Anti-Inflammatory Effect of Fermented *Artemisia princeps* Pamp in Mice

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**Abstract** — Essential oil-excluded *Artemisia princeps* Pamp var Ssajuarissuk (AP) was fermented with *Lactobacillus brevis* K-1, which was isolated from cabbage Kimchi, and the anti-inflammatory effects of AP and fermented AP (FAP) on lipopolysaccharide (LPS)-induced inflammatory response in peritoneal macrophages were investigated. AP and FAP inhibited LPS-induced TNF-α, IL-1β, COX-2, iNOS and COX-2 expression, as well as NF-κB activation. AP and FAP also reduced ear thickness, inflammatory cytokine (TNF-α, IL-1β and IL-6) expression and NF-κB activation with 12-O-tetradecanoylphorbol-13-acetate (TPA) induced dermatitis in mice. Furthermore, AP and FAP also reduced exudate volume, cell number, protein amount, inflammatory cytokines (TNF-α, IL-1β and IL-6) expression and NF-κB activation in carrageenan-induced air pouch inflammation in mice. The inhibitory effects of FAP were more potent than those of non-fermented AP. Based on these findings, we propose that FAP can improve inflammatory diseases, such as dermatitis, by inhibiting the NF-κB pathway.

**Keywords:** *Artemisia princeps* Pamp var Ssajuarissuk, Inflammation, Dermatitis, Fermentation

**INTRODUCTION**

*Artemisia princeps* Pamp (Family Asteraceae), which contains eupatilin, acacetin and eudesmane as major components (Ryu et al., 2005), has been used for the treatment of inflammation, diarrhea, gastric ulcer and many circulatory disorders (Kim et al., 1997). *Artemisia princeps* Pamp var Ssajuarissuk (AP) cultivated in Ganghwado has a high eupatilin content compared to that cultivated in other places such as China (Ryu et al., 2005). AP inhibits the passive cutaneous anaphylaxis (PCA) reaction in mice but doses not inhibit the scratching behaviors stimulated by compound 48/80 (Shin et al., 2006b). However, fermentation of AP by *Bifidobacterium infantis* K-525 increases its inhibitory effects against the PCA reaction and against asthma and scratching behavior (Lee et al., 2006; Bae et al., 2007). However, the anti-inflammatory effects of fermented AP (FAP) have not been studied. Therefore, we prepared essential oil-excluded AP, fermented it with *Lactobacillus brevis* K-1, a lactic acid bacterium found in cabbage Kimchi, and investigated the inhibitory effects against 12-O-tetradecanoylphorbol-13-acetate-induced dermatitis and carrageenan-induced air pouch inflammation in mice.

**MATERIALS AND METHODS**

**Materials**

Betamethasone, indomethacin, TPA, carrageenan and RPMI16450 were purchased from Sigma Co. (USA). Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories, Inc. (CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-1β, and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies for iNOS, COX-2, NF-κB (pp64 and p65) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, LA, USA). Radio-immunoprecipitation assay (RIPA) lysis buffer was purchased from Sigma Co. (St Louis, MO, USA).

*Lactobacillus brevis* K-1 was isolated from cabbage Kimchi, according to previously reported (Bae et al., 2007).

**Extraction of AP and fermentation**

AP artificially cultured in GangHwa-Do, Korea, was collected and dried. A voucher specimen (KHOPS-08-07) was deposited at College of Pharmacy, Kyung Hee University, Seoul, Korea. AP (1 kg) was distilled in a Clevenger-type apparatus for 5 h and the residue (1 kg, wet weight)
was immersed in 0.5 liter of water, sterilized by 100°C boiling, and incubated with and without Lactobacillus brevis K-1 (1×10¹⁰), which is isolated from cabbage Kimchi, at 30°C for 3 days. The reaction mixture was extracted with 0.5 liter of MeOH and the supernatant was collected and concentrated in vacuo [Yields of 50% MeOH extract of AP and fermented AP (FAP) were 25.1 and 27.5%, respectively.]: the contents of scopoletin, eupatilin and jaceosidin were 0.6%, 3.1% and 1.1% in AP, respectively, and 6.6%, 2.9% and 1.0% in FAP, respectively, by HPLC analysis (Fig. 1). The concentrates were used in experiment.

HPLC analyses were performed on the HPLC (Younglin high performance liquid chromatography system): column, Develosil ODS-UG-5 (4.6 mm i.d. × 150 mm, 5.8 μm particle diameter); mobile phase, linear-gradient mixture of 100% water and 100% acetonitrile for 0-7 min, 100% acetonitrile for 7-10 min, linear-gradient mixture of 100% acetonitrile and 100% water for 10-12 min and 100% water for 12-20 min; injection volume, 10 μl; flow rate, 1 ml/min; and detection, UV at 256 nm.

The essential oil was dried over anhydrous sodium sulfate and stored at 4°C. The quali-quantitative analysis of the essential oil (2 μl) was carried out by gas chromatography-mass spectrometry (GC-MS, Varian GC3800, MS Saturn1200), equipped with a VF-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was held at 70°C for 1 min and then programmed to 250°C at a rate of 10°C/min; injector temperature, 200°C; transfer line temperature, 200°C; carrier gas, He (1 ml/min); energy ionization, 70 eV. The essential oils consisted of eucalyptol (21.1%), borneol (12.2), α-terpinen-4-ol (8.7%), α-terpineol (8.2%), camphore (6.2%) and others.

Isolation and identification of lactic acid bacteria

One gram of cabbage Kimchi was homogenized in a stomacher blender for 2 min and then inoculated in MRS agar plates, and then aerobically incubated at 37°C for 2 days. Of the colonies grown, five colonies were identified by gram staining and 16S rDNA sequence analyses. 16S rDNA sequence analyses were performed using a Big Dye Terminator Cycle Sequencing Kit with an automatic DNA kit from Applied Biosystems (Model 310, Perkin-Elmer, Foster City, CA, USA). The 16S rDNA sequences of the isolated strains were aligned with the 16S rDNA of lactic acid bacteria. Among them, Lactobacillus brevis K-1 is gram-positive, aerotolerant and unsusceptible for AP.

Animals

Male ICR mice (20-25 g) were supplied from Charles River Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20-22°C, a relative humidity of 50 ± 10% humidity, a frequency of air ventilation of 15-20 times/h, and 12 h illumination (07:00-19:00; intensity, 150-300 Lux), fed standard laboratory chow (Charles River Orient Experimental Animal Breeding Center, Seoul Korea) and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

TPA-induced dermatitis

TPA-induced dermatitis was measured according to the previous method of Shin et al. (2006b). Each group contained 6 mice (20-25 g). TPA (3 μg/20 μl acetone) was ap-
Effects of AP and FAP on the inflammatory mediators in LPS-stimulated peritoneal macrophages. LPS-treated group (LC) received the vehicle alone instead of test agents. Normal control group (NC) received the vehicle alone instead of LPS and test agents. After 20-h incubation with LPS in absence or presence of AP or FAP, (A) Effect on proinflammatory cytokine expression. TNF-α, IL-1β and IL-6 in the culture medium was measured using ELISA kit. (B) Effect on COX-2 and iNOS expression and NF-κB activation levels. Their levels were measured by immunoblot analysis after incubation with LPS in absence or presence of AP or FAP (10 and 20 μg/ml) for 20 h. (C) Effect on cytotoxicity. The cytotoxicity of AP and FAP (20 μg/ml) in the presence of LPS (50 ng/ml). Cell viability was measured with crystal violet. All data are expressed as mean ± S.D. (n=3 in each experiment). #p < 0.05 normal control group, *p < 0.5 vs. LPS-treated control.

Carrageenan-induced inflammation in mouse air pouch

Air pouches were produced by a subcutaneous injection of 2 ml of sterile air into the intra-scapular area of the back of mice (Min et al., 2009). To maintain the space, 1 ml of air was injected into the cavity every 2 days. Carrageenan solution (1 ml, 2 w/v% dissolved in saline) was injected into the pouch 7 days after the first air injection. AP and FAP (30 and 60 mg/kg), or indomethacin (5 mg/kg) was orally administered once a day from 5th to 7th day after the first air injection. Fourteen hours after the carrageenan injection, mice were sacrificed, the pouches were flushed with 2 ml of phosphate buffered saline (PBS), and exudates were harvested and volume measured. The protein concentration of the exudates was measured using a Bio-Rad Protein Assay kit. Aliquots were diluted 1:3 with trypan blue (0.01% w/v in PBS), and cells were counted in a standard hemacytometer.

Histopathologic examination

The ear specimen of mice treated with TPA was post-fixed in 50 mM phosphate buffer (pH 7.4) containing 4% paraformaldehyde overnight and then immersed in 30% sucrose solution (in 50 mM phosphate buffered saline). Frozen specimen was sectioned in a cryostat at 30 μm and stained with hematoxylin-eosin, and then assessed under light microscopy.

Isolation and culture of peritoneal macrophages

Mice were intraperitoneally injected with 2 ml of 4% thioglycolate solution. Mice were sacrificed 4 days after the injection and the peritoneal cavities were flushed with 10 ml of RPMI 1640. The peritoneal lavage fluids were centrifuged at 200×g for 10 min and the cells were resuspended with RPMI 1640 and plated. After incubation for 1 h at 37°C, the cells were washed three times and nonadherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.5×106 cells/well) at 37°C in RPMI 1640 plus 10% FBS. The attached cells were used as peritoneal

Fig. 2. Effects of AP and FAP on the inflammatory mediators in LPS-stimulated peritoneal macrophages. LPS-treated group (LC) received the vehicle alone instead of test agents. Normal control group (NC) received the vehicle alone instead of LPS and test agents. After 20-h incubation with LPS in absence or presence of AP or FAP, (A) Effect on proinflammatory cytokine expression. TNF-α, IL-1β and IL-6 in the culture medium was measured using ELISA kit. (B) Effect on COX-2 and iNOS expression and NF-κB activation levels. Their levels were measured by immunoblot analysis after incubation with LPS in absence or presence of AP or FAP (10 and 20 μg/ml) for 20 h. (C) Effect on cytotoxicity. The cytotoxicity of AP and FAP (20 μg/ml) in the presence of LPS (50 ng/ml). Cell viability was measured with crystal violet. All data are expressed as mean ± S.D. (n=3 in each experiment). #p < 0.05 normal control group, *p < 0.5 vs. LPS-treated control.
macrophages (Park et al., 2009). To examine the anti-inflammatory effects of AP and FAP, peritoneal macrophages were incubated in the absence or presence of AP or FAP with 50 ng/ml LPS.

**ELISA and immunoblot analysis**

Levels of TNF-α, IL-1β, and IL-6 in the supernatant of the exudates from carrageenan-treated air pouches and TPA-treated ear of mice, which was homogenized in RIPA lysis buffer, were determined by ELISA with commercially available kits according to the manufacturer’s instructions. Cell lysates were subjected to electrophoresis on a 8-10% sodium dodecyl sulfate-polyacrylamide gel, as previously described (Joh et al., 2010), and then transferred to a nitrocellulose membrane. COX-2, iNOS, NF-κB (pp65 and p65) and β-actin levels were assayed using their corresponding antibodies. Immunodetection was performed with an enhanced chemiluminescence detection kit.

**Statistics**

All the data were expressed as the mean ± standard deviation, and statistical significance was analyzed by one way ANOVA followed by Student-Newman-Keuls test.

![Fig. 3. Effect of AP and FAP against TPA-induced ear dermatitis in mice. (A) The increased ear thicknesses. It was measured 3 h after the final treatment with test compounds (AP 0.01, 0.01% AP; AP0.05, 0.05% AP; FAP0.01, 0.01% FAP; FAP0.05, 0.05% FAP and Dex0.05, 0.05% dexamethasone). The normal control group (NC) received the vehicle alone. The TPA-treated control group (TPA) received TPA and the vehicle. (B) Histopathological photograph. Mouse ears were excised after the measurement of ear thickness and stained with hematoxylin-eosin. All values are means ± S.D. (n=2). *p < 0.05 normal control group. **p < 0.05 vs. TPA-treated control.](image-url)
Fig. 4. Effect of AP and FAP against TPA-induced ear dermatitis in mice. (A) The increased ear thicknesses. It was measured 3 h after the final treatment with test compounds (AP 0.01, 0.01% AP; AP0.05, 0.05% AP; FAP0.01, 0.01% FAP; FAP0.05, 0.05% FAP and Dexa0.05, 0.05% dexamethasone). The normal control group (NC) received the vehicle alone. The TPA-treated control group (TPA) received TPA and the vehicle. (B) Histopathological photograph. Mouse ears were excised after the measurement of ear thickness and stained with hematoxylin-eosin. All values are means ± S.D. (n=2). *p < 0.05 normal control group. #p < 0.05 vs. TPA-treated control.

Fig. 5. Effect of AP and FAP on volume (A), cell number (B) and protein content (C) in exudates from the air pouches treated with carrageenan. The control group (CC) received only vehicle before carrageenan injection. The samples (AP30, 30 mg/kg AP; AP60, 60 mg/kg AP; FAP30, 30 mg/kg FAP; FAP60, 60 mg/kg FAP and IM, 10 mg/kg indomethacin) were orally administered 1 h before the carrageenan injection. The animals were sacrificed 12 h later, and exudates from each air pouch were collected. The volume, the number of cells and protein amount in the exudates were assessed. Each value is expressed as the mean ± S.D. (n=6). *p < 0.05 vs. carrageenan-treated control.

Inhibitory effect of AP and FAP on TPA-induced mouse ear dermatitis

We investigated the anti-inflammatory effect of AP and FAP on TPA-induced ear dermatitis in mice (Fig. 3A). When TPA was applied to the mouse ear, erythema (reddening of ear thickness). LPS induced these enzymes; however, AP or FAP significantly inhibited the LPS-induced expression of these enzymes. LPS also activated the transcription factor NF-κB (p-p65). Treatment with AP or FAP significantly reduced the level of p-p65. However, AP and FAP did not exhibit cytotoxicity against peritoneal macrophages (Fig. 2e).
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Fig. 6. Effect of AP and FAP on inflammatory mediators in exudates from air pouches treated with carrageenan. The samples (AP30, 30 mg/kg AP; AP60, 60 mg/kg AP; FAP30, 30 mg/kg FAP; FAP60, 60 mg/kg FAP and IM, 10 mg/kg indomethacin) were orally administered before the carrageenan injection. The carrageenan-treated control group (CC) received vehicle alone instead of samples. The animals were sacrificed 24 h later, and exudates from each air pouch were collected. (A) The levels of TNF-α, IL-1β and IL-6 in ear tissues were measured by ELISA analysis. (B) The expression levels of COX-2, iNOS, p-p65, p65 and β-actin were by immunoblot analysis. Each value is expressed as the mean ± S.D. (n=6). *p < 0.05 vs. carrageenan-treated group.

DISCUSSION

The constituents of orally administered herbal medicines are inevitably brought into contact with human intestinal microflora and may therefore, be transformed by the intestinal microflora before absorption from the intestinal tract into the blood. Therefore, studies on the metabolism of the constituents by intestinal microflora are important for understanding their biological effects (Kobashi and Akao, 1997; Kim, 2002). However, the pharmacological activities may be significantly different between individuals due to the diversity of intestinal microflora. Therefore, fermentation technologies are currently being developed to increase the pharmacological effects of herbal medicines (Bae et al., 2004; Lee et al., 2006). The anti-scratching behavioral, anti-PCA reaction and anti-asthmatic effects of AP were increased by LAB fermentation (Lee et al., 2006; Bae et al., 2007). However, the inhibitory effects of FAP on inflammatory diseases, such as dermatitis, have not been studied. In addition, fermentation of herbal medicines by Bifidobacterium sp. is troublesome, because these bacteria are anaerobic and sensitive to AP essential oil. Therefore, we developed an aerotolerant lactic acid bacterium, Lactobacillus brevis K-1, and used it to
ferment essential oil-excluded AP. We then investigated the anti-inflammatory effects of the fermented product in mice.

Allergic reactions including rhinitis, asthma and anaphylaxis produced many inflammatory mediators and caused inflammation, scratching, pain and increased vascular permeability (Plaut et al., 1989; Stevens and Austen, 1989; Wuthrich, 1989; Biely, 2004). Steroids, anti-histamines, and immunosuppressants have potent anti-inflammatory effects, but can cause intense side reactions (Simons, 1992; Schafer-Korting et al., 1996; Sakuma et al., 2001; Friedman et al., 2002). Therefore, herbal medicines including AP have been advanced for use in inflammatory and allergic diseases, and AP has received increasing attention because of its effectiveness. We developed FAP fermented by Lactobacillus brevis K-1 to increase the anti-inflammatory and anti-allergic activities of AP. In the present study, we evaluated the inhibitory effects of AP and FAP on TPA-induced ear dermatitis in mice. TPA-induced dermatitis was accompanied by sustained swelling and redness, as previously reported (Reynolds et al., 1998; Park et al., 2001). COX-2 and iNOS, markers of acute inflammatory disease (Reynolds et al., 1998; Hernandez et al., 2001; Park et al., 2001), were also induced. AP and FAP significantly inhibited the sustained swelling (thickness) caused by TPA, as well as COX-2 and iNOS expression, in the mouse ears. AP and FAP also inhibited inflammatory markers, such as increased exudate volume, cell number, protein content, and leukocyte number, in carrageenan-injected air pouches in mice. Carrageenan also increased the levels of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6. AP and FAP inhibited the carrageenan-induced COX-2 and iNOS expression, as well as NF-κB activation, which promotes COX-2 and iNOS expression (Fig. 3). The anti-inflammatory effects of FAP were more potent than those of AP. These results suggest that fermentation may activate the anti-inflammatory effect of AP, and FAP may inhibit inflammatory reactions by inhibiting pro-inflammatory cytokines and the NF-κB pathway. This hypothesis was supported by the findings that AP and FAP significantly inhibited the expressions of TNF-α and IL-1β as well as the expression of COX-2 and iNOS and the activation of transcription factor NF-κB in LPS-induced peritoneal macrophages. Based on these findings, FAP can improve inflammatory disorders, such as dermatitis, by inhibiting the NF-κB pathway.

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