The water-eluting component of *Anemarrhena asphodeloides* Bge. laxative effects on loperamide-induced constipation rats

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Research

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Abstract

Background

Slow transit functional constipation (STC) is the most common type of chronic constipation,with seriously affecting people's survival and quality of life in the recent years. Based on the safety and small side effects of traditional Chinese medicine, it is urgent to research and develop new medicine for the treatment of constipation. As a traditional Chinese medicine, *Anemarrhena asphodeloides* Bge. has been reported treat constipation in a long time, and carbohydrate is its active ingredient.

Methods

ELISA used for detection of serum gastrointestinal hormones and neurotransmitters levels. HE stain was analyzed to evaluate the colon injury. Western blot and RT-PCR detected interstitial cells of Cajal markers (SCF/c-Kit) and aquaporins (AQPs).

Results

The mainly compositions of oligosaccharide and amino acid from AABW was determined by HPLC. Based on the laxative activities of AABW in the study, AABW obviously enhanced the contents of VIP, Gas, MTL, SP and 5-HT and decreased the levels of NO from loperamide-induced rats by Elisa kits. Additionally, AABW could up-regulate the expressions of SCF, c-Kit, AQP3, VIP and down-regulate AQP8 to repair the damage of colon.

Conclusion

These findings suggested that AABW, which could treat constipation through mediating intestinal motility and water metabolism, were laxative active constituents from *Anemarrhena asphodeloides* Bge..

1. Background

Slow transit functional constipation (STC) is the most common type of chronic constipation, and also a frequent clinical disease. It is often a functional bowel disorder characterized by persistent hardship, reduced frequency of defecation or a feeling of endless defecation [1–3]. With the changes of human lifestyle and life structure, the incidence of STC is on the rise, and it has seriously affected people's survival and quality of life in the recent years [4, 5]. The pathophysiology of constipation is not well understood, but it has been suggested that the pathogenesis of constipation is likely to be multi-factor, resulting from inflammation, secretory dysfunction, gastrointestinal motility disorders, and changes in gastrointestinal innervation [6, 7]. To date, a range of traditional and modern medicines, including Senokot, Correctol, Dulcolax and Tegacero, were often used to treat constipation because of their

cathartic effects. These drugs increase the contraction of the smooth muscles of the gut, promote gastrointestinal motility and promote defecation. However, high costs and adverse reactions limit their use in the treatment of constipation. Prokinetic agents, especially Tigacelo, are still commonly used today as laxatives, but are prone to life-threatening coronary artery constriction and myocardial infarction [8]. Based on the safety and small side effects of traditional Chinese medicine, it is urgent to research and develop new medicine for the treatment of constipation.

As a traditional Chinese medicine, *Anemarrhena asphodeloides* Bge. has been reported various pharmacological effects including senile dementia improvement, anti-tumor, anti-platelet aggregation, anti-osteoporosis, anti-inflammation, antibiosis and antidiabetics [9]. There is increasing evidence to indicate that it has potential for use in the treatment of constipation. It is reported that polysaccharides of *Anemarrhena asphodeloides* treat constipation via enhancing gastrointestinal motility [10]. However, the precise effects on the water-eluting component of *Anemarrhena asphodeloides* Bge. (AABW) on constipation have not been investigated thus far, at least to the best of our knowledge.

In this study, AABW was studied to explore the effects and potential mechanism for the laxative treatment, by evaluating the basic indicators of faeces number, faeces wet weight water content, and some futher characteristics of the intestinal motility, muscle abnormality, intestinal nervous system disorder, intestinal stromal cell abnormality and water channel abnormality. The above researches could make some contributions to the development of healthy and non-toxic laxatives.

2. Materials And Methods

2.1. Materials

Loperamide was obtained from Sigma chemicals Co (MO, Louis, USA). The remaining chemicals were of analytical grade.

2.2. Plant material collection and HPLC analysis

The rhizomes of *Anemarrhena asphodeloides* Bge. were collected from Hebei province of China in 2016. After botanical identification by comparative macroscopic and microscopic studies by A/Prof. Ruifeng Fan (Medicinal Botany, Heilongjiang University of Chinese Medicine), the specimens (No.20160901) were identified as the rhizomes of *Anemarrhena asphodeloides* Bge. (*Liliaceae*) and deposited in the herbarium of the Department Chinese Medicine Chemistry of Heilongjiang University of Chinese Medicine.

The dried *Anemarrhena asphodeloides* rhizomes (1 kg) were fractured into pieces and extracted three times with boiling water (1:10, wt/wt) for 3 h. The combined extracts were centrifuged to remove impurities. The supernatant was concentrated, precipitated with 95% ethanol and stored at 4 °C overnight. After centrifugation (3000 rpm for 10 min, at 25 °C), the supernatant was vacuum-dried and reserved at 4 °C until use. The supernatant was subjected to column chromatography on a D101 type macroporous adsorption resin, and eluted with water, 20% ethanol, 60% ethanol, and 95% ethanol in turn. Water eluting

component was obtained and determined by high performance liquid chromatography (HPLC) analysis and identify the composition. The water eluting fraction was dissolved in water at a concentration of 1.00 mg/mL. HPLC analysis was performed on a Atlantis T3 (4.6 mm × 250 mm, 3 mm) maintained at 30 °C, the mobile phase A was water and the mobile phase was methanol. the total run time was 30 min the gradient was: 0–30 min (A: 95% – 0%) The injection volume was 10 mL and the flow rate was set at 1.0 mL/ min. Detection wavelength was set at 254 nm. Figure 1 showed the HPLC chromatograms of the fructose samples, the content of fructose detected by HPLC-UV analysis was more than 90%.

2.3. Experimental animals

Adult Wistar rats (male, 200 ± 20 g, single cage breeding, Certificate: SDXK (Liao) 2017-0065) were purchased from Harbin Medical University. All animal experiments were implemented according to the Animal Care Committee of the School of Medicine, Heilongjiang University of Chinese Medicine (Approval Number, 2017050801). Rats were housed under a constant temperature of 25 ± 2 °C, a relative humidity of 60 ± 10%, and a natural light-dark photoperiod.

2.4. Animal experimental design

Constipation was induced in the animals by injecting 1 mL of 4 mg/kg loperamide twice a day for 3 days, while the control rats were administered normal saline [11]. Successful modeling was performed at day 0 (D0).

AABW was given at a dose of (20 mg/kg, 40 mg/kg and 80 mg/kg, b. w., p. o.) for 3 days (D1-D3) after modeling success. Other groups were treated with 1.2 g/kg Maren Pills and 5.0 mg/kg Mosapride, which were used as the positive control drugs [12].

2.5. Gastrointestinal function test

After successful modeling and completion of treatment, feces of rats in each group were collected within 2 h, weighed by electronic balance, and the quantity, wet weight and water content of feces of rats were recorded. GIP transit was measured through gavage activated carbon suspension 2 mL (concentration: 100 g/L) [13]. The gastrointestinal propulsion was calculated based on the following formula:

GIP = (Distance travelled by charcoal (cm)/total intestinal length (cm)) × 100%

2.6. Histopathological analysis

Paraffin sections of 5 μm thick were taken from the colon of rats. The sections were dewaxed in xylene and then dehydrated in gradient ethanol. After rehydration, colon tissue sections were stained with HE according to the instructions of HE staining kit, and the morphological changes of colon tissue were observed under a Olympus BX60 research microscope (Olympus Corporation, Tokyo, Japan) [11].

2.7. Elisa analysis

After blood samples were extracted from the abdominal aorta, the upper serum was collected by centrifugation at low temperature. Corresponding ELISA kit was used to detect the contents of NO, VIP,MTL, Gas, SP and 5-HT in peripheral blood [14].

2.8. Western blot analysis

After conventional homogenization, total protein was extracted from colon tissue with RIPA lysate. Protein quantification was performed using the BCA kit. 30 μg protein was taken from each sample, denatured by boiling, and 10% SDS-PAGE was performed. The electrophoretic protein was electrotransferred to PVDF membrane. After transfer, the membrane was sealed with 5% skim milk powder at room temperature for 1 h, followed by primary antibody (SCF: AB_1542939, c-Kit: AB_10618857, AQP3: AB_11188824 and AQP8: AB_800001, 1:1000), and incubated at 4℃ overnight. The next day, after washing with TBST, the secondary IRDye® 800CW Goat anti-Rabbit/Mouse lgG (H + L) antibody was added and incubated at room temperature for 1.5 h. After washing with PBST, the blots were developed using an Odyssey® CLx Imaging System with image lab software [15, 16].

2.9. Real-time quantitative PCR analysis

The colon tissue was removed from the RNA Later liquid and the total RNA was extracted by Trizol method. Primer Premier3.0 software was used to design the PCR primers, and primer sequences were shown in Table 1. RNA from colon tissue was extracted according to the experimental procedure provided by the reagent description. After the total RNA was successfully extracted, 2 μL RNA was added into 98 μL DEPC water, and the absorbance (OD) at 260 nm and 280 nm was determined on NanoDrop ND-8000 (Thermo, Waltham, MA, USA) instrument, with a ratio of 1.8 ~ 2. Between 0.20 μL cDNA was obtained by reverse transcription of 1 μg RNA. According to the instructions of real-time quantitative PCR kit, 1 μL cDNA was configured into a 10 μL reaction system. The reaction conditions were pre-denaturation at 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, and cycling 40 times. Finally, the dissolution curve program was added (95 °C for 15 s, 60 °C for 1 min, and 95 ° C for 15 s). Thresholdcycles (Ct) of the sample was obtained by using the RQ Manager data analysis software to calculate the relative copy levels and then compare the samples. Ct values measured using $2^{-\Delta\Delta Ct}$ method [17].

Table 1
primers sequences

Indicators	primers sequences
SCF	F: 5'-CTC AGT TTT GTG GCT TCG TTT A-3'
	R: 5'-CTA CCA TGT CCG ATA CTA CGA C-3'
c-Kit	F: 5'-CAG AAA CCC ATG TAT GAA GT-3'
	R: 5'-CTT TCC AAA ACT CAG CCT GT-3'
AQP3	F: 5'-GCC ATT GTT GAC CCT TAT AAC AAC-3'
	R: 5'-AGT GAA AAG GCG AGG TCC AA-3'
AQP8	F: 5'-CTG TGT GTA TGG GTG CCG TCA A-3'
	R: 5'-AGA TCC CAC CAC CTG CCA GGA-3'
PGE2	F: 5'-CTC GGC TGC AAC ATC AGT GT-3'
	R: 5'-AGT CCT TTC TCC TCT CCG GC-3'
GAPDH	F: 5'-TGG GTG TGA ACC ACG AGA A-3'
	R: 5'-GGC ATG GAC TGT GGT CAT GA-3'

2.10. Statistical analysis

All data were subjected to a one-way ANOVA and a Dunnett's T test and the data were expressed as the mean ± SEM by SPSS 20.0. *P* values less than 0.05 or 0.01 indicated significant or extremely significant differences, respectively, and these differences were labeled with * or ** (extremely significant difference).

3. Results

3.2. Effect of AABW on stool parameters

Compared with the control group, loperamide significantly reduced the fecal quantity, wet weight and water content of rats. AABW could improve this abnormal change, as shown in Fig. 2.

3.3. Effect of AABW on small intestinal transit rates (GIP)

Compared with the control group, the GIP of the model group was significantly lower ($P < 0.01$). Compared with the model group, AABW 80 mg/kg could improve or even restore intestinal peristalsis in Fig. 3.

3.4. Effects on Histopathology

In the normal group, the colonic mucosa was intact, and the four layers of the mucosal layer, submucosa, muscular layer and outer layer of the intestinal wall were clear, the columnar cells were arranged neatly, and the goblet cells were mixed in the middle. No inflammatory cells were found and the glands were arranged neatly. In the model group, a large number of inflammatory cells infiltrated, interstitial vasodilation and hyperemia; AABW 80 mg/kg and positive control groups showed obvious improvement of inflammatory cell infiltration, and intestinal mucosa was arranged neatly in Fig. 4.

3.5. Gastrointestinal hormone levels

Compared with the control group, loperamide significantly decreased levels of Gas, MTL, SP, 5-HT and VIP and noticeably increased the content of NO ($P < 0.05$, $P < 0.01$). Compared with the model group, treatments with Maren Pills, Mosapride and AABW 80 mg/kg were shown to improve abnormal levels of gastrointestinal hormones (Fig. 5) ($P < 0.05$, $P < 0.01$).

3.6. Expressions of SCF and c-Kit, AQP3 and AQP8 proteins

Compared with the normal group, the expression levels of SCF and c-Kit protein in the model group were significantly lower; compared with the model group, the expression levels of positive control groups and AABW 80 mg/kg were significantly increased (Fig. 6).

Compared with the normal group, the expression of AQP3 in the constipation group clearly decreased; however, constipation rats treated with AABW 80 mg/kg showed an increase in AQP3 expression. Nonetheless, the expression of AQP8 was opposite to that of AQP3 (Fig. 6).

3.7. SCF, c-Kit, VIP, AQP3 and AQP8 mRNA expression levels of the constipation-related genes

As shown in results, the levels of mRNA expression of SCF, c-Kit, AQP3 and VIP were significantly downregulated, and the level of mRNA expression of AQP8 was significantly upregulated in the loperamide-induced rat colon ($P < 0.01$). However, the levels of mRNA expression in the AABW-treated constipation rat groups showed that AABW could significantly enhance the mRNA expression levels of SCF, c-Kit, AQP3 and VIP, and AABW could significantly decrease the mRNA expression level of AQP8 ($P < 0.05$, $P < 0.01$) (Fig. 7).

4. Discussion

STC is the most common type of chronic functional constipation, which has a high incidence in the population and is difficult to treat. STC has become one of the important factors affecting people's quality of life worldwide. Data show that the prevalence of constipation in Chinese adult population is 3% ~ 17%, which is higher in females, 4 times as high as that in males [18, 19]. AABW, as a component from *Anemarrhena asphodeloides* Bge., has been reported to play multiple effects, including relieving

constipation. However, the detailed mechanisms through which AABW exerted laxative effects remain to be elucidated. The results of this study showed that AABW could promote defecation and increase fecal water content in constipated rats, and reduce fecal passage time in the intestine. These results suggested that AABW has the effect of relieving constipation (Fig. 2–3).

Loperamide directly stimulates enteric-wall μ-receptors, inhibits the production of Ache and PEG2, and reduces intestinal peristalsis and secretion of gastrointestinal hormones, thus playing an anti-diarrhea role [20]. The rat constipation model induced by loperamide has a short establishment period and a long duration of symptoms, and can well simulate the pathophysiological characteristics of constipation. It should be pointed out that gender had a great influence on the establishment of the constipation model, and female rats had a more obvious difference in modeling effect. In order to avoid the interference of unknown factors, male rats were finally selected [21, 22]. In this study, it was found that rats were injected with loperamide, combined with symptoms, colonic fecal volume and fecal water content, to preliminarily determine the establishment of loperamide induced constipation. Above all, it could be concluded that the rat model of constipation induced by lomeramide was successfully replicated (Fig. 2).

Constipation is associated with an enteric nervous System (ENS) disorder. In ENS, 5-hydroxytryptamine (5-HT), vasoactive intestinal peptide (VIP) and NO are all neurotransmitters related to peristaltic activity of gastrointestinal tract [23–25]. 5-HT is an excitatory neurotransmitter, which can directly act on the 5-HT_4 receptor in the intestinal pheochromocytoma cells in the superior colonic mucosa to cause intestinal smooth muscle contraction and promote intestinal peristalsis to cause defecation [23]. VIP could promote gastric empties, promote digestive secretion, inhibit gastrointestinal smooth muscle relaxation, and play a promoting role in gastrointestinal movement [24, 29, 30]. MTL and Gas could promote the secretion of pepsin, contract gastrointestinal smooth muscle, promote gastric peristalsis, and play an exciting role in gastrointestinal movement [25]. NO is a non-specific inhibitory neurotransmitter in ENS. Excessive NO could be diffused into intestinal smooth muscle cells to reduce intracellular Ca^{2+} concentration, thus causing excessive relaxation of intestinal stage smooth muscle and excessive intestinal stage peristalsis (intestinal spasm) [26, 27]. AABW could improve the symptoms of constipation by relieving the above gastrointestinal hormone and neurotransmitter abnormalities (Fig. 5)

At present, the etiology of STC is not clear, but slow intestinal peristalsis and excessive absorption of intestinal water are two important factors for the occurrence of constipation. Recent studies have shown that ICC and AQPs are closely related to intestinal dynamics and intestinal fluid metabolism, respectively. ICC is closely related to intestinal peristalsis and is considered as a pacemaker cell of intestinal peristalsis, which can initiate rhythmic intestinal myoelectric activity and simultaneously produce contraction rhythm of gastrointestinal smooth muscle [30]. A study showed that the ICC density of constipation patients was lower than that of normal people, and the decrease of ICC density resulted in the loss of ICC spontaneous rhythmic slow wave effect and colonic motor disorder. C-Kit and its ligand SCF are the main factors for ICC growth, development and maintenance [31–34]. AABW significantly upregulated the levels of both c-Kit and SCF in mice with Lop-induced constipation (Fig. 6). These results suggested that AABW increased the numbers of ICCs in mice with Lop-induced constipation.

Aquaporin (AQPs) is closely related to intestinal water absorption. Abnormal expression of AQPs in the intestinal tract will have an impact on intestinal hydrologic metabolism, and is closely related to the occurrence and development of constipation [35]. At present, 13 subtypes of AQP family have been found (AQP0-AQP12), among which AQP3 plays an important role in intestinal hydrohydration metabolism. VIP is a neuropeptide regulating intestinal smooth muscle contraction and intestinal fluid metabolism, while AQP3 is mainly distributed in human intestinal and colon epithelial cells, and plays an important role in intestinal epithelial cell fluid reabsorption. VIP might bind to the receptor of intestinal epithelial cells and regulate the expression of AQP3 in the intestine of rats through the cAMP-PKA signaling pathway [36]. Recent studies have found that a variety of inflammatory factors have regulation on AQP3, such as IL-6, PEG2. PEG2 is associated with increased AQP endocytosis and degradation [37]. Stimulating colon to release PEG2 and VIP and down-regulating AQP3 expression in epithelial cytoplasm may be the key link of AABW in regulating the water metabolism of hemp canal. By regulating the content of functional AQP3 in the plasma membrane of colonic epithelial cells, the efficiency of water transport can be regulated, which further influences the hydrologic metabolism of colon. This might be the biological basis for the role of AABW (Figs. 1D, 6 and 7).

5. Conclusion

In conclusion, AABW might improve intestinal water liquid metabolism by regulating the colon absorption and colon secretion of both ICCs and AQPs in the enteric nervous system, so that its function can be improved or even restored to normal, accelerate colonic peristalsis, promote fecal discharge, relieve and treat constipation symptoms.

List of abbreviations

HPLC	High Performance Liquid Chromatography		
UV	Ultraviolet Rays	NO	Nitric oxide
p.o.	per os	HE	Hematoxylin-eosin
ELISA	enzyme linked immunosorbent assay	ICC	interstitial cells of Cajal
IL-6	Interleukin-6	PEG2	prostaglandin E2

Declarations

Declarations

Ethics approval and consent to participate

All animal experiments were implemented according to the Animal Care Committee of the School of Medicine, Heilongjiang University of Chinese Medicine (Approval Number, 2017050801).

Consent for publication

All authors listed have approved the publication of this manuscript.

Availability of data and materials

The research data generated from this study is included within the article.

Competing interests

The authors of the paper have no financial or personal relationships with other people or organizations that would create a conflict of interest.

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Authors' contributions

Contributions of Xiaomao Li and Yan Liu were equal for designing the experiment, conducting the experiment, processing the data and writing the manuscript. Yonggang Xia and Yanping Sun touched up the manuscript. Bingyou Yang and Haixue Kuang provided funding and supervised the experiment.

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Figures

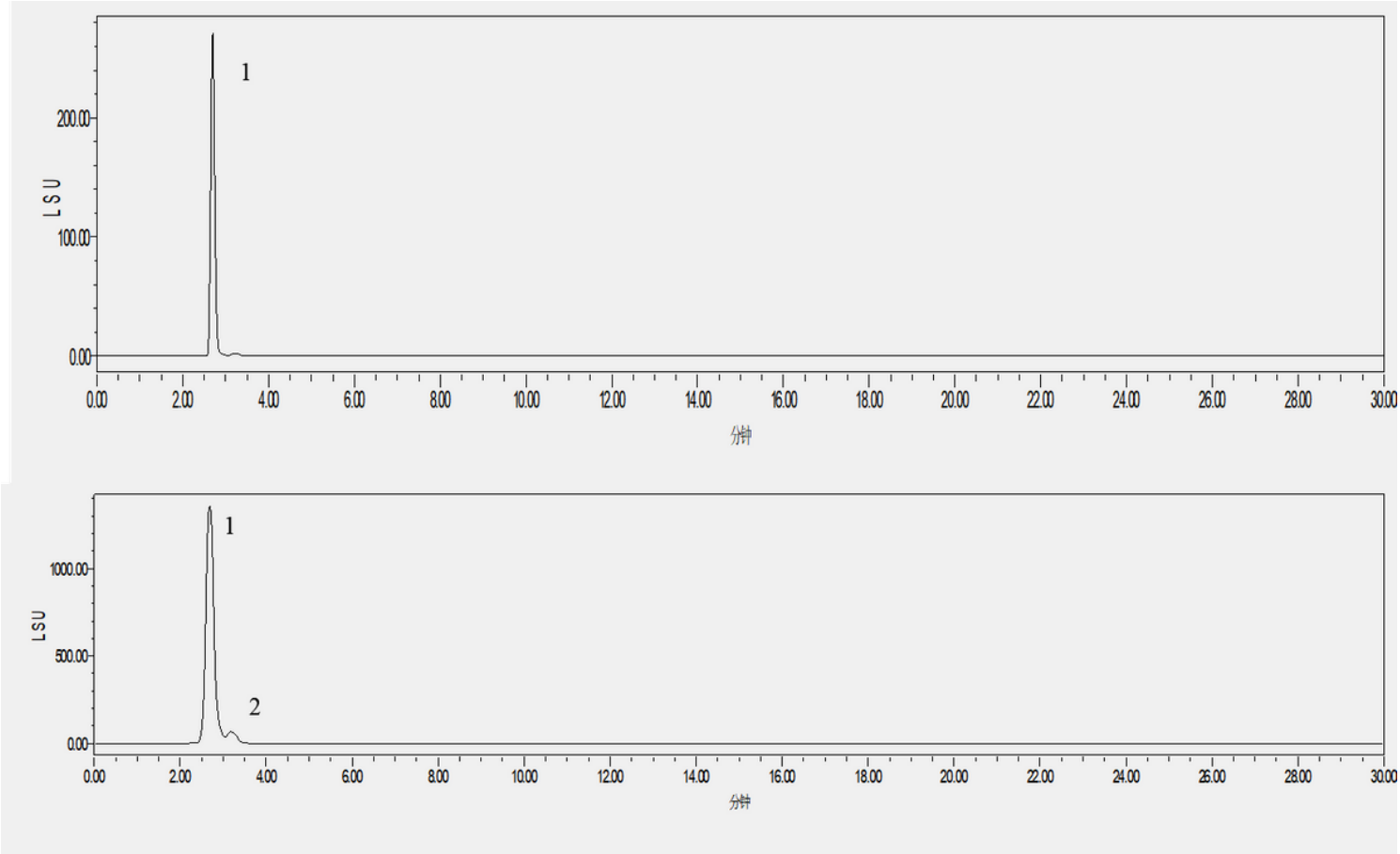

Figure 1

HPLC analysis of AABW and fructose standard

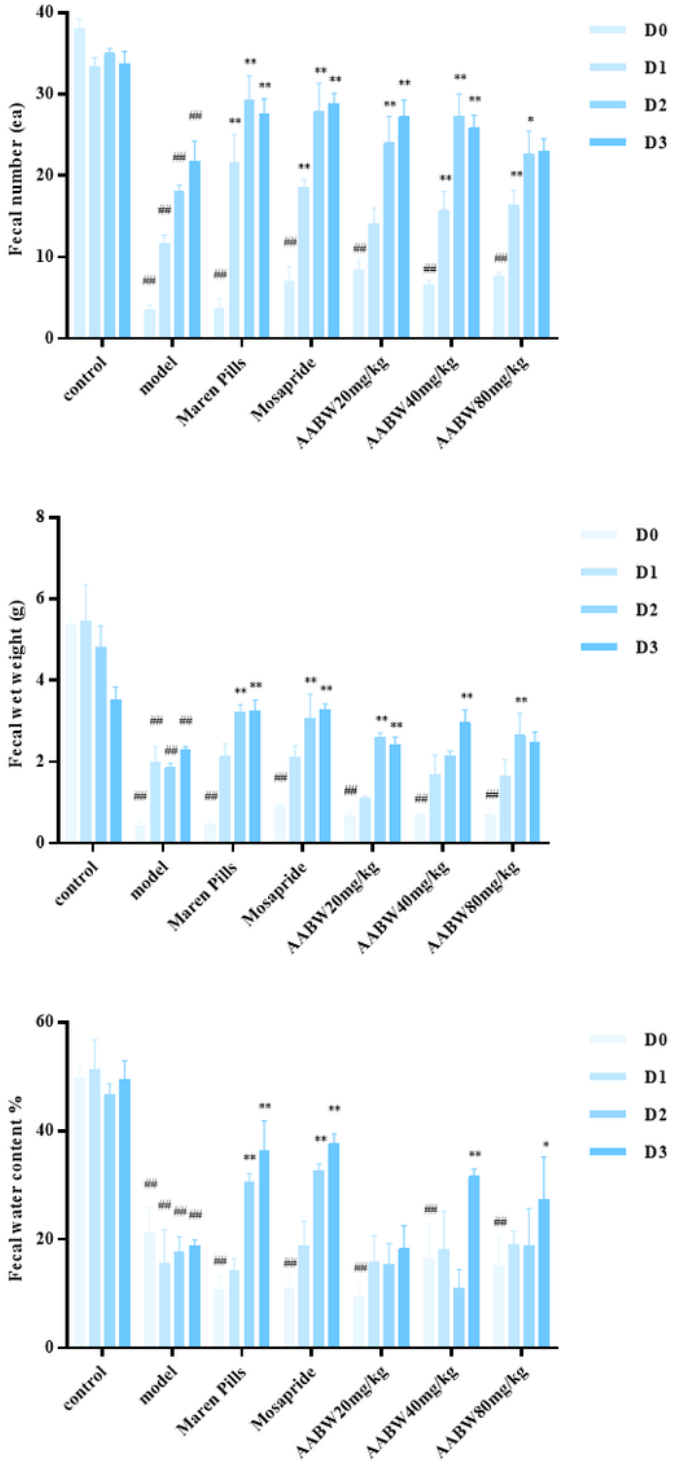

Figure 2

Effects of AABW on faecal output character in loperamide-induced rats. (A) number of faeces; (B) wet weight of faeces; (C) water content. Mean ± SEM, n = 8 ##P < 0.01 compared to the control group, while *P < 0.05 and ** < 0.01 compared to loperamide-induced group.

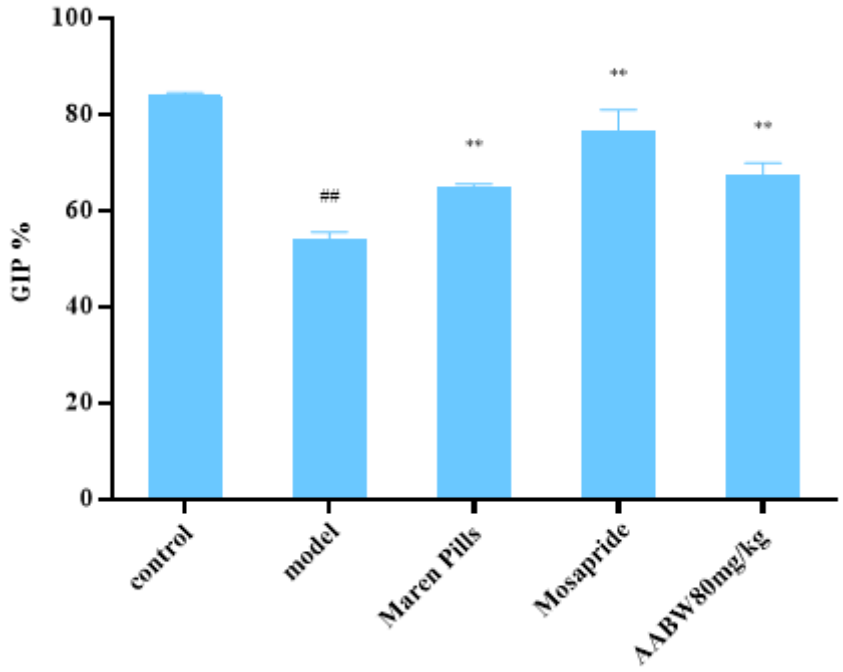

Figure 3

Small intestinal transit rate of rats. Mean ± SEM, n = 8 ##P < 0.01 compared to the control group, while *P < 0.05 and ** < 0.01 compared to loperamide-induced group.

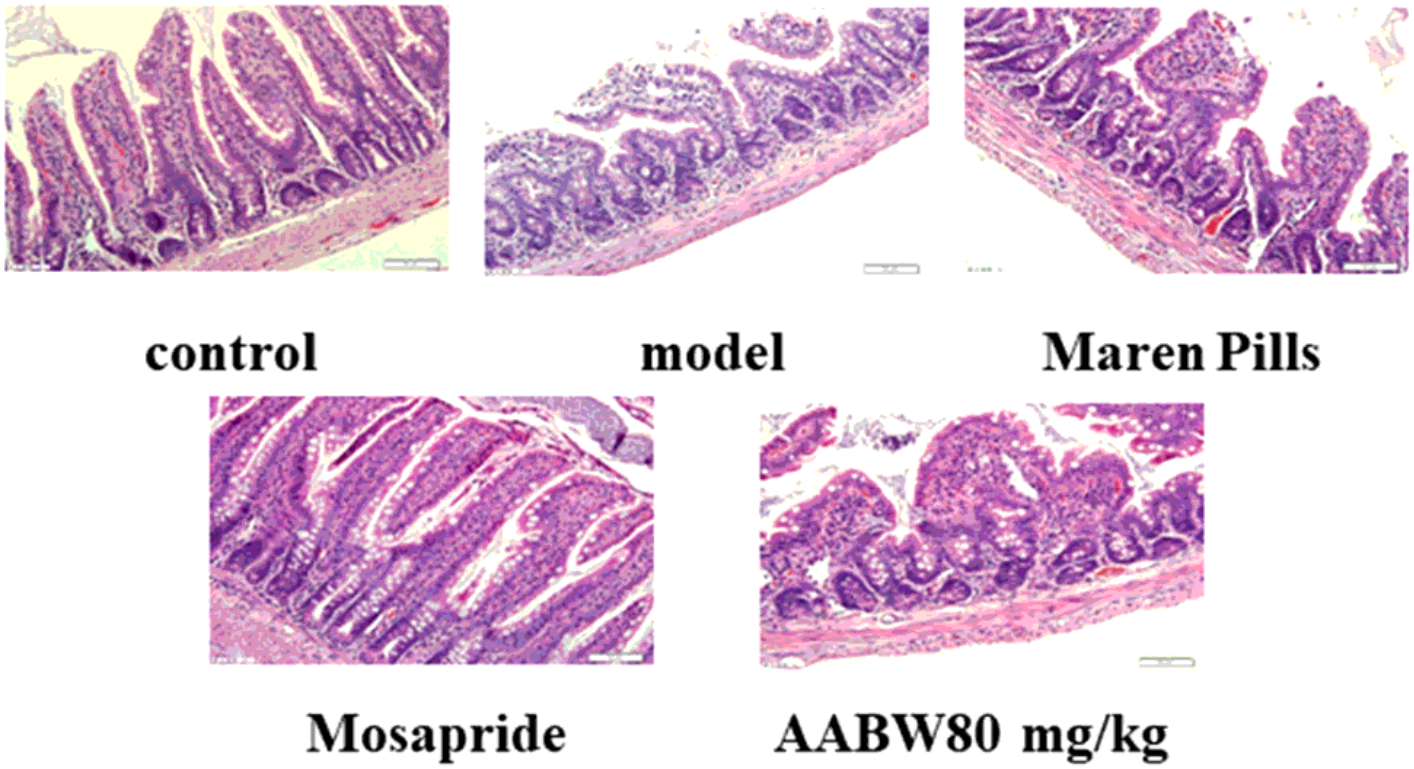

Figure 4

Representative slides of HE stain results for colon sections (magnification, ×200).

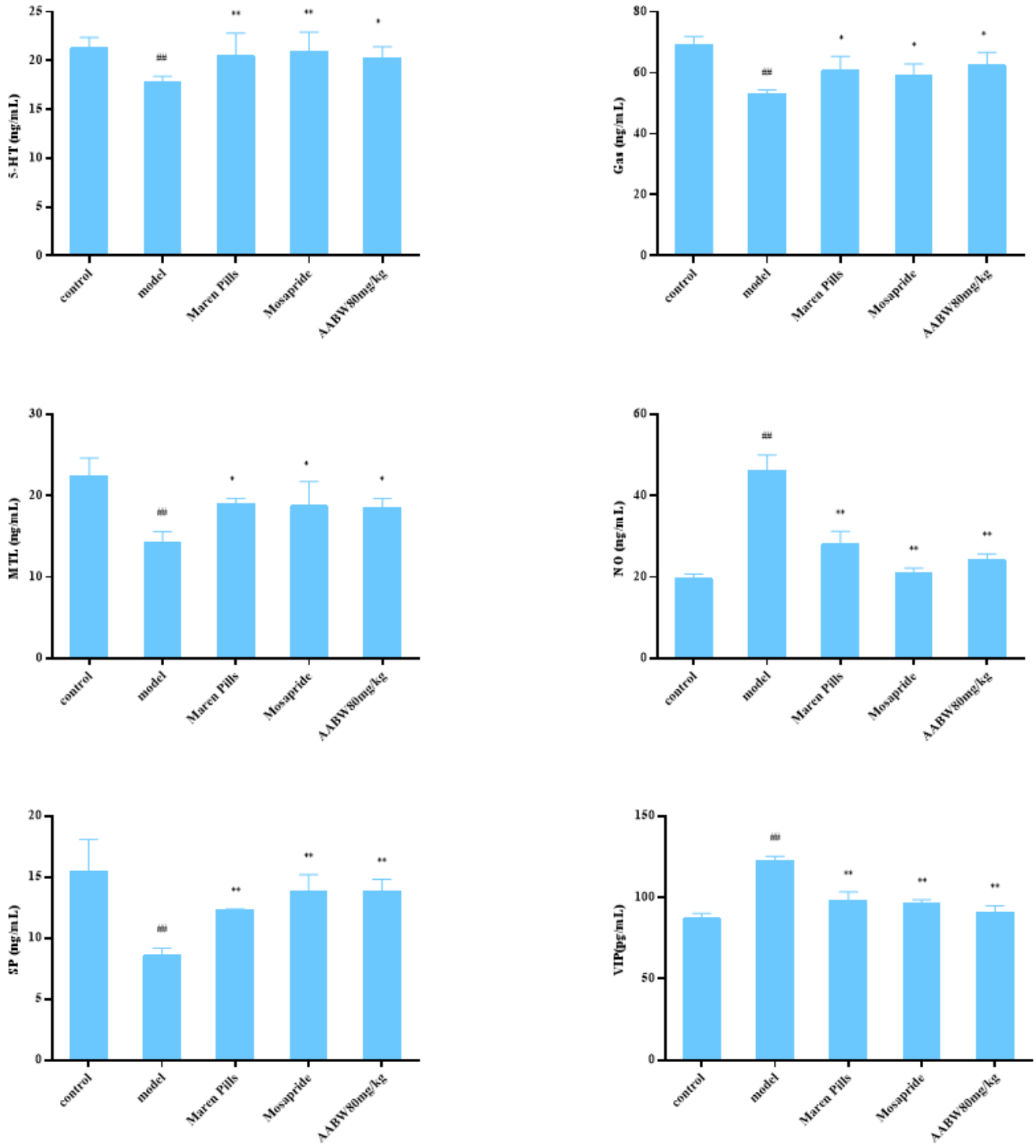

Figure 5

Effect of AABW on serum levels of Gas, MTL, SP, 5-HT, VIP and NO. Mean ± SEM, n = 8 ##P < 0.01 compared to the control group, while *P < 0.05 and ** < 0.01 compared to loperamide-induced group.

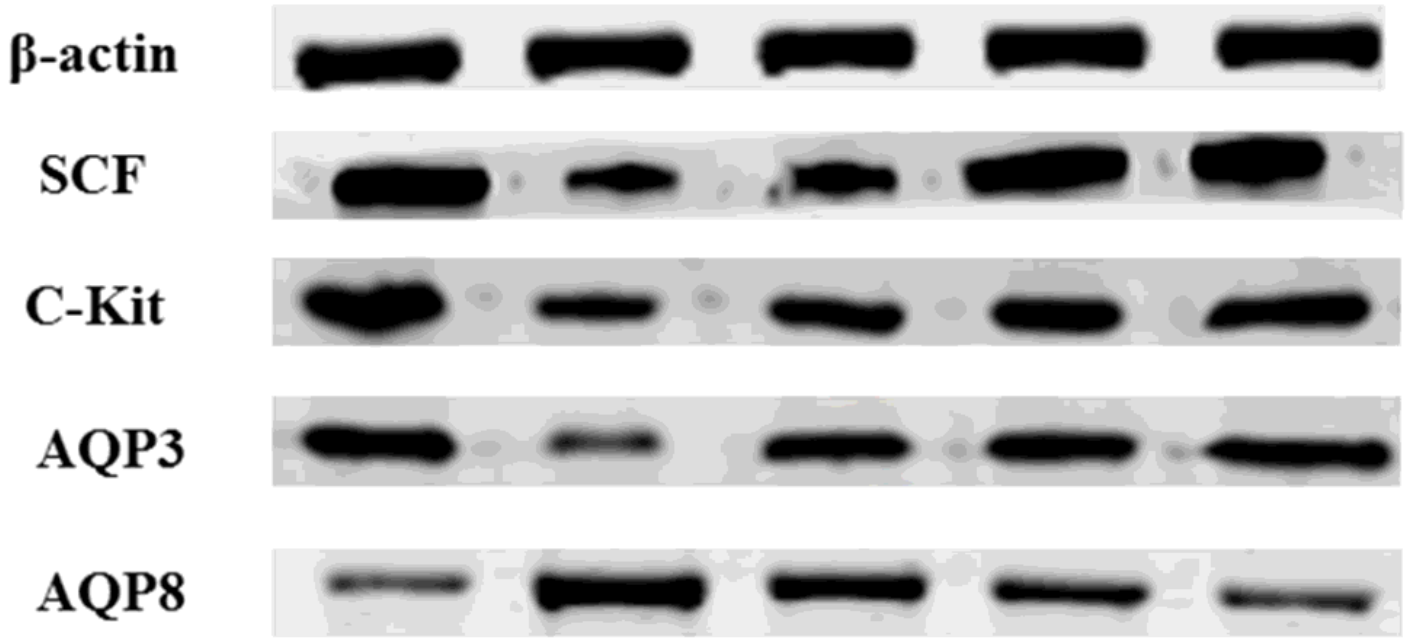

Figure 6

SCF, c-Kit, AQP3 and AQP8 protein expression levels in the rat colon. Mean ± SEM, n = 8 ##P < 0.01 compared to the control group, while *P < 0.05 and ** < 0.01 compared to loperamide-induced group.

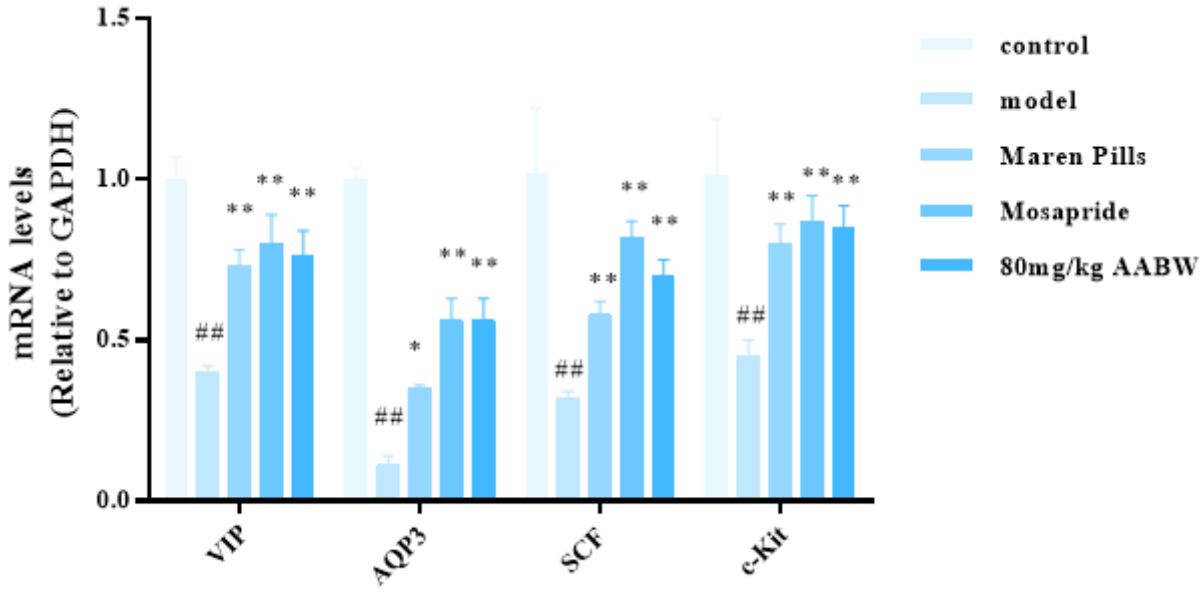

Figure 7

Effects of AABW on the mRNA levels of SCF, c-Kit, AQP3, VIP, in rat colons. Mean ± SEM, n = 8 ##P < 0.01 compared to the control group, while *P < 0.05 and ** < 0.01 compared to loperamide-induced group.