



Molecular mechanisms of anti-inflammatory effect of chrysophanol, an active component of AST2017-01 on atopic dermatitis in vitro models

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ABSTRACT

AST2017-01 mainly consists of *Rumex crispus* and *Cordyceps militaris* and has been widely consumed as an herbal medicine or functional food in Korea. Here we investigated the influences of AST2017-01 and its active component, chrysophanol on human mast cell (HMC-1 cell) and human keratinocyte (HaCaT cell)-mediated inflammatory reactions. Pretreatment with AST2017-01 or chrysophanol suppressed intracellular calcium levels and histamine release in phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-treated HMC-1 cells. Levels of phosphorylated-mitogen-activated protein kinase increased by PMACI stimulation were reduced by AST2017-01 or chrysophanol pretreatment. Protein levels of IκB kinaseβ and receptor-interacting protein 2 in PMACI-treated HMC-1 cells were decreased by AST2017-01 or chrysophanol pretreatment. Pretreatment with AST2017-01 or chrysophanol significantly blocked PMACI-induced activation of caspase-1 and nuclear factor-κB. In addition, pretreatment with AST2017-01 or chrysophanol significantly decreased the PMACI-induced levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and thymic stromal lymphopoietin (TSLP) on HMC-1 cells. In activated HaCaT cells, pretreatment with AST2017-01 or chrysophanol significantly reduced production of TSLP and activation of caspase-1. In conclusion, these findings indicate that chrysophanol is an active component of AST2017-01 and AST2017-01 acts as a novel potent anti-inflammatory herbal medicine or functional food.

1. Introduction

Allergic inflammatory disorders, including atopic dermatitis (AD), allergic rhinitis, anaphylaxis, and allergic asthma are caused by type 2 immune responses and characterized by the presence of IgE and Th2 cells [1]. Th2 cell-derived interleukin (IL)-4 is thought to promote IgE production by B cells in response to a particular allergen, and the allergen-specific IgE antibodies bind to the high-affinity Fcε receptor 1 (FcεR1) on mast cells [2]. Mast cell activation by IgE interaction with FcεR1 triggers the release of inflammatory mediators with diverse biological effects to control allergic inflammation through degranulation and accelerates the development of AD [2–4]. In addition, keratinocytes play a key pathogenic role in development of AD via abnormal releasing cytokines and chemokines as well as thymic stromal lymphopoietin (TSLP) [5]. In our previous study, we have reported that TSLP is a proliferative factor for mast cell [3].

Mast cell stimulation by extracellular stress triggers the increase of intracellular calcium, which is proposed as a second messenger for mast cell activation [6]. Increased intracellular calcium is required for activation of IκB kinase (IKK), receptor-interacting protein 2 (RIP2),

mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and caspase-1 [7]. MAPKs play a significant role in cytokine expression and are activated to control allergic inflammatory reactions [8]. In particular, NF-κB and caspase-1 are candidates for transcription and production of inflammatory cytokines including IL-1β, IL-6, tumor necrosis factor (TNF)-α, and TSLP [9,10]. Overexpression of these mediators in AD-skin lesion has been reported to correspond with disease severity [11]. For this reason, signaling pathway of inflammatory mediator production is an obvious target for emerging anti-allergic inflammatory therapies.

In Korean medicine, various processing methods (Beopje) have been applied to herbal medicines in order to improve their therapeutic effects and safety in clinical trials. AST2017-01 mainly consists of processed-*Rumex crispus* and processed-*Cordyceps militaris* and has been widely consumed as an herbal medicine or functional food in Korea. *Cordyceps militaris* shows anti-inflammatory and anti-cancer effects [12,13]. *Rumex crispus* is widely used to treat inflammation, edema, jaundice, stroke, disinfestation, and diarrhea [14–16]. However, anti-inflammatory properties of AST2017-01 and its specific chemical compound have not yet been demonstrated. In this study, we tested

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anti-inflammatory effect of AST2017-01 and its major compound, chrysophanol on human mast cell and human keratinocytes.

2. Materials and methods

2.1. Reagents

Chrysophanol (purity: $\geq 98\%$), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fura-2/AM, poly(I:C), phorbol 12-myristate 13-acetate (PMA), A23187, and bicinchoninic acid were obtained from Sigma Chemical Co (St. Louis, MO, USA). TSLP, IL-1 β , IL-6, and TNF- α antibodies were from R&D Systems, Inc. (Minneapolis, MN, USA). Phosphorylated (p)-c-Jun N-terminal kinases (JNK, SC-6254), JNK (SC-571), p-p38 (SC-7973), p38 (SC-7149), p-extracellular signal-regulated kinases (p-ERK, SC-7383), ERK (SC-94), p-I κ B α (SC-8404), NF- κ B (SC-8008), Poly-ADP-ribose polymerase (SC-8007), RIP2 (SC-22763), IKK β (SC-7607), and GAPDH (SC-32233) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), and Isocove's modified Dulbecco's medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA).

2.2. Preparation of AST2017-01

AST2017-01 was provided by Gahwa Well Food Co. (Chungbuk, Republic of Korea). AST2017-01 was composed of processed-*Rumex crispus* and processed-*Cordyceps militaris* in a ratio of 6:4. Processed-*Rumex crispus* and processed-*Cordyceps militaris* were prepared in order of washing, steam, dehydration, parch, and then dehydration. AST2017-01 was boiled with distilled water at 80 °C for 3 h. The AST2017-01 extracts were filtered and concentrated in vacuo at 60 °C. And then these were lyophilized. The extract yields of herbs were about 15–20% (w/w). The powders were dissolved in distilled water and filtered using a 0.22 μ m syringe filter and kept at 4 °C. The concentration of chrysophanol in the AST2017-01 was approximately 0.13% [17]. Chrysophanol was dissolved in DMSO and diluted in distilled water (DMSO final concentration $\leq 0.1\%$). DMSO (0.1%) did not affect levels of inflammatory mediators as compared with PMA and A23187 (PMACI) stimulation [10].

2.3. Human mast cell line (HMC-1 cell) and human keratinocyte cell line (HaCaT cell) culture

HMC-1 cells were incubated in IMDM supplemented with 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 10% FBS at 37 °C in 5% CO₂ atmosphere at 95% relative humidity. HaCaT cells were cultured in DMEM supplemented with 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 10% FBS at 37 °C in 5% CO₂ with 95% humidity.

2.4. Fluorescent measurements of intracellular calcium levels

To evaluate the intracellular calcium level, HMC-1 cells suspensions were pretreated with Fura-2/AM for 30 min and then were harvested. After washing twice with medium containing extracellular calcium chelator EGTA (0.5 mM), the cells (1×10^5) were placed into a 96-well plate and pretreated with AST2017-01 or chrysophanol for 1 h. HMC-1 cells were stimulated with PMACI for 100 s. Phosphate-buffered saline (PBS) was treated as a negative control. The intracellular calcium levels were determined at 440 nm (excitation 360 nm) in a spectrofluorometer.

2.5. Histamine assay

Histamine levels were measured using a histamine assay kit (Oxford Biomedical Research, Oxford, MI, USA).

2.6. Western blot analysis

Stimulated cells were lysed and proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes, which were then blocked and incubated with primary (1:500 dilution) and secondary (1:3000 dilution) antibodies. Finally, protein bands were visualized using an enhanced chemiluminescence assay (Amersham Co. Newark, NJ, USA).

2.7. Caspase-1 assay

The enzymatic activities of caspase-1 were assayed using a colorimetric assay kit (R&D Systems).

2.8. MTT assay

Cell viability was measured using a MTT assay. Briefly, 500 μ l of cell (3×10^5) were pretreated with AST2017-01 or chrysophanol for 1 h and then stimulated with PMACI for 8 h. MTT solution (5 mg/ml) then added and incubated at 37 °C for 4 h. After removing the supernatant by washing, the insoluble formazan product so obtained was dissolved in DMSO. Optical densities were measured using an ELISA reader at 590 nm.

2.9. Cytokines assay

Levels of TSLP, IL-1 β , IL-6, and TNF- α were determined using a sandwich ELISA method according to the manufacturer's instructions (R&D Systems).

2.10. RNA isolation and quantitative real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from HMC-1 cells using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, Korea). Concentrations of total RNA were determined by NanoDrop spectrophotometry (Thermo Scientific, Worcester, MA, USA). Total RNA (2.5 μ g) was heated at 75 °C for 5 min and then chilled on ice. Samples were reverse-transcribed to cDNA for 60 min at 42 °C using a cDNA synthesis kit (iNtRON Biotech, Sungnam, Korea). Quantitative real-time PCR was performed using a SYBR Green master mix with primers (Table 1) and mRNA was analyzed using an ABI StepOne real-time PCR System (Applied Biosystems, Foster City, CA, USA). Expression levels of cytokine were normalized versus GAPDH. All data were analyzed using the $\Delta\Delta$ CT method.

2.11. Statistics

All results are representative of three independent experiments conducted in duplicate and are expressed as the mean $\pm$ standard errors of means (SEMs). The analysis was performed using an

Table 1
The sequence of primers used for real-time PCR analysis.

Genes		Sequences
TSLP	Sense	TATGAGTGGGACCAAAAGTACCG
	Antisense	GGGATTGAAGGTTAGGCTCTGG
IL-1 β	Sense	AAACAGATGAAGTGCTCCTT
	Antisense	TGGAGAACACCACTTGTTC
IL-6	Sense	AAATTCGGTACATCTCGACGGCA
	Antisense	AGTGCTCTTTGCTGCTTCACAC
TNF- α	Sense	AGGACGAACATCCAACCTTC
	Antisense	TTTGAGCCAGAAGAGGTTGA
GAPDH	Sense	TCGACAGTCAGCCGATCTTCTTT
	Antisense	CCAAATCCGTTGACTCCGACCTT

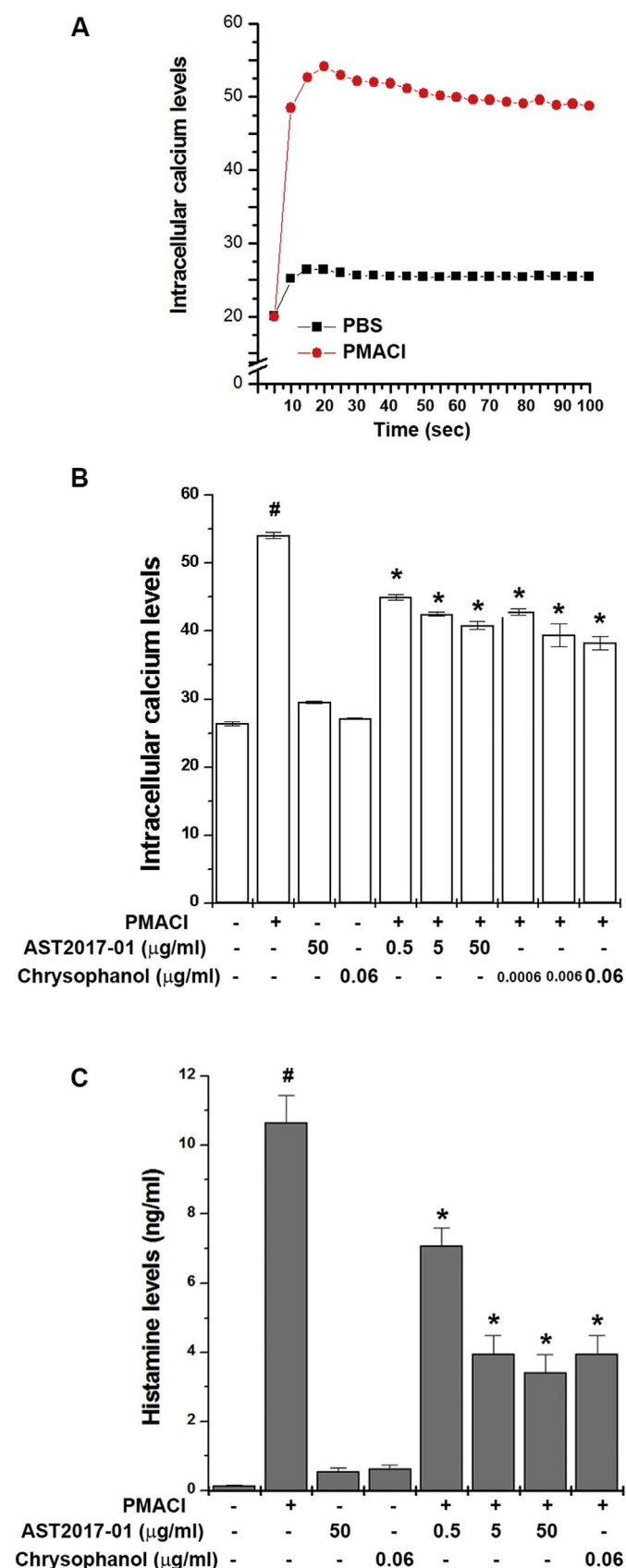


Fig. 1. AST2017-01 and chrysophanol diminished the levels of intracellular calcium and histamine on activated HMC-1 cells. (A) HMC-1 cells (1×10^5) were stimulated with PMACI for 100 s. (B) HMC-1 cells (1×10^5) were treated with AST2017-01 or chrysophanol and stimulated with PMACI for 1 min. The intracellular calcium levels were determined using the fluorescence. (C) HMC-1 cells (3×10^5) were treated with AST2017-01 or chrysophanol and stimulated with PMACI for 8 h. Histamine contents were measured using histamine assay kit. Data represent mean $\pm$ S.E.M. of three independent experiments. $^{\#}P < 0.05$; significantly different from the unstimulated cells, $^*P < 0.05$, significantly different from PMACI-stimulated cells. PBS, phosphate-buffered saline; PMACI, phorbol myristate acetate plus calcium ionophore A23187.

independent *t*-test or by ANOVA with Tukey's post hoc test using SPSS (IBM Corporation, Armonk, NY, USA). Statistical significance was accepted for *P*-values of < 0.05 .

3. Results

3.1. AST2017-01 and chrysophanol diminished the levels of intracellular calcium and histamine on activated HMC-1 cells

In our previous study, we found that PMACI exaggerated inflammatory reaction through increasing intracellular calcium levels in HMC-1 cells [18]. Thus, we sought to determine whether level of intracellular calcium was regulated by AST2017-01 or chrysophanol in PMACI-stimulated HMC-1 cells. The levels of intracellular calcium were increased by PMACI stimulation (Fig. 1A and B). The results revealed that pretreatment with AST2017-01 or chrysophanol significantly attenuated the levels of intracellular calcium in PMACI-stimulated HMC-1 cells (Fig. 1B, $P < 0.05$). Next, we tested whether AST2017-01 or chrysophanol would have a regulatory effect on degranulation of mast cells. The results showed that AST2017-01 or chrysophanol significantly reduced the histamine releases from the activated HMC-1 cells (Fig. 1C, $P < 0.05$). AST2017-01 (50 μg/ml) and chrysophanol (0.06 μg/ml) alone did not affect the levels of intracellular calcium and histamine compared with the PBS controls (Fig. 1B and C).

3.2. AST2017-01 and chrysophanol diminished the markedly MAPK activation in activated HMC-1 cells

It is clear that the levels of intracellular calcium are regulated by AST2017-01 in activated mast cells. MAPK is a downstream regulator of intracellular calcium and our previous reports showed that PMACI induced the phosphorylation of MAPK family in HMC-1 cells [19]. Thus, we tested whether AST2017-01 or chrysophanol could attenuate p-JNK, p-ERK, and p-p38 levels in PMACI-stimulated HMC-1 cells. The results showed that levels of p-JNK, p-ERK, and p-p38 increased by PMACI stimulation were reduced by AST2017-01, chrysophanol, or dexamethasone pretreatment (Fig. 2, $P < 0.05$).

3.3. AST2017-01 and chrysophanol diminished the levels of IKKβ and RIP2 in activated HMC-1 cells

IKKβ and RIP2 signaling pathways play central roles in mast cell-mediated inflammatory reactions [7]. Thus, we tested whether AST2017-01 or chrysophanol could influence IKKβ and RIP2 protein expression levels by PMACI stimulation. As was expected, protein levels of IKKβ and RIP2 were increased by PMACI stimulation in HMC-1 cells (Fig. 3A), but pretreatment with AST2017-01, chrysophanol, or dexamethasone reduced these increases (Fig. 3A).

3.4. AST2017-01 and chrysophanol diminished the powerful caspase-1 and NF-κB activities in activated HMC-1 cells

Next, we tested the regulatory effects of AST2017-01 or chrysophanol on the activities of caspase-1 and NF-κB, the pathways of which have been implicated in cytokine expression [20,21]. First, we analyzed

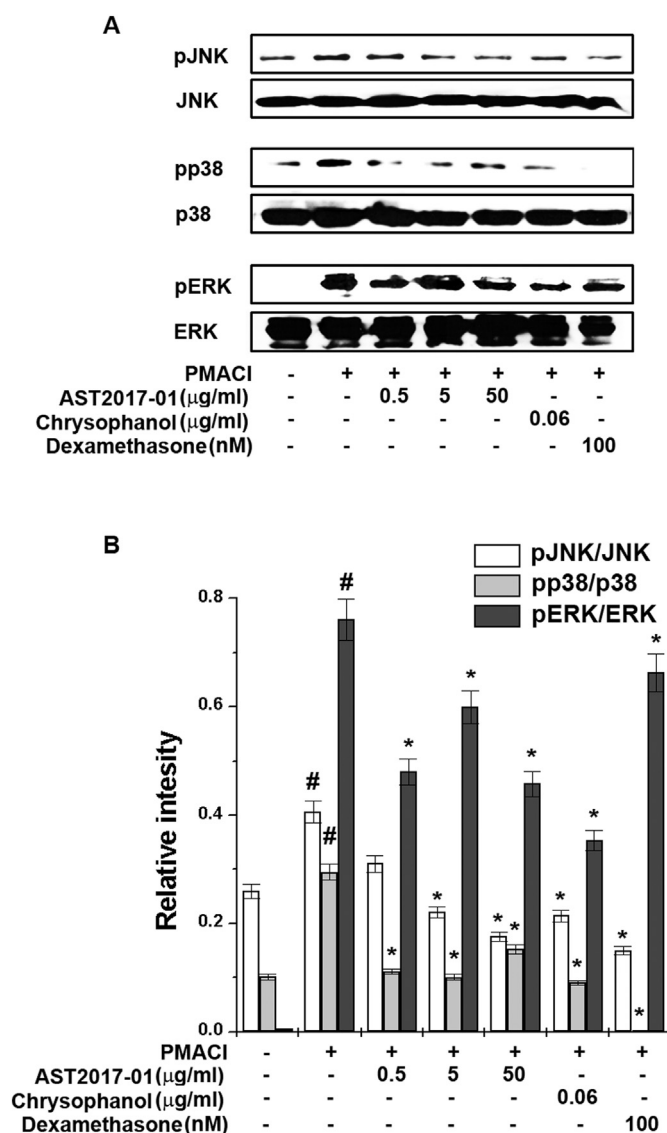


Fig. 2. AST2017-01 and chrysophanol diminished the markedly MAPK activation in activated HMC-1 cells. HMC-1 cells (3×10^6) were pretreated with AST2017-01, chrysophanol, or dexamethasone for 1 h and then treated with PMACI as indicated for 30 min. (A) Levels of p-MAPKs were analyzed with Western blotting. (B) Relative intensities of protein levels were quantified by densitometry means pJNK/JNK, pp38/p38, and pERK/ERK, and JNK, p38, and ERK were used as loading control for pp38, pERK, and pJNK, respectively. Data represent mean $\pm$ S.E.M. of three independent experiments. # $P < 0.05$; significantly different from the unstimulated cells, * $P < 0.05$, significantly different from PMACI-stimulated cells. PMACI, phorbol myristate acetate plus calcium ionophore A23187.

the caspase-1 activity. The caspase-1 activities were significantly reduced by AST2017-01 pretreatment in PMACI-stimulated HMC-1 cells (Fig. 3B, $P < 0.05$). The IC_{50} value of AST2017-01 with respect to caspase-1 activation was approximately 0.12 μg/ml. Pretreatment with chrysophanol or dexamethasone also significantly reduced the caspase-1 activities in PMACI-stimulated HMC-1 cells (Fig. 3B, $P < 0.05$). Next, we tested the regulatory effects of AST2017-01 or chrysophanol on NF-κB activation. As shown in Fig. 3C, pretreatment with AST2017-01 (50 μg/ml), chrysophanol, or dexamethasone decreased the levels of nuclear-NF-κB and cytosol-p-IκBα in PMACI-stimulated HMC-1 cells.

3.5. AST2017-01 and chrysophanol diminished the levels of inflammatory cytokine by activated HMC-1 cells

From the above results, AST2017-01 and chrysophanol effectively

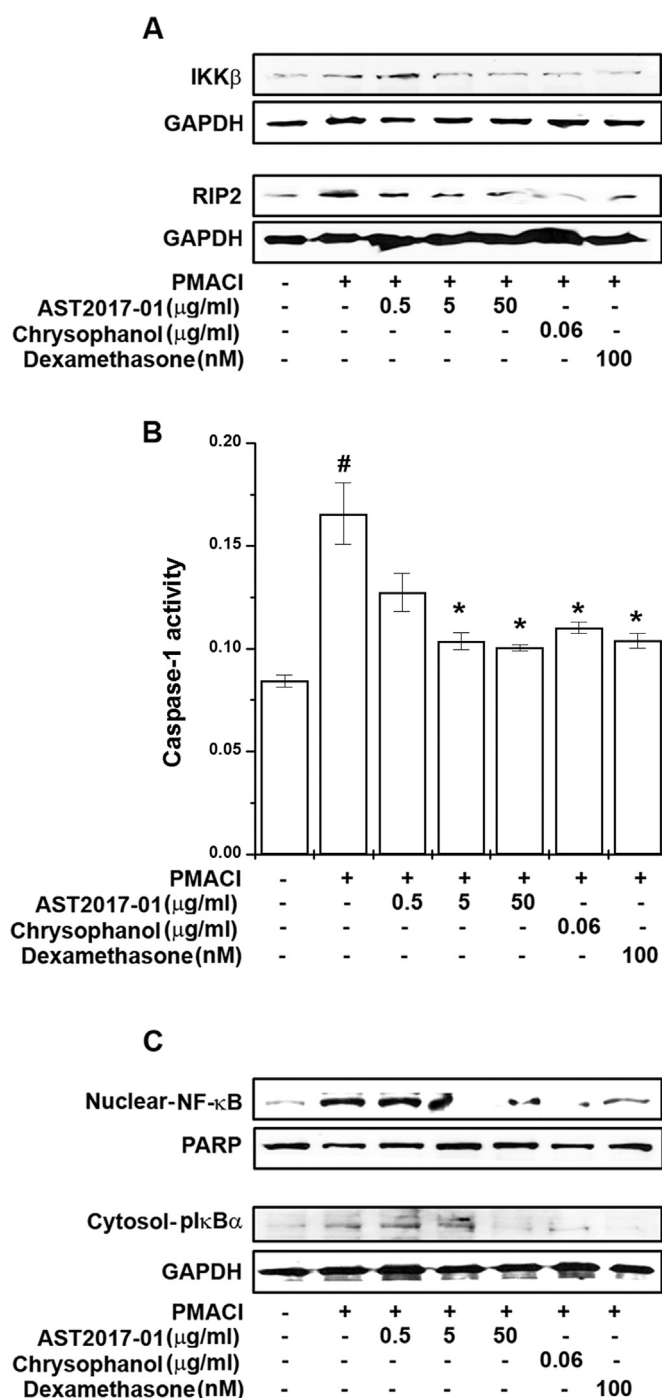


Fig. 3. AST2017-01 and chrysophanol diminished the levels of IKKβ and RIP2 and activities of caspase-1 and NF-κB in activated HMC-1 cells. HMC-1 cells (3×10^6) were pretreated with AST2017-01, chrysophanol, or dexamethasone for 1 h and then treated with PMACI for 2 h. (A) Levels of IKKβ and RIP2 were analyzed with Western blotting. GAPDH was used as a loading control. (B) Caspase-1 activities were analyzed with caspase-1 colorimetric assay kit. (C) Levels of nuclear-NF-κB and cytosol-p-IκBα were analyzed with Western blotting. PARP was used as a nuclear marker and loading control. GAPDH was used as a loading control. Values are means $\pm$ SEMs. # $P < 0.05$; significantly different from the unstimulated cells, * $P < 0.05$, significantly different from PMACI-stimulated cells. PMACI, phorbol myristate acetate plus calcium ionophore A23187.

reduced the caspase-1 and NF-κB activities in activated HMC-1 cells. The activities of caspase-1 and NF-κB play an important role in production and transcription of inflammatory cytokine. Thus, we tested whether AST2017-01 could affect the protein levels of TSLP, IL-1β, IL-6,

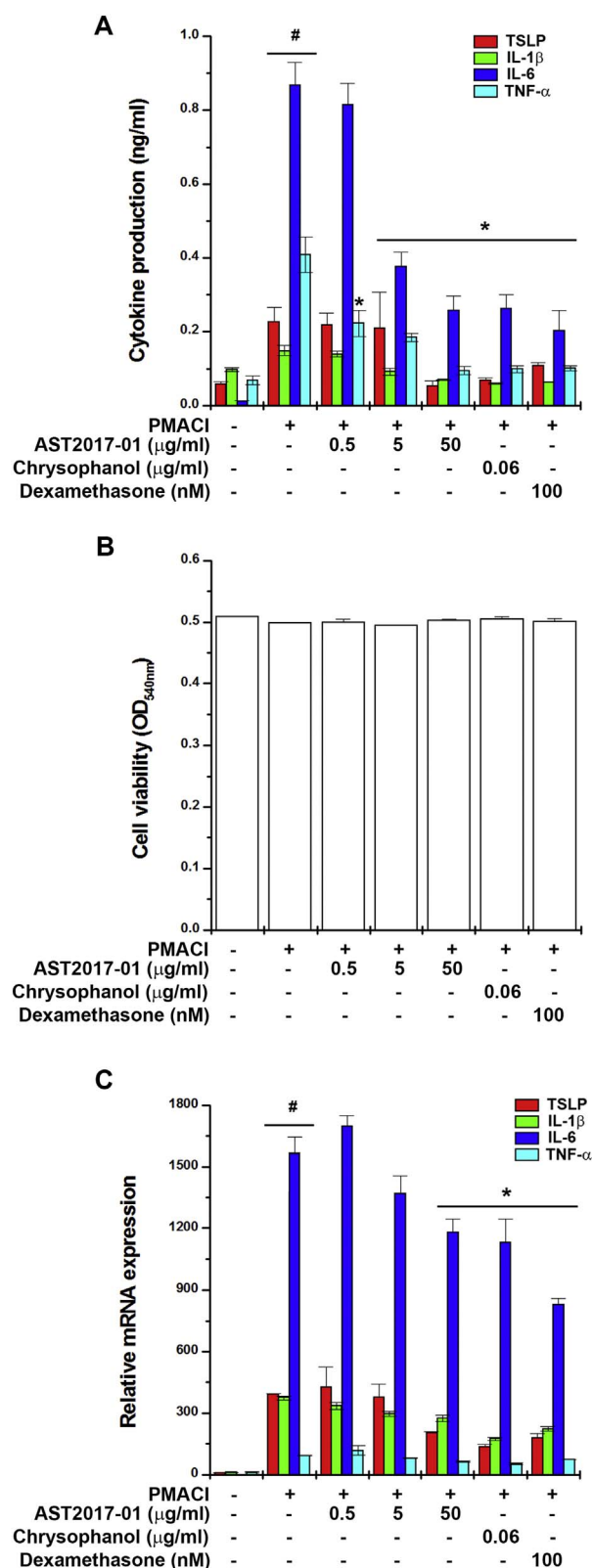


Fig. 4. AST2017-01 and chrysophanol diminished the levels of inflammatory cytokine by activated HMC-1 cells. (A) HMC-1 cells (3×10^5) were pretreated with AST2017-01, chrysophanol, or dexamethasone for 1 h and then treated with PMACI for 8 h. Levels of inflammatory cytokine were analyzed with ELISA. (B) Cell viability was determined with an MTT assay. (C) HMC-1 cells (3×10^5) were pretreated with AST2017-01 or chrysophanol for 1 h and then treated with PMACI for 5 h. Levels of inflammatory cytokine mRNA expression were analyzed with quantitative real-time PCR analysis. Data represent mean $\pm$ S.E.M. of three independent experiments. $^{\#}P < 0.05$; significantly different from the unstimulated cells, $^*P < 0.05$; significantly different from PMACI-stimulated cells. PMACI, phorbol myristate acetate plus calcium ionophore A23187.

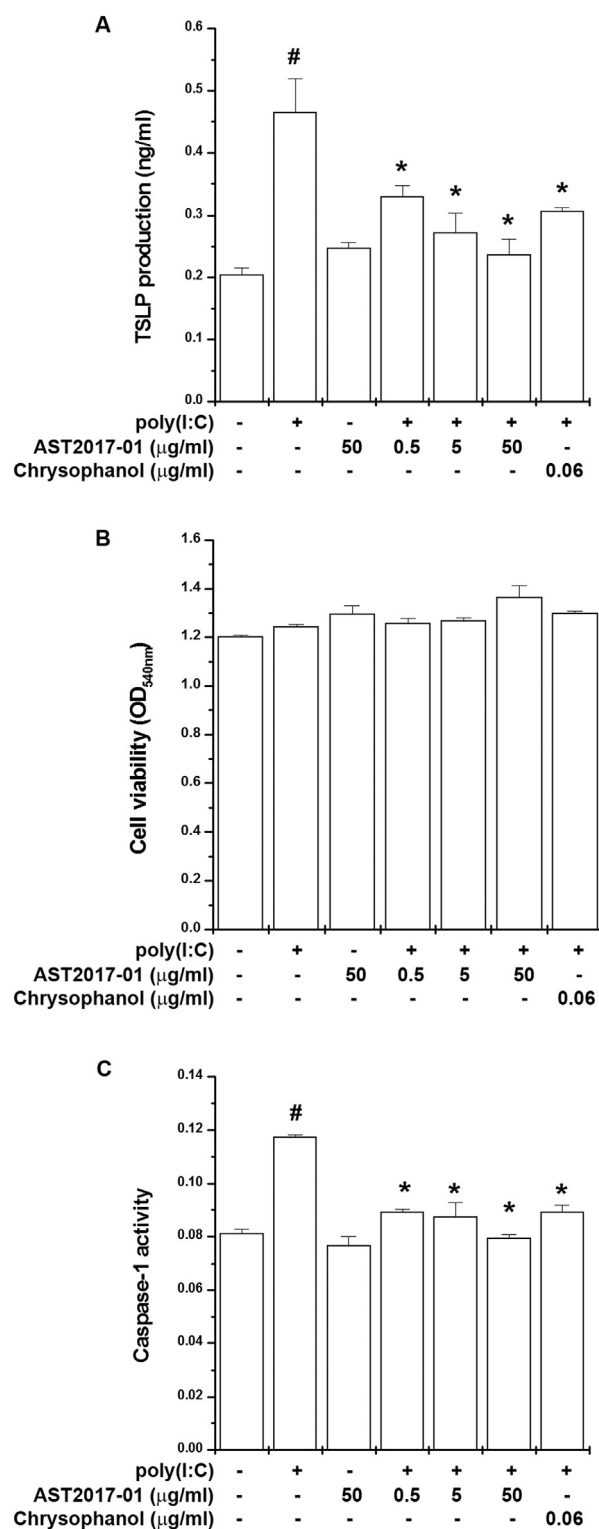


Fig. 5. AST2017-01 and chrysophanol diminished the levels of TSLP and activities of caspase-1 on activated HaCaT cells. (A) HaCaT cells (1×10^5) were pretreated with AST2017-01, chrysophanol, or dexamethasone for 1 h and then treated with poly(I:C) for 24 h. Levels of TSLP were analyzed with ELISA. (B) Cell viability was determined with an MTT assay. (C) HaCaT cells (1×10^5) were pretreated with AST2017-01, chrysophanol, or dexamethasone for 1 h and then treated with poly(I:C) for 4 h. Caspase-1 activities were analyzed with caspase-1 colorimetric assay kit. Data represent mean $\pm$ S.E.M. of three independent experiments. $^{\#}P < 0.05$; significantly different from the unstimulated cells, $^*P < 0.05$; significantly different from poly(I:C)-stimulated cells.

and TNF- α by PMACI stimulation. The results revealed that protein levels of TSLP, IL-1 β , IL-6, and TNF- α were significantly increased by PMACI stimulation (Fig. 4A, $P < 0.05$). However, pretreatment with AST2017-01, chrysophanol, or dexamethasone significantly decreased the protein productions of these cytokines (Fig. 4A, $P < 0.05$). When MTT assay was performed, there was no effect on cell viability with increasing concentrations (Fig. 4B). In addition, real-time PCR was used to assess mRNA expression of these cytokines. As a result, pretreatment with AST2017-01 (50 μ g/ml), chrysophanol, or dexamethasone significantly reduced the levels of mRNA expression in PMACI-stimulated HMC-1 cells (Fig. 4C, $P < 0.05$).

3.6. AST2017-01 and chrysophanol diminished the levels of TSLP and activities of caspase-1 on activated HaCaT cells

Epithelial-derived TSLP has emerged as a potent inducer of allergic inflammation at skin barrier [22]. Thus, we finally tested whether AST2017-01 could affect production of TSLP and activation of caspase-1 on poly(I:C)-stimulated HaCaT cells. Pretreatment with AST2017-01, chrysophanol, or dexamethasone significantly decreased the TSLP production on activated HaCaT cells (Fig. 5A, $P < 0.05$) and there were no obvious changes in cell viability (Fig. 5B). In addition, caspase-1 activities were significantly reduced by AST2017-01, chrysophanol, or dexamethasone pretreatment (Fig. 5C, $P < 0.05$).

4. Discussion

Inhibition of the mechanisms underlying the activation of mast cell and keratinocyte is of potential clinical value for the development of therapeutic strategies against AD. AST2017-01 consists of many different herbal medicines. The specific function of herbal medicine is that each medical formula often contains multiple medicinal herbs, which have several chemical components, inducing many pharmacological effects with multiple targets and pathways [23]. This characteristic of herbal medicine makes it a potential treatment to simultaneously target multiple cytokines and signaling pathways [24]. An important finding in this study is that AST2017-01 and chrysophanol diminished the levels of intracellular calcium and histamine, phosphorylation of MAPKs, activation of IKK β , RIP2, caspase-1, and NF- κ B, and expression of inflammatory cytokines increased by PMACI stimulation on HMC-1 cells. In addition, AST2017-01 and chrysophanol reduced the production of TSLP and activation of caspase-1 induced by poly(I:C) stimulation on HaCaT cells.

Mast cells have been recognized as key effector cells in the allergic inflammatory response of AD because of releasing various allergic inflammatory mediators such as histamine and inflammatory cytokines [3]. Mast cell activation signaling cascades require the activation of protein kinase C (PKC)/calcium mobilization signaling pathways [6]. PMACI, which is PKC activator (PMA) and calcium ionophore (A23187), activates PKC and increases intracellular calcium levels. PMACI induces the release of preformed or de novo synthesized inflammatory mediators through the activation of MAPKs, IKK β , phosphatidylinositol-3 kinase, caspase-1 and NF- κ B pathways [7]. In our previous study, we reported that a decrease of intracellular calcium by calcium chelator, 2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid acetoxymethyl ester, diminished the RIP2 expression, caspase-1 activation, I κ B α phosphorylation, and NF- κ B activation as well as inflammatory cytokine production on PMACI-stimulated HMC-1 cells [18]. Therefore, we can hypothesis that blockade of intracellular calcium can help us to treat AD because the increase of intracellular calcium induces mast cell activation. In this study, we showed that AST2017-01 and chrysophanol blocked the calcium signaling pathways in activated mast cells. Therefore, these results suggest that AST2017-01 and chrysophanol inhibit the mast cell activation through blocking of calcium influx.

Keratinocytes are not only the outermost components of mucous

membranes and skin but also a forefront of the defense system by sensing biological, chemical, or mechanical stimulation [25] and participate the initiation, amplification, and persistence of allergic inflammatory and immune responses [26]. Keratinocytes produce proinflammatory mediators, including TSLP in response to a variety of stimuli [25,27]. Keratinocyte-derived TSLP induces activation and differentiation of Th2 cells and innate lymphoid cells, important effectors in pathophysiology of AD [25,28]. Abnormal cytokine production by keratinocytes was mainly involved in the pathophysiology of atopic diseases. Previous studies reported that TSLP was produced and expressed by activation of caspase-1 and NF- κ B on mast cells and keratinocytes [21,28]. For that reason, caspase-1 and TSLP in AD have been considered as target molecules for therapy. In this study, we showed that AST2017-01 and chrysophanol reduced the production of TSLP through inhibition of caspase-1 activation. Therefore, these results suggest that AST2017-01 and chrysophanol have an anti-AD effect by inhibiting keratinocytes activation.

Jang et al., [17] reported that chrysophanol is a marker compound of AST2017-01 and the concentration of chrysophanol in processed-*Rumex crispus* was higher than that in non-processed-*Rumex crispus*. In this study, chrysophanol also diminished the mast cell and keratinocyte activation signaling pathway. Therefore, we suggest that chrysophanol is a marker compound and an active component of AST2017-01 on inflammatory responses.

In the present study, it was found for the first time that AST2017-01 and chrysophanol diminished the PMACI-induced mast cell activation by down-regulating calcium signaling pathway. Furthermore, AST2017-01 and chrysophanol decreased the poly(I:C)-induced TSLP production and caspase-1 activation. These findings demonstrate that AST2017-01 should be considered a potentially useful anti-inflammatory agent. However, an anti-inflammatory effect of AST2017-01 will be investigated in animal studies in the future.

Conflict of interest disclosure

None declared.

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