



## Original Article

# *Rumex japonicus* Houtt. alleviates dextran sulfate sodium-induced colitis by protecting tight junctions in mice

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## ABSTRACT

**Background:** *Rumex japonicus* Houtt. (RJ) is widely distributed in Korea, Japan, and China. The root of RJ has traditionally been used to treat constipation, jaundice, hematemesis, dysfunctional uterine bleeding, and gastrointestinal diseases. According to recent studies, plants of the genus *Rumex* have beneficial functionalities such as anti-microbial, antioxidative, and anti-inflammatory effects. Inflammatory bowel disease, including Crohn's disease and ulcerative colitis, is a chronic inflammatory disease characterized by an abnormal immune response and epithelial barrier dysfunction. This study evaluates the protective effect of RJ against dextran sulfate sodium (DSS)-induced colitis.

**Methods:** Male 8-week-old C57BL/6N mice were treated with methanolic extract of RJ for 14 days, and DSS-induced groups were administered 2.5% DSS for last 7 days. After sacrifice, the length and weight of the colon were measured, and colon sections were subjected to H&E staining, immunohistochemistry and Western blotting to investigate the changes of inflammatory cytokines, tight junction and apoptosis-related factors.

**Results:** The colon of DSS-treated mouse was significantly shorter and heavier than the normal mouse. Moreover, DSS exposure induced an increase of tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, occludin, zonula occludens-1, p21, p53 and Bcl-2, and decreased the expressions of IL-10, claudin-2 and cleaved caspase-3 in the colon tissue. These DSS-induced changes were inhibited by RJ treatment.

**Conclusion:** Our results indicate that RJ effectively suppresses DSS-induced colitis by protecting tight junction connections in the colonic tissue. We therefore infer that RJ has the potential as a medicine or ingredient for treating colitis.

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## 1. Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis (UC), is a chronic inflammatory disease characterized by an abnormal immune response, epithelial barrier dysfunction, enterohemorrhage, diarrhea, and abdominal pain.<sup>1-3</sup> The pathogenesis and etiology of IBD are not clearly elucidated, but recent studies have indicated that an abnormal mucosal immune system and gut barrier dysfunction are major factors.<sup>4,5</sup>

While IBD is frequently known to occur in Europe and North America, the incidence has recently increased in Asia because of industrialization and westernization.<sup>6</sup> Environment, food intake, genetic problems, microbiome, and immune responses are considered as major factors contributing to IBD.<sup>7</sup> Various pharmaceuticals, including immunosuppressive drugs, antibiotics, sulfasalazine and 5-aminosalicylic acid (5-ASA) drugs, are administered for the treatment of IBD, but these drugs have numerous accompanying side effects such as abdominal pain, headache and diarrhea.<sup>8-10</sup> Recently, patients wanting to minimize side effects have preferred alternative medicines, natural drugs, or functional foods; especially, the interest in herbal medicines has increased as they are known to have low toxicity<sup>11</sup> and are cheaper than Western medicine.<sup>12</sup>

A perennial herb, *Rumex japonicus* Houtt. (RJ) is widely distributed in Korea, Japan, and China. The yellow colored root of RJ

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has traditionally been used to treat jaundice, hematemesis, dysfunctional uterine bleeding, dermatopathy, and digestive system diseases such as constipation.<sup>13,14</sup> The genus *Rumex* contains a large number of anthraquinones, oxanthrones, and flavonoids;<sup>15,16</sup> the anthraquinones especially contribute to the bioactivities such as anti-microbial, purgative, antioxidative, and anti-inflammatory effects.<sup>14,17</sup> The RJ have long been used for digestive system diseases in Korea, however, its effects have not been scientifically proven. Therefore, the present study evaluates the effects of RJ in the colon of dextran sulfate sodium (DSS)-induced UC mouse model, focusing on several changes in immune activity, epithelial barrier, and apoptosis-related factors. In addition, we used 5-ASA as a positive control to compare the effects of RJ.

## 2. Methods

### 2.1. Sample preparation

The dried root of RJ was purchased from GwangMeongDang Medicinal Herbs (Uusan, Korea). The herb was harvested in Gyeongsanbukdo, South Korea in 2015. The crude material was authenticated by Prof. Hyungwoo Kim (Division of Pharmacology, Pusan National University, School of Korean Medicine, Republic of Korea). A total of 100 mg RJ was immersed in 500 mL of methanol and sonicated for 15 min, followed by extraction at room temperature for 24 h. The resultant supernatant was transferred, and fresh RJ was again extracted with 500 mL of methanol for 24 h. The two extracts were then combined and filtered through Whatman filter paper (No. 20, Toyo Roshi Kaisha, Tokyo, Japan), after which the filtered extract was evaporated under reduced pressure using a vacuum evaporator. Next, the concentrated extract was lyophilized using a freeze dryer (Labconco, Kansas City, MO, USA), which produced 1.27 g of lyophilized powder (yield, 12.74%). A sample of the specimen was deposited in the herbarium at the Division of Pharmacology, School of Korean Medicine, Pusan National University (Voucher no. MH2012-006, Supplementary data 1). Freeze-dried RJ was resolved in water for every single oral administration (p.o.).

### 2.2. Animal study design

Male C57BL/6N mice were obtained from Samtaco Bio Korea (Osan, Korea) at 5 weeks of age. Animals were maintained under controlled conditions (RH 55 ± 5%, 22 ± 2 °C, 12-h light/dark light cycle) with feed and drinking water supplied *ad libitum*. The study protocol was approved by the Institutional Animal Care and Use Committee of Pusan National University (Busan, Korea, approval number PNU-2018-1847).

The animals were adapted for 1 week, then randomly divided into 4 groups: normal, DSS, RJ and 5-ASA. Mice in the normal and DSS groups were administered the vehicle (water), and the animals in RJ and 5-ASA groups were administered RJ (100 mg/kg) or 5-ASA (75 mg/kg) for 14 days (p.o.). The proper administration doses of RJ and 5-ASA were determined by pilot test and references.<sup>18–20</sup>

Mice in the DSS and RJ groups were subsequently given 2.5% DSS (w/v; MP Biomedical, Solon, OH, USA) in drinking water, from 8th day to 14th day. All mice were euthanized by isoflurane and sacrificed, after a 12 h fasting period.

### 2.3. Hematoxylin and eosin (H&E) staining

Colon tissues were harvested and fixed in 10% (v/v) neutral buffered formalin, followed by embedding in paraffin. Sections were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA), dehydrated, and mounted. Images were acquired using

an Axio Scope A1 microscope (Carl ZEISS, Oberkochen, Germany) equipped with an AxioCam ICC3 camera (Carl ZEISS).

### 2.4. Immunohistochemistry

Sections of colon tissue were incubated with 3% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS) for 15 min, and washed with PBS. Next, sections were blocked with 1% BSA at room temperature for 30 min, followed by incubation with anti-occludin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-zonula occludens (ZO)-1 (Santa Cruz Biotechnology) primary antibody, overnight at room temperature. Sections were then incubated with Dako envision kit (Dako, Glostrup, Denmark) at room temperature for 1 h, and subsequently incubated with a diaminobenzidine substrate kit (Dako) for 1–2 min. The sections were counter stained using Mayer's hematoxylin solution (Sigma-Aldrich), and then dehydrated and mounted. Histological images were acquired using an Axio Scope A1 microscope (Carl ZEISS) and AxioCam ICC3 camera (Carl ZEISS).

### 2.5. Western blot

Colon tissues were lysed with RIPA buffer (Invitrogen Life Technologies, Carlsbad, CA, USA) and centrifuged at 13,000 rpm for 15 min at 4 °C. Total protein concentration was determined using a BioRad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electrotransferred to a 0.45 μm nitrocellulose blotting membrane (GE Healthcare UK Ltd, Little Chalfont, UK). The blots were incubated overnight at 4 °C with anti-tumor necrosis factor (TNF)-α, anti-interleukin (IL)-1β, anti-IL-6, anti-IL-10, anti-occludin, anti-ZO-1, anti-claudin-2, anti-cyclooxygenase (COX)-2, anti-p21, anti-p53, anti-Bcl-2, anti-caspase-3 or anti-actin primary antibody, while immersed in 5% skim milk or 5% bovine serum albumin. The probed blots were subsequently incubated with secondary antibodies for 1 h at room temperature. The membranes were then washed three times with PBS containing 0.05% (v/v) Tween 20, and visualized with enhanced chemiluminescence reagent (Thermo Fisher Scientific Inc., Rochford, IL, USA). All primary antibodies for Western blotting were purchased from Santa Cruz Biotechnology, except anti-TNF-α (Cell Signaling Technology Inc., Beverly, MA, USA), anti-IL-10 (Abcam, Cambridge, UK), and anti-COX-2 (Abcam). The blots were quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA, [www.imagej.nih.gov](http://www.imagej.nih.gov)).

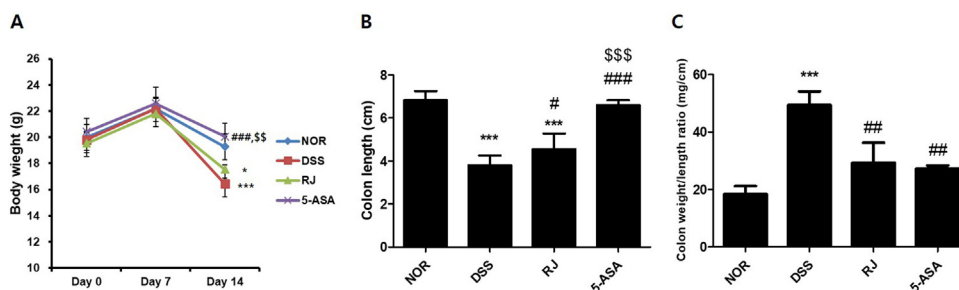
### 2.6. Statistical analysis

All data are presented as the means ± standard deviation. Differences between the mean values are assessed by one-way analysis of variance with Tukey's multiple-range tests using Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Differences with a *p* < 0.05 are considered statistically significant.

## 3. Results

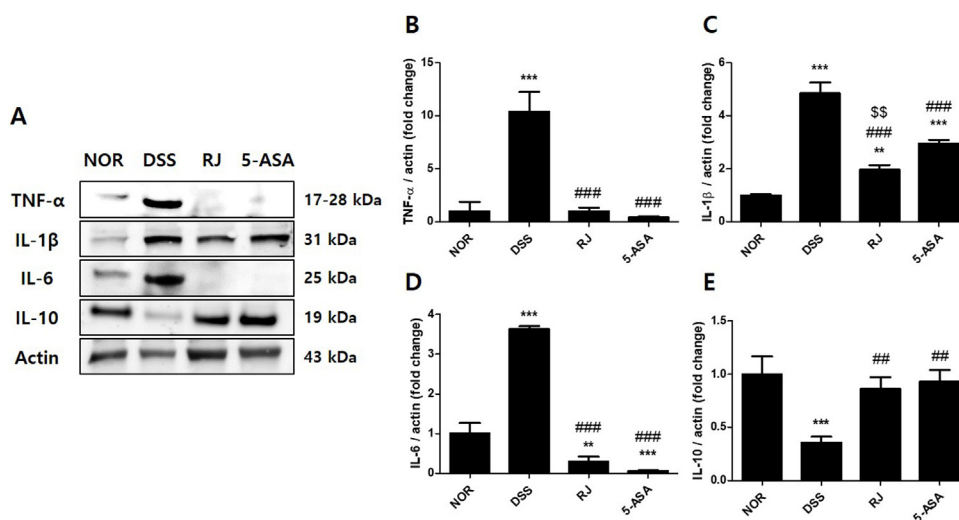
### 3.1. Effects of DSS and RJ on body weight, colonic length, and colon weight/length ratio

Body weights were measured on days 0, 7, and 14. Decreased body weight was observed in all groups on day 14, due to the fasting period before sacrifice. Mice in the DSS-treated groups (DSS and RJ) showed a significant decrease in body weight as compared to mice in the normal group; however, no significant difference was observed between mice in the DSS and the RJ groups. Meanwhile, 5-ASA treated group showed higher bodyweight than the normal group (Fig. 1A).



**Fig. 1.** Effects of dextran sulfate sodium and *Rumex japonicus* Houtt. on body weight, colonic length, and colon weight/length ratio.

(A) Dextran sulfate sodium (DSS) significantly decreases body weights, regardless of *Rumex japonicus* Houtt. (RJ) treatment. (B) Treatment with DSS results in decreased colon length, which is prevented by RJ treatment. (C) DSS significantly increases colonic weight/length ratio, and RJ significantly inhibits the edematous change. Values are the means  $\pm$  SD ( $n=6$ ). \* $p < 0.05$  and \*\*\* $p < 0.001$  versus normal group, # $p < 0.05$ , ## $p < 0.01$ , and ### $p < 0.001$  versus DSS group, \$\$\$ $p < 0.001$  versus RJ group.



**Fig. 2.** Changes in inflammatory cytokines in the colon of DSS- or RJ-treated mouse.

Changes in inflammatory cytokines were confirmed by Western blotting (A). DSS treatment significantly increases the levels of TNF- $\alpha$  (B), IL-1 $\beta$  (C) and IL-6 (D) and decreases the level of IL-10 (E) in the colon, which is suppressed by RJ treatment. Values are the means  $\pm$  SD ( $n=3$ ). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus normal group, ## $p < 0.01$  and ### $p < 0.001$  versus DSS group, \$\$ $p < 0.01$  versus 5-ASA group. TNF, tumor necrosis factor. IL, interleukin.

Colon length reduction is a typical change due to inflammation in a DSS-treated mouse. Colon length in the DSS group ( $3.80 \pm 0.40$  cm) was significantly shorter than that obtained in the normal group ( $6.82 \pm 0.39$  cm). However, RJ ( $4.56 \pm 0.65$  cm,  $p < 0.05$ ) and 5-ASA ( $6.74 \pm 0.42$  cm,  $p < 0.001$ ) treatment prevented shortening of the colon length (Fig. 1B). Colonic weight/length ratio in the DSS group was significantly higher ( $49.44 \pm 3.80$  mg/cm) than that obtained in the normal group ( $18.38 \pm 2.29$  mg/cm), due to the presence of edematous changes. However, treatment with RJ ( $29.33 \pm 5.69$  mg/cm) and 5-ASA ( $27.33 \pm 0.84$  mg/cm) significantly prevented the edematous change ( $p < 0.01$ ; Fig. 1C).

### 3.2. Effect of RJ in inflammatory cytokines in the colon of DSS-treated mouse

Protein expressions of pro- or anti-inflammatory cytokines in the colon tissues are presented in Fig. 2. In the DSS group, the expressions of pro-inflammatory cytokines TNF- $\alpha$  ( $p < 0.001$ ), IL-1 $\beta$  ( $p < 0.001$ ) and IL-6 ( $p < 0.001$ ) are significantly greater than those in the normal group, whereas the expressions are significantly suppressed in the RJ group, when compared to the DSS group ( $p < 0.001$  for all cytokines, Fig. 2A–D). The expression of the anti-inflammatory cytokine IL-10 in the DSS group are lower than levels obtained in the normal group ( $p < 0.001$ ), but are significantly higher in the RJ group than in the DSS group ( $p < 0.01$ , Fig. 2A, E). The levels of TNF- $\alpha$ , IL-6 and IL-10 in the RJ group are similar to

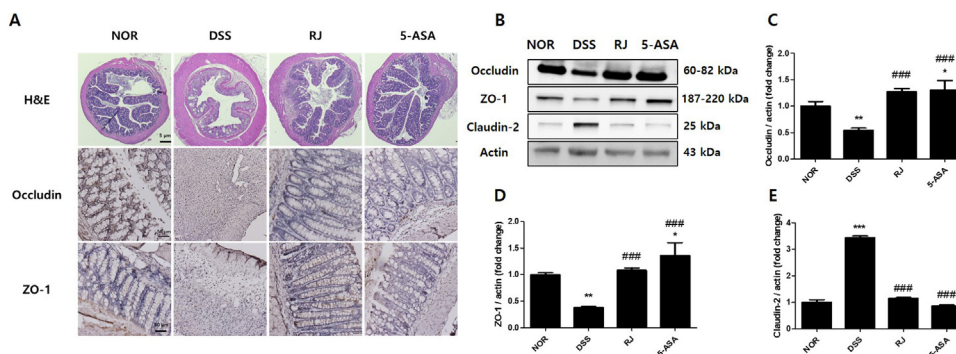
those in the 5-ASA group (Fig. 2A, B, D, E), however, the level of IL-1 $\beta$  is significantly lower in the RJ group than in the 5-ASA group ( $p < 0.01$ , Fig. 2A, C).

### 3.3. Effect of RJ in DSS-mediated weakness of tight junction in the colon

Previous studies have reported structural disruption, ulceration, and widespread inflammatory cell infiltration in the colon of DSS-induced colitis mouse model. In the present study, erosive lesions, crypt loss, and severely transformed cells in mucous layers were observed in the DSS group, but these DSS-induced changes were suppressed in the RJ and the 5-ASA groups. The structure of the colonic cells and villi were disrupted due to DSS treatment, which was observed to be reduced after RJ or 5-ASA treatment (Fig. 3A). Moreover, DSS treatment significantly suppressed the levels of occludin ( $p < 0.001$ ) and ZO-1 ( $p < 0.05$ ) and increased the level of claudin-2 ( $p < 0.05$ ) in the colon; however, RJ or 5-ASA treatment significantly prevented alterations of the levels ( $p < 0.001$ ,  $p < 0.01$  and  $p < 0.05$ , respectively; Fig. 3B–E).

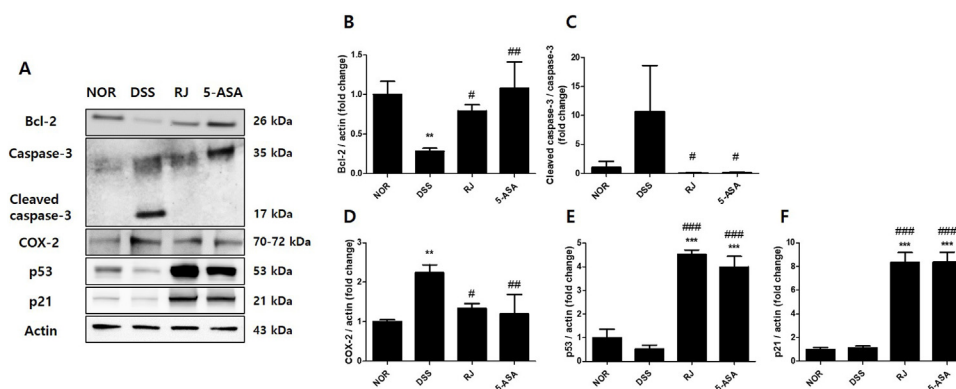
### 3.4. Anti-apoptotic effects of RJ in DSS-induced colon

Changes in apoptosis-related factors in the colon are presented in Fig. 4. The Bcl-2 expression in the DSS group is significantly decreased compared to that in the normal group ( $p < 0.01$ ), but



**Fig. 3.** Histological changes and expressions of tight junction-related proteins in the colon.

(A) Hematoxylin and eosin (H&E) staining and immunohistochemical staining of occludin and ZO-1 in the colon. RJ suppresses the DSS-induced erosive lesions, crypt loss, and severely transformed cells in the mucous layers. Moreover, RJ suppresses the DSS-induced decrease expressions of occludin and ZO-1 in the colon. (B) Confirmation of changes in occludin, ZO-1, and claudin-2 expressions in the colon. RJ treatment significantly prevents the DSS-induced decrease of occludin (C) and ZO-1 (D), and increase of claudin-2 (E) in the colon. Values are the means  $\pm$  SD ( $n=3$ ). Scale bar indicates 5  $\mu$ m in H&E staining, and 50  $\mu$ m in immunohistochemistry staining. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  versus normal group, ### $p < 0.001$  versus DSS group. ZO, zonula occludens.



**Fig. 4.** Changes in apoptosis-related proteins in the colon.

Changes in apoptosis-related proteins were confirmed by Western blotting (A). DSS induces the decrease of Bcl-2 (B) and increase of cleaved caspase-3 (C) in the colon, whereas RJ alleviates these changes. DSS also induces alterations in factors of colitis-associated colorectal cancer, namely, increased levels of COX-2 (D) and decreased levels of p21 (E) and p53 (F). These changes are also suppressed by RJ. Values are the means  $\pm$  SD ( $n=3$ ). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus normal group, # $p < 0.05$ , ## $p < 0.01$ , and ### $p < 0.001$  versus DSS group. COX, cyclooxygenase.

is significantly increased in the RJ and the 5-ASA groups as compared to the DSS group ( $p < 0.05$  and  $p < 0.01$ , respectively, Fig. 4A, B). DSS treatment activates caspase-3, but RJ or 5-ASA treatment significantly inhibits the activation ( $p < 0.05$  at each group, Fig. 4A, C).

The expression of COX-2 is significantly increased in the DSS group compared to the normal group ( $p < 0.01$ ); however, these expressions are significantly lower in the RJ and the groups as compared to the expressions obtained in the DSS group ( $p < 0.05$  and  $p < 0.01$ , respectively, Fig. 4A, D). In addition, RJ or 5-ASA treatment significantly increases the expressions of p53 and p21 than normal and DSS groups ( $p < 0.001$  at each group, Fig. 4A, E, F).

#### 4. Discussion

The present study demonstrates that RJ effectively suppresses DSS-mediated histological change, inflammation, TJ weakness, and apoptosis in the colon, using a DSS-induced UC mouse model. The DSS-induced colitis model is a widely used experimental method for understanding various colonic diseases including UC, inflammation, and tight junction (TJ) destruction. In general, DSS-treated mice show loss of bodyweight, diarrhea, and bloody excrement<sup>21,22</sup>; the colon tissue of the DSS-treated mouse is shorter and heavier than normal control due to the DSS-induced inflammation in the colon,<sup>23</sup> resulting in a higher weight/length ratio

in DSS-treated mice. In this study, diarrhea, bloody excrement, bodyweight loss, shortening of colon length, and increase of colon weight were experienced after DSS treatment, similar to previously reported studies. However, diarrhea and bloody excrement were not observed in RJ-administrated mice (data not shown), and the colon tissues of RJ-treated mice were significantly longer and lighter than the DSS-treated mice, thereby indicating that RJ suppresses the DSS-induced colitis.

In colitis-induced mice, immune cells and epithelial cells in the gut produce much of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6.<sup>24,25</sup> In this study, DSS treatment increased the expressions of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, which were significantly suppressed after exposure to RJ. Moreover, DSS significantly suppressed the expression of anti-inflammatory cytokine IL-10 in the colon, which was significantly alleviated by RJ treatment. These results indicate that DSS induces an inflammatory reaction in the mouse colon, and RJ effectively inhibits the reaction.

TJs are intercellular junctions adjacent to the apical ends of paracellular spaces.<sup>26</sup> The major role of TJ proteins is maintaining intact intestinal epithelium.<sup>27</sup> Under inflammatory conditions, a compromised TJ barrier increases paracellular permeability and triggers intestinal damages such as erosion, ulceration, and apoptosis. In addition, inflow of immune cells into the intestinal mucosa through a permeable TJ barrier stimulates the release of pro-inflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$  in the mucosa, which even-



tually worsens the inflammatory condition.<sup>27</sup> This negative cycle (inflammation-TJs reduction-leaky gut-inflammation) is observed in IBD patients.<sup>27</sup>

TJs are composed of several proteins such as occludin, claudins, junctional adhesion molecules, and ZO proteins.<sup>26</sup> Occludin regulates the paracellular permeability by binding ZO-1,<sup>26</sup> which mediates protein-protein interactions and links to the actin cytoskeleton.<sup>26</sup> Among the claudins, claudin-2 is mostly increased in leaky epithelial cells, and is known as a channel-forming TJ protein permeable to water and small cations.<sup>28,29</sup> IBD induces the decrease of ZO-1 and occludin, and increases the expression of claudin-2.<sup>26,30</sup> Several studies have shown that intestinal inflammation is closely associated with decreased ZO-1 and occludin expressions in both humans and animals having IBD,<sup>31–33</sup> and increased levels of claudin-2 in IBD-associated dysplasia and sporadic adenomas as compared to non-dysplastic IBD.<sup>34</sup> In addition, pro-inflammatory cytokines induce an alteration of TJ proteins, apoptosis, and bacterial translocation in the IBD affected gut.<sup>35</sup> Among the cytokines, TNF- $\alpha$  is especially associated with TJs and epithelial barrier functions,<sup>26</sup> and TNF- $\alpha$  therapeutics (such as etanercept and infliximab) attenuate changes of ZO-1, occludin, and claudin-2 expressions in colitis-induced mice.<sup>36</sup> In this study, RJ treatment effectively mitigated the abnormalization of TJs by regulating the expressions of ZO-1, occludin, and claudin-2 (Fig. 3), which may be related to inhibition of pro-inflammatory cytokines, especially TNF- $\alpha$  (Fig. 2A, B).

A recent study has reported that increase of apoptosis and decrease of normal cell proliferation results in abnormalization of the epithelial barrier function in colon of DSS-treated mouse.<sup>37</sup> Bcl-2 is an anti-apoptotic protein, and caspase-3 plays a major role in the apoptotic pathway. In this study, exposure to RJ treatment resulted in suppression of apoptosis by controlling Bcl-2 and caspase-3 activities, indicating that the anti-apoptotic effect of RJ also contributes to maintain TJs in the colon.

Chronic colitis causes neoplasia and adenocarcinoma, leading to the progression of colitis-associated colorectal cancer (CAC). UC patients have an 8-times higher risk for developing colon cancer than normal people, and the cumulative risk for CAC rises continuously with an extended duration of colitis.<sup>38</sup> Both COX-2 and p53 are affected by pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6,<sup>39,40</sup> and overexpression of COX-2 and loss of p53 may result in cancer.<sup>38,41,42</sup> In colon cancer, the expression of COX-2 is increased in premalignant lesions, thereby increasing the possibility of a transition to malignancy of the colon.<sup>43</sup> The tumor suppressor p53 is related to cell cycle arrest and apoptosis,<sup>44</sup> and the cyclin-dependent kinase (CDK) inhibitor p21 is controlled by p53. Thus, p53 and p21 suppress the G1 to S phase transition of the cell cycle, and inhibit proliferation of abnormal cells in cancer progression.<sup>45</sup> According to a recent report, the risk for CAC can be reduced by early diagnosis, by confirming the p53 and p21 activities.<sup>46</sup> In the present study, because of the mice not yet go to the carcinogenesis, there may not be significant change between normal and DSS group in p53 and p21. However, RJ effectively regulates the activation of CAC related factor COX-2, and enhances p53 and p21 (Fig. 4A, D–F), indicating that RJ not only treats colitis but also prevents it from advancing to CAC.

Taken together, the present study determines that RJ suppresses DSS-induced colitis in mice. DSS-mediated histological change, inflammation, TJ weakness, and apoptosis in the colon are suppressed after exposure to RJ, indicating that RJ prevents colitis by protecting the TJs. Based on these results, we believe that RJ has potential use in the prevention or treatment of colitis. RJ may be a good medicine to the people who want to be treated by Korean medicine or who have side effects of chemical medicine such as 5-ASA. However, the clinical effect and the key compounds of RJ are

still elusive. Therefore, further studies are required to find the key compounds responsible for exerting the efficacy of RJ using high performance liquid chromatography and the clinical evidence of RJ on colitis.

### Author contributions

Conception: HYK. Methodology: HK and SK. Investigation: HYK and HK. Analysis: HJ, CHB, YL. Writing - Original Draft: HYK. Writing - Review & Editing: HYK, HK and SK. Supervision: SK.

### Conflict of interest

The authors declare no conflict of interest.

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### Ethical statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Pusan National University (Busan, Korea, approval number PNU-2018-1847).

### Data availability

All data used to support the findings of this study are included in the article.

### Supplementary material

Supplementary information on the dried root of *Rumex japonicus* Houtt can be found, in the online version at doi:<https://doi.org/10.1016/j.imr.2020.02.006>.

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