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Anti-inflammatory properties of Fu brick tea water extract contribute to the improvement of diarrhea in mice

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Abstract

Fu brick tea, a special kind of dark tea fermented dominantly by *Eurotium cristatum*, is traditionally used for diarrhea therapy in China. However, limited reports are available on the anti-diarrhea of Fu brick tea water extract (FTE) and its potential mechanisms. In the present study, the treatment effects of FTE on the senna-induced diarrhea in mice were investigated. We found that FTE effectively improved diarrhea index and inhibited gut peristalsis. Additionally, histopathological examination revealed that FTE protected the integrity and reduced inflammatory infiltration of the ileum mucosal barrier. Furthermore, FTE significantly decreased the levels of the pro-inflammatory factor 5-hydroxytryptamine (5-HT) and increased the expression of sodium–hydrogen exchanger 3 (NHE-3). The association among both intestinal damage and electrolyte balance and inflammation has been reported by many studies. Collectively, our study showed that FTE had anti-diarrhea activity, which may be associated with anti- inflammatory properties.

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INTRODUCTION

Diarrhea is usually defined as the passage of liquid or loose stools more frequently compared with normal. The hallmark of diarrhea is characterized by a decrease in the absorption of fluid and electrolytes and increased gut motility and secretion[1,2]. However, watery diarrhea may begin to develop due to an inflammatory response, which might develop further into profuse bloody diarrhea[3]. At present, antibiotics are commonly used for the therapy of diarrhea diseases. Research also points to antibiotics having anti-inflammatory properties[4]. Nevertheless, incorrect antibiotic use or overuse increases resistance and side effects[5,6]. Of concern, natural products with anti-inflammation and anti-diarrhea effects have the characteristics of safety and uncommon side effects. In most cases, they are also relatively safe and affordable. Therefore, the prevention and treatment of diarrhea using natural active substances is increasingly advocated.

As is widely known, the high expression of 5-hydroxytryptamine (5-HT) leads to diarrhea[7]. More importantly, intestinal 5-HT acts as a promoter of intestinal inflammation. Abundant evidence indicates that the role of the gut 5-HT is in mucosal maintenance, inflammation and translocation[8,9]. Ninety-five percent of the 5-HT in the body is secreted by enterochromaffin cells located in the intestinal mucosa[10]. 5-HT as a potent gut secretagogue, simultaneously plays an essential role in maintaining the balance of gut electrolyte and fluid[11]. Na^+/H^+ exchanger (NHE) acts in a net uptake of sodium chloride and water from the gut tract and maintains the water-electrolyte balance[12]. NHE-1 at a low level may reduce an uptake of sodium chloride and water from gut lumen and thus balance electrolytes[13,14]. The disorder of water-electrolyte of NHE-3 deletion mice could cause diarrhea[15,16]. Therefore, the inhibition of 5-HT expression is beneficial to improving intestinal inflammation and diarrhea.

Fu brick tea, is a special kind of dark tea fermented dominantly by *Eurotium cristatum*[17] (Fig. 1). It is traditionally used for diarrhea therapy in China. However, little work has been performed on the efficacy evaluation of anti-diarrhea activity and the mechanism in Fu brick tea water extract (FTE). The main chemical components in Fu brick tea are polyphenols and polysaccharides[18], which provide health beneficial properties, such as anti-inflammation[19–21]. More recently, an FTE-supplemented diet showed its great potential in the reduction of inflammation associated with metabolic disorders[22–24]. Additionally, FTE improved colitis induced by experimental dextran sodium sulfate in mice[25]. Therefore, we hypothesized that FTE protects against the development of diarrhea via its anti-inflammatory properties.

In the present study, a mouse model of diarrhea induced by senna was established to evaluate the anti-diarrhea activity of FTE. In addition, damage was assessed by histopathology analysis of intestinal samples. Furthermore, the expression of 5-HT was detected by immunosorbent assay (ELISA) and immunohistochemical staining (IHC). The expression of NHE-1 and NHE-3 was verified by IHC and reverse transcription-

Fig. 1 Fu brick tea.

quantitative PCR (RT-qPCR). Hopefully, this study will provide new experimental evidence of antidiarrhea activity and potential for further development and utilization of Fu brick tea.

RESULTS

Effect of FTE on diarrhea

In order to examine the anti-diarrhea effect of FTE on senna-induced diarrhea mice, male Kunming mice were supplemented with 2,530, 1,260, 630 mg kg^{-1} of FTE for 7 days. As is shown in Fig. 2a–d, mice that were induced by senna exhibited severe diarrhea symptoms compared with the normal control group. It was manifested at the loose stools volume and diarrhea index. Notably, all doses of the FTE treatment groups produced significant and sustained anti-diarrhea effects, and showed significant dose-efficacy dependence ($P < 0.05$). Furthermore, high dose FTE played a greater role than berberine. The results of this study confirmed that FTE possesses good anti-diarrhea activity.

Effect of FTE on histopathological change of ileum

To investigate the impact of FTE on the intestinal pathology, histopathological changes of the ileum were examined. As is shown in Fig. 2e, the distinct infiltration of inflammatory cells in the ileum was observed in the MC group, as well as broken intestinal villi with enlarged gaps. This indicates that increased inflammation and intestinal permeability in diarrhea mice. However, the pathological changes were clearly improved by the administration of FTE. Therefore, the FTE supplementation may help with the improvement of inflammatory infiltration and protection of intestinal integrity.

Effect of FTE on pro-inflammation factor 5-HT levels

To assess the level of systemic inflammation, the levels of serum and gut pro-inflammatory factor 5-HT were assessed by ELISA and IHC. Diarrhea was accompanied by an increased level of 5-HT. Experimentally, the levels of 5-HT in the MC group increased significantly compared with the NC group. The results indicated that the mice have displayed organism inflammation. As is shown in Fig. 3, the level of 5-HT in serum and its positive expression in the ileum and colon decreased after FTE administration. In addition, the downward trend of the FTE-M group was even more pronounced than that in the other groups and closest to the NC group. In short, the results indicate that FTE could effectively reduce the secretion of pro-inflammatory factor 5-HT to improve inflammation.

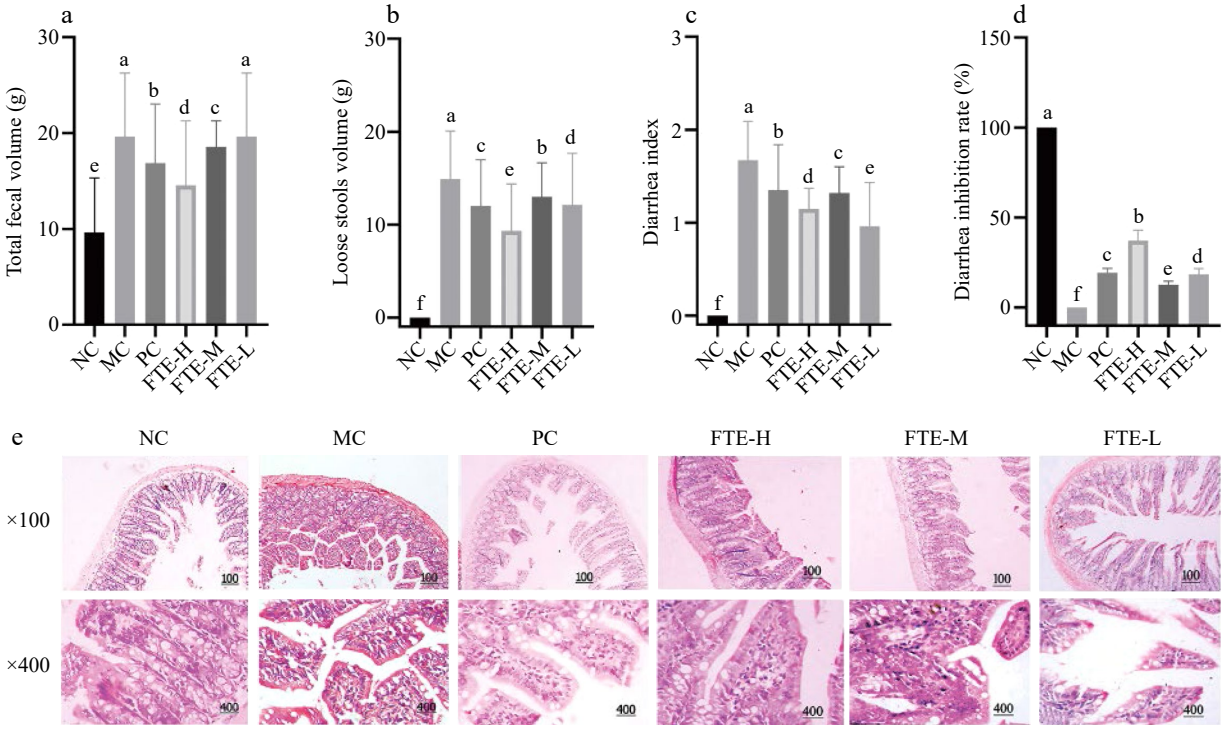

Fig. 2 Diarrhea parameters: (a) total fecal volume, (b) loose stools volume, (c) diarrhea index, (d) diarrhea inhibition rate, and (e) pathological photomicrographs of ileum. NC: normal control group; MC: model control group; PC: positive control group; FTE-H: high dose FTE group; FTE-M: middle dose FTE group; FTE-L: low dose FTE group. Lowercase letters represent significant differences in different types of samples by one-way ANOVA followed by Duncan post hoc test ($P < 0.05$).

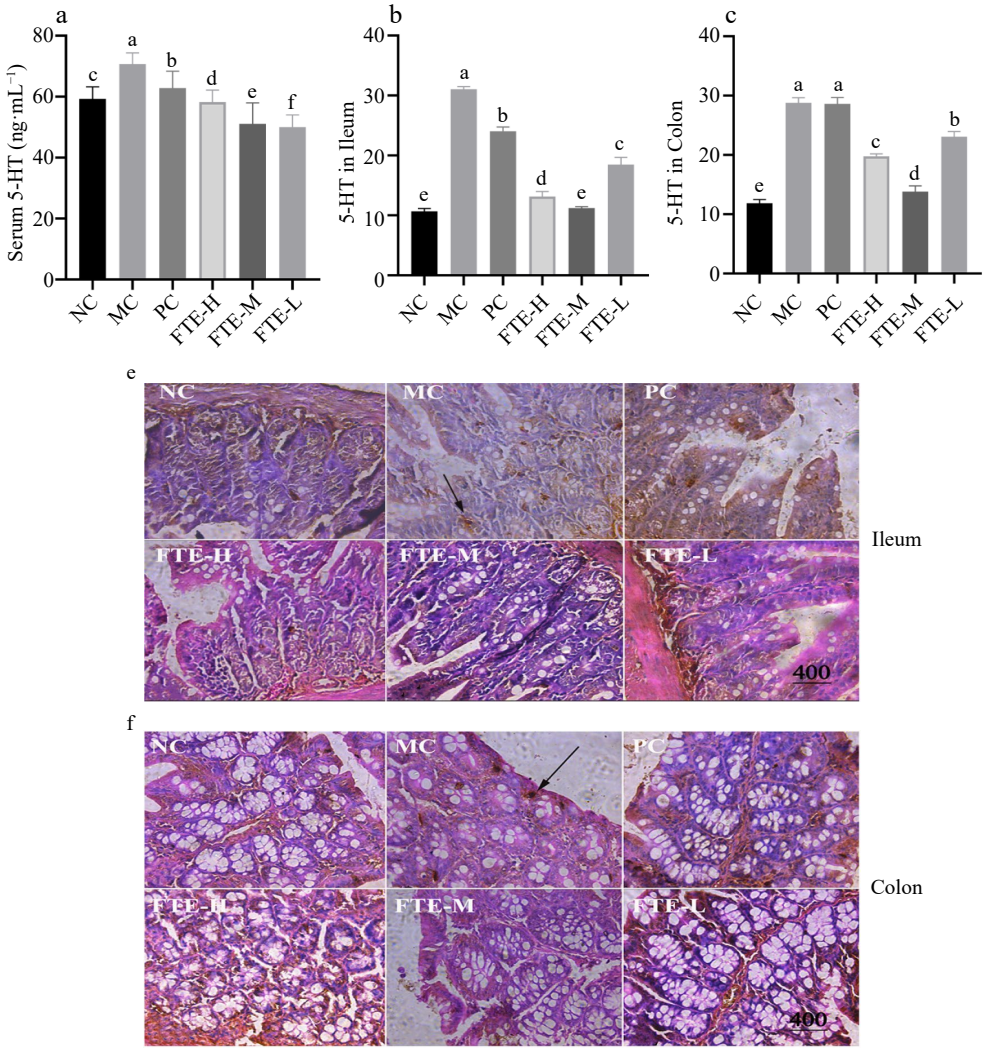

Fig. 3 Analysis of regulation of the level of 5-HT in the serum (a) and microscopy, immunohistochemistry, and image analysis of 5-HT in ileum and colon (b–f). Arrows indicate 5-HT antigens (brown).

Effect of FTE on the expression of NHE-1 and NHE-3 in ileum and colon

In order to explore the exact effect of FTE on electrolyte disorder, the levels of ileum and colon NHE-1and NHE-3 were assessed by IHC and qRT-PCR. As is shown in Fig. 3, the expression of NHE-1 and NHE-3 genes in the colon were down-regulated (Fig. 4e & f), but ileum NHE-3 protein abundance was up-regulated after FTE administration (Fig. 4a & b). NHE-3 gene expression in the colon was consistent with protein expression. Extrapolating from the differences in NHE-3 expression, the regulatory site of FTE may be in the ileum, but not functional in the colon. FTE significantly downregulated colon NHE-1, and upgraded ileum NHE-3. There is currently no definitive evidence that the chemical compositions of FTE regulate expression of NHE-1 and NHE-3 directly. However, the current experimental findings suggest that NHE-1 and NHE-3 expressions are indeed regulated by FTE. In addition, the strength of gene expression is related to functional importance. Collectively, according to these results, we speculated that FTE may regulate expression of NHE-1 and NHE-3, thus maintaining electrolyte balance.

DISCUSSION

Our study found that medium and high-doses of FTE had good anti-diarrhea function, which may be related to its improvement of intestinal inflammation. This study provided new experimental evidence of antidiarrhea activity and potential for further development and utilization of Fu brick tea.

Intestinal inflammation may be an important cause of diarrhea development. The burst of inflammatory activation can disrupt integrity of the mucosa, water-electrolyte balance, eventually resulting in diarrhea development[26,27]. It has been reported that 5-HT can activate the TLR4/MyD88/NF-κB signaling pathway, leading to the release of pro-inflammatory factors[28–30]. In this study, the integrity of ileum mucosa of mice in the model group was damaged and inflammatory infiltration was serious (Fig. 2). This results in ileal brake failure, reduced retention time of intestinal contents in the intestine and increased frequency of defecation. Interestingly, FTE intervention reduced intestinal inflammatory infiltration and protected the integrity of the ileum. There is now sufficient evidence to suggest that functional compounds of FTE can reduce the level of pro-inflammatory factors thereby

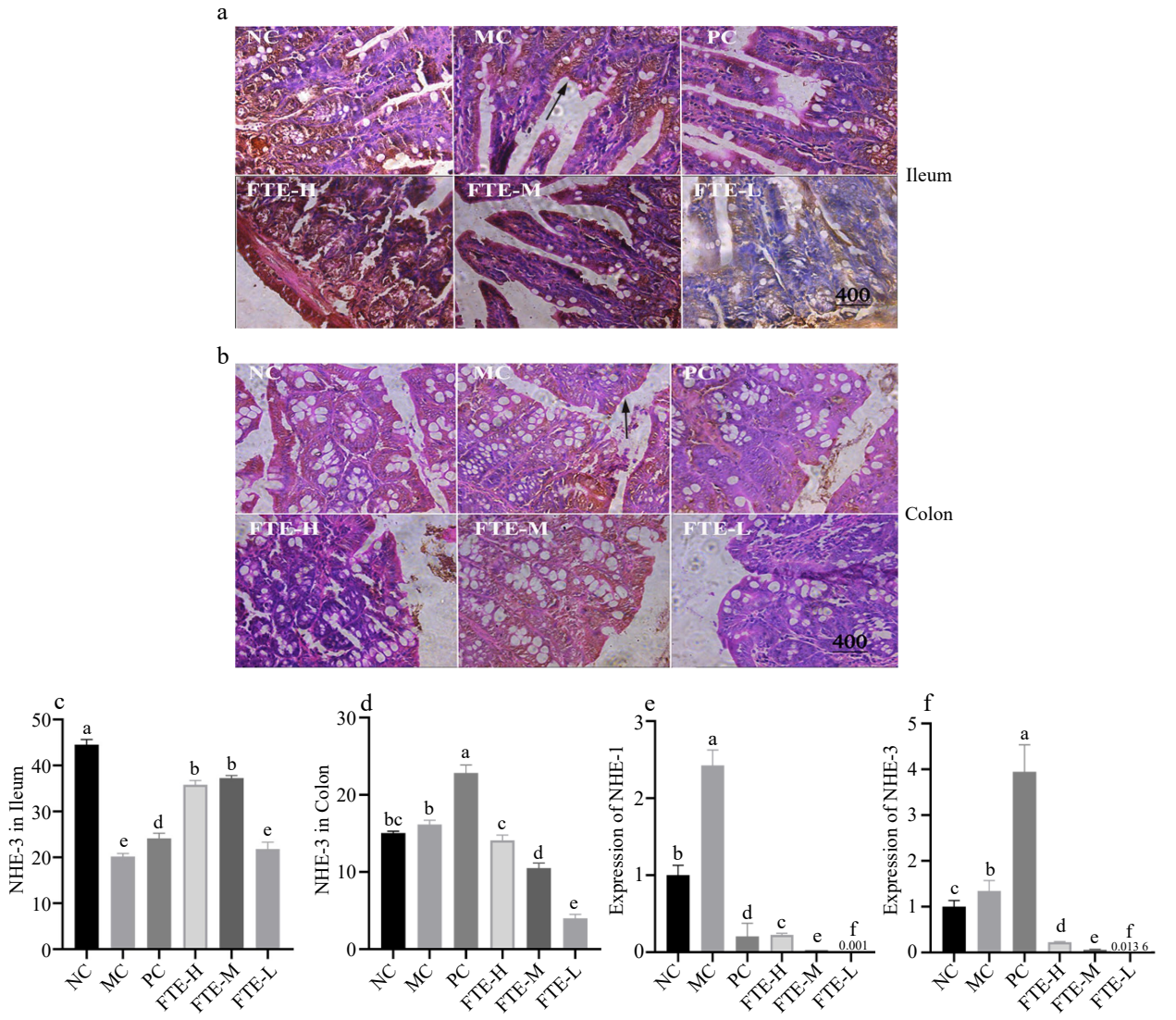

Fig. 4 Microscopy, immunohistochemistry, and image analysis of NHE-3 in the ileum and colon (a–d), and analysis of regulation of gene expression of NHE-1, NHE-3 by FTE (e, f). Arrows indicate NHE-3 antigens (brown).

suppressing the development of inflammation[20,31–33]. Many microbes can utilize carbohydrates to produce short chain fatty acids, which attenuate inflammation to maintain gut health[34,35]. Recent reports also provided evidence that polysaccharides of Fu brick tea regulate intestinal immunity, which indicated it can be effectively utilized by gut microbiota[21]. Therefore, we speculated that the promising anti-diarrhea effect of FTE may be ascribed to its anti-inflammation activity.

The results of this study show that Fu brick tea can effectively improve the expression of NHE-3 in the ileum and colon. Intestinal NHE-3 not only contributes significantly to electrolyte balance, but also affects the integrity of intestinal structure when NHE-3 is lacking[36]. More importantly, intestinal fluid secretion can also be promoted by intestinal inflammation. 5-HT-induced water and electrolyte secretion is mediated by pathways involving 5-HT2, 5-HT3, and 5-HT4 receptors subtypes. In addition, 5-HT couples G protein and calmodulin to regulate NHE-1 and NHE-3 activity through different receptors[37–39]. Therefore, our hypothesis is that NHE-1 and NHE-3 are activated through this pathway (Fig. 5). Collectively, improvement of water-electrolyte disorder may be associated with the anti-inflammatory effects of FTE.

This study showed that medium and high doses of FTE had a significant improvement effect on diarrhea. According to the acute toxicology report, the water extract of fu brick tea of 14,700 mg kg^{-1} had no adverse effect on healthy mice[40]. Thus, combined with the report and previous experiments, the dose used in this study is safe. For healthy adults, the recommended intake of dark tea is 10 g dry tea. Based on body surface area calculations, the effective therapeutic dose used in this trial was 5–10 times the recommended dose. Therefore, we suggest patients with mild diarrhea drink Fu brick tea reasonably according to their own conditions, which can improve diarrhea to a certain extent.

CONCLUSIONS

Collectively, our work has shown that FTE has promising antidiarrhea activity, which may reduce intestinal injury and balance electrolytes through its anti- inflammation properties.

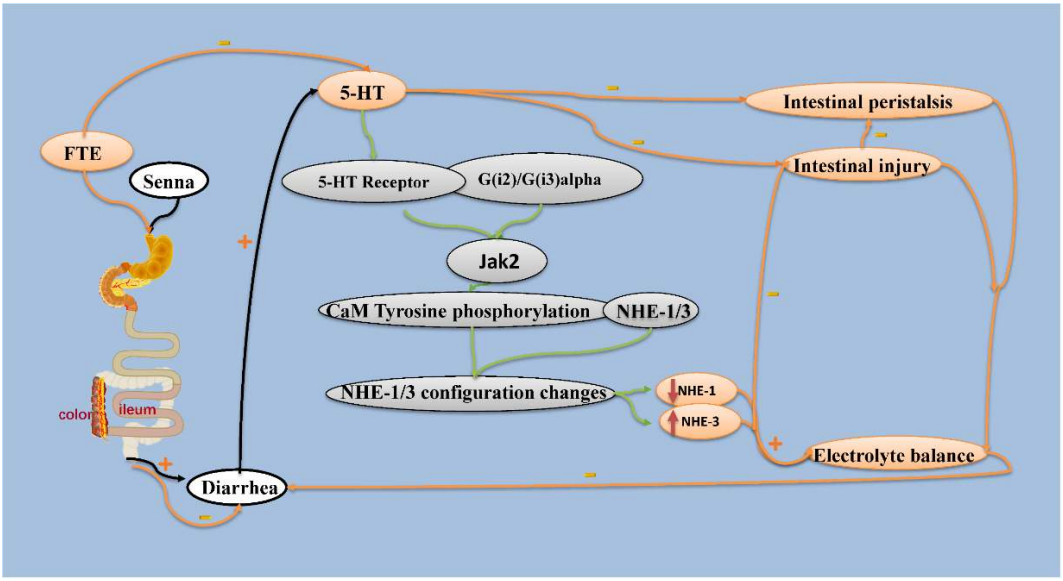

Fig. 5 The potential mechanisms of the anti-diarrhea effect of FTE. First, pro-inflammation 5-HT level was significantly elevated due to senna-induced diarrhea, resulting in an increased inflammatory reaction. Moreover, chronic intestinal inflammation results in damage of the intestinal epithelium along with the dysregulation of intestinal mucosal immunity. Additionally, 5-HT binds with various 5-HT receptors which are coupled with G protein to regulate the expression of NHE-1 and NHE-3 as above. Intestinal damage and electrolyte disturbances are improved with the decreased level of 5-HT of diarrhea mice, thus alleviating diarrhea symptoms. Collectively, anti-diarrhea activity of FTE may be attributed to the maintainance of barrier integrity and electrolyte balance by anti-inflammation.

The findings provide new insights into the mechanisms underlying FTE anti-diarrhea activity. It may also promote the development and utilization of Fu brick tea as an anti-diarrhea resource.

MATERIALS AND METHODS

Materials and chemicals

Fu brick tea was obtained from Hunan Yiyang Tea Factory Co., Ltd., (Yiyang, Hunan Province, China). Senna was purchased from Chengxin Chinese Medicinal Materials Co., Ltd., (Shijiazhuang, Hebei Province, China). Berberine was purchased from Northeast Pharmaceutical Group Co., Ltd., (Shenyang, Liaoning Province, China). Sennoside A (98.75% purity) was purchased from Chengdu Manster Biotechnology Co., Ltd., (Chengdu, Sichuan Province China). Fu brick tea and senna were crushed and boiled in water (100 °C) for 45 min with a material–liquid ratio of 1:10, and then filtered separately through three layers of gauze. The two extracts were concentrated under reduced pressure and then freeze-dried. Senna leaves contained 8.44 mg ml^{-1} sennoside A. FTE contained 22.28 ± 1.04% polysaccharides, 24.01 ± 0.21% polyphenols, 9.24 ± 0.06% flavonoids.

Experimental design

A total of 60 male and female Kunming mice (5–6 weeks of age, body weight of 25.0 ± 2.0 g), were obtained from Hunan SJA Laboratory Animal Co., Ltd., (Changsha, Hunan Province, China). The mice were housed under standard laboratory conditions (23–25 °C, 12 h light/dark cycle). Five mice were housed in one cage with ad libitum access to food and purified water. After one week of acclimatization, mice were randomly divided into the normal control group (NC, n = 10), the model control group (MC, n = 10), the positive control group (PC, n = 10), the high dose FTE group (FTE-H; n = 10), middle dose FTE group (FTE-M; n = 10), low dose FTE group (FTE-L; n = 10). All mice, except those in the normal group, were fed with senna aqueous extract (6,000 mg kg^{-1}) to establish the diarrhea model, and then given corresponding doses of drugs after 1 h. Mice in the normal and model groups were supplemented daily with 0.3 ml of distilled water (vehicle) by intragastric gavage. Mice in the positive control group were given an administration of berberine (60 mg kg^{-1}) by intragastric gavage. Mice in the FTE-H, FTE-M, FTE-L groups were given an administration of FTE (2,530, 1,260, 630 mg kg^{-1}) daily by intragastric gavage respectively. After 7 days of treatment, all mice were anesthetized with 4% chloral hydrate and sacrificed after overnight fasting. The blood, ileum and colon were harvested and the intestinal tissue fixed in 4% paraformaldehyde, or stored at −80 °C for further analysis. Mice feces from metabolic cages were collected and weighed daily. Diarrhea inhibition rate and diarrhea index were according to the literature[41,42]. The loose stool grade was calculated according to the diameter (cm) of the area of the contamination on the filter paper. It graduated into four grades: grade 1 (1 cm), grade 2 (1–1.9 cm), grade 3 (2–3 cm), and grade 4 (3 cm). The diameter of feces blots was measured by a ruler to quantify differences and an average value in each group was taken. Diarrhea rate was calculated as the ratio of the times of defecations per mouse to the total number of defecations. In addition, each granule or pile of defecation on the filter paper was counted as one. The diarrhea index was the product of diarrhea rate and the loose stool grade. Calculating the diarrhea inhibition rate uses the following formula: Diarrhea inhibition rate (%) = 1 – Diarrhea rate. All mice procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hunan Agricultural University.

Histological and immunohistochemical analysis

Ileum and colon tissues were fixed in 4% neutral formalin solution for 24 h, embedded in paraffin, and sectioned (4–5 μm

thick). Hematoxylin and eosin (H&E) staining was performed for pathological examination. Immunohistochemical (IHC) staining was conducted according to the manufacturer's instructions (Immunostain SP kit, Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd.; Beijing, China)[43]. Images of all sections were captured using a camera-equipped light microscope (Olympus, Tokyo, Japan). The positive area of the ileum and colon section was quantified using the Image-pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Measurement of serum 5-HT

Serum was separated after the blood samples were centrifuged at 2,500 r min^{-1} for 10 min., An ELISA kit (Sigma Chemical Company, St. Louis, MO, USA) was employed to detect serum 5-HT level according to the manufacturer instructions.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from the ileum and colon using the TRIZOL reagent and treated with DNase I from the DNA-free™ kit (Ambion, United States) according to manufacturer's instructions. The RNA was used to perform a two-step reverse-transcription polymerase chain reaction as previously described according to manufacturer's instructions (Tiangen Biochemical Technology, China). The primers were synthesized by Nanjing Genscript (Nanjing, Jiangsu Province, China): NHE-1: forward 5'-TCTGCCGTCTCAACTGTCTCTA-3'; reverse 5'-CCC TTCAACTCCTCATTCACCA-3', NHE-3: forward 5'-CCACACACTG CAACAGTACC-3' and reverse 5'-ATAGGCAGTTTCCCATTAGG-3'.

Statistical analysis

Data were presented as means ± SD (standard deviation). The significant differences between groups were determined by Duncan's multiple range test. A p-value < 0.05 was statistically significant.

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Conflict of interest

The authors declare that they have no conflict of interest.

Dates

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