ORIGINAL ARTICLE

The ameliorating potential of Citrus aurantifolia peel extract in the 2, 4, 6-trinitrobenzenesulfonic acid model of mice colitis

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ABSTRACT

Introduction: The number of inflammatory bowel diseases cases has increased throughout the years. Since, the current therapeutic methods have their adverse effects, this is leading to the development of alternative therapy derived from natural products.

Materials and Methods: In the present study, our objective was to explore the potential of Citrus aurantifolia peel extract (CAPE) on 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) induced colitis in mice. Twenty-eight male Balb/c mice were divided into four groups: (1) normal group, (2) TNBS group, (3) 125 mg/kg CAPE group and (4) 250 mg/kg CAPE group. Colitis was induced through rectal administration of TNBS. The anti-inflammatory effects of CAPE against colitis were assessed by body weight, DAI score, colonic length, weight-to-length ratio, haematology profile and histopathological examinations.

Results: Our results showed that CAPE maintained the body weight of mice, repressed the increase of DAI score, maintained mice colonic length and weight, improved blood profile and suppressed the excessive production of TNF-α, IL-6 and IL-1β. Furthermore, CAPE improved the histopathological score of colitis mice.

Conclusion: All the findings of this study suggested that Citrus aurantifolia peel extract may be a potential natural agent for protecting mice against TNBS-induced colitis.

KEYWORDS:
Citrus aurantifolia peel extract, phenolic compound, colitis, TNBS, anti-inflammatory, mice

INTRODUCTION

Inflammatory bowel diseases (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a chronic remitting and relapsing inflammation that affects the gastrointestinal tract. The symptoms include acute abdominal pain, diarrhoea, mucosal inflammation of the colon and rectum, tissue damage, bloody stools, mucosa and pus.[1] Though the cause of IBD remains unknown, it is believed that IBD is associated with genetic and environmental abnormalities that cause the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, leading to uncontrolled mucosa inflammation.[2,3]

According to the epidemiological study from Asia, the Asia-Pacific Crohn's and Colitis Epidemiologic Study (ACCESS), IBD incidence has increased in number specifically in Asian countries including Indonesia. From 2011 to 2013, the corresponding incidence rate was 1.50 per 100,000 of IBD, and the number was predicted to grow over the year.[4] Current medications for IBD have been palliative therapies, such as anti-inflammatory drugs, immunosuppressants and corticosteroids. However, functional food has currently become a healthy alternative to maintain the disease progression and it is derived from natural products. Epidemiological evidence shows that regular consumption of functional foods, value-added food products, and nutraceuticals is associated with a lowered risk of some chronic disease progression, including ulcerative colitis.[5]

Prior studies reported that natural bioactive compounds, such as phenolic, have anti-inflammation and antioxidant properties.[6] *Citrus aurantifolia* is a medicinal plant native to Southeast Asia and often used in various traditional medicine. It is one of the bitter orange species which possesses higher flavonoid and phenolic content compared to other bitter orange species such as grapefruit and lemon.[7] The fruits are extremely rich in phenolic compounds, but the peel contains an abundance of bioactive compounds that demonstrated potent anti-inflammatory activities. Well-established assays have also demonstrated that *Citrus aurantifolia* peel acts as a very powerful antioxidant.[8] However, the effect of *Citrus aurantifolia* peel on colitis has yet to be reported.

The study aimed to investigate the ameliorating effect of *Citrus aurantifolia* peel extract (CAPE) in a colitis mice model. The experimental colitis was induced by TNBS prior to treatment with CAPE. The anti-inflammatory effect was evaluated by DAI scores, histopathological observations, and determination of inflammation markers, including IL-1β, TNF-α and IL-6.

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MATERIALS AND METHODS

Citrus Aurantifolia Peel Extraction

Citrus aurantifolia were obtained from Colombo Market, Yogyakarta. The fruit was fully ripened, with green peel and 4 to 5 cm in diameter. Peels were manually separated using knife. The peel was freeze-dried at -48°C for 36 hours until the moisture content reached 10% and ground to obtain a Citrus aurantifolia peel powder (CAPP). CAPP was extracted using ultrasound-assisted extraction using 70% ethanol with the ratio of 1:30 (w/v) to yield CAPE. The extract was then concentrated under vacuum condition using rotary evaporator at 110 mbar for 2 hours and then suspended in 5% Na-CMC.

In-vivo experimental design and colitis induction

This study was approved by the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada (certificate number 00006/04/LPPT/IV/2022) and was carried out in accordance with the guidelines for the care and use of laboratory animals. A total of 28 adult male BALB/c mice (initial weight 30–40 g) were acclimated for 7 days to adapt to a new environment in a communal cage, under a controlled condition of 22–25°C with RH of 60–65%, AIN-93M feed and water available ad libitum. After acclimated, 28 mice were randomly divided into four groups with seven mice each. The groups were as follow: normal group (I), TNBS group (II), low dose CAPE group (III) and high dose CAPE group (IV). CAPE suspension in 0.5% Na-CMC was given at a volume of 10 ml/kg body weight. The CAPE dosage was determined according to the conversion of hesperidin dietary intake for human, resulting in 125 mg CAPE/kg body weight for low dosage and 250 mg CAPE/kg body weight for high dosage.[9,10] The normal group and TNBS group received water solution while CAPE groups received CAPE suspension once every day from day 1 to 7 days. The mice have then fasted for 24 hours after CAPE gavage.

On day 8, 100 mg/kg TNBS (Sigma Aldrich, USA) was used to induce acute colitis through enema. Mice were anesthetised using diethyl ether and a catheter (polyethylene, 1 mm diameter fixed on 1 ml syringe) was inserted into the anus about 3.5 cm deep, before 100 µl TNBS was injected into the colon. After the catheter was pulled out, mice were positioned vertically for 2 min. Throughout colitis period, all groups received the same treatment as pretreatment. Body weight, diarrhoea incidence, bloody stool and food intake were recorded daily. On day 18, mice were all sacrificed, and the colon was excised for further analysis.

Disease Activity Index

The severity of colitis was assessed daily in the 9 days after the induction of colitis using the disease activity index (DAI). DAI score was calculated as the sum of the damage scores for the criteria body weight loss, stool consistency, and faecal bleeding, in accordance with Liu et al.[11] The weight loss following induction of colitis was calculated relative to the initial weight of each animal prior to induction.

Blood Sampling Collection

Blood was collected through sinus orbitalis according to method by Parasuraman et al.[12] A capillary tube was inserted into the medical canthus of the eye (30° angle to the nose). Slight thumb pressure was applied to puncture the plexus/sinus so the blood will come through the capillary tube. 5 mL blood was then collected in heparin tube to be analysed immediately for haematology profile using haematology analyser KX-21 (Sysmex Corporation, Kobe, Japan).

Tissue collection and Biochemical Analysis

The colonic tissues were cut and then homogenised in phosphate buffered solution (1:9 [w/v]). The homogenised samples were subjected to centrifugation (5000 g) for 5 minutes. The cytokine levels of TNF-α, IL-6 and IL-1β were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (FineTest).[13]

Histological Analysis

The colonic tissues were embedded in paraffin, cut at 5 mm, then mounted on clean glass slides. After the slicing was deparaffinised and rehydrated, the tissue was dyed with haematoxylin and eosin (H&E). All colon specimens were examined with an Olympus CX23 microscope to evaluate the ameliorating effect of CAPE on TNBS-mice colonic tissues. Histological gradings were assessed based on the criteria as reported by Bonfiglio et al.[14]

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 23. The statistical significance of any difference in each parameter among groups was evaluated by one-way analysis of variance (ANOVA). All data was presented as the means ± S.E.M (standard error of mean).

RESULTS

CAPE Ameliorated TNBS-induced Colitis in Mice

Compared to normal group, body weight loss and an increase in the DAI score were observed in the TNBS group right after the day of induction. However, administration of CAPE in low and high dose managed to maintain the mice body weight and attenuate the DAI score, making them significantly lower than the TNBS group (Figure 1A-B). Shortening of colon length and increasing weight to length ratio were also observed in the TNBS group, which were used as indirect indicators of the severity of TNBS-mice colitis (Figure 1C-D). Compared with the TNBS group, CAPE intake attenuated colon shortening and reduced colon weight to length ratio.

CAPE Suppresses Inflammation in TNBS-Induced Colitis Mice

The level of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in the homogenate supernatant of colonic tissues was determined by ELISA. Compared to the normal group, TNBS group showed a significant increase in TNF-α, IL-6 and IL-1β levels. However, the expression of the pro-inflammatory cytokines in the CAPE groups were significantly decreased compared to that in the TNBS group although the IL-6 levels of low dose and high dose group did not show a significant difference (Figure 2A-C).

Table I: Haematology profile of all mice groups.

Parameter	Unit	Normal	TNBS	Low Dose	High Dose
White blood cells	$\times10^3/\mu l$	6.07 ± 0.73[a]	16.58 ± 4.83[a]	7.03 ± 1.93[a]	6.82 ± 1.01[a]
Red blood cells	$\times10^6/\mu l$	10.47 ± 0.22[a]	8.79 ± 0.39[c]	9.95 ± 0.30a[b]	9.29 ± 0.22b[c]
Haemoglobin	g/dl	15.15 ± 0.38[a]	12.25 ± 0.70[b]	14.73 ± 0.30[a]	14.40 ± 0.56[a]
Haematocrit	%	52.08 ± 1.58[a]	42.68 ± 2.34[b]	50.78 ± 0.79[a]	49.77 ± 1.74[a]
Mean corpuscular volume	fl	49.72 ± 0.88[a]	48.5 ± 0.54[a]	51.03 ± 0.86[ab]	53.50 ± 0.88[b]
Mean corpuscular haemoglobin	pg	14.50 ± 0.41[ab]	13.93 ± 0.30[a]	14.80 ± 0.32[ab]	15.50 ± 0.42[b]
Mean corpuscular haemoglobin concentration	g/dl	29.12 ± 0.61[a]	28.68 ± 0.47[a]	29.00 ± 0.34[a]	28.93 ± 0.45[a]
Platelet	$\times10^3/\mu l$	1218 ± 60.20[a]	1046 ± 149.78[a]	1137.5 ± 33.91[a]	1134.83 ± 4.47[a]
Lymphocytes	%	75.73 ± 15.41[ab]	24.97 ± 15.29[a]	90.47 ± 2.85[b]	46.00 ± 20.69[ab]
Neutrophils	%	24.27 ± 15.42[ab]	75.03 ± 15.29[b]	9.53 ± 20.69[a]	40.54 ± 20.69[ab]

Different subscripts notated significant difference. Data (means ± SEM) was analysed by ANOVA.

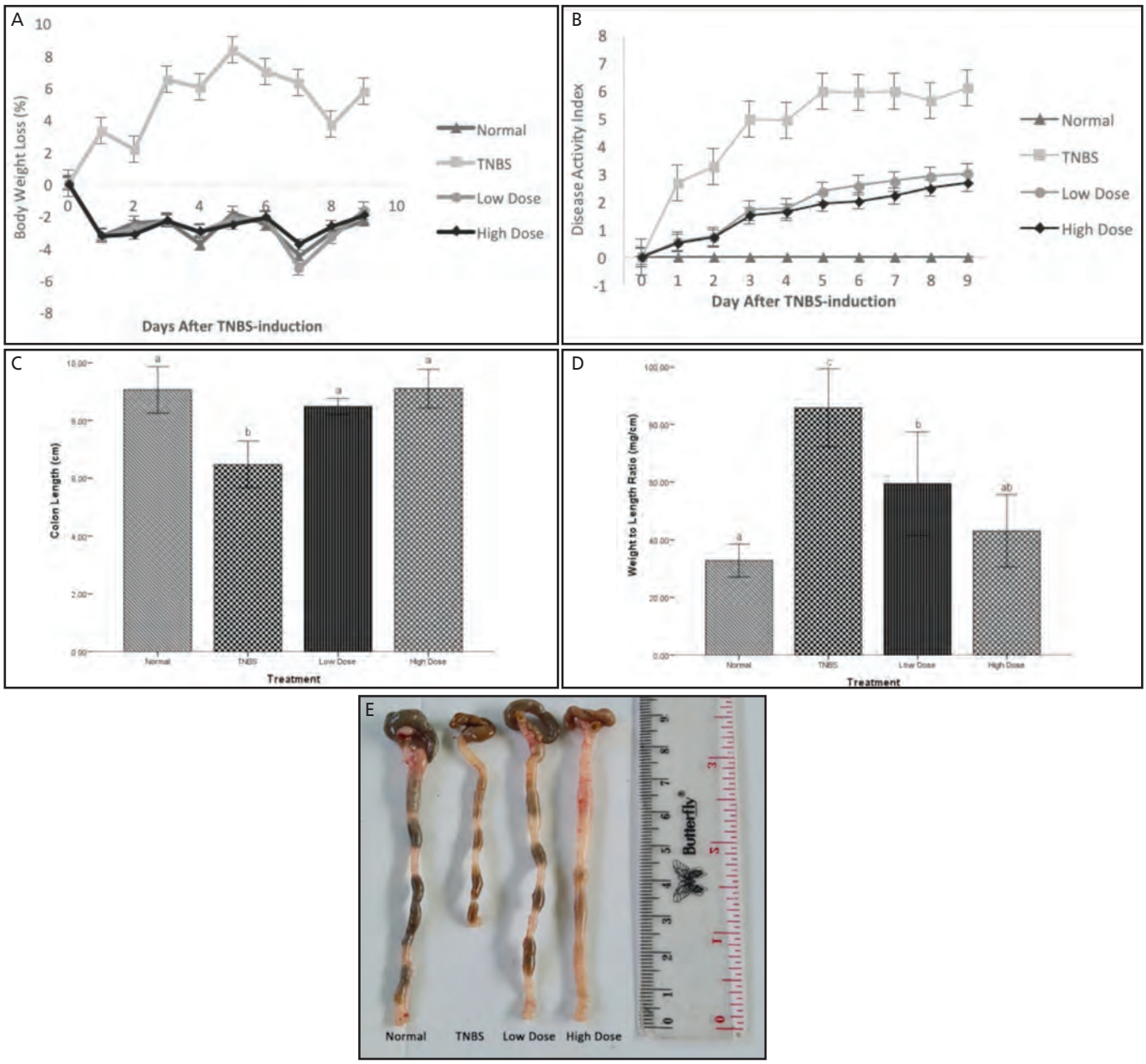

Fig. 1: Citrus aurantifolia peel extract (CAPE) ameliorates 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice. (A) Body weight loss compared to the previous day. (B) Change in DAI score. (C) Colon length of mice at day 9 after TNBS administration. (D) Weight to length ratio of mice colon tissues. (E) Representative pictures of colons. Normal: negative control; TNBS: positive control; low dose: 125 mg/kg CAPE; high dose: 250 mg/kg CAPE. Different scripts notated significant difference. Data (mean±SEM) was analysed by ANOVA

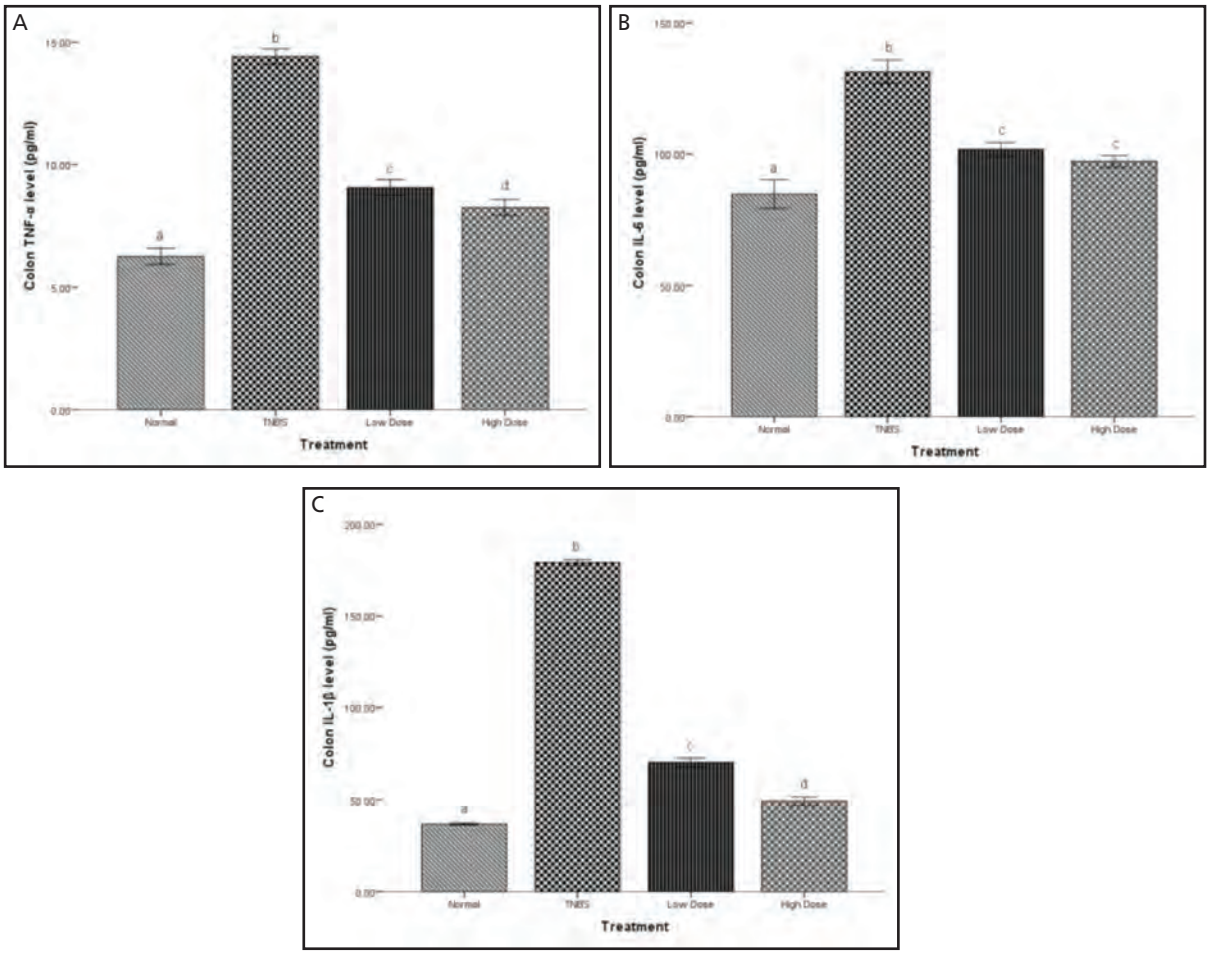

Fig. 2: CAPE suppressed the over-expression of pro-inflammatory cytokines (A) TNF-α, (B) IL-6 and (C) IL-1β in colonic tissues. Normal: Negative control; TNBS: Positive control; 125 mg/kg BW: 125 mg/kg BW CAPE; 250 mg/kg BW: 250 mg/kg BW CAPE. Different scripts notated significant difference. Data (mean±SEM) was analysed by ANOVA

CAPE Improves the Haematology Profile of TNBS-Induced Colitis Mice

An abnormality was observed in the blood profile of TNBS mice, as displayed in Table I. Compared to the normal group, TNBS group had higher white blood cells count and neutrophile while having lower absolute lymphocyte count and red blood cells count. The results emphasised the occurrence of inflammation in TNBS group. Administration of CAPE improved the blood profile of mice, as shown by the decrease of white blood cells count and neutrophile and the increase of red blood cells and absolute lymphocyte count. Therefore, it could be surmised that CAPE ameliorated TNBS-induced colitis mice indirectly through ameliorating their haematology profile.

CAPE Alleviates the Histological Damage in the Colon of TNBS-Induced Colitis Mice

Colitis was successfully induced in the TNBS group as the animals exhibited commonly found colitis symptoms, as shown by the decrease in body weight and an increase in DAI score. Compared to the normal group, the H&E-stained sections of colonic tissue of the TNBS group (Figure 3A-B) showed a serious inflammation with a scattered infiltration of monocytes, loss of histological structure and lesions throughout the mucosa. However, CAPE intake significantly reduced the inflammatory cell infiltration and morphological alteration, thus reducing the histological damage all over (Figure 3C-E).

DISCUSSION

IBD is a chronic remitting disorder of the gastrointestinal tract associated with mucosal inflammation that leads to the increased activation of adhesion, molecules and mucosal damage. While the exact cause of IBD is yet to be known, many studies have reported that oxidative stress is heavily involved in the exacerbation of colitis. Excessive pro-oxidant substances may cause mucosal injury and cause dysregulation of redox signalling that leads to an over-expression of pro-inflammatory cytokines.[15]

Over the past decade, functional food has emerged as a promising means to prevent and attenuate the disease progression of IBD. It affects the disease in various positive ways, for example, the consumption of quinoa, an edible grain-like crop, reduces the severity of histological damage in DSS-induced colitis mice due to the high amount of polysaccharide in quinoa thus promoting the growth of

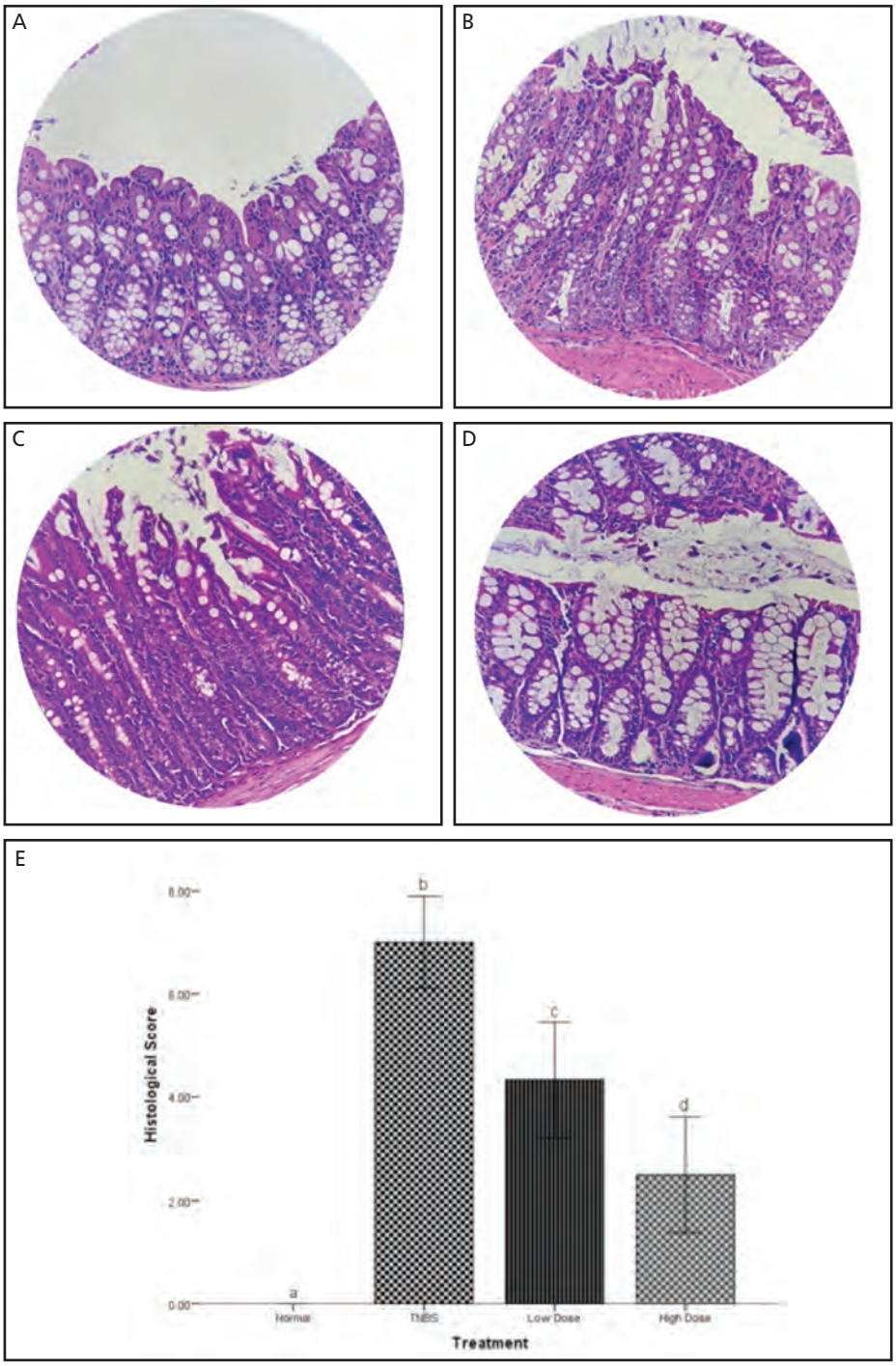

Fig. 3: CAPE attenuates histological injury in TNBS-induced colitis in mice. Representative images of haematoxylin and eosin (H&E) staining of colon tissue from (A) normal group, (B) TNBS group, (C) 125 mg/kg CAPE group, and (D) 250 mg/kg CAPE group. (E) Colonic histological score. Different scripts notated significant differences. Data (mean± SEM) was analysed by ANOVA

beneficial bacteria and the production of short chain fatty acids (SCFA).[16] Resveratrol, a polyphenol commonly found in grapes and peanuts, is reported to mitigate one of the main symptoms of IBD which is the disruption of the intestinal barrier in a Caco-2 cell model.[17] Several flavonoid compounds found in black ginger, namely, 3, 5, 7, 3′, 4′-pentamethoxyflavone and 5,7-dimethoxyflavone, also served the same effect.[18,19] In another case, the powder form of Citrus limon peel is able to repair the damage done to the colon tissue of DSS-induced mice and also alter the faecal SCFA composition which implies the change in intestinal microflora compositions.[20] On top of its various mechanisms, functional food is readily available in our daily lives, making it a more convenient choice as an alternative method to decrease the severity of IBD.

In this study, TNBS was chosen as a colitis inducer agent due to its significant similarities with human IBD. It is also one of

the most appropriate and successful models of experimental colitis. The pathology of TNBS-induced colitis is as such: transmural granulomatous inflammation associated with diarrhoea, rectal prolapse, weight loss and colonic wall thickening, all observed in human CD.[21] Infiltration of polymorphonuclear and mononuclear into the intestinal tissue, release of inflammatory cytokines (IL-1 and TNF-α) and an increase of inducible nitric oxide synthase (iNOS) and COX-2 were observed in TNBS-model of mice colitis.[22]

The over-expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β is a common marker in IBD. It was induced by Toll-like receptor (TLR) ligands in monocytes, macrophages, T and B lymphocytes and is a sign of a compromised immune system. TNF-α is identified as the primary regulator of inflammatory responses involved in the pathogenesis of IBD.[23] Its over-expression result in a decrease in colonic mucosa layer thickness thus exposing colonic mucosa to luminal antigens.[24] IL-6 is another common marker in IBD, with its over-production heavily involved in dysregulation of immune responses and B-cell malignancies.[25] It is known to aggravate inflammation by directly inducing lymphocyte proliferation and differentiation through the nervous system.[26] The induction of TNBS acts as a potent oxidative agent that stimulates the T-cell, mimicking that of a human, thus causing an excessive expression of TNF-α, IL-6 and IL-1β.[27]

In our experiment, we administered CAPE orally before and during the colitis induction with TNBS and evaluated the therapeutic effects of CAPE. Induction of TNBS caused the loss of body weight, pathological changes of colonic tissue, and an increase in the tissue level of inflammatory mediators. However, daily oral gavage of CAPE protected BALB/c from various damages caused by TNBS-induction and ameliorated the body weight loss, DAI score, colonic weight and length, haematology profile, and histological scores. Moreover, the data showed that CAPE could decrease the production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in colonic tissues. The number of studies regarding the ameliorating effect of CAPE on colitis-induced inflammation is limited, but the results obtained are in accordance with studies regarding the anti-inflammatory properties of other citrus species. However, CAPE improved several parameters better compared to other citrus species peel extract, such as Citrus unshiu peel extract, which gave no improvement to the colon length of the treated DSS- induced mice and Citrus aurantium L. peel extract, which did not improve the DAI of the treated TNBS-induced mice.[28,29] Regardless, they were able to decrease the gene expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β, thus reducing their concentrations in colon tissue, which could also be seen in the treatment results using CAPE.

CAPE contained various bioactive compounds, one of them being phenolic. However, phenolic is a broad group. Therefore, it is difficult to identify the specific compound in CAPE that attributes to the attenuation of TNBS-induced colitis in mice. Previously, we studied the phenolic compounds in CAPE and found that CAPE contains high concentration of quercetin. Quercetin is a flavanol known for its anti-inflammation properties. It works as an anti-inflammation agent by blocking TNF-α-mediated inflammation by preventing TNF-α from directly activating extracellular signal-related kinase (ERK), c-Jun NH2-terminal kinase (JNK) and Nf-κB, which are potent inducers of inflammatory gene expression and protein secretion.[30] Quercetin may also indirectly prevent inflammation by increasing peroxisome proliferator-activated receptor c (PPAR-γ) activity, therefore antagonising Nf-κB or activator protein-1 (AP-1) transcriptional activation of inflammatory genes.[31] Other phenolic compound found in CAPE in an insignificant amount may also contribute to its anti-inflammatory properties thus making CAPE a remarkable anti-inflammation agent. Further identification is required, and the phenolic compounds contained in CAPE will need to be studied on their effects of TNBS-induced colitis in mice.

CONCLUSION

Our study showed that Citrus aurantifolia peel extract showed an ameliorating effect in TNBS-induced colitis mice. This effect is at least associated with its ability in maintaining relative balance between pro-inflammatory and anti-inflammatory cytokines namely the suppression of several pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6. Our results provide a new nutritional supplement perspective and a potential therapeutic remedy for attenuating the pathological conditions in IBD.

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