

Effects of the Rhizomes of *Atractylodes japonica* and Atractylenolide I on Allergic Response and Experimental Atopic Dermatitis

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(Received April 24, 2012/Revised August 22, 2012/Accepted September 19, 2012)

Although some anti-allergic activities of the rhizome of *Atractylodes japonica* have been previously reported, the active principle(s) for anti-allergic action is not fully elucidated and the effect of this plant material on atopic dermatitis (AD) is not known. In this study, the 70% ethanol extract of the rhizome of *A. japonica* was found to significantly inhibit 5-lipoxygenase (5-LOX)-catalyzed leukotrienes (LT) production from rat basophilic leukemia (RBL)-1 cells. From the extract of *A. japonica*, three major sesquiterpene derivatives including atractylenolide I, atractylenolide III and eudesma-4,7-dien-8-one were successfully isolated. Among these compounds, only atractylenolide I was shown to strongly inhibit 5-LOX from RBL-1 cells ($IC_{50} = 18.6 \mu M$). To evaluate the effects of experimental AD, the ethanol extract of *A. japonica* (200 mg/day) was administered orally to hapten-treated NC/Nga mice which is an animal model of AD. It was firstly found that the extract significantly inhibited AD-like symptoms in mice, as judged by severity score and scratching behavior. Taken together, it is concluded that *A. japonica* possesses the inhibitory activity on 5-LOX and an animal model of AD, and atractylenolide I may contribute, at least in part, to these anti-allergic actions of *A. japonica*.

Key words: *Atractylodes japonica*, Atractylenolide I, 5-Lipoxygenase, Anti-allergy, Atopic dermatitis

INTRODUCTION

Atopic dermatitis (AD), a chronic allergic skin disorder, is characterized by frequent itching with flare, hemorrhage, and hyperplasia on the affected skin (Leung, 1995; Rudikoff and Lebowitz, 1998). Although several classes of drugs including steroidal anti-inflammatory drugs, anti-histamines, and immunosuppressants are currently used to treat AD (Oyoshi et al., 2009; Murota and Katayama, 2011), the control and cure of AD is still difficult, due to the complex and unknown etiology of AD. Nonetheless, many pathological molecules involved in this disease have been identified to date (Novak, 2009; Oyoshi et al., 2009). Among these, leukotrienes (LT) produced by 5-lipoxygenase (5-LOX) are found to be deeply involved in AD (Rubin and

Mollison, 2007). Therefore, it is believed that the 5-LOX inhibitor may alleviate the symptoms of AD. In this regard, herbal drugs having a 5-LOX inhibitory action can potentially treat human AD.

Atractylodis Rhizoma Alba is a rhizome of *Atractylodes japonica* Koidz. This species of plant, belonging to the Compositae family, is distributed in some Asian countries such as Korea, China, and Japan. It has been used as an aromatic stomachic and diuretic, and used as an immune-boosting, anti-microbial, and anti-inflammatory agent (Pu et al., 1996; Jang et al., 2004; Hong et al., 2010; Jeong et al., 2010; Choi et al., 2011a). Phytochemical investigations have revealed a number of chemical components such as sesquiterpenes, sesquiterpene glycosides, and polyacetylenes. Monoterpene glycosides, aromatic glycosides, sucrose esters, and steroids were also isolated from *A. japonica* rhizomes (Yamamoto et al., 1993; Kitajima et al., 2003; Jeong et al., 2010; Choi et al., 2011b).

Previously, the aqueous extract of the rhizome of *A. japonica* was reported to inhibit PGE_2 and nitric oxide

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(NO) production from lipopolysaccharide-treated RAW 264.7 cells mainly, via the down-regulation of cyclooxygenase-2 and inducible NO synthase (Jang et al., 2004). Recently, the ethanol extract has shown some anti-inflammatory and anti-allergic activities. The ethanol extract inhibited proinflammatory and allergy-related cytokine production from mast cells (Hong et al., 2010). However, it did not affect the immediate hypersensitivity reaction. In addition, the herbal combination having *Achyranthes bidentata* and *A. japonica* had demonstrated inhibitory activity on animal subjects with arthritis, collagen-induced arthritis and adjuvant-induced arthritis (Han et al., 2005). These previous studies have shown that *A. japonica* possesses some anti-inflammatory and anti-allergic activities, and *A. japonica* may have a potential to treat human allergic disorder(s).

In our preliminary experiment, the 70% ethanol extract of *A. japonica* was found to inhibit 5-LOX. From these findings, we chose to investigate the inhibitory activity of *A. japonica* and its constituents on 5-LOX and on immediate hypersensitivity reaction, β -hexosaminidase release, from the mast cells. In addition, the inhibitory activity of the ethanol extract of *A. japonica* on an animal model of AD was studied.

MATERIALS AND METHODS

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), p-nitrophenyl-N-acetyl- β -D-glucosaminide, nordihydroguaiaretic acid (NDGA), carboxymethylcellulose (CMC), quercetin, dinitrophenyl (DNP)-BSA and anti-DNP mouse IgE were purchased from Sigma Chem. A23187 was obtained from Biomol. 2,4,6-Trinitro-1-chloro benzene (TNCB, picryl chloride) was purchased from Nacalai Tesque. Dulbecco's Modified Eagle Medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were products of Gibco BRL. A protein assay kit was purchased from Bio-Rad. Clobetasol 17-propionate ointment (0.05% w/w, Dermovate[®]) was obtained from GlaxoSmithKline Korea.

Animals

Male NC/Nga mice (6 weeks old, specific pathogen free) were purchased from SLC. The animals were maintained under the conditions of 20–22°C, 40–60% relative humidity and a 12 h/12 h (light/dark) cycle. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KIACUC-11-0007). The animals were handled according to the guidelines described in the KFDA Guide for

the Care and Use of Laboratory Animals throughout the experiments.

Preparation of the extract of *A. japonica* and isolation of the major constituents

The plant material was provided and authenticated by Prof. J. H. Lee at Dongkuk University. For pharmacological evaluation, a portion of air-dried and chopped *A. japonica* rhizomes were extracted separately with hot water and 70% ethanol, two times under reflux. The water and 70% ethanol extracts were obtained after vacuum evaporation and the yields were 11.9% and 12.4%, respectively. For isolation of the active principle(s), the dried rhizomes of *A. japonica* (1.5 kg) were extracted with MeOH, and a residue (268 g) was obtained after removing the solvent under reduced pressure. This MeOH extract was suspended with water and continuously partitioned by *n*-hexane, CHCl_3 , EtOAc, and *n*-BuOH. The *n*-hexane fraction (71 g) was chromatographed on a silica gel column eluted with *n*-hexane-EtOAc (100:0 $\rightarrow$ 5:1) to give fourteen fractions (H1–H14). Fraction H3 precipitate (5.42 g) was applied to reversed-phase HPLC (MeOH- H_2O (70:30)), compound **1** (20 mg, 0.007% w/w) was separated during this process. Fraction H9 (5.22 g) was applied to a silica gel column and eluted with *n*-hexane-EtOAc (60:1 $\rightarrow$ 1:1) to afford twenty fractions (H9-1–H9-20). Using reversed-phase HPLC (MeOH- H_2O (73:27)), compound **2** (53 mg, 0.020% w/w) was separated from fraction H9-5. Fraction H10 (861.3 mg) was applied to a silica gel column and eluted with *n*-hexane-EtOAc (80:1 $\rightarrow$ 5:1), compound **3** (77 mg, 0.029% w/w) was separated during this process. The compounds **1**, **2**, and **3** were identified with spectroscopic data analysis by comparison with literature values (Ding et al., 2000; Chang et al., 2007; Duan et al., 2008) and they were revealed as atractylenolide III (**1**), eudesma-4(14),7(11)-dien-8-one (**2**) and atractylenolide I (**3**) (Fig. 1). It was confirmed that three isolated compounds from methanol extract were also found in 70% ethanolic extract through TLC comparison of anisaldehyde- H_2SO_4 color development, R_f values, and UV absorption patterns.

Rat basophilic leukemia-1 (RBL-1) cell culture and measurement of LT

To evaluate the 5-LOX inhibitory activity, RBL-1 cells purchased from the American Type Culture Collection (ATCC) were cultured in RPMI 1640 with 10% FBS, 2 mM glutamine and 1% antibiotics under 5% CO_2 at 37°C. The test compounds dissolved in DMSO were added to the cells. The cells were then pre-incubated with the test compounds for 10 min. A23187 (3 μM) was added to activate the 5-LOX and the cells were

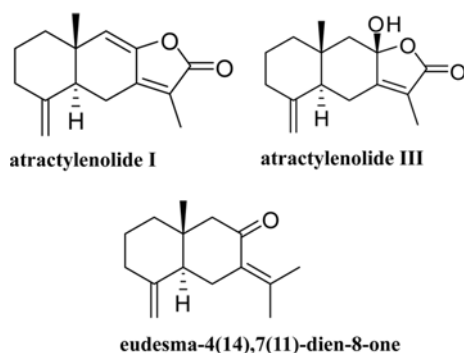


Fig. 1. Chemical structures of the constituents isolated from the rhizomes of *A. japonica*.

incubated for 15 min as previously described, with slight modification (Tries et al., 2002). The media was then collected and the concentration of the 5-LOX product, cysteinyl leukotrienes ($\text{LTC}_4/\text{D}_4/\text{E}_4$), was measured using an ELISA kit (Enzo Life Sciences) as recommended by the manufacturer. The cell viability was assessed using an MTT assay as previously described (Mossman, 1983).

RBL-2H3 cell culture and antigen-induced degranulation of β -hexosaminidase

According to the previously described procedure (Choi et al., 1996), RBL-2H3 cells (ATCC) were cultured in 24-well plates (2×10^5 cells/well) using DMEM with 10% FCS. Anti-DNP mouse IgE was added for sensitization and incubated overnight. Twenty four hours later, the cells were washed and test compounds were added. After 10 min incubation with DNP-BSA ($1 \mu\text{g}/\text{mL}$), the reaction was stopped by cooling in an ice bath for 10 min. The supernatant was transferred into 96-well plates. The substrate (1 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide) was added and incubated for 1 h at 37°C . The reaction was stopped by adding 0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ ($200 \mu\text{L}/\text{well}$). The absorbance was measured at 405 nm .

Hapten-induced atopic dermatitis (AD) in NC/Nga mice

To the hair-removed backs of mice, $200 \mu\text{L}$ of 1% trinitrochlorobenzene (TNCB) solution in acetone:olive oil (3:1) was topically applied according to the previously described procedures (Matsuda et al., 1997; Suto et al., 1999). Three days later, TNCB solution was applied again. One week after the initial treatment, 0.4% TNCB solution ($150 \mu\text{L}$) was applied three times per week for 4 weeks to induce dermatitis. According to the slightly modified criteria of the previously described procedure (Leung et al., 1990; Suzuki et al., 2002), the skin severity was scored based on the skin clinical

symptoms, dry skin and lichenification, edema and excoriation, and erythema on the last day. Scratching behavior was measured by observing each mouse for 20 min. And mice were sacrificed and blood was withdrawn. The IgE concentration of the serum was measured using an ELISA kit (Shibaya Co.). The ethanol extract of *A. japonica* dissolved in 0.4% CMC was administered orally at $200 \text{ mg}/\text{kg}/\text{day}$, 5 days/week for 5 weeks during the experiment. Clobetasol 17-propionate ointment ($30 \text{ mg}/\text{day}/\text{mouse}$, 5 days/week, 5 weeks) was applied topically as a reference drug.

Statistical analysis

All data were represented as arithmetic mean $\pm$ S.D. One-way analysis of variance (ANOVA), followed by Dunnett's test was used to determine the statistical significance.

RESULTS

Treatment of A23187 (ionophore) on RBL-1 cells activated 5-LOX, produced high amounts of cysteinyl-LTs ($1,609.0 \pm 129.8 \text{ pg}/\text{mL}$) following 15 min of incubation. The level of the cysteinyl-LTs without A23187 was $337.2 \pm 13.7 \text{ pg}/\text{mL}$ ($n = 3$). The water and ethanol extracts of *A. japonica* were treated and it was found that the ethanol extract showed much higher 5-LOX inhibitory activity (Fig. 2A). The IC_{50} of the ethanol extract was $< 12.5 \mu\text{g}/\text{mL}$, while the water extract reduced LT production (44.0%) at $200 \mu\text{g}/\text{mL}$. Based on these findings, the major three compounds including atractylenolide I, III and eudesma-4,7-dien-8-one were isolated to find for active principles. Among these derivatives, only atractylenolide I showed strong inhibition against 5-LOX-catalyzed LT production (Fig. 2B). IC_{50} of atractylenolide I was $18.6 \mu\text{M}$. Atractylenolide III showed only a 13.1% reduction of LT production at $50 \mu\text{M}$. As expected, the NDGA ($1 \mu\text{M}$) that was used as a reference strongly inhibited the LT production (98%). These results indicate that atractylenolide I may contribute to the 5-LOX inhibitory action of *A. japonica*. No cytotoxic effect to RBL-1 cells was observed in all groups of the test compounds as revealed by the MTT bioassay.

It is well-known that RBL-2H3 cells degranulate β -hexosaminidase along with histamine release (Schwartz et al., 1981; Marquardt and Wasserman, 1983). In the present study, when an antigen was treated, β -hexosaminidase was degranulated into the media from RBL-2H3 cells, for a 10 min incubation period (from $0.0 \pm 1.3\%$ to $100.0 \pm 5.0\%$ release, $n = 3$). The water extract did not show the inhibitory activity against this parameter (Fig. 2C). The ethanol extract showed some in-

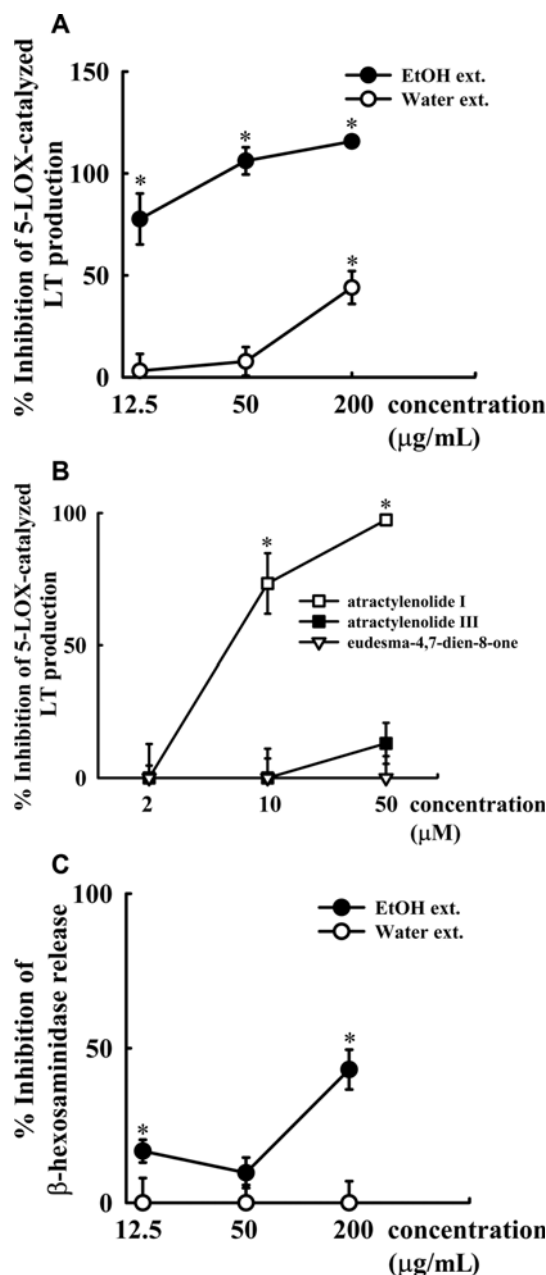


Fig. 2. Inhibition of LT production and β-hexosaminidase release by *A. japonica* and the constituents. (A) Effects of the water and ethanol extracts on 5-LOX-catalyzed LT production in A23187-treated RBL-1 cells. (B) Effects of the constituents on 5-LOX-catalyzed LT production in A23187-treated RBL-1 cells. (C) Effects of the water and ethanol extracts on β-hexosaminidase release in antigen-treated RBL-2H3 cells.

hibitory action, but it was cytotoxic at 200 μg/mL (data not shown). On the other hand, the reference agent, quercetin (degranulation inhibitor, 20 μM), inhibited β-hexosaminidase release (92.1%) as expected. These results suggest that *A. japonica* possesses 5-LOX inhibitory action, but does not show considerable inhibi-

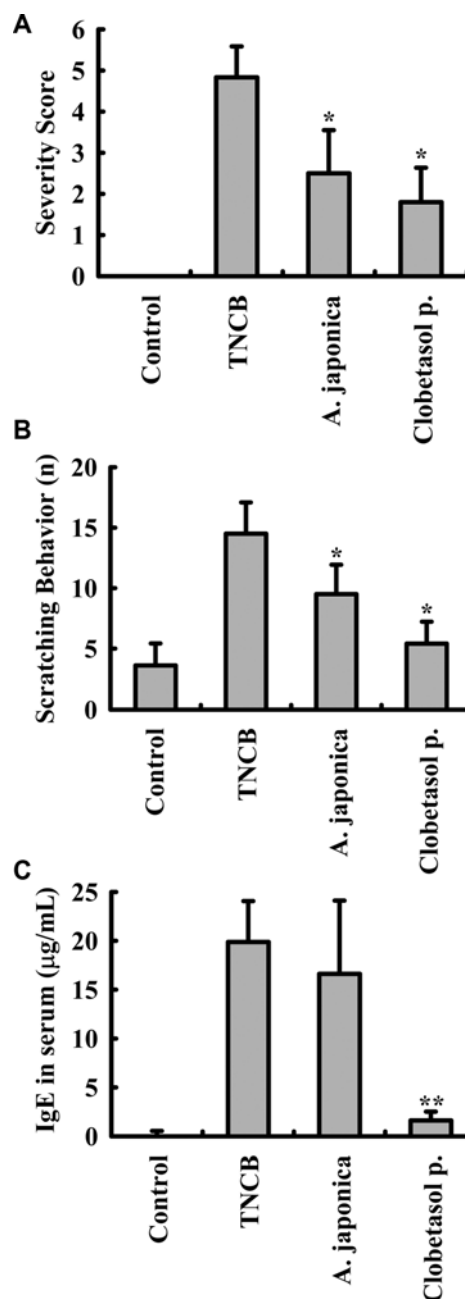


Fig. 3. Inhibition of experimental AD in NC/Nga mice by the ethanol extract of *A. japonica*. (A) Skin severity score, three categories were checked on the skin of NC/Nga mice (dry skin and lichenification, edema and excoriation, erythema), and each category was scored based on the severity as follows: no symptom (0), weak (1), medium (2), severe (3). The scores were combined in each animal. (B) Scratching behavior, number of scratching was counted for 20 min period. (C) Serum IgE concentration, data and bars represented the arithmetic mean ± S.D. ($n = 5$). * $p < 0.01$, significantly different from the TNCB-treated control group.

tory action on a degranulation reaction.

Since 5-LOX and its reaction products (LTs), are deeply associated with the symptoms of AD (Rubin

and Mollison, 2007), the ethanol extract of *A. japonica* was examined in an animal model of AD, a hapten-induced dermatitis in NC/Nga mice. Following the topical application of hapten, NC/Nga mice develop human AD-like symptoms (Matsuda et al., 1997; Suto et al., 1999). In this study, a repetitive application of the hapten, TNCB, for 5 weeks produced skin lesions of the affected area. The severity score on the affected skin area increased dramatically (4.8 ± 0.8), and scratching behavior also significantly increased (14.5 ± 2.6 from the basal level of 3.6 ± 1.8 , $n = 5$) as shown in Fig. 3A and 3B. Serum IgE concentration increased considerably with TNCB treatment (Fig. 3C). Against these parameters, the ethanol extract of *A. japonica* showed some reduction at 200 mg/kg/day. It significantly alleviated skin severity (47.9% reduction) as well as the scratching behavior (45.9% reduction), although the serum IgE concentration was not significantly reduced (16.6%). Clobetasol 17-propionate, the reference drug, also showed significant reduction of skin severity, scratching behavior, hyperplasia and inflammatory cell recruitment as expected, being more potent than *A. japonica*. It also reduced IgE concentration.

DISCUSSION

The present investigation clearly demonstrated that *A. japonica* possesses an inhibitory activity on 5-LOX-catalyzed LT production from a mast cell line, and *in vivo* experimental AD in mice. Among the major constituents isolated, atractylenolide I was found to inhibit 5-LOX.

This study also found that the extracts of *A. japonica* did not show the significant inhibition on a degranulation reaction. This result correlated with a previous study (Hong et al., 2010) that *A. japonica* did not inhibit compound 48/80-induced histamine release from the mast cells and *in vivo* passive cutaneous anaphylaxis reaction (immediate hypersensitivity). Since Compound 48/80 is an ionophore to influx calcium ion to degranulate histamine to the media, it is suggested that *A. japonica* does not affect calcium influx as well as the degranulation reaction.

In the present investigation, three sesquiterpene derivatives were isolated. Previously, several pharmacological actions of these compounds were reported. In particular, atractylenolide I and III were demonstrated to possess anti-inflammatory activity *in vivo* (Li et al., 2007a; Dong et al., 2008). Atractylenolide I and III inhibited TNF- α and NO production in macrophages (Li et al., 2007b). It was further demonstrated that atractylenolide III inhibited IL-6 secretion from human mast cells (Kang et al., 2011). Recently, we have found

that atractylenolide I inhibited matrix metalloproteinase-13 expression in chondrocytes and carrageenan-induced paw edema in mice (Park et al., 2011). But, anti-allergic action of the major constituents including atractylone, atractylenolide I and III has been rarely elucidated. Here it was found for the first time that atractylenolide I is a 5-LOX inhibitor and may contribute to the 5-LOX inhibitory action and AD inhibitory action of *A. japonica*, at least in part.

In conclusion, the results of the present study clearly demonstrated that *A. japonica* possesses the inhibitory activity on AD-like symptoms. The ethanol extract and one of the constituents, atractylenolide I, reduced LT production by 5-LOX, and these inhibitory actions could contribute to the inhibitory action of AD by *A. japonica*. Moreover, our results provide a scientific basis for the anti-allergic use of *A. japonica* in traditional medicine.

ACKNOWLEDGEMENTS

This study was financially supported by the research fund of Studies on the Identification of the Efficacy of Biologically Active Components from Oriental Herbal Medicines from Korean Food and Drug Administration (2009-2011) and post-BK21 project from the Ministry of Education, Korea.

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