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Phycocyanin ameliorates mouse colitis *via* phycocyanobilin-dependent antioxidant and anti-inflammatory protection of the intestinal epithelial barrier†

Wei Guo,[a,b] Mingyong Zeng,[b] Suqin Zhu,[c] Shiyang Li,[b] Yilin Qian[b] and Haohao Wu *[b]

Phycocyanin is a typical microalgal active compound with antioxidant and anti-inflammatory efficacy, and the pigment moiety phycocyanobilin has been recently proposed as its active structural component. Here, to explore the structural basis for phycocyanin's intestinal protective action, we evaluated the therapeutic effects and mechanism of action of phycocyanin and phycocyanobilin in dextran sodium sulphate (DSS)-induced colitis mice and in Caco-2 and RAW 264.7 cells. Phycocyanobilin was obtained by solvothermal alcoholysis of phycocyanin and characterized by spectroscopy and mass spectrometry methods. Phycocyanin, phycocyanobilin and a positive drug mesalazine were intragastrically administered to C57BL/6 mice daily for 7 days during and after 4-day DSS exposure. Clinical signs and colon histopathology revealed that phycocyanin and phycocyanobilin had an equivalent anti-colitis efficacy that was even superior to mesalazine. Based on biochemical analysis of colonic tight junction proteins, mucus compositions and goblet cells, and colonic and peripheral proinflammatory cytokines, phycocyanin and phycocyanobilin displayed equivalent intestinal epithelial barrier-protecting and anti-inflammatory potential that was evidently superior to that of mesalazine. Flow cytometry analysis of phycocyanobilin fluorescence in Caco-2 cells unveiled a similar uptake efficacy of phycocyanin and phycocyanobilin by intestinal epithelial cells. According to lactic dehydrogenase release, 2′,7′-dichlorodihydrofluorescein fluorescence and methylthiazolyldiphenyl-tetrazolium bromide assay in Caco-2 cells, phycocyanin and phycocyanobilin could equally and effectively protect the intestinal epithelial barrier from oxidant-induced disruption. Phycocyanin and phycocyanobilin also showed equivalent anti-inflammatory effects in tumor necrosis factor-α-stimulated Caco-2 cells and in lipopolysaccharides- and tumor necrosis factor-α-activated RAW264.7 cells. Overall, our results demonstrate the phycocyanobilin-dependent anti-colitis role of phycocyanin *via* antioxidant and anti-inflammatory mechanisms.

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Introduction

Inflammatory bowel disease (IBD), which encompasses ulcerative colitis and Crohn's disease, has become more prevalent in the West since the middle of the 20th century, and its incidence has risen rapidly over the past few decades due to the westernization of lifestyle and industrialization.[1] According to the Global Burden of Diseases, Injuries, and Risk Factor Study 2017, there were 6.8 million cases of IBD worldwide with the highest prevalence (0.422%) observed in high-income North America, and since 1990, there has been a rapid rise in newly industrialized countries in South America, Eastern Europe, Asia, and Africa.[2] IBD is a relapsing and incurable disorder characterized by non-infectious chronic inflammation of the gastrointestinal tract, and displays various symptoms like abdominal cramping pain, persistent diarrhea, and even colorectal cancer, causing 1.85 million disability-adjusted life-years in 2017. Treatment options for IBD include untargeted therapies using drugs like 5-aminosalicylic acid and corticosteroids, and targeted biologic therapies employing antibodies against targets like tumor necrosis factor (TNF)-α and interleukin (IL)-23;[3] nevertheless, side effects and occasional futility to some

[a]*School of Pharmacy, Binzhou Medical University, 346 Guanhai Road, Yantai, Shandong, 264003, China*
[b]*College of Food Science and Engineering, Ocean University of China, 5 Yushan Road, Qingdao, Shandong, 266003, China. E-mail: wuhaohao@ouc.edu.cn; Tel: +86-532-8203-2400*
[c]*Institute of Nutrition and Health, School of Public Health, Qingdao University, Qingdao 266021, China*

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patients made many therapeutic needs remain unmet.[4] Attention has therefore been devoted to developing alternative strategies to alleviate IBD symptoms in a mild way without any complications by utilizing nutraceuticals in recent years.

Phycocyanin is a photosynthetic pigment protein unique to cyanobacteria for light harvesting, and consists of the protein moiety and a linear tetrapyrrole chromophore, *i.e.* phycocyanobilin (PCB), linked by a thioether bond. Phycocyanin could constitute up to 20% of the dry weight of *Spirulina*, a well-known and widely commercialized microalgae for food and nutrition, and has been documented to confer antioxidant and anti-inflammatory protection to the heart, kidneys, liver, lungs, eyes, brain and intestine in several animal models.[5] However, the material basis for phycocyanin's beneficial health effects remains poorly understood. Pleonsil, Soogarun & Suwanwong (2013) unveiled an extraordinarily higher antioxidant capacity of phycocyanin than its apoprotein moiety,[6] implying a predominant contribution of the pigment moiety to phycocyanin's antioxidant potential. Garcia-Pliego *et al.* (2021) demonstrated that PCB is responsible for antioxidant nephroprotection by phycocyanin in the $HgCl_2$-induced kidney injury in mice.[7] Therefore, PCB might be an essential material basis for phycocyanin's nutraceutical functions.

Phycocyanin has been previously shown to exert intestinal protective effects in acetate- and radiation-induced rodent models of acute colitis.[8,9] To explore the structural basis for phycocyanin's intestinal protection, the present study evaluated the beneficial effects of phycocyanin and PCB in dextran sodium sulphate (DSS)-induced mouse colitis and in cellular models of intestinal inflammation and barrier function.

Experimental section

Reagents and materials

Methylthiazolyldiphenyl-tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), lactic dehydrogenase (LDH) cytotoxicity assay kit, radioimmunoprecipitation assay lysis buffer (10×), phenylmethanesulfonyl fluoride, 3% H_2O_2 solution, lipopolysaccharides (LPS), sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride monomethanolate, sodium nitrite, the enhanced chemiluminescence horseradish peroxidase (HRP) substrate, and the primary mouse monoclonal anti-β-actin antibodies were provided by Sigma-Aldrich LLC (Shanghai, China). Human TNF-α and IL-1β recombinant proteins, Pierce™ BCA protein assay kit, phosphate buffered saline (PBS), enzyme-linked immunosorbent assay kits for human IL-8 and mouse IL-6, TNF-α and IL-1β were purchased from Thermo Fisher Scientific Co., Ltd (Shanghai, China). The primary rabbit monoclonal anti-claudin-4, anti-occludin, anti-nuclear factor kappa B (NF-κB) p65, anti-cyclooxygenase (COX)-2 and anti-inducible nitric oxide (NO) synthase (iNOS) antibodies, and the secondary antibodies of rabbit anti-mouse IgG H&L (HRP) and goat anti-rabbit IgG H&L (HRP) were obtained from Abcam (Cambridge, MA, USA). Dulbecco's modified Eagle's medium and fetal bovine serum were supplied by Biological Industries (Beth Haemek, Israel). SDS-PAGE sample loading buffer (5×) was acquired from Beyotime Biotechnology Co., Ltd (Shanghai, China). DSS (36–50 kDa) was provided by Yisheng Biotechnology Co., Ltd (Shanghai, China). PureZOL™ RNA isolation reagent and iScript™ cDNA synthesis kit were purchased from Bio-Rad Laboratories, Inc. (Shanghai, China). Mesalazine (5-aminosalicylic acid) was provided by Ethypharm (Saint Cloud, France). Phycocyanin from *Spirulina platensis* with an A620/A280 value of 1.9 was purchased from Fuqing King Dnarmsa Spirulina Co., Ltd (Fuqing, China). The other reagents used were commercially available and of reagent grade.

Cleavage of PCB from phycocyanin

PCB was cleaved from phycocyanin according to the procedure described by Roda-Serrat *et al.* (2018).[10] Briefly, commercial phycocyanin was purified by successive washes with methanol until no further loss of weight was detected. The recovered phycocyanin cake was mixed with 100 volumes of 96% ethanol in a Teflon-lined autoclave, followed by heating at 120 °C for 30 min, and then the resulting mixture was centrifuged at 8000*g* for 10 min. The resulting supernatant was rotary evaporated to obtain a deep blue solid as PCB.

The absorbance spectrum of PCB was measured over a wavelength range of 300–750 nm on a UV-1780 spectrophotometer (Shimadzu, Tokyo, Japan). Chromatographic analysis was performed on a Shimadzu LC-20AT HPLC system equipped with a Zorbax Eclipse Plus C18 column (2.1 × 150 mm, 1.8 μm, Agilent Technologies, CA, USA) and an SPD-20A UV-VIS detector (Shimadzu, Tokyo, Japan). Elution was conducted with a linear gradient of 40–55% acetonitrile in water (containing 0.1% formic acid) at a flow rate of 0.8 mL min^{-1} over 15 min with the eluent monitored at 666 nm.

Liquid chromatography-mass spectrometry (LC-MS) analysis was carried out on an Agilent 1290 Infinity UPLC system coupled to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC-MS system with an electrospray ionization source (Agilent Technologies, CA, USA). The separation was achieved using an ACQUITY UPLC® BEH C18 column (2.7 × 100 mm, 1.7 μm, Waters Corporation, London, UK). The gradient used for the analysis consisted of a mobile phase A (0.1% formic acid in Milli-Q water) and a mobile phase B (0.1% formic acid in acetonitrile) pumped at 0.4 mL min^{-1}. The elution was conducted with a gradient of 100–85% B in 3.0 min, 85–50% B in 6.5 min, 50–20% B in 8.0 min, 20–0% B in 10.5 min and 100% A in 15.0 min. Data were collected in positive ion mode in a mass range of 100 to 1000 *m*/*z*. The capillary voltage was set to 4000 V, and the drying gas was at the flow rate of 11 L min^{-1} at 325 °C with a gas nebulizer, fragment voltage, and skimmer set at 40 psi, 175 V, and 65 V, respectively.

Animals

Male C57BL/6 mice (6–7-week-old, weighing 19.0–21.0 g) were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd (Jinan, China). All mice were individually housed in

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an air-conditioned room maintained at 20–24 °C with 55–65% relative humidity and a lighting schedule of 12 h light:12 h dark. Animals were fed on an AIN-93 purified diet (Trophic Animal Feed High-Tech Co., Ltd, Nantong, China) *ad libitum*. All experiments were performed in accordance with the principles of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and were approved by the Committee on the Ethics of Animal Experiments of Ocean University of China (No. SPXY20200829).

Colitis induction and treatment protocol

Following a 5-d acclimation period, mice were randomized into five groups (n = 8 in each group), comprising normal control, DSS control, DSS + Mes, DSS + PC, and DSS + PCB groups. The normal control group received distilled water *ad libitum* throughout an experimental period of 7 d (Fig. 2a). To induce colitis in the other groups, the mice received 3% DSS in distilled water (w/v) with *ad libitum* access for 4 d before changing to distilled water for the remaining 3 d. During the 7-d experimental period, the groups of normal control and DSS control received daily intragastric administration of 100 μL PBS, while the DSS + Mes, DSS + PC, and DSS + PCB groups received daily intragastric administration of 100 μL PBS containing mesalazine (positive control drug), phycocyanin, and PCB, respectively. Mesalazine and phycocyanin were administered at the dose of 100 mg per kg body weight, while the dose for PCB was 5 mg per kg body weight, which was equivalent to the amount of PCB contained in the dose of phycocyanin.[11] The human equivalent dose of phycocyanin could be calculated to be 488 mg for adults,[12] and considering the content of phycocyanin in *Spirulina* (15%–20% of dry weight), this dose corresponds to approximately 3 g of dry *Spirulina* biomass per day. In fact, a recommended dosage for adults is usually in the range of 3–10 g of *Spirulina* per day on a dry matter basis.[13]

Colitis evaluation

Food intake, body weight, and typical colitis symptoms (*i.e.*, diarrhea and rectal bleeding) were recorded daily. To evaluate the severity of colitis, the ulcerative colitis disease activity index (DAI) was determined by scoring the trait of stool, rectal bleeding conditions, and body weight loss, which were calculated following the criteria listed in Table S1.†[14]

Animal sacrifice and necropsy

At the end of the experiment, blood was drawn by cardiac puncture at the time of euthanasia. After the blood was clotted for 30 min at room temperature, serum was obtained by centrifugation (3000*g*, 15 min) for IL-6 analysis with the ELISA kit according to the manufacturer's instructions. The entire colon was removed from the abdominal cavity and the length of each colon was immediately measured at rest, without stretching. Following a thorough rinse with PBS, the colon was cut into 0.5 cm long pieces for histological and biochemical analysis.

Histopathological examination

For histopathological analysis, a 0.5 cm piece of the distal colon was fixed in 4% paraformaldehyde, dehydrated in a series of ethanol, and embedded in paraffin. The embedded tissues were cut into 5 μm thin sections. The sections were stained with hematoxylin and eosin (H&E) for histomorphometry and with alcian blue/periodic acid–Schiff (AB/PAS) to observe the goblet cells. All sections were visualized under a Nikon Ni-E upright fluorescence microscope (Tokyo, Japan). Blind scorings of histologic lesions and goblet cell numbers were performed with H&E and AB/PAS staining images, respectively, as described previously.[15,16]

Measurement of cytokines and protein expressions in the colon

For cytokine quantification, colon samples were homogenized in 25 volumes of radioimmunoprecipitation assay lysis buffer containing protease inhibitors with a TissueLyser, and the extracts were cleared by centrifugation at 12 000*g* for 15 min at 4 °C. Protein concentrations in the tissue extracts were quantified with the BCA protein assay kit as per the manufacturer's instructions. The concentrations of TNF-α, IL-1β and IL-6 in the tissue extracts were measured using ELISA kits according to the manufacturer's instructions. The levels of claudin-4, occluding and NF-κB in the tissue extracts were determined by western blot analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total cellular RNA was extracted from colon segments with the mixed solution of Trizol reagent, chloroform, isopropanol, and 75% ethanol solution (10 : 2 : 5 : 10, v/v/v/v). The cDNA synthesis kit (Vazyme Biotech, Nanjing, China) was used for reverse transcription following the manufacturer's instructions. The amplification, detection, and data analysis were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA). All the primer sequences are shown in Table 1. The results were analyzed using the $2^{\Delta\Delta C_T}$ method.

Caco-2 cell experiments

Caco-2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), routinely maintained in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium at passage levels of 55 to 65. All cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere with the medium renewed every 2 d.

For the cellular uptake experiment, cells seeded in 12-well plates at the density of 5.0×10^5 cells per well were cultured in complete medium for 21 d to form a polarized epithelial cell monolayer. After being washed three times with pre-warmed Dulbecco's PBS, the cell monolayers were incubated in HBSS with or without 20 μg mL^{-1} phycocyanin or 1 μg mL^{-1} PCB for 12 h, followed by fixation with 4% formaldehyde solution for 15 min, and then the cells suspended in flow cytometry buffer were analyzed on a BD FACSVerse™ flow cytometer (CA, USA). Data were analyzed using FlowJo software 10.6.1 (Treestar, MA, USA).

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Table 1 Primer sequences used for qRT-PCR analysis

Primer	Forward primer	Reverse primer
GAPDH	TGGAGAAACCTGCCAAGTATGA	TGGAAGAATGGGAGTTGCTGT
MUC2	GCTGACGAGTGGTTGGTGAATG	GATGAGGTGGCAGACAGGAGAC
TFF3	CCGTGGTTGCTGTTTTGAC	GCCTGGACAGCTTCAAAATG
ZO-1	GCTTTAGCGAACAGAAGGAGC	TTCATTTTTCCGAGACTTCACCA

Intracellular reactive oxygen species (ROS) were measured using the cell-permeable fluorogenic probe DCFH-DA. The cells seeded in 96-well plates at the density of 1.0×10^4 cells per well were cultured for 72 h before treatment with or without 20 μg mL^{-1} phycocyanin or 1 μg mL^{-1} PCB in complete medium for 12 h. The cells were then loaded with 10 μM DCFH-DA in HBSS for 40 min, and after the excess fluorescent dye was removed, the DCF-loaded cells were treated with or without 500 μM H_2O_2 for 30 min. The fluorescence was then immediately analyzed using a SpectraMax i3x microplate reader (Molecular Devices, CA, USA) at 485 nm excitation and 528 nm emission.

To test the oxidant-induced cytotoxicity, Caco-2 cells were seeded in 96-well plates at a density of 1.0×10^4 cells per well and cultured for 72 h in complete media, followed by incubation with or without 20 μg mL^{-1} phycocyanin or 1 μg mL^{-1} PCB for 12 h. For the LDH release assay, the cells were exposed to 1.0 mM H_2O_2 for 30 min before the media supernatant was harvested for LDH determination with a commercial assay kit as per the manufacturer's instructions. For apoptosis assay, the cells were exposed to 2 mM H_2O_2 for 30 min before cell viability was measured by the MTT assay. Briefly, the cells were washed with Dulbecco's PBS three times, followed by a 4 h incubation with 0.5 mg mL^{-1} MTT in Dulbecco's PBS, and then dimethyl sulfoxide was used to dissolve the resulted formazan crystals with the absorbance at 570 nm read on a SpectraMax i3x microplate reader (Molecular Devices, CA, USA).

To test the epithelial inflammation and tight junction reorganization, the cells seeded in 12-well plates at a density of 5.0×10^5 cells per well were cultured in complete medium for 21 d to form a polarized epithelial cell monolayer, followed by the treatment with or without 20 μg mL^{-1} phycocyanin, or 1 μg mL^{-1} PCB in complete medium for 12 h, and were then stimulated with TNF-α (10 ng mL^{-1}) for another 24 h. The media supernatant was collected for IL-8 detection with an ELISA kit according to the manufacturer's instructions. The cells were lysed in radioimmunoprecipitation assay lysis buffer containing 1 mM phenylmethanesulfonyl fluoride at 4 °C for 30 min, followed by centrifugation at 12 000*g* for 15 min at 4 °C, and the resulting supernatant was used as whole cell lysate for western blot analysis of claudin-4, occludin, COX-2 and iNOS.

RAW 264.7 cell experiments

RAW 264.7 cells were obtained from the Cell Bank of Chinese Academy of Sciences and routinely maintained in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium at 37 °C in a humidified 5% CO_2 atmosphere at passage levels of 10 to 20. To assay the production of NO and IL-8, the cells seeded into a 12-well plate at the density of 5.0×10^5 cells per well were cultured for 24 h in complete media, followed by the treatment with or without 5 μg mL^{-1} phycocyanin or 0.25 μg mL^{-1} PCB in complete medium for another 12 h, and after triple rinsing with Dulbecco's PBS, cells were stimulated with LPS (1 μg mL^{-1}) or TNF-α (10 ng mL^{-1}) for 12 h. The culture supernatants were then harvested for NO and IL-8 determination by the Griess reaction assay and a commercial ELISA kit, respectively, and the whole cell lysates were prepared in radioimmunoprecipitation assay lysis buffer containing 1 mM phenylmethanesulfonyl fluoride for western blot analysis of iNOS and NF-κB. For the Griess reaction assay, the culture supernatant was mixed with an equal volume of freshly prepared Griess reagent consisting of 1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine, and 5% phosphoric acid in a 96-well plate, and the reaction mixture was left for 10 min before the absorbance at 540 nm was measured on a microplate reader.[17]

Western blot

After being heat-denatured in SDS-loading buffer and electrophoresed by 10% SDS-PAGE, proteins in the samples were blotted to a PVDF transfer membrane (0.45 μm, Thermo Scientific™, USA), blocked with 5% skimmed milk dissolved in Tris-buffered saline for 1 h at room temperature, and immunoblotted with the primary antibodies overnight at 4 °C, followed by a second incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. The imaging was performed with an enhanced chemiluminescence detection kit on a Tanon-5200 Multi automatic image analyzer (Tanon, Shanghai, China).

Statistical analysis

The results were statistically analyzed using IBM SPSS Statistics for Windows version 20 (IBM Corp., NY, USA) and Origin version 8.0 (OriginLab, Northampton, USA). Data in histograms were presented as means ± standard deviations and compared by one-way analysis of variance (ANOVA) followed by Duncan's *post hoc* test. Significant differences were defined at *p*-values of $p < 0.05$.

Results

Solvothermal alcoholysis preparation of PCB from phycocyanin

Solvothermal alcoholysis in a sealed vessel has been recently demonstrated as a fast and high-purity method for the prepa-

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ration of PCB from phycocyanin.[10] In the present study, PCB was obtained as a deep blue solid by solvothermal alcoholysis of phycocyanin (Fig. 1a, inset), and yielded an absorption spectrum with two peaks at around 375 and 620 nm at pH 7, which are the characteristic short and long wavelength absorptions of PCB.[18] The chromatogram of the PCB product is shown in Fig. 1b. According to Beale and Cornejo (1984) and Roda-Serrat *et al.* (2018),[10,19] the peaks at the retention times of 5.1 and 6.8 min could be assigned to (3*E*)- and (3*Z*)-PCB isomers, respectively, which are the products of two competing elimination reaction pathways of E2 and S_N2 during solvolysis of phycocyanin, while the peak at a retention time of 6.1 min should be an ethanol adduct of PCB (PCB-OEt). A major peak (7.085 min) and a minor peak (7.493 min) in the total ion chromatogram of LC-MS/MS analysis (Fig. 1c) showed the quasi-molecular ion for PCB at *m*/*z* 587 (Fig. 1d and S1a†) and its major MS/MS fragment ions $[C_{17}H_{19}N_2O_3]H^+$ at *m*/*z* 299 and $[C_{26}H_{30}N_3O_5]H^+$ at *m*/*z* 464 (Fig. 1e, and S1b†) due to the successive loss of two pyrrole rings from the parent ion (Fig. 1h),[10] confirming the presence of two PCB isomers in the PCB product. The peak at the retention time of 7.268 min in Fig. 1c showed a quasi-molecular ion for PCB-OEt at *m*/*z* 633 (Fig. 1f) and its major MS/MS fragment ions of $[PCB]H^+$ at *m*/*z* 587, $[C_{17}H_{19}N_2O_3]H^+$ at *m*/*z* 299 and $[C_{26}H_{30}N_3O_5]H^+$ at *m*/*z* 464 (Fig. 1g) due to the successive loss of the ethanol group and two pyrrole rings from the parent ion (Fig. 1h),[10] validating the formation of PCB-OEt during solvothermal alcoholysis of phycocyanin. As calculated by integrating areas of the corresponding peaks shown in Fig. 1b, (3*E*)-PCB, (3*Z*)-PCB and PCB-OEt were present in the PCB product at a ratio of 91.6%.

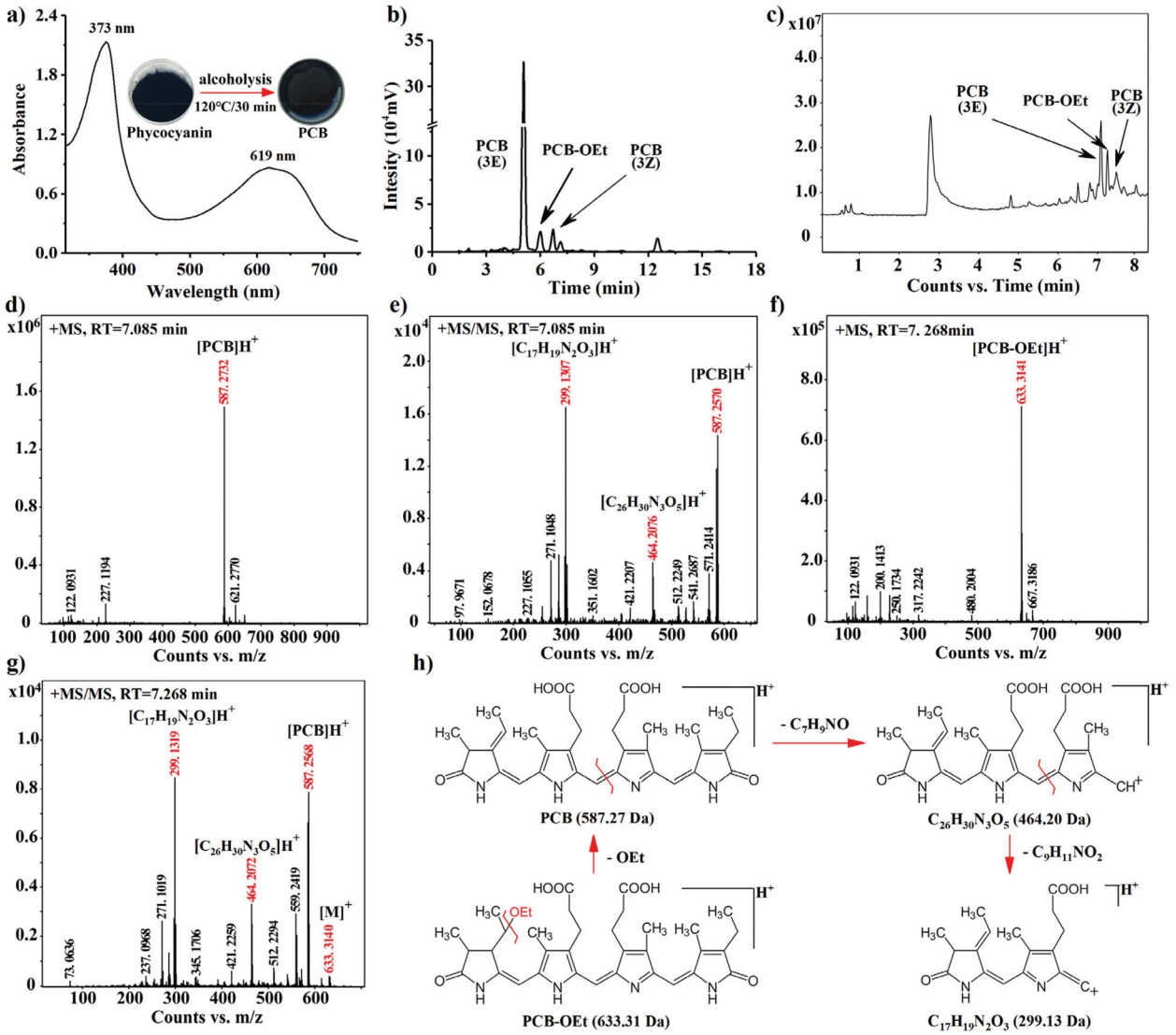

Fig. 1 Characterization of the phycocyanobilin (PCB) product: (a) ultraviolet–visible absorption spectrum; (b) high performance liquid chromatography profile; (c) total ion current chromatogram of LC-MS analysis; (d) primary mass spectra of PCB (3E); (e) secondary mass spectra of PCB (3E); (f) primary mass spectra of the ethanol adduct of PCB (PCB-OEt); (g) secondary mass spectra of PCB-OEt; and (h) schematic presentation of the formation of major fragment ions from PCB and PCB-OEt.

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Taken together, PCB was successfully obtained by solvothermal alcoholysis of phycocyanin.

Effects of phycocyanin and PCB on DSS-induced mouse colitis

To assess their therapeutic efficacies on acute colitis, phycocyanin and PCB were intragastrically administered to mice for 7 d during and after 4-d DSS exposure (Fig. 2a). As shown in Fig. 2b, the kinetics of body weight unveiled that the DSS control group suffered from significant body weight loss in comparison with the normal control group from day 3 ($P < 0.01$). Intragastric treatments with mesalazine, phycocyanin and PCB significantly attenuated the DSS-induced weight loss from day 7, day 5, and day 6, respectively ($P < 0.05$). Fig. 2c shows the total food intake during the 7 d of experiment, and remarkable appetite loss was observed for DSS control animals compared to the normal controls ($P < 0.01$). Oral adminis-

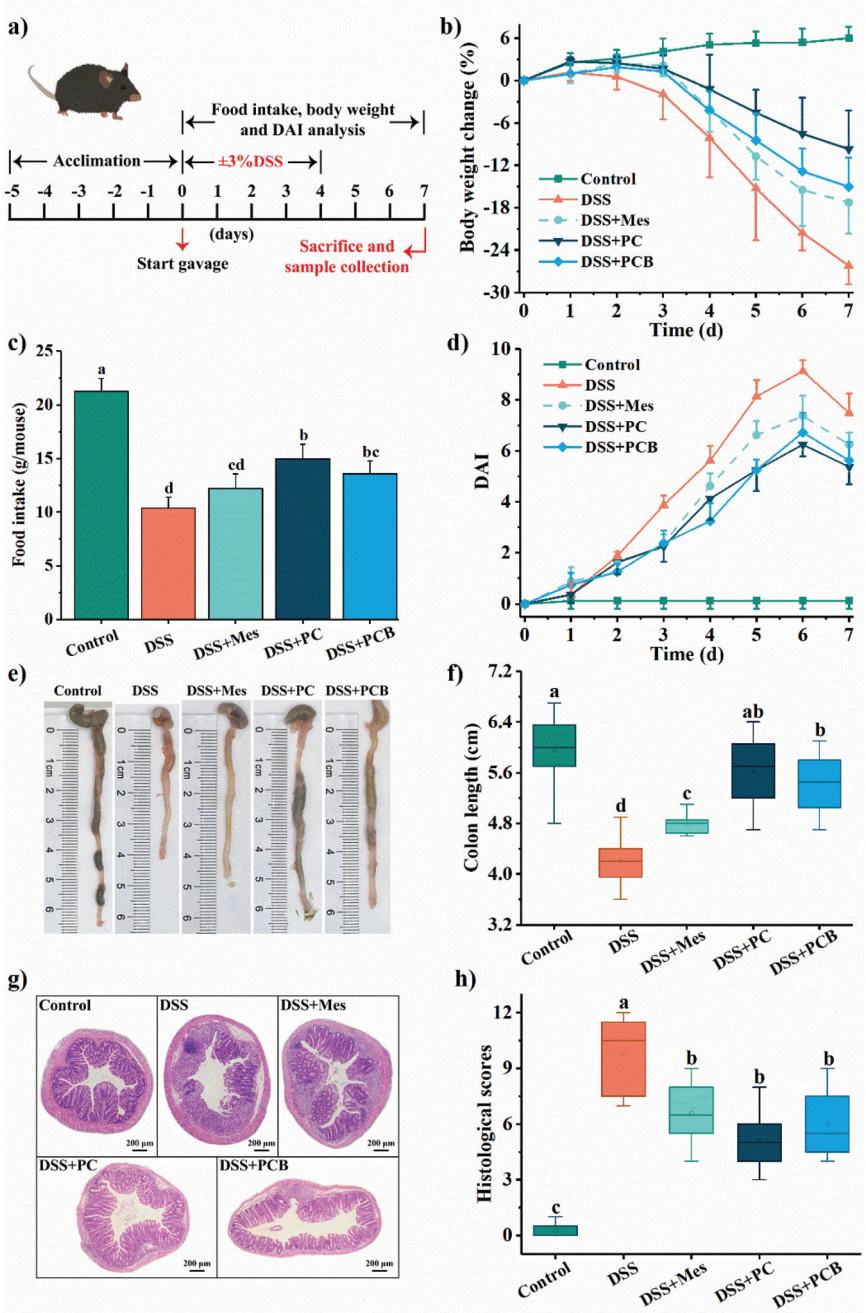

Fig. 2 Effects of oral administration of phycocyanin (PC) and phycocyanobilin (PCB) on clinical parameters of DSS-induced colitis: (a) experimental design; (b) body weight changes; (c) food intake; (d) disease activity index (DAI); (e) typical images of colon length measurement; (f) colon length; (g) typical images of hematoxylin and eosin (H&E)-stained colonic sections and (h) histological scores. Data in b–d were expressed as means ± standard deviations ($n = 8$). Different superscript letters denote statistically significant differences ($p < 0.05$).

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tration of phycocyanin and PCB effectively ameliorated the DSS-induced appetite loss ($P < 0.05$).

Fig. 2d shows the DAI values that were obtained based on the pathological conditions of colitis (*i.e.* weight loss, loose stool, and fecal blood). The DSS control-treated mice had remarkably increased DAI from day 2 in comparison with the normal control-treated ones ($P < 0.01$), suggesting evident acute pathological conditions of colitis in response to free drinking of DSS. Orally administered mesalazine, phycocyanin and PCB significantly attenuated the DSS-induced increase in DAI from day 5, day 3, and day 3, respectively ($P < 0.05$).

Colon length is a common indicator of the severity of acute colitis in mice. As shown in Fig. 2e and f, DSS-induced colon shortening was effectively prevented by the treatments with mesalazine, phycocyanin, and PCB ($P < 0.05$), and mesalazine displayed a significantly weaker protecting effect than phycocyanin and PCB ($P < 0.05$). Histological analysis of H&E-stained colon tissue samples, as shown in Fig. 2g, revealed typical colitis lesions (*e.g.*, crypt damage, edema, mucosal ulceration, and infiltration of inflammatory cells) in the DSS control mice, while those treated with mesalazine, phycocyanin, and PCB displayed remarkably less histological lesions. By using histological scoring (Fig. 2h), phycocyanin and PCB were found to be more efficient at ameliorating DSS-induced histological lesions than mesalazine ($P < 0.05$).

As evidenced by the above results observed for body weight, food intake, DAI, colon length and histopathology, phycocyanin and PCB had an equivalent anti-colitis efficacy that was even superior to mesalazine. A leaky intestinal epithelial barrier is the triggering mechanism for DSS-induced colitis, and could lead to secondary colonic and systematic inflammation by facilitating the invasion of luminal antigens or microorganisms into lamina propria and systemic circulation.[20,21] Intestinal epithelial barrier protection and anti-inflammation are thus typical anti-colitis mechanisms. In the following section, to elucidate the mechanism of action of phycocyanin and PCB, we will examine their potential in intestinal epithelial barrier protection and anti-inflammation in the DSS-treated mice.

Effects of phycocyanin and PCB on the intestinal epithelial barrier and proinflammatory response in DSS-induced colitis mice

Intestinal intercellular tight junctions and mucus layer work together to maintain the barrier function of the intestinal epithelium.[21] According to the results of western blot analysis (Fig. 3a and b), the DSS control mice had markedly reduced colonic levels of occludin and claudin-4 compared to the normal controls, while orally administered mesalazine, phycocyanin and PCB effectively prevented the DSS-induced loss of these tight junction proteins in the colon. The results of qRT-PCR analysis also revealed 3.1, 5.1 and 5.5 times increased colonic transcriptional levels of zonula occluden-1 (ZO-1) by oral treatments with mesalazine, phycocyanin and PCB, respectively, in DSS-induced colitis mice ($P < 0.01$) (Fig. 3c). In colonic mucus, mucin 2 (MUC2) is the major gel-forming mucin establishing a protective polymer barrier with a net-like structure, in which trefoil factor family peptide 3 (TFF3) links mucins by forming disulfide-bonded crosslinks, and an increased production of MUC2 and TFF3 in the colon has been observed in IBD, representing a colonic stress response to compensate for mucus impairment.[22] As shown in Fig. 3d and e, qRT-PCR analysis revealed significantly increased colonic mRNA levels of MUC2 and TFF3 in the DSS control group than in the normal control group ($p < 0.05$), while oral administration of mesalazine, phycocyanin and PCB markedly suppressed the DSS-induced increase in colonic MUC2 and TFF3 transcription ($p < 0.05$). Intestinal MUC2 and TFF3 are synthesized by goblet cells that are specialized epithelial cells participating in mucosal regeneration and repair.[23] The results of AB/PAS staining (Fig. 3f and g) revealed evidently less intestinal goblet cells in the DSS control group than in the normal control group ($p < 0.05$), and the oral treatments with mesalazine, phycocyanin and PCB significantly inhibited the DSS-induced loss of intestinal goblet cells ($P < 0.05$). Based on the above results, phycocyanin and PCB protected the intestinal epithelial barrier in colitis mice, and they generally displayed an equivalent protecting potential that is significantly higher than that of mesalazine (Fig. 3).

Proinflammatory cytokines, *e.g.*, TNF-α, IL-1β and IL-6, are key players in the initiation and propagation of inflammation during the progression of IBD, and NF-κB is a critical regulator for the intracellular signal transduction pathways of these cytokines.[24] In this study, the DSS control group had a markedly increased protein level of NF-κB in the colon (Fig. 3a and b), and displayed approximately 2, 10 and 6 times higher levels of colonic TNF-α, IL-6 and IL-1β, respectively, than the normal control group (Fig. 4a–c). The oral treatments with mesalazine, phycocyanin and PCB notably suppressed the DSS-induced increase in the colonic levels of NF-κB and proinflammatory cytokines ($p < 0.05$). As shown in Fig. 4d, the DSS control mice also displayed about a 10 times higher level of serum IL-6 than the normal control ones ($p < 0.05$), while orally administered mesalazine, phycocyanin and PCB greatly suppressed the DSS-induced burst of this cytokine in the systemic circulation. Based on the above results of proinflammatory cytokines, phycocyanin and PCB exerted inhibitory effects on both colonic and systemic inflammation in colitis mice, and they displayed an equivalent anti-inflammatory potential that is evidently superior to that of mesalazine (Fig. 4).

Apparently, phycocyanin and PCB alleviated DSS-induced colitis *via* a mechanism involving intestinal epithelial barrier protection and anti-inflammation. However, the intestinal epithelial barrier and host proinflammatory response could interact with each other and involve a cause-and-effect relationship on both sides. In order to gain further insight into the anti-colitis mechanism of phycocyanin and PCB, their direct effects on the intestinal epithelial barrier and proinflammatory responses were evaluated in the *in vitro* cell lines of Caco-2 and RAW264.7, respectively, in the following sections.

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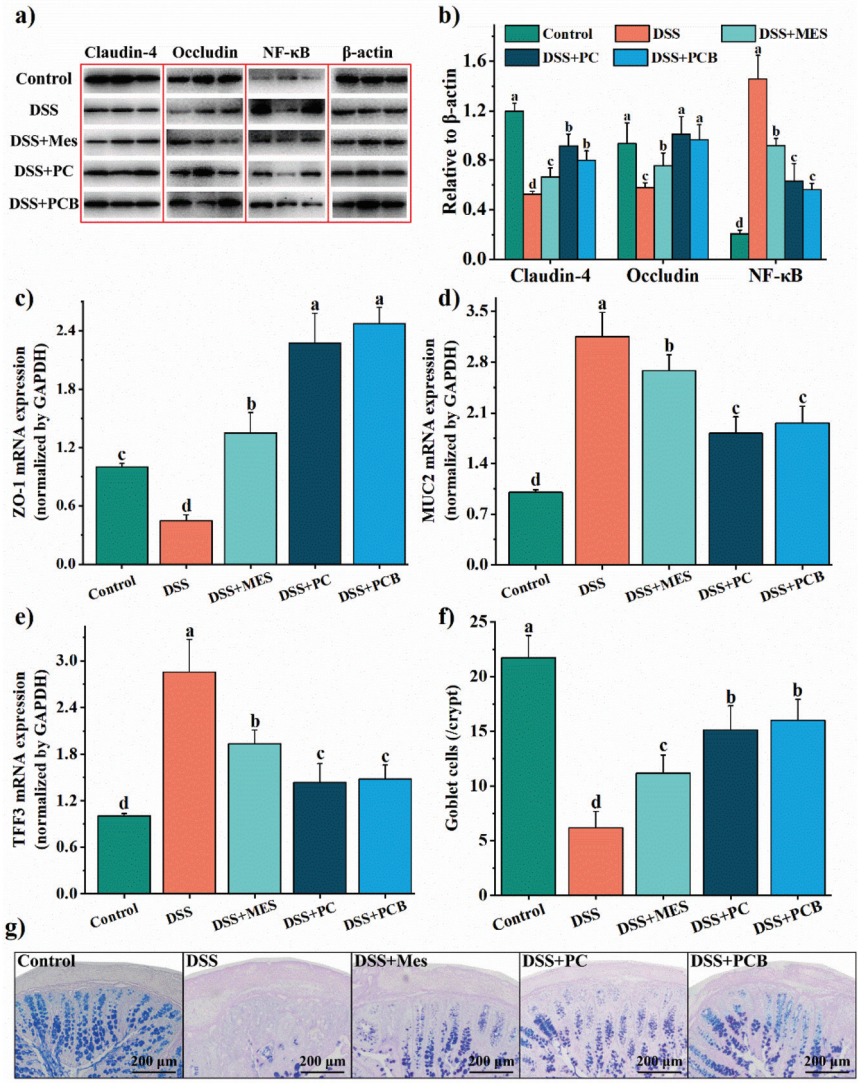

Fig. 3 Effects of orally administered phycocyanin (PC) and phycocyanobilin (PCB) on the intestinal epithelial barrier in DSS-induced colitis mice: western blot (a) imaging and (b) densitometric analysis of the expression levels of claudin-4, occluding and nuclear factor kappa B (NF-κB); mRNA expression levels of (c) zonula occludens-1 (ZO-1), (d) mucin 2 (MUC2) and (e) trefoil factor family peptide 3 (TFF3); (f) goblet cells number; (g) the alcian blue/periodic acid–Schiff stained colonic sections. Data in (b)–(f) were expressed as means ± standard deviations (n = 8). Different superscript letters denote statistically significant differences ($p < 0.05$).

Protective effects of phycocyanin and PCB against H_2O_2-induced epithelial barrier disruption in Caco-2 cells

To determine their bioavailability to intestinal epithelial cells, phycocyanin and PCB were incubated with polarized Caco-2 cells for 12 h before their natural fluorescence (excitation 633 nm, emission 660 nm) intensities within cells were measured by flow cytometry. According to the flow cytometry spectra shown in Fig. 5a, there was an obvious increase of fluorescence in Caco-2 cells treated with phycocyanin and PCB compared to the negative controls, so phycocyanin and PCB seem to be efficiently taken up by intestinal epithelial cells. There was no significant difference in the fluorescence intensity between phycocyanin- and PCB-treated Caco-2 cells, suggesting similar cellular uptake efficacy of phycocyanin and PCB.

Oxidative stress has been implicated to mediate mucosal injury and immune activation during the pathogenesis of many gastrointestinal mucosal diseases, *e.g.* ulcerative colitis and Crohn's disease.[25,26] In this study, the 0.5 h treatment with 500 μM H_2O_2 caused significantly increased DCF fluorescence in Caco-2 cells (Fig. 5b), and this was effectively prevented to a similar extent by 12 h pretreatments of cells with phycocyanin and PCB ($p < 0.05$). These results suggest that phycocyanin and PCB were equally effective at attenuating the H_2O_2-induced burst of intracellular ROS.

As shown in Fig. 5c, treatment with 500 μM H_2O_2 for 1 h caused a 48% increase in LDH release, suggesting a severely

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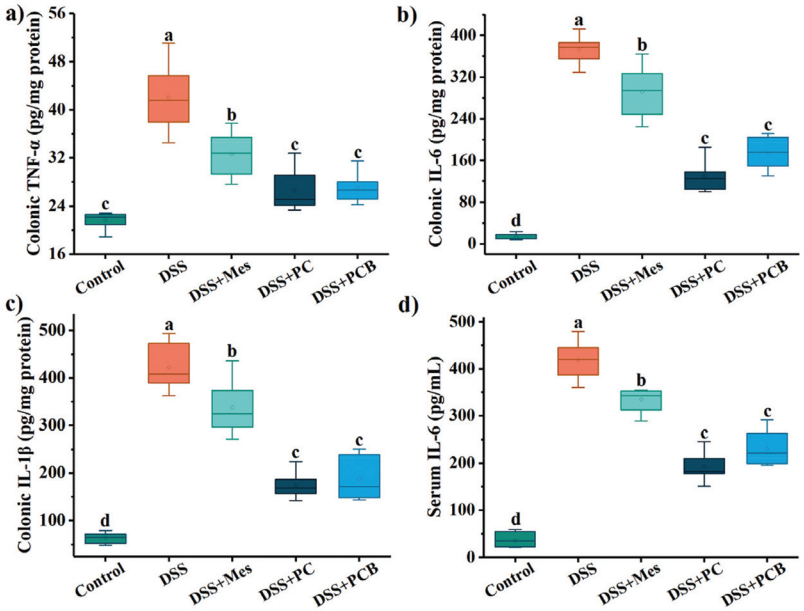

Fig. 4 Effects of orally administered phycocyanin (PC) and phycocyanobilin (PCB) on the levels of proinflammatory cytokines in DSS-induced colitis mice: the colonic levels of (a) tumour necrosis factor (TNF)-α, (b) interleukin (IL)-6 and (c) IL-1β; and (d) the serum level of IL-6. Different superscript letters denote statistically significant differences ($p < 0.05$).

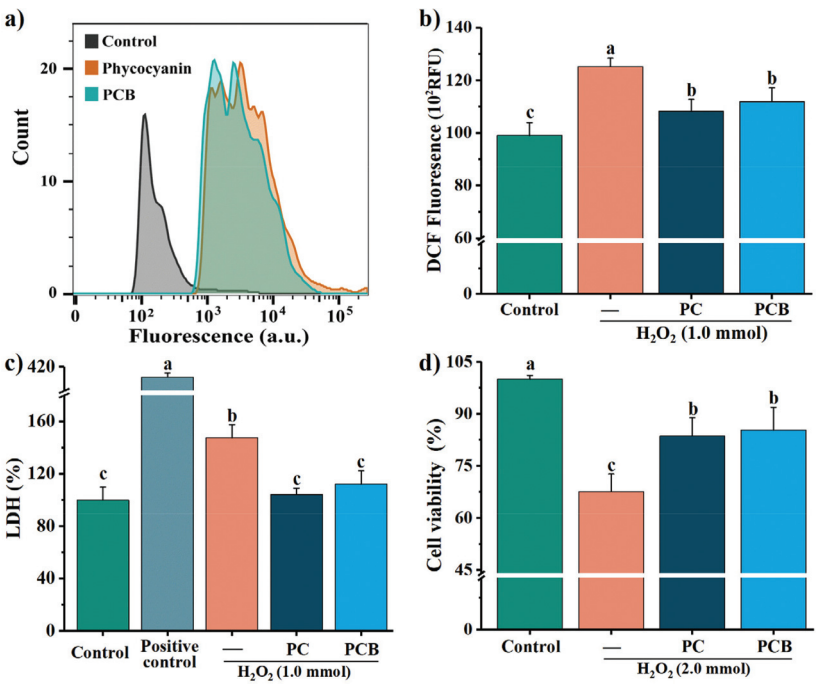

Fig. 5 Beneficial effects of phycocyanin (PC) and phycocyanobilin (PCB) on H_2O_2-induced Caco-2 cells: (a) cellular uptake of PC and PCB; (b) intracellular reactive oxygen species (ROS); (c) lactate dehydrogenase (LDH) release; and (d) cell apoptosis ($n = 6$). Data in (b)–(d) were expressed as means ± standard deviations ($n = 6$). Different superscript letters denote statistically significant differences ($p < 0.05$).

impaired plasma membrane integrity by H_2O_2-induced oxidative stress, and this was effectively prevented to a similar extent by 12 h pretreatments of cells with phycocyanin and PCB ($p < 0.05$). These results indicated an equivalent cytoprotective potential of phycocyanin and PCB against ROS-induced plasma membrane leakage of intestinal epithelial cells.

As shown in Fig. 5d, Caco-2 cells suffered from about one-third loss of viability following the treatment with 2 mM H_2O_2

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for 30 min, and this was significantly attenuated to a similar extent by 12 h pretreatments of cells with phycocyanin and PCB ($p < 0.05$). These results reveal that phycocyanin and PCB exerted equivalent cytoprotective effects against oxidant-induced apoptosis of intestinal epithelial cells.

Based on the above results of flow cytometry, DCF fluorescence, LDH release, and cell viability, phycocyanin and PCB could confer direct antioxidant protection to the intestinal epithelial barrier, which should at least in part explain their anti-colitis activity, and PCB seems to be responsible for the anti-oxidant potential of phycocyanin in Caco-2 cells.

Anti-inflammatory effects of phycocyanin and PCB in TNF-α-stimulated Caco-2 cells

In response to proinflammatory cytokines, intestinal epithelial cells can directly modulate host immune responses by secreting immunological mediators especially IL-8.[27] As shown in Fig. 6a, treatments of Caco-2 cells with TNF-α for 24 h significantly stimulated IL-8 production, and this was remarkably attenuated to a similar extent by 12 h pretreatments with phycocyanin and PCB ($p < 0.05$). These results indicated an equivalent anti-inflammatory potential of phycocyanin and PCB in intestinal epithelial cells.

COX-2 and iNOS are important contributors to the cascade of epithelial inflammatory responses induced by certain pathogens or diseases. In the present study, we observed markedly increased expression of COX-2 and iNOS in response to TNF-α stimulation in Caco-2 cells (Fig. 6b and c). The cells pretreated with phycocyanin and PCB were equally less sensitive to TNF-α stimulation for the expression of COX-2 and iNOS ($p < 0.05$), which again implied an equivalent anti-inflammatory potential of phycocyanin and PCB in intestinal epithelial cells.

Tight junction (TJ) proteins between lumen-lining cells play crucial roles in the maintenance of the gut barrier function.[28] As shown in Fig. 6b and c, TNF-α significantly downregulated the expression of two typical tight junction proteins claudin-4 and occludin in Caco-2 cells as evaluated by western blot analysis and subsequent densitometry ($p < 0.05$), and this was prevented to a similar extent by 12 h pretreatments with phycocyanin and PCB, suggesting that phycocyanin and PCB conferred equally effective anti-inflammatory protection against TJ dysfunction in an intestinal inflammatory milieu.

According to the above results of IL-8 production and expression of COX-2, iNOS, claudin-4 and occludin, phycocyanin and PCB could confer direct anti-inflammatory protection to the intestinal epithelial barrier, which should partly explain their anti-colitis activity, and PCB seems to play a crucial role in the anti-inflammatory effects of phycocyanin in intestinal epithelial cells.

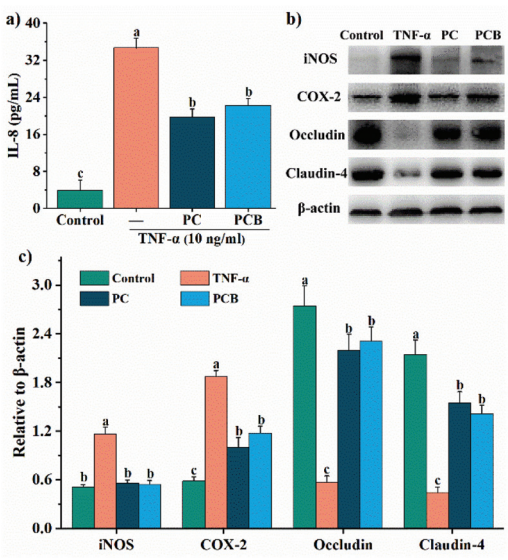

Fig. 6 Anti-inflammatory effects of phycocyanin (PC) and phycocyanobilin (PCB) in Caco-2 cells: (a) IL-8 production with TNF-α stimulation ($n = 6$); western blot (b) imaging and (c) densitometric analysis of claudin-4, occludin, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) when stimulated with TNF-α. Data were expressed as means ± standard deviations ($n = 3$). Different superscript letters denote statistically significant differences ($p < 0.05$).

Anti-inflammatory effects of phycocyanin and PCB in LPS- and TNF-α-activated RAW264.7 cells

Overproduction of NO derived from iNOS plays an important role in the pathogenesis of inflammation.[29] As shown in Fig. 7a, LPS significantly increased the production of NO in RAW264.7 macrophages ($p < 0.05$), and this was markedly attenuated to a similar extent by 12 h pretreatments with phycocyanin and PCB ($p < 0.05$). As revealed by western blot analysis (Fig. 7c), LPS significantly activated the expression of iNOS in RAW264.7 cells, and this was significantly blocked to a similar extent by the 12 h pretreatments with phycocyanin and PCB ($p < 0.05$). Phycocyanin and PCB thus seem to be equally effective at alleviating LPS-induced NO production by suppressing the activation of iNOS in RAW264.7 cells.

NF-κB is a key regulator for the production of many proinflammatory cytokines, and has been demonstrated as an important target for anti-inflammatory therapy.[30] As illustrated in Fig. 7b and d, the treatment of RAW264.7 macrophages with 10 ng mL^{-1} TNF-α for 12 h caused over 2 times increase in IL-8 secretion and NF-κB activation, and this was effectively prevented to a similar extent by 12 h pretreatments with phycocyanin and PCB. These results suggested that phycocyanin and PCB could exert equivalent anti-inflammatory effects in RAW264.7 macrophages by blocking TNF-α-stimulated NF-κB activation.

Based on the above results of NO and IL-8 production and the activation of iNOS and NF-κB, PCB seems to be responsible for the anti-inflammatory potential of phycocyanin in RAW264.7 macrophages. Apparently, phycocyanin and PCB could directly counteract the proinflammatory cascade in an intestinal inflammatory milieu, which may provide an additional explanation for their therapeutic effects against colitis.

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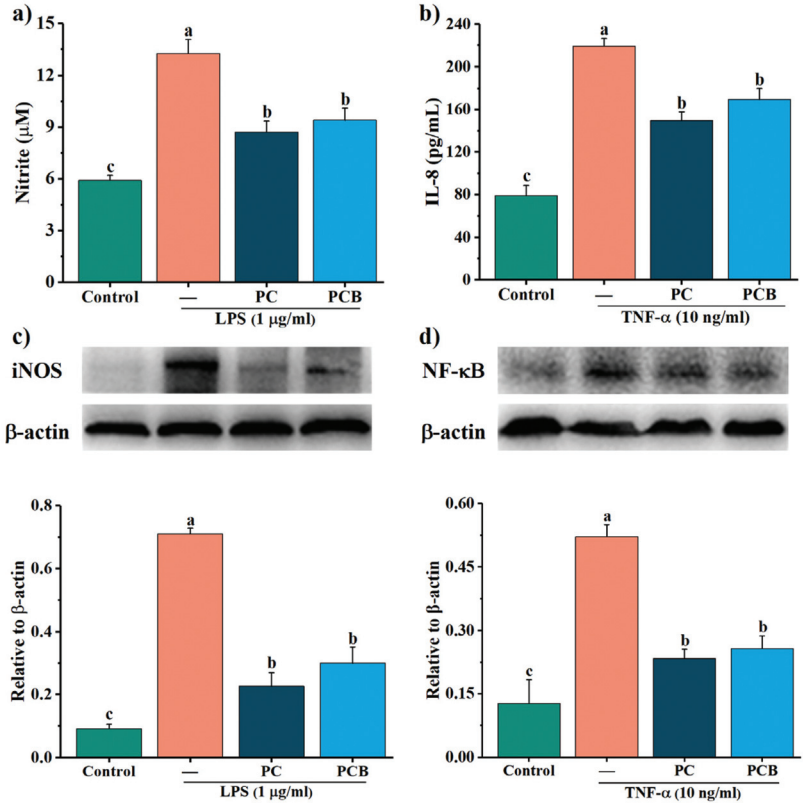

Fig. 7 Anti-inflammatory effects of phycocyanin (PC) and phycocyanobilin (PCB) in RAW264.7 cells: (a) nitric oxide (NO) production with lipopolysaccharide (LPS) activation (n = 6); (b) interleukin (IL)-8 production with tumour necrosis factor (TNF)-α activation (n = 6); (c) western blot imaging and densitometric analysis (n = 3) of inducible nitric oxide synthase (iNOS) when stimulated with LPS; and (d) western blot imaging and densitometric analysis (n = 3) of nuclear factor kappa B (NK-κB) when stimulated with TNF-α. Data were expressed as means ± standard deviations. Different superscript letters denote statistically significant differences ($p < 0.05$).

Discussion

Oxidative stress is believed to play an important role in the pathogenesis and progression of IBD.[25] Antioxidants from natural dietary sources, *e.g.* polyphenols, carotenoids and vitamin A, have been found to protect the colon against ulcers by improving antioxidant nutritional status.[25,31] Phycocyanin has been well documented for its strong anti-radical activity against alkoxyl, hydroxyl, peroxyl, peroxynitrite, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), hypochlorous acid, and 1,1′-diphenyl-2-picrylhydrazyl radicals.[32,33] Earlier studies have shown the bleaching of the chromophore in phycocyanin as a result of the attack by the peroxyl and hydroxyl radicals, suggesting the involvement of PCB in the radical scavenging activity of phycocyanin.[34] Bhata and Madyastha (2001) reported that the radical treatments of phycocyanin could result in oxidative degradation of the covalently linked chromophore into mesobiliviolin and biliviolins possibly *via* a radical addition reaction to conjugated double bonds in the tetrapyrrole structure of PCB.[35] According to Hirata *et al.* (1999, 2000) and Pleonsil, Soogarun & Suwanwong (2013), PCB accounted for the majority of phycocyanin's antioxidative capacity against radical-initiated lipid peroxidation.[6,36,37] Zheng *et al.* (2012) demonstrated that phycocyanin and PCB were equally effective at inhibiting renal oxidative stress in diabetic nephropathy.[38] In the present study, phycocyanin and PCB exerted equivalent protective effects against H_2O_2-induced intracellular ROS burst, plasma membrane leakage, and apoptosis in polarized Caco-2 cells (Fig. 5b–d), suggesting the PCB-dependent radical-scavenging role of phycocyanin in protecting the intestinal epithelial barrier in colitis. In addition, tetrapyrroles including bilirubin, hemin, biliverdin, protoporphyrin, and PCB have been previously reported to inhibit the activation of superoxide-producing NADPH oxidase through hydrophobic interactions,[36,38] so phycocyanin might also exert anti-oxidant effects through PCB-mediated attenuation of NADPH dependent superoxide production.

Phycocyanin has been well documented to exert anti-inflammatory effects by inhibiting NF-κB activation in a variety of animal and cellular models of inflammation.[39–41] In the present study, PCB showed an equivalent potency to phycocyanin against NF-κB activation and downstream inflammatory responses in human intestine and RAW264.7 cells (Fig. 6, 7b and d), suggesting the critical structural role of PCB in phycocyanin's anti-inflammatory activity. In fact, several studies have reported the anti-inflammatory properties of PCB in

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rodents with neuroinflammatory disorders such as autoimmune encephalomyelitis and acute cerebral hypoperfusion.[42,43] PCB has been revealed to interact with the DNA binding site of NF-κB p65 by molecular docking, and may hinder the inducible binding of NF-kB to DNA regulatory elements.[44] PCB may also inhibit the activation of NF-κB, at least in part, *via* scavenging ROS that are considered as an important category of NF-κB activators.[45] The structure of phycocyanobilin is homologous to the structure of biliverdin, the intermediate product of heme catabolism in mammals, which is reduced by biliverdin reductase into bilirubin, the end product of heme degradation. Bilirubin has been reported to exert anti-inflammatory effects by regulating heme oxygenase, inducible nitric oxide synthase, and cyclooxygenase, so phycocyanobilin might also modulate inflammation through phycocyanorubin, its possible mammalian metabolism end product homologous to bilirubin.[46]

Patients with IBD present several defects in the specialized components of the mucosal barrier, from mucus compositions to adhesion molecules that regulate paracellular permeability.[47] In the present study, phycocyanin and PCB protected the intestinal mucosal barrier by preserving tight junction proteins, mucus compositions and goblet cells in colitis mice (Fig. 3d–g). This might be, at least in part, explained by their potency to inhibit the augmented release of proinflammatory cytokines (Fig. 4a–d), which could result in perturbed regulation of tight intercellular junctions, leading to augmented intestinal permeability and increased exposure of the intestine to exotics.[48] Cumulative evidence indicates that ROS can directly disrupt the mucosal barrier integrity by inducing apoptosis of intestinal epithelial cells.[49] In the present study, phycocyanin and PCB showed evident protective activities against H_2O_2-induced epithelial barrier disruption in Caco-2 cells (Fig. 5c), so their protective activity on the intestinal mucosal barrier in colitis mice (Fig. 3a–e) may also be in part explained by an antioxidant mechanism.

Conclusions

In conclusion, our *in vivo* and *in vitro* results demonstrate the PCB-dependent anti-colitis role of phycocyanin *via* antioxidant and anti-inflammatory mechanisms. Our study elucidates the structural and mechanistic basis for phycocyanin's intestinal protective action, which will help in developing new remedies based on PCB, phycocyanin and/or *Spirulina* for ameliorating IBD. Further investigation is needed to evaluate the clinical efficacy of phycocyanin and PCB in human subjects.

Abbreviations

AB/PAS	Alcian blue/periodic acid–Schiff
COX-2	Cyclooxygenase 2
DAI	Disease activity index
DSS	Dextran sodium sulphate
IBD	Inflammatory bowel disease
IL	Interleukin
iNOS	Inducible nitric oxide synthase
H&E	Hematoxylin and eosin
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
MUC2	Mucin 2
NF-κB	Nuclear factor kappa B
PCB	Phycocyanobilin
PCB-OEt	An ethanol adduct of PCB
ROS	Reactive oxygen species
TFF3	Trefoil factor family peptide 3
TJ	Tight junction
TNF	Tumour necrosis factor

Conflicts of interest

There are no conflicts to declare.

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