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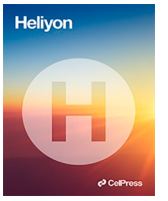

Citrus peel polyphenols alleviate intestinal inflammation in mice with dextran sulfate sodium-induced acute colitis

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ABSTRACT

Citrus peel polyphenols have possess the distinct anti-inflammatory activities. However, its underlying mechanism on ulcerative colitis have not been elucidated. The aim of this research was to investigate the anti-inflammatory effect and action mechanisms of citrus peel polyphenols. Total citrus peel polyphenols were concentrated using macroporous resins and separated into water-soluble citrus polyphenols and ester-soluble citrus peel polyphenols. These extracts were then gavaged to acute colitis mice induced by dextran sulfate sodium for 14 days using a dose of 300 mg/kg■bw. High performance liquid chromatography results showed that the extracts contained flavanones, flavonoids, and phenolic acids. Compared to the dextran sulfate sodium group, total citrus peel polyphenols, water-soluble citrus polyphenols, and ester-soluble citrus peel polyphenols significantly ameliorated the severity of colitis symptoms. Additionally, citrus peel polyphenols reduced the activity of myeloperoxidase, lowered secretion of tumor necrosis factor-α and interleukin-6, and increased interleukin-10. Meanwhile, total citrus peel polyphenols, water-soluble citrus polyphenols, and ester-soluble citrus peel polyphenols effectively blocked the activation of the nuclear factor-kappa B. These results demonstrated that citrus peel polyphenols alleviated ulcerative colitis in mice by damping pro-inflammatory cytokine secretion and suppressing the nuclear factor-kappa B pathway activation.

1. Introduction

Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease (CD) often cause diarrhea, abdominal pain, bloody stools, and fatigue [1]. UC is a persistent inflammation occurring in the rectum and colonic mucosa. CD affects the entire digestive tract, and is a non-continuous full-thickness inflammation. The UC is more common than CD, and the incidence of UC is higher. The occurrence and development of UC relates to various factors, including genetic makeup, environmental factors (diets, smoking, drugs, etc.) and immune disorders [2]. Surgery and various pharmacological interventions are used to treat UC. Common therapeutic agents such as aminosalicylates, immunosuppressants, and corticosteroids have limited efficacy but significant side effects including liver damage, increased blood pressure, nausea, and vomiting [3]. In recent years, polyphenols are suggested as a safe and

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efficient supplementation for the management and mitigation of UC. For example, nobiletin inhibited colitis induced by trinitrobenzene sulfonic acid in rats by lowering inducible nitric oxide syhthase (iNOS) and cyclooxygenase 2 (COX-2) and modulating nuclear factor-kappa B (NF-κB) and myosin light chain kinase (MLCK) pathway [4]. Hesperetinand naringenin can induce tight junction protein expression in cells and positively affect the intestinal barrier function [5,6]. Sinapic acid was shown to up regulate the activity of antioxidant enzymes, maintain the intestinal inflammation, and decrease inflammation in ulcerative colitis [7].

Citrus peel residue could represent one of the major sources of plant polyphenols. With the development of the citrus industry, solid wastes such as seeds and peel residues generated during citrus processing account for about 30%–50% of the total processed fruit. Citrus waste is rich in plant polyphenols. Citrus peel contains four types of polyphenols according to their structure and polarity. The first type is the low-polarity polymethoxylflanones, including nobiletin, tangeretin, sinensetin, etc. The second type is the polar flavanone glycosides, including hesperedin, narirutin, quercetin and naringin. The third type is the medium polar flavanones and flavones including, hesperetin, naringenin, and luteolin. The fourth type are the medium polar phenolic acids, including ferulic acid and caffeic acid, etc. Many studies have shown that polyphenols from citrus had anti-inflammatory, antioxidant, and anticancer activities [8–10]. It was suggested that citrus peel extracts are more effective than individual polyphenols in alleviating inflammation due to synergism [11]. However, it is not yet clear whether citrus peel polyphenols affect ulcerative colitis, nor is it known which type of citrus peel polyphenols has better activity for UC alleviation.

Therefore, the purpose of this research was to explore the anti-inflammatory effect of citrus peel polyphenols on C57BL/6J mice with dextran sulfate sodium (DSS)-induced acute colitis and further explore its underlying mechanism. Total citrus peel polyphenols were extracted and fractionated into water soluble and ester soluble polyphenols before they were gavaged to mice. The colitis symptoms and colonic histology were used to assess the preventive and shielded effects of citrus peel polyphenols. The activity of myeloperoxidase, the secretion and gene expression of inflammatory cytokines, NF-κB signaling pathway proteins p65 and IκB-α were measured as consequences to investigate the potential mechanisms of citrus peel polyphenols.

2. Materials and methods

2.1. Chemicals and reagents

Dextran sulfate sodium (DSS, molecular weight 36–50 kDa) was bought from MP Biomedical Corporation (Santa Ana, California, USA). Carboxyl methyl cellulose-Na (CMC-Na) was gained from Shanghai Changguang Enterprise Development Co., Ltd. (Shanghai, China). AIN93G purified rodent diet was acquired from Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd. (Jiangsu, China). Hemoccult Kits, MPO test kits, BCA test kits were received from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies for NF-κB p65, IκB-α and phosphor-IκB-α were obtained from Thermo Scientific Co., Ltd. (Waltham, MA, USA). Trizol reagents were acquired from Invitrogen (Carlsbad, CA, USA). Revert Aid reverse transcription kits, PCR SuperMix, RNase-Free Water were purchased from Thermo Scientific Co., Ltd. (Waltham, MA, USA).

2.2. Extraction and fractionation of citrus peel polyphenols

Citrus peel polyphenols (Qingjian citrus) were extracted using a published method with modification [12]. Briefly, citrus peels of Qingjian cultivar were freeze-dried, crushed, and ultrasonically extracted in 95% ethanol for 45 min. The extracts were concentrated using a rotary evaporator before they were partitioned between water and hexane to remove essential oils. Afterward, the extracts were loaded on a glass column (10 cm × 70 cm) packed with 2 L of preconditioned AB-8 macroporous resin. Then the column was eluted with 10 L of water to remove sugar and other water-soluble impurities after resin absorbed citrus polyphenols. The total citrus peel polyphenols were recovered by eluting the resin column with 8 L of methanol. Part of the total citrus peel polyphenols (TCP) was partitioned between water and ethyl acetate to obtain water-soluble citrus peel polyphenols (WCP) and ester-soluble citrus peel polyphenols (ECP). TCP, WCP, and ECP were concentrated, freeze-dried, and reserved at −80 °C before further analysis.

2.3. Analysis of flavanones, flavones, and phenolic acids

Flavanones and phenolic acids were analyzed by high performance liquid chromatography (HPLC) equipped with two pumps, an automatic sampler and an ultraviolet detector (Dionex, Sunnyvale, California, USA). For flavanones, TCP, WCP or ECP were extracted twice in ethyl acetate, re-dissolved in 65% methanol, and filtered before injection. The extracts were separated on a Venusil MP C18 column (4.6 mm × 250 mm, 5 μm). 1% formic acid aqueous solution (phase A) and acetonitrile (phase B) were used as the mobile phases. The gradient elution consisted of 22–55% B in 0–25 min, 55–100% B in 25–38 min, 100% B in38–48 min, and 100-22% in 48–60 min. The wavelength of detection was 283 nm and 330 nm and the flow rate was 1.0 mL/min. For phenolic acids, citrus peel extracts were hydrolyzed in 8 mol/L NaOH at 25 °C for 1 h. The solution pH was coordinated to 1–2 by appending HCl after hydrolysis. The acidified solution was extracted half-volume of ethyl acetate followed by half-volume of diethyl ether. Ethyl acetate and diethyl ether were combined and concentrated on a rotary evaporator. Concentrate was dissolved in 80% methanol and filtered before injection. The mobile phase for elution included 0.5% formic acid aqueous solution (phase A) and methanol (phase B). The gradient elution consisted 20% B in 0–22 min, 20–70% B in 22–52 min, 70–100% B in 52–60 min, 100-20% B in 60–66 min. The wavelength of detection was 260 nm and 320 nm. The flow rate was 1.0 mL/min.

2.4. Mouse model of acute colitis

Forty 7-week-old male C57BL/6J mice were acclimated to the experimental condition for a week on AIN93G diet. The mice were maintained in temperature (23 ± 2 °C) and photoperiod cycle (12 h light and 12 h dark) during the experiment. After the acclimation, the mice were grouped into five groups with eight mice per group at random. Healthy control group mice were fed with AIN93G diet and ultrapure water for 14 days. DSS group mice were received AIN93G diet and ultrapure water for the first 7 days before DSS (2%, w/v) in drinking water to induced acute colitis in the last 7 days. From the first day of the experiment, TCP, WCP and ECP were used for preventive treatment. Mice in TCP + DSS group received the same feeding scheme as DSS group and were gavaged with TCP (300 mg/kg of mice weight) for whole 14 days. Mice in WCP + DSS group received the same feeding scheme as DSS group and were gavaged with WCP (300 mg/kg of mice weight) for whole 14 days. Mice in ECP + DSS group received the same feeding scheme as DSS group and were gavaged with ECP (300 mg/kg of mice weight) for whole 14 days. All procedures were conducted respecting the ethical precepts of experimental animals and the study was approved by the Commission for the Laboratory Animal Ethics Committee of Southwest University (IACUC-201909-03).

2.5. Disease activity index

The disease activity index was defined as disease activity index (DAI) using a published method [13].

2.6. Histological and pathological analysis of colon

The colon was explanted from mice, and the colon weight and length were determined. A part of colon was fixed with 10% paraformaldehyde for 24 h, then stained using hematoxylin and eosin (H&E). The scores for histological damage of HE-stained colon sections are assessed using a previous histopathological scoring standard [13].

2.7. Determination of myeloperoxidase activity

Colons were homogenized in 0.9% NaCl solution before centrifugation at 10,000 g at 4 °C for 10 min to gather the supernatant. The myeloperoxidase (MPO) activity of the colon mucosa was measured utilizing a kit following the manufacturer's manuals.

2.8. Measurement of IL-6, TNF-α and IL-10 in colon and serum

The secretions of IL-6, TNF-α and IL-10 in colon tissue and serum were measured by utilizing ELISA kits (Shanghai Enzymelink Biotechnology Co., Ltd. Shanghai, China).

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The total RNA was extracted from the colon tissue with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Further, the RNA extract reverse transcripted into cDNA by utilizing RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). A 20 μL reverse transcription reaction mixture consisted of RNA (1 μL), primer dT (1 μL), water (10 μL), Ribolock RNase inhibitor (1 μL), reaction buffer (4 μL), Revert Aid M-muLV (1 μL) and 10 mM dNTP mix (2 μL). The cDNA was used for relative quantification of various gens in the NF-κB pathway such as IL-6, TNF-α, IL-8 and NF-κB p65. Moreover, the real-time PCR (RT-PCR) was conducted utilizing SYBR Green PCR Master Mix on a 7500 Real-time PCR System (Applied Biosystems, Foster city, California, USA). The cycle program was set to pre-denaturation at 95 °C for 10 min once, and at 95 °C for 15 s and at 60 °C for 60 s for 40 cycles. The semi-quantitative calculations were carried out according to formula 2-ΔΔCt using β-actin as an internal reference. The primer sequence sets used are listed in Supplemental Table 1S.

2.10. Western blot analysis

Colon was crushed in radioimmunoprecipitation (RIPA) assay lysis buffer containing protease and phosphatase inhibitors. After centrifugation (12,000 rpm at 4 °C, 5 min), the supernatant was gathered to measure protein concentration utilizing BCA kit (Servicebio, Wuhan, China). Next, 30 μg proteins were loaded in SDS-PAGE gel wells and transferred to PDVF membrane by transfer buffer. After blocked with 5% skimmed milk powder in tris-buffered saline with Tween 20 (TBST), the PDVF membranes were incubated with the mouse monoclonal antibodies (NF-κB p65, *p*-IKB-α, IKB-α, 1:1000) diluted in TBST solution at 4 °C overnight. The membrane was cleaned for 3 times with TBST, then, incubated with the secondary antibody bound to Horseradish Peroxidase (HRP) at 1: 1000 dilution in TBST solution at room temperature for 1 h. After washing with TBST for 5 times, the proteins in the membrane were displayed by ECL Western Blotting Substrate (Thermo Fisher, Waltham, MA, USA) and developed with a Tano Luminescent Imaging Workstation (Shanghai Tanon Technology CO., Ltd, Shanghai, China). The relative expression of the target proteins was figured through comparing with the β-actin as an internal reference.

2.11. Statistical analysis

The SPSS software (SPSS.19.0. SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) with Ducan's multiple range tests were used to compare mice in five groups. A value of $P < 0.05$ reveals a significant difference between the groups.

3. Results and discussion

3.1. Phenolic composition in TCP, WCP, and ECP

TCP contained all four types of polyphenols (Table 1). After partition between water and ethyl acetate, all polymethoxylflavanones were extracted in the ethyl acetate phase. Their concentration in ECP was 5-6-fold of those in TCP, whereas they were undetectable in WCP. This can be explained by the low polarity of polymethoxylated flavones [14].

Hesperidin is the major flavanone glycoside in TCP and has much high polarity than polymethoxylated flavones. Its concentration in ECP was more than WCP and both contained less hesperidin than TCP. However, the opposite was seen for narirutin, because its content in TCP was lower than WCP and ECP. This is because the hesperidin contained an additional methoxy group on its B ring compared to narirutin. As a result, hesperidin is slightly less polar than narirutin [15]. Dydimin's content in ECP was significantly higher than TCP and ECP. The content of naringin has a similar distribution in the three citrus peel polyphenols to dydimin. Contrary to dydimin, WCP contained greater level of eriocitrin than TCP and ECP.

The polarity of flavanones and flavonoids including hesperetin, naringenin and lutelin is between that of polymethoxylated flavones and flavanone glycosides. They were extracted in the ethyl acetate phase and were concentrated in ECP, This is likely due to ethyl acetate has a stronger solubility for this type of polyphenols [16]. The partition behavior of phenolic acids was comparable to flavanone glycosides due to similar polarity. Ferulic acid and sinapic acid were the primary bound phenolic acid in citrus peels. The content of ferulic acid and sinapic acid in the TCP is only 12.4 mg/g and 6.3 mg/g. After extraction in the ethyl acetate phase, the ECP concentration reached 14.5 mg/g and 9.21 mg/g. Hydroxybenzoic acid such as gallic acid is basically absent in this type of citrus fruits, which partially depends on the citrus variety [17].

The total flavonoids content of ECP (143.3 mg/g) was significantly higher than TCP (47.0 mg/g) and ECP (27.2 mg/g). Moreover, ECP had significantly higher total phenolic acids content (30.8 mg/g) than TCP (24.0 mg/g) and WCP (15.2 mg/g). The phenolic acids have a lipophilic benzene ring with a size much larger than the hydrophilic hydroxyl groups. Hydroxyls and carboxyl on phenolic acid tends to form hydrogen bonds which further decrease their polarity. As a result, phenolic acid compounds are more soluble in the organic phase. This partially explained the significantly higher contents of phenolic acids in the ethyl acetate phase than those in the water phase [18].

Table 1
The polyphenol content in TCP, WCP, and ECP.

Polyphenols	TCP (mg/g dry weight)	WCP (mg/g dry weight)	ECP (mg/g dry weight)
	Polymethoxylated Flavones		
Sinensetin	7.08 ± 0.46^{b}	nd	41.5 ± 0.27^{a}
Nobiletin	5.65 ± 0.55^{b}	nd	34.2 ± 2.34^{a}
		Flavanone glycosides	
Hesperidin	14.8 ± 6.35^{a}	5.61 ± 1.28^{b}	11.0 ± 0.85^{a}
Narirutin	6.53 ± 0.84^{c}	15.9 ± 2.54^{a}	9.73 ± 0.64 b
Didymin	4.92 ± 0.82^{b}	3.19 ± 0.59^{b}	14.0 ± 1.36^{a}
Naringin	2.04 ± 0.50^{b}	nd	10.6 ± 1.64^{a}
Eriocitrin	1.63 ± 0.05^{b}	1.80 ± 0.11^{a}	nd
	Flavanones and flavone		
Hesperetin	3.32 ± 0.95^{b}	0.66 ± 0.07^{c}	15.1 ± 0.75^{a}
Naringenin	0.82 ± 0.08^{b}	nd	4.40 ± 1.25^{a}
Luteolin	0.16 ± 0.02^{b}	nd	2.66 ± 0.76^{a}
		Phenolic acids	
Ferulic acid	12.4 ± 0.92^{a}	5.16 ± 0.37^{b}	14.5 ± 2.76^{a}
Sinapic acid	6.33 ± 0.19^{b}	5.68 ± 0.66^{b}	9.21 ± 0.94^{a}
Coumaric acid	2.65 ± 0.20^{a}	1.67 ± 0.14^{b}	2.84 ± 0.52^{a}
Chlorogenic acid	2.44 ± 0.34^{b}	2.70 ± 0.21^{b}	4.02 ± 0.94^{a}
Caffeic acid	0.17 ± 0.01^{b}	nd	0.29 ± 0.08^{a}
Gallic acid	nd	nd	nd
	Total flavonoids and phenolic acids		
Total flavonoids	47.0 ± 8.97^{b}	27.2 ± 3.76^{c}	143.3 ± 8.00^{a}
Total phenolic acids	24.0 ± 1.53^{b}	15.2 ± 1.25^{c}	30.8 ± 5.00^{a}

Note: Data are mean ± standard deviation for triplicate tests; Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$) in the same row. nd, not detected.

3.2. TCP, WCP or ECP alleviate symptoms of DSS-induced acute colitis

The symptoms, including weight loss, thick stool, and bleeding, are common hallmarks of UC [19]. Healthy mice maintained normal weight gain. There was a drastic drop of mice weight in the DSS colitis group on day 5–7 due to diarrhea of mice. Adding TCP, WCP or ECP in diet significant prevented the body weight loss of colitis mice (Fig. 1A). Mice in healthy control showed no symptoms of colitis. DAI in mice of the DSS group continued to progress to reach 8 at day 7. Major symptoms of colitis mice were diarrhea and rectal bleeding. Supplementing citrus peel polyphenols significantly decreased DAI in colitis mice (Fig. 1B). Several studies suggested that polymethoxylated flavones were more effective than other flavanones in decrease inflammation and against cancer [20–22]. ECP has the highest polymethoxylated flavones content, about 53%, and the rest are other flavanones and phenolic acids, while WCP has the lowest polymethoxylated flavones content. However, ECP, WCP, and TCP appeared equally effective in alleviating the symptoms of acute UC. A possible reason is that all these polyphenols including flavanone glycosides, flavanones and flavonoids and phenolic acids in citrus peel extracts both contributed to the anti-inflammatory effect of citrus peel polyphenols extracts [23]. Another possible reason for the similar activity among ECP, WCP, and TCP was the high dose used in the study. In previous studies, 5–100 mg/kg citrus extracts or individual phenol were administered to prevent or treat UC in animal colitis models [24].

3.3. TCP, WCP or ECP attenuated histological damage of colon tissues

Healthy mice showed normal colon length (Fig. 2A). Compared to mice in healthy control group, mice in DSS group were significantly shortened and decreased ($P < 0.05$). This was because progression of inflammation induced colon to swell and shorten [19]. The TCP significantly preserved the colon weight and length. WCP and ECP preserved the length of the colon but did not affect

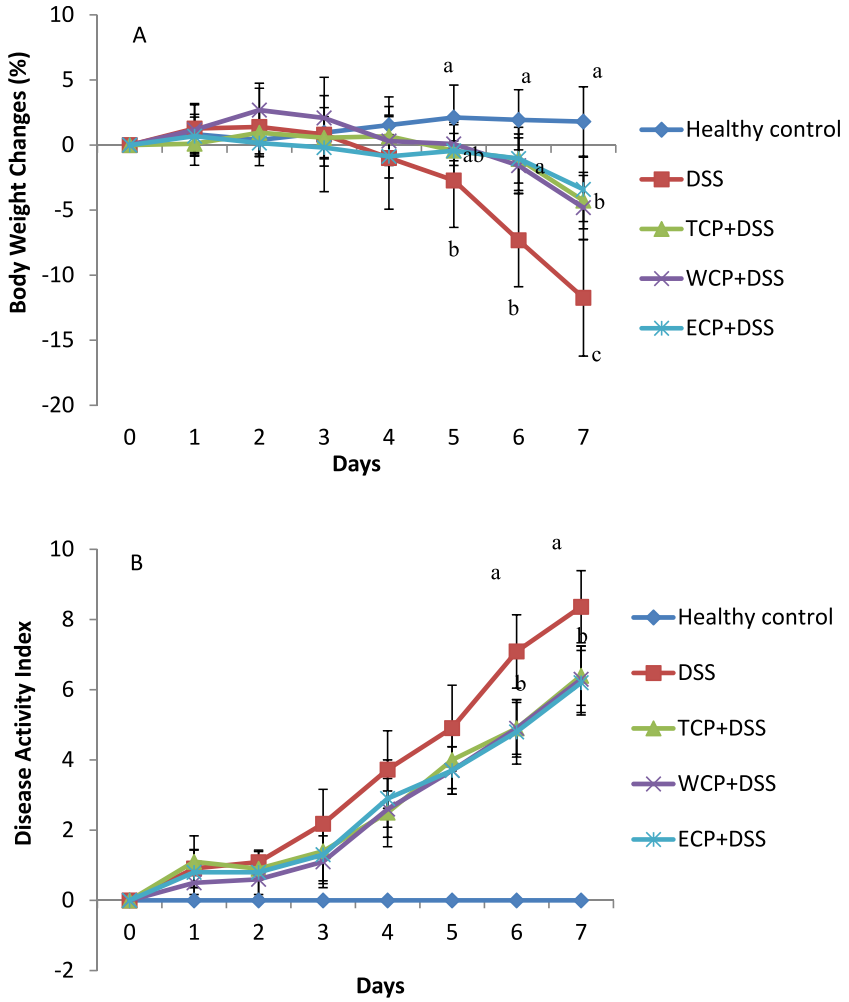

Fig. 1. TCP, WCP, and ECP supplementation reduced body weight loss (A) and symptoms reduction (B) of DSS-induced colitis in mice in the recent 7 days. Results are the mean ± standard deviation for 8 mice per group. Different letters (a, b, c) indicate significant differences among groups ($P < 0.05$).

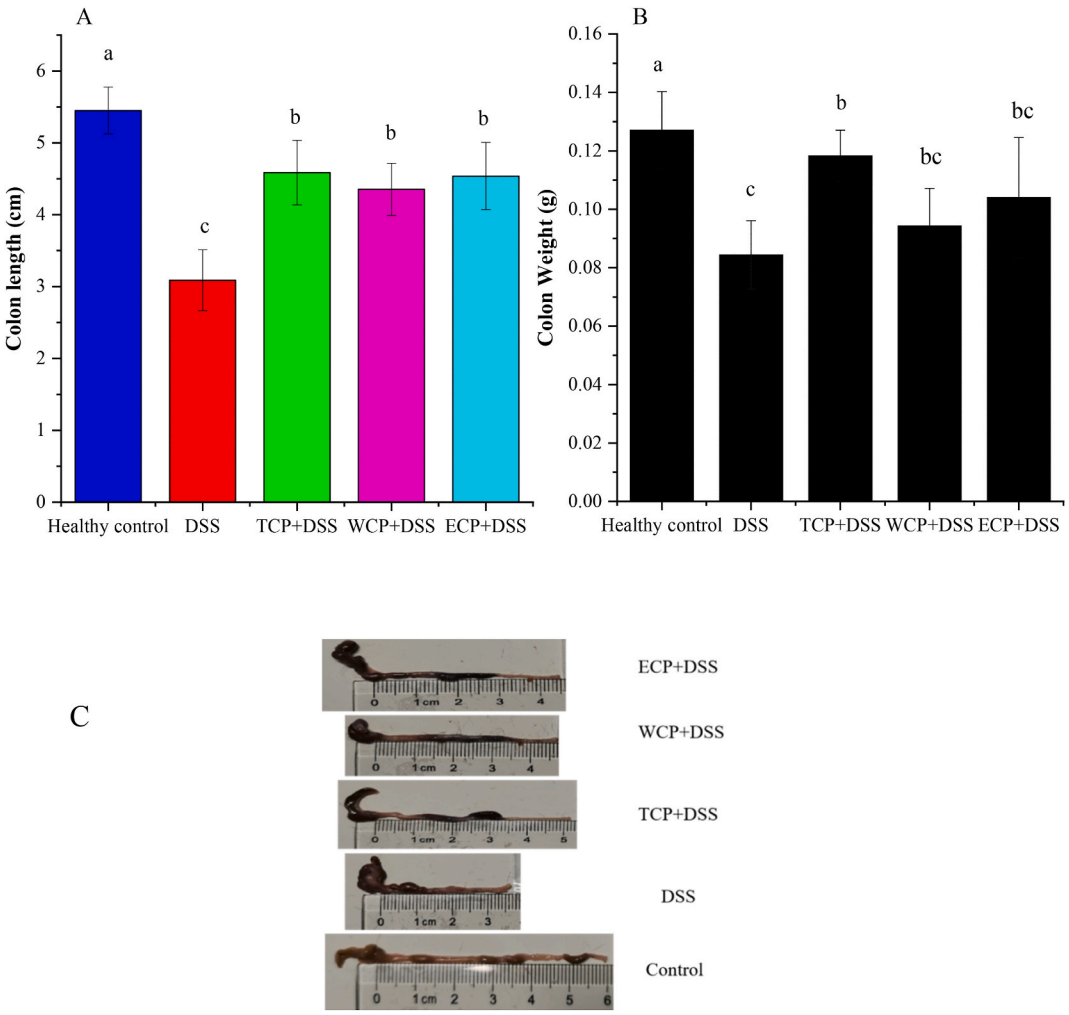

Fig. 2. Citrus polyphenols increased the length (A, C) and weight (B) of colon in colitis mice. Bars are mean ± standard deviation for 8 mice per group. Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$).

colon weight (Fig. 2A–C).

The severity of colitis was further assessed by colonic histology. The healthy control mice had a healthy colonic architecture (Fig. 3A). DSS induced serious damage to the colon tissue, include damaging to the intestinal epithelial mucosa and decreasing in the amount of necrotic epithelial cells, neutrophils, crypts and inflammatory cells in the intestinal lumen (Fig. 3A). In the TCP + DSS, WCP + DSS, ECP + DSS experimental groups, the degree of intestinal epithelial edema and inflammation was declined. The degree of inflammation was reduced with significantly less neutrophil infiltration (Fig. 3A). Fig. 3B shows the histological score of colonic pathological damage. The mice gavaged with TCP, WCP or ECP had lower histological damage scores of colon mucosa compared with the DSS group ($P < 0.05$). TCP, WCP, and ECP appeared equally effective.

3.4. TCP, WCP or ECP reduced MPO activity

MPO is a heme protease mainly secreted by neutrophils. Its activity indirectly reflects the degree of neutrophil infiltration in the tissue [25]. In colon with ulcerated colitis, a large number of neutrophils release MPO outside the cell or in the phagosome during the migration into the colon cavity, thereby damage the intestinal mucosal [26]. An elevated activity of MPO in the colon indicates that neutrophils inflow into the colon tissue, and a reduced MPO activity is a manifestation of decreased neutrophil aggregation in the inflamed tissue. As shown in Fig. 4, compared to healthy control group, DSS remarkably enhanced the MPO activity to 6.96 U/g ($P < 0.05$). Compared to DSS group, the experimental groups gavaged with TCP (3.27 U/g), WCP (3.86 U/g), or ECP (3.62 U/g) significantly decrease MPO activity in the colon. Results showed that TCP, WCP or ECP extracts suppressed the neutrophil cells infiltration into the damaged colon tissue.

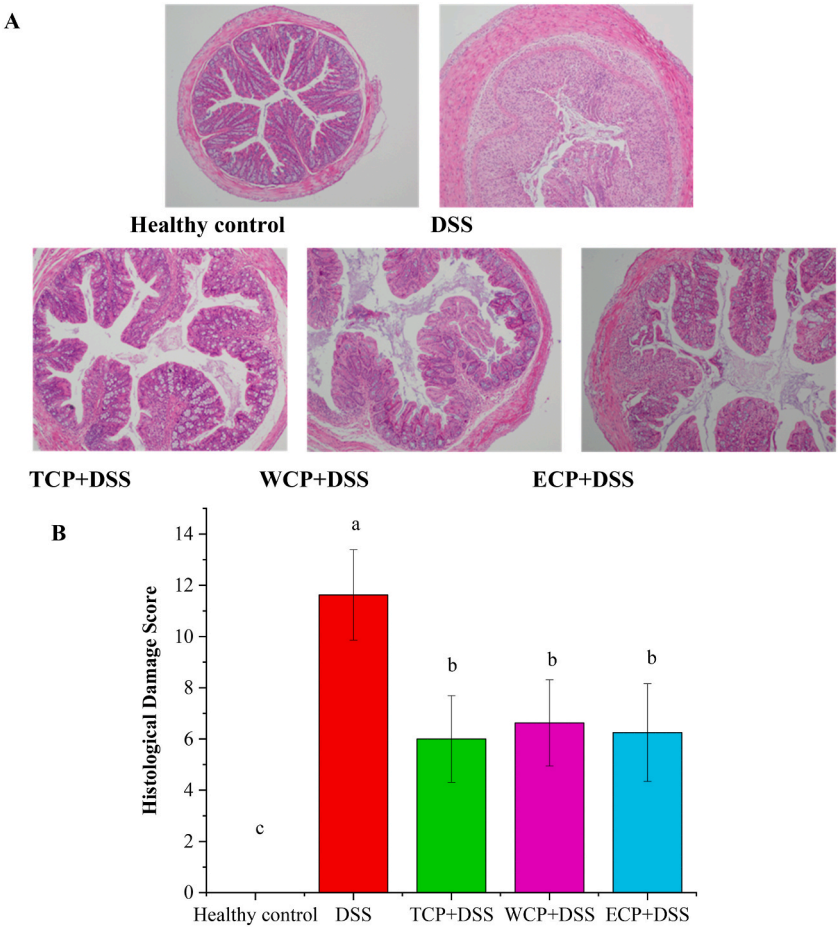

Fig. 3. Representative histopathological image (magnification: 40 ×) of H&E stained colons (A) and the histological damage score of colons (B). Bars are mean ± standard deviation (n = 8 mice per group). Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$).

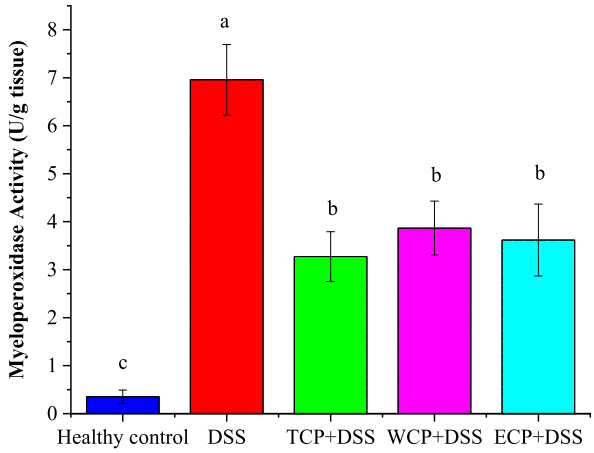

Fig. 4. TCP, WCP and ECP decreased myeloperoxidase activity in colitis mice. Data are mean ± standard deviation for 8 mice per group. Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$).

3.5. TCP, WCP or ECP suppressed overproduction of inflammatory cytokines in colitis mice

Inflammatory cytokines such as IL-6 and TNF-α play significant roles in ulcerative colitis disease. TNF-α is mainly excreted by mononuclear macrophage. It mediates a variety of inflammatory reactions, such as the activation of the coagulation-fibrinolysis system, and recruitment of inflammatory sites [27]. IL-6 is a multifunctional pro-inflammatory cytokines, which plays a profound role in monocytes and promotes T-cells proliferation and B-cells differentiation and replication. It accelerates intestinal inflammation from acute to chronic [28]. IL-10 is mainly produced by Th2 cells and monocyte macrophages. As an immunosuppressive cytokine, IL-10 inhibits inflammation via multiple mechanisms including blocking the secretion of pro-inflammatory cytokines and decreasing the immune cells activation. IL-10 down-regulates the secretion of COX-2 and iNOS, and reduces prostaglandin E2 (PGE2) levels [29].

Therefore, the inflammatory biomarkers including IL-6, IL-10 and TNF-α, were determined in the serum and colon tissue to investigate the preventive effects of TCP, WCP or ECP on inflammatory bowel disease. DSS damages the mucosal membrane in the intestine and stimulates macrophages on the intestinal surface, thereby promote IL-6 and TNF-α secretion. Hence, in contrast to the healthy control group, DSS remarkably increased ($P < 0.05$) the expression of TNF-α and IL-6 in mice serum and colon tissue (Table 2, Table 3). TCP, WCP or ECP remarkably suppressed the secretion of TNF-α and IL-6 (Table 2, Table 3). There were no differences in reduction of pro-inflammatory cytokines level among three citrus extracts. The level of IL-10 in the serum and colon tissue in DSS group mice was higher than that of healthy control mice ($P < 0.05$). After TCP, WCP, and ECP treatments, the IL-10 secretion level was significantly increased (Table 2, Table 3). These consequences indicated that the three extracts of citrus peel inhibited the secretion of pro-inflammatory cytokines induced by DSS, increased the secretion of anti-inflammatory cytokines, and suppressed the inflammatory response. These observations agreed with previous findings about preventative and anti-inflammatory actions of polyphenols from fruits and vegetables in IBD animal models [30].

3.6. TCP, WCP or ECP inhibited the mRNA expression of inflammatory cytokines

To investigate the mechanism of TCP, WCP or ECP anti-inflammatory effect, the expression of IL-6, TNF-α, IL-8, NF-κB p65 mRNA in the colon tissue was determined. Compared with the healthy mice, the relative expressions of IL-6 (Fig. 5A), IL-8 (Fig. 5B), TNF-α (Fig. 5C), NF-κB p65 (Fig. 5D) mRNA in the colon tissue in DSS group mice were remarkably increased ($P < 0.05$). The mice gavaged with TCP, WCP or ECP had decreased mRNA expression for IL-6 (Fig. 5A), IL-8 (Fig. 5B), TNF-α (Fig. 5C), NF-κB p65 (Fig. 5D) compared to mice in DSS group. These consequences showed that citrus polyphenol extracts decreased the content of IL-6, TNF-α and IL-8 in colon tissue and serum by down-regulation the gene expression of inflammatory cytokines IL-6, TNF-α and IL-8.

3.7. TCP, WCP, and ECP reduced inflammation by regulating the NF-κB pathway

The NF-κB pathway is the significant signal pathway that induces the pathogenesis of UC. The NF-κB is made up of RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) [31]. NF-κB plays a profound role in conserving intestinal barrier function, intestinal homeostasis, and modulating the secretion of cellular inflammatory mediators [32]. Toll like receptors 4 (TLR4) and mitogen-activated protein kinases (MAPK) pathways were the upstream signaling pathway. TLR4 non-specifically recognize and bind pathogen-related molecular patterns, MAPK regulates immune response and promote inflammation, thereby activating NF-κB-related protein channels [33]. In addition, there are many extracellular stimulating factors such as IL-1, TNF-α, T cell receptor, Toll-like receptor can activate the NF-κB pathway. After NF-κB is stimulated by the environment and activated, IKB-α protein is phosphorylated. This leads to the release and transport of p65 from cytosol to the karyon. Then the p65 binding to specific genes leads to the transcriptions of cytokines and attachment molecules, which further promote the secretion of inflammatory cytokines, inflammatory proteins iNOS and COX-2 in intestinal mucosal cells, thereby inducing the intestinal inflammatory responses [34].

To determine whether TCP, WCP or ECP extracts have an effect on the DSS-mediated colitis via NF-κB pathway, the p65 (Fig. 6D), the IKB (Fig. 6B) and the phosphorylation of IKB (Fig. 6C) protein were measured. The outcomes indicated (Fig. 6A) that DSS induced phosphorylated IKB and p65 and triggered IKB degradation, while TCP, ECP or WCP significantly suppressed the expression of NF-κB p65 and *p*-IκB-α. These results suggested that the activation of the NF-κB pathway was inhibited, thereby decreasing the secretion of pro-inflammatory cytokines. It showed that citrus peel polyphenols extracts down-regulated the NF-κB pathway activation, and suggested possible impacts on its down-stream pathways such as signal transducer and activator of transcription (STAT) pathway, TLR4 pathway and NLRP3. Several previous researches have shown that polyphenols regulate the NF-κB pathway in intestinal

Table 2
The secretion of IL-6, TNF-α, and IL-10 in serum of C57BL/6J mice.

Treatment groups	IL-6 (pg/mL)	TNF-α (ng/L)	IL-10 (pg/mL)
Healthy control	146.3 ± 28.6^{c}	64.6 ± 4.9^{c}	324.8 ± 23.7^{c}
DSS	676.8 ± 101.6^{a}	257.4 ± 10.2^{a}	483.6 ± 64.6^{b}
TCP + DSS	287.9 ± 29.7^{b}	147.0 ± 18.9^{b}	889.3 ± 254.5^{a}
WCP + DSS	303.7 ± 29.7^{b}	136.4 ± 23.4^{b}	935.4 ± 179.5^{a}
ECP + DSS	297.5 ± 52.1^{b}	156.3 ± 23.2^{b}	876.4 ± 142.3^{a}

Note: Data are mean ± standard deviation for 8 mice per group. Different letters (a, b, c) indicate significant differences among groups in the same column ($P < 0.05$).

Table 3
The secretion of IL-6, TNF-α, and IL-10 in the colon tissue of C57BL/6J mice.

Treatment group	IL-6 (ng/g)	TNF-α (ng/g)	IL-10 (ng/g)
Healthy control	0.56 ± 0.11^{c}	4.64 ± 0.22^{d}	3.65 ± 1.93^{c}
DSS	2.06 ± 0.20^{a}	13.5 ± 0.59^{a}	7.46 ± 0.37^{b}
TCP + DSS	1.23 ± 0.34^{b}	9.34 ± 0.47^{b}	11.1 ± 1.41^{a}
WCP + DSS	1.60 ± 0.07^{b}	10.1 ± 0.61^{bc}	12.7 ± 1.97^{a}
ECP + DSS	1.33 ± 0.08^{b}	10.5 ± 0.86^{c}	13.5 ± 3.67^{a}

Note: Data are mean ± standard deviation for 8 mice per group. Different letters (a, b, c) indicate significant differences among groups in the same column ($P < 0.05$).

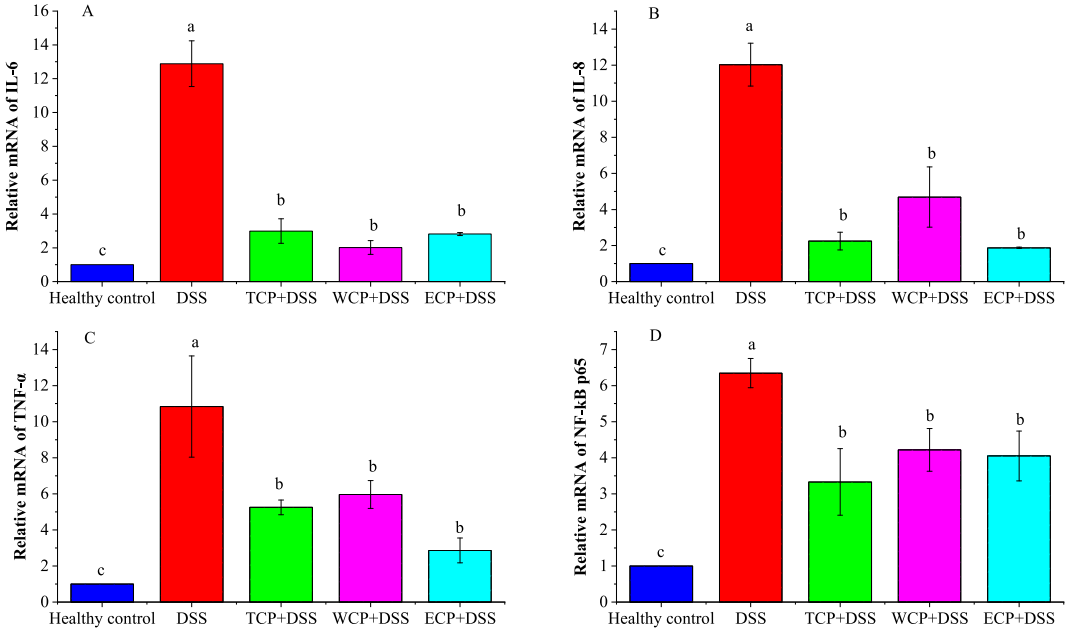

Fig. 5. TCP, WCP, and ECP significantly decrease the mRNA expression of IL-6 (A), IL-8 (B), TNF-α (C), and NF-κB p65 (D) in the colon of colitis mice. Data are mean ± standard deviation (n = 8 mice per group). Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$).

inflammation. For example, citrus extracts such as orange juice extracts and Yuzu extracts hindered NF-κB activation in mice [35,36]. Individual phenolic acids such as caffeic acids, ellagic, and vanillic alleviated colitis symptoms via the reduction of NF-κB activation [37]. Flavonoids of different polarity such as luteolin, naringenin and hesperidin restrained NF-κB activation through reducing the phosphorylation of p65 and IκB-α in animal models or cell models [38]. TCP, WCP or ECP were equally effective to alleviate intestinal inflammation in DSS-induced mice and anti-inflammatory activity of each extract was likely due to a combined action of multiple bioactive polyphenols and high dose TCP, WCP and ECP used in the study. Moreover, in addition to NF-κB, other studies suggested citrus polyphenols affect multiple other inflammation related pathways. For example, citrus polyphenols decreased inflammatory response by reducing oxidative stress [39], inducing the Nrf2 signaling pathway [40], stimulating STAT pathway [41], protecting gut barrier [42], and regulating gut microbiota [43]. Altogether, anti-inflammatory activities of citrus peel polyphenols resulted from the collective effects of various types of phenolic compounds and their influences on multiple biological pathways.

4. Conclusions

This research showed that total citrus polyphenols, water-soluble citrus polyphenols or ester-soluble citrus polyphenols mitigated DSS-induced acute ulcerative colitis in C57BL/6J mice. These citrus polyphenols effectively reduced colon mucosa damages and the secretion of inflammatory cytokines. The anti-inflammatory activity of citrus polyphenols was potentially related to their capacity to inhibit NF-κB activation. Therefore, the bioactive polyphenols from citrus may be a safety and valid supplementation in preventing or managing inflammatory bowel diseases. This study only examined the effects of TCP, WCP or ECP on an animal model, thus further clinical studies are demanded to test their efficacy in human.

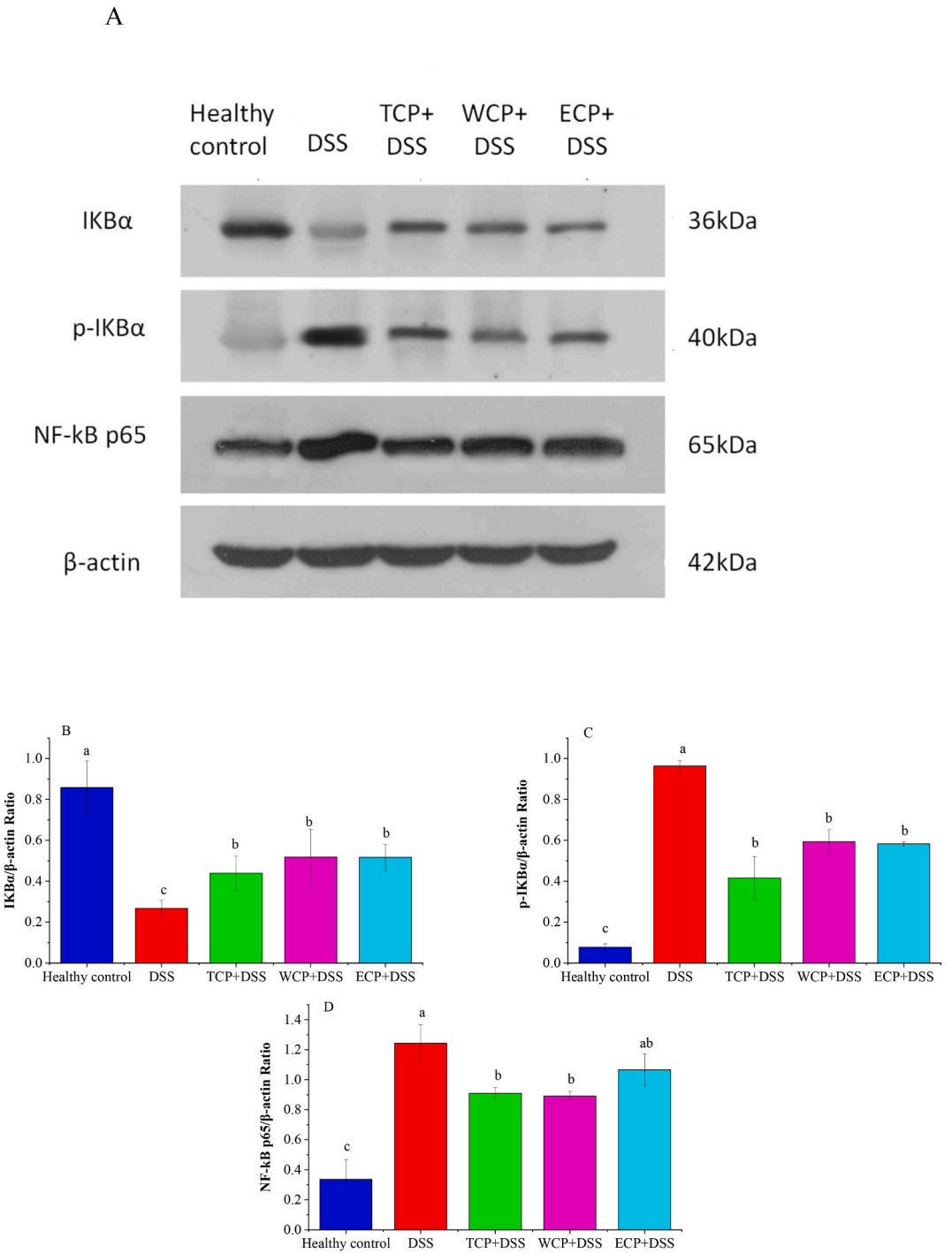

Fig. 6. The effect of TCP, WCP and ECP on NF-κB signaling pathway in the colon tissue of colitis mice. A: Band diagram of IκBα, *p*-IκBα and p65 determined by Western blot; B: the relative content of *p*-IκBα; C: the relative content of IκB-α; D: the relative content of NF-κB p65.

Author contribution statement

Yajing He: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Zhigao Sun: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. JunYing Bai: Contributed reagents, materials, analysis tools or data; Wrote the paper. Yu Zhang, Yu Qian, Xin Zhao: Conceived and designed the experiments; Contributed

reagents, materials, analysis tools or data. Shanshan Chen: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Data availability statement: Data included in article/supp. material/referenced in article. Declaration of interest's statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement

All procedures were conducted respecting the ethical precepts of experimental animals and the study was approved by the Commission for the Laboratory Animal Ethics Committee of Southwest University (IACUC-201909-03).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18137.

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