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Anti-colitic effects of the modified *Bojanggunbi-tang* on dextran sulfate sodium-induced mouse colitis model

Hong-Geol Kim[1*], Seok-Jae Ko[2*], Burmgi Hong[1], Jinsung Kim[2], Beomjoon Lee[2], Hyejin Joo[3], Hye Hyun Yoo[5], In Sook Kim[5], Hwan-Su Jung[6], Won Kyu Chang[7], Hyun-Chul Kim[7], Youngmin Bu[4] and Jae-Woo Park[2]

[1]Department of Clinical Korean Medicine, Graduate School, Kyung Hee University, Seoul, Korea
[2]Department of Internal Medicine, College of Korean Medicine, Kyung Hee University, Seoul, Korea
[3]Department of Science in Korean medicine, Graduate School, Kyung Hee University, Seoul, Korea
[4]Department of Herbal Pharmacology, College of Korean Medicine, Kyung Hee University, Seoul, Korea
[5]Institute of Pharmaceutical Science and Technology and College of Pharmacy, Hanyang University, Gyeonggi-do, Korea
[6]Department of Pediatrics, College of Korean Medicine, Sang-Ji University, Gangwon-do, Korea
[7]Department of Korean Medicine Graduate School Kyung Hee University, Seoul, Korea

Abstract: *Bojanggunbi-tang* (BGT) is a well-known and widely used herbal prescription in Korea for colon diseases, with well-documented pharmacological effects on the digestive system. The current study aimed to develop a new simple and effective prescription using the original prescription. mBGT, a modified BGT, was developed by mixing the extracts of *Lonicera japonica* Thunb., *Alisma orientalis* and *Atractylodes macrocephala* based on a literature review and screening of 16 kinds of component herbs of BGT. A colitis mouse (Male, BALB/c) model was induced using dextran sulfate sodium (5%). The effects of BGT and mBGT on body weight, histological damage, clinical score, macroscopic score and colon length were compared. The mechanisms of action were analyzed based on cytokine production in colon tissue. mBGT at 300mg/kg showed similar effectiveness to that of BGT on colon shortening (P<0.01), clinical score (P<0.05), macroscopic score (P<0.01) and histological damage (P<0.01). In addition, mBGT decreased cytokines, including Interleukin 1 beta, tumor necrosis factor alpha and Interleukin 17, in a dose-dependent manner. In conclusion, mBGT could be a substitute prescription for BGT in clinics and a candidate for the development of a new BGT-based therapeutic agent against colitis.

Keywords: *Bojanggunbi-tang,* colitis, cytokines, dextran sulfate sodium, inflammation.

INTRODUCTION

Several therapeutic agents, including anti-inflammatory, immunosuppressive and biological agents (e.g. TNF-a inhibitor), have been developed (Fujisawa *et al*., 2005) and used to relieve the clinical symptoms of inflammatory bowel disease (IBD), such as abdominal pain, weight loss, diarrhea, hematochezia and fever (Siegel, 2011). However, these agents are known to have some limitations in clinics due to their side effects, which include allergic reaction, gastrointestinal discomfort and lymphoma (Lee *et al*., 2009, Siegel, 2011). Lots of natural products are considered safe and are used to prevent intestinal inflammation via immunomodulation with low side effects (Hou *et al*., 2011). Therefore, previous studies have investigated the application of natural products, including herbal medicine, as alternative treatments for IBD (Cheifetz *et al*., 2017, Curro *et al*., 2017).

The traditional medicine of East Asia also has treatments for colon diseases including colitis and IBD (Ke *et al*., 2012). We previously reported the protective effects of several herbal medicines and prescriptions on chemical-induced colitis (Kim *et al*., 2013, Ko *et al*., 2014, Park *et al*., 2011). *Bojanggunbi-tang* (BGT) is a representative and widely used herbal prescription in Korea for colon disorders. Various effects of this preparation on gastrointestinal tract have been reported, including dilatory effects on intestinal smooth muscle, anti-ulcer and anti-diarrhea effects (Joun *et al*., 1994). Moreover, we have recently reported the protective effects of BGT on colitis mouse models induced by dextran sulfate sodium (DSS) and 2,4,6-Trinitrobenzenesulfonic acid (TNBS) (Ryu *et al*., 2011). In addition, we also reported the effects of *Lonicera japonica*, a major herb component of BGT, on a DSS-induced colitis mouse model and the Th17-related mechanism thereof (Park *et al*., 2013).

The number of components making up a prescription is an important factor in determining its quality control, production cost and safety. BGT, which is composed of 16 herbs, has a high cost of manufacture and is therefore difficult to standardize and assert quality control. Thus, a modified BGT (mBGT) made of only the effective herbs of BGT might be beneficial for development and manufacture.

**Corresponding author:* e-mail: pjw2907@khu.ac.kr; ymbu@khu.ac.kr

In the current study, we selected 8 herbs through literature review and then conducted a screening test using a DSS-induced colitis model. Subsequently, we synthesized 2 candidates of mBGT by mixing the most effective herbs, namely *Lonicera japonica* and *Alisma orientalis*, with another herb, *Atractylodes macrocephala*, at a ratio of 3:1:1, which is in line with the original prescription and at an equivalent ratio of 1:1:1. We found that the original ratio of mBGT (3:1:1) had more potent effects on colitis in the mouse model than those of equivalent ratio (1:1:1) (data not shown).

In the current study, we compared the efficacy of mBGT with the original BGT and investigated the mechanisms of mBGT on a DSS-induced colitis mouse model.

MATERIALS AND METHODS

Sample preparation

Sixteen dried herbal medicines of BGT were obtained from Kyung Hee University Hospital at Gangdong (Seoul, Korea) and identified by Prof. Hoyoung Choi (department of herbal pharmacology, College of Korean medicine, Kyung Hee University). BGT (total 216g (16 dried herbs)) was prepared by same method with our previous study (Ryu *et al*., 2011). The mBGT was prepared with 3 selected-herbs, namely the flower buds of *Lonicera japonica* Thunb. (LJ (#PSS01)), the roots of *Atractylodes macrocephala* Koidz. (AM (#PSS02)) and roots of *Alisma orientalis* Juz. (AO (#PSS10)) (200 g each). Each herb was boiled for 1.5h at 100°C in 2,000mL water, respectively. The yields were 2.37% (BGT), 6.00% (aqua extract of LJ (LJE)), 10.80% (aqua extract of AM (AME)) and 4.56% (aqua extract of AO (AOE)). All dried materials and extracts were stored in cold storage room for herbal medicine of department of herbal pharmacology, College of Korean medicine, Kyung Hee University with code number (#PSS No.). Before experiment, each aqueous extract was dissolved to a working concentration in distilled water. mBGT was prepared by mixing the extracts of LJE, AME and AOE at 3:1:1 ratio (original ratio of BGT).

Standardization using high pressure liquid chromatography

The extracts of LJE, AME and AOE were standardized with chlorogenic acid, atractylenolide III and alisol B acetate, respectively, using high pressure liquid chromatography (HPLC). Chromatography was performed by an Agilent 1260 HPLC system (Palo Alto, USA) using Xterra C18 analytical column (250 × 4.6 mm, 5µm; Waters, USA). The mobile phase was a gradient of 0.1% acetic acid in DW and 100% acetonitrile with different condition for each extract. Chromatograms were acquired by UV detection at 237nm for atractylenolide III, 327nm for chlorogenic acid and 205 nm for alisol B acetate (fig. 1). We found that LJE, AME and AOE contained 42.2µg/mg chlorogenic acid, 75.1ng/mg atractylenolide III and 624ng/mg alisol B acetate, respectively.

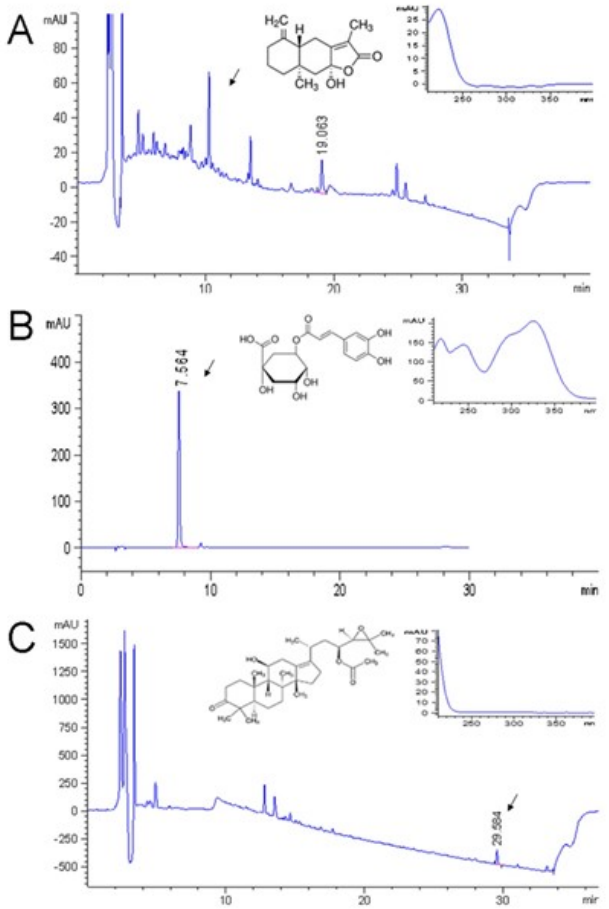

Fig. 1: Representative HPLC chromatograms of the aqueous extract of (A) Atractylodes macrocephala, (B) Lonicera japonica and (C) Alisma orientalis. The chemical structure is that of (A) atractylenolide III, (B) chlorogenic acid and (C) alisol B acetate each standard. The square box in the chromatogram indicates the UV absorbance spectrum of each marker compound.

Animals

All animal experimental procedures were conducted according to the guideline of Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-16-136). A total of 77 Male BALB/c mice (9 weeks, 22±2g, DaeHan BioLink, Eumseong, Korea) were housed on a 12h light/dark cycle and provide standard rodent food (Biopia, Korea) and water for 1 weeks before the experiment.

Colitis induction

Mouse colitis model was made by freely drinking DSS according to the method of our previous study (Ryu *et al*., 2011). In brief, the mice of the normal control group were provided with normal drinking water, whereas the mice of the colitis control and experimental groups were provided

with drinking water containing 5% DSS (Molecular weight 36,000 ~ 50,000 kD, MP Biomedicals, USA) ad libitum for 7 days.

Experimental design

Mice (n = 42) were randomly divided into normal control (n=7), colitis control (n=7), BGT 300 mg/kg-treated (n=7) and mBGT 30, 100 and 300mg/kg-treated groups (n= each). Mice in the normal control group were not exposed to DSS nor treated with any extract. BGT- and mBGT-treated group was orally-administered with water suspension (100μL/mouse) of BGT and mBGT, respectively, twice a day for 7 days. The colitis control group was administered with the same volume of water. For the mechanism study, mice (n=35) were divided into normal control (n=7), colitis control (n=7) and mBGT 30, 100 and 300mg/kg-treated groups (n=7 each).

Measurement

Clinical score

Clinical scores were measured at 9 a.m. daily by two independent observers in a blinded manner. The clinical scores were spontaneous behavior (posture change and hunching; 0- points), coat and piloerection (cleanness, color and piloerection; 0–4 points), cleaning of perianal region (cleanness, stool and blood; 0- points) (Johswich, Martin *et al*., 2009, Park, Bu *et al*., 2011). The data of each mouse was obtained by summarizing all the scores.

Body weight measurement and macroscopic analysis

Food intake and body weight were measured at 9 a.m. daily for 7 days. Mice were anesthetized with urethane (1.2g/kg i.p. Daejung chemicals & metals, Korea) after the last body weight measurement. The colon was isolated and measured the length from the caecum to the anus using a Vernier caliper (Mitutoyo, Japan). Macroscopic score was measured by two observers in a blinded manner based on the edema and thickness of colorectal tissue (0–5 points), as well as overall health and state of feces (0-5 points) (Hyun *et al*., 2008, Park *et al*., 2011). Data was obtained by modifying the sum of 2 scores into 0-5 points (from 0 point = severe edema and thickness and bleeding status to 5 point = normal).

Histological analysis

The colon was isolated at 7 days after DSS administration. The isolated colon tissue was fixed in 10% buffered formalin, embedded in paraffin and cut into sections. The appropriate section was stained with hematoxylin and eosin (H&E). Two independent observers evaluated the histological score by previous method with minor modification (Kitajima, Takuma *et al*., 2000). The inflammatory cell infiltration was assessed in each layer of the colon, including surface epithelium, cryptal glands, stroma, submucosa, and transmural layer and graded from 0 to 4 (none (0); minimal (1); mild (2); moderate (3); and severe (4)). The severity of ulceration was graded from 0 to 4 (none (0); mild and focal surface (1); mucosal layer (2); submucosal layer (3); and transmural layer (4)). These two scores were obtained by averaging the scores of three parts in the colon (proximal, middle, and distal). Lesion distribution in the colon: none (0); focal (1); 1/3 of colon (2); 2/3 of colon (3); the entire colon (4). The data (total 12 points) were obtained by summing the mean value of the first two scores (total scores of 8 points each) and the scores of lesions in the whole colon (4 points).

Biometric multiplex cytokine profiling

To further analyze the mechanism of anti-colitic effect of mBGT, the colon was isolated at 7 days after DSS administration. The production of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, IL-17 and IFN-γ were measured in the colonic mucosal tissues of the normal control, colitis control, and experimental groups. The extracted colon was snap-frozen at -70°C and mucosal tissue was collected by scraping the mucosal layer of the colon. Afterwards, 10mg of mucosal tissue was dissolved in triple-detergent lysis buffer (50mM Tris-HCl, pH 8.0; 150mM NaCl; 0.1% sodium deoxycholate; 1 mM phenylmethylsulfonyl fluoride) and homogenized. The levels of 6 cytokines were quantified using a biometric multiplex cytokine assay (Merck Millipore, MA, USA).

STATISTICAL ANALYSIS

All data were expressed as mean ± standard error of the mean (SEM) and analyzed by one-way ANOVA. Dunnett's post-hoc test was used for comparison with colitis control group. Student t-test was used for comparison between BGT-and mBGT 300mg/kg-treated groups. Differences were regarded as statistically significant at $p<0.05$.

RESULTS

Body weight

Contrary to the increase in body weight in the normal control group during the 7 days of experiment, the body weight of the colitis control, BGT and mBGT-treated groups decreased continuously during the experiment period. There was no statistically significant difference between colitis control and experimental groups (fig. 2A). The food intake of the colitis control group decreased continuously during the experimental period by a difference of more than 1g per day from the normal group. mBGT and BGT treated groups were not different from the colitis control group (fig. 2B).

Colon length

The colons of the normal control group were healthy and intact, whereas those of the DSS-induced groups were shortened (fig. 3A). mBGT showed a dose-dependent

protective effect on colon shortening, with the 300mg/kg dose showing a significant difference in colon length than that of the colitis control group (fig. 3B). Meanwhile, BGT exhibited no ameliorative effect on colon shortening.

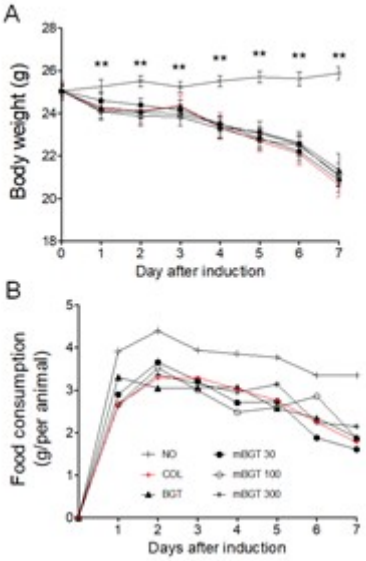

Fig. 2: Changes in body weight (A) and food intake (B) of each group. NO is normal control group and COL is colitis control group and BGT is BGT 300 mg/kg- treated group. Values are mean ± SEM (n=7) at each day and analyzed by one-way ANOVA with Dunnett's post-hoc test. *Significantly different from colitis control group (**p <0.01).

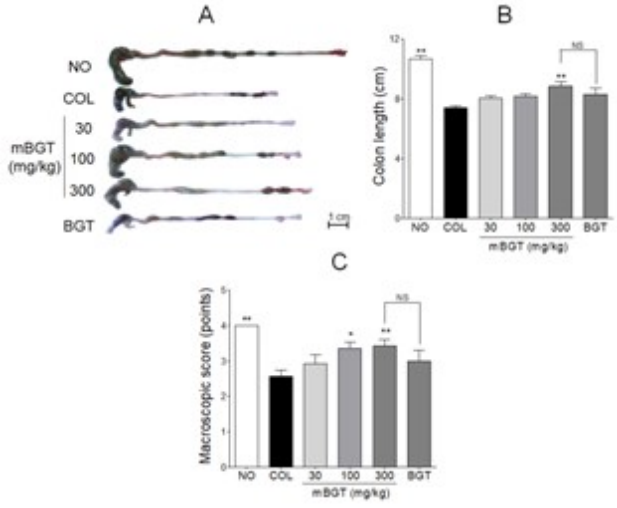

Fig. 3: Representative photographs of isolated mouse colon of each group (A), graph showing the colon length (B) and graph showing the macroscopic score (C). NO is normal control group and COL is colitis control group, BGT is BGT 300mg/kg-treated group. All data are presented as mean ± SEM (n=7) and analyzed by one-way ANOVA with Dunnett's post-hoc test. The difference between BGT and mBGT 300mg/kg was analyzed by Student t-test. NS indicates no significant difference between groups. *Significantly different from colitis control group (*p<0.05 and **p<0.01).

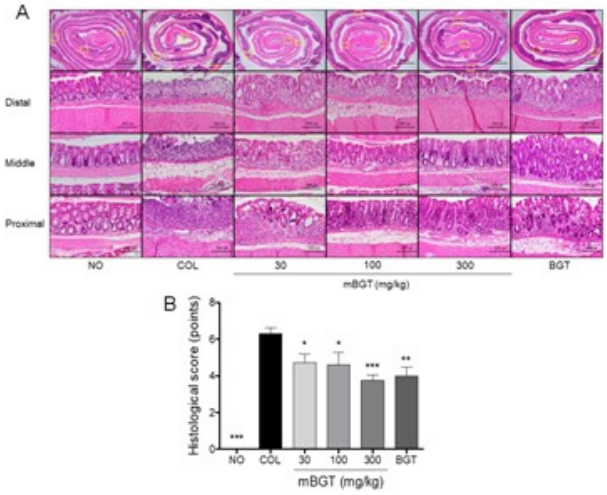

Fig. 4: Representative photographs of H&E-stained mouse colon tissue from each group (A) and graph showing the data (B). The colon tissue was rolled and stained. The edge is the proximal part of the colon and the central part is the distal part of the colon. Lower photos (200 x) were the magnification of the square of upper photo (20 x) which is the proximal, middle and distal colon. NO is normal control group and COL is colitis control group, BGT is BGT 300mg/kg-treated group. All data are presented as mean ± SEM (n=7) and analyzed by one-way ANOVA with Dunnett's post-hoc test. The difference between BGT and mBGT 300 mg/kg was analyzed by Student t-test. NS indicates no significant difference between groups. *Significantly different from colitis control group (*p <0.05, **p <0.01 and ***p <0.001).

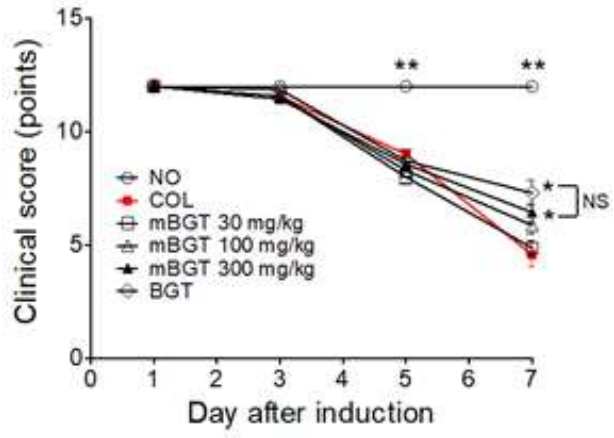

Fig. 5: Changes in clinical score of each group. NO is normal control group and COL is colitis control group, BGT is BGT 300mg/kg-treated group. All data are presented as mean ± SEM (n=7) and analyzed by one-way ANOVA with Dunnett's post-hoc test. The difference between BGT and mBGT 300mg/kg was analyzed by Student t-test. NS indicates no significant difference

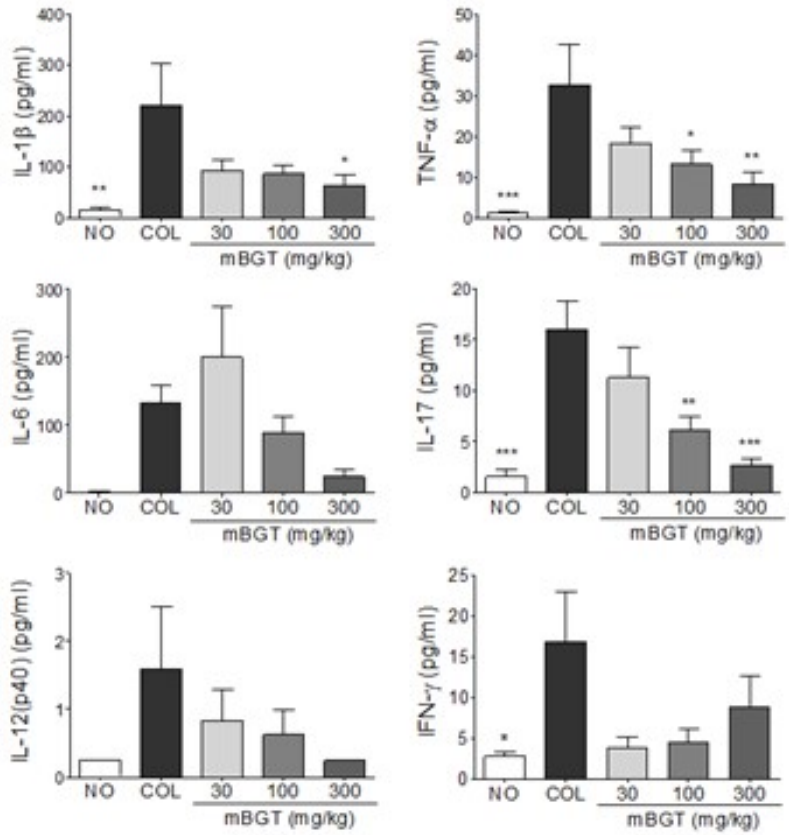

Fig. 6: Cytokines production in colon mucosa of each group. NO is normal control group and COL is colitis control group. All data are presented as mean ± SEM (n=7) and analyzed by one-way ANOVA with Dunnett's post-hoc test. NS indicates no significant difference between groups. *Significantly different from colitis control group (*p <0.05, ** p <0.01 and ***p <0.001).

between groups. *Significantly different from colitis control group (*p<0.05 and **p<0.01).

Macroscopic score

The macroscopic score of the normal control group were 4 points, and the scores of the colitis-induced groups were decreased at 7 days after DSS treatment compared with that of the normal control group (fig. 3C). mBGT showed dose-dependent effects, with mBGT 100 and 300mg/kg showing significantly higher scores than those of the colitis control group (fig. 3C). However, BGT showed no protective effects on macroscopic score at 7 days.

Histological damage

The colonic mucosa, submucosa and smooth muscle layer of in distal colon the normal control group was intact, while colitis control group showed severe damage to the epithelium, ulceration and infiltration of the inflammatory cells throughout the colon (fig. 4A). mBGT treated groups showed localized histological damage to the distal colon and intact histological condition compared with those of the colitis control group (fig. 4A). The histological score of the normal control group were 0 points, whereas those of the colitis-induced groups were increased at 7 days after DSS treatment (fig. 4B). mBGT showed dose-dependent effects, with all treatment groups showing significant decrease in score compared to that of the colitis control group (fig. 4B). In addition, there is no difference between the scores of BGT and mBGT 300 mg/kg.

Clinical score

The clinical score of the normal control group were 12 points throughout the experiment and the scores of the other groups abruptly decreased from 3 days. mBGT showed dose-dependent effects, with mBGT 300mg/kg showing significantly higher scores than those of the colitis control group (fig. 5). In addition, there is no difference between the scores of BGT and mBGT 300 mg/kg.

Cytokine levels

The levels of all cytokines in colitis control group was higher than those of normal control group. Compared with those of the colitis control group, mBGT decreased the level of IL-1β, TNF-α and IL-17 in a dose-dependent manner but did not decrease the level of IL-6, IL-12 and IFN-γ (fig. 6).

DISCUSSION

In the present study, mBGT showed the protective effects against the colon shortening and the worsening of clinical score, macroscopic score, and histological damage, but not against colitis-associated weight loss. mBGT 300 mg/kg produced effects that are similar to BGT 300 mg/kg. In addition, mBGT decreased the levels of IL-1β, TNF-α and IL-17.

DSS induced colitis mice model is a mostly used model for human ulcerative colitis (Maharshak *et al*., 2010). DSS administration to mouse is known to induce the acute pathological change in colon including weight loss, colon shortening, diarrhea, and bloody stool (Egger *et al*., 2000). The severity of colitis is known to be mainly dependent on DSS concentration. We used same concentration of DSS (5%) of previous study that might be the most appropriate for pharmacological evaluation (Ryu *et al*., 2011). BGT and mBGT exhibited no beneficial effects on loss of body weight in this study, which was inconsistent with previous studies that reported the inhibitory effects of BGT and LJE on body weight loss. We speculated that the doses of BGT and LJE used in the present study (300 and 180mg/kg, respectively) was not enough to elicit comparable effects with that of the previous studies (450 and 500mg/kg, respectively) (Park *et al*., 2013, Ryu *et al*., 2011).

In the current study, mBGT showed dose-dependent protective effects on structural changes of colon in colitis mouse model, including colon shortening, macroscopic score, and histological damage. The DSS-induced colitis mouse model is a well-established and the most frequently used colitis model (Egger *et al*., 2000). DSS is well known to induce damage to intestinal epithelium of basal crypt followed by mucosal barrier damage (Cooper *et al*., 1993, Kitajima *et al*., 2000, Park *et al*., 2013), which results in epithelial damage, ulceration of mucosal layer, inflammatory cell infiltration, cryptal damage, smooth muscle thickening and edema (Dardalhon *et al*., 2008).

Gross anatomical changes, colon shortening, and edema in colon tissue, influence the overall health of colon, and fecal bleeding status could be a valid measurement of the severity of histological colon damage (Hyun *et al*., 2008). In the current study, mBGT produced the ameliorative effects on colon shortening, histological damage and macroscopic score change in dose-dependent manner. BGT 300mg/kg did not present significant effects in colon shortening and macroscopic score change except histological damage, even though BGT 450 mg/kg showed the ameliorating effects on body weight loss, colon shortening and histological damage in a previous study (Ryu *et al*., 2011). It was suggested that BGT 300mg/kg might not have a sufficiently protective effect against colitis-induced damage on the colon. Thus, the optimal therapeutic dosage of BGT on colitis might be at least over 300mg/kg or about 450 mg/kg. Taken together, the effect of mBGT against DSS-induced colon shortening and histological damage might be more potent than that of the original BGT, which indicates that selecting and combining only the most effective herbs of BGT is promising in the developmental research of colitis medication and mBGT might be the better choice than BGT for colitis patients.

Both BGT and mBGT showed improvement in clinical score on DSS-induced colitis. Another characteristic of the DSS-induced colitis mouse model besides the main histological change is clinical symptoms including spontaneous behavior and posture, coat and piloerection, and cleaning of perianal region (Johswich *et al*., 2009). Colitis patients have suffered from abdominal discomfort/pain, indigestion, diarrhea, fever, fatigue, hematochezia, and anal bleeding, which are known to have a strong influence on IBD patients' quality of life (Conley *et al*., 2017). Thus, improvement in clinical score observed in this study showed the therapeutic potential of mBGT for relieving these colitis symptoms in patients.

In the current study, BGT–E showed inhibitory effects on the productions of pro-inflammatory cytokines IL-1β and TNF-α. It also inhibited IL-17 production in a dose-dependent manner. It also showed the tendency of inhibiting IL-6 and IL-12. The production and pathological roles of pro-inflammatory cytokines, such as IL-1B, IFN-y, TNF-a, IL-6 and IL-12 (p40), are well documented. Those cytokines are known to be produced by activated macrophages or dendritic cells and are involved in the inflammatory change, erosion and ulceration in colonic mucosa at the acute phase of DSS-induced colitis (Cooper *et al*., 1993, Kitajima *et al*., 2000). While, IL-17 has been known to generate inflammation via the induction of inflammatory cytokines and chemokines (Dardalhon *et al*., 2008, Liu *et al*., 2009). The current results might be supported by our previous study which described that LJE, a major component herb of mBGT, showed inhibitory effects on the production of Th17 cytokine (Park *et al*., 2013). Thus, the current results indicated that the inhibition of inflammatory cytokine production might be the main mechanism behind the ameliorative effects of mBGT on colitis.

CONCLUSION

mBGT, a modified BGT, was effective on DSS-induced colitis similar to BGT, indicating that mBGT might be a potent candidate for the development of new BGT-based colitis therapeutic agents. Further studies will be required to demonstrate the anti-colitic effects of mBGT on the other colitis models, such as TNBS-induced colitis and specific immunodeficiency colitis. In addition, studies on

the appropriate dosage decision and toxicity of mBGT are also required for clinical trials.

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