence and development of cancer$^{[44]}$. However, the function and activation mechanism of TRPM8 in other tissues is largely unknown. In clinical practice, visceral pain in the distal colon and rectum is one of the most common causes of irritable bowel syndrome. Moreover, the expression of multiple TRP channels in gut sensory neurons has been confirmed. Research shows that TRPA1 and TRPM8 can cooperate in the nociceptive response. In order to study the relationship between the expression of TRPM8 and IBS with spleen-stomach deficiency cold syndrome, also to clarify the expression sites of TRPM8 in rats, we also selected three sites for this study: DRG, COL and STO, and we only found that TRPM8 expressed in DRG. Compared with the normal group, the expression levels of TRPM8 mRNA and protein in the dorsal root ganglia of the model group were significantly increased, Alpinia group and Euodia group significantly inhibited this overexpression ($P<0.05$ and $P<0.01$) (Fig. 7, Fig. 8).

4 Discussion

Transient receptor potential (TRP) channels play a central role in the transduction of thermal and nociceptive stimuli, with the development of life sciences, it was found that TRP channels (TRPV1, TRPV3, TRPA1, TRPM2, TRPM5 and TRPM8) were expressed in multiple cells, and these channels play an important role in intestinal peristalsis, cell secretion, mucosal homeostasis,

![Fig. 7 Effect of Alpinia Officinarum and Euodia Rutaecarpa on the expression of TRPM8 mRNA and protein in DRG.](image)

A. Expression of TRPM8 mRNA; B. Expression of TRPM8 protein. Values were expressed as mean±SD. ▲$P<0.05$, ▲▲$P<0.01$, compared with normal group; ★$P<0.05$, ★★$P<0.01$, compared with model group. DRG: dorsal root ganglion; IOD: integral optical density.

![Fig. 8 Immunohistochemical detection of TRPM8 protein expression in DRG (400×).](image)

DRG: dorsal root ganglion.
tissue protection, epithelial repair and other links, therefore TRP channels have become drug targets for diseases of the digestive system\textsuperscript{[45]}. Among them, TRPA1 and TRPM8 are two channels also participating in the generation of cold-feeling\textsuperscript{[46-48]}. Some studies have shown that TRPA1 is highly expressed in the intestinal tract of irritable bowel syndrome model rats. In traditional Chinese medicine theory, interior-warming medicinal are mostly pungent in flavor and warm or hot in nature. Their warm nature is most appropriate for dispelling internal invasion of pathogenic cold, and warming the channels to relieve pain, reinforcing and reviving Yang. Alpinia Officinarum and Euodia Rutaecarpa are two commonly used medicines in clinical practice, they can expel wind and dispel cold, warming the middle and relieve pain. There can cure the cold symptoms of spleen and stomach such as belching and acid swallowing, vomiting, stomachache, dyspepsia, diarrhea, acute gastroenteritis and so on. Studies have found that they are clearly effective in treating spleen and stomach with cold syndrome, and literature research has found that they can regulate gastrointestinal motility, anti-inflammatory, and anti ulcer, etc\textsuperscript{[49-51]}. Our studies have found that Alpinia Officinarum and Euodia Rutaecarpa could inhibit the over-expression of TRPA1 \textit{in vitro}\textsuperscript{[52]}. Therefore, this experiment established an IBS rat model of spleen and stomach deficiency-cold syndrome, to study the effects of Alpinia Officinarum and Euodia Rutaecarpa on TRPA1 and TRPM8 \textit{in vivo}. The result showed the warm nature of Alpinia Officinarum and Euodia Rutaecarpa obviously improved energy metabolism of cold syndrome such as ATP enzyme activity, they also clearly inhibited symptoms of diarrhea, therefore this study proved its efficacy in IBS disease with spleen and stomach deficiency-cold. The tests of mRNA and protein showed that the model group significantly increased the expression of TRPA1 in spinal ganglia and colon compared to the normal group, but Alpinia Officinarum and Euodia Rutaecarpa significantly inhibited this overexpression. Furthermore, Alpinia Officinarum and Euodia Rutaecarpa significantly inhibited overexpression of TRPM8 in spinal ganglia. TRP ion channel is a non-selective Ca\textsuperscript{2+} ion channel on the cell membrane, the activation of this channel causes Ca\textsuperscript{2+} ion to flow rapidly. TRPM8 starts to show obvious inward current when it is below 28 ℃, while TRPA1 can be directly activated by low temperature activation below the threshold of TRPM8 (below 17 ℃), which leads to muscle contraction and spasm, or plays a pro-inflammatory effect by increasing the gene expression of inflammatory factors, then causes the feeling of cold or pain, in this model, it may be manifested as intestinal spasm and diarrhea, visceral pain, etc. Some research data show that TRPA1, TRPV1 and TRPM8 are most likely to coexist in the same neuron and can cooperate in the nociceptive response\textsuperscript{[53]}. Through this experiment, we surprisingly found TRPA1 expressed in the stomach, distal colon and dorsal root ganglion of rats, but TRPM8 not found in the stomach and distal colon. In sum, this study proved the effect of Alpinia Officinarum and Euodia Rutaecarpa on IBS with spleen-stomach deficiency-cold, the result indicated that interior-warming medicinal (Alpinia Officinarum, Euodia Rutaecarpa) is likely to achieve its effect of warming the middle and analgesic action through influencing TRPA1 or TRPM8 abnormally expression. It provides a scientific basis for clarifying the mechanism of warming characteristics of interior-warming medicinal, and also provides an indication for developing new traditional Chinese medicine as an antagonist of TRPA1 or TRPM8. The signal transduction mechanism of TRP channels is complex and requires further in-depth research.

5 Conflicts of interest
The authors have no conflict of interest to declare.
6 Author contribution statement

CHEN Yanfen: Conceptualization, Methodology, Data curation, Validation, Writing–Original draft preparation, Visualization. ZHANG Yuying: Methodology, Data curation, Validation. YANG Chaoyan: Methodology, Data curation, Validation. HE Jiahui: Writing–Original draft preparation, Visualization. ZHANG Zihong: Writing–Original draft preparation, Visualization. LI Kunping: Writing–review and editing. YUAN Xujiang: Writing–review and editing. GU Chiming: Writing–review and editing.

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References


[43] Ulrich M, Wissenbach U, Thiel G. The supercooling compound icilin stimulates c-Fos and Egr-1 expression and activity involving TRPM8
channel activation, Ca\(^{2+}\) ion influx and activation of the ternary complex factor Elk-1[J]. Biochem Pharmacol. 2020; 177:113936.


