

# Inhibition of Mast Cell-Mediated Allergic Responses by Arctii Fructus Extracts and Its Main Compound Arctigenin

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## Supporting Information

**ABSTRACT:** The *Arctium lappa* seeds (Arctii Fructus) and its major active compound, arctigenin (ARC), are known to have anticancer, antiobesity, antiosteoporosis, and anti-inflammatory activities. However, the effect of Arctii Fructus and ARC on mast cell-mediated allergic inflammation and its associated mechanism have not been elucidated. Therefore, we attempted to investigate the antiallergic activity of Arctii Fructus and ARC on mast cells and experimental mouse models. Arctii Fructus water extract (AFW) or ethanol extract (AFE) and ARC reduced the production of histamine and pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  in mast cells. AFW, AFE, and ARC inhibited phosphorylation of MAPKs and NF- $\kappa$ B in activated mast cells. Moreover, IgE-mediated passive cutaneous anaphylaxis and compound 48/80-induced anaphylactic shock were suppressed by AFW, AFE, and ARC administration. These results suggest that Arctii Fructus and ARC are potential therapeutic agents against allergic inflammatory diseases.

**KEYWORDS:** Arctii Fructus, arctigenin, allergic inflammation, mast cells, MAPK

## INTRODUCTION

Allergic diseases, such as allergic rhinitis, allergic eczema, and atopic dermatitis, are hypersensitivity disorders of the immune system. The number of allergy patients has consistently increased with approximately 10–20% of the world's population suffering from allergic diseases.<sup>1</sup> Thus, allergic diseases are important public health problems that can influence quality of life and socioeconomic burden. Antihistamines, steroids, nonsteroidal anti-inflammatory drugs, and immunosuppressants are common drugs currently used to treat and prevent allergic diseases. However, these medications can show central side effects, and in certain cases do not result in efficient recovery.<sup>2</sup>

Mast cells play a critical role as regulators of allergic diseases due to their wide distribution in various tissues.<sup>3</sup> Allergic reactions result in mast cell degranulation, which leads to the rapid release of histamine and various inflammatory mediators such as leukotrienes, serotonin, chemokines, and proinflammatory cytokines, including interleukin (IL)-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ . These mediators aggravate allergic responses through the infiltration, differentiation, and activation of immune cells.<sup>4</sup> Therefore, modulation of mast cell degranulation and release of allergic mediators, is an effective treatment strategy for allergic diseases.

The mitogen-activated protein kinases (MAPKs) signaling pathway is associated with several pivotal physiological processes such as proliferation, activation, degranulation, and migration. Extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 are major components of the MAPK signaling pathway.<sup>5–7</sup> Extracellular stimuli activate MAPKs, which induce the degradation of I $\kappa$ B proteins and the nuclear translocation of nuclear factor-kappaB (NF- $\kappa$ B). Activation of NF- $\kappa$ B causes the transcription of target genes,

including inflammatory mediators, which are crucial factors in the development of allergic responses.<sup>6,8,9</sup>

*Arctium lappa* seeds (Arctii Fructus) have been used as anti-inflammatory, diuretic, and detoxifying drugs in oriental medicine.<sup>10</sup> The Arctii Fructus extract improves high fat/cholesterol diet-induced endothelial dysfunction and dermal extracellular matrix metabolism, which reduces wrinkles.<sup>11,12</sup> Arctigenin (ARC) is one of the major active constituents present in the Arctii Fructus water extract (AFW) and ethanol extract (AFE).<sup>13</sup> ARC has antihypertension, antitumor, antiosteoclastogenesis, and neuroprotective activities.<sup>14–19</sup> Specifically, fermented Arctii Fructus extract can suppress IgE-mediated allergic reaction in RBL-2H3 cells.<sup>20</sup> Moreover, ARC successfully inhibited ovalbumin (OVA)-induced anaphylaxis, compound 48/80-induced histamine release, and proinflammatory enzyme activities.<sup>21</sup> However, the antiallergic activities of Arctii Fructus and ARC on human mast cells, and the underlying mechanisms are unclear. The aim of this study was to determine the effect of Arctii Fructus and ARC on mast cell-mediated allergic inflammation.

## MATERIALS AND METHODS

**Preparation of Extracts and ARC Isolation from Arctii Fructus.** Dried and ground Arctii Fructus powder were obtained from Omniherb (Uiseong, Republic of Korea). AFW was prepared by extracting the water-soluble components of the Arctii Fructus powder in boiling water (100 g/L of water) at 100 °C for 3 h. The solution was filtered using Whatman filter paper and lyophilized. To prepare the AFE, 100 g of Arctii Fructus was boiled in 70% ethanol (1 L) for 3 h and evaporated using a Rotary evaporator. The extract was freeze-

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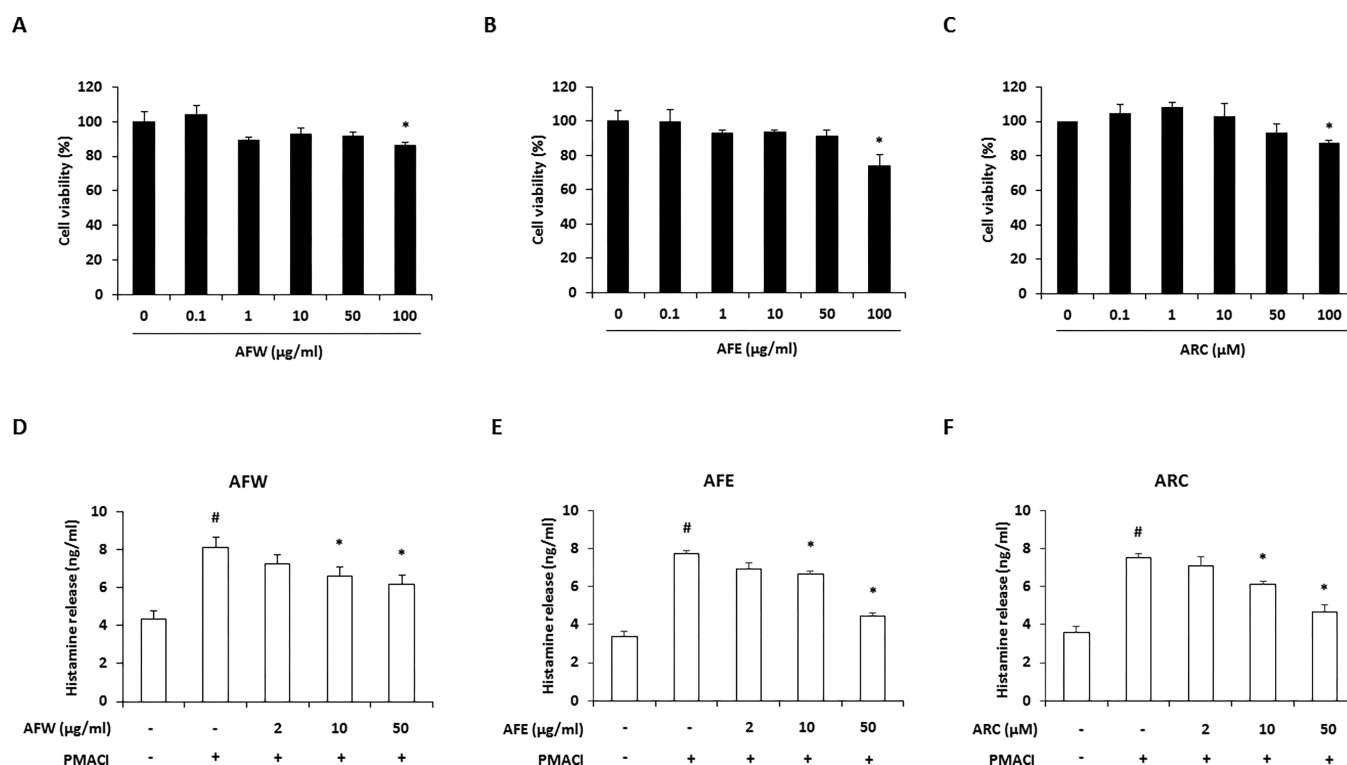


**Figure 1.** Effect of AFW, AFE, and ARC on cell viability and histamine release in HMC-1 cells. (A–C) Viability of HMC-1 cells after AFW (A), AFE (B), and ARC (C) treatment for 24 h. (D–F) HMC-1 cells ( $5 \times 10^4$  cells/well) were seeded in a 24-well plate and pretreated with AFW (D), AFE (E), and ARC (F) for 1 h. After stimulation with PMACI for 24 h, the supernatant was used for analysis using the histamine ELISA kit. Each bar represents the mean  $\pm$  SD of three independent experiments. # $p < 0.05$  versus blank and \* $p < 0.05$  versus PMACI alone.

dried at  $-56^\circ\text{C}$  and 9 mm Torr. ARC was isolated from *Arctii Fructus* as reported previously.<sup>21</sup> ARC was identified<sup>22</sup> by comparing the  $^1\text{H}$  NMR spectrum data.<sup>23</sup> AFW was dissolved in distilled water, whereas AFE and ARC were dissolved in DMSO (final concentration of DMSO is less than 0.1%).

**Reagents.** Antiphospho-ERK, phospho-JNK, phospho-p38, phospho-I $\kappa$ B $\alpha$ , NF- $\kappa$ B, and receptor interacting protein-2 (RIP2) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Antihistone,  $\alpha$ -tubulin, ERK, JNK, p38, and caspase-1 antibodies were purchased from Santa Cruz Biotechnology (CA, U.S.A.). Thiazolyl Blue Tetrazolium Bromide (MTT), PMA, A23187, DNP-IgE, DNP-BSA, and compound 48/80 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.).

**Cell Culture.** The human mast cell line (HMC-1) was provided by Eichi Morri (Osaka University, Japan), and the rat basophilic leukemia mast cell line RBL-2H3 was purchased from Korean Cell Line Bank (Seoul, Republic of Korea). These cell lines were cultured in 10% FBS, 100 U/mL of penicillin and streptomycin in Iscove's Modified Dulbecco's Medium and Dulbecco's Modified Eagle's Medium, respectively. Cells were cultured in an incubator with 5%  $\text{CO}_2$ /95% air at  $37^\circ\text{C}$ .

**Cell Viability.** Cells were seeded in 24-well microplates at a density of  $5 \times 10^4$  cells/well and stabilized overnight. After 24 h of treatment with AFW, AFE, and ARC, cells were treated with MTT reagent (0.5 mg/mL) and incubated for 4 h. Formazan was lysed with DMSO, and colorimetric changes were measured using a VersaMax ELISA Microplate Reader at 540 nm.

**Histamine Assay.** Histamine production from mast cells was measured using the human histamine ELISA kit (ALPCO Diagnostics, NH, U.S.A.) as per the manufacturer's protocol. HMC-1 cells were pretreated with AFW, AFE, and ARC for 1 h and stimulated with PMA plus A23187 (PMACI; PMA 50 nM and A23187  $1 \mu\text{M}$ ) for 24 h. RBL-2H3 cells were sensitized with DNP-IgE (100 ng/mL) for 12 h, and the samples were added to cells 1 h before DNP-BSA treatment. The conditioned medium was collected and used as a sample.

**Real-Time RT-PCR.** Total RNA was isolated from cells, and cDNA was synthesized using RNA-spin, Total RNA Extraction Kit, and Power cDNA Synthesis Kit (iNtRon Biotech, Seoul, Republic of Korea). Expression specific genes were determined by step-one plus real-time PCR systems (Applied Biosystems, Foster City, CA, U.S.A.). All data were normalized to GAPDH mRNA. The primer sequences are summarized in the Supporting Information, SI.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Release of proinflammatory cytokines was measured using a BD OptEIA ELISA kit (BD Pharmingen, San Diego, CA, U.S.A.) according to the manufacturer's protocol. Briefly, 96-well microplates were precoated with capture antibodies at  $4^\circ\text{C}$  overnight. After blocking with 5% FBS for 1 h, samples and standards were added and incubated for at least 3 h. The plates were washed with washing buffer and incubated with detection antibodies and streptavidin HRP for 1 h 30 min. This was followed by washing with 0.05% PBST five times, and the plates were incubated with TMB substrate reagent at  $37^\circ\text{C}$  for 30 min. Colorimetric development was blocked with a stop solution and measured using a microplate reader at 405 nm.

**Western Blot Analysis.** Collected cells were washed and lysed using pro-prep protein extraction solution (iNtRon Biotech, Seoul, Republic of Korea) to extract total protein. Cell lysates were centrifuged at 13 000 rpm for 5 min, and the supernatants containing the proteins were obtained. Nuclear extracts were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Thermo Scientific, Rockford, IL, U.S.A.). Total protein was quantified using BCA protein assay, mixed with a 5 $\times$  sample buffer. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to a PVDF membrane. Specific proteins were detected using primary antibodies and Horseradish Peroxidase secondary antibodies. Protein bands were photographed using the FluorChem M system (ProteinSimple, San Jose, CA, U.S.A.).

**Caspase-1 Activity Assay.** Caspase-1 colorimetric assay kit (Biovision, CA, U.S.A.) was used to determine caspase-1 activity according to the recommended instructions. Briefly, PMACI was

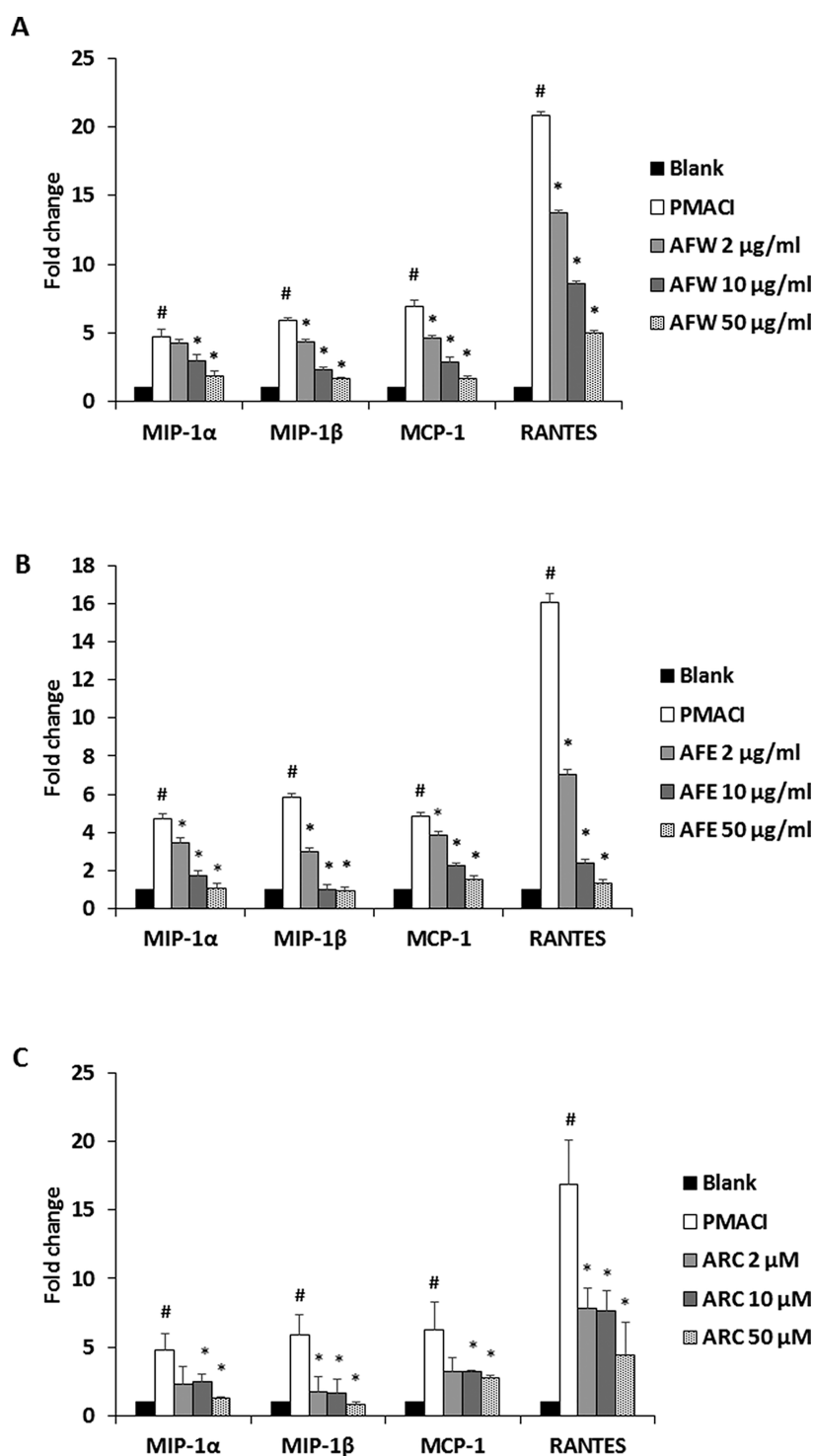


**Figure 2.** Effect of AFW, AFE, and ARC on mRNA expression of chemokines in PMACI-stimulated HMC-1 cells. The expression of chemokines was measured using real-time RT-PCR. HMC-1 cells ( $5 \times 10^4$  cells/well) were seeded in a 24-well plate and pretreated with AFW, AFE (2–50  $\mu\text{g}/\text{mL}$ ) and ARC (2–50  $\mu\text{M}$ ) for 1 h. After stimulation with PMACI for 24 h, cells were collected and used for real-time RT-PCR. Each bar represents the mean  $\pm$  SD of three independent experiments.  $\#p < 0.05$  versus blank and  $*p < 0.05$  versus PMACI alone.

added to AFW-, AFE-, and ARC-pretreated HMC-1 cells for 24 h, and harvested cells were lysed with lysis buffer. Caspase-1-specific peptide YVAD-pNA was used for the measurement of caspase-1 activity. Colorimetric changes were quantified using a microplate reader at 405 nm.

**Animals.** ICR mice (5 weeks, male) were purchased from Samtaco Korea (Osan, Republic of Korea). Mice were maintained in a laminar airflow room at  $22 \pm 1$  °C. An in vivo experiment was performed in

accordance with the regulations issued by Wonkwang University guidelines (WKU14–100).

**Compound 48/80-Induced Anaphylactic Shock.** AFW, AFE (25–100 mg/kg), and ARC (10–50 mg/kg) were orally administered 1 h prior to injecting compound 48/80. Control mice received the same volume of 0.05% carboxymethyl cellulose. After intraperitoneal injection of compound 48/80 (8 mg/kg), mortality by anaphylactic shock was monitored for 1 h.

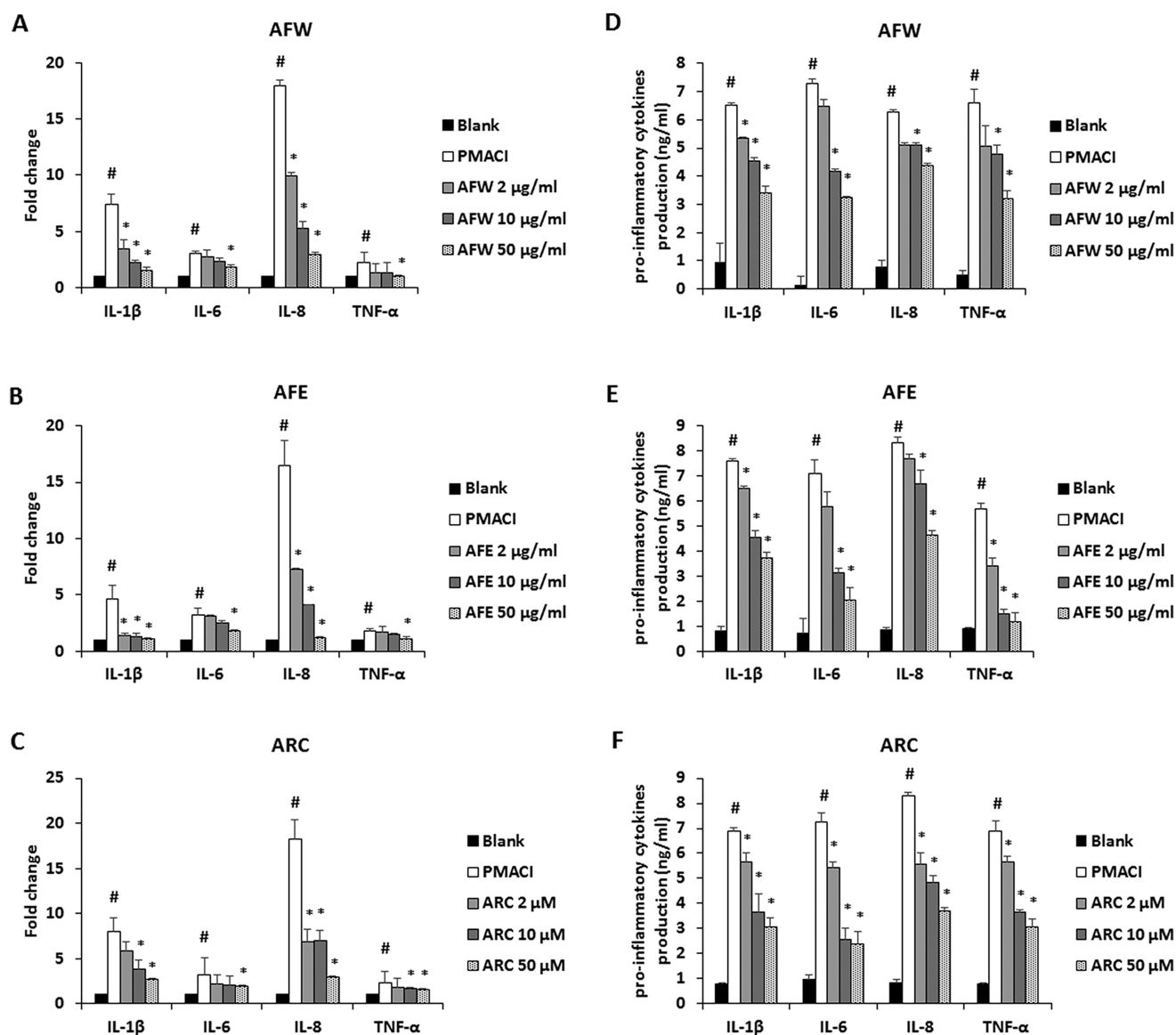


**Figure 3.** Effect of AFW, AFE, and ARC on the production of proinflammatory cytokines in PMACI-stimulated HMC-1 cells. HMC-1 cells ( $5 \times 10^4$  cells/well) were seeded in a 24-well plate and pretreated with AFW, AFE (2–50  $\mu\text{g/mL}$ ), and ARC (2–50  $\mu\text{M}$ ) for 1 h. After stimulation with PMACI for 24 h, media and cells were collected separately. The supernatant was used for ELISA, and the cells were used for real-time RT-PCR analysis. (A–C) The mRNA expression of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  was determined using real-time RT-PCR. (D–F) The production of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  was measured using the ELISA kit. Each bar represents the mean  $\pm$  SD of three independent experiments. # $p < 0.05$  versus blank and \* $p < 0.05$  versus PMACI alone.

**Passive Cutaneous Anaphylaxis (PCA).** A day prior to the experiment, hair was completely removed from the back of mice, and antidinitrophenyl (DNP)-IgE (0.5  $\mu\text{g}$ ) was injected into the dorsal skin. AFW, AFE (100 mg/kg), and ARC (50 mg/kg) were orally administered 1 h before the antigen challenge. DNP-BSA (1  $\mu\text{g}$ ) in saline containing 1% Evans blue was intravenously injected into each mouse via the tail vein. After killing, the dorsal skin was resected to evaluate the effect of the compounds on PCA. Blue dye in the dorsal skin was dissolved with formaldehyde for 24 h and colorimetric change was measured using a microplate reader at 620 nm. The inhibition rates of the compounds were calculated using the following equation: Inhibition rate (%) =  $100 - (a/b \times 100)$ ; “a” is the average absorbance of the sample group, and “b” is the average absorbance of the control group.

**Statistical Analysis.** Results are shown as the mean  $\pm$  SD from at least three experiments. All statistical analyses were performed using the Student’s *t*-test, and  $p < 0.05$  indicated statistical significance.

## RESULTS

**Arctii Fructus Extracts and ARC Decrease Histamine Release in Mast Cells.** Mast cell degranulation results in histamine release by antigen stimulation causing allergic reactions.<sup>4</sup> Therefore we investigated whether AFW, AFE, and ARC can suppress the release of histamine from activated mast cells. MTT assay was performed to determine the effect of AFW, AFE, and ARC on the viability of HMC-1 cells. As shown in Figure 1A and B, AFW and AFE (2–50  $\mu\text{g/mL}$ ) did not result in any cytotoxicity after 24 h of incubation. Doses of  $<50 \mu\text{M}$  ARC had no effect on HMC-1 cell viability; however, use of 100  $\mu\text{M}$  ARC resulted in 20% cytotoxicity (Figure 1C). Therefore, we estimated the effects of AFW, AFE (2–50  $\mu\text{g/mL}$ ), and ARC (2–50  $\mu\text{M}$ ) on mast cell degranulation. AFW and AFE suppressed histamine release in PMACI-stimulated mast cells in a dose-dependent manner (Figure 1D and E).

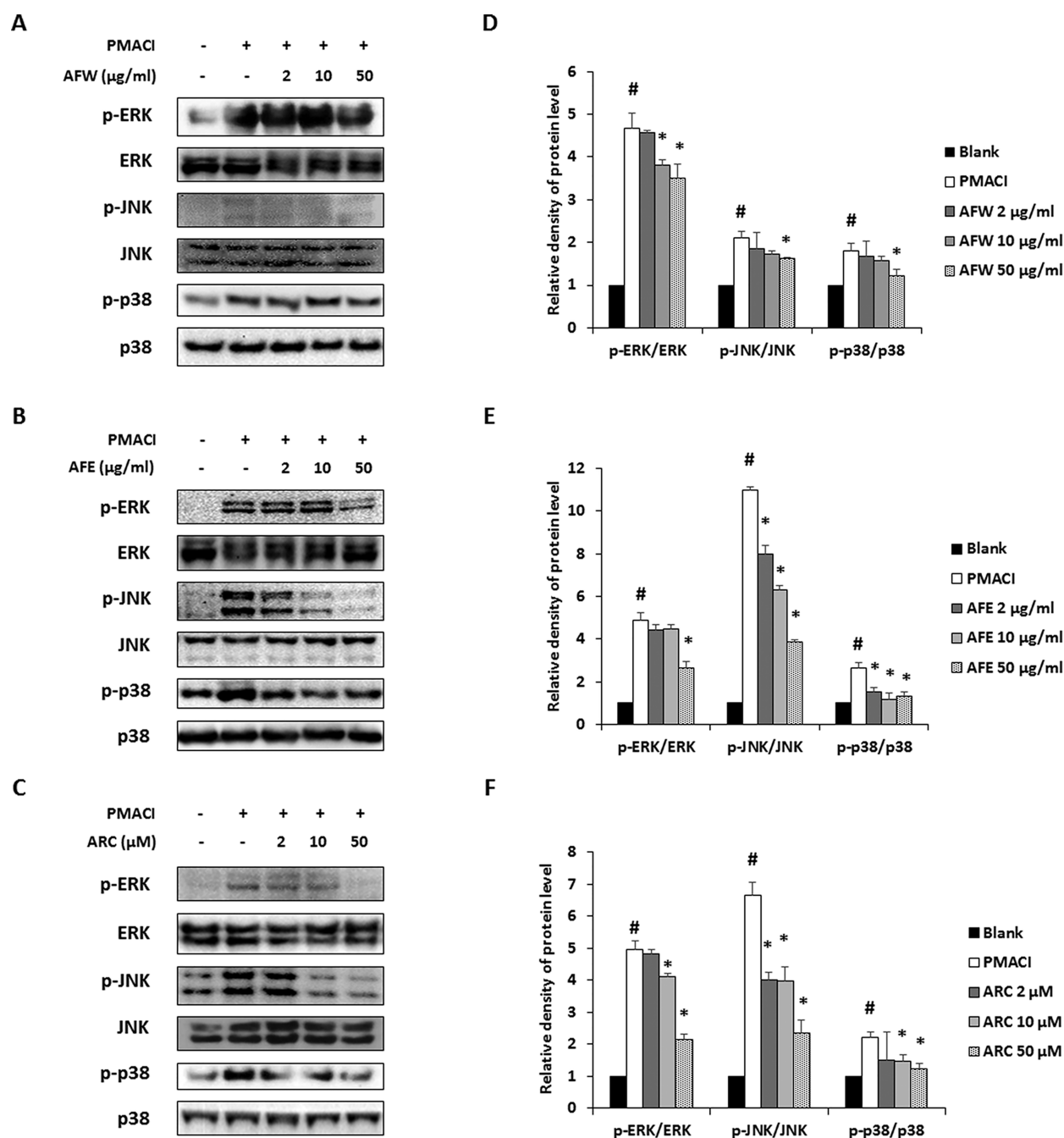


**Figure 4.** Effect of AFW, AFE, and ARC on phosphorylation of MAPKs in PMACI-stimulated HMC-1 cells. HMC-1 cells ( $1 \times 10^6$  cells/well) were pretreated with various concentrations of AFW, AFE, and ARC for 1 h and stimulated with PMACI for 30 min. (A–C) Phosphorylation of ERK, JNK, and p38 was detected by Western blot analysis. (D–F) Relative protein levels of ERK, JNK, and p38 phosphorylation. These data are representative of three independent experiments. <sup>#</sup> $p < 0.05$  versus blank and <sup>\*</sup> $p < 0.05$  versus PMACI alone.

ARC (2–50 μM) also reduced PMACI-induced histamine release in a dose-dependent manner (Figure 1F). The highest concentrations of AFW, AFE (50 μg/mL), and ARC (50 μM) significantly decreased histamine release from IgE-sensitized and BSA-stimulated RBL-2H3 cells (Figure S1).

**Arctii Fructus Extracts and ARC Reduce Chemokine Expression and Proinflammatory Cytokine Production in Mast Cells.** Mast cells play an important role in allergic reactions through the release of several mediators such as chemokines and pro-inflammatory cytokines.<sup>24</sup> Therefore, we determined whether AFW, AFE, and ARC could regulate the expression of chemokines and pro-inflammatory cytokines in

mast cells. As shown in Figure 2, AFW, AFE, and ARC decreased mRNA levels of mast cell-derived chemokines, such as MIP-1α, MIP-1β, MCP-1, and RANTES, in HMC-1 cells. The mRNA expression of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α, was reduced by both extracts and ARC in activated HMC-1 and RBL-2H3 cells (Figure 3A–C, Figure S2). Additionally, treatment with AFW, AFE, and ARC also suppressed the release of these cytokines in HMC-1 cells in a dose-dependent manner (Figure 3D–F). These results indicate that AFW, AFE, and ARC have an inhibitory effect on the expression of chemokines and the production of proinflammatory cytokines in mast cells.

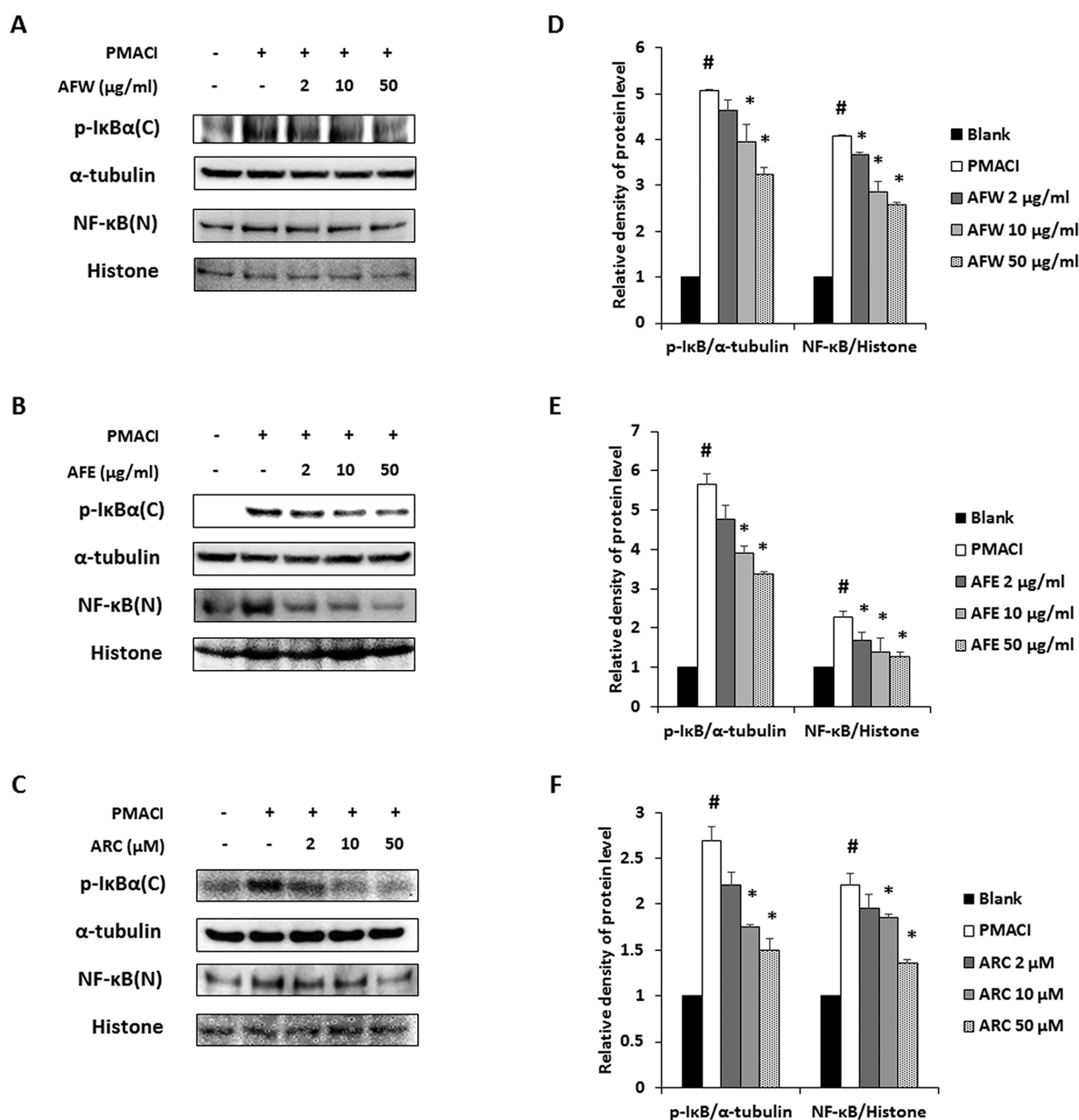


**Figure 5.** Effect of AFW, AFE, and ARC on degradation of IκBα and translocation of NF-κB in PMACI-stimulated HMC-1 cells. HMC-1 cells ( $1 \times 10^6$  cells/well) were pretreated with AFW, AFE, and ARC for 1 h and stimulated with PMACI for 2 h. (A–C) The phosphorylation of IκBα and translocation of NF-κB were detected by Western blot analysis. (D–F) Relative protein levels of IκB phosphorylation and NF-κB expression. These data are representative of three independent experiments. C, cytosol extracts; N, nuclear extracts. # $p < 0.05$  versus blank and \* $p < 0.05$  versus PMACI alone.

**Arctii Fructus Extracts and ARC Inhibit Phosphorylation of MAPKs and the Translocation of NF-κB in HMC-1 Cells.** Allergen stimulation produces proinflammatory cytokines via the activation of the MAPKs/NF-κB signaling pathway.<sup>6,8</sup> The phosphorylation of IκBα leads to IκB degradation and the release of NF-κB/Rel complexes. Translocation of NF-κB initiates transcription of target genes, including proinflammatory cytokines and chemokines.<sup>9</sup> To determine molecules that are regulated by AFW, AFE, and ARC treatment, we investigated the phosphorylation of ERK, JNK, and p38 in PMACI-stimulated HMC-1 cells. AFW extracts and ARC decreased the PMACI-induced phosphorylation of ERK, JNK, and p38 in HMC-1 cells (Figure 4). Activation of MAPKs was inhibited by AFW, AFE, and ARC pretreatment in IgE and BSA-stimulated RBL-2H3 cells (Figure

S3). Additionally, AFW, AFE, and ARC treatment suppressed PMACI-induced phosphorylation of IκBα and nuclear localization of NF-κB (Figure 5). These results demonstrate that AFW, AFE, and ARC reduce the proinflammatory cytokines via the inhibition of the MAPK/NF-κB signaling pathway in mast cells.

**Arctii Fructus Extracts and ARC Decrease RIP2 and Caspase-1 Activation in HMC-1 Cells.** RIP2 interacts with caspase-1 and plays a crucial role in NF-κB activation.<sup>25</sup> Activation of RIP2 promotes caspase-1 activity, which subsequently induces NF-κB activation leading to production of proinflammatory cytokines.<sup>25</sup> Therefore, we performed Western blot analysis to determine the effects of the extracts and ARC on RIP2/caspase-1 complex. As shown in Figure 6, AFW, AFE, and ARC significantly suppressed PMACI-induced

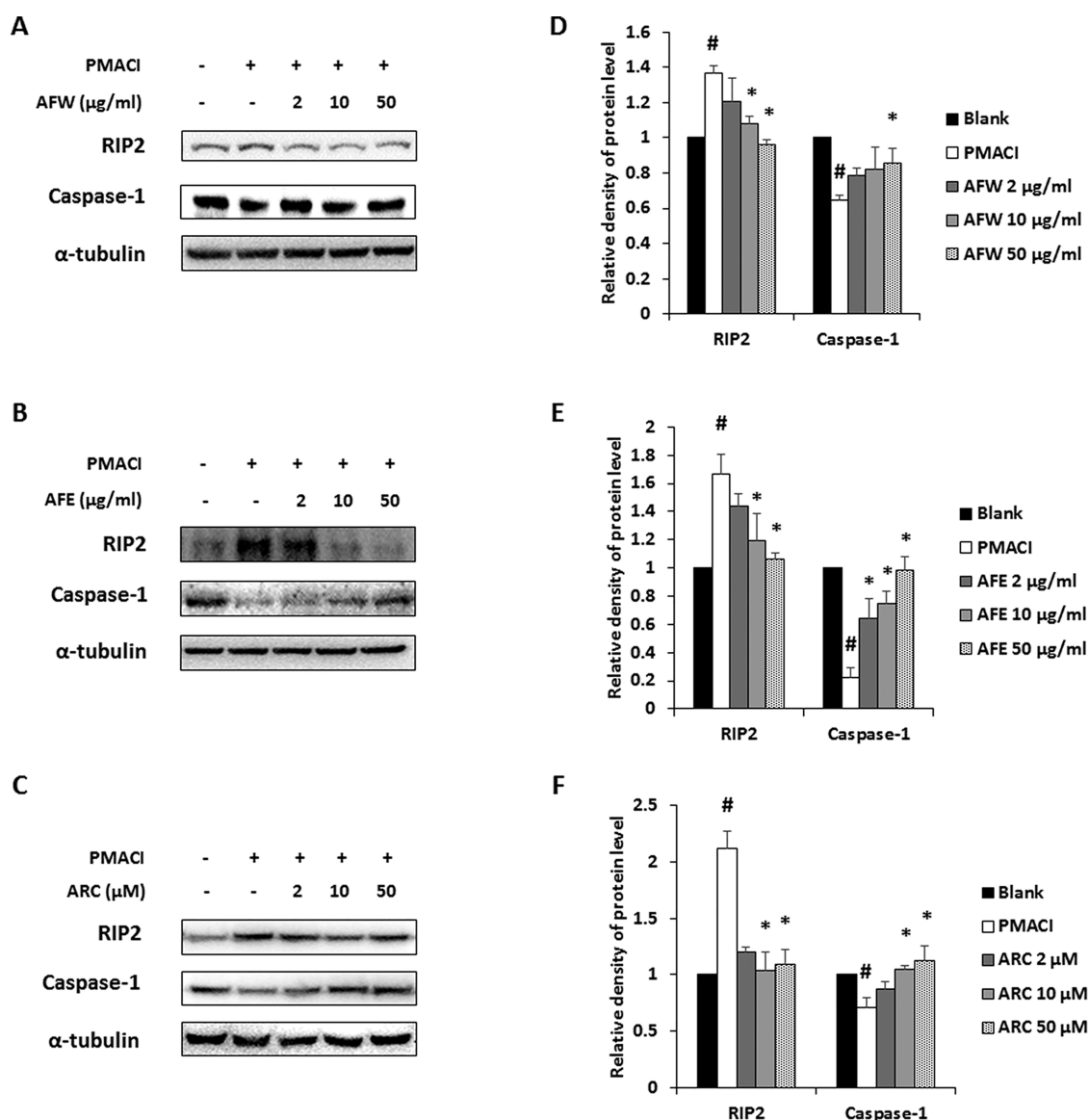


**Figure 6.** Effect of AFW, AFE, and ARC on RIP2/caspase-1 activation in PMACI-stimulated HMC-1 cells. HMC-1 cells ( $1 \times 10^6$  cells/well) were pretreated with AFW, AFE, and ARC for 1 h and stimulated with PMACI for 2 h. (A–C) Protein expression of RIP2 and caspase-1 was detected by Western blot analysis. (D–F) Relative protein levels of RIP2 and caspase-1 expression. These data are representative of three independent experiments. <sup>#</sup> $p < 0.05$  versus blank and <sup>\*</sup> $p < 0.05$  versus PMACI alone.

RIP2 and caspase-1 activation in HMC-1 cells. These results suggest that AFW, AFE, and ARC inhibited RIP2/caspase-1 activation in PMACI-stimulated mast cells.

**Arctii Fructus Extracts and ARC Suppress Anaphylactic Shock and PCA Reaction.** Compound 48/80-induced anaphylactic shock is the standard in vivo model for allergic reaction.<sup>26</sup> In order to investigate the effect of Arctii fructus extracts and ARC on local allergic reaction, anaphylactic shock was induced by the intraperitoneal injection of compound 48/80 followed by oral administration of AFW, AFE (25–100 mg/kg), and ARC (10–50 mg/kg). Compound 48/80 induced 100% mortality in the control group. At the highest concentration (100 mg/kg), the mortality rates in the AFE-treated and AFW-treated groups were 42.5% and 20%, respectively, whereas in the 50 mg/kg ARC-treated group it was 22.5%. Extracts of Arctii fructus and ARC inhibited compound 48/80-induced anaphylactic shock in a dose-dependent manner (Table 1).

PCA is also a standard method used to confirm local allergic reactions in vivo.<sup>27</sup> Local sensitization of anti-DNP IgE and intravenous injection of DNP-BSA induces allergic inflammation. Injection of 1% Evans blue mixed with DNP-BSA results in a blue coloration at the injection site. To determine whether AFW, AFE, and ARC repressed immediate allergic reactions, we sensitized DNP-IgE in the dorsal skin of mice for 12 h and injected DNP-BSA containing 1% Evans blue. Inhibition rates of IgE-mediated PCA reaction at the highest doses of AFW, AFE, and ARC were  $37.95 \pm 1.49\%$ ,  $56.11 \pm 1.89\%$ , and  $59.36 \pm 2.85\%$ , respectively (Figure 7). These inhibitory effects were found to be dose dependent. These results indicate that AFW, AFE, and ARC efficiently suppressed allergic responses via the inhibition of anaphylactic shock and IgE-mediated PCA reaction.

**Table 1. Effect of AFW, AFE, and ARC on the Compound 48/80-Induced Systemic Anaphylactic Reaction<sup>a</sup>**

| group         | dose (mg/kg) | compound 48/80<br>(8 mg/kg) | mortality (%) |
|---------------|--------------|-----------------------------|---------------|
| none (saline) | —            | —                           | 0             |
| none (saline) | —            | +                           | 100           |
| AFW           | 25           | +                           | 73.3          |
|               | 50           | +                           | 53.3          |
|               | 100          | +                           | 42.5          |
| AFE           | 25           | +                           | 66.7          |
|               | 50           | +                           | 46.7          |
|               | 100          | +                           | 20            |
| ARC           | 10           | +                           | 53.3          |
|               | 25           | +                           | 40            |
|               | 50           | +                           | 22.5          |

<sup>a</sup>The different mice groups ( $n = 5$ ) were orally administrated with saline, AFW, AFE or ARC given at various doses 1 h before the compound 48/80 injection. The compound 48/80 solution was intraperitoneally injected to the different mice groups. Mortality (%) within 1 h following compound 48/80 injection is represented as the 'Number of dead mice  $\times 100$ /Total number of experimental mice'.

## DISCUSSION

Allergic inflammation causes several diseases including anaphylaxis, asthma, eczema, and atopic dermatitis. The first phase of an allergic response is triggered by mast cell mediators, which are activated by allergens. Allergen-stimulated mast cells release histamine and proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ . Additionally, activated mast cells produce chemokines, including MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES, which induce proliferation and activation of immune cells such as leukocytes.<sup>28</sup> Thus, these mediators can potentially cause immune reactions and promote allergic responses.<sup>29</sup> In this study, we demonstrated that AFW, AFE, and ARC decreased the release of histamine and expression of MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES in HMC-1 cells (Figure 1). Additionally, production and expression of proinflammatory cytokines IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  reduced after treatment with AFW, AFE, and ARC in PMACI-stimulated HMC-1 cells (Figure 2).

Various intracellular signaling pathways are associated with the production of immune mediators including histamine, chemokines, and proinflammatory cytokines in activated mast cells. Signaling pathways resulting from Kit and Fc $\epsilon$ RI are most commonly associated with mast cell responses. Aggregation of IgE-occupied Fc $\epsilon$ RI induces Lyn, Fyn, and Syk phosphorylation, which leads to GAB2/RAS/MAPKs activation in mast cells.<sup>6,7,30–32</sup> Subsequently, activation of MAPKs signaling results in the separation of the transcription factor NF- $\kappa$ B from I $\kappa$ B. NF- $\kappa$ B then translocates into the nucleus leading to transcription of allergic inflammation-related genes including mediators of allergic inflammation.<sup>8,33</sup> Therefore, MAPKs/NF- $\kappa$ B signaling is a major therapeutic target in allergic diseases. Recent studies have reported that fermented *Arctii Fructus* extract inhibits the Lyn, Fyn, and Syk signaling cascade and its downstream targets including ERK, JNK, and p38 phosphorylation in IgE-sensitized RBL-2H3 cells.<sup>20</sup> 90% ethanol extract of *Arctium lappa* L. and its active compound oleamide suppressed Lyn-Syk and JNK/p38 pathway in RBL-2H3 cells.<sup>34</sup> ARC also regulates MAPKs signaling associated with apoptosis of colorectal cancer cells.<sup>35</sup> Thus, we have identified the regulatory mechanism underlying the activity of AFW, AFE,

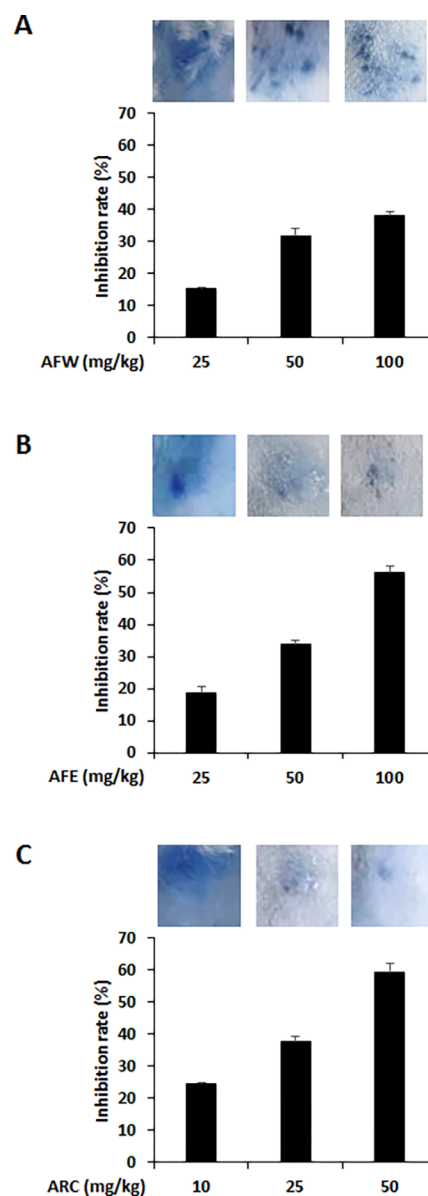


**Figure 7.** Effect of AFW, AFE, and ARC on IgE-mediated PCA reaction. The mice were sensitized using injected anti-DNP IgE (0.5  $\mu$ g/spot) on dorsal skin for 24 h. Following administration of saline, AFW (A), AFE (B), and ARC (C); DNP-BSA containing 1% Evans blue was injected via the tail vein. Blue areas on the dorsal skin were collected and photographed. Samples were dissolved in formaldehyde and measured using a microplate reader. Results were represented as the mean  $\pm$  SD of three independent experiments. The intensity of absorbance was measured at 620 nm using a microplate reader. These data are representative of three independent experiments.

and ARC via the MAPKs signaling pathway in the PMACI-stimulated HMC-1 cells. As expected, extracts of *Arctii fructus* and ARC decrease the degradation of I $\kappa$ B $\alpha$  and nuclear translocation of NF- $\kappa$ B via the inhibition of ERK, JNK, and p38 phosphorylation (Figures 4 and 5).

The RIP2/caspase-1 complex activates the MAPKs (ERK, JNK and p38)/NF- $\kappa$ B signaling pathways. Depletion of RIP-2 reduces the production of pro-inflammatory cytokines including TNF- $\alpha$  and IL-6. RIP2 regulates the activation of caspase-1 by binding to the caspase-1 prodomain.<sup>36</sup> Caspase-1 exists as an inactive proenzyme and is activated only when cleaved into two subunits. It is also known as the IL-1 $\beta$  converting enzyme,

which is associated with the biological activity of pro-inflammatory cytokine IL-1 $\beta$ . Activated caspase-1 converts the inactive pro-IL-1 $\beta$  to mature IL-1 $\beta$ , which can then bind to receptors and activate cells.<sup>37</sup> Therefore, the RIP2/caspase-1 complex plays an important role in the progress of allergic diseases. In our results, PMACI-induced RIP2 and caspase-1 activation was suppressed by AFW, AFE, and ARC in mast cells (Figure 6). These results indicated that the Arctii fructus extracts and ARC inhibit the release of mediators during allergic inflammatory responses through the inhibition of the MAPKs signaling pathway and regulation of RIP2/caspase-1 activation. These results indicate that Arctii Fructus and ARC can inhibit the release of proinflammatory cytokines via the MAPKs/NF- $\kappa$ B and RIP2/caspase-1 pathway.

Passive systemic anaphylaxis (PSA) and passive cutaneous anaphylaxis (PCA) are the most widely used mice models for the study of immediate allergic reactions. Compound 48/80 is one of the nonimmunologic stimulators that induce the degranulation of mast cells.<sup>26</sup> PCA reaction is induced by the injection of anti-DNP IgE antibody and antigens such as DNP-BSA on the back or ear of the mouse. Antigen injected site adopts a blue coloration, which indicates a positive allergic reaction. Thus, these in vivo experiments are appropriate to elicit and evaluate acute allergic responses. As reported in previous studies, oral administration of 50 mg/kg ARC as well as 100 mg/kg AFW and AFE exhibited antiobesity and antitumor activities without any toxicity in mice.<sup>13,38,39</sup> On the basis of these results, AFW, AFE (25–100 mg/kg), and ARC (10–50 mg/kg) were used to investigate the antiallergic activity after anaphylactic shock and PCA reaction. AFW, AFE, and ARC significantly inhibited allergic reactions in compound 48/80-induced PCA and IgE-mediated PCA reaction in mice (Table 1 and Figure 7).

Furthermore, we validated these inhibitory effects of Arctii Fructus extracts and ARC on allergic reactions using RBL-2H3 cells, which exhibit characteristics of mucosal-type mast cells and IgE receptor response. This cell line is extensively used for studying the comprehensive events associated with mast cell activation.<sup>40</sup> Extracts of Arctii Fructus and ARC inhibited the mRNA expression of proinflammatory cytokines, including IL-1 $\beta$ , IL-6, TNF- $\alpha$  as well as histamine release in antigen-stimulated RBL-2H3 cells (Figures S1 and S2). Moreover, AFW, AFE, and ARC also reduced the IgE BSA-induced phosphorylation of ERK, JNK, and p38 (Figure S3). These results reinforced the antiallergic activity of AF and ARC in an in vivo model.

Inhibitory activity of AFW on allergic responses is less than that of AFE and ARC treatments in this study. Notably, in our previous study we demonstrated that the concentration of ARC is higher in AFE than that in AFW.<sup>13</sup> ARC inhibits picryl chloride and dinitrofluorobenzene-induced contact dermatitis and heterologous passive cutaneous anaphylaxis induced by OVA injection. It also reduces the activities of pro-inflammatory enzymes, such as 5-lipoxygenase, phospholipase A2, cyclooxygenase (COX)-1, COX-2, and phosphodiesterase in rat peritoneal mast cells (RPMCs).<sup>21</sup> Furthermore, ARC shows anti-inflammatory activity through the inhibition of lipopolysaccharide-stimulated nitric oxide production and pro-inflammatory cytokine secretion, including TNF- $\alpha$  and IL-6, in RAW 264.7 cells.<sup>41</sup> Thus, ARC-enriched AFE might be a more efficient inhibitor of allergic inflammation than AFW.

In conclusion, Arctii Fructus extracts and ARC successfully suppressed the production of histamine and proinflammatory

cytokines in HMC-1 cells. The inhibitory activities of Arctii Fructus and ARC on proinflammatory cytokines were mediated by the MAPKs/NF- $\kappa$ B and RIP2/caspase-1 pathways. Furthermore, PCA reaction and anaphylactic shock were also suppressed by Arctii Fructus extracts and ARC administration. Therefore, this study provides experimental evidence that Arctii Fructus and ARC can treat allergic diseases.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02965.

Table S1: Sequences of the real-time RT-PCR primers for human genes; Table S2: sequences of the real-time RT-PCR primers for mouse genes; Figure S1: effect of AFW, AFE, and ARC on histamine release in activated RBL-2H3 cells; Figure S2: effect of AFW, AFE, and ARC on the mRNA expressions of proinflammatory cytokines in activated RBL-2H3 cells; and Figure S3: effect of AFW, AFE, and ARC on phosphorylation of MAPKs in activated RBL-2H3 cells (PDF)

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### Notes

The authors declare no competing financial interest.

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**Supporting information** to the article

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**Table S1:** Sequences of the Real-time RT-PCR primers for human genes

**Table S2:** Sequences of the Real-time RT-PCR primers for mouse genes

**Figure S1:** Effect of AFW, AFE, and ARC on histamine release in activated RBL-2H3 cells

**Figure S2:** Effect of AFW, AFE and ARC on the mRNA expressions of proinflammatory cytokines in activated RBL-2H3 cells

**Figure S3:** Effect of AFW, AFE and ARC on phosphorylation of MAPKs in activated RBL-2H3 cells

**Table S1: Sequences of the Real-time RT-PCR primers for human genes**

| Genes          | Forward (5'-3')          | Reverse (5'-3')           |
|----------------|--------------------------|---------------------------|
| MIP-1 $\alpha$ | GTCTGTGCTGATCCCAGTGA     | TTGTCACCAGACGCGGTGTG      |
| MIP-1 $\beta$  | GCTGCTCAGAGACAGGAAGTCTT  | ACAGGAACTGCGGAGAGGAGT     |
| MCP-1          | GTCTCTGCCGCCCTTCTGT      | TTGCATCTGATGGCAGTAGCT     |
| RANTES         | CCGCGGCAGCCCTCGCTGTCATCC | CATCTCCAAAGAGTTGATGTACTCC |
| IL-1 $\beta$   | TACCTGTCCTGCGTGTTGAA     | TDTTTGGGTAATTTTGGGATCT    |
| IL-6           | GATGAGTACAAAAGTCCTGATCCA | CTGCAGCCACTGGTTCTGT       |
| IL-8           | AAGCTGGCCGTGGCTCTCTTG    | AGCCCTCTTCAAAAACCTTCTC    |
| TNF- $\alpha$  | CGCTCCCCAAGAAGACAG       | AGAGGCTGAGGAACAAGCAC      |
| GAPDH          | TGCACCACCACCTGCTTAGC     | GGCATGGACTGTGGTCATGAG     |

**Table S2: Sequences of the Real-time RT-PCR primers for mouse genes**

| Genes         | Forward (5'-3')      | Reverse (5'-3')      |
|---------------|----------------------|----------------------|
| IL-1 $\beta$  | CACAGCAGCACATCAACAAG | GTGCTCATGTCCTCATCCTG |
| IL-6          | CTCTGGGAAATCGTGGAAAT | CCAGTTTGGTAGCATCCATC |
| TNF- $\alpha$ | ATGAGAAGTTCCCAAATGGC | CTCCACTTGGTGGTTTGCTA |
| GAPGH         | GACATGCCGCCTGGAGAAAC | AGCCCAGGATGCCCTTTAGT |

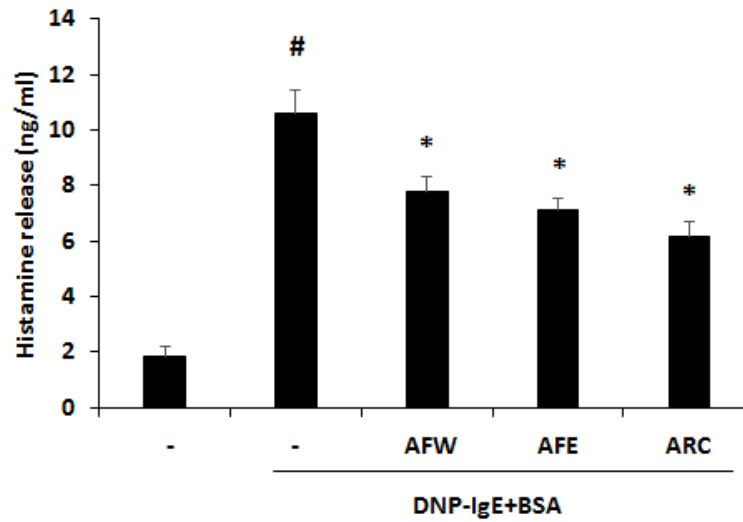


**Figure S1:** Effect of AFW, AFE, and ARC on histamine release in activated RBL-2H3 cells. RBL-2H3 cells ( $5 \times 10^4$  cells/well) were seeded in 24-well plate and sensitized with DNP-IgE (100 ng/ml) for 12 h. After pretreatment of AFW (50  $\mu$ g/ml), AFE (50  $\mu$ g/ml) and ARC (50  $\mu$ M) for 1 h, DNP-BSA (1  $\mu$ g/ml) was treated to cells for 24 h. Conditioned medium was used for analysis using the histamine ELISA kit. Each bar represents the mean  $\pm$  SD of three independent experiments. # $p < 0.05$  versus blank and \* $p < 0.05$  versus DNP-IgE+BSA alone.

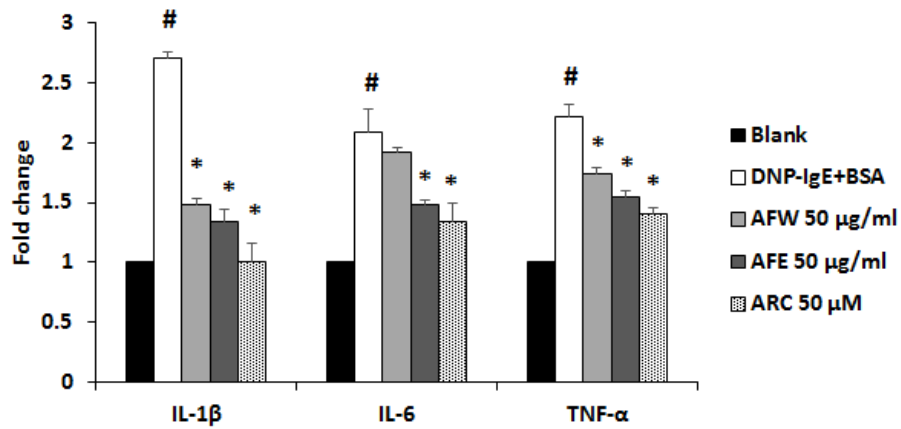


**Figure S2:** Effect of AFW, AFE and ARC on the mRNA expressions of proinflammatory cytokines in activated RBL-2H3 cells. RBL-2H3-1 cells ( $5 \times 10^4$  cells/well) were seeded in 24-well plate and DNP-IgE (100 ng/ml) was added to cells for 12 h. AFW, AFE (50 µg/ml) and ARC (50 µM) were pretreated for 1 h and stimulated by DNP-BSA (1 µg/ml) for 24 h. Cells were used for real-time RT-PCR analysis. Each bar represents the mean  $\pm$  SD of three independent experiments. <sup>#</sup> $p < 0.05$  versus blank and <sup>\*</sup> $p < 0.05$  versus DNP-IgE+BSA alone.

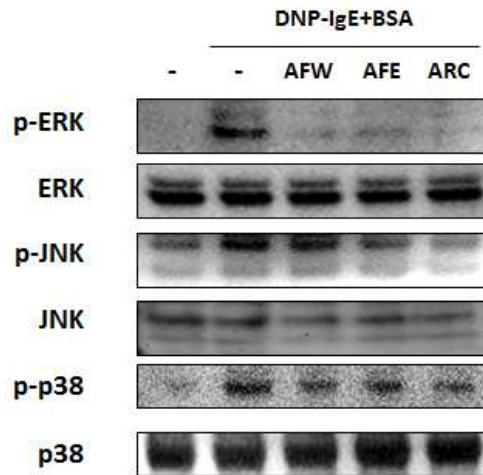


**Figure S3:** Effect of AFW, AFE and ARC on phosphorylation of MAPKs in activated RBL-2H3 cells. RBL-2H3 cells ( $5 \times 10^5$  cells/well) were seeded to 6-well plate and sensitized with DNP-IgE (100 ng/ml) for 12 h. Cells were pretreated with AFW, AFE (50  $\mu$ g/ml) and ARC (50  $\mu$ M) for 1 h and stimulated with DNP-BSA (1  $\mu$ g/ml) for 30 min. These data are representative of three independent experiments. # $p < 0.05$  versus blank and \* $p < 0.05$  versus DNP-IgE+BSA alone.