



Acteoside inhibits type I allergy through the down-regulation of Ca/NFAT and JNK MAPK signaling pathways in basophilic cells

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Abstract We have previously reported that acteoside inhibits the release of β -hexosaminidase from immunoglobulin E (IgE)-sensitized and bovine serum albumin-stimulated rat basophilic leukemia cells as well as the intracellular calcium level, release of histamine from, and production of tumor necrosis factor-alpha and interleukin-4 in human basophilic (KU812) cells. However, the molecular mechanism underlying the anti-allergic effects of acteoside has not yet been elucidated. Here, we used microarray analysis to determine the global gene expression profile of KU812 cells treated with acteoside and calcium ionophore A23187 plus phorbol-12-myristate 13-acetate (A23187+PMA), and the results were validated by real-time polymerase chain reaction (PCR) and Western blotting. Microarray analysis results showed that of the 201 genes in the microarray, 149 genes were up-regulated, while 52 genes were down-regulated. The significantly down-regulated genes have functions as chemokine and IgE receptors, as well as for immune response. Results of the validation of the microarray results using real-time

PCR showed a significant decrease in the expressions of Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide (*FCERIA*) and nuclear factor of activated T cell, cytoplasmic, calcineurin-dependent 1 (*NFATC1*) genes. Furthermore, Western blotting showed a decrease in the phosphorylation of mitogen-activated protein kinase (MAPK) Jun N terminal kinase (JNK), revealing the role of JNK MAPK in acteoside-mediated allergy inhibition. We determined that the anti-allergy effects of acteoside were due to the down-regulation of the expressions of the chemokine ligand 1 (*CCL1*), *CCL2*, *CCL3*, *CCL4*, *FCERIA* and *NFATC1* genes and the inhibition of the MAPK pathway through decreased JNK phosphorylation.

Keywords Anti-allergic activity · Acteoside · *Cistanche tubulosa* · Gene expression · Microarray · MAPK JNK

Abbreviations

IgE	Immunoglobulin E
BSA	Bovine serum albumin
RBL-2H3	Rat basophilic leukemia
$[Ca^{2+}]_i$	Intracellular calcium
TNF- α	Tumor necrosis factor-alpha
Fc ϵ RI	High-affinity IgE receptors
IL	Interleukin
KU812	Human basophilic
A23187+PMA	Calcium ionophore A23187 plus phorbol-12-myristate 13-acetate
PCR	Polymerase chain reaction
<i>FCERIA</i>	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
<i>NFATC1</i>	Nuclear factor of activated T cell, cytoplasmic, calcineurin-dependent 1
MAPK	Mitogen-activated protein kinase
JNK	c-Jun N terminal kinase

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CCL Chemokine ligand
ERK Extracellular signal-regulated kinase

Introduction

Acteoside [β -(3,4-dihydroxyphenylethyl)- O - α -L-rhamnopyranosyl-(1 $\rightarrow$ 3)- β -D-(4- O -caffeoyl)-glucopyranoside] and other phenylpropanoid glycosides are abundant in plants that are widely used in traditional herbal medicine, such as *Cistanche tubulosa* (Schenk) R. Wight (Orobanchaceae). *C. tubulosa*, a parasitic plant growing on the roots of *Salvadora* or *Calotropis* species and distributed in North Africa and Arabia as well as in Asia, is traditionally used as a blood circulation-promoting agent for treatment of male impotence, sterility, lumbago, and body weakness, and as a tonic. The structure of acteoside is a complex of components that include caffeic acid, 3,4-dihydroxyphenylethanol, glucose, and rhamnose. Previous studies have indicated that acteoside has various biological activities, such as anti-oxidative activities [1, 2], and anti-apoptotic effects [3]. Moreover, it has been reported that the anti-inflammatory action of acteoside is related to the inhibition of lipopolysaccharide-induced prostaglandin E_2 , NO, and tumor necrosis factor- α (TNF- α) production in mouse peritoneal macrophages [4].

Type I allergies are caused by antigens derived from environmental allergens such as foods, dust, medicine, cosmetics, and pollen. This class of antigen induces production of antibodies that bind to mast cells or basophils. When antigen-specific immunoglobulin E (IgE) antibodies bind strongly to high-affinity IgE receptors (Fc ϵ RI) on the surface of mast cells or basophils, the receptors are cross-linked to transform the cell surfaces, various enzymes are activated, and the intracellular calcium ($[Ca^{2+}]_i$) levels increase. The mast/basophil cells release β -hexosaminidase, histamine, cytokines, prostaglandins, leukotrienes, and chemokines, which cause allergy [5]. Among these mediators, chemokines have been traditionally defined as small chemoattractant peptides that provide directional cues for cell trafficking and thus are vital for protective host response. It is now generally accepted that some chemokines and their receptors play essential roles in the pathogenesis of allergic rhinitis and atopic dermatitis [6, 7].

The mitogen-activated protein kinase (MAPK) signaling pathway is a network of interacting proteins that mediates the transmission of extracellular signals into their intracellular targets to regulate a large number of cellular processes [8]. There are three major classes of MAPK in mammals—the c-Jun N terminal kinase (JNK), the extracellular signal-regulated kinase (ERK), and the p38 MAPK [9]. The complex roles of MAPKs in allergic reactions, as

well as in cellular programs like inflammation, cell differentiation, cell proliferation, and apoptosis, have been reported. It appears that the activation of JNK, ERK, and p38 by environmental stimuli plays a significant role in cytokine expression, in addition to its important regulatory role in most inflammatory diseases [10, 11].

In our previous study, we investigated the inhibitory effect of three phenylethanoid glycosides derived from *C. tubulosa* on β -hexosaminidase release by IgE-mediated RBL-2H3 cells as a type I allergy model. Our data showed that acteoside extracted from *C. tubulosa* can inhibit the β -hexosaminidase release and $[Ca^{2+}]_i$ level from immunoglobulin E (IgE)-sensitized and bovine serum albumin (BSA)-stimulated rat basophilic leukemia (RBL-2H3) cells. In addition, acteoside can decrease the levels of histamine, TNF- α , and interleukin (IL)-4 in calcium ionophore A23187 plus phorbol-12-myristate 13-acetate (A23187+PMA)-stimulated human basophilic (KU812) cells [12]. In the present study, we determined the mechanism underlying the observed suppression of cell degranulation following acteoside treatment on the type I allergic reaction in KU812 cells.

Materials and methods

Sample preparation, cell culture, and treatment condition

Acteoside was isolated from stems of *Cistanche tubulosa* which were purchased from Shinwa Bussan Co., Ltd (Osaka, Japan). The procedure for the isolation of acteoside was as previously described [12], and its chemical structure is shown in Fig. 1 [13]. Pure acteoside was dissolved in PBS, and the stock solution was stored at -80°C prior to use. To obtain the desired final concentration, the stock solution was diluted in cell culture medium. The human basophilic KU812 cells were purchased from Riken Cell Bank (Tsukuba, Ibaraki, Japan) and were maintained in RPMI1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (Hyclone Co., Ltd) containing 50 U/ml of penicillin

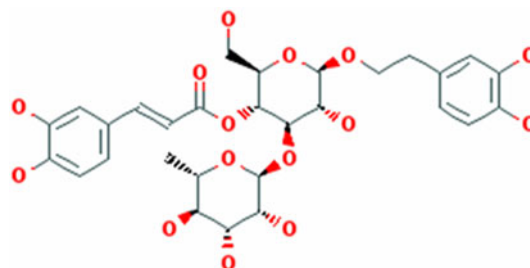


Fig. 1 Chemical structure of acteoside

and 50 µg/ml of streptomycin, and cultured at 37 °C in a humidified atmosphere with 5 % CO₂. KU812 cells were treated with A23187 (1 µM) + PMA (20 nM) after acteoside treatment (0.1 or 1.0 µg/ml). Calcium ionophore A23187 and PMA were obtained from Sigma Japan (Tokyo) and dissolved in dimethyl sulfoxide.

Total RNA isolation

KU812 cells at a concentration of 1×10^6 cells/ml were treated with or without 0.1 or 1.0 µg/ml acteoside for 15 min and incubated at 37 °C, after which A23187+PMA was added, and the cells incubated further for 4 h. DNA-free total RNA was isolated from the cells using Isogen (Nippon Gene Co., Tokyo, Japan) following the manufacturer's instructions.

DNA microarray analyses

To elucidate the molecular mechanisms underlying the acteoside effect, DNA microarrays spotted with 201 genes were used to determine the gene expression profile of KU812 cells treated with acteoside (0.1 and 1.0 µg/ml) plus A23187+PMA or KU812 cells treated with A23187+PMA alone. Synthetic DNA oligonucleotides were installed as probes onto Genopal® (Mitsubishi Rayon), which is made up of hollow plastic fibers. In this system, oligonucleotide DNA probes are attached to a gel within the three-dimensional space of each hollow fiber [14]. First, RNA was amplified using the MessageAmpII biotin-enhanced amplification kit (Applied Biosystems Japan, Tokyo, Japan), according to the manufacturer's instructions. Fragmentation reagents (Applied Biosystems Japan) were applied to 5 µg of biotinylated antisense RNA (aRNA), which was then incubated at 94 °C for 7.5 min. The fragmentation reaction was terminated by the addition of stop solution. Hybridization was carried out with a DNA microarray (Genopal, Mitsubishi Rayon) and 5 µg of fragmented biotinylated aRNA at 65 °C overnight. After hybridization, the DNA microarray was washed, and was then labeled with streptavidin-Cy5 (GE Healthcare Bio-Science KK, Tokyo, Japan). Hybridization signals were acquired using a DNA microarray reader with multibeam excitation technology (Yokogawa Electric Co., Tokyo, Japan) [15]. The DNA microarrays were scanned at multiple exposure times from 0.1 to 40 s and the intensity values with the best exposure conditions for each spot were selected. Genes with 2-fold change in expression (23 genes) were analyzed using the hierarchical clustering program TIGR MultiExperiment Viewer v. 4.0 [16].

Real-time PCR analysis

To validate the results of microarray and Western blot analyses, real-time polymerase chain reaction (PCR) was performed using specific and validated Taqman Gene Expression Assay (Applied Biosystems) primers for nuclear factor of activated T-cells (*NFAT*), Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide (*FCERIA*), *MAPK8*, and interleukin (*IL*)-13 genes. The complementary DNA obtained by reverse transcription of total RNA, with oligo (dT) primer bearing a T7 promoter, was used as a template for real-time PCR. The real-time PCR reactions were performed following TaqMan (Applied Biosystems) protocol. Briefly, conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate to confirm the data's reproducibility and the amount of target mRNA in each sample was normalized using that of the mean β-actin gene expression levels.

Western blot analysis

KU812 cells at a density of 1×10^6 cells/ml were seeded onto 100-mm dishes and cultivated by the method described above. After overnight incubation, the medium was then replaced with medium containing 0.1 or 1.0 µg/ml acteoside followed by incubation for 15 min. The cells were then treated with A23187+PMA for 30 min, after which they were washed with PBS, and extraction of total protein was carried out using Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma) following the manufacturer's instructions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to resolve 15 µg of extracted protein sample, which was then transferred onto nitrocellulose membrane. Membranes were blocked with blocking buffer containing skim milk powder in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T), and probed with 1:1000 diluted primary antibodies (Cell Signaling Technologies, Santa Cruz Biotechnology) specific for the target protein. After reaction with horseradish peroxidase (HRP)-labelled anti-rabbit IgG antibody (Santa Cruz Biotechnology, Inc.), the signal was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Nihon Millipore K.K., Tokyo, Japan) or ECL plus (GE Healthcare Bioscience, USA).

Statistical analysis

Student's *t* test was used to determine the statistical significance between the treated and control groups in each experiment. Differences were considered statistically significant at a value of $p < 0.05$.

Results

Acteoside suppresses A23187+PMA-induced allergic response in KU812 cells

Based on the results of microarray analysis, out of the 201 genes in the DNA chip, 149 genes were up-regulated while 52 genes were down-regulated in cells that had been pre-treated with 0.1 µg/ml of acteoside for 15 min. Increasing the acteoside concentration from 0.1 to 1.0 µg/ml raised the number of up-regulated genes to 167 and decreased the number of down-regulated genes to 34 (Fig. 2).

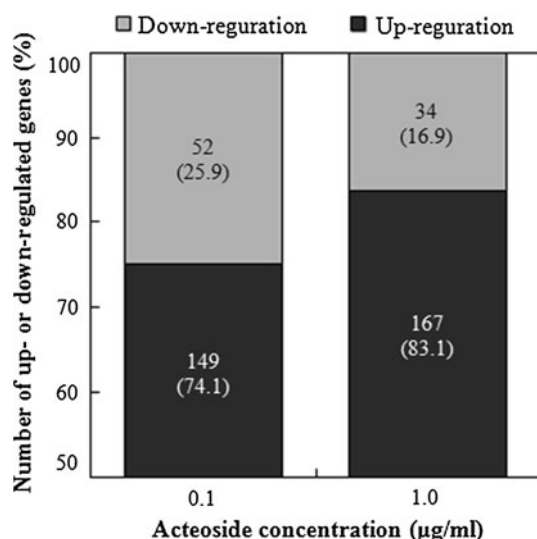


Fig. 2 Proportion of up- or down-regulated genes in KU812 cells after treatment with acteoside plus A23187+PMA compared to A23187+PMA alone. The numbers shown in parentheses indicate the up- or down-regulated gene ratio with acteoside plus A23187+PMA treatment compared to A23187+PMA alone in the total of 201 genes in the DNA chip

To understand how the genes interact, hierarchical clustering analysis was performed on the 23 differentially expressed genes, which showed a 2-fold change in gene expression in A23187+PMA-treated cells compared with control. The hierarchical clustering grouped 6 genes in one cluster (*CCL1*, *CCL2/MCP1*, *CCL3/MIP-1α*, *CCL4/MIP-1β*, *FCERIA*, *NFATC1*), and these are the genes that had been significantly down-regulated by acteoside treatment (data not shown). The KEGG PATHWAY database [17] was used to identify the enriched function-related gene groups and the results are presented in Table 1.

Acteoside inhibited the mRNA expressions of *FCERIA* and *NFATC1*

The microarray results presented in Table 1 were validated using real-time PCR (Fig. 3). The real-time PCR results confirmed the DNA microarray results, showing clearly that *FCERIA* and *NFATC1* gene expressions in the cells were both significantly decreased by acteoside treatment.

Acteoside inhibited the activation of JNK

The role of MAPK in allergy has been well described [18]. Here, to determine the role of MAPK on the anti-allergic effects of acteoside, the total cellular protein was extracted from KU812 cells that were stimulated with A23187+PMA for 15 min and treated without or with 0.1 µg/ml acteoside, and subjected to Western blotting using specific antibodies for p38, ERK1/2, and JNK. Results show that although acteoside treatment did not cause any significant change in the activation of p38 or ERK1/2, the expression of phosphorylated JNK was decreased (Fig. 4). Determination of the expression of JNK at the transcription level reveals a down-regulated *MAPK8* mRNA expression (Fig. 5a). It has been reported that inhibition of JNK-dependent NFATC1

Table 1 Ratio of down-regulated gene expression in KU812 cells that were treated with acteoside plus A23187+PMA

Functional category and gene name	Gene symbol	Accession no.	Gene expression ratio (treatment/control)		
			A23187+PMA	0.1 µg/ml	1.0 µg/ml
Chemokine					
Chemokine (C–C motif) ligand 1	<i>CCL1</i>	NM_002981	5.14	4.77	4.93
Chemokine (C–C motif) ligand 2	<i>CCL2/MCP1</i>	NM_002982	22.74	20.44	20.27
Chemokine (C–C motif) ligand 3	<i>CCL3/MIP-1α</i>	NM_002983	73.21	67.34	65.82
Chemokine (C–C motif) ligand 4	<i>CCL4/MIP-1β</i>	NM_002984	73.76	65.93	66.91
IgE receptor					
Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	<i>FCERIA</i>	NM_002001	2.64	2.33	2.35
Immune response					
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	<i>NFATC1</i>	NM_006162	6.45	6.59	5.82

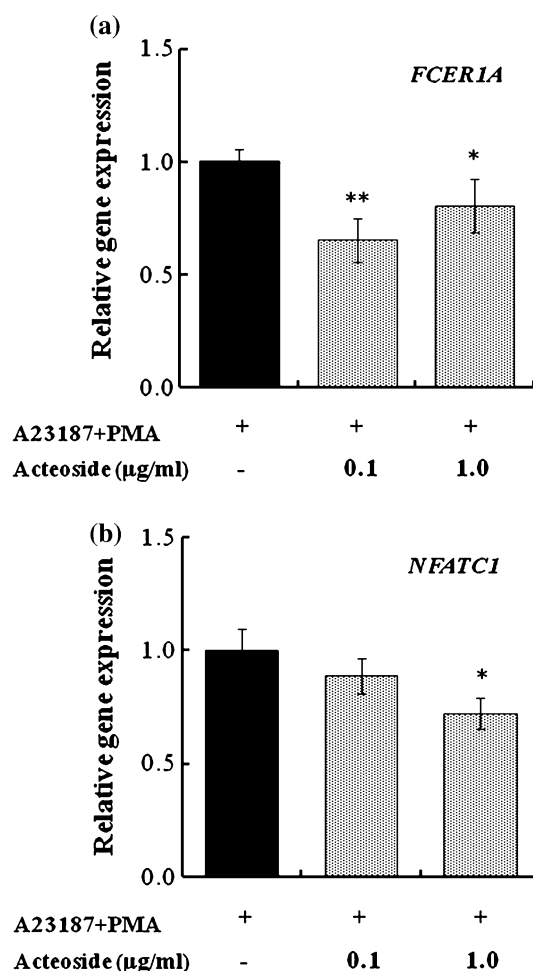


Fig. 3 Effect of acteoside on the gene expression levels of **a** *FCER1A* and **b** *NFATC1* in KU812 cells quantified using real-time PCR. KU812 cells at a concentration of 1×10^6 cells/ml were treated with or without 0.1 or 1.0 µg/ml acteoside and incubated at 37 °C for 15 min, after which A23187+PMA was added and the cells incubated further for 4 h. * $p < 0.05$ A23187+PMA versus acteoside treatment; ** $p < 0.01$ A23187+PMA versus acteoside treatment. Each bar represents the mean of three replicates $\pm$ SEM

expression can cause the down-regulation of the expression of *IL-13* gene in KU812 cells [19]. Similarly, the results of this study showed that *IL-13* gene expression in KU812 cells was also significantly decreased when JNK phosphorylation was inhibited by acteoside treatment (Fig. 5b).

Discussion

We have previously reported that acteoside can inhibit the release of β -hexosaminidase from IgE-sensitized BSA-stimulated RBL-2H3 cells, and decrease the histamine release and TNF- α and IL-4 production by lowering the $[Ca^{2+}]_i$ level in A23187+PMA-stimulated KU812 cells [12]. To evaluate the global patterns of allergy-related gene expressions in KU812 cells, microarray analysis was

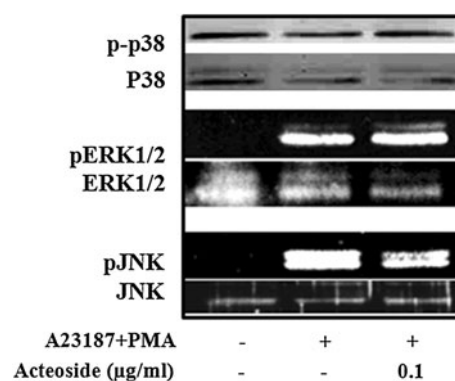


Fig. 4 Effect of acteoside on the activation of p38, ERK1/2, and JNK in human KU812 cells. KU812 cells (1×10^6 cells/ml) were treated with acteoside (0.1 µg/ml) for 15 min and stimulated with A23187 (1 µM) + PMA (20 nM) for 30 min, after which the total protein samples were extracted using RIPA buffer (Sigma) and detected using specific antibodies obtained from Santa Cruz Biotechnologies

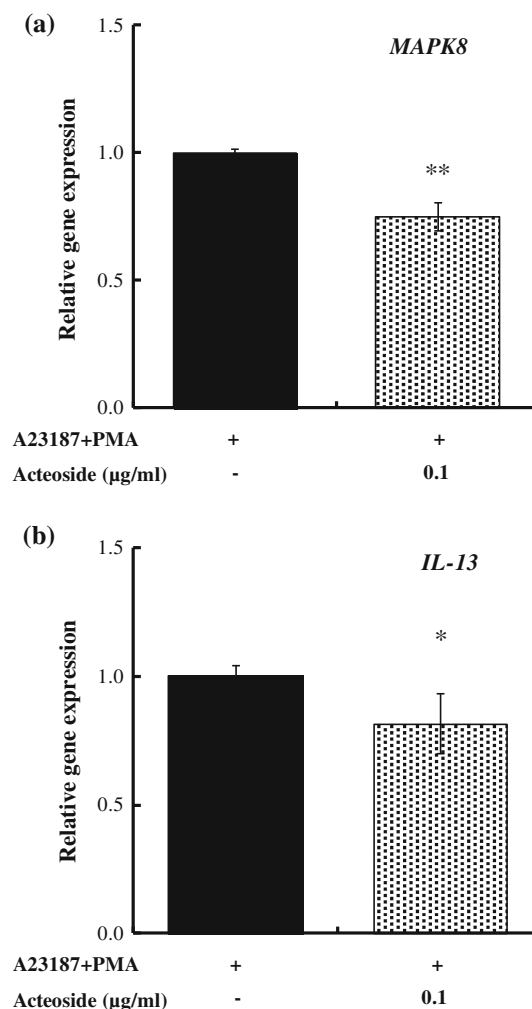


Fig. 5 Effect of acteoside on the gene expression levels of **a** *MAPK8* and **b** *IL-13* in KU812 cells quantified using real-time PCR. * $p < 0.05$ A23187+PMA versus acteoside treatment; ** $p < 0.01$ A23187+PMA versus acteoside treatment

performed and the results revealed that treatment with acteoside affected the expressions of genes that function in the following pathways: chemokine, IgE receptor, and immune response (Table 1).

Chemokines are a group of cytokines that are responsible for the influx of blood cells, including T and B lymphocytes, monocytes, neutrophils, eosinophils and basophils, in allergic and other inflammatory conditions [20, 21]. Chemokine (C–C motif) ligand 1 (CCL1) is the chemotactic cytokine implicated in a variety of biological responses, namely inflammation and chemotaxis, in many cell types. Chemokine (C–C motif) ligand 2 (CCL2), a potent regulator of monocyte/macrophage chemotaxis and invasion in various disease states, is induced in response to CCL1 in human HeLa cells [22]. CCL2 and CCL3 induce both immediate and delayed skin hypersensitivity reactions in human atopic and non-atopic subjects [23]. Our results showed that acteoside treatment suppressed the expressions of *CCL1*, *CCL2*, *CCL3*, and *CCL4* genes in KU812 cells.

The binding of IgE to FcεRI on the surface of the mast/basophil cells and its subsequent crosslinking to an allergen is the first step in the cascade of reactions leading to the release of cellular pro-inflammatory mediators [24]. On these cells, FcεRI has a heterotetrameric form and consists of an α subunit (FcεRIα) and β chain (FcεRIβ), and two γ chains (FcεRIγ) [25]. A recent genome-wide scan identified the gene encoding the alpha chain of the high affinity receptor for IgE (*FCERIA*) as a susceptibility locus at which genetic variation influences total serum IgE levels [26].

Acteoside suppresses the $[Ca^{2+}]_i$ influx in the IgE-sensitized BSA-stimulated RBL-2H3 cells, indicating that the intracellular $[Ca^{2+}]_i$ level was lower in the acteoside-treated cells than in DNP-BSA-stimulated RBL-2H3 cells. Therefore, it is possible that acteoside inhibits *NFATC1* by decreasing the $[Ca^{2+}]_i$ level in basophilic cells. NFAT proteins are a family of five inducible transcription factors that control numerous cellular differentiation processes, including immune cell activation, heart valve formation, and the growth of cartilage and stem cells. The NFAT protein family is comprised of four closely related members (NFATC1–4) that are activated by Ca^{2+} signaling [27].

Based on the microarray results, acteoside treatment showed a significant inhibitory effect on the expressions of *CCL1*, *CCL2*, *CCL3*, and *CCL4* genes but the effect on the expressions of *FCERIA* and *NFATC1* genes was not as significant as the effect on the chemokine genes. Previous studies have reported that the activation of NFAT proteins is induced by the engagement of receptors that are coupled to the calcium signaling pathway, such as the antigen receptors (T and B cells), the Fcγ receptors (monocytes and natural killer cells), or the Fcε receptors (mast cells) [28].

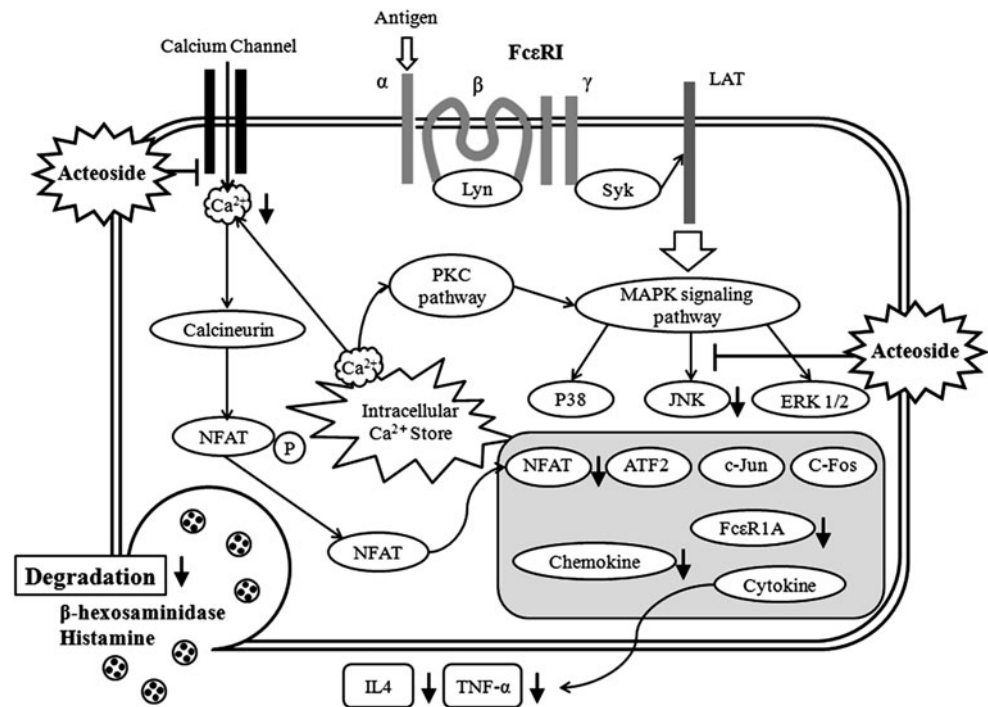
It should be noted that microarray results often vary when compared to techniques that are relatively more sensitive, such as real-time PCR [29]. It was, therefore, important to validate microarray results using real-time PCR to confirm the inhibitory effect of acteoside on the expressions of *FCERIA* and *NFATC1* genes in KU812 cells (Fig. 3). Although the expressions of the *FCERIA* and *NFATC1* genes were also significantly down-regulated by acteoside treatment, the effect on *FCERIA* expression was not dose-dependent.

Acteoside is a phenylethanoid glycoside and its ^1H -NMR data analysis indicated the presence of a hydroxytyrosol, caffeic acid, and two sugar (rhamnose and glucose) moieties on acteoside and isoacteoside [30]. Acteoside is an antioxidant which has a radical oxygen scavenging activity in activated human leukocytes [1, 2]. Furthermore, both hydroxytyrosol and caffeic acid show a broad spectrum of biological properties due to its strong antioxidant and radical-scavenging properties [31–33]. Recently, there have been many reports on the important role of reactive oxygen species (ROS) in several signaling pathways, thus strengthening the view that they are not only associated with toxicity and pathology but also have significant controlling influences in many physiological processes such as allergy and acute inflammatory response. ROS stimulates the release of β-hexosaminidase and histamine by activating protein kinase C [34]. The sustained elevation of intracellular calcium through store-operated calcium entry was totally abolished when the production of ROS was blocked [35]. The MAPK signaling pathway is recognized as one of the important signaling pathways that are involved in immune responses [36], and, as such, they are considered to be appropriate targets for pharmacological treatment of inflammatory disorders [37]. Studies have demonstrated that ROS can induce or mediate the activation of the MAPK pathway [38, 39].

The tea catechin (–)-epigallocatechin-3-*O*-gallate (EGCg) is a major mediator of the antioxidative effect of green tea. As Sugisawa et al. [40] demonstrated, a high concentration of EGCg induced chromosomal damage caused by the production of H_2O_2 in WIL2-NS cells; on the other hand, a low concentration of EGCg prevented the chromosomal damage induced by ROS [H_2O_2 , *tert*-butyl hydroperoxide (*tert*-BuOOH), and superoxide] in the cells [40]. The presence of EGCg either inside or outside the cells can protect the cells against ROS-induced chromosomal damage, but the effect was not dose-dependent.

Hyaluronic acid is a glycosaminoglycan found in the extracellular matrix of connective tissues. The structure of the sugar moiety of acteoside is similar to that of hyaluronic acid. Hyaluronic acid exerts a negative effect on β-hexosaminidase secretion and histamine release in antigen-stimulated RBL-2H3 cells. In addition, hyaluronic acid

Fig. 6 Intracellular signal transduction pathways regulated by acteoside on A23187+PMA-induced type I allergy in basophilic cells. Acteoside significantly suppressed *NFATC1* activation by blocking the $[Ca^{2+}]_i$ and reduced phosphorylation of MAPK signaling (JNK), leading to the inhibition of basophilic cell degranulation



was also reported to target CD44 and inhibit FcεRI signaling involving PKCδ, Rac1, ROS, and MAPK in its anti-allergic effect [41]. The inhibitory effect of hyaluronic acid on FcεRI protein production was not dose-dependent based on Western blot results [41]. These reports indicate that low levels of antioxidants may show a stronger antioxidant activity than that of high levels in some cases. In this study, acteoside at 0.1 μg/ml was also more effective than at 1.0 μg/ml (Fig. 2). Here, one of the possible reasons for this observed concentration-specific inhibitory effect of acteoside on *FCER1A* gene expression could be its antioxidant effect on KU812 cells.

NFATC1 is a key transcriptional factor reported to be activated by JNK [42]. The expression of *NFATC1* gene was decreased by acteoside treatment, based on our microarray results. To determine the intracellular signaling events that occurred, the effect of acteoside on the phosphorylation of MAPKs p38, ERK1/2, and JNK was investigated using Western blotting. The significant decrease observed in the activation of the JNK protein prompted us to determine its expression at the genetic level by determining the expression of the *MAPK8* and *IL-13* genes using real-time PCR. In this study, the phosphorylation of JNK as well as the mRNA expression of its gene, *MAPK8*, was observed to be decreased following treatment with acteoside (Figs. 4, 5a), revealing the role of JNK in acteoside's anti-allergy effect. A related study on allergy, in which KU812 cells were treated with epigallocatechin to induce allergic reactions by positively regulating *IL-13* gene expression in KU812 cells, found that the allergy was

induced via the JNK-dependent NFATC1 pathway [19] and that inhibition of JNK suppresses pro-inflammatory cytokine expression and mast cell activation [11]. In this study, treatment with acteoside also inhibited the expression of *IL-13* gene through the suppression of JNK phosphorylation (Fig. 5b). IL-13 is the major cytokine for IgE production, facilitating T-helper type 2 differentiation and allergic responses together with IL-4 [43]. In our previous study, we reported that acteoside inhibited the expression of IL-4 in KU812 cells at the protein level. Therefore, it is possible that the observed inhibition of *IL-13* gene expression in KU812 cells could be one of the key factors in the anti-allergy activity of acteoside. The anti-allergy effects of acteoside could be regulated through several different pathways, either through chemical mediator release, intracellular calcium level, regulation of the allergy-related gene expressions, or through the regulation of the MAPK JNK in basophilic cells. These results suggested the complexity of its action via its antioxidative activity.

Previously, we showed that acteoside can inhibit the release of β-hexosaminidase and $[Ca^{2+}]_i$ level from IgE-sensitized and BSA-stimulated RBL-2H3 cells, as well as inhibit histamine release and TNF-α and IL-4 production in A23187+PMA-stimulated KU812 cells. Here, we determined the molecular mechanism underlying the anti-allergic effect of acteoside on KU812 cells. In this study, microarray results showed that acteoside decreased the A23187+PMA-enhanced chemokine, IgE receptor, immune response, and apoptosis inducer gene expression

levels in KU812 cells. Notably, acteoside inhibited the gene expression of chemokines which play a major role in immune and inflammatory responses. Real-time PCR results validated the observed inhibition of *FCER1A* and *NFATC1* gene expressions by acteoside treatment. In addition, acteoside suppressed the A23187+PMA-induced phosphorylation of JNK in KU812 cells. The anti-allergic effects of acteoside, therefore, were due to the down-regulation of the expression of *NFATC1* gene through the decrease in the $[Ca^{2+}]_i$ level and the inhibition of JNK phosphorylation (Fig. 6). An investigation into the efficacy of acteoside in an in vivo experiment is recommended to further characterize its effect as an anti-allergy drug.

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