Inhibitory Effect of Acteoside Isolated from *Cistanche tubulosa* on Chemical Mediator Release and Inflammatory Cytokine Production by RBL-2H3 and KU812 Cells

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Key words
- antiallergic
- acteoside
- β-hexosaminidase
- *Cistanche tubulosa*
- Orobanchaceae
- cytokines
- histamine

Abstract

The immediate-type allergic reaction is involved in many allergic diseases such as asthma, allergic rhinitis, and sinusitis. In this study, we investigated the effect of acteoside extracted from *Cistanche tubulosa* (Schrenk) R. Wight on the basophilic cell-mediated allergic reaction. The effect of acteoside on β-hexosaminidase release and intracellular $[Ca^{2+}]i$ level from rat basophilic leukemia (RBL-2H3) cells was determined. Also, ELISA was used to determine the level of histamine, tumor necrosis factor (TNF)-α, and interleukin (IL)-4 on human basophilic (KU812) cells. The effect of acteoside on basophilic cell viability was determined using the 3-[4,5-dimethylthiazolyl]-2,5-diphenyltetrazolium bromide (MTT) assay. These results indicated that 0.1–10.0 µg/mL acteoside inhibits the release of β-hexosaminidase and $[Ca^{2+}]i$ influx from IgE-mediated RBL-2H3 cells. Moreover, acteoside inhibited histamine release, TNF-α, and IL-4 production in a dose-dependent manner from calcium ionophore A23187 plus phorbol 12-myristate 13-acetate (PMA) or compound 48/80-stimulated KU812 cells. Our findings provide evidence that acteoside inhibits basophilic cell-derived immediate-type and delayed-type allergic reactions. This is the first report describing antiallergic activity of acteoside extracted from *Cistanche tubulosa* on basophilic cells.

Abbreviations

IL-4:	interleukin-4
KU812:	human basophilic cells
MTT:	3-[4,5-dimethylthiazolyl]-2,5-diphenyltetrazolium bromide
PKC:	protein kinase C
PMA:	phorbol 12-myristate 13-acetate
RBL-2H3:	rat basophilic leukemia cells
TNF-α:	tumor necrosis factor-α

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Introduction

Herba Cistanchis is the pharmaceutical name of a whole herb of the Orobanchaceae family and *Cistanche* genus; it is also calledd Da Yun, Cong Rong, and Cun Rong. The herb of the same genus named *Cistanche tubulosa* (Schrenk) R. Wight (Orobanchaceae) is a perennial parasitic plant growing on the roots of *Salvadora* or *Calotropis* species and distributed in North Africa and Arabia as well as in Asian countries. It has been traditionally used as a blood circulation-promoting agent and in the treatment of impotence, sterility, lumbago, body weakness, and as a tonic [1,2]. The *Cistanche tubulosa* extract has been shown to have an effect on various brain diseases, antiaging functions, fat metabolism, and hair growth [3–6]. Recently, several compounds including iridoids, monoterponenoids, phenylethanoid glycosides such as acteoside, echinacoside, and cistanoside A, and lignans were isolated from the Chinese and Pakistani *Cistanche tubulosa* [1,7]. Some of these phenylethanoid glycosides appear to have various biological activities, such as anti-inflammatory, antioxidant, and relaxing properties [8–10]. Moreover, acteoside extracted from *Cistanche tubulosa* regulated immunity on aging mice [11], as well as acteoside isolated from *Clerodendron trichotomum* Thunberg had anti-inflammatory effect on melittin-stimulated RBL-2H3 cells [12] and lipopolysaccharide-stimulated mouse peritoneal macrophage [13]. Considerable data on the acteoside and echinacoside extracted from *Cistanche*

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tubulosa have been reported with the few studies on their antioxidant effect. However, their antiallergic effects have not been studied.

The type I allergy is induced by certain types of antigens such as foods, dust mites, medicines, pollen, and cosmetics. These types of antigens induce the production of antigen-specific IgE antibodies that bind to receptors on mast cells or basophilic cells. Recently, the early phase and late phase reactions have been reported in type I allergy. The early phase reaction occurs within a few minutes after allergen exposure, and the cells release mediators such as histamine and serotonin. The late phase reaction occurs hours after the early phase reaction in type I allergy; mediators such as inflammatory cytokines TNF-α, IL-4, IL-6, IL-8, and IL-13 are secreted from the cells [14–16]. β-Hexosaminidase, which is stored in the secretory granules of the mast cells, is released concomitantly with histamine when the mast cells are immunologically activated. Thus, β-hexosaminidase activity in the medium is used as a marker of mast cell degranulation [17, 18]. RBL-2H3 cells have been extensively used for studying IgE-FcεRI (the high affinity IgE receptor) interactions [19], signaling pathways for degranulation [20] and gene expression of inflammatory cytokines [21]. These cells are therefore considered as a good tool for studying the effect of unknown compounds on histamine release or β-hexosaminidase release activity.

Basophils and mast cells types originate from the hematopoietic stem cells and share several biochemical and functional properties. Human mast cells are useful cells for studying synthesis of mediators and the cytokine activation pathway because they secrete histamine and many inflammatory cytokines when stimulated with phorbol esters and calcium ionophore A23187 [22, 23]. Both basophils and mast cells play a major role in the pathogenesis of inflammatory diseases by releasing several pro-inflammatory mediators [24]. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines such as TNF-α, IL-6, IL-4, IL-8, and IL-13. Therefore, modulation of the secretion of these cytokines from mast cells can provide a useful therapeutic strategy for allergic inflammatory disease [23, 25].

In this study, we investigated the inhibition effect of three phenylethanoid glycosides derived from *Cistanche tubulosa* on β-hexosaminidase release by IgE-mediated RBL-2H3 cells as a type I allergy model, and inhibition effect of acteoside on histamine release from A23187 plus PMA or compound 48/80-stimulated KU812 cells, and on cytokines production by the same cell line.

Materials and Methods

Cell lines, chemicals, and biochemicals

RBL-2H3 cells were purchased from the JCRB Cell Bank and maintained in MEM supplemented with 10% FBS and 2 mM L-glutamine, while KU812 cells were obtained from the Riken Cell Bank, maintained in a RPMI 1640 medium supplemented with 10% FBS and incubated at 37 °C in a 5% CO_2 incubator. Dinitrophenylated bovine serum albumin (DNP-BSA) was purchased from Cosmo Biotechnology Co., Ltd. Anti-DNP-IgE, ketotifen fumarate salt (> 99%), L-glutamine, calcium ionophore A23187, PMA, and compound 48/80 were from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from Hyclone Co., Ltd. and Eagle's Minimum Essential Medium (MEM) from Nissui Pharmaceutical Co., Ltd. 3-[4,5-Dimethylthiazolyl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Dojindo Co., Ltd.

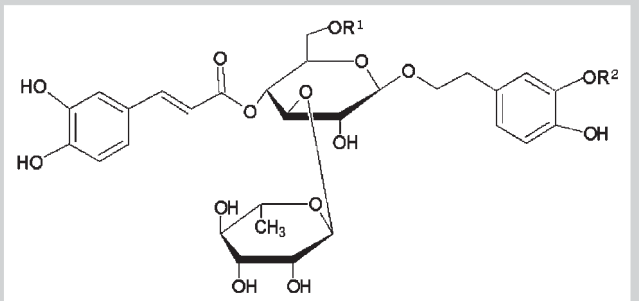

Fig. 1 Chemical structure of phenylethanoid glycosides extracted from *Cistance tubulosa*. Acteoside: R^1 = H, R^2 = H; Echinacoside: R^1 = Glc, R^2 = H; Cistanoside A: R^1 = Glc, R^2 = CH_3.

Plant material

The stems of *Cistanche tubulosa* (Orobanchaceae) were purchased from Shinwa Bussan Co., Ltd. in Osaka, Japan and identified by a botany expert. Their voucher specimens (UT-CT040401) were deposited in the Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan.

Extraction and isolation

The stems of *Cistanche tubulosa* (500 g) were extracted with MeOH (1.5 L) and evaporated to dryness *in vacuo* at 30 °C. The MeOH extract was partitioned between EtOAc (1.0 L × 3) and H_2O (1.0 L), and the H_2O layer was partitioned with *n*-BuOH (1.0 L × 3). The *n*-BuOH-soluble portion (3.1 g) was subjected to a ODS column (Cosmosil ODS; 2.2 × 30 cm, MeOH/H_2O, 3 : 7 → 1 : 0) to separate twelve fractions (CT-BU-1 ~ 12) including echinacoside (CT-BU-9, 25.4 mg). CT-BU-10 (491 mg) was applied to a silica gel column (2.2 × 30 cm, $CHCl_3$/MeOH/H_2O, 80 : 25 : 3) to afford thirteen fractions (CT-BU-10–1 ~ 13) including cistanoside A (CT-BU-10–11, 86.2 mg). A silica gel column (2.2 × 30 cm, $CHCl_3$/MeOH/H_2O, 80 : 25 : 3) was used to obtain acteoside (12.1 mg) from the CT-BU-10–9 fraction. The purity (> 90%) of these three phenylethanoid glycosides, acteoside, echinacoside, and cistanoside A, was determined by the high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectra. The three compounds were identified by comparisons of their ^1H and ^{13}C NMR data with those previously reported in the literature. Chemical structures of the three phenylethanoid glycosides extracted from *Cistanche tubulosa* are shown in **Fig. 1**.

MTT assay

The MTT assay is a sensitive and quantitative colorimetric assay that is used to determine cell viability [26]. RBL-2H3 and KU812 cells were harvested at approximately 60–80% confluence and seeded onto a 96-well plate at 5.0×10^4 cells/well in 100 µL medium. After an overnight incubation, the cells were washed twice with PBS (−), and 100 µL of medium with the three phenylethanoid glycosides (0.1–100.0 µg/mL) was added. The cells were incubated for 48 h, before 10 µL of 5 mg/mL of MTT was added. After 24 h of incubation, 180 µL of 10% sodium dodecyl sulfate (SDS) was added, this being followed by another 24 h of incubation to completely dissolve the formazan produced by the cells. The absorbance was then determined at 570 nm with a microplate reader (Power Scan HT; Dainippon Pharmaceutical Co., Ltd.). Blanks

were also prepared with the same treatment. The optical density of the formazan produced by the untreated control cells was considered as representing 100% viability.

β-Hexosaminidase release assay

The β-hexosaminidase release inhibition assay using RBL-2H3 cells was performed as previously described [26]. RBL-2H3 cells were seeded onto 96-well plates (Falcon Co.) at 5.0×10^4 cells/well in 100 µL of medium. The cells were incubated for 24 h at 37 °C and sensitized with 0.3 µg/mL anti-DNP-IgE, then washed twice with PBS (−) to eliminate free IgE. After incubating the cells at 37 °C for 10 min in 60 µL per well of a releasing mixture containing 5 µL of the three phenylethanoid glycosides (0.1–10.0 µg/mL), the cells were exposed to 0.3 µg/mL DNP-BSA in PBS (−) followed by incubation at 37 °C for 1 h. For positive and negative controls, 3 mM ketotifen fumarate salt (Keto.) and PBS (−) were respectively used. Then, 80 µL of substrate solution was added to 20 µL of the supernatant followed by incubation at 37 °C for 30 min. After adding 100 µL/well of stop buffer, the absorbance at 405 nm was obtained using a microplate reader. The percentage of β-hexosaminidase released was calculated using the following equation:

$$\beta\text{-hexosaminidase release (\%)} = \{(T-B_t)/(C-B_c)\} \times 100$$

Control (C): Cell (+), DNP-BSA (+), test sample (−); Test (T): Cell (+), DNP-BSA (+), test sample (+); $Blank_t$ (B_t): Cell (−), DNP-BSA (+), test sample (+); $Blank_c$ (B_c): Cell (−), DNP-BSA (+), test sample (−).

Determination of intracellular $[Ca^{2+}]i$ level

$[Ca^{2+}]i$ measurement was performed by the method of Aase and Arna [27] with some modifications. RBL-2H3 cells (5.0×10^4 cells per well in 100 µL of medium) were precultured at 37 °C for 24 h in 96-well plates using a medium containing 10% FBS and 0.3 µg/mL anti-DNP IgE. The cells were washed twice with 200 µL of PBS (−) to eliminate free IgE. The cells were then incubated with 100 µL per well of loading buffer containing Fluo3-AM (Calcium Kit-Fluo3; Dojindo Co., Ltd.) at 37 °C for 1 h. The cells were then washed twice with 200 µL of PBS (−) to eliminate free Fluo3-AM, followed by incubation with 60 µL per well of recording medium at 37 °C for 1 h in a 5% CO_2 incubator with different concentrations of the sample (0.1, 1.0, and 10.0 µg/mL acteoside). For positive and negative controls, 3 mM Keto. and PBS (−) were used, respectively. The fluorescence intensity (FI) was determined 150 sec after adding 0.3 µg/mL of DNP-BSA antigen. FI was measured at an excitation wavelength of 490 nm and emission wavelength of 530 nm using a microplate reader.

Histamine release assay

The histamine release inhibition assay using KU812 cells was performed as previously described [28] and according to the method described by Hosoda et al. [23] with some modifications. KU812 cells were suspended at 2.0×10^5 cells/well in 200 µL of Tyrode buffer A (30 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.03% BSA) for the treatment with acteoside. The cells were next incubated with or without 25 µL/well of various concentrations of acteoside (0.1–10.0 µg/mL) for 15 min and then stimulated with A23187 (1 µM) plus PMA (20 nM) or compound 48/80 (10 µg/mL) for 30 min at 37 °C. The cells were separated from the released histamine by centrifugation at 400 × *g* for 5 min at 4 °C. The supernatant (50 µL) was transferred to a 96-well ELISA system, and the histamine concentration was determined by ELISA kit according to the manufacturer's instructions. Histamine EIA kit (Oxford Biomedical Research) was used to detect histamine content. The percentage of histamine released was calculated by using the following equation:

$$\text{Histamine release (\%)} = \{(T-N)/(C-N)\} \times 100$$

Control (C): A23187 + PMA (+) or compound 48/80 (+), test sample (−); Test (T): A23187 + PMA (+) or compound 48/80 (+), test sample (+); Normal (N): A23187 + PAM (−) or compound 48/80 (−), test sample (−).

TNF-α and IL-4 production assay

The TNF-α and IL-4 production inhibition assay using KU812 cells was performed as previously described [28]. KU812 cells were seeded onto 96-well plates at 2.0×10^5 cells/well in 200 µL of medium, incubated with or without acteoside for 15 min and then stimulated with A23187 plus PMA for 16 h at 37 °C, 5% CO_2 incubator. The cells were separated from the secreted cytokine by centrifugation at 400 × *g* for 5 min at 4 °C. The supernatant (50 and 100 µL) was transferred to a 96-well ELISA system, and the TNF-α level and IL-4 concentration were determined by ELISA kit according to the manufacturer's instructions. Human TNF-α and IL-4 ELISA kit (Biosource International) were used to detect TNF-α and IL-4, respectively. The absorbance at 450 nm was obtained using a microplate reader. To estimate the production of TNF-α or IL-4 by the cells, the same procedure was followed, but without the addition of A23187 plus PMA (Normal). Thus, the percentage of TNF-α or IL-4 production was calculated using the following equation:

$$\text{Cytokine production (\%)} = \{(T-N)/(C-N)\} \times 100$$

Control (C): A23187 + PMA (+), test sample (−); Test (T): A23187 + PMA (+), test sample (+); Normal (N): A23187 + PMA (−), test sample (−).

Statistical analysis

Our results are expressed as means ± SD. The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by Duncan's post hoc test.

Results

We used the MTT assay to assess the cytotoxicity of three phenylethanoid glycosides on RBL-2H3 and KU812 cells. The cells were treated with the three phenylethanoid glycosides for 48 h at final concentrations of 0.1–100.0 µg/mL. As shown in **Fig. 2**, they did not cause cytotoxicity at 0.1–10.0 µg/mL, but the cell viability of the KU812 cells was decreased to 90% with the treatment of 100.0 µg/mL acteoside, this effect being significant ($p < 0.01$, vs. the control value).

Three phenylethanoid glycosides were screened for their inhibitory effect on β-hexosaminidase release by RBL-2H3 cells. The β-hexosaminidase release from IgE-sensitized RBL-2H3 cells was induced by DNP-BSA as a stimulatory antigen. The β-hexosaminidase release from RBL-2H3 cells as affected by the three phenylethanoid glycosides is shown in **Fig. 3**. All three compounds showed inhibitory effect on the β-hexosaminidase release, the effect being significant in all treatments with acteoside and in the

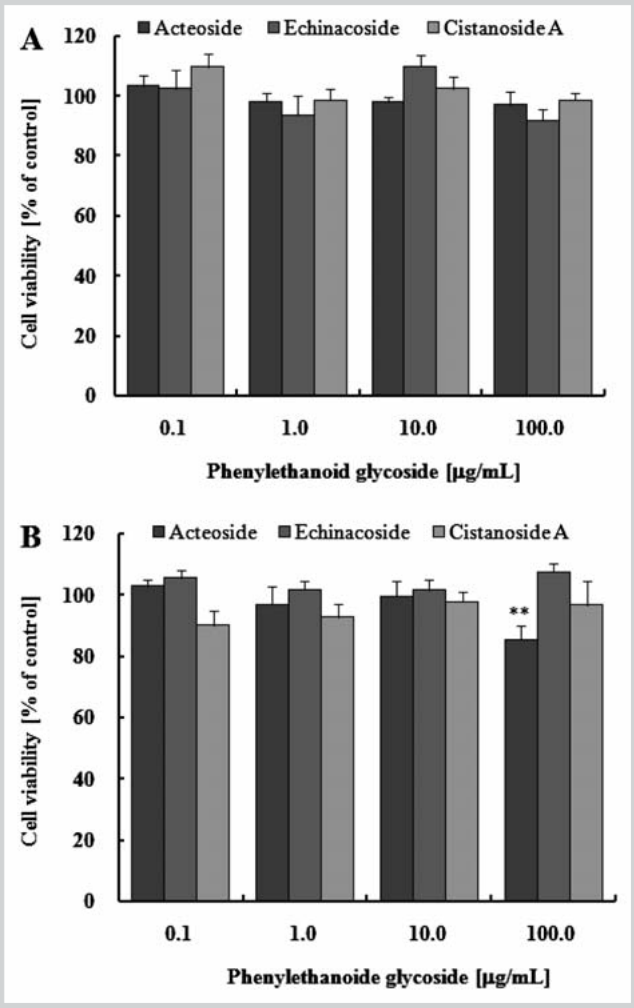

Fig. 2 Cytotoxic effect of three phenylethanoid glycosides from *Cistanche tubulosa* on RBL-2H3 and KU812 cells. The percent cell viability was calculated relative to the untreated cells. **A** The cell viability of RBL-2H3 cells. **B** The cell viability of KU812 cells. Results represent one trial (n = 8). Three additional trials show similar results. * * Statistically significant compared to the control at $p < 0.01$.

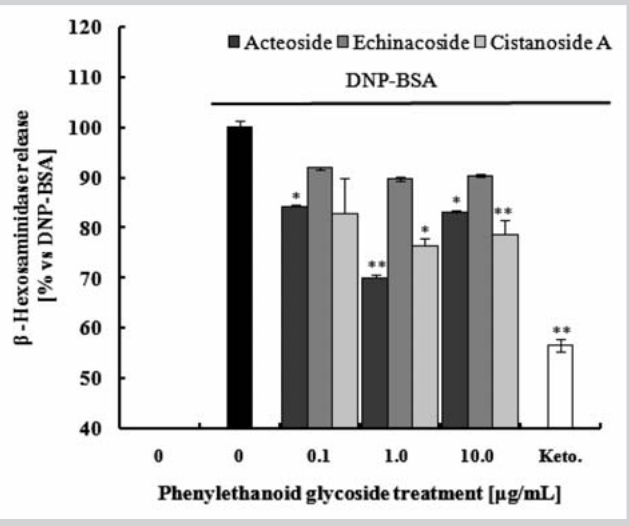

Fig. 3 Inhibitory effect of three phenylethanoid glycosides from *Cistanche tubulosa* on β-hexosaminidase release from RBL-2H3. Ketotifen fumarate (Keto.) was used as a positive control of β-hexosaminidase release. The cells (5.0×10^4 cells/well) in 100 μL were preincubated with three kinds of phenylethanoid glycosides at 37 °C for 10 min prior to their incubation with DNP-BSA for 1 h. Results represent one trial (n = 6–8). Three additional trials show similar results. * Statistically significant compared to the control at $p < 0.05$. * * Statistically significant compared to the control at $p < 0.01$.

1.0–10.0 μg/mL treatments with cistanoside A ($p < 0.05$ and $p < 0.01$ vs. the BSA value). The echinacoside showed an inhibition effect in all treatments, but the effect was not significant compared with the negative control (BSA). In the present study, we compared the effect of the three phenylethanoid glycosides with the clinically available antiallergic drug, Keto., which is known as a mast cell stabilizer, H1-receptor antagonist, and eosinophil inhibitor [26]. Kim et al. [29] demonstrated that Keto. decreases the β-hexosaminidase release and cytoplasm ROS level by IgE-mediated RBL-2H3 cells. The acteoside was the most potent among the three compounds with the 1.0 μg/mL treatment, showing 29.9% inhibitory effect compared to the 3 mM Keto. treatment (final 214 μM, IC_{50} = 200–300 μM; inhibition rate was 43.5%; **Fig. 2**).

To examine the relationship between β-hexosaminidase release and $[Ca^{2+}]i$ levels in RBL-2H3 cells, we focused on acteoside, which induced the highest inhibitory effect on β-hexosaminidase release, to determine the change in the $[Ca^{2+}]i$ levels. The increased rate of the $[Ca^{2+}]i$ level in IgE-mediated RBL-2H3 cells as affected by acteoside is shown in **Fig. 4**. Results reveal that after DNP-BSA stimulation, the cells showed a decrease in $[Ca^{2+}]i$ level with each acteoside treatment (**Fig. 4**). These results suggest that acteoside suppressed $[Ca^{2+}]i$ influx in the IgE-sensitized BSA-stimulated RBL-2H3 cells. The reduction of $[Ca^{2+}]i$ levels with acteoside in the 1.0–10.0 μg/mL treatments was higher than Keto. as a positive control.

To investigate the inhibitory effect of acteoside on human basophilic cells, we examined A23187 plus PMA- or compound 48/80-induced KU812 cell activation. First of all, inhibitory effects of acteoside on A23187 plus PMA-induced KU812 cell degranulation were examined. Light microscopy photo showed that control KU812 cells generally are spherical or oval (**Fig. 5A**). After stimulation with A23187 plus PMA, the cells became swollen and showed many vacuoles and extruded granules near the cell surface and in the surrounding medium, which is interpreted as mast cell degranulation (**Fig. 5B**). The cells became swollen with an irregular boundary, but the degranulation was reduced (**Fig. 5C** and **5D**) by preincubation with acteoside in the A23187 plus PMA-stimulated KU812 cells.

The inhibitory effects of acteoside on A23187 plus PMA- or compound 48/80-mediated histamine release from KU812 cells are shown in **Fig. 6**. Acteoside dose-dependently inhibited A23187 plus PMA- (**Fig. 6A**) or compound 48/80- (**Fig. 6B**) mediated histamine release in a concentration range of 0.1–10.0 μg/mL. The histamine release was inhibited approximately 63.2, 100.0, and 100.0% after treatment with the acteoside at 0.1–10.0 μg/mL, and this effect was significant ($p < 0.05$) when compared to the A23187 plus PMA value. The histamine release was inhibited approximately 100.0, 51.6, and 77.0% after treatment with the acteoside at 0.1–10.0 μg/mL, this effect being also significant ($p < 0.05$) when compared to the value for compound 48/80.

We examined whether acteoside could regulate inflammatory cytokines such as TNF-α and IL-4 in KU812 cells. The inhibition rate of TNF-α and IL-4 production in A23187 plus PMA-stimu-

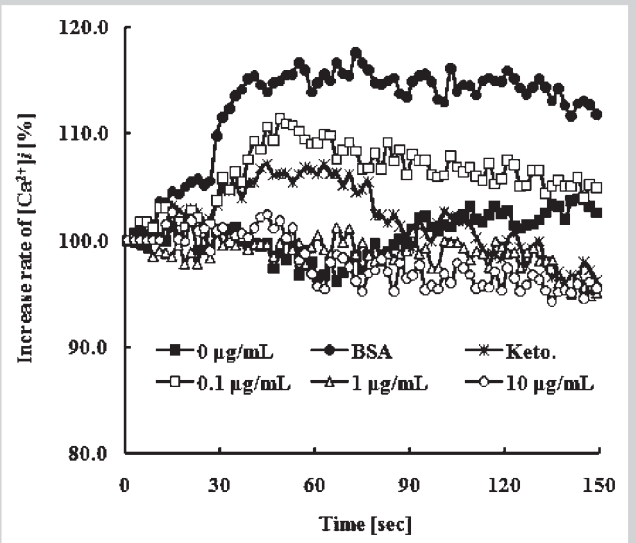

Fig. 4 Effect of acteoside extracted from *Cistanche tubulosa* on the $[Ca^{+2}]i$ levels in IgE-mediated RBL-2H3 cells. Ketotifen fumarate salt (Keto.) was used as a positive control of $[Ca^{+2}]i$ influx. IgE-sensitized RBL-2H3 cells (5.0×10^4 cells/well) were incubated with 100 µL of loading buffer including Fluo-3AM for 1 h. The treated cells were incubated with 60 µL recording buffer for 30 min. Changes in $[Ca^{2+}]i$ level induced by DNP-BSA were measured with a microplate reader. Results represent one trial (n = 3). Two additional trials show similar results. BSA: Stimulated by DNP-BAS; Keto.: Treatment by ketotifen fumarate salt as a positive control.

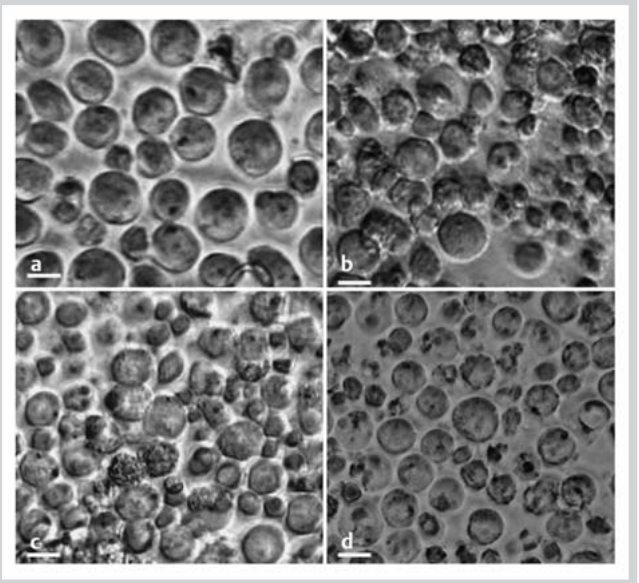

Fig. 5 Light microscopy photo of KU812 cells in medium (**a**), after stimulation with A23187 (1 µM) plus PMA (20 nM) (**b**), 1.0 µg/mL acteoside prior to the stimulation with A23187 plus PMA (**c**), 10.0 µg/mL acteoside prior to the stimulation with A23187 plus PMA (**d**). Normal KU812 cells are generally characterized by round shape with fine granules and regular surface. Degranulated basophilic cells become swollen and have many vacuoles, an irregular surface, and extruded granules. However, pretreatment with acteoside can reduce A23187 plus PMA-induced degranulation of KU812 cells. The magnification of the photo was × 200. Bar = 10 µm.

lated KU812 cells as affected by the acteoside is shown in ▶ **Fig. 7**. The acteoside dose-dependently inhibited the production of TNF-α (▶ **Fig. 7A**) and IL-4 (▶ **Fig. 7B**) after treatment for 16 h. The production of the TNF-α was inhibited by about 32.1 and 52.1%, and the effect was significant ($p < 0.05$, vs. the A23187 plus PMA). Furthermore, the production of the IL-4 was inhibited by about 14.2 and 80.7% with 1.0 and 10.0 µg/mL treatments, the effect being significant ($p < 0.05$, vs. the A23187 plus PMA) for the 10.0 µg/mL treatment.

Discussion

In this study, phenylethanoid glycosides obtained from *Cistanche tubulosa* did not affect cell viability on RBL-2H3 and KU812 cells with the 1.0–10.0 µg/mL treatments. However, acteoside was cytotoxic at the 100.0 µg/mL treatment (▶ **Fig. 2B**), as has been reported by Saracoglu et al. [30] who have shown that acteoside isolated from *Phlomic armeniaca* and *Scutellaria salviifolia* displayed cytotoxic effects on dRLh-84, S-180, and P-388/D1 cell lines with the IC_{50} 30–221 µg/mL at different cell numbers. Our result suggests that the inhibition effect of acteoside on cell viability with the 100 µg/mL treatment may be affected by its apoptotic activity.

The immediate-type allergic reaction is involved in many allergic diseases such as asthma, allergic rhinitis, and sinusitis. Mast cells play a crucial role in inflammatory and immediate allergic responses. Our data shows that the acteoside has the highest inhibitory effect on the β-hexosaminidase release from IgE-sensitized, antigen-stimulated RBL-2H3 cells compared with other phenylethanoid glycosides with 1.0 µg/mL treatment (▶ **Fig. 3**). The inhibition effect of acteoside on β-hexosaminidase release with the 10.0 µg/mL treatment was lower than that with the 1.0 µg/mL treatment. As Sugisawa et al. [31] demonstrated, this may be due to the H_2O_2 induction at high concentrations of acteoside, which might regulate the calcium signals and degranulation on the RBL-2H3 cells [32], influencing the β-hexosaminidase release with the 10.0 µg/mL acteoside treatment.

The degranulation of mast cells is closely related to $[Ca^{2+}]i$. The inhibition of Ca^{2+} influx by antiallergic drugs plays a crucial role in the suppression of degranulation in mast cells [33,34]. Our results indicate that the intracellular $[Ca^{2+}]i$ level was lower in the acteoside-treated, DNP-BSA-stimulated RBL-2H3 cells (▶ **Fig. 4**), which is consistent with other reports [34,35], and these results agree with those of ▶ **Fig. 3**. We consider from these observations that the decrease in intracellular $[Ca^{2+}]i$ is involved in the inhibitory effect of acteoside on β-hexosaminidase release. Nitric oxide (NO) and H_2O_2 are two major reactive oxygen species (ROS) known in regulation of calcium signal and degranulation of mast cells. ROS are necessary for the secretion of β-hexosaminidase and calcium influx, and nicotinamide adenin dinucleotide phosphate (NADPH) oxidase is mainly responsible for ROS production in the IgE-mediated RBL-2H3 cells [29,32]. The sustained elevation of cytosolic calcium through store-operated calcium entry was totally abolished when the ROS production was blocked. In addition, ROS have some relationship with β-hexosaminidase and histamine release by protein kinase C (PKC) activation and IgE stimulation in RBL-2H3 cells [34]. Furthermore, Suzuki et al. [36] found that there was a significant correlation between inhibition effects on histamine release and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) or superoxide anion radical scavenging activities of curcumin-related antioxidant compounds. On the other hand, acteoside has free radical scavenging properties on NO and DPPH

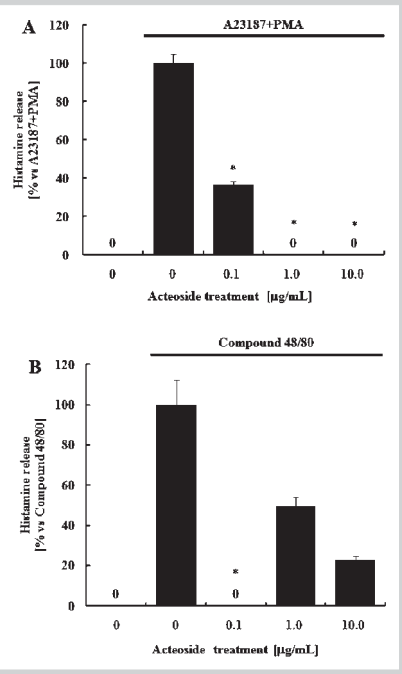

Fig. 6 Inhibitory effect of acteoside on histamine release from KU812 cells after stimulation with A23187 (1 μM) plus PMA (20 nM) or compound 48/80 (10 μg/mL). The cells (2.0×10^5 cells/well) in 200 μL were preincubated with acteoside at 37 °C for 15 min prior to their incubation with A23187 plus PMA (**A**) or compound 48/80 (**B**). Results represent one trial (n = 3). Two additional trials showed similar results. * Statistically significant compared to A23187 plus PMA at $p < 0.05$.

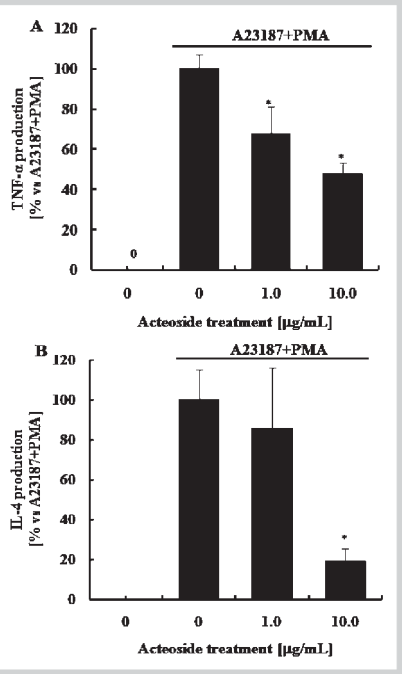

Fig. 7 Inhibitory effect of acteoside on TNF-α and IL-4 production by KU812 cells after stimulation with A23187 plus PMA. The KU812 cells (2.0×10^5 cells/well) in 200 μL were preincubated with or without acteoside for 15 min and then stimulated with A23187 (1 μM) plus PMA (20 nM) for 16 h. The production of TNF-α (**A**) and IL-4 (**B**) was quantified by ELISA assay. Results represent two trials (n = 4). * Statistically significant compared to A23187 plus PMA at $p < 0.05$.

radicals [9, 37]. It has also shown scavenging activity in activated human leucocytes [38]. One of the possibilities is that the inhibition effect of acteoside on intracellular calcium release might be affected by its free radical scavenging activities or NADPH oxidase activities.

Acteoside dose-dependently inhibited histamine release on A23187 plus PMA-stimulated KU812 cells (**Fig. 4A**). Acteoside inhibited glutamate-induced intracellular Ca^{2+} influxes resulting in overproduction of NO and reduced formation of ROS [37]. Both PKC and Ca^{2+} signaling pathways are required for histamine and leukotrienes release from mast cells as well as rodent systems [39]. We hypothesized that acteoside may have an inhibitory effect on histamine release through Ca^{2+} influx. For this purpose, we used A21387 plus PMA to stimulate KU812 cells and demonstrated histamine release inhibition effect with acteoside. This result supported our hypothesis that acteoside may decrease histamine release from KU812 cells by inhibition of Ca^{2+} influx or PKC activation.

It is believed without doubt that stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway, which leads to histamine release. Senyshyn et al. [40] identified that the recombinant G subunit markedly synergized phospholipase D activation by compound 48/80 in permeabilized basophilic cells. Compound 48/80-induced secretion is associated with a transient increase in cytosolic Ca^{2+}. This secretion was blocked by the calcium chelator and PKC inhibitor. In the present study, we observed that acteoside inhibited the compound 48/80-induced degranulation from basophilic cells, but the inhibition effect of acteoside on histamine release with the 0.1 μg/mL treatment was higher than that with the 1.0–10.0 μg/mL treatments (**Fig. 4B**). As Lau et al. [41] demonstrated, the inhibition effect of acteoside on compound 48/80-induced histamine release may be involved in the anti-inflammation effect of acteoside against vascular permeability-associated edema. Possibly, acteoside may have several different ways of inhibiting chemical mediator release from basophilic cells, suggesting the complexity of its action. However, in order to reveal the mechanism of histamine release inhibition of this compound, further studies should be performed.

Among the cytokines produced by basophilic cells, TNF-α, IL-4, IL-13, and IL-5 are the key molecules. The reduction of pro-inflammatory cytokines from mast cells or basophilic cells is one of the key indicators of reduced allergic symptoms [42]. With regard to TNF-α, the production is mainly regulated by Ca^{2+} influx, but the release process is regulated by additional mechanisms possibly involving activation of PKC in KU812 cells. Inhibition of $[Ca^{2+}]i$ influx is involved in the expression of cytokines in mast cells and basophilic cells [34, 43]. Our result showed that 1.0 and 10.0 μg/mL acteoside reduced TNF-α and IL-4 production from A23187 plus PMA-stimulated KU812 cells after treatment for 16 h. Acteoside might inhibit TNF-α and IL-4 production by decreasing the $[Ca^{2+}]i$ level in A23187 plus PMA-stimulated KU812 cells. Moreover, our results suggested that acteoside has an antiallergic effect at the late phase. The confirmation of this effect using FcεRI expressing mast cells or basophilic cells is necessary in the future.

In conclusion, we report for the first time that acteoside, echinacoside, and cistanoside A extracted from *Cistanche tubulosa* can inhibit the release of β-hexosaminidase from IgE-sensitized BSA-stimulated RBL-2H3 cells. Furthermore, acteoside can inhibit the histamine release, as well as TNF-α and IL-4 production in a dose-dependent manner on A23187 plus PMA-stimulated KU812 cells. These results suggest that acteoside could be a good candidate for the therapeutic treatment of various allergic diseases. The detailed mechanism behind the antiallergic effect of acteoside will be the subject of a future study.

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