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Original Research Article

Supercritical fluid carbon dioxide extract of *Alpinia oxyphylla* ameliorates dextran sulfate sodium-induced intestinal barrier damage in mice

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Abstract

***Purpose**: Inflammation and oxidative stress are the leading causes of intestinal barrier dysfunction and a wide range of diseases. The purpose of this study was to explore the protective effect and elucidate potential mechanisms of supercritical carbon dioxide extract from Alpinia oxyphylla Miq. (AOE) on intestinal inflammation.*

***Methods**: The AOE was extracted by supercritical carbon dioxide extraction and its components were determined. Cytotoxicity of the extract (0.05 to 20μg/mL) was determined on Caco-2 cells. In vitro assessment of whether AOE protected against LPS-induced intestinal barrier dysfunction was done on Caco-2 cells pretreated with non-cytotoxic concentrations (0.5μg/mL and 1 μg/mL) of the extract. Experimental colitis of mouse model was induced by drinking water containing 3% dextran sulfate sodium (DSS). The intestinal permeability was assessed by TEER and fluorescein isothiocyanate conjugated dextran (FITC). Pathological evidence and possible mechanism were verified by tissue staining and molecular methods including quantitative real-time polymerase chain reaction (qRT-PCR) and western blot.*

***Results**: TEER and FITC testing indicated that AOE pretreatment significantly improved intestinal barrier hemostasis ($p<0.05$). Furthermore, AOE pretreatment counteracted DSS-induced upregulation of pro-inflammatory cytokines and oxidative stress ($p<0.05$). Lastly, two crucial signal pathways regarding hemostasis of intestinal barrier, NF-κB and NOX1-LCN2, were significantly attenuated upon AOE pretreatment ($p<0.05$).*

***Conclusion**: This study sheds lights on the protective effect of AOE for intestinal hemostasis and supports the development of AOE-based intestinal protective agents.*

***Keywords**: Alpinia oxyphylla extract; Intestinal inflammation; Dextran Sulfate Sodium Salt; NF-κB; Oxidative stress*

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INTRODUCTION

In addition to digestion and absorption, the intestine forms a natural barrier protecting the body and gut environment as well as being potentially hostile for microorganisms, toxins, and antigens [1,2]. The intestinal epithelium, albeit only a single-cell layer, acts as the most component of barrier against the external environment because it not only covers the whole gut tract but also establishes a selectively permeable barrier through tandem with individuals by intracellular tight junctions (TJs) [3,4]. Inflammation and oxidative stress are the leading causes of intestinal barrier dysfunction. Defected intestinal barrier could result in both intestinal and systemic diseases, such as inflammatory bowel disease (IBD), diabetes, and even cancers [2,4]. Therefore, safeguarding epithelial barrier hemostasis denotes a new therapy strategy for disease prevention.

Alpinia oxyphylla (family: Zingiberaceae) fruit, a species of ginger native to East Asia, is a traditionally dietary Chinese herb in use for hundreds of years. As the Chinese name (Yizhi) suggests, it is mainly available for intelligence improvement in history in spite of other beneficial effects which includes alleviation of a variety of diseases such as diarrhea and salivation [5,6]. *Alpinia oxyphylla* Miquel extract is believed to possess a variety of biological and pathological activities including neuroprotection [7], anti-inflammatory [8], and antioxidant [9,10]. Chemical analysis indicated that p-cymene and nootkatone are the representative compounds from *Alpinia oxyphylla* Miquel essential oil [11,12]. Quantitative analysis of the volatile profiles of *A. oxyphylla* oil with various origins and harvest times by gas chromatography-flame ionization detection indicated that the concentration of p-cymene ranges from 2.31 to 77.30 μL/mL while that of nootkatone ranges from 12.38 to 99.34 mg/mL [12]. Interestingly, p-cymene and nootkatone have been well documented to be good at modulation of oxidative stress and inflammation [13-16]. For example, nootkatone repressed reactive oxygen species (ROS)-induced inflammasome activation to alleviate airway inflammation and attenuated carbon tetrachloride-induced acute liver injury through targeting oxidative stress and NF-κB pathways [15,17]. P-cymene have been reported to significantly reduce carrageenan-induced hyperalgesia involving in inhibition of leukocyte migration and inflammatory cascade [18].

In this study, given the pathogenesis of intestinal barrier damage and the abundant anti-inflammatory active substances of *Alpinia oxyphylla* Miquel essential oil, we utilized supercritical fluid extraction with carbon dioxide (CO_2) to obtain the essential oil from *Alpinia oxyphylla* Miquel (AOE) and explored the protective effect of AOE on intestinal barrier dysfunction. The mechanism of action of AOE was also studied via its modulatory role on the inflammatory NF-κB and oxidative NOX1-LCN2 signals axis which are keys to maintaining intestinal barrier integrity. It is hoped that our study will lay a theoretical foundation for health care and pharmacological effects of *Alpinia oxyphylla* Miquel, and thus provide a new possibility for the prevention and treatment of intestinal injury.

EXPERIMENTAL

Materials

Alpinia oxyphylla fruits without pericarp identified by a taxonomist (Dr. Peng Zhang of the College of Tropical Crops, Hainan University, Haikou, China) were provided by Zhongminjian Agricultural Development Co. Ltd (Hainan, China). Human Caco-2 colon cells were purchased from China Center for Type Culture Collection (Wuhan, China). Fluorescein isothiocyanate (FITC) and 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Solarbio (Beijing, China). Antibodies ZO-1, Occludin, Claudin-4, Lipocalin-2 (LCN2) and Nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX-1) were purchased from Abcam (Cambridge, UK). Antibodies for conical NF-κB signals IκBα, pIκBα, p65, and p-p65 were purchased from Cell Signaling Technology (Danvers, USA).

Preparation of *Alpinia oxyphylla* Extract (AOE) by Supercritical Fluid Extraction with Carbon Dioxide

AOE was obtained according to our previous method [19,20]. Briefly, the dried fruits were cut and ground into powder. Some of the powder (200 g) was put in an extraction tank under a temperature of 40 °C, flow rate of CO_2 at 0.8 Liter min^{-1}, and pressure of 15 MPa for 4 hours. The resultant extract was collected and stored for subsequent analysis and bioactive investigation.

Gas chromatography-Mass spectrophotometer (GC-MS) Analysis

GC-MS analysis of AOE was performed using a Trace1310, TSQ 8000 Evo GC–MS (Thermo Fisher Scientific Inc., MA, USA) with a capillary column (Thermo TR-5MS, 30 m × 0.25 mm × 0.25 μm) according to previous method [21]. The

temperature was programmed from 50 to 200 ℃ by a gradient of 3 ℃/min, from 200 to 280 ℃ at a rate of 20 ℃/min/ and subsequently maintained at 10 min. Helium was the carrier gas at a flow rate of 1 mL/min. The shunt ratio was 60:1, and the solvent delay was 3 mins. Ionization energy was 70 eV, ion source temperature was 230 ℃, interface temperature was 250 ℃, and mass scanning ranged from 50 to 300 m/z.

Measurement of Cytotoxicity

Cytotoxicity of AOE was assessed according to previous method [21]. Exactly, human Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin mixture and incubated at 37 ℃ in a humid atmosphere of 5% CO_2. Cytotoxicity was measured with MTT [3-(4, 5-dimethyl tiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method. Caco-2 cells were inoculated at 2 × 10^4 cells/well in a 96-well sterile culture plate, and the culture medium was changed daily. When the cells grew to confluence, AOE at different concentrations（0.5 μg/mL to 20 μg/mL）were added and pretreated for 4h, and 0.1μg/mL lipopolysaccharide (LPS) was added to stimulate the cells. MTT solution (5 mg/mL) was added and cultured for another 4 hours. Sterile dimethyl sulphoxide (DMSO) was added and left for 15 mins at 37 ℃ and the absorbance value was measured at 490 nm against the control which consisted of the Caco-2 cells not treated with the extract. The cell viability was determined as follows:

$$\text{Cell viability} = \frac{\textit{absorbance value of cells treated with extract}}{\textit{absorbance value of cell not treated with extract (control)}}$$

Transepithelial Electrical Resistance (TEER)

TEER of AOE pretreated Caco-2 cells was measured as previously reported [4,22]. Briefly, Caco-2 cells inoculated in transwell chambers were consecutively cultured for 21 days to fulfill differentiation prior to experiments. Epithelial voltammeter electrode (Millicell, Massachusetts, USA) was utilized to measure the values of TEER at 2 h, 4 h, 8 h, 12 h, 24 h, 36 h and 48 h after 0.5 μg/mL and 1 μg/mL of AOE pretreatment.

Fluorescein Isothiocyanate Conjugated Dextran (FITC) for *in vitro* Paracellular Permeability Measurement

Paracellular permeability of AOE pretreated Caco-2 cells was measured using FITC as previously reported [4,22]. Dulbecco's modified eagle medium (DMEM) was removed, in the upper transwell chamber and it was replaced with 0.1 mg/mL fluorescein isothiocyanate isomer (FITC) (MW 4000), and in the lower medium it was changed to phosphate buffer saline (PBS). The plate was put into the incubator for 1 h and then multifunctional microplate meter was used to measure the fluorescence absorbance with the excitation wavelength set at 480 nm and the emission wavelength set at 520 nm.

Animals Studies

All animal experimental procedures were approved by the Experimental Animals Ethics Committee of Tianjin University of Commerce (TKLFB-2020012) according to the Regulation Guide from Ministry of Science and Technology, China. C57BL/6 mice (age: 6~8-weeks; weight: 18~20 g) were purchased from Beijing HFK Bioscience Co. Ltd (Beijing, China). The mice were raised under the conditions of 12-hour light/dark cycling, 22 ± 3 °C, relative humidity of 55 ± 5%, and freely obtain food and water for one week prior to experiment.

DSS-induced mouse colitis was performed as previously reported [4,22]. The mice were randomly divided into four groups and each group consisted of 8 mice. Exactly, as displayed in Figure 2A, mice in control group (CON) received drinking water only, mice in colitis model group (DSS) received the drinking water with 3% DSS, and mice in low dose administration group (AOEL) and in high dose administration group (AOEH) were intragastrically administrated 50 mg/kg and 200 mg/kg AOE respectively in addition to 3% DSS treatment. Body weight, water intake, and disease activity index (DAI) were recorded daily for 7 days as previously reported [4,22]. Blood samples and intestinal samples were collected and preserved for further pathological examination on day 7.

FITC for In Vivo Intestinal Epithelial Permeability Measurement

In vivo intestinal epithelial permeability was assessed as previously reported [22]. Mice in all four groups received gastric infusion of 0.5 mg/g FITC and blood samples were withdrawn by ocular venous plexus 4 h later. The fluorescence absorbance of serum from collected blood samples was measured at the excitation and emission wavelengths of 485 nm and 528 nm, respectively.

Histological Analysis

Colon tissues from mice of the 4 groups were soaked in 4% paraformaldehyde (PFA) for 2 days before paraffin embedding. Paraffin-

Table 1: The primers of GAPDH, IL-1β, IL-6, and IL-10 sequences

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	AGAGTGGGAGTTGCTGTTG	GCCTTCCGTGTTCCTACC
IL-1β	TTGTGCAAGTGTCTGAAGCA	TAGCCCTCCATTCCTGAAAGC
IL-6	CACCAGGCAAGTCTCCTCATTG	TACATCCTCGACGGCATCTCA
IL-10	TGCTAACCGACTCCTTAATGCA	TCATGGCCTTGAGACACCTTG

embedded colon sections were cut into 5μm thickness and sequentially dried, dewaxed, rehydrated, and eventually stained with hematoxylin and eosin. Sections were examined with an inverted optical microscope (Nikon CLIPSECi-L, Tokyo, Japan).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from colon tissues obtained from the mice of the 4 groups using Trizol reagent (Thermo Fisher Scientific, USA). The Light Cycler 96 Real-time PCR system (Roche, Basel, Switzerland) was used reverse transcription and quantitative real-time PCR. The primers of GAPDH, IL-1β, IL-6, and IL-10 were indicated as shown in Table 1. The results were calculated according to the 2ˆ(–delta delta CT) method [23].

Western Blot Analysis

Western Blot was assessed according to previous method [24]. The mice colon tissues were cut into small pieces and the total protein was extracted using Radio-Immunoprecipitation Assay Lysis (RIPA) buffer with phenylmethylsulfonyl fluoride (Solarbio, Beijing, China) and protease inhibitor on ice. Nuclear protein was isolated according to the instruction of cytoplasm and nuclear protein extraction kit (Solarbio, Beijing, China). The protein concentration was determined by Bicinchoninic Acid (BCA) protein assay kit (Solarbio, Beijing, China). 8%, 10%, and 12.5% Bis-Tris Gel was utilized to separate target proteins which were then transferred to topolyvinylidene difluoride membranes (PVDF) by wet-transfer. The membranes were originally blocked for 1 h soaked in skim milk and then incubated with primary antibodies overnight at 4 ℃. After that, the membranes were incubated with the second-antibodies for 1 h at room temperature. Image formation of the membranes were processed with ImageQuant LAS 500 (General Electric Company, Boston, America) and the results were measured by Image-J.

Intestinal Reactive Oxygen Species (ROS) and Myeloperoxidase (MPO) Detection

The colon tissues obtained from mice were fragmentized under ice bath conditions, and the supernatant was extracted by centrifugation. Next, the supernatant was incubated for 30 min with DCFH-DA fluorescent probe to test ROS levels by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. To further confirm the change of oxidative stress, intestinal MPO activity was determined by a commercialized Detection Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the product manual, as is broadly utilized by published papers [25,26].

Statistical Analysis

Data was presented from at least triplicated independent experiments and represented as mean ± standard deviation (SD). One-way ANOVA was used with the software GraphPad Prism to statistically analyze or otherwise indicated. When the *P* value was less than 0.05, the data was considered statistically significant.

RESULTS

Compounds Identified by GC/MS in AOE

There were 38 compounds, accounting for 68.79% of the total components, found in AOE (Table 2). Among them, Nootkatone (13.17%), Valencen (9.8%), Gingerol (7.48%), Vetiverol (6.55%), α-Maaliene (4.43%), and (-)-Globulol (3.07%) were the major compounds.

AOE Prevents Lipopolysaccharide (LPS)-induced Damage of Caco-2 Cell Permeability

On the basis of the compounds identified in our experiments and previous researches, we then tried to explore whether AOE could protect intestinal epithelial barrier utilizing human intestinal Caco-2 cells. We primarily established the optimal concentration of AOE to exclude AOE toxicity effect on cells. There was no impairment of cell viability when cells were pretreated with AOE at less than 2 μg/mL (Figure 1A). Therefore, 0.5 μg/mL and 1 μg/mL concen-

Table 2: The chemical compositions of AOE

NO.	Components	RT	Relative content	Molecular Formula
1	p-Cymene	7.49	0.92	$C_{10}H_{14}$
2	β-Ocimene	7.87	0.57	$C_{10}H_{16}$
3	α-Terpinene	8.11	1	$C_{10}H_{16}$
4	Linalool	8.87	0.29	$C_{10}H_{18}O$
5	Terpinen-4-ol	10.28	0.27	$C_{10}H_{18}O$
6	Bicyclo(3.1.1)hept-2-ene-2-carboxaldehyde, 6,6-dimethyl	10.58	0.5	$C_{10}H_{14}O$
7	2,6-dimethylocta-3,5,7-trien-2-ol	10.74	0.11	$C_{10}H_{16}O$
8	2-Methyl-3-phenylpropanal	11.3	0.1	$C_{10}H_{12}O$
9	Megastigma-4,6(E),8(Z)-triene	11.75	0.24	$C_{13}H_2O$
10	α-Copaene	13.29	1.26	$C_{15}H_{24}$
11	(+)-Aromadendrene	13.5	0.36	$C_{15}H_{24}$
12	Lemnalol	13.76	0.17	$C_{15}H_{24}O$
13	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-(1R-(1a,3ab,4a,7b))-	14.49	3.56	$C_{15}H_{24}$
14	Aristolochene	15.06	1.96	$C_{15}H_{24}$
15	Valencen	15.22	9.8	$C_{15}H_{24}$
16	α-Maaliene	15.59	4.43	$C_{15}H_{24}$
17	Caryophyllene oxide	16.57	1.78	$C_{15}H_{24}O$
28	(-)-globulol	17.6	3.07	$C_{15}H_{26}O$
19	8-Isopropyl-1,5-dimethyltricyclo(4.4.0.02,7)dec-4-en-3 –one	17.85	2.56	$C_{15}H_{22}O$
20	Vetiverol	18.16	6.55	C15H24O
21	Isoshyobunone	18.36	1.16	$C_{15}H_{24}O$
22	7-Isopropenyl-1,4a-dimethyl-4,4a,5,6,7,8-hexahydro-3 H-naphthalen-2-one	18.74	2.04	$C_{15}H_{22}O$
23	Nootkatone	19.52	13.17	$C_{15}H_{22}O$
24	n-Hexadecanoic acid	20.91	1.08	$C_{16}H_{32}O_2$
25	Oleic acid	22.65	0.79	$C_{18}H_{32}O_2$
26	Androstan-3-ol	23.12	0.34	$C_{19}H_{32}O$
27	Heneicosane	23.94	0.49	C21H44
28	Tetracosane	24.78	0.14	$C_{24}H_{50}$
29	Phenol, 2,2'-methylenebis6-(1,1-dimethylethyl)-4-methyl	25.18	0.22	$C_{23}H_{32}O_2$
30	heptacos-1-ene	25.41	0.21	$C_{27}H_{54}$
31	Octacosane	25.59	0.45	$C_{28}H_{58}$
32	Diisooctyl phthalate	26.35	0.39	$C_{24}H_{38}O_4$
33	Tectochrysin	26.83	0.15	$C_{16}H_{12}O_4$
34	Gingerol	27.43	7.48	$C_{17}H_{26}O_4$
35	3-Benzofuranmethanol, 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hyd roxy-1-propenyl)-7-methoxy	27.97	0.25	$C_{20}H_{22}O_6$
36	E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)hept-4-en-3-o ne	28.13	0.12	$C_{21}H_{24}O_5$
37	(10]-Gingerdione	28.39	0.48	$C_{21}H_{32}O_4$
38	Others		31.21	N/A

trations were selected for subsequent *in vitro* assessment of whether AOE protected against LPS-induced intestinal barrier dysfunction. As shown in Figure 1B, TEER results suggested that intestinal barrier dysfunction induced by LPS was significantly counteracted when cells were pretreated with either 0.5 μg/mL or 1 μg/mL AOE for 12 h prior to exposure to LPS (p<0.05). Additionally, cells treated with 0.5 μg/mL of AOE displayed better barrier protection than those treated with 1 μg/mL of AOE (p<0.05) (Figure 1A).

Consistent with TEER tests, the fluorescence values of FITC-dextran indicated that LPS-induced damage of intestinal permeability was significantly impeded when Caco-2 cells were pretreated with either 0.5 μg/mL or 1 μg/mL of AOE (p<0.001) (Figure 1C).

AOE Prevents DSS-induced Intestinal Damage in Mice

We evaluated the survival rate (Figure 2B), anatomy including colonic appearance (Figure 2C) and length of colon (Figure 2D), and disease activity index (DAI) (Figure 2E). There were no dead mice found in groups with AOE pretreatment but a mouse died in the DSS group (model group) during the 7-day observation period. As per the quality of life, mice with AOE intervention showed reduced DAI scores (p<0.05). In addition, the AOE pretreatment

prevented DSS-induced shortened intestine (Figure 2C) together with the degree of loose stools and bloody stool as well as the intestinal wall thickness.

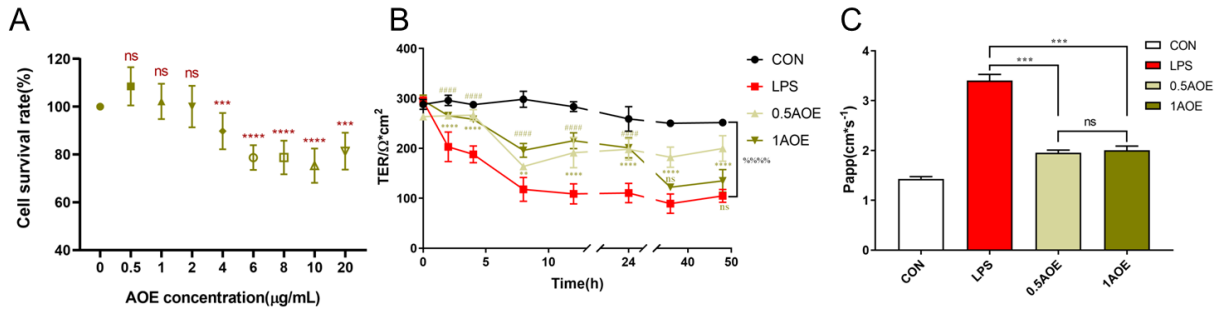

Figure 1: *Alpinia oxyphylla* extract (AOE) protects against damage of the intestinal Caco-2 cell barrier caused by LPS (n=3). *(A) Cytotoxicity assessment of AOE treatment on Caco-2 cells. The differences of the indicated c oncentration of AOE were ccompared with cells without AOE treatment. (B) TEER assessments suggested that AOE counteracted LPS-induced damage of Caco-2 permeability. (C) AOE retarded the permeability change of monolayer barrier on Caco-2 cells stimulated by LPS.* $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, $^{d}p < 0.0001$ *compared to LPS group*

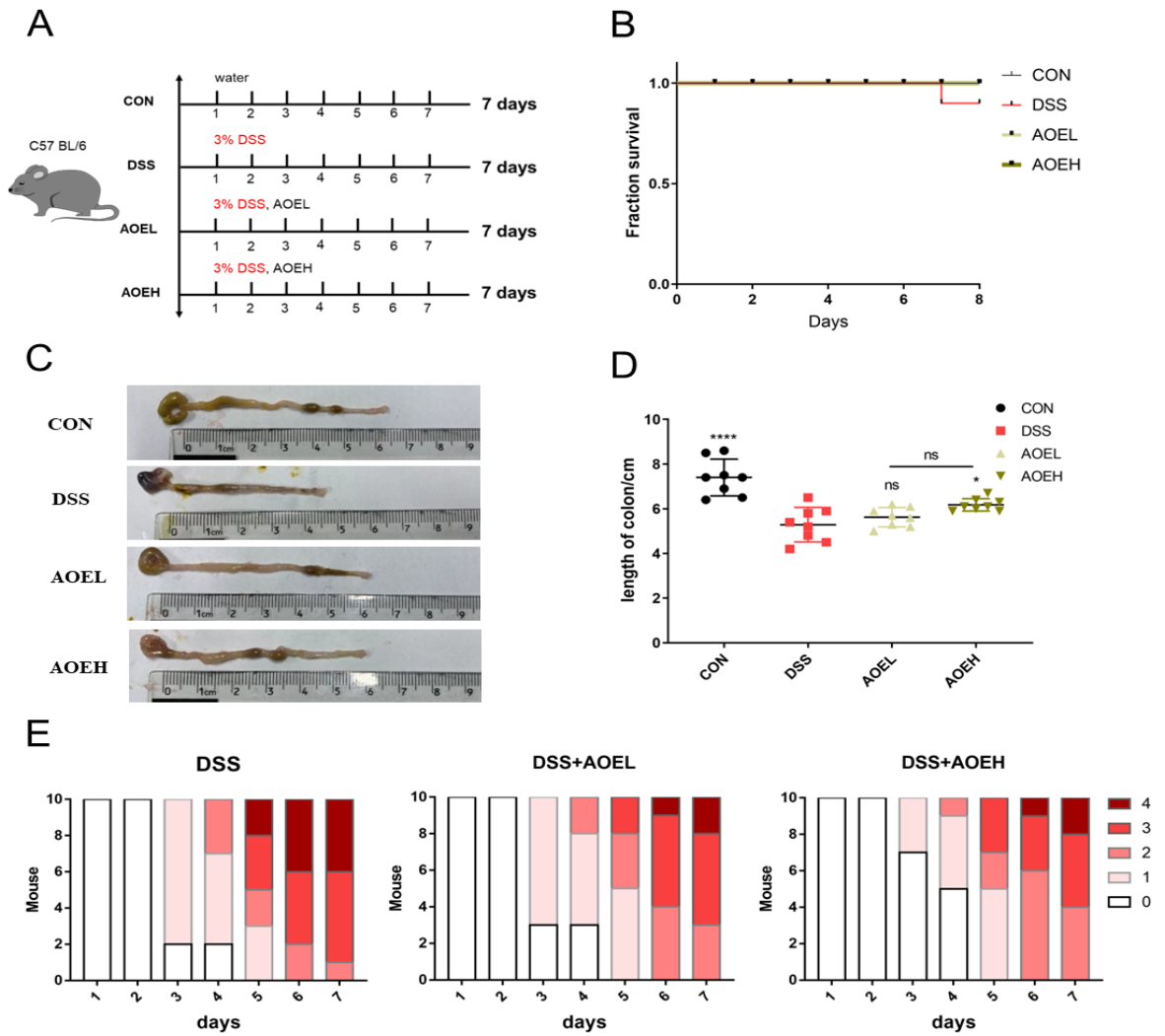

Figure 2: *Alpinia oxyphylla* extract (AOE) improved DSS-induced intestinal inflammation in mice. (A) *Animal experimental design diagram; (B) Survival rate of mice; (C) Appearances of colon samples; (D) Lengths of colon samples; (E) DAI scores of mice during 7 days' observation, 0 to 4 indicates bleeding in the colon of the mice, 0 indicates normal mice, and 1-4 indicates increasing bleeding*

AOE Prevented Against DSS-induced Damage of Intestinal Barrier in Mice

On the basis of the observation aforementioned, we subsequently examined whether AOE could offer protection against DSS-induced damage of intestinal epithelial integrity. Indeed, DSS treatment resulted in significantly increased FITC-dextran flux. However, DSS-induced gut leaking was significantly (p<0.0001) blocked by AOE pretreatment (Figure. 3C).

Histological analysis revealed (Figure. 3A) that mice exposed to DSS exhibited severely defected structures of intestinal villi (with shorter length) and crypt in addition to markedly inflammatory cell infiltration. Pretreating mice with AOE significantly ameliorated DSS-induced inflammatory cell infiltration and intestinal epithelial incompleteness (p<0.05). TOverall, the experiment indicated that AOE protected the integrity of intestinal barrier.

We detected several representative TJs including Occludin, ZO-1, and Claudin-4 in the mice colon samples (Figure 3D-G). As expected, mice pretreated with AOE showed significant improvement of the checked TJs (p<0.05), which were dramatically lost upon DSS challenge.

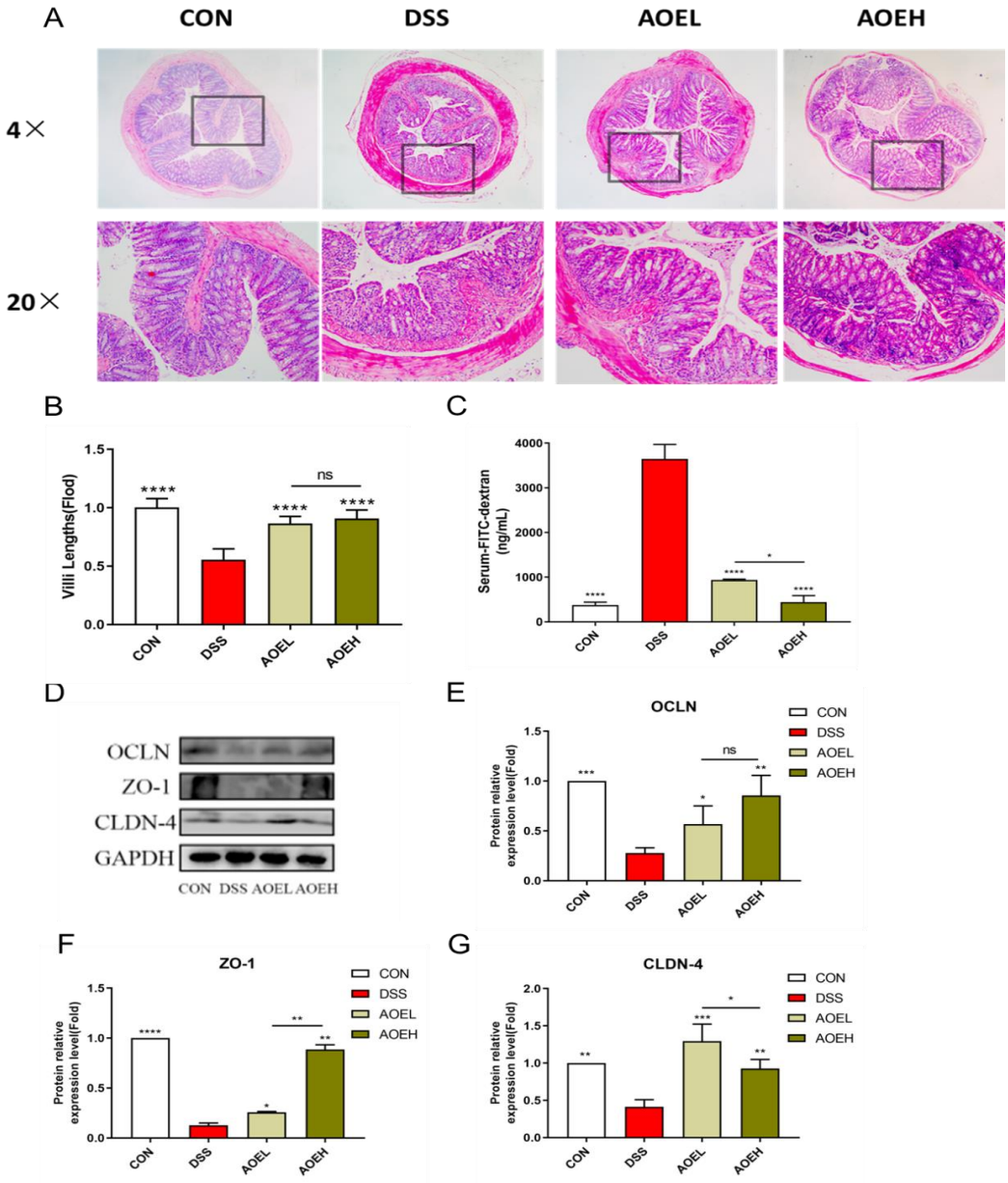

Figure 3: Intestinal barrier protection of *Alpinia oxyphylla* extract (AOE). (A) *Histology of the intestinal tissue assessed by HE staining. (B) Longer villi lengths in AOE-pretreated mice compared with DSS group. (C) The serum concentrations of FITC in mice. (D~G) TJs of OCLN, ZO-1, and CLDN-4 relative to GAPDH in intestine samples. All error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001*

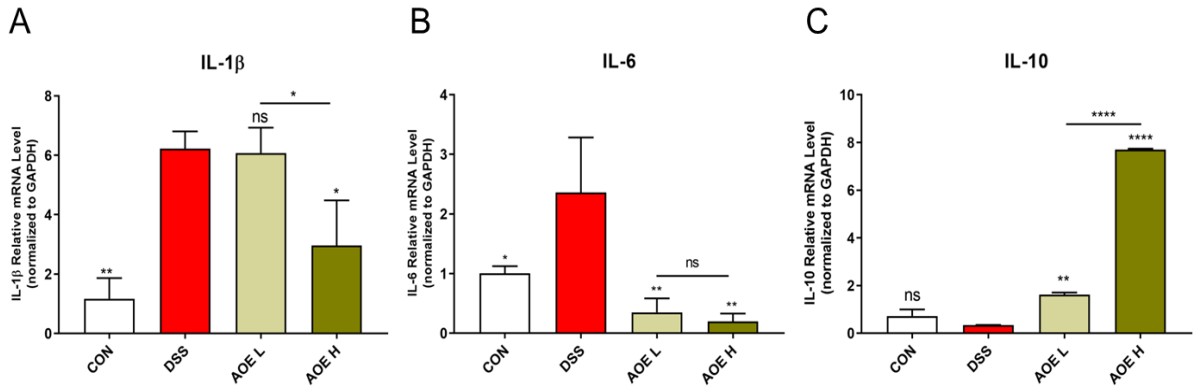

Figure 4: Effect of *Alpinia oxyphylla* extract (AOE) on the DSS-induced intestinal cytokine profiles in mice. mRNA levels of IL-1β(A), IL-6 (B) and IL-10 (C) cytokines in intestinal samples. *Data are shown as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns=not significant or p>0.05 versus DSS group*

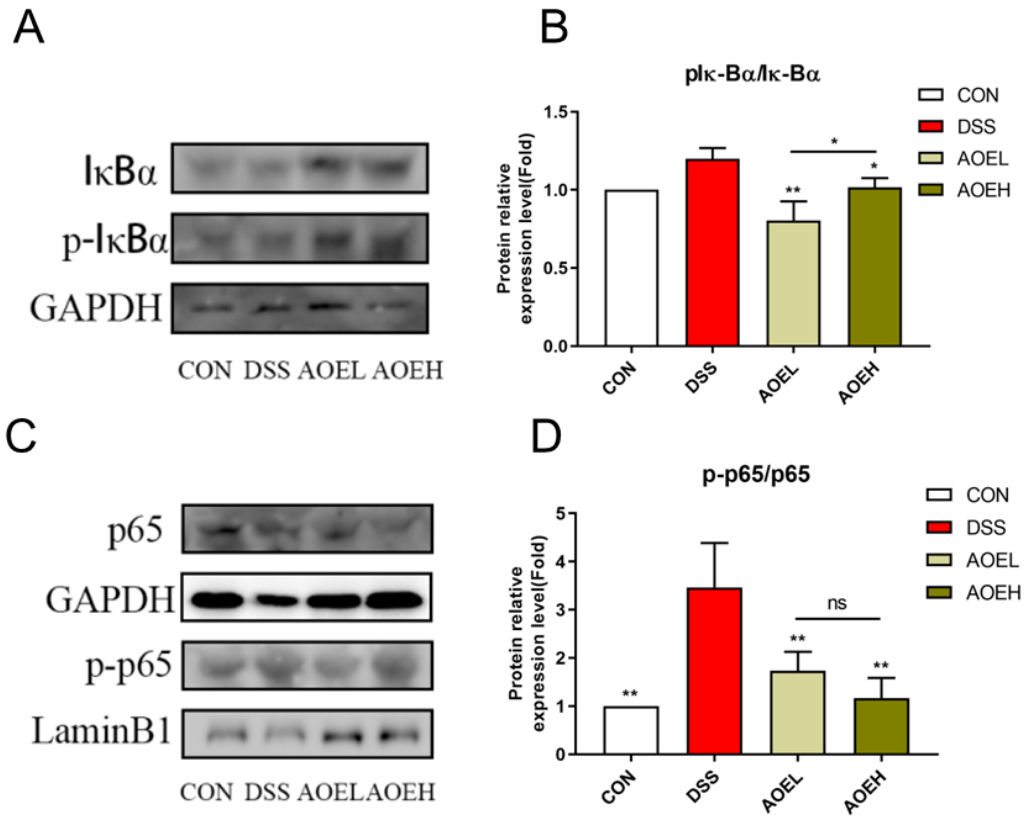

Figure 5: Effect of *Alpinia oxyphylla* extract (AOE) on DSS-induced intestinal inflammatory related NF-κB signal pathways in mice intestinal tissues. *(A and B) Western blotting analysis of IκBα expression in intestinal tissues relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C and D) Expression of NF-κB/p65 relative to GAPDH in mice intestinal tissues. All error bars represent SD. *p < 0.05, **p < 0.01 versus DSS group*

AOE Targeted Against DSS-induced Inflammatory Pathogenesis

To confirm whether AOE pretreatment is linked to alteration of inflamed intestine, real time PCR measurement was performed to systematically investigate the cytokine profiles in mice. The AOE pretreatment effectively counteracted cytokine profiles associated with DSS challenge including the elevated IL-1β (p<0.05) (Figure 4A) and IL-6 (p<0.001) (Figure 4B) and significantly reduced IL-10 (p<0.001) (Figure 4C).

AOE Inhibited Intestinal Activation of NF-κB

As shown in Figure 5 (A-D), AOE pretreatment obviously stunted DSS-activated NF-κB signal elements including phosphorylated cytoplasmic IκBα and nuclei p65 in colon samples (p<0.05). These results suggested that AOE prevented the DSS-induced intestinal barrier injury associated with targeting NF-κB.

AOE Inhibit Oxidative Response by Inhibiting LCN2 Expression Regulated by NOX1 in DSS-induced Mice Intestine

To confirm whether there was excessive production of Reactive Oxygen Species (ROS) and Myeloperoxidase (MPO), the colon samples of mice were determined (Figure 6A-B). Indeed, the ROS and MPO levels in mice stimulated by DSS were significantly increased (p<0.05). However, mice pretreated with AOE showed significantly lower ROS and MPO levels compared with mice in the DSS group (p<0.01). These data demonstrate that AOE suppressed oxidative stress in the colon. On whether the effect of AOE pretreatment on DSS-induced intestinal damage was associated with NOX1-LCN2 axis, intestinal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (NOX1) and Lipocalin-2 (LCN-2) were significantly higher in the DSS group than those in the CON group (Figure 6C-E). AOE pretreatment disrupted DSS-induced activation of NOX1-LCN2 axis to favor intestinal epithelial hemostasis as DSS-induced activation of NOX1-LCN2 axis was significantly lowered on AOE pretreatment (p<0.05).

A summary of our findings is presented in Figure 7.

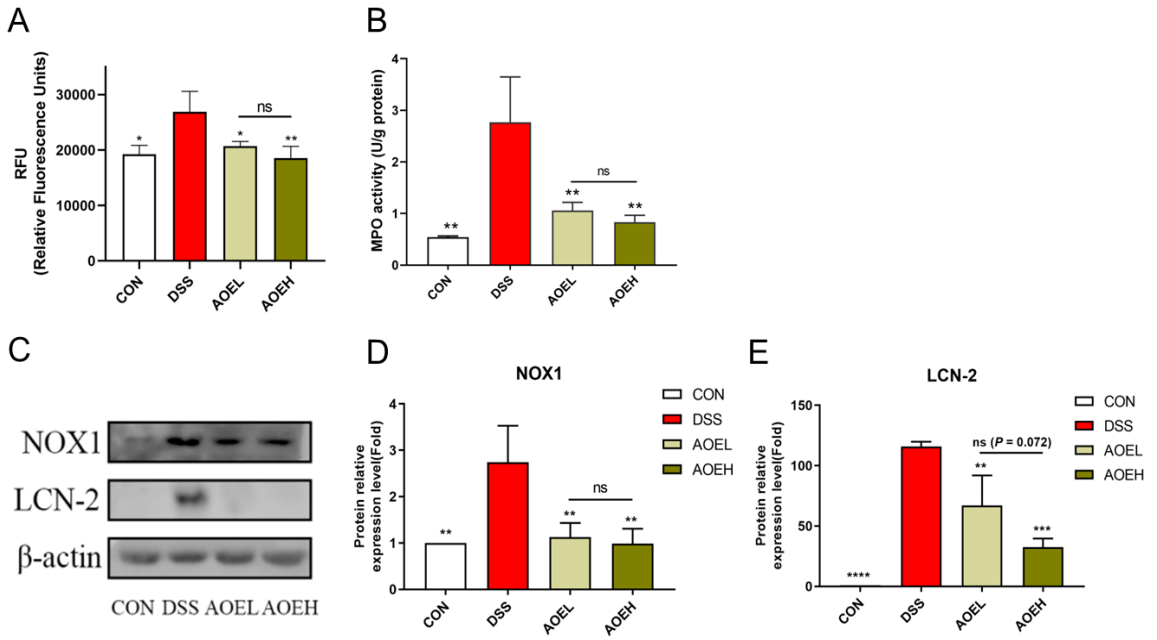

Figure 6: Effects of *Alpinia oxyphylla* extract (AOE) on ROS-related NOX1 and LCN2 expression in the inflamed intestines. *(A) AOE pretreatment alleviated DSS-induced intestinal reactive oxygen species (ROS) activity. (B) AOE reduced DSS-induced intestinal myeloperoxidase (MPO) activity. (C) Western blotting analysis of nicotinamide adenine dinucleotide phosphate oxidase 1(NOX1) (D) and Lipocalin-2 (LCN2) (E) relative to β-actin in intestinal tissues. All error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus DSS group*

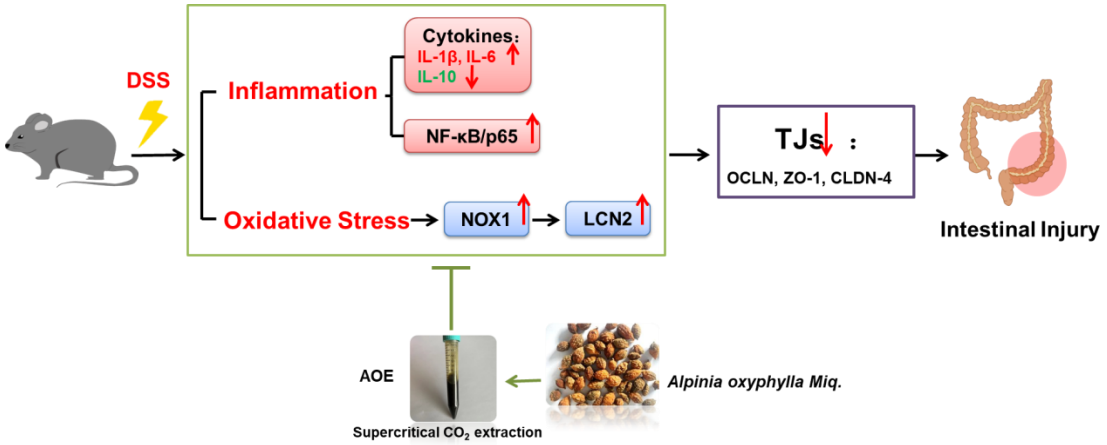

Figure 7: *Alpinia oxyphylla* extract safeguards intestinal hemostasis involving inhibition of inflammation and oxidative stress

DISCUSSION

Amounting evidence suggests that the gastrointestinal tract is not only a specialized organ for food digestion but also a barrier against external harmful factors which are associated with diseases through disrupting or creation of an imbalance in hemostasis of intestinal barriers [4,29]. Nowadays, life styles and chronic stress ranks high in disrupting intestinal hemostasis and it is impractical to fully rely on quick interventions in such situations.

To create and establish feasible solutions for maintaining intestinal hemostasis, development of diet has been strongly recommended in the recent decades. As a matter of fact, food is not only indispensable for nutrition maintenance but also closely associated with gut health. Traditional Chinese medicine and Mediterranean diet have coincidentally proven that plant foods are vital agents needed in maintenance of intestinal hemostasis [30-32].

In this study, we demonstrated that oily extract of *Alpinia oxyphylla* by supercritical CO_2 safeguards intestinal hemostasis via inhibition of inflammation and oxidative stress (Figure 7). Upon pretreatment with AOE, intestinal Caco-2 cells resisted inflammatory stimuli-induced damage of permeability. Inspired by the *in vitro* experimental finding [33], we then analyzed whether AOE ameliorated intestinal inflammation and barrier dysfunction *in vivo u*sing the widely recognized DSS-induced intestinal damage model [4,34]. Mice showed alleviated phenotypes in quality of life and pathological alterations. Tight junction molecules (TJs) are composed of multiple transmembrane barrier proteins and cytoplasmic scaffolding proteins through which the intracellular cytoskeleton is directly connected and intestinal epithelial permeability is modulated [35]. Dysregulation of TJs leads to loss of epithelial barrier integrity [22,36]. Based on the findings that AOE preserved the integrity of intestinal barrier, AOE's effect was further confirmed on intestinal TJs. Given that leaky gut disrupts the hemostasis of intestinal barrier [30,37], we further observed that AOE affected integrity of intestinal barrier via restoration of tight junction proteins. In theory, TJ molecules, including occludins, claudins, and zonula, are assembled in an organized manner to seal the lateral side of the two adjacent intestinal epithelial in order to maintain the epithelial barrier integrity [38]. As a result, the well-established barrier allows the transport of essential molecules but restricts harmful substances and loss of TJs resulting in leaky gut along with enhancement of intestinal permeability [38]. Mice pretreated with AOE showed restored intestinal TJs accompanied by reduced severity of pathological alterations that may explain why AOE is effective in improvement of clinical phenotypes in mice.

TJs are mainly regulated by the intracellular signaling transduction system which is dynamically affected by a number of extracellular stimuli such as pro-inflammatory cytokines and ROS [22,39]. Elevated proinflammatory cytokines are responsible for activation of some key inflammatory signal pathways and eventually result in deregulation of TJs and dysfunction of intestinal barrier [22,40,41]. Of the signal pathways, NF-κB pathway plays a central role on intestinal homeostasis because it not only promotes various pro-inflammatory genes but also mediates the different cell-type specific effects within gut [42-45]. In addition to altered patterns of cytokine synthesis, inflammatory bowel damage is characterized by excessive ROS and MPO. The NADPH oxidase NOX1 is the major regulator of redox in intestinal epithelial cells [46]. Intestinal epithelial NOX1 is activated by key inflammatory cytokines, e.g. TNFα and IL-17, which induces ROS production and consequently drive the expression of Lipocalin-2 (LCN-2) which binds gelatinase B to blunt its enzymatic activity in favor of inflammatory pathogenesis [47]. Therefore, controlling NOX1-LCN2 axis might also be a promising way to preventing inflammation-induced intestinal mucosal dysfunction. Our results confirm that AOE lowered pro-inflammatory cytokines and counteracted NF-κB and NOX1-LCN2 signal pathways which are crucial to TJs and intestinal hemostasis. These findings elucidated the underlying regulatory mechanisms of AOE and suggested potential development of AOE-based approaches on loss of TJs. According to Chinese Pharmacopoeia, *Alpinia oxyphylla* has been broadly applied in southern part of China as food and medicinal substance [5]. Additionally, increasing number of studies have documented anti-inflammatory and anti-oxidant effects of the extract and phytochemicals of *Alpinia oxyphylla* in addition to neuroprotective, anti-cancer, anti-diarrheal, anti-aging, and anti-diabetes effects [5]. For example, the AOE fractioned by various methods have showed 1,1-diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) scavenging activity [48]. Extracts of the fruit of *Alpinia oxyphylla* have also been reported to inhibit nitric oxide (NO) release in activated Macrophages [55]. Main pharmacological constituents of *Alpinia oxyphylla* include sesquiterpenes, flavones, diarylheptanoids, and glycosides [5]. Consistently with other studies, AOE in our study

was shown to be high in phenolic compounds like gingerol and sesquiterpenes such as nootkatone and vetiverol and these compounds are well verified as anti-inflammatory and anti-oxidant agents [15,16,49]. Most of these compounds have been shown to be essential in food and drug development [50]. For example, the compounds with the highest proportion in AOE in previous studies, e.g., nootkatone, valencen, and vetiverol, are reported as improving oxidative stress and inflammation in rodent models [51-54]. These compounds might contribute to understanding how AOE possesses the capacity of targeting intestinal barrier damage through regulating NF-κB and NOX1-LCN2 signal pathways. Of note, our study indicated that most of the key indicators of intestinal hemostasis including the *in vivo* permeability, TJs, and cytokine profiles were significantly better in high-dose AOE group than in low-dose AOE despite similar trends in counteracting DSS-induced activation of NF-κB and NOX1-LCN2 signal pathways found in the two groups. Taken together, our findings suggested that AOE in general dose-dependently protects intestinal tissue.

CONCLUSION

Collectively, our data provide a novel discovery that AOE pretreatment restored DSS-induced damaged integrity of intestinal barrier. We further confirmed that AOE is rich in anti-inflammatory and anti-oxidant constituents and the protective mechanisms of AOE are associated with attenuation of activated NF-κB and redox-associated NOX1-LCN2 signal pathways. These results shed light on development of AOE-based intestinal protective agents.

DECLARATIONS

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Ethical approval

All animal experimental procedures were approved by the Experimental Animals Ethics Committee of Tianjin University of Commerce (TKLFB-2020012) according to the Regulation Guide from Ministry of Science and Technology, China.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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