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Gallic acid serves as an effective therapeutic agent of inflammatory bowel disease: Pharmacological impacts on tight junction-dependent intestinal permeability *in vivo* and its related intracellular signaling

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ABSTRACT

Intestinal tight junction disruption contributes to the pathogenesis of inflammatory bowel diseases (IBD). We have recently reported that gallic acid was able to enhance intestinal tight junction assembly via CaMKK-β/AMPK/SIRT-1/ERK-dependent mechanisms with unknown possible therapeutic applications in IBD. The main aims of this study are to investigate the *in vivo* effects of gallic acid in experimental colitis mice and to search for feasible mechanisms of action. Here, we found that gallic acid attenuated weight loss, reduced disease activity index, and reversed colon length shortening in DSS-induced colitis mice. Importantly, gallic acid also significantly increased survival rates of DSS-induced colitis mice. Based on histopathological analyses, gallic acid diminished immune cell infiltration and neutrophil activity in colitis tissues. Of particular interest, gallic acid significantly reduced gene expressions of proinflammatory cytokines, including TNF, IFN-γ, IL-1β, IL-6, and IL-8. In addition, gallic acid suppressed MLCK gene transcription and protein expression in DSS-induced colitis mice. Furthermore, gallic acid also enhanced the expression of tight junction proteins, including ZO-1 and occludin. Consistently, gallic acid reduced tight junction-dependent leak pathway permeability and was shown to increase SIRT-1 activity, AMPK, and ERK phosphorylation in colon tissues of DSS-induced colitis mice. This study not only explores anti-colitogenic impacts of gallic acid, but also sheds some light on the mechanisms of its action. According to our findings, gallic acid may be useful as an anti-colitogenic nutraceutical.

1. Introduction

Inflammatory bowel disease (IBD) is an autoimmune-induced chronic intestinal inflammation (Chen et al., 2024; Kaser et al., 2010). It is characterized into two major types, including ulcerative colitis (UC) and Crohn's disease (CD), according to regions of the gastrointestinal tissue damage (Kazemifard et al., 2024; Kobayashi et al., 2020; Roda et al., 2020). In UC, inflamed tissue lesions are mainly restricted in the colon to rectum areas. On the other hand, inflamed tissue lesions of patients with CD can be sporadically distributed throughout the whole gastrointestinal tract. At a subcellular level, pathophysiological mechanisms of IBD are mediated by genetic predisposition, dysbiosis, submucosal immune hyperresponsiveness, and increased intestinal tight junction-dependent tissue permeability (Jauregui-Amezaga et al., 2024; Cicerone et al., 2024; Ciorba et al., 2024a; Xin, 2024; Pereira et al., 2024; Rudbaek et al., 2024; Nishida et al., 2018; Odenwald et al., 2013; Edelblum et al., 2009). At present, a local, non-absorbed anti-inflammatory drug 5-aminosalicylate (5-ASA) and corticosteroid treatment are the gold standard options for the treatment of IBD with enhanced infectious susceptibilities and various severe side effects (Jairath et al., 2024; Papamichael et al., 2019). In addition, IBD is known to be incurable because of its complex pathogenesis (Ciorba et al., 2024b). Hence, novel therapeutic choices for IBD treatments need to be further identified.

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Cytokines including interferon gamma (IFN-γ), tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6, IL-8, and LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T cells) were found to be elevated in colonic tissues of IBD patients and were known to determine disease pathogenesis (Neurath, 2024; Abraham et al., 2022; Wang et al., 2005a). The rate of tight junction-dependent intestinal permeability was also increased in healthy first-degree relatives of Crohn's disease patients (Turpin et al., 2020; Michielan et al., 2015; Buhner et al., 2006). At a subcellular level, IFN-γ sensitized the effects of TNF-induced, myosin light-chain kinase (MLCK) expression-mediated MLC phosphorylation, contraction of the actin cytoskeleton, and tight junction endocytosis (Zuo et al., 2023; Graham et al., 2019; Wang, 2005b, 2006). Of note, based on previous studies on many models of experimental colitis, suppression of cytokine-induced tight junction disruption was known to sufficiently diminish the severity of disease progression at an early phase of colitis (Zuo et al., 2023; Graham et al., 2019; Su et al., 2013). Hence, tight junction-associated intestinal permeability is well accepted as the therapeutic target for IBD (Moonwiriyakit et al., 2023). Nevertheless, an FDA-approved drug that can effectively promote intestinal tight junction-dependent intestinal barrier function is clinically unavailable currently. MLCK has long been considered as the therapeutic target for IBD, but it is impossible to use a MLCK inhibitor for the treatment of this disease because of its possible lethal adverse effects (Moonwiriyakit et al., 2023).

Our recent findings have strongly revealed that gallic acid isolated from *Ocimum sanctum* L. flower aqueous extract was able to enhance intestinal tight junction assembly via the calcium/calmodulin-dependent protein kinase kinase-beta (CaMKK-β)/AMP-activated protein kinase (AMPK)/sirtuin-1 (SIRT1)/extracellular signal-regulated kinase (ERK)-dependent mechanism (Wachiradejkul et al., 2024). In addition, a recent study reported that gallic acid downregulated pro-inflammatory cytokine expression in lipopolysaccharide (LPS)-treated intestinal epithelial Caco-2/RAW 264.7 macrophage co-culture model (Mu et al., 2024). However, *in vivo* pharmacological effects of gallic acid on clinical outcomes of colitis, histopathology of colon parts, tight junction-dependent intestinal barrier permeability, and expression levels of associated intracellular signaling in colon parts of the experimental colitis mouse model have never been fully elucidated yet. The main aims of this study are to investigate the therapeutic effects of gallic acid in dextran sulfate sodium (DSS)-induced colitis in mice and to also search for possible mechanisms of its action.

2. Materials and methods

2.1. Animal ethical approval

All C57BL/6 mice were purchased from the Nomura Siam International Co.,Ltd. And care at the Central Animal Facility (MUSC-CAF), Faculty of Science, Mahidol University based on the regulations of the Institutional Animal Care and Use Committee (IACUC) (Approved Protocol No. MUSC66-055-685). All mice were six weeks of age with body weight of approximately 20–25 g. In each experimental group, six mice were used.

2.2. Animal housing protocol

All mice used in this study were housed in an individually ventilated cage with the standard of the strict hygienic conventional system. Mice were kept in social housing condition of five adult mice per one cage according to MUSC–IACUC policy. For this housing and care, the environmental temperature was maintained at 22 ± 1 °C with 30–70 % relative humidity and standard light cycle (12:12 h).

2.3. DSS-induced experimental colitis mouse model and treatment with gallic acid

All experiments conducted in this study were done using DSS-induced colitis model in C57BL/6 male mice. In order to induce colitis in these mice, dextran sulfate sodium (DSS; 5 %) was dissolved in drinking water (molecular weight of batch of DSS is approximately 40 kDa, Cat. #J63606.22, Thermo Fisher Scientific Inc., Waltham, MA, USA) was orally administrated to C57BL/6 mice for 8 days to evaluate disease activity, molecular and histopathological analyses. For survival study, mice were fed 5 % DSS in drinking water for 2 weeks. Of note, more detailed protocol of DSS-induced colitis was explained previously (Yousef et al., 2012). To evaluate *in vivo* the effects of gallic acid on experimental colitis, the DSS-induced colitis mice were intra-gastrically administered with gallic acid (Sigma-Aldrich co.; Cat. #91215) at various concentrations (10, 40, and 100 mg/kg) along with DSS administration. Indeed, clinical symptoms and survival rate of mice were observed. Furthermore, colon tissues from DSS-induced colitis mice were further collected to investigate expression levels of related molecular signaling. Therefore, to conduct experiments based on detailed protocol mentioned above, mice were divided into five groups for dose-dependent experiments including control group (mice fed with drinking water without DSS), mice fed with 5 % DSS in drinking water, mice fed with 5 % DSS in drinking water plus oral gavage of gallic acid (10 mg/kg/day) in 100 μl in volume, mice fed with 5 % DSS in drinking water plus oral gavage of gallic acid (40 mg/kg/day) in 100 μl in volume, and mice fed with 5 % DSS in drinking water plus oral gavage of gallic acid (100 mg/kg/day) in 100 μl in volume. Indeed, the effective dose that can produce the maximal beneficial effects was selected for the subsequent experiments.

2.4. Clinical observation of DSS-induced colitis mice treated with gallic acid

All clinical outcomes of DSS-induced colitis mice were recorded daily according to characteristics of diarrhea, fur texture, posture, and motor activity as a disease activity index (DAI), which was each scored from 0 to 2 (Graham et al., 2019; Su et al., 2013; Raju et al., 2020). The severity of colitis of DSS-fed mice treated with or without gallic acid in this study were determined by measurement of inflamed colon shortening and pathohistological evaluation based on tissue architectural change, epithelial damage, mucin depletion, crypt abscess, and colonic submucosal edema (Supplemental Table 1). Furthermore, colonic immune cell infiltration was examined and evaluated using Nancy index (Supplemental Table 2) as previously described (Vespa et al., 2022). In fact, evaluation of Nancy index and histopathological analysis were performed by an anatomical pathologist who is experimentally blinded to control and treatment groups.

2.5. Myeloperoxidase (MPO) activity assay

MPO is considered as an enzyme highly expressed in neutrophils. In case of intestinal mucosal neutrophil infiltration, enzymatic activity of MPO can be observed in colonic tissue samples. Therefore, to evaluate neutrophil infiltration in colonic parts of DSS-fed mice, MPO activity assay (Cat. #ab105136, Abcam, Cambridge, MA, USA.) was performed. Based on assay instruction, MPO was able to produce hypochlorous acid (HClO), resulting in enhanced production of taurine chloramine. Indeed, taurine chloramine generally oxidizes a yellow chromogenic TNB probe to reduce its chromatic signals. The optical density (O.D.) for absorbance of TNB color was quantitatively detected at wavelength of 412 nm. Of note, O.D. value of TNB is inversely proportional to the amount and activity of MPO enzyme.

2.6. Investigation of gene expression of cytokine-associated IBD and tight junctions

Quantitative real-time PCR (qRT-PCR) was used to scrutinize the effects of gallic acid treatment on colonic cytokine and tight junction gene transcription in DSS-induced colitis mice. Total RNA extraction from colonic tissues derived from DSS-induced colitis mice was performed using RNeasy Mini Kit (Cat #74106) (Qiagen, Hilden, Germany). Concentration of RNA samples were determined by NanoDrop (NanoDrop Technologies Inc.,Wilmington, DE, USA). The process of cDNA synthesis from RNA templates was performed using an iScript Reverse Transcription Supermix (Cat. #1708841, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gene amplifications were done by the CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with PowerTrack™ SYBR™ Green Master Mix (Cat. #A46109, Applied Biosystem, Foster City, CA, USA). The primer sets used in this study were shown in Supplemental Table 1.

No.	Transcripts	Primer Sequences (5′ → 3′)	Length	Tm	%GC
1.	*Tjp1*	TGGAATTGCAATCTCTGGTG (Forward)	20	56.01	45
		CTGGCCCTCCTTTTAACACA (Reverse)	20	57.71	50
2.	*Tjp2*	ATGGGAGCAGTACACCGTGA (Forward)	20	60.9	55
		TGACCACCCTGTCATTTTCTTG (Reverse)	22	58.78	45.45
3.	*Ocln*	GCTGTGATGTGTGTGAGCTG (Forward)	20	59.48	55
		GACGGTCTACCTGGAGGAAC (Reverse)	20	59.18	60
4.	*Cldn3*	AAGCCGAATGGACAAAGAA (Forward)	19	54.99	42.11
		CTGGCAAGTAGCTGCAGTG (Reverse)	19	58.83	57.89
5.	*Cldn4*	CGCTACTCTTGCCATTACG (Forward)	19	56.24	52.63
		ACTCAGCACACCATGACTTG (Reverse)	20	58.11	50
6.	*Cldn7*	AGGGTCTGCTCTGGTCCTT (Forward)	19	59.84	57.89
		GTACGCAGCTTTGCTTTCA (Reverse)	19	56.86	47.37
7.	*Cldn8*	GCCGGAATCATCTTCTTCAT (Forward)	20	55.59	45
		CATCCACCAGTGGGTTGTAG (Reverse)	20	57.89	55
8.	*Il1b*	GAGTGTGGATCCCAAGCAAT (Forward)	20	57.87	50
		TACCAGTTGGGGAACTCTGC (Reverse)	20	59.31	55
9.	*Il6*	TAGTCCTTCCTACCCCAATTTCC (Forward)	23	59.22	47.83
		TTGGTCCTTAGCCACTCCTTC (Reverse)	21	59.37	52.38
10.	*Tnf*	AGCCCCCAGTCTGTATCCTT (Forward)	20	59.96	55
		GGTCACTGTCCCAGCATCTT (Reverse)	20	59.67	55
11.	*MYLK*	TCTCCTGCAAGATCACTGGC (Forward)	20	62.2	55
		CTCAGACACAGACACACGGG (Reverse)	20	63.6	60
12.	*Il8*	CGCCCAGACAGAAGTCATAG (Forward)	20	57.78	55
		TCCTCCTTTCCAGGTCAGTTA (Reverse)	21	57.73	47.62
13.	*GAPDH*	GCCAAGAGGGTCATCATCTC (Forward)	20	57.75	55
		CCTTCCACAATGCCAAAGTT (Reverse)	20	56.79	45

2.7. Western blotting analysis

Colon parts of normal, vehicle- and gallic acid-treated, DSS-induced mice were surgically removed to be further digested in lysis buffer and were homogenized in TissueLyser LT system (Qiagen, Hilden, Germany). Supernatants of all homogenized samples were collected. In addition, western blotting analysis was performed to measure the expression levels of MLCK, zonula occludens-1 (ZO-1), occludin, claudin-1, claudin-4, and β-actin. Briefly, supernatants containing protein samples were loaded and separated into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and further transferred to a nitrocellulose membrane by the Trans-Blot Turbo transfer system (Bio-Rad Laboratories, Hercules, CA, USA). To diminish the non-specific binding background of the antibodies, membrane containing proteins was blocked for few minutes with BlockPRO™ 1 Min Protein-Free Blocking Buffer (Cat. #BM10-100, Visual Protein, Neihu Dist., Taipei, Taiwan). Furthermore, membrane containing protein samples were stained with specific primary antibodies against MLCK (Clone: K36, Sigma-Aldrich, Cat# M7905), ZO-1 (Clone: ZO1-1A12, ThermoFisher scientific, Cat# 33–9100), occludin (Clone: OC-3F10, ThermoFisher scientific, Cat# 33–1500), claudin-1 (Clone: D3H7C, Cell Signaling, Cat# 13995 S), claudin-4 (Clone: 3E2C1, ThermoFisher scientific, Cat# 32–9400), and β-actin (Clone: 13E5, Cell Signaling, Cat# 4970 S) overnight (4 °C). Membrane containing protein samples were also stained with secondary antibodies against IgG domain of the primary antibodies for a few hours (room temperature). For detecting protein signals, luminol/enhancer solution and peroxide (Cat. #1705060) (Bio-Rad Laboratories, Hercules, CA, USA) was used. Of note, β-actin was used as a loading control for validating equal protein loading. Band densitometry of protein expression of MLCK, ZO-1, occludin, claudin-1, and claudin-4 were evaluated by Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. SIRT-1 activity assay

Enzymatic deacetylase activity of SIRT-1 of normal, vehicle- and gallic acid-treated, DSS-induced colitis mice was investigated using a SIRT1 Activity Assay Kit (ab156065, Abcam, MA, USA) based on assay instruction and partly adjusted from previous study (Sukmak et al., 2024). In brief, supernatants containing protein lysates of normal, vehicle- and gallic acid-treated colitis mice were dissolved in SIRT1 Assay Buffer, NAD, and Fluoro-Substrate Peptide. The fluorescent signals of sample reactions were determined using a microplate reader at excitation/emission wavelength of 350 nm/450 nm.

2.9. In vivo paracellular permeability assay

To differentiate between tight junction disruption-mediated leak pathway permeability and tight junction-independent, mucosal erosion-associated barrier loss in DSS-induced colitis mice, *in vivo* multiplex permeability assay was used with slight adjustment from previous original protocol (Chanez-Paredes et al., 2021). Briefly, permeability probe cocktail containing 4-kDa FITC-dextran (80 mg/ml) (Cat. #FD4, Millipore-Sigma) and 70-kDa rhodamine-dextran (40 mg/ml) (Cat. #R9379, Millipore-Sigma) was dissolved together as a stock in ultrapure water as fluorescent probes for tight junction-dependent leak pathway permeability and tight junction-independent tissue erosion-associated barrier loss. Of note, 4-kDa FITC-dextran can be transported across paracellular space in case of tight junction disruption and tissue erosion but 70-kDa rhodamine-dextran can pass through paracellular space in case of tissue damage because its molecular diameter is larger than leak pathway permeability. To clearly eliminate the remaining food in the GI tract, fasting was performed 4 h prior to the experiment. Permeability probe cocktail was gently administrated to normal, vehicle- and gallic acid-treated, DSS-induced colitis mice by intragastric gavage. At 4 h after oral gavage, mice were anesthetized thiopental (150 mg/kg, i. p.) with xylazine (10 mg/kg, i. p.) and blood samples were collected by cardiac puncture. Fluorescent intensity of mouse serum samples from each experimental group were measured using Biotek Synergy HT. Fluorescein and rhodamine B fluorescence were read at Excitation/Emission wavelength of 495 nm/525 nm and 555nm/585 nm, respectively.

2.10. Statistical analysis

All presented results in this study were expressed as means ± S.E.M. Statistical analysis for multiple comparisons in this study was determined by the analysis of variance (one- or two-way ANOVA) followed by

the Bonferroni analysis using Prism 5.0, where appropriate. For analysis of histological score, a two-tailed Kruskal-Wallis test was used. For some experiment regarding survival studies, Log-rank (Mantel-Cox) test was used. If *P*-value was <0.05 is statistically significant. Statistical analyses for all experiments of this study were performed using GraphPad Prism software.

3. Results

3.1. *In vivo pharmacological effects of gallic acid as an anti-colitogenic agent in mice*

Induction of intestinal tight junction recovery has been proposed as one of the therapeutic strategies to treat colitis (Graham et al., 2019). Since our recent findings suggested that gallic acid was able to induce intestinal tight junction in the intestinal epithelial-like T84 cell monolayers (Wachiradejkul et al., 2024), but its therapeutic impacts on colitis *in vivo* have never been fully explored in term of both clinical symptoms and molecular mechanisms of its actions. We first induced experimental colitis in mice by dissolving 5 % DSS in drinking water for 8 and 14 days to evaluate all IBD-related clinical outcomes and survival rate, respectively. Gallic acid at various concentrations (10, 40, and 100 mg/kg) were orally given to DSS-induced colitis mice daily by intragastric gavage, one time a day. In vehicle-treated, DSS-induced colitis mice, the body weight of these mice was significantly decreased when compared to normal mice and gallic acid (100 mg/kg) slightly elevated body weight of colitis mice with statistically significant differences when compared to vehicle-treated group (Fig. 1A). Gallic acid was capable of reducing disease activity index (DAI) significantly (Fig. 1B). In addition, gallic acid reversed colon shortening in DSS-induced colitis mice (Fig. 1C and D). Of particular importance, gallic acid (100 mg/kg) also significantly increased the survival rate of DSS-induced colitis mice (Fig. 1E).

3.2. *Investigation of the histopathological and immune cell activity in colonic tissues from colitis mice treated with gallic acid*

After mouse experiment was terminated, colon parts from normal and DSS-induced colitis mice with or without gallic acid treatment were collected and carefully fixed with paraformaldehyde (4 % in PBS) and further embedded in paraffin blocks. All paraffin blocks containing tissue samples were 5-μm thinly sectioned and were put onto the slide. All sample slides were stained by hematoxylin and eosin (H&E) or periodic-acid Schiff (PAS) reagents. According to our results, we found tissue immune cell infiltration, submucosal edema, and other inflammation-associated characteristics (Fig. 2A). Surprisingly, gallic acid attenuated DSS-induced colonic histopathological damage and immune infiltration in mice (Fig. 2A). Furthermore, it was also found that the ratio of PAS-positive goblet cells per crypt was obviously increased in DSS-induced colitis mice (Fig. 2B). Of particular importance, gallic acid reduced PAS-positive goblet cells in DSS-induced experimental colitis mice (Fig. 2B). In addition, we also found that gallic acid treatment was able to significantly reduce pathohistological score and Nancy index in DSS-fed mice (Fig. 2CandD). Colonic submucosal neutrophil infiltration has been widely accepted as one of the pathohistological characteristics of IBD patients (Drury et al., 2021). It is noticed that MPO enzyme that is known to be highly expressed in neutrophil enzymatically decreases the color of TNB chromogen in tissues (Yousef et al., 2012; Rodrigues et al., 2022). Here, we found that gallic acid significantly increased TNB intensity in colonic protein lysate samples isolated from DSS-induced mice (Fig. 2E).

3.3. *Effects of gallic acid treatment on expression of pro-inflammatory cytokines and MLCK*

We further analyzed the transcriptional expression levels of IBD-related pro-inflammatory cytokines. Indeed, we found that, compared to normal mice, gene expressions of pro-inflammatory cytokines including TNF, IFN-γ, IL-1β, IL-6, and IL-8 were upregulated in colitis mice (Fig. 3A–E). On the other hand, gallic acid significantly suppressed transcriptional expressions of these cytokines (Fig. 3A–E). It is noticed that MLCK is considered as an upstream regulator of inflammation-induced intestinal tight junction disruption and serves as the therapeutic target for colitis (Graham et al., 2019). Therefore, we next measured gene and protein expression of MLCK in normal and DSS-fed mice with

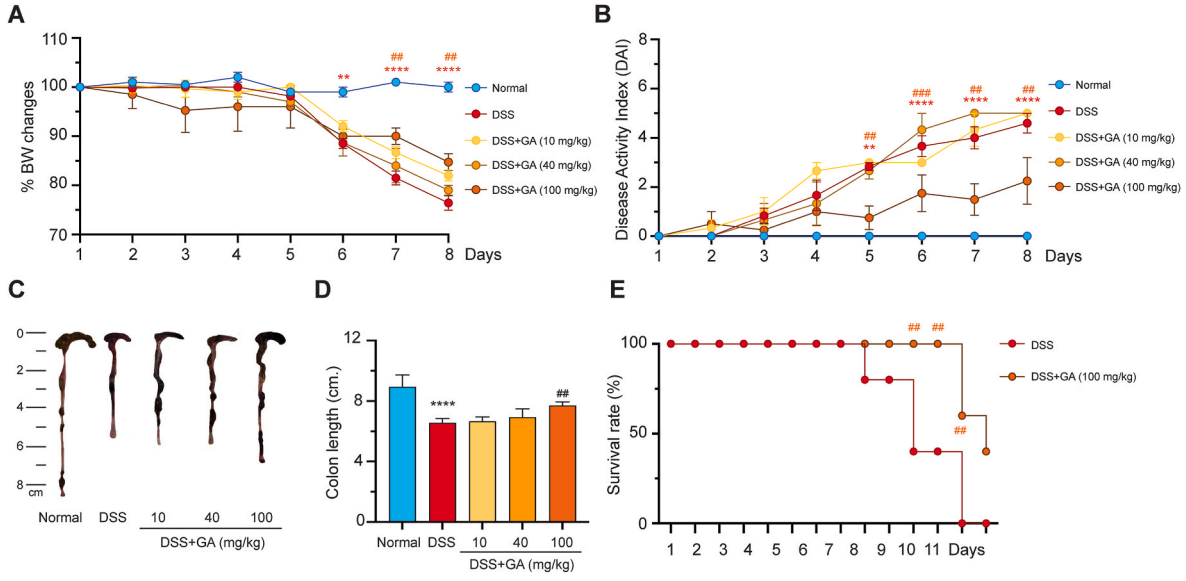

Fig. 1. Oral administration of gallic acid reduced all clinical IBD-related symptoms of DSS-induced colitis mice. Gallic acid at various concentrations (10, 40, and 100 mg/kg/day) was orally administered by daily intragastric gavage to mice fed with 5 % dextran sulfate sodium (DSS) in drinking water. (A) Observation of percentage body weight changes over time in mice treated with vehicle and DSS with or without gallic acid. (B) Disease activity index (DAI) was calculated based on apparent phenotypes and behaviors of DSS-induced colitis mice orally treated with gallic acid compared to normal and DSS-fed mice. (C) Representative gross images of the effect of gallic acid treatment on the colon length in DSS-induced colitis mice. (D) Summary of colon shortening in all experimental groups at day 8. (E) Effect of gallic acid treatment on survival rate of mice at 14 days after induction of DSS-induced colitis. Data were expressed as mean ± SEM (n = 6). $^{**}p < 0.01$; $^{****}p < 0.0001$ compared with normal mice. $\#\#p < 0.01$; $\#\#\#\#p < 0.0001$ compared with DSS-treated mice.

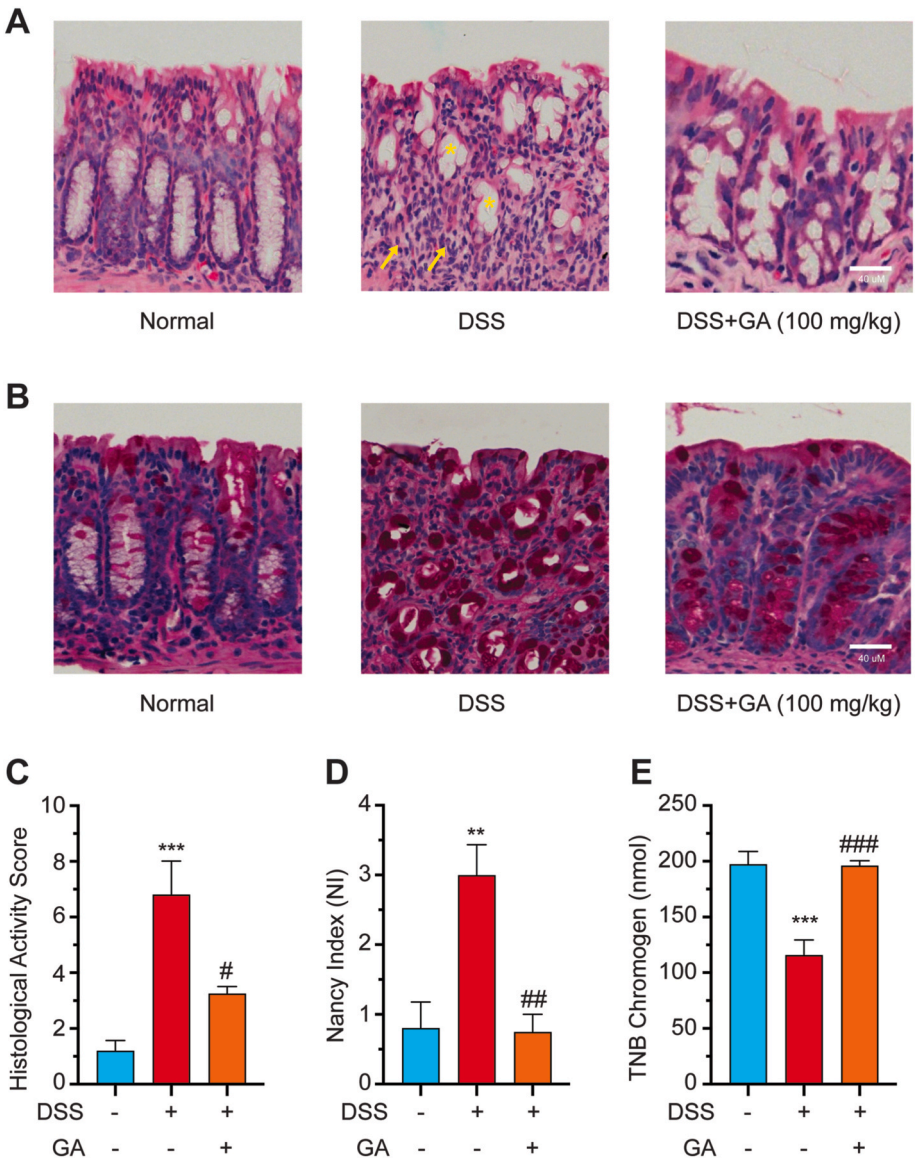

Fig. 2. Pathohistological analyses of colonic tissue architecture of colitis mice. (A) Representative images of H&E staining of colon parts obtained from DSS-induced colitis mice treated with or without gallic acid as shown (20 ×). (B) Representative images of PAS staining of colon sections from DSS-induced colitis mice treated with or without gallic acid. (C) Summary of the histopathological scores according to morphological changes and immune infiltration of the colonic tissue architecture. (D) Summary of the histopathological scores based on Nancy index indicated by immune cell infiltration. (E) Measurement of TNB chromogen levels in protein lysates obtained from colonic segments of DSS-induced colitis mice that recapitulated the function of neutrophil infiltration. Arrows indicate immune infiltration whereas asterisks indicates crypt distortion. Data were expressed as mean ± SEM (n = 6). $^{**}p < 0.01$; $^{***}p < 0.001$ compared with normal mice. $\#p < 0.05$; $\#\#p < 0.01$; $\#\#\#p < 0.001$ compared with DSS-treated mice.

or without gallic acid treatment. It was found that, in colitis mice, MLCK mRNA and protein expression were significantly increased when compared to normal mice (Fig. 3FandG). Interestingly, gallic acid was capable of diminishing the expression of MLCK at both transcriptional and translational levels, demonstrated in colonic tissues isolated from DSS-induced colitis mice (Fig. 3FandG).

3.4. Effects of gallic acid on intestinal tight junction expression and permeability in experimental colitis

As mentioned above, intestinal tight junction disruption contributes to colitis pathogenesis (Edelblum et al., 2009). Here, we performed experiments to quantitatively scrutinize expressions of various barrier-forming tight junctions in normal and DSS-fed mice with or without gallic acid treatment. In fact, we found that, in addition to *Ocln* mRNA downregulation, almost all tight junction-related mRNAs including *Tjp1*, *Cldn3*, *Cldn4*, *Cldn7*, and *Cldn8* were not changed in colonic tissues from all experimental groups (Fig. 4A–F), suggesting that inflammatory lesions in experimental colitis may not affect transcriptional modification, stability, and integrity. Furthermore, our western blotting analyses revealed that ZO-1, occludin, and claudin-4, but not claudin-1 were significantly decreased in colon parts from colitis mice compared to normal (Fig. 5A–D). Surprisingly, Gallic acid recovered ZO-1 and occludin protein expression in DSS-induced colitis mice (Fig. 5AandB). However, no effects on protein expression levels of claudin-1 and claudin-4 in response to gallic acid treatment were observed (Fig. 5CandD). Based on protein expression profiles, we found that barrier-forming tight junctions were attenuated in experimental colitis mice. Therefore, we further investigated the effects of gallic acid on colonic mucosal-to-serosal permeabilities in colitis mice. As expected, the permeability rate of 4-kDa FITC-dextran, but not 70-kDa rhodamine-dextran, was significantly increased in DSS-fed mice when

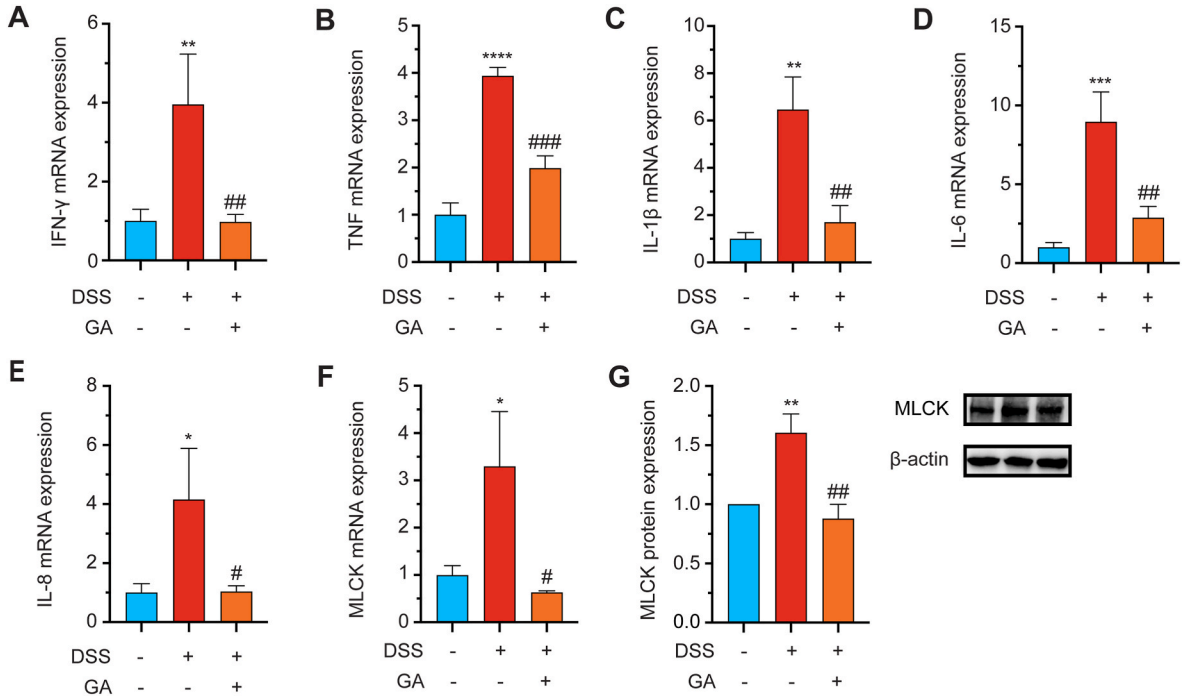

Fig. 3. Effects of Gallic acid on expressions of inflammatory cytokine and MLCK in colitis mice. Effect of gallic acid on gene expressions of (A) IFN-γ, (B) TNF, (C) IL-1β, (D) IL-6, and (E) IL-8. (F) Effect of gallic acid on mRNA expression of MLCK. (G) Western blotting analysis of protein expression of MLCK. Data were expressed as mean ± SEM (n = 6). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ compared with normal mice. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ compared with DSS-treated mice.

compared to normal mice (Fig. 6A).

3.5. *Possible mechanisms of the anti-colitogenic effects of gallic acid*

We recently reported that AMPK, SIRT-1, and ERK contributed to gallic acid-induced intestinal tight junction assembly in intestinal epithelial-like T84 cell monolayers (Wachiradejkul et al., 2024). Here, we also carefully analyzed whether gallic acid activates these enzymes in colonic tissues of colitis mice in concordance with what we have found *in vitro*. Indeed, we found that, in DSS-fed mice, intensity of AMPK phosphorylation in colonic tissues was attenuated, but gallic acid reversed AMPK phosphorylation in DSS-induced colitis mice (Fig. 6B). Furthermore, gallic acid was also able to enzymatically activate SIRT-1 in colonic tissues of colitis mice (Fig. 6C). Surprisingly, we also found that gallic acid increased ERK phosphorylation in colitis mice.

4. Discussion

In this study, we showed that gallic acid treatment predominantly reduced all colitis-related clinical symptoms and significantly enhanced the survival rate of DSS-fed mice. We also found that gallic acid functionally suppressed pro-inflammatory expression and MLCK signaling. Furthermore, gallic acid treatment was demonstrated to improve intestinal barrier function, at least in part, by activating AMPK/SIRT-1/ERK-dependent mechanisms. These findings may lead to the successful development of a novel, inexpensive, effective small molecule for IBD patients in the future.

Approaches that can induce intestinal tight junction assembly and suppress tight junction-dependent leak pathway permeability have long been widely accepted as effective therapeutics for colitis (Wang et al., 2024a; Chen et al., 2018; Sharma et al., 2018). Recently, we have reported that gallic acid promoted intestinal tight junction assembly in T84 cell monolayers (Wachiradejkul et al., 2024), leading to the generation of our hypothesis that gallic acid may be capable of treating colitis. Therefore, we conducted DSS-induced mouse colitis experiments to prove our hypothesis. Indeed, gallic acid treatment was shown to attenuate all IBD-associated clinical outcomes and increase the survival rate of DSS-induced colitis mice as expected. These sets of data strongly indicated that gallic acid that attenuated all pathogenic parameters in DSS-induced colitis mice can possibly be used as a therapeutic agent for the treatment of IBD. Although gallic acid treatment significantly improved survival rates, it slightly increased the body weight of DSS-fed mice. Normally, significant weight loss in IBD might be due to intestinal inflammation-associated diarrhea, malabsorption, and reduced food intake (Bruner et al., 2023). Our results indicated that gallic acid suppressed diarrhea, but it may not be able to reverse appetite loss in this model. Of note, we found that PAS-positive goblet cells were increased in DSS-induced colitis. This effect is known as an inducible nitric oxide synthase (iNOS)-dependent compensatory mechanism to restore the compromised protective barrier in IBD by secreting mucus covering the colonic mucosal layers (Schreiber et al., 2013; Agawa et al., 1988). Our findings indicated that gallic acid treatment reduced PAS-positive goblet cells in DSS-induced colitis, which was consistent with previous studies revealing that gallic acid suppressed iNOS activity in various cell types (Umadevi et al., 2013; Shaban et al., 2024; Liu et al., 2020). In addition, we also found that gallic acid was able to significantly reduce colonic gene expression of IBD-related pro-inflammatory cytokines, including IFN-γ, TNF, IL-1β, IL-6, and IL-8 in DSS-fed mice. These data strongly suggested that gallic acid treatment was capable of attenuating inflammation and its pathogenic consequences in DSS-induced colitis mice. These findings were consistent with a previous study in which gallic acid functionally inhibited lipopolysaccharide (LPS)-induced NF-κB activity in the hippocampus of male Wistar rats with memory loss and in testicular tissues isolated from Spraque Dawley male rats with testicular inflammation (Delen et al., 2025; Dastan et al., 2024). Of note, NF-κB is considered a master regulator of pro-inflammatory cytokine expression (Liu et al., 2025a; Kannan et al., 2025; Ghiasi, 2024).

MLCK is known to mediate TNF-induced intestinal tight junction disruption, contributing to induced intestinal barrier loss (Zuo et al., 2023; Graham et al., 2019; Su et al., 2013; Chanez-Paredes et al., 2024).

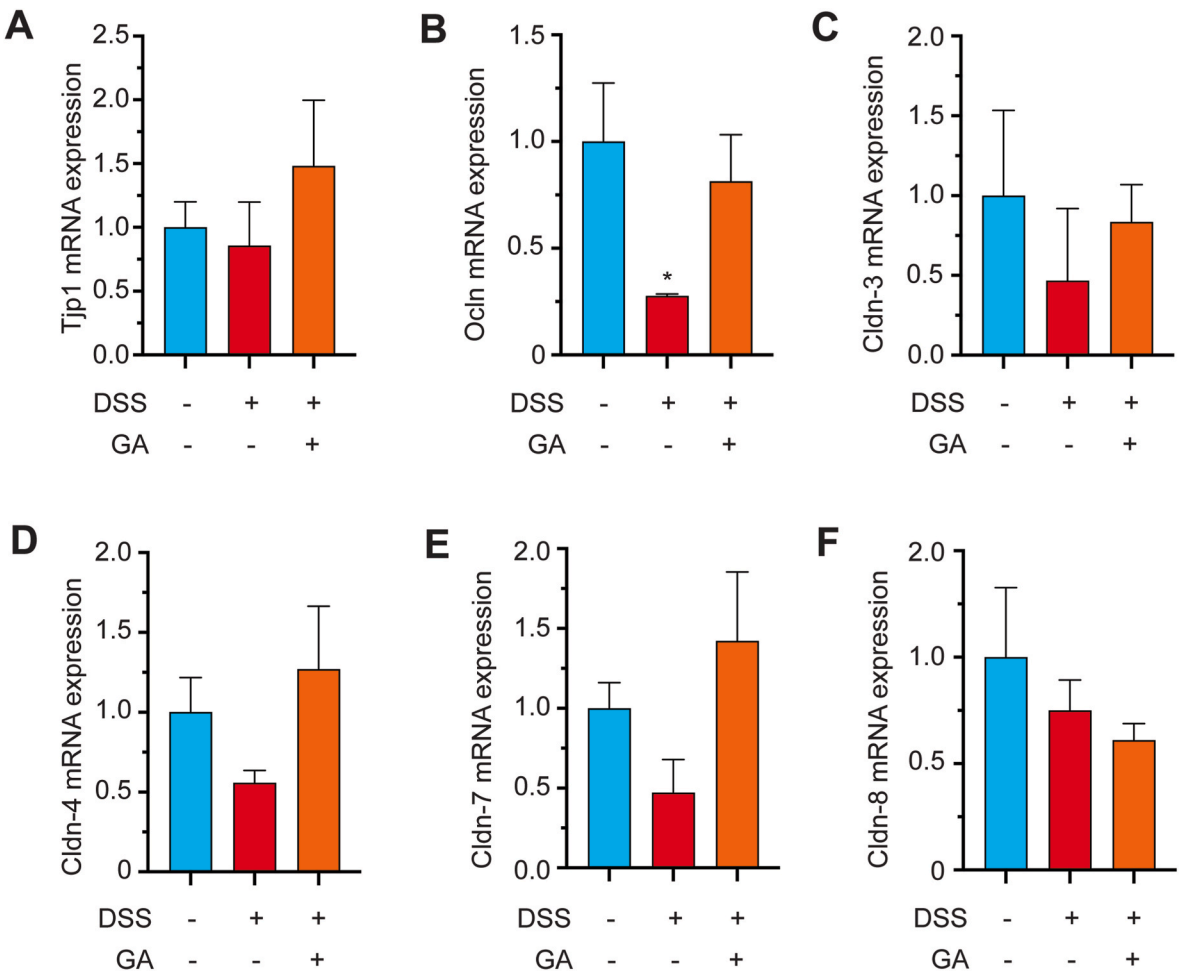

Fig. 4. Effects of gallic acid on tight junction-related transcript expression in colitis mice. The qRT-PCR indicated the colonic mRNA expression levels of (A) *Tjp1*, (B) *Ocln*, (C) *Cldn-3*, (D) *Cldn-4*, (E) *Cldn-7*, and (F) *Cldn-8* in normal and DSS-fed mice with or without gallic acid. Data were expressed as mean ± SEM (n = 6). *$p < 0.05$ compared with normal mice.

Here, we found that gallic acid treatment suppressed MLCK transcription and translation in DSS-induced colitis mice. Of note, MLCK has been reported to be a downstream signaling of NF-κB in intestinal epithelia (Wang, 2023, 2024b; Zhang et al., 2024). Therefore, gallic acid might diminish MLCK expression via inhibiting NF-κB activity, but we have not experimentally validated this hypothesis that is a limitation of this study. In addition, gallic acid improved ZO-1 and occludin protein expression in colonic tissues from DSS-induced colitis mice with stimulating AMPK/SIRT-1/ERK pathways. Indeed, LPS-mediated MLCK expression was shown to induce ZO-1 and occludin degradation (Liang et al., 2024). We found that gallic acid was able to maintain stability of ZO-1 and occludin expression that is possibly due to the inhibitory effect of gallic acid on MLCK expression. Our data suggested that enhanced paracellular permeability in our experimental colitis model was mediated mainly by tight junction-dependent paracellular permeability. Interestingly, gallic acid significantly suppressed mucosal-to-serosal permeability of 4-kDa FITC-dextran in DSS-fed mice (Fig. 6A), indicating that gallic acid inhibited leak pathway permeability. Hence, we found upregulation of all intracellular signaling related to our previous *in vitro* results. It is possible that gallic acid suppressed tight junction-dependent colonic mucosal leak pathway permeability by inhibiting inflammation and upregulating tight junction proteins, at least in part, by stimulating AMPK/SIRT-1/ERK-dependent mechanisms. These findings were consistent with our previous study in intestinal epithelial-like T84 cells (Wachiradejkul et al., 2024). Indeed, gallic acid was reported to activate AMPK/SIRT-1 pathway in liver, muscle, and brown adipose tissue, resulting in preventing insulin resistance and suppressing diet-induced body weight gain in mice (Doan et al., 2015). Of particular importance, AMPK/SIRT-1 pathway has been widely accepted as drug targets for colitis since activation of these enzymes exhibited both anti-inflammatory and antioxidant effects, not only in colonocytes, but also in various cell types (Gu et al., 2025; Chen et al., 2025; Yang et al., 2025; Singla et al., 2023). In contrast, the roles of ERK as therapeutic target for colitis are currently controversial. For example, previous study suggested that inhibition of ERK in macrophages, being considered as an upstream positive regulator of NF-κB, was proposed as a strategy to attenuate severity of colitis, at least in part, by suppressing intestinal inflammation (Liu et al., 2025b). On the other hands, activation of ERK in intestinal epithelial cells suppressed apoptosis and inflammation in DSS-induced colitis mice (Vukelic et al., 2020), contributing to attenuating IBD-related clinical symptoms. Based on our previous findings, the activation of ERK also promoted intestinal tight junction assembly in T84 cell monolayers. In addition, ERK has been demonstrated to be important for the proliferation and differentiation processes of intestinal epithelial cells, possibly leading to healing and recovery of inflammation-associated intestinal lesions (Konno et al., 2019). Therefore, ERK activation may produce the beneficial impacts on intestinal health and may also serve as a therapeutic target for IBD. On the other hand, ERK signaling was reported to be crucial for the differentiation and polarization of macrophage, resulting in promoting inflammatory cytokine releases (Li et al., 2024; Lee et al., 2023). Indeed, the effect of ERK activation in macrophages may worsen the clinical

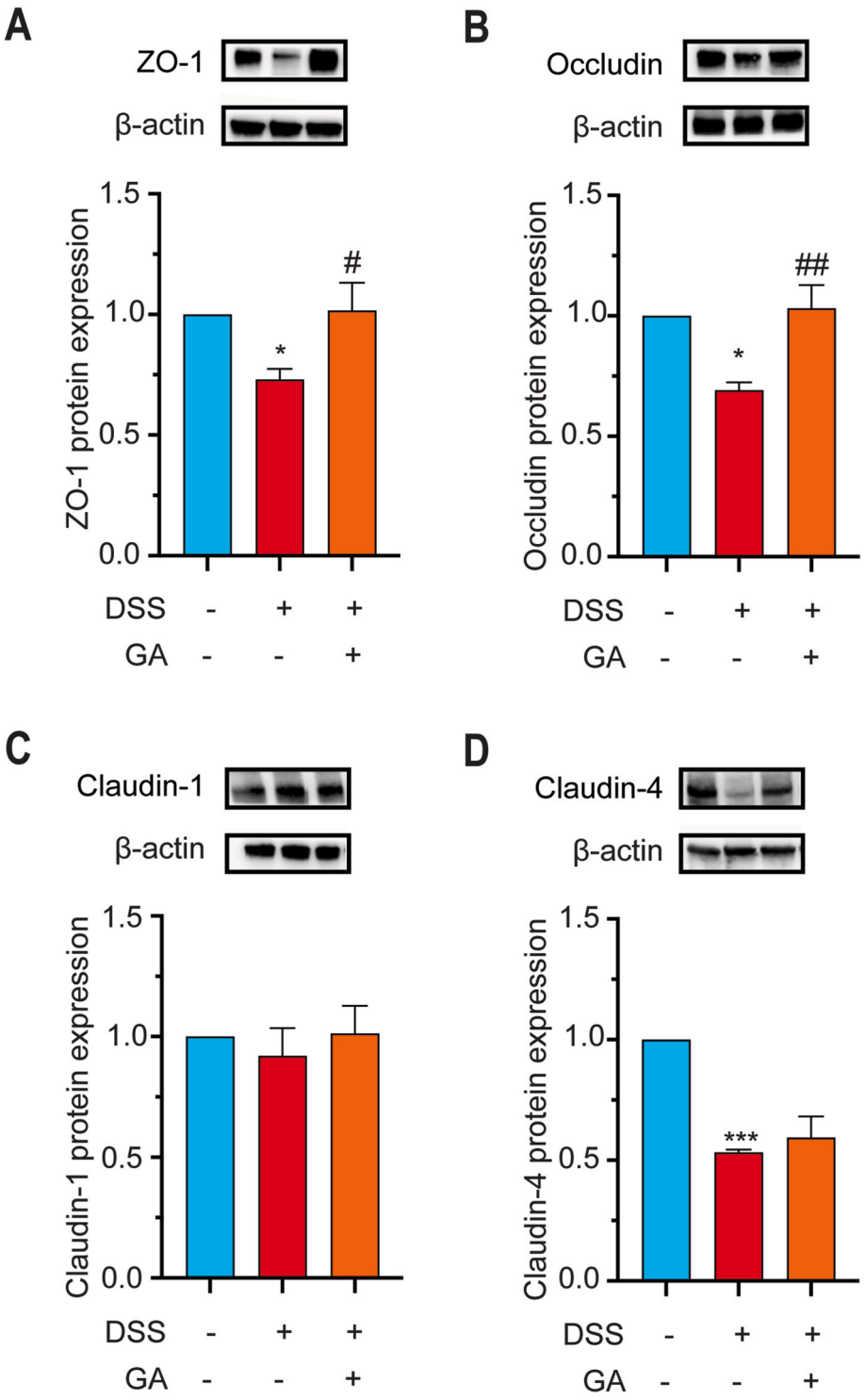

Fig. 5. Effects of gallic acid on tight junction protein expression in colitis mice. Western blot analyses revealed the colonic protein expression levels of (A) ZO-1, (B) occludin, (C) claudin-1, (D) claudin-4 in normal and DSS-fed mice with or without gallic acid. Data were expressed as mean ± SEM (n = 6). *$p < 0.05$; ***$p < 0.001$ compared with normal mice. #$p < 0.05$; ##$p < 0.01$ compared with DSS-treated mice.

symptoms of IBD patients. Therefore, the effect of pharmacological activation of ERK may be either organ- or cell type-specific.

All experimental data conducted in this study were restricted to a mouse model of colitis induced by DSS. Of note, DSS-induced colitis model recapitulates epithelial injury and innate immune activation in a late stage of IBD. Therefore, effects of gallic acid should also be investigated in other colitis models including IL-10 receptor knock-out mice and T-cell transfer-induced colitis that better represent chronic inflammation and adaptive immunity in the future. Although mouse models can represent *in vivo* environments that have microbiota and enteric nervous system, they may not fully mimic human-relevant internal environment and phenotypes. To complete this gap, effects of gallic acid on intestinal tight junction assembly and its anti-inflammatory impacts should also be performed in human colonoids. Furthermore, gallic acid derivatives should be tested on these perspectives as well. Lastly, this study mainly focused on the effects of gallic acid

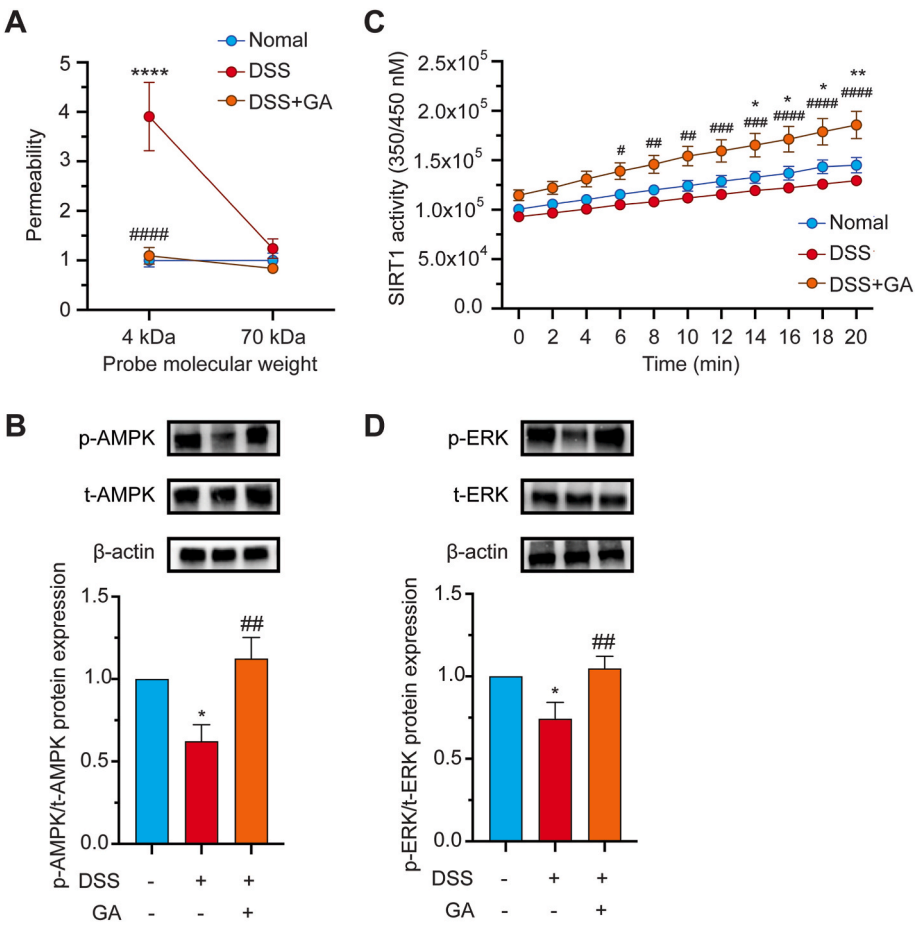

Fig. 6. Effects of gallic acid on intestinal mucosal-to-serosal paracellular permeability and expressions of its related intracellular signaling. (A) Mucosal-to-serosal permeability rate of FITC-dextran (4-kDa) and rhodamine-dextran (70-kDa) in normal and DSS-fed mice with or without gallic acid treatment. (B) Western blot analysis of ratio of phosphorylated AMPK to total AMPK in colonic tissues of normal and DSS-fed mice with or without gallic acid treatment. (C) Comparison of colonic SIRT-1 enzymatic activity in normal and DSS-fed mice with or without gallic acid treatment. (D) Western blot analysis of ratio of phosphorylated ERK to total ERK in colonic tissues of normal and DSS-fed mice with or without gallic acid treatment. Data were expressed as mean ± SEM (n = 6). *$p < 0.05$; **$p < 0.01$; ****$p <$ 0.0001 compared with normal mice. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$; ####$p <$ 0.0001 compared with DSS-treated mice.

on tight junction-dependent leak pathway permeability or barrier-forming tight junction defects. Conversely, claudin-2-dependent pore pathway permeability was reported to contribute to pathogenesis of T-cell transfer-induced experimental colitis (Raju et al., 2020), and its expression level was positively correlated with disease severity of IBD patients (Villanacci et al., 2025). Nevertheless, its effect on claudin-2-mediated, pore pathway permeability or overexpression of claudin-2 tight junction cation channel is currently unknown. Lastly, for clinical translation of these findings, effectiveness, pharmacokinetics, bioavailability, and safety considerations of gallic acid treatment in human should be concerned and are necessary for the potential use of this bioactive compound in the treatment of IBD.

5. Conclusion

We explore the pharmacological effects and possible mechanism of action of gallic acid on DSS-induced colitis mouse models and furnish a proof-of-concept that gallic acid is capable of reducing disease progression of experimental colitis. This study can lead to successful development of gallic acid as a cost-effective small molecule for IBD patients in the future.

CRediT authorship contribution statement

Apiwan Arinno: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Validation, Writing – original draft. **Pichayapa Sukmak:** Methodology, Investigation. **Purisha Kulworasreth:** Methodology, Investigation. **Thaniya Sricharunrat:** Conceptualization, Methodology, Investigation, Data curation, Validation. **Chutima S. Vaddhanaphuti:** Resources, Supervision. **Pawin Pongkorpsakol:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Ethics approval and consent to participate

All experiments in this study used C57BL/6 male mice. Indeed, C57BL/6 mice were obtained from the Nomura Siam International Co., Ltd. And maintained at the Central Animal Facility (MUSC-CAF) Faculty of Science, Mahidol University in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC) (Protocol No. MUSC66-055-685). No human studies have been conducted in this study.

Availability of data and materials

Data will be made available upon reasonable request.

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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.crphar.2025.100223.

Data availability

Data will be made available on request.

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