

Prophylactic effects of *Lonicera japonica* extract on dextran sulphate sodium-induced colitis in a mouse model by the inhibition of the Th1/Th17 response

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Abstract

Inflammatory bowel diseases (IBD) are chronically relapsing inflammatory disorders of the intestine. Although some therapeutic agents, including steroids, are available for the treatment of IBD, these agents have limited use. Therefore, dietary supplements have emerged as possible interventions for IBD. Japanese honeysuckle flower, the flower of *Lonicera japonica*, is a well-known dietary supplement and has been used to prevent or treat various inflammatory diseases. In the present study, we investigated the effects of *L. japonica* on experimental murine colitis. Colitis was induced by 5% dextran sulphate sodium (DSS) in Balb/c mice. The water extract of *L. japonica* (LJE) at doses of 20, 100 or 500 mg/kg was orally administered to mice twice per day for 7 d. Body weight, colon length and a histological damage score were assessed to determine the effects on colitis. Cytokine profiles were assessed to examine the effects on helper T (Th) cell-related immunological responses. In addition, CD4⁺CD25⁺Foxp3⁺T cells were analysed *in vivo* and *in vitro* for investigating the effects on regulatory T (T_{reg}) cells. LJE showed dose-dependent inhibitory effects against colon shortening, weight loss and histological damage. LJE down-regulated IL-1β, TNF-α, interferon-γ, IL-6, IL-12 and IL-17. However, LJE did not show any significant effects on IL-10, IL-23, transforming growth factor-β1 and T_{reg} cell populations. In conclusion, LJE showed protective effects against DSS-induced colitis via the Th1/Th17 pathway and not via T_{reg} cell-related mechanisms.

Key words: Inflammatory bowel disease: *Lonicera japonica*: Dextran sulphate sodium: Regulatory T cells: Th1/Th17 pathway

Inflammatory bowel diseases (IBD) are defined as chronic inflammatory conditions of the intestine and are generally divided into Crohn's disease and ulcerative colitis⁽¹⁾. The prevalence of IBD is increasing worldwide, with recent annual increases of about 42.1 and 439 patients per 10⁵ people in South Korea and North America, respectively⁽¹⁾. Although current treatments, including non-steroidal anti-inflammatory drugs and corticosteroids, have been administered to reduce symptoms, they have potential side effects including hypersensitivity, adrenal suppression and lymphoma⁽²⁾.

Various diets or dietary ingredients such as fruits, vegetables and fibre are considered safe and are used to prevent the

initiation or aggravation of gut inflammation through immune interactions and the alteration of the gut microbiota⁽³⁾. Therefore, many studies have been performed on the use of dietary supplements and natural products as alternative therapies for IBD^(4,5).

Japanese honeysuckle flower (*Lonicera japonica* Thunb.; LJ) is a well-known dietary supplement that has long been used in Asia⁽⁶⁾. LJ is the main ingredient of the herbal formula 'Bojanggubni-tang', which has widely been used to treat IBD in Korea and has demonstrated protective effects against dextran sulphate sodium (DSS)- and trinitrobenzene sulfonic acid-induced colitis in mice⁽⁷⁾. LJ has been reported to

Abbreviations: CGA, chlorogenic acid; DSS, dextran sulphate sodium; IBD, inflammatory bowel disease; IFN-γ, interferon-γ; LJ, *Lonicera japonica*; LJE, water extract of *Lonicera japonica*; TGF-β1, transforming growth factor-β1; T_{reg}, regulatory T cell.

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have various bioactivities including anti-inflammatory⁽⁸⁾, anti-atherogenic⁽⁹⁾, anti-carcinogenic⁽¹⁰⁾, analgesic⁽¹¹⁾ and neuroprotective⁽¹²⁾ effects. It contains various compounds including chlorogenic acid (CGA), luteolin, ferulic acid, quercetin and caffeic acid that are responsible for these effects^(8–12). To our knowledge, the effect of LJ on IBD has not yet been reported.

Of the many existing animal models of IBD, chemically induced models including DSS- and trinitrobenzene sulfonic acid-induced colitis are among the most common⁽¹³⁾. DSS-induced murine colitis is well characterised by clinical symptoms (diarrhoea, bloody stools, weight loss and colon shortening), histological changes (cryptal damages, erosion, ulceration and infiltration of the inflammatory cells) and changes in inflammatory mediators including chemokines and cytokines^(13–15). Therefore, DSS-induced colitis is a well-established model for testing the effects of the suggested agents.

Cytokines, the inflammatory mediators, have a key role in innate and adaptive immune reactions. They regulate immune cell functions that trigger signalling pathways and mediate proliferation and differentiation of immune cells in inflammatory diseases including colitis⁽¹⁵⁾. Cytokines can be classified along the helper T (Th)1, Th2, Th17 and regulatory T (T_{reg}) cell spectra, and cytokines in each class are responsible for immune reactions specific to that subset⁽¹⁵⁾. Thus, it is important to identify the related cytokine changes according to the Th subsets. Acute DSS-induced colitis is considered analogous to Crohn's disease, which is known to involve the Th1/Th17/T_{reg}-dependent pathological process^(16,17).

Considering both the traditional use and bioactivities of LJ, we postulated that LJ could show preventive effects against colitis. In the present study, we investigated the effects of LJ in a DSS-induced IBD mouse model by assessing body weight, colon length and histological damage. Mechanistic studies were also conducted *in vivo* and *in vitro* by (1) assessing the promotion of specific T cell lineages, (2) profiling cytokine expression via a biometric multiplex signalling method and (3) analysing the prevalence of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells.

Materials and methods

Animals

Male Balb/c mice (7 weeks old, 20–24 g) were supplied by Daehan Bio Link for *in vivo* experiments. Foxp3^{EGFP} Balb/c (C. *Cg-Foxp3tm2Tcb/J*) mice were purchased from the Jackson Laboratory for *in vitro* experiments. Mice were housed at 20–22°C and in 40–60% humidity under a 12h light–12h dark cycle, and were provided food and water *ad libitum*. All procedures were conducted according to the animal welfare guidelines of the National Institute of Health, and the present study was approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(se)-09-036).

Sample preparation and HPLC analysis

Flowers of LJ (Caprifoliaceae, 390 g) were purchased from the Kyung Hee Hanyak Company, and extracts were prepared by

boiling the flowers in water at 100°C for 2 h. LJ water extract (LJE, yield 6.83%) was obtained by evaporating and freeze-drying. LJ (PS003) and LJE (PSE003) were deposited in the Department of Herbal Pharmacology at the College of Oriental Medicine, Kyung Hee University. For the HPLC analysis of LJE, 2 mg of LJE were dissolved in 50% methanol (1 ml) and then filtered through a 0.22 μm membrane filter (Millipore). Then, 10 μl of the sample or standard (CGA (3-*O*-caffeoylquinic acid, C₁₆H₁₈O₉), purity >95%; Sigma-Aldrich) were injected into the HPLC system (Agilent Technologies). The sample was analysed on a Capcell Pak UG120 C₁₈ analytical column (150 × 4.6 mm, 5 μm; Shiseido). The oven temperature was maintained at 35°C. The mobile phase comprised 0.1% formic acid (A) and acetonitrile–methanol–formic acid (0.1%) (45:45:10) (B); the flow rate was 1 ml/min. The gradient elution programme was used as follows: initially, A:B = 84%:16%, which was linearly changed to A:B = 60%:40% at 25 min, and finally maintained up to 27 min. At 27.5 min, the composition of the mobile phase returned to the initial conditions, which were maintained for 7.5 min for column re-equilibration. Chromatograms were acquired at 245 nm by UV detection. In the HPLC chromatogram, CGA (retention time = 11.3 min) was found to be a major component of the extract sample. The content of CGA was 10.51 (SD 0.02)% (Fig. 1).

Induction of colitis and experimental design

Colitis was induced by DSS as described previously⁽⁷⁾. Mice were provided with drinking-water containing 5% DSS (MP Biomedicals) *ad libitum* for 7 d. In the present study, we selected the dosages of LJE in consideration of standard human dosages. LJE at a dosage of 20 mg/kg in mice corresponds to 1.0 g LJE/50 kg-weighted human subject, where LJE is extracted from approximately 14.7 g of the LJ raw material; this concentration is similar to the dosage of LJE in honeysuckle drinking tea (10–15 g/d)⁽¹⁸⁾. Finally, we decided the dosages of LJE, i.e. 20, 100 and 500 mg/kg, separated by five time intervals. In the case of CGA, as a main component of LJE, we calculated the dosage using HPLC analysis. We chose a dosage of 52.5 mg/kg of CGA, which corresponded to 500 mg/kg of LJE. To investigate the effects of the co-treatment of LJE, mice were randomly allocated into five groups (*n* 8): normal, DSS and three DSS + LJE (20, 100 and 500 mg/kg) groups. To compare the effects of CGA (52.5 mg/kg) and LJE (500 mg/kg), mice were randomly allocated into four groups (*n* 8): normal, DSS, DSS + CGA 52.5 mg/kg and DSS + LJE 500 mg/kg groups. Mice were administered distilled water (10 ml/kg) in the DSS group or each dose of LJE in the DSS + LJE group by a feeding needle twice per day from day 1 to day 7. To compare the effects of delayed treatment with the co-treatment of LJE, mice were randomly allocated into three groups (*n* 8): normal, DSS and DSS + LJE 500 mg/kg groups. Mice were administered distilled water (10 ml/kg) in the DSS group or LJE 500 mg/kg in the DSS + LJE 500 mg/kg group, twice per day from day 4 to day 7.

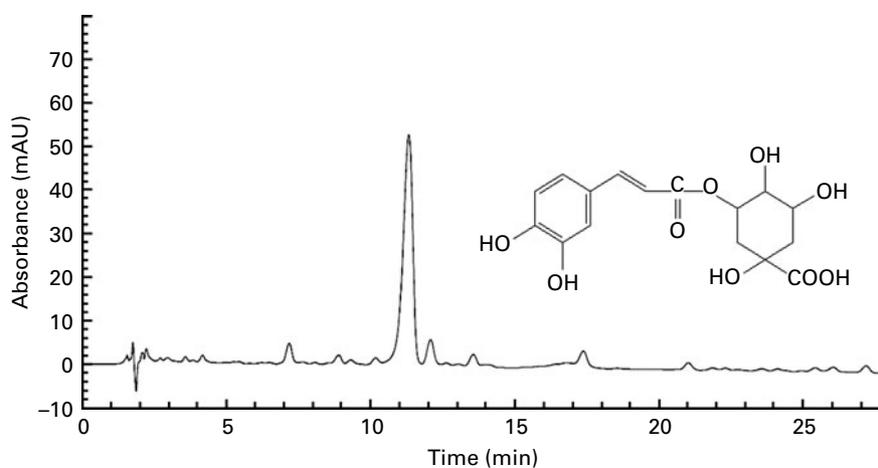


Fig. 1. A representative HPLC chromatogram of the water extract of *Lonicera japonica*. Chlorogenic acid (retention time = 11.3 min). AU, arbitrary units.

Assessment of mouse weight and colon length

Mouse weight was measured daily from day 0 to day 7. The colon was isolated immediately after the last weight check. Colon length was measured from the caecum to the anus using a vernier caliper (Mitutoyo).

Histological scoring

Colons were fixed in 10% buffered formalin and embedded in paraffin. Histological sections cut from the paraffin blocks were stained with haematoxylin and eosin. In a blind fashion, the scoring of histological damage was divided into two categories: inflammatory cell infiltration and ulceration. Inflammatory cell infiltration was graded on a scale of 0–3 (0 = none; 1 = mild; 2 = moderate; 3 = severe), obtained from each layer of the colon, including surface epithelium, cryptal glands, stroma, submucosa and transmural layer. The severity of ulceration was graded histologically on a scale of 0–4 (0 = none; 1 = mild and focal surface; 2 = mucosal layer; 3 = submucosal layer; 4 = transmural layer)^(7,19). We modified and established a 0–19-point system by using the sum of all scores.

Collection of colonic mucosa and biometric multiplex cytokine profiling

Isolated colons were snap-frozen and stored at -70°C . The mucosa was scraped from the muscle layer of the colon. Next, it was weighed, and 10 mg mucosa were dissolved in triple-detergent lysis buffer (50 mM-Tris-HCl, pH 8.0, 150 mM-NaCl, 0.1% SDS, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate and 1 mM-phenylmethylsulfonyl fluoride) and homogenised. The levels of nine cytokines covering a broad spectrum of immune and inflammatory mechanisms were measured in parallel following the induction of colitis⁽¹⁶⁾. The cytokine levels (interferon (IFN)- γ , TNF- α , IL-1 β , IL-6 and IL-12 as Th1 panels; IL-10 and transforming growth factor (TGF)- β 1 as T_{reg} panels; IL-17 and IL-23 as Th17 panels) in the mucosa samples were analysed according to the manufacturer's manual of biometric multiplex cytokine assay (Millipore).

Regulatory T cell measurement in dextran sulphate sodium-induced colitis

To investigate the effect of LJE on T_{reg} cell promotion, mice were divided into four groups (non-treated normal group, LJE 500 mg/kg group, DSS group and DSS + LJE 500 mg/kg group; n 8 for each group). Spleens were isolated and ground over a wire mesh screen at day 7 of the DSS treatment. Erythrocytes were lysed in 0.85% NH_4 in Tris-HCl buffer. Next, splenocyte suspension was centrifuged at 300 **g** for 5 min and resuspended in Roswell Park Memorial Institute 1640 medium (WelGENE, Inc.) supplemented with 10% fetal bovine serum, penicillin (30 $\mu\text{g}/\text{ml}$) and streptomycin (50 $\mu\text{g}/\text{ml}$) (Hyclone). For evaluating T_{reg} populations, splenocytes were analysed by flow cytometry. Antibodies that were used for flow cytometry included fluorescein isothiocyanate-conjugated anti-mouse CD4, R-phycoerythrin (PE)-conjugated anti-mouse CD25 and PE-Cy5 anti-mouse Foxp3 (eBioscience). Multiple-colour immunofluorescence staining was performed using a mouse T_{reg} staining kit (eBioscience) following the manufacturer's instructions. All samples were analysed on a FACSCalibur and CellQuest (BD Bioscience) and data were calculated as the percentage of CD25⁺Foxp3⁺ cells among the total CD4⁺ cells.

Fluorescence-activated cell sorting analysis for Foxp3 in vitro

The method of splenocyte isolation from Foxp3^{EGFP} Balb/c mice has been described above. To examine the effect of LJE on T_{reg} cells *in vitro*, splenocytes were treated with LJE (1 and 10 $\mu\text{g}/\text{ml}$), bee venom (a positive control, 1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) or PBS in the presence of anti-CD3 ϵ (10 $\mu\text{g}/\text{ml}$) and anti-CD28 anti-mouse monoclonal antibodies (2 $\mu\text{g}/\text{ml}$) (BD Biosciences). After 72 h, cells were stained for flow cytometric analyses. Antibodies that were used for flow cytometry included antigen-presenting cell-conjugated anti-mouse CD4 (eBioscience). The methods used for performing multiple-colour immunofluorescence staining and analysis were the same as those used in the *in vivo* study. Data were

obtained and calculated by setting the DSS group as 100% and expressing the remaining samples as a percentage of the DSS group.

Statistical analysis

All results are expressed as mean values with their standard errors for each group. Data were analysed statistically using one-way ANOVA followed by Dunnett's test for comparative analysis with the DSS group and by Tukey's test for comparative analysis between the groups. $P < 0.05$ was regarded as statistically significant.

Results

Effects of the water extract of *Lonicera japonica* on weight loss in dextran sulphate sodium-induced colitis

Body weights of mice in the normal group continuously increased until day 7; however, the body weight of those in the DSS group continuously decreased. The DSS + LJE administration increased mouse weight in a dose-dependent manner compared with the DSS group. In particular, the DSS + LJE 500 mg/kg group presented a significant protective effect against weight loss induced by DSS on days 5, 6 and 7 ($P < 0.05$; Fig. 2).

Effects of the water extract of *Lonicera japonica* on colon shortening in dextran sulphate sodium-induced colitis

The colons from the normal group were thin and healthy with intact caeca. However, the colons from the DSS group were short, oedematous and hyperaemic, with damaged caeca. The DSS + LJE groups showed lesser short and oedematous colons with lesser-damaged caeca than the DSS group (Fig. 3(a)). The administration of 5% DSS for 7 d induced colon shortening in the DSS group compared with the

normal group. The LJE treatment significantly inhibited colon shortening in a dose-dependent manner ($P < 0.05$ in the DSS + LJE 20 mg/kg group and $P < 0.001$ in the DSS + LJE 100 mg/kg and 500 mg/kg groups; Fig. 3(b)).

Effects of the water extract of *Lonicera japonica* on the histological damage of colonic mucosa in dextran sulphate sodium-induced colitis

Surface epithelium, cryptal glands, stroma and submucosa from the normal group were intact, while the DSS group showed severe damage to the overall surface epithelium, disruption of the cryptal glands and infiltration of the inflammatory cells (Fig. 4(a) and (b)). The DSS + LJE groups showed protective effects against histological damage of the colonic mucosal layer induced by DSS. The DSS + LJE 20 mg/kg group showed a relatively intact surface epithelium compared with the DSS group; however, disruption of the cryptal glands and infiltration of the inflammatory cells were similar to those observed in the DSS group (Fig. 4(c)). The DSS + LJE 100 mg/kg group presented a more intact surface epithelium and cryptal glands than those in the DSS and DSS + LJE 20 mg/kg groups (Fig. 4(d)). In the DSS + LJE 500 mg/kg group, a small amount of inflammatory cell infiltration was found, while cryptal glands and surface epithelium were intact, which was similar to that observed in the normal group (Fig. 4(e)).

The histological score of the normal group was set to 0 points, and the DSS group scored over 10 points. However, the DSS + LJE groups showed dose-dependent protective effects against DSS-induced histological damage. The DSS + LJE 100 mg/kg and 500 mg/kg groups presented significant protection from histological damage ($P < 0.05$ and $P < 0.01$, respectively; Fig. 4).

Effects of the water extract of *Lonicera japonica* on cytokine levels in colonic mucosa

To analyse the influence of LJE on cytokine production in colonic mucosa during colitis, we investigated representative Th1 (IFN- γ) and Th17 (IL-17 and IL-23) cytokine levels through multiplex profiling after 7 d of DSS administration. LJE inhibited IFN- γ in a dose-dependent manner ($P < 0.05$ in the DSS + LJE 500 mg/kg group compared with the DSS group; Fig. 5). The level of IL-17 in the DSS group showed a significant increase compared with the normal group, and the LJE treatment reduced IL-17 levels in a dose-dependent manner. In particular, the level of IL-17 in the DSS + LJE 500 mg/kg group was as low as that in the normal group ($P < 0.05$; Fig. 5). LJE also inhibited other Th1/Th17 response-related cytokines including TNF- α , IL-1 β , IL-6 and IL-12 in a dose-dependent manner (Fig. 5). IL-23, which is related to Th17 differentiation, IL-10 and TGF- β 1 did not show any significant differences in all groups (Fig. 5).

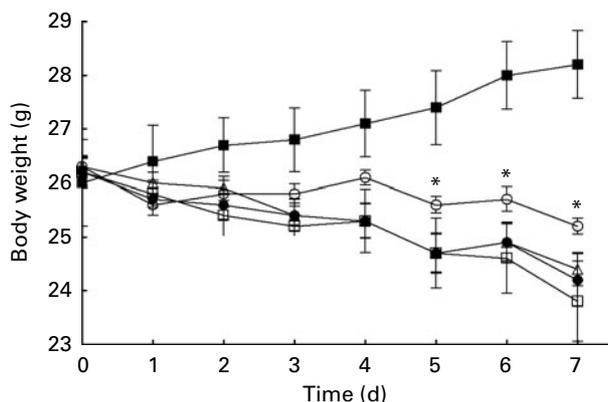


Fig. 2. Effects of the water extract of *Lonicera japonica* (LJE) on mouse weight in dextran sulphate sodium (DSS)-induced colitis. The normal group (■) was not treated with DSS or LJE. Values are means ($n = 8$ for each group), with their standard errors represented by vertical bars. *Mean values were significantly different compared with the DSS group at each day ($P < 0.05$). DSS, group treated with 5% of DSS (●); DSS + LJE, DSS group treated with 20 mg/kg (□), 100 mg/kg (△) and 500 mg/kg (○) of LJE.

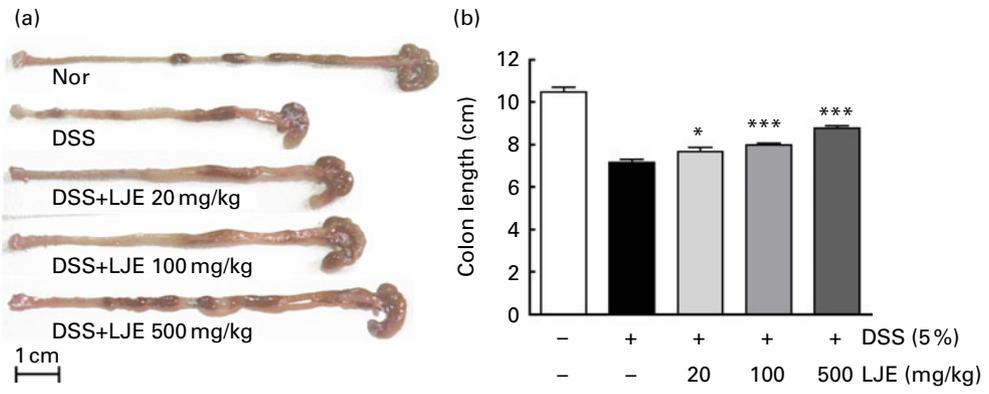


Fig. 3. Effects of the water extract of *Lonicera japonica* (LJE) on colon length in dextran sulphate sodium (DSS)-induced colitis. (a) Representative images of a typical colon morphology and the length of each group. (b) A graph showing the data of colon length. Values are means (n 8 for each group), with their standard errors represented by vertical bars. Mean values were significantly different compared with the DSS group: * $P < 0.05$, *** $P < 0.001$. Nor, normal group; DSS, a group treated with DSS; DSS + LJE, DSS group treated with each dosage of the water extract of *Lonicera japonica* (a colour version of this figure can be found online at www.journals.cambridge.org/bjn).

Effects of the water extract of Lonicera japonica on regulatory T cell populations in dextran sulphate sodium-induced colitis

The administration of 500 mg/kg of LJE, as the effective dosage, for 7 d did not significantly increase T_{reg} populations in the spleen compared with the non-treated normal group. While DSS administration increased T_{reg} populations ($P < 0.05$; Fig. 6), the DSS + LJE 500 mg/kg group did not show any effect on T_{reg} populations compared with the DSS group (Fig. 6). To confirm the effects of LJE on T_{reg} populations, we performed fluorescence-activated cell sorting analysis for Foxp3 *in vitro*. T_{reg} populations in cells treated with bee venom, as a positive control, increased two times more than the DSS group. However, LJE (1 and 10 μ g/ml) did not increase T_{reg} populations in splenocyte cultures (Fig. 7).

Effects of the delayed treatment of the water extract of Lonicera japonica on colon damage

The delayed treatment of 500 mg/kg of LJE from day 4 to day 7 showed only 18.7% of the protective effect on colon shortening compared with the DSS group ($P < 0.05$; Fig. 8), which was far less effective than the co-treatment of 500 mg/kg of LJE (48.5% of protection; Fig. 2).

Effects of the water extract of Lonicera japonica and chlorogenic acid on colon damage

The treatment of 500 mg LJE/kg and 52.5 mg CGA/kg from day 1 to day 7 showed 40.8 and 24.9% of the protective effects against colon shortening compared with the DSS group, respectively ($P < 0.001$ for the LJE 500 mg/kg treatment,

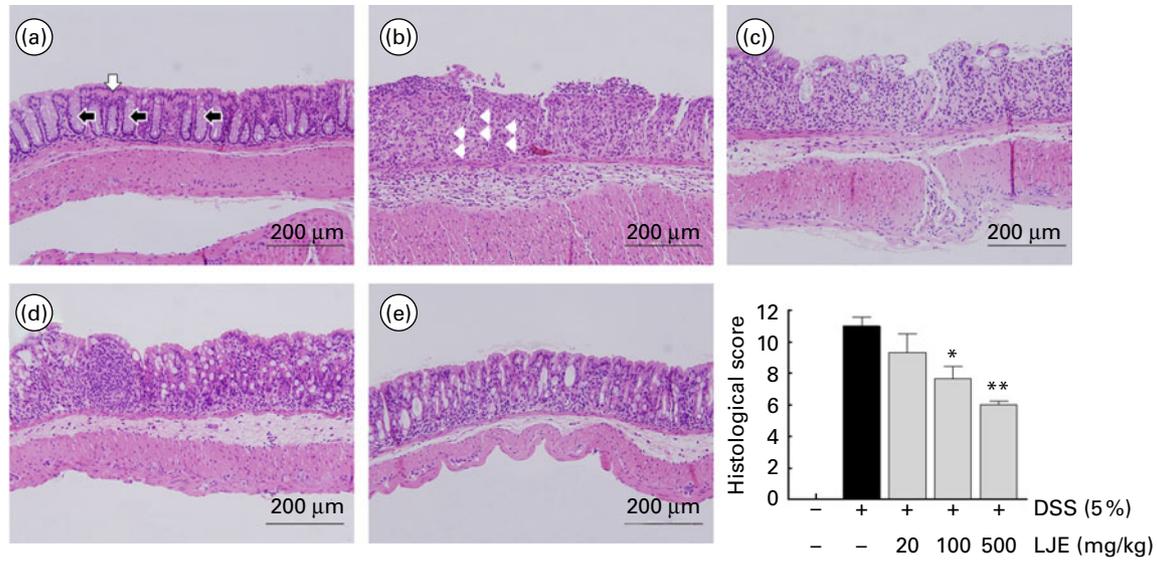


Fig. 4. Effects of the water extract of *Lonicera japonica* (LJE) on histological findings and score in dextran sulphate sodium (DSS)-induced colitis. The images (a–e) are the representative histological findings of all groups: (a) normal, (b) DSS, (c) DSS + LJE 20 mg/kg, (d) DSS + LJE 100 mg/kg and (e) DSS + LJE 500 mg/kg groups. ◀, cryptal grand; ↓, surface of the epithelium; <, neutrophils. The graph shows the histological scores. The score of the normal group is zero. Values are means (n 8 for each group), with their standard errors represented by vertical bars. Mean values were significantly different compared with the DSS group: * $P < 0.05$, ** $P < 0.01$ (a colour version of this figure can be found online at www.journals.cambridge.org/bjn).

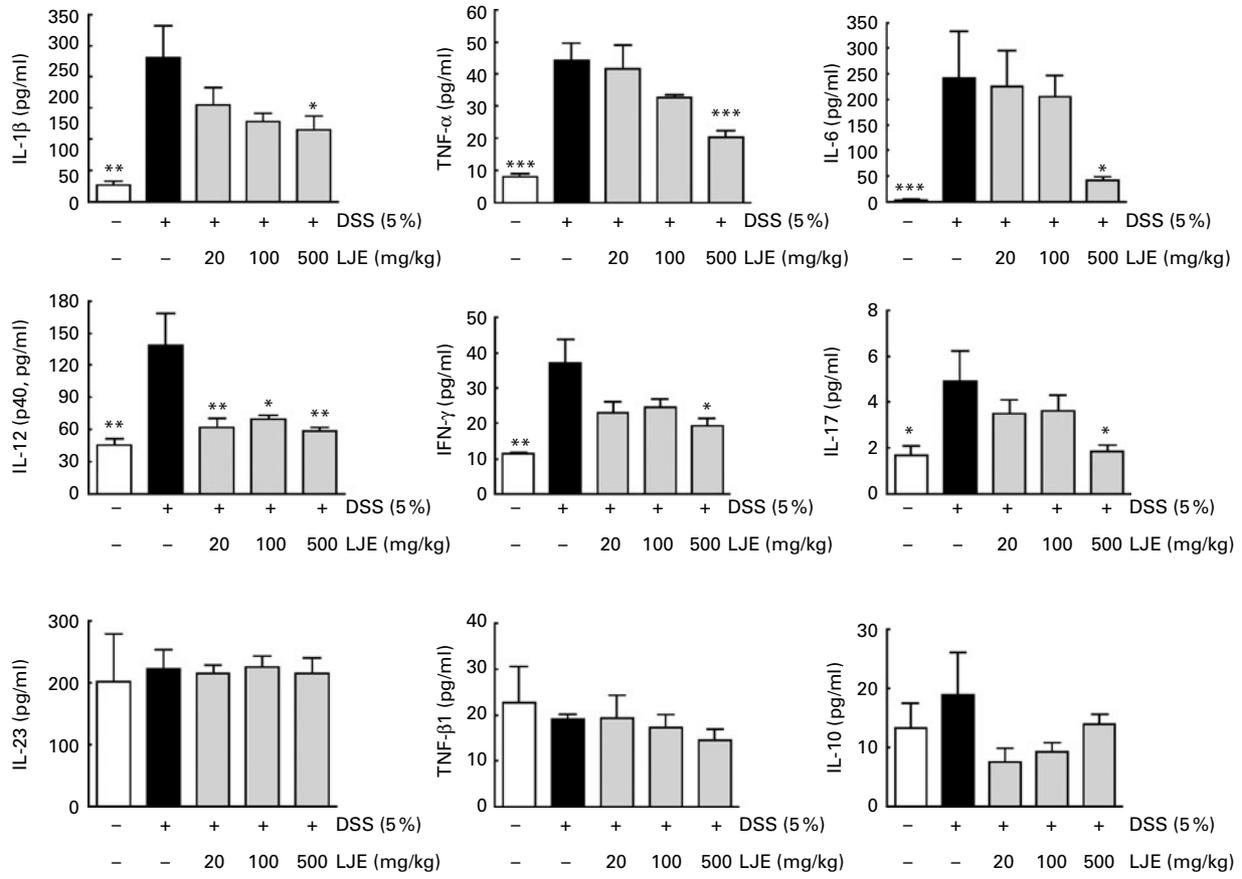


Fig. 5. Effects of the water extract of *Lonicera japonica* (LJE) on cytokine production induced by dextran sulphate sodium (DSS) in mouse colonic mucosa. Values are means ($n = 8$ for each group), with their standard errors represented by vertical bars. Mean values were significantly different compared with the DSS group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IFN- γ , interferon- γ ; TGF- $\beta 1$, transforming growth factor- $\beta 1$.

$P < 0.01$ for the CGA 52.5 mg/kg treatment; Fig. 8(b)). The LJE 500 mg/kg treatment showed more protective effect than the CGA 52.5 mg/kg treatment ($P < 0.05$; Fig. 8(b)).

Discussion

In the present study, oral administration of LJE at a dose of 20, 100 and 500 mg/kg showed dose-dependent inhibitory effects against colon shortening, weight loss and histological damage associated with DSS-induced colitis. LJE down-regulated Th1/Th17 pathway-related cytokines. However, IL-10, IL-23 and TGF- $\beta 1$ were not affected. LJE also did not show any significant effects on T_{reg} populations. The effect of the delayed treatment of LJE was less effective than that of the co-treatment of LJE.

LJE administration inhibited the shortening of colon length in a dose-dependent manner, and the maximal protective effect of LJE was achieved at a dose of 500 mg/kg. In general, DSS administration in mice is known to induce colitis similar to that observed in human IBD. Oral administration of DSS, a heparin-like polysaccharide, induces inflammatory structural changes, which have been reported to be initiated by direct damage to gut epithelial cells of the basal crypts and subsequent damage of the mucosal barrier integrity^(19,20). Lumen bacteria produce inflammatory cytokines including

IL-6 and TNF- α at the damaged site, and cause colonic mucosal inflammation, erosion and ulceration. As a result of these inflammatory reactions, the colon is known to be shortened^(19,20). The effects of LJE on colon shortening might be supported by the results from the present study, which revealed that LJE treatment protected against DSS-induced cryptal gland loss and epithelial damage and inhibited inflammatory cytokines. Therefore, it could be suggested that LJE showed anti-inflammatory and preventive potential by inhibiting initial structural damage to the intestine in DSS-induced colitis. Inflammatory structural changes in a DSS-induced colitis model lead to various clinical symptoms such as diarrhoea, bloody stool, weight loss and behavioural changes^(13–15,21). Among these symptoms, weight loss is considered one of the major systemic symptoms due to colonic structural damage^(13,21). In previous studies, mice treated with DSS for 7 d tended to rapidly lose weight from day 4^(22,23). The present results showed a similar pattern of weight loss in the DSS group. Hence, significant inhibition of weight loss by 500 mg/kg of LJE administration from day 4 might arise from a protective effect of LJE against structural damage and functional insufficiency of the inflamed colon induced by DSS.

To evaluate the effects of samples on IBD in *in vivo* models, two kinds of protocols can be used: one protocol is pre- or co-treatment of the sample with DSS to investigate

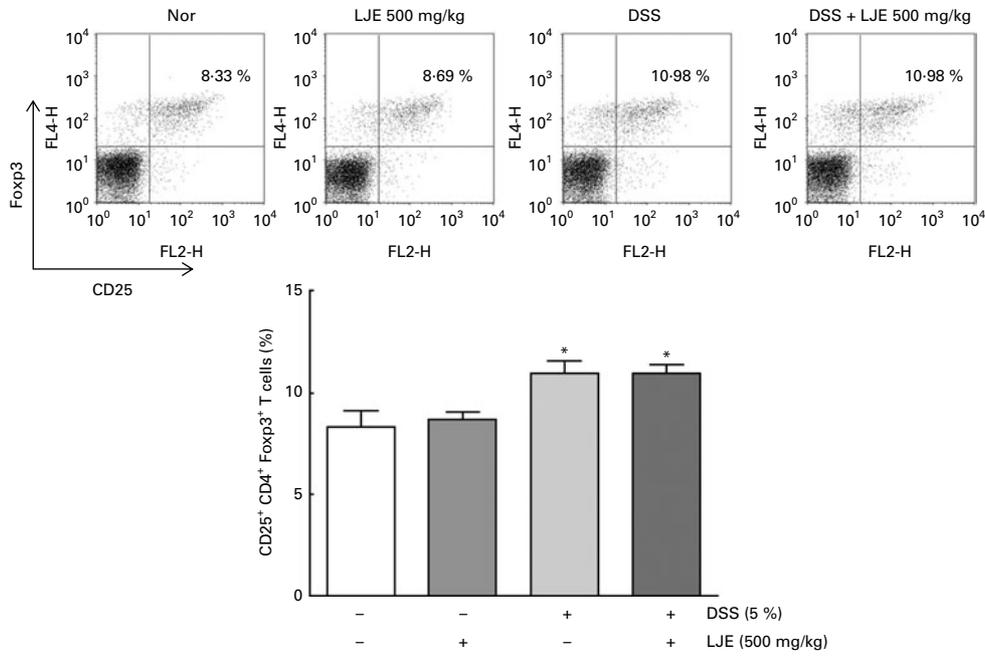


Fig. 6. Effects of the water extract of *Lonicera japonica* (LJE) on CD25⁺CD4⁺Foxp3⁺T cell populations in dextran sulphate sodium (DSS)-induced mouse colitis. The upper images indicate representative flow cytometry analysis of splenocytes in each group. The graph shows the data of flow cytometry analysis in each group ($n = 8$). Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different compared with the DSS group ($P < 0.05$). Nor, normal group; LJE 500 mg/kg, group treated with 500 mg/kg of LJE without DSS treatment; DSS, group treated with DSS from day 1 to day 7; DSS + LJE 500 mg/kg, DSS group treated with 500 mg/kg of LJE; FL2-H, fluorescence intensity 2-height; FL4-H, fluorescence intensity 4-height.

the preventive effects, while the other is delayed treatment to investigate the therapeutic effects^(24,25). In the present study, day 4 might be the time point at which structural changes caused by DSS administration exceeded a critical

point, disturbing normal intestinal function and leading to systemic symptoms. In the delayed-treatment design (LJE administration from day 4 to day 7 of the DSS treatment), the LJE treatment was less effective than the co-treatment

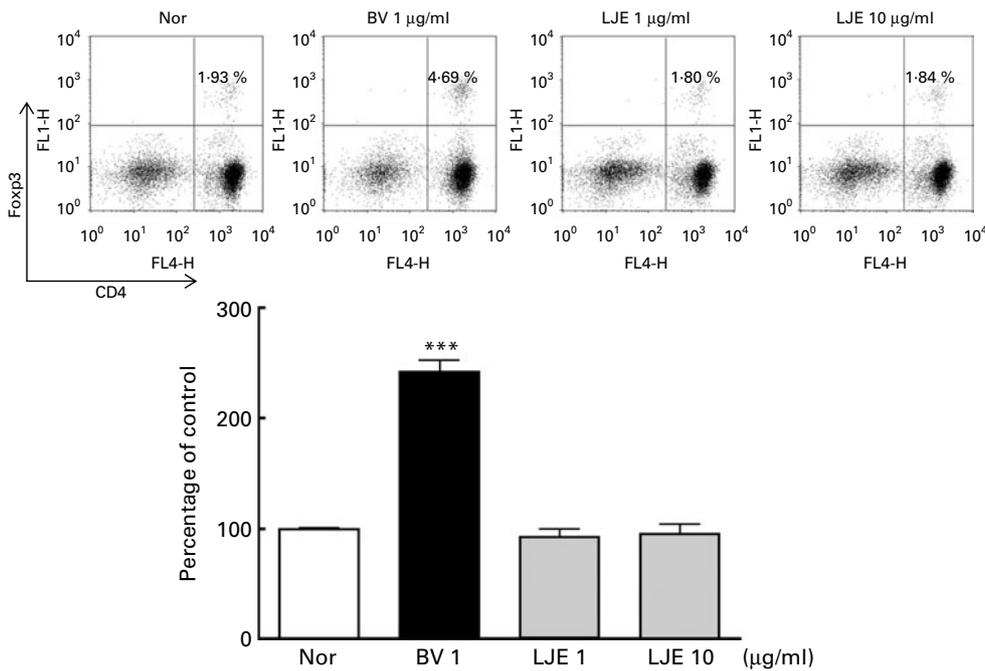


Fig. 7. Effects of the water extract of *Lonicera japonica* (LJE) on CD4⁺Foxp3⁺T cell populations *in vitro*. The upper images indicate representative flow cytometry analysis of splenocytes in each group. The graph shows the data of flow cytometry analysis in each group (in triplicate). BV, bee venom (1 µg/ml). Data of the dextran sulphate sodium (DSS) group was set at 100%, and data of the other groups were calculated and expressed as a percentage of the DSS group. Values are means, with their standard errors represented by vertical bars. *** Mean value was significantly different compared with the DSS group ($P < 0.001$). Nor, normal group; BV 1 µg/ml, group treated with 1 µg/ml of BV; LJE 1 µg/ml, group treated with 1 µg/ml of LJE; LJE 10 µg/ml, group treated with 10 µg/ml of LJE; FL1-H, fluorescence intensity 1-height; FL4-H, fluorescence intensity 4-height.

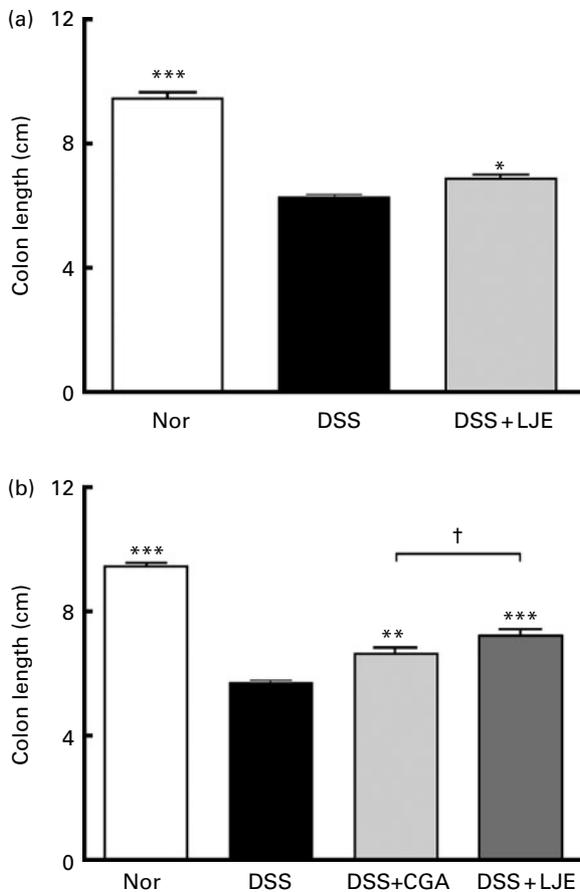


Fig. 8. Effect of the delayed treatment of (a) the water extract of *Lonicera japonica* (LJE) (500 mg/kg) and (b) the comparison between LJE (500 mg/kg) and chlorogenic acid (CGA) (52.5 mg/kg) on colon length in dextran sulphate sodium (DSS)-induced mouse colitis. Values are means (n 8 for each group), with their standard errors represented by vertical bars. Mean values were significantly different compared with the DSS group: * P < 0.05, ** P < 0.01, *** P < 0.001. † Mean values were significantly different from each other. Nor, normal group; DSS, group treated with DSS from day 1 to day 7; DSS + CGA, DSS group treated with 52.5 mg/kg of CGA from day 1 to day 7; DSS + LJE, DSS group treated with 500 mg/kg of LJE from day 4 to day 7 (a) and from day 1 to day 7 (b).

beginning at day 1. In addition, allowing the sample to mix freely with the diet is a common method for investigating the effects of the food ingredients^(26,27). In the present study, LJE was administered orally by a feeding tube to investigate the effective dosage more precisely.

From the present results, it is noteworthy that 20 mg/kg of LJE, known as the general human dosage, also showed the protective effects on colitis, and the maximal effect of LJE on colitis was 500 mg/kg (although we did not present the data, the DSS + LJE 250 mg/kg group (LJE concentration 12.5 times the normal human dosage) showed 8.5 (SD 0.29) cm of colon length, which was similar to that observed in the DSS + LJE 500 mg/kg group). Since the metabolic capacity of mice has been reported to be about 10-fold that of humans, a safe and maximal effective dosage might be approximately 200 mg/kg, and the present results are supported by these facts⁽²⁸⁾. Therefore, the levels of effective dosages in

the present study correspond to possible dosages to be administered in humans.

The main components of LJE in the present study are CGA (10.5% of LJE), an ester of caffeic acid and quinic acid, which is a well-known compound that has antioxidant⁽²⁹⁾, anti-inflammatory⁽⁸⁾, anti-carcinogenic⁽¹⁰⁾, analgesic⁽¹¹⁾ and neuroprotective⁽¹²⁾ effects. Along with LJ, it is also present in blueberries⁽³⁰⁾ and coffee⁽³¹⁾. Recently, coffee has been reported to have anti-carcinogenic effects on various colon cancer models, and the major active components are CGA and its metabolites (comprising up to 12% of CGA in coffee), which have also been reported to have protective effects on colitis models^(32–37). In the present results, we also found that CGA showed protective effects on the DSS-induced colitis mice model, and that the LJE 500 mg/kg treatment showed more protective effect than the CGA 52.5 mg/kg treatment that corresponds to 500 mg/kg of LJE. Thus, the protective effects of LJE in the present study might arise from the additional effects of the components of LJE along with CGA.

In the present study, LJE treatment generated a dose-dependent decrease in histological score that reflects cryptal damages and the severity and extent of inflammation⁽¹⁹⁾. DSS administration induces direct gut epithelium damage and affects the integrity of the mucosal barrier⁽²¹⁾, which result in severe cryptal depletion, epithelial cell damage, ulceration of mucosa and submucosa, inflammatory cell infiltration, muscle thickening and tissue oedema⁽¹³⁾. Thus, the protective effects of LJE might result from the inhibitory effects against inflammation-induced structural changes.

Deleterious effects of pro-inflammatory cytokines in IBD have been well documented⁽¹⁵⁾. Thus, cytokine inhibition could be the target of the mechanistic studies of IBD; recent studies on IBD have focused on agents that can block cytokine production⁽¹⁵⁾. In particular, analysis of specific cytokine changes related to various Th-subset pathways could provide key mechanisms for the protective effects of putative therapeutic agents in colitis. Therefore, we tried to elucidate the effects of LJE on Th subset-specific cytokine production after the DSS treatment. A biometric multiplex cytokine signalling method was applied to analyse a broad spectrum of cytokines in colonic mucosa⁽¹⁶⁾. The DSS treatment in the present study up-regulated pro-inflammatory cytokines and Th1- and Th17-related cytokines in inflamed mucosa, in accordance with recent studies⁽¹⁶⁾. LJE inhibited representative Th1 (IFN- γ) and Th17 (IL-17) cytokines, and Th1/Th17 response-related pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6 and IL-12, but not IL-23, in a dose-dependent manner, in colonic mucosa. Th17 has been reported to be one of the most deleterious immune cells that orchestrate tissue inflammation by inducing pro-inflammatory cytokines, chemokines and matrix metalloproteases, and by subsequently recruiting neutrophils or other Th1 cells to the target tissue^(38,39). Th1 cells are similar to most key immune cells in that they induce infiltration and activation of effector T cells or mononuclear cells, which results in direct tissue damage through the massive production of inflammatory cytokines, including IFN- γ ^(15,38,39). Thus, it could be suggested that the severity of histological damage in colitis might be closely related to the activation

of the Th1/Th17 pathway. The present results showing the inhibitory effects of LJE on Th1/Th17 cytokines (IFN- γ and IL-17), tissue damage of colonic mucosa and neutrophil infiltration could suggest that LJE attenuates colitic damage by modulating Th1/Th17 lineage development. Previous studies of caffeic acid in DSS-induced colitis have shown this compound to inhibit inflammatory damage to the colon and decrease *IL-17* gene expression⁽³⁶⁾. These findings are in agreement with the present results and could indicate that the protective effects of LJE may originate from CGA metabolites.

In the present study, LJE did not inhibit IL-23 production in spite of Th17 inhibition. In general, IL-23 plays an important role in the differentiation of naive CD4⁺T cells into Th17⁽¹⁵⁾. However, the function of IL-23 on Th17 differentiation is exhibited only in the presence of IL-6 and TGF- β 1^(10,15,40). Moreover, it has also been reported to involve in only sustained IL-17 production and promote effector function in the chronic stage of colitis and not to be a necessary factor in the early commitment to Th17 lineage development^(15,23). Thus, the present results suggest that the Th17-inhibitory effect of LJE might be caused by factors other than IL-23.

In the present study, LJE did not show any inhibitory effects on IL-10, TGF- β 1 or T_{reg} promotion. IL-10 attenuates experimental colitis (and other T cell-mediated inflammatory responses) and induces T_{reg} populations⁽⁴¹⁾. TGF- β 1 is known to be secreted by T_{reg} cells and plays an important role in the activation and differentiation of T_{reg} cells, in conjunction with IL-6; T_{reg} cells then act as anti-inflammatory and immunomodulatory cells⁽⁴¹⁾. IL-10 and TGF- β 1 coordinately regulate homeostatic mucosal immune responses. IL-10 has been shown to facilitate the expression of TGF- β -secreting T_{reg} cells and maintain Foxp3 expression and regulatory activity in an inflammatory milieu^(41,42). IL-10 and TGF- β 1 expression in the DSS group were not significantly higher than those in the normal group. Further, the levels of IL-10 and TGF- β 1 in the acute or subacute stages of colitis after DSS administration were not significantly elevated^(16,42). Therefore, the anti-inflammatory effects of LJE in the present study do not depend on increased IL-10 and TGF- β 1 in colonic mucosa, which indicates that the anti-inflammatory effects of LJE may not be related to T_{reg} cell differentiation or activation. The effects of LJE on these cytokines and T_{reg} cells are in parallel. T_{reg} cell differentiation was more enhanced in the DSS group than in the normal group, and LJE administration did not show any inhibitory effects in the *in vivo* and *in vitro* studies. Therefore, the effects of LJE on DSS-induced colitis might not be due to T_{reg} cell-mediated immunomodulation.

In the present results, the inhibitory effects of LJE on the production of whole pro-inflammatory cytokines in colitis could be considered to be the immunosuppressive effects such as corticosteroids. Corticosteroids, including dexamethasone and methylprednisolone, have been reported to show immunosuppressive and protective effects in experimental colitis by enhancing the T_{reg} population^(43,44). Thus, the fact that LJE does not enhance T_{reg} populations suggests that the effects of LJE are not steroid-like.

In conclusion, LJE showed protective effects against DSS-induced colitis via the Th1/Th17 pathway and not via T_{reg} cell-related mechanisms. This suggests the potential for using LJE as a preventive application against intestinal inflammation. Further investigation including studies of the chronic phase of inflammation is needed.

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