

Original Article

Therapeutic Effect of Ershen Pill (二神丸) Extract on Pi (Spleen)-Shen (Kidney) Yang Deficiency-Induced Diarrhea in Rat Model*

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ABSTRACT **Objective:** To investigate whether Ershen Pill (ESP, 二神丸) could alleviate the symptom of Pi (Spleen)-Shen (Kidney) yang deficiency (PSYD)-induced diarrhea in rat model and explore its anti-diarrhea mechanism. **Methods:** Seventy-five Sprague-Dawley rats were divided into 5 groups by a random number table, including control, positive, model, low-dose (LD) and high-dose (HD) ESP groups, 15 rats in each group. All the rats, except those in the control group, were developed PSYD induced-diarrhea based on its pathology and etiology. The rats in positive, LD and HD ESP groups were treated with Shenling Baizhu Pill (参苓白术丸), LD (1.05 g/kg) or HD (3.50 g/kg) ESP petroleum ether extract once a day for 2 weeks, respectively. Body weight change and diarrhea index were measured. The histology scores of the kidney were evaluated via hematoxylin and eosin (HE) staining. Aquaporin-3 (AQP3) expression in the colon was analyzed by immunofluorescence, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot, respectively. **Results:** Compared with the model group, oral administration of LD and HD ESP prevented body weight loss and inhibited diarrhea after 2-week treatment ($P < 0.05$). Kidney deterioration was impeded, and the histology score in LD and HD ESP groups were 8.2 and 10.5, respectively, which were both higher than those in the model group ($P < 0.05$). In addition, ESP treatment alleviated rat colitis, and HD ESP significantly improved the AQP3 positive staining intensity in the colon tissue compared with the model group. The result from Western blot revealed that AQP3 protein synthesis in colon tissue of LD and HD ESP groups increased by 2.1- and 5.9-fold compared with the model group ($P < 0.05$). qRT-PCR result showed that AQP3 gene expression in the HD ESP group was also up-regulated by 2.5-fold normalized to the model group ($P < 0.05$). **Conclusion:** ESP extract effectively alleviates the symptoms of PSYD and relieves PSYD-induced diarrhea by improving AQP3 synthesis in the colon.

KEYWORDS Ershen Pill, Chinese medicine, petroleum ether extract, anti-diarrhea, AQP3, colon

Pi (Spleen)-Shen (Kidney) yang deficiency (PSYD) is one of the most common syndromes in Chinese medicine (CM) clinics. The characteristic symptoms of PSYD include diarrhea, feeling cold, loss of appetite, lethargy, and sexual dysfunction.^(1,2) In CM, Shen has a special relationship with Pi. Huangdi's Internal Classic recorded that Pi mainly controlled movement and transformation of multiple systems including digestion, absorption, energy conversion, and the immune system.⁽³⁾ It is the foundation of acquired constitution and includes not only Pi in modern anatomy but also the pancreas and lymphatic system. PSYD would disrupt normal metabolism and digestive functions and lead to severe diarrhea.^(4,5) Thus, the investigation of PSYD-induced diarrhea is of significant value. Many relatively mature models of Shen yang deficiency⁽⁶⁾ and Pi yang deficiency⁽⁷⁾ have been generated successfully by hydrocortisone

injection,⁽⁸⁾ CM,⁽⁹⁾ or surgical treatments.⁽¹⁰⁾ In our previous study, PSYD model was developed according to its pathological and etiological characteristics.⁽¹¹⁾

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The Chinese herbal medicine formula Ershen Pill (ESP, 二神丸) comprises processed *Myristica Fragrans* (MF) and *Fructu Psoraleae* (FP) and has been used for treating PSYD-induced diarrhea. It was reported that FP had been widely used to treat a variety of bone disorders,⁽¹²⁻¹⁵⁾ skin diseases,⁽¹⁶⁾ and also used as an anti-fungal agent.⁽¹⁷⁾ In clinical CM, MF was mainly used to treat various intestinal diseases.^(18,19) Its extracts were considered to have many biologically useful activities including antioxidant and inflammatory,⁽²⁰⁾ anti-tumor,⁽²¹⁾ anti-bacterial,⁽²²⁾ and others. Until now the therapeutic mechanisms of ESP for PSYD diarrhea remain unclear.

In this study, a rat PSYD diarrhea model was established. Body weight change, diarrhea index, and kidney histology were analyzed to evaluate the therapeutic effect of ESP petroleum ether extract on PSYD-induced diarrhea symptoms. Aquaporin-3 (AQP3) expression in colon was measured to analyze the possible anti-diarrheal mechanism.

METHODS

Preparation of ESP Petroleum Ether Extract

Dried FP and MF were purchased from Sichuan Province Traditional Chinese Medicine Hospital (lot No. 20140212, 20140124) and both were authenticated by Prof. LU Xian-ming from Chengdu University of Traditional Chinese Medicine, China. After traditional Chinese slicing and stir-fry processing, FP and MF were prepared as CM decoction pieces, then mixed proportionately (2:1) and decocted by refluxing twice with 75% ethyl alcohol (1:8, w/v), followed by water (1:10, w/v). The filtrates were combined and condensed to form a raw extract. The raw extract was further extracted with petroleum ether by Soxhlet extraction and dried under reduced pressure to form the ESP petroleum ether extract, subsequently referred to as ESP extract (Yield: 7.96% ± 0.35%).

High Performance Liquid Chromatography Analysis

The quality of ESP extract was measured using high performance liquid chromatography (HPLC). In brief, 0.01 g was dissolved in methanol and filtered through 0.45 μm filters for HPLC analysis. Chromatographic conditions were as follows: Shimadzu LC-20AT-DAD HPLC system (Shimadzu Co. Japan), C18 column (4.6 mm × 250 mm, 5 μm), injected volume of 10 μL, column temperature of 30 °C, flow velocity at 1 mL/min, UV wavelength

at 230 nm, and gradient elution of acetonitrile/water as follows: 0–15 min, acetonitrile 30%–45%, water 70%–55%; 15–45 min, acetonitrile 45%–65%, water 55%–35%; 45–55 min, acetonitrile 65%–100%, water 35%–0%, and keep 10 min.

Model Development and Grouping

Healthy male Sprague-Dawley rats (weight: 300 ± 20 g, n=75) were commercially purchased from Dashuo Biological Science and Technology Co., Ltd. [Certificate No. 0002568, SCXK (Chuan) 2013-24, Sichuan, China]. All the animals were all fed under the normal condition with temperature: 25 °C, relative humidity: 50%–65%, light/dark cycle: 12 h light and 12 h dark. The animal experiments were performed at the Key Laboratory of State Administration of Traditional Chinese Medicine, Chengdu University of Traditional Chinese Medicine (No.TCM-03-043), and the animal experiment protocols were approved by Chengdu University of Traditional Chinese Medicine Animal Care and Use Committee. Seventy-five rats were randomly divided into 5 groups (15 rats per group) by a random number table including control, model, low-dose (LD) ESP, high-dose (HD) ESP and positive group. All the rats, except those in the control group, were treated with 0.2 g/(kg·d) of adenine by oral gavage for 4 weeks to simulate Shen yang deficiency. Additionally, in order to weaken Pi yang during this period of time, the model rats were given cabbage (10 g/each) orally on odd-numbered days and refined lard oil (2 mL/each) on even-numbered days to create a diet disorder. Also, all the rats were exhausted by swimming every afternoon. At the third week of modeling, the rats were drenched with chilled *Folium Sennae* (2 mL/each) to induce Pi yang deficiency diarrhea. The period of modeling was 4 week totally. The experimental protocol is shown in Figure 1.

Drug Administration

The ESP extract was mixed with Tween-80, ground to sufficient emulsification standards, and combined with distilled water to form a suspension. The suspension was administered daily via gavage to mimic its use based on CM pharmacology research method.⁽²³⁾ Low-dose (6 times of normal: 1.05 g/kg) and high-dose (20 times of normal: 3.50 g/kg) ESP extract were chosen for oral administration to rats. The positive group received Shenling Baizhu Pill (参苓白术丸, lot No. 20120515, Shandong Fangjian Pharmaceutical Co., Ltd., China), and the model

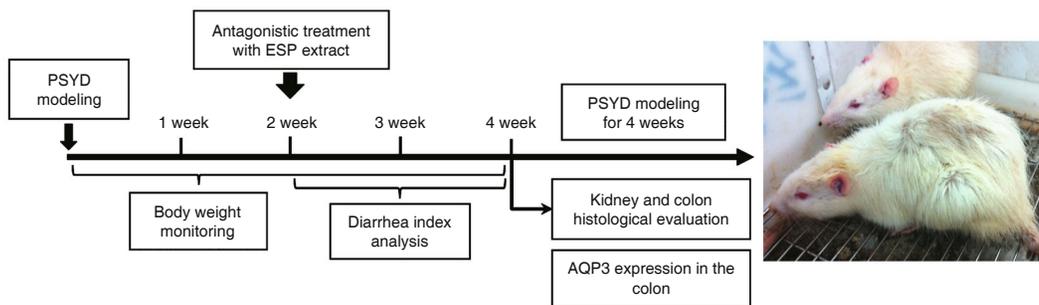


Figure 1. Experimental Protocol

group was given an equivalent volume of Tween-80. ESP extract was orally administered to all the treated rats starting at week 2.

Measurement of Body Weight and Stool Condition

During the entire treatment, body weight change was measured every 3 days. Stool condition was analyzed via diarrhea index. The loose stools grade was represented according to the splotch diameter: grade 1, diameter ≤ 1 cm; grade 2, diameter 1 to 1.9 cm; grade 3, diameter 2 to 3 cm and grade 4, diameter ≥ 3 cm. Afterwards, the diarrhea index was calculated using the following formula:

$$\text{Diarrhea index} = \frac{\text{loose stools}}{\text{total feces}} \times \text{loose stools grade}$$

Histological Analysis

After treatment with ESP extract, the rats were sacrificed by euthanasia with carbon dioxide, and the kidney and colon were removed for macroscopic observation and histological analysis. Kidney and colon were fixed with 4% paraformaldehyde (PFA) at 4 °C overnight, dehydrated with ethyl alcohol, and prepared in 5 μ m thick paraffin-embedded sections. All the samples were stained with hematoxylin and eosin (HE) and examined using a microscope. Kidney histological scoring is presented in Appendix 1.

Immunofluorescent Analysis

The colon tissue in each group was collected and prepared into 10 μ m thick frozen sections. The sections were then blocked with 2% bovine serum albumin (BSA) for 30 min at 37 °C, then incubated with anti-AQP3 primary antibody (1:50, Santa Cruz Biotechnology, Inc, sc-20811) at 4 °C overnight. After washing the sections with phosphate buffered solution (PBS) for 3 times, Alexa Fluor 488 conjugated secondary antibody was applied for targeting AQP3. The sections were counter-stained with DAPI and observed under

fluorescent microscope.

Western Blotting Assay

All colon samples were collected and washed with PBS. Total protein was extracted using Tissue Protein Extraction kit (CWBiotech, CW0891A, lot No. 20140812). Protein concentration was determined by BCA Protein Assay kit (Beyotime, China). All the protein samples were separated by electrophoresis with 10% separation gel (Bio-Rad, California, USA) at 120 V for 40 min. The proteins were transferred to a polyvinylidene fluoridemembrane (Millipore) using the wet-transfer method at 90 V for 90 min. After transfer, the membrane was washed with Tris-buffered saline with Tween-20 (TBST) and then blocked with 5% BSA for 1 h at 37 °C. It was then incubated with rabbit anti-AQP3 (1:500, Santa Cruz Biotechnology, Inc., sc-20811, California, USA) and β -actin primary antibodies overnight at 4 °C. Afterwards, the membrane was incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody for 1 h at 37 °C. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo-Scientific) was used for detecting targeted proteins, and imaging was taken with VersaDoc Imaging System (Bio-Rad).

Quantitative Reverse Transcription-Polymerase Chain Reaction Assay

In order to analyze the AQP3 gene expression of the colon, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed. Sprague-Dawley rats specific primer was used for targeted AQP3 (Forward: 3'-ACCCTCATCCTTGTGATGTTTC-5'; Reverse: 3'-TTCAAGTGGGCTCCAGACT-5', 155 bp), and GAPDH was used as an internal control. All primers were tested with NCBI Primer Blast and synthesized by Sangon Biotech. Briefly, all the samples were ground to powder in liquid nitrogen. Total RNA

was extracted with Total RNA Preparation kit (RP1202, BioTeke) according to the manufacturer's instructions. The SYBR Premix Ex Taq II kit (BK402, TaKaRa) and 96-well PikoREAL qPCR System (TCR0096, ThermoFisher Scientific) were used to perform qPCR. The data was analyzed by the $2^{-\Delta\Delta ct}$ method.

Statistical Analysis

All data was expressed as mean \pm standard deviation ($\bar{x} \pm s$). Statistical evaluation was conducted using SPSS 17.0 and one-way analysis of variance (ANOVA) followed by LSD was used to compare the differences between groups. Differences were considered statistical significant when $P < 0.05$.

RESULTS

Identification of Main Components in ESP Extract

According to HPLC analysis, 6 known components in the ESP extract were found and identified (Figure 2). The relative content of psoralen, isopsoralen, methyleugenol, methylisoeugenol, dehydrodiisoeugenol, and bakuchiol in the extract were 7.55%, 5.93%, 1.66%, 1.76%, 2.74%, and 28.60%, respectively.

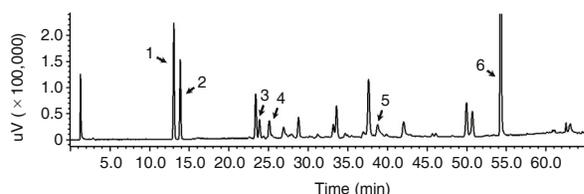


Figure 2. HPLC Chromatogram for Petroleum Ether Fraction Extract of ESP

Notes: 1: Psoralen; 2: Isopsoralen; 3: Methyleugenol; 4: Methylisoeugenol; 5: Dehydrodiisoeugenol; 6: Bakuchiol

Effect of ESP Extract on Body Weight and Diarrhea Index

In this study, we found that the PSYD modeling rats exhibited coldness, depilation, lethargy, fatigue, and sleepiness. As illustrated in Figure 3, compared with the control group, the body weight of rats in the model group decreased and diarrhea index increased after 2 week ($P < 0.05$). The body weight had a significant increase (day 12–28) and diarrhea index (day 18–28) decreased in the LD ESP, HD ESP and positive groups compared with the model group ($P < 0.05$). On day 28 (after 14 days of treatment), the diarrhea index declined by 0.61, 0.77, and 0.80-fold in LD ESP, HD ESP and positive groups, respectively ($P < 0.05$). HD ESP and positive treatment both showed significant differences compared with the

LD ESP group after 14 days treatment ($P < 0.05$). HD ESP group showed a similar anti-diarrheal effect as a positive drug ($P > 0.05$).

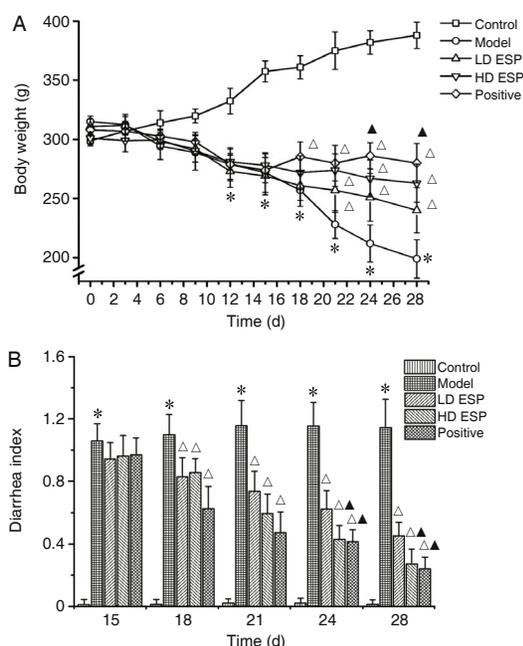


Figure 3. Effect of ESP Extract on Body Weight (A) and Diarrhea Index (B)

Notes: * $P < 0.05$ vs. control group; $\Delta P < 0.05$ vs. model group; $\blacktriangle P < 0.05$ vs. LD ESP group

Effect of ESP Extract on Kidney Histology

After 2-week treatment, macroscopic morphology of the kidney was observed (Figure 4A). The kidney showed swelling and wanness in the model group. LD ESP, HD ESP and positive groups significantly reversed these effects. Pathological examination showed that PSYD modeling rendered severe renal damage in the rats, which included glomerulus atrophy or necrosis, serious hyperplasia, fibrosis, tubular cell desquamation, and inflammatory cell infiltrates. Treatment with ESP extract reduced these lesions (Figure 4B). The histology scores of LD ESP, HD ESP and positive groups were 8.2, 10.5, and 11.8, respectively, all higher than the model group ($P < 0.05$, Figure 4C).

Effects of ESP Extract on Rat Colon Histology and AQP3 Expression

In the colon histological examination, we found that PSYD modeling resulted in colitis and a slight detachment of colon chorioepithelium. ESP extract treatment effectively alleviated colitis by reducing immunocyte infiltration and reversed colon physiological structure changes (Figure 5A). ESP extract significantly increased AQP3 expression in

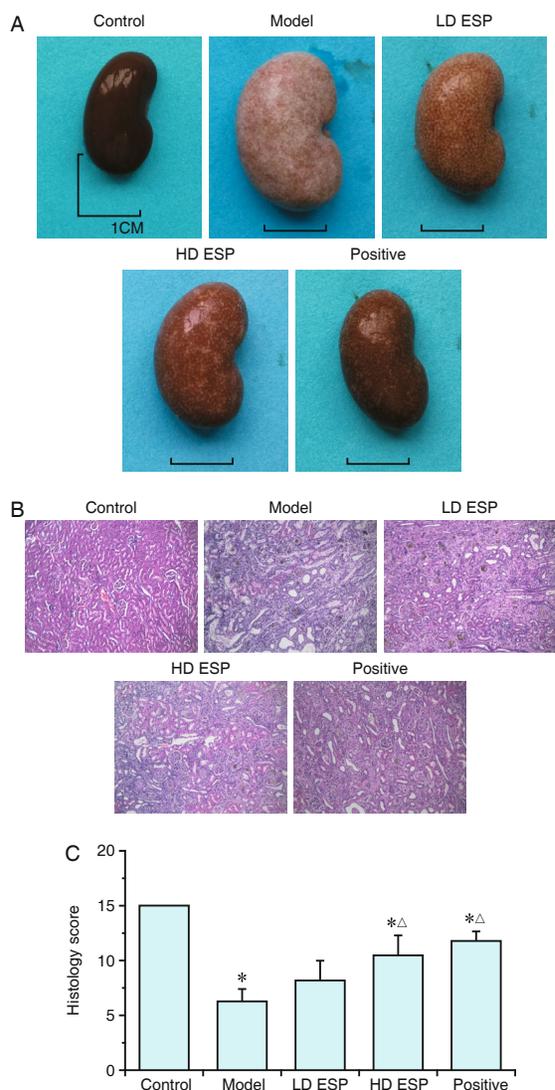


Figure 4. Effect of ESP Extract on Kidney Appearance and Histology of PSYD Rats

Notes: A: Macroscopic evaluation of kidney; B: HE staining analysis ($\times 40$); C: Histological score. * $P < 0.05$ vs. control group; $\Delta P < 0.05$ vs. model group

the rat colon inhibited by PSYD modeling. In AQP3 immunofluorescent staining, the positive staining intensity of LD ESP, HD ESP, and positive groups were stronger than the model group (Figure 5B). Based on immunofluorescence, the expressed protein of AQP3 in the colon was detected with Western blotting (Figure 5C). After treatment, AQP3 synthesis in the HD ESP and positive groups were enhanced by 1.1- and 2.4-fold, respectively, compared with the model group ($P < 0.05$). As shown in Figure 5D, the change in expression levels of the HD ESP and positive groups were 2.5- and 2.9-fold, normalized to the model group ($P < 0.05$). Compared with the LD ESP group, the HD ESP group had up-regulated levels by 0.7-fold ($P < 0.05$).

DISCUSSION

It is reported that *Fructus Psoraleae* strengthened Shen,⁽²⁴⁾ meanwhile, *Myristica Fragrans* alleviated colitis⁽¹⁷⁾ and regulated Pi and stomach functions.^(25,26) In our preliminary study, we sifted through the anti-diarrheal effect of different solvent extracts of ESP and found petroleum ether portion of ESP extract was the best. Thus, we performed quality control and found the yield to be relatively stable. In addition, according to HPLC analysis, the chemical components were stable, and there were 6 known active compounds including psoralen, isopsoralen, methyleugenol, methylisoeugenol, dehydrodiisoeugenol, and bakuchiol. The relative content was 7.55%, 5.93%, 1.66%, 1.76%, 2.74%, and 28.60%, respectively. Therefore, the quality of the petroleum ether fraction of ESP extract was stable, controllable, and feasible for the study of its therapeutic effects on PSYD-induced diarrhea. After quality control, rats were orally given ESP extract after 2 weeks of PSYD modeling. The results demonstrated that ESP extract effectively inhibited body weight loss, relieved diarrhea, prevented kidney lesions, and alleviated colitis.

Based on these results, we further investigated the anti-diarrheal mechanism of ESP extract on PSYD-induced diarrhea rats. AQPs are membrane channels that transport water into the intestinal tract,⁽²⁷⁾ dominantly expressed in the colon and regulated the absorption of water.⁽²⁸⁾ It is reported that the inhibition of AQP3 expression in the colon caused diarrhea.⁽²⁹⁾ In this study, we found that AQP3 synthesis was inhibited in the colon of PSYD rat. We considered that the inhibition of AQP3 expression in the colon may directly reduce the re-absorption of water from the colon, then indirectly lead to diarrhea. However, after treatment with the ESP extract, AQP3 synthesis improved and the diarrhea was gradually alleviated. It indicated that AQP3 might be a target of the ESP extract to regulate absorption of water in the rat colon, thus preventing diarrhea.

Taken together, ESP petroleum ether extract effectively alleviates the symptoms of PSYD including body weight loss, kidney lesions, colitis, and PSYD-induced diarrhea by improving AQP3 synthesis in the colon.

Conflict of Interest

All the authors declare that there is no conflict of interests

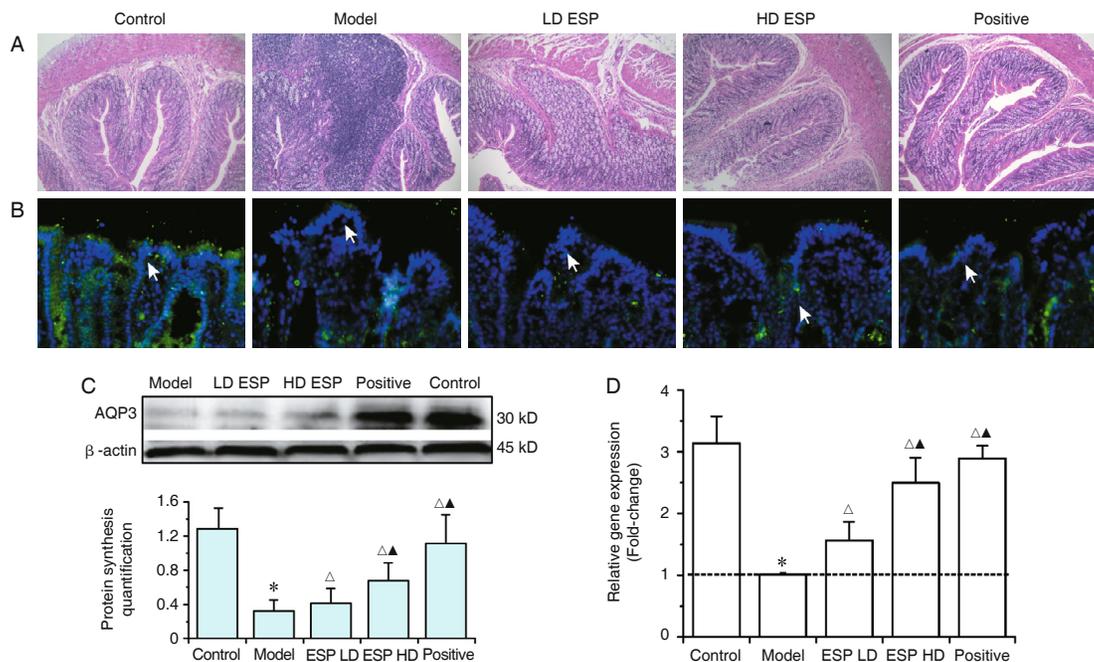


Figure 5. Effects of ESP Extract on Histology and AQP3 Expression of PSYD Rat Colon

Notes: A: HE staining of rat colon section (40 \times); B: AQP3 immunofluorescent staining in rat colon, cell nucleus stained with DAPI showed blue, and AQP3 showed green (indicated by arrow, 100 \times); C: AQP3 expression by Western blotting analyses, the graphs represent band intensity; D: AQP3 gene expression analyses, the graphs represent fold changes. * $P < 0.05$ vs. control group; $\Delta P < 0.05$ vs. model group; $\blacktriangle P < 0.05$ vs. LD ESP group

regarding the publication of this paper.

Author Contributions

Pan X and Xu K designed the project, performed the experiments, collected the data, and wrote the manuscript; Xiong R, Chen ZM and Feng J analyzed the data, wrote and revised the manuscript. LI WB and Hu CJ gave financial support and wrote and revised the manuscript. All authors participated in the review of the manuscript.

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Electronic Supplementary Material: Supplementary material (Appendix 1) is available in the online version of this article at <https://doi.org/10.1007/s11655-017-2766-4>.

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