Kalopanaxsaponin B Ameliorates TNBS-Induced Colitis in Mice

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Abstract

The stem-bark of *Kalopanax pictus* (KP, family Araliaceae), of which main constituent is kalopanaxsaponin B, has been used for asthma, rhinitis, and arthritis in Chinese traditional medicine. To clarify anticolitic effect of KP, we examined anti-inflammatory effect of KP extract and kalopanaxsaponin B in lipopolysaccharide (LPS)-stimulated peritoneal macrophage and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitic mice. Of KP extracts, KP BuOH-soluble fraction most potently inhibited LPS-induced IL-1β, IL-6 and TNF-α expression, as well as NF-κB activation. However, KP BuOH fraction increased IL-10, an anti-inflammatory cytokine. KP BuOH fraction also inhibited colon shortening and myeloperoxidase activity in TNBS-induced colitic mice. KP BuOH fraction also potently inhibited the expression of the pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α as well as the activation of NF-κB. Kalopanaxsaponin B, a main constituent of KP, inhibited TNBS-induced colonic inflammation, including colon shortening, and TNBS-increased myeloperoxidase activity pro-inflammatory cytokine expression and NF-κB activation in mice. Based on these findings, KP, particularly its main constituent, kalopanaxsaponin B, may ameliorate colitis by inhibiting NF-κB pathway.

Key Words: Kalopanaxsaponin B, *Kalopanax pictus*, Colitis, NF-κB

INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are chronically relapsing disorders of the intestine (Shanahan, 2002; Binder, 2004). Their pathogenic mechanism is assumed to be a dysregulation of the intestinal immune response to intestinal environmental antigens, such as intestinal microflora, and is characterized by several factors, such as T cell-rich infiltrates, and IL-6 increment in the inflamed mucosa of the terminal ileum and colon, where many intestinal microbes reside (Rafii *et al.*, 1999; Atreya *et al.*, 2000). These findings implicate that intestinal microflora play an important role in initiating and perpetuating colonic inflammation. Intestinal bacterial endotoxins, such as lipopolysaccharide (LPS), penetrate the epithelial barrier, either due to damage or via paracellular pathways, and directly stimulate the mucosal immune system (Radema *et al.*, 1991; Rafii *et al.*, 1999). This results in the production of pro-inflammatory cytokines and other mediators, causing the inflammatory activation of the mucosal immune system via distinct signaling pathways through Toll-like receptors (TLRs) and/or cytokine receptors (Jung *et al.*, 1995; Chow *et al.*, 1999; Cario and Podolsky, 2000).

The stem-bark of *Kalopanax pictus* (KP, family Araliaceae), which contains kalopanaxsaponins as main constituents, has been used for asthma, rhinitis, and arthritis in Chinese traditional medicine (Park *et al.*, 1998; Choi *et al.*, 2002). Of its constituents, kalopanaxsaponin A exhibits anti-nociceptive, anti-rheumatoid, anti-diabetic, anti-colitic, anti-carcinogenic, memory and deficit-ameliorating effects (Park *et al.*, 1998; 2001; Choi *et al.*, 2002; Joh and Kim, 2011) and kalopanaxsaponin B, which is a major constituent in KP, has anti-inflammatory effect in LPS-stimulated macrophages (Kim *et al.*, 2002; Joh and Kim, 2011). These kalopanaxsaponins ameliorate inflammation by inhibiting TLR4-IRAK1-NF-kB pathway (Joh and Kim, 2011). Nevertheless, anti-colitic effect of kalopanaxsaponin B and its contained KP extract have not been studied.

Therefore, we investigated the anti-inflammatory effects of KP extracts and its main constituent, kalopanaxsaponin B, in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitic mice.

MATERIALS AND METHODS

Materials

RPMI1640, TNBS, hexadecyl trimethyl ammonium bromide, and radio-immunoprecipitation assay (RIPA) lysis buffer

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were purchased from Sigma Co. (St Louis, MO, USA). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology, Inc., (Rockford, IL, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, USA).

Isolation of kalopanaxsaponin B

The dried stem-bark of KP (2 kg) was extracted five times with MeOH under heating. The MeOH extracts were combined and evaporated to dryness under reduced pressure, which yielded 72 g. The dried extract was suspended in distilled water and extracted with EtOAc (5.3 g) and BuOH (9 g), successively. Kalopanaxsaponin B (420 mg; purity, >95%) was isolated from KP BuOH fraction (9 g) according to the previous method of Joh *et al.* (2012).

Animals

Male ICR mice (specific pathogen-free, 20-25 g, 5 weeks-old) were supplied by Orient Experimental Animal Breeding Center (Seoul, Korea) and housed in wire cages at 20-22°C and 50 ± 10% humidity, fed standard laboratory chow (Samyang, Seoul, Korea) and allowed water ad libitum. All procedures relating to animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85-23 revised 1985 and Kyung Hee University, animal experiment guideline 2006).

Isolation and culture of peritoneal macrophages

Male ICR mice were intraperitoneally injected with 2 ml of 4% sodium thioglycolate solution. Mice were sacrificed 4 days after injection and the peritoneal cavities were flushed with 10 ml of RPMI 1640. The peritoneal lavage fluids were centrifuged at 500× g for 10 min and the cells were resuspended with RPMI 1640 and plated. After incubation for 2 h at 37°C, the cells were washed three times and nonadherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.5×10^6 cells/well) at 37°C in RPMI 1640 plus 10% FBS. The attached cells were used as peritoneal macrophages. To examine the anti-inflammatory effect of KP water extract, KP MeOH extract and KP BuOH fraction, peritoneal macrophages were incubated in the absence or presence of kalopanaxsaponin B with 50 ng/ml LPS. The cytoxicity of these agents in the cell viability was measured using the crystal violet method.

Preparation of experimental colitic mice

The mice were randomly divided into 7 groups for the evaluation of anti-colitic effect of KP extracts (normal and TNBS-induced colitic groups treated with or without KP MeOH extract, KP BuOH fraction or sulfasalazine) and 5 groups for that of kalopanaxsaponin B (normal and TNBS-induced colitic groups treated with or without kalopanaxsaponin or sulfasalazine). Each group is consisted of 6 mice. TNBS-induced colitis was induced by the administration of 2.5% (w/v) TNBS solution (100 μl) in 50% ethanol into the colon of lightly anesthetized mice via a thin round-tip needle equipped with a 1-ml syringe (Joh and Kim, 2011). The normal group was treated with vehicle alone. The needle was inserted so that the tip was 3.5-4 cm proximal to the anal verge. To distribute the agent within the entire colon and cecum, mice were held in a vertical posi-
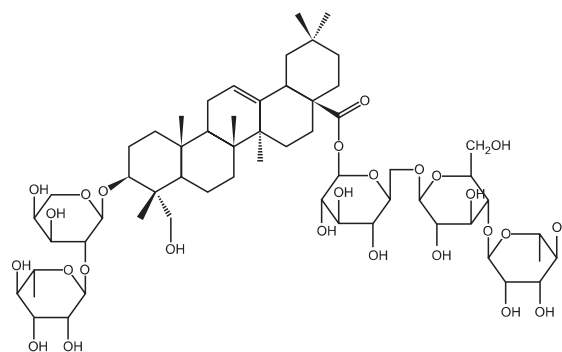

Fig. 1. Structure of kalopanaxsaponin B.

tion for 30 s after the injection. Using this procedure, >95% of the mice retained the TNBS enema. If an animal quickly excreted the TNBS-ethanol solution, it was excluded from the remainder of the study. KP MeOH extract (10 and 20 mg/kg), KP BuOH fraction (10 and 20 mg/kg) or kalopanaxsaponin B (10 and 20 mg) or vehichle alone was orally administered once a day for 3 days after TNBS treatment. Test agents were dissolved in 2% tween 80 solution. The mice were anesthetized with ether and sacrificed. The colon was quickly removed, opened longitudinally, and gently cleared of stool by PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulcer and no inflammation; 1, ulceration without hyperemia; 2, ulceration and local hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm, and the colon tissue was then used for ELISA and immunoblotting.

For the histological exam, the colons were fixed in 10%-buffered formalin solution, embedded in paraffin using standard methods, cut into 5-μm sections, stained with hematoxylin-eosin, and then assessed under light microscopy.

Colon tissue preparation

The colon tissues were excised, perfused with ice-cold perfusion solution (0.15 M KCl, 2 mM EDTA, pH 7.4), and homogenized in 50 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 10,000 × *g* at 4°C for 30 min. The supernatant was used for the estimation of antioxidant defense system.

Assay of myeloperoxidase activity

An aliquot (50 μl) of the colon supernatant was added to a reaction mixture of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2 and incubated at 37°C; the absorbance was obtained at 650 nm over time (Joh and Kim, 2011). Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37°C and expressed in unit/mg protein. The protein content was assayed by the method of Bradford (1976).

ELISA and immunoblot

For the ELISA of IL-1β, IL-6, IL-10 and TNF-α, colons or cell-cultured supernatants were homogenized in 1 ml of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (15,000x *g*, 4°C) for 15 min, and the supernatant

was transferred to 96-well ELISA plates. IL-1β, IL-6, IL-10 and TNF-α concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, USA) (Joh and Kim, 2011).

For the immunoblot analyses of p-IRAK1, p-IKKβ, p-p65 and β-actin, the colon tissue homogenates or the colleted cells were resuspended in 1 ml of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, the supernatant was used for the immunoblot assay. The proteins from collected cells were subjected to electrophoresis on 8-10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose membrane. Levels of p-IRAK1, p-IKKβ, p-p65 and β-actin were assayed as previously described (Joh and Kim, 2011). Immunodetection was performed using an enhanced chemiluminescence detection kit.
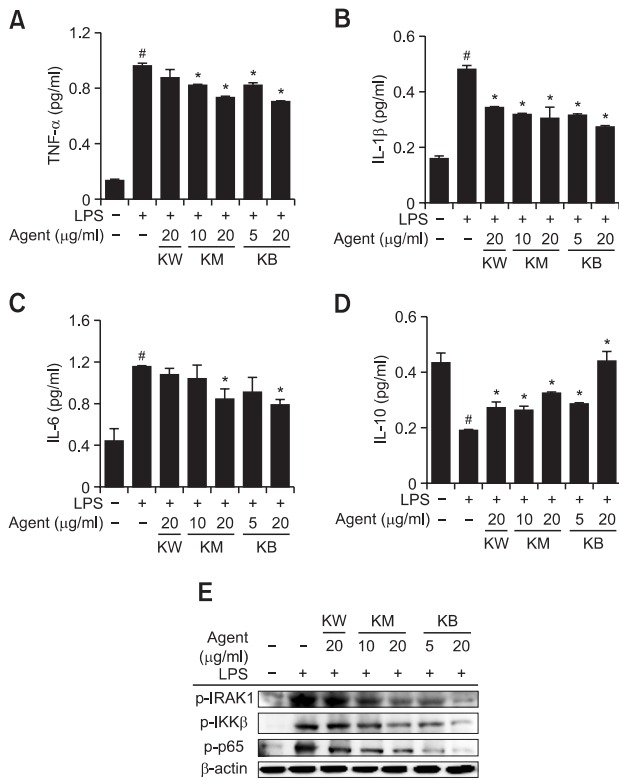
Statistical analysis

All data are expressed as the mean ± standard deviation, with statistical significance analyzed using one-way ANOVA followed by a Student-Newman-Keuls test.

RESULTS AND DISCUSSION

During a screening program to evaluate the anticolitic activities of herbal medicines, KP water extract was found to inhibit NF-κB activation in LPS-stimulated peritoneal macrophages (Fig. 1). To isolate active component(s), we extracted it with MeOH and prepared its BuOH fraction and then investigated
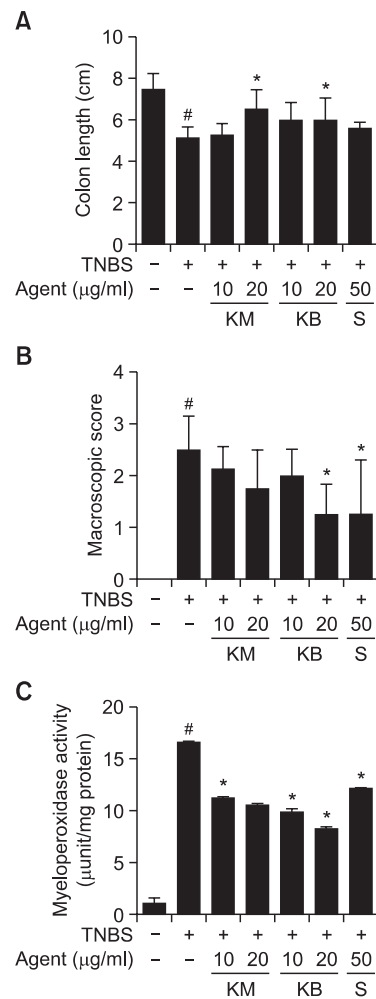
Fig. 2. Inhibitory effects of KP extracts on the expression of pro-inflammatory cytokines and the phosphorylation of IRAK1, IKKβ and p65 in LPS-stimulated peritoneal macrophages. The peritoneal macrophages (0.5×10^6 cells) were treated with 50 ng/ml LPS in absence (LPS) or presence of KP water extract (KW, 20 μg/ml), MeOH extract (KM, 10 and 20 μg/ml) or BuOH fraction (KB, 5 and 20 μg/ml) for 20 h. Levels of TNF-α (A), IL-1β (B), IL-6 (C) and IL-10 (D) in culture supernatants were measured by ELISA. p-IRAK1, p-IKKβ, p-p65 and β-actin were measured by immunoblotting. Normal control was treated with vehicle alone instead of LPS and test agents. (E) LPS stimulated peritoneal macrophages were treated with and without LPS in the absence or presence of KP extracts for 60 min. Immunoblotting for on p-IRAK1, p-IKKβ, p-p65, and β-actin was performed for their lysates. All data are expressed as mean ± S.D. (n=4 in a single experiment). #Significantly different vs. normal control group (p<0.05). *Significantly different vs. group treated with LPS alone (p<0.05).

Fig. 3. The effects of KP extracts on colon length (A), macroscopic disease (B) and colonic myeloperoxidase activity (C) in TNBS-induced colitic mice. TNBS was intrarectally administered in TNBS, KP MeOH extract (KM, 10 and 20 mg/kg), KP BuOH fraction (KB, 10 and 20 mg/kg) and S (50 mg/kg) groups. Test agents (suspended in 2% Tween 80) were orally administered once a day for 3 days after TNBS treatment. Normal and TNBS groups were treated with vehicle alone. The mice were anesthetized with ether and sacrificed 12 h after the final administration of test agents. All values are the mean ± S.D. (n=6). #Significantly different vs. normal control group (p<0.05). *Significantly different vs. group treated with TNBS alone (p<0.05).

their inhibitory effects against the expression of proinflammatory cytokines in LPS-stimulated peritoneal macrophages (Fig. 2). LPS increased TNF-α, IL-1β and IL-6 expression and NF-κB activation. However, treatment with LPS in the presence of KP water, MeOH or BuOH extract reduced TNF-α, IL-1β and IL-6 expression and NF-κB activation. Of them, KP BuOH extract most potently inhibited the expression of proinflammatory cytokines and the activation of NF-κB.

Next we tested the ability of KP MeOH and its BuOH fraction to inhibit TNBS-induced colitis in mice. Intrarectal injection of TNBS caused significant colitis in mice (Fig. 3). TNBS caused severe inflammation, manifested by shortened, thickened, and erythematous colons. TNBS increased myeloperoxidase activity. Treatment with KP MeOH extract or BuOH fraction in TNBS-induced colitic mice inhibited colon shortening and myeloperoxidase activity. We also measured their inhibitory effects on the pro-inflammatory cytokines, TNF-α,
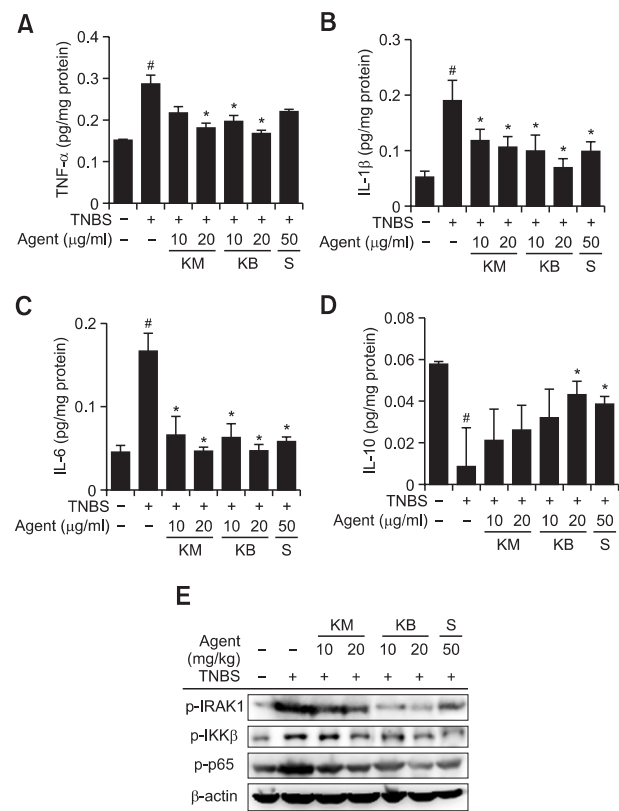
IL-1β, and IL-6 in the colons of TNBS-induced colitic mice (Fig. 4). TNBS increased protein expression of IL-1β IL-6 and TNF-α in mice, respectively. KP MeOH and BuOH extracts inhibited the expression of these proinflammatory cytokines and the activation of NF-κB. KP BuOH extract at a dose of 20 mg/kg inhibited TNBS-induced TNF-α, IL-1β, and IL-6 expression by 72%, 81% and 98%, respectively. However, it reversed IL-10 to 69% of the normal control group. TNBS also phosphorylated IRAK1, IKKβ and p-65. KP BuOH potently inhibited their phosphorylations.

To ensure the anticolitic effect of KP BuOH fraction, we isolated kalopanaxsaponin B, a main constituent of KP BuOH fraction (Joh *et al.*, 2012), in TNBS-induced colitic mice (Fig.
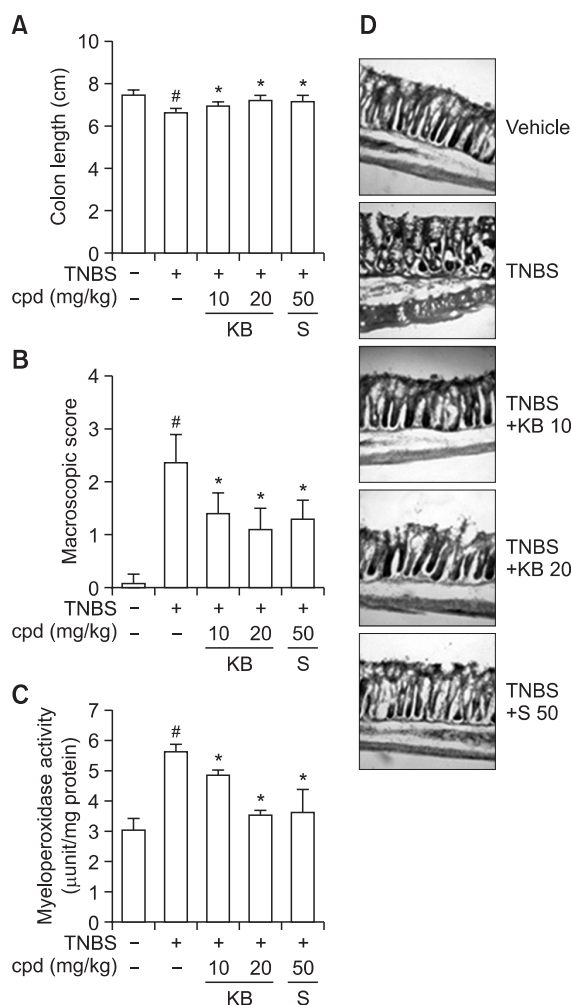

Fig. 4. The effects of KP extracts on the expression of pro-inflammatory cytokines and the phosphorylation of IRAK1, IKKβ and p65 in TNBS-induced colitic mice. TNBS was intrarectally administered in TNBS, KM (10 and 20 mg/kg), KB (10 and 20 mg/kg) and S (50 mg/kg) groups. Test agents (suspended in 2% Tween 80) were orally administered once a day for 3 days after TNBS treatment. Normal and TNBS groups were treated with vehicle alone. The mice were anesthetized with ether and sacrificed 12 h after the final administration of test agents. Levels of TNF-α (A), IL-1β (B), IL-6 (C) and IL-10 (D) in the colon homogenate were measured by ELISA. p-IRAK1, p- IKKβ, p-p65 and β-actin were measured by immunoblotting. (E) Colons were collected and p-IRAK1, p-IKKβ, p-p65, and β-actin expression were measured by immunoblot analysis. All values are the mean ± S.D. (n=6). #Significantly different vs. normal control group (*p*<0.05). *Significantly different vs. group treated with TNBS alone (*p*<0.05).

Fig. 5. The effects of kalopanaxsaponin B (KB) on colon length (A), macroscopic disease (B), colonic myeloperoxidase activity (C) and histology (D) in TNBS-induced colitic mice. TNBS was intrarectally administered in TNBS, KB (10 and 20 mg/kg) and S (50 mg/kg) groups. Test agents (suspended in 2% Tween 80) were orally administered once a day for 3 days after TNBS treatment. Normal and TNBS groups were treated with vehicle alone. The mice were anesthetized with ether and sacrificed 12 h after the final administration of test agents. The colons were stained with hematoxylin-eosin and then assessed by light microscopy. All values are the mean ± S.D. (n=6). #Significantly different vs. normal group (*p*<0.05). *Significantly different vs. group treated with TNBS alone (*p*<0.05).

5). TNBS caused severe colonic inflammation, including colon shortening, increased myeloperoxidase activity and proinflammatory cytokine expression, and NF-kB activation. Treatment with kalopanaxsaponin B in TNBS-induced colitic mice inhibited colon shortening and myeloperoxidase activity. Furthermore, kalopanaxsaponin B (10 mg/kg) inhibited TNF-α, IL-1β, and IL-6 expressions by 86%, 98% and 97%, respectively (Fig. 6). Kalopanaxsaponin B inhibited the expression of COX-2 and iNOS and the activation of NF-κB. Anti-colitic effect of kalopanaxsaponin B was comparable to that of sulfasalazine.

TNBS, a colitis inducer, induces gram-negative bacteria, such as Enterobacteriaceae, in the intestine of mice (Lee et al., 2010a). Gram-negative bacteria produce endotoxins, such as LPS. LPS increases serum IL-1β and TNF-α levels and causes inflammation, although blood IL-1β and TNF-α levels are barely detectable in mice without any stimuli or treatment (Blanqué et al., 1996; Ingalls et al., 1999). Suppression of IL-1β and/or TNF-α productions by other constituents, such as luteolin, quercetin, ginsenosides, berberine and kalopanax-

saponin A are suggested to ameliorate several inflammatory disorders, including endotoxemia, colitis, sand rheumatoid arthritis (Huang et al., 1999; Kotanidou et al., 2002; Joh et al., 2011; Lee et al., 2010b). Their potential mechanisms have been suggested to inhibit signal transduction pathways, such as the activation of NF-κB.

In the present study, KP extracts exhibited anti-inflammatory effects in LPS-induced peritoneal macrophages, like the previously reported (Kim et al., 2002). Of KP extracts, KP BuOH extract showed the most potent inhibition against LPS-stimualted peritoneal macrophages and TNBS-induced murine colitis. Furthemore, kalopanaxsaponin B, a main constituent of KP, potently inhibited LPS-stimulated inflammation in peritoneal macrophages and TNBS-induced colitis in mice, like kalopanaxsaponin A. It is suggested that kalopanaxsaponin A may exhibit more potent anti-inflammatory effect than kalopanaxsaponin B (Kim et al., 2002). Nevertheless, anticolitic effect of KP extracts may be dependent on kalopanaxsaponins, particularly kalopanaxsaponin B. Because kalopanaxsaponin B is a major constituent in KP and, if orally administered, it is partially metabolized to kalopanaxsaponin A in the intestine by intestinal microflora.

Based on these findings, KP, particularly its main constituent, kalopanaxsaponin B, may ameliorate colitis by inhibiting NF-κB pathway.

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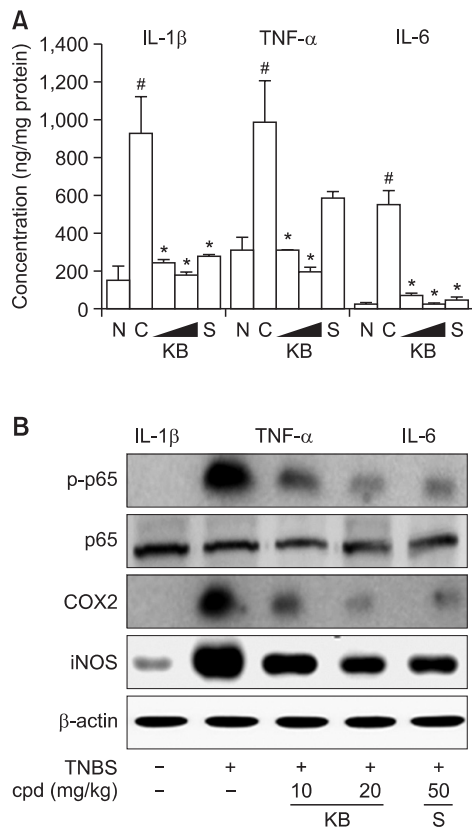
Fig. 6. The effect of kalopanaxsaponin B (KB) on the expression of pro-infl ammatory cytokines (A), COX-2 and iNOS and the activation of NF-κB (B) in TNBS-induced colitic mice. TNBS was intrarectally administered in TNBS, KB (10 and 20 mg/kg) and S (50 mg/kg) groups. Test agents (suspended in 2% Tween 80) were orally administered once a day for 3 days after TNBS treatment. Normal and TNBS groups were treated with vehicle alone. The mice were anesthetized and sacrified 12 h after the final administration of test agents. Levels of IL-1β, TNF-α and IL-6 in the colon homogenate were measured by ELISA. p-IRAK1, p-IKKβ, p-p65 and β-actin were measured by immunoblotting. All values are the mean ± S.D. (n=6). #Significantly different vs. normal (N) group (p<0.05). *Signifi cantly different vs. control (C) group (p<0.05).

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