Gwon *et al. Applied Biological Chemistry* (2025) 68:4
https://doi.org/10.1186/s13765-025-00980-4

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The Korean Society for Applied Biological Chemistry

ARTICLE

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Anti-allergic effect of 5,7-dihydroxy-4-methylcoumarin in IgE-mediated RBL-2H3 cells and PCA murine model

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Abstract

Allergy is an immune-mediated disorder characterized by an exaggerated response of the immune system to non-hazardous substances, resulting in allergic symptoms such as rash, itching, and runny nose. Current therapeutic interventions include antihistamines and steroids; however, they induce several side effects. Although 5,7-dihydroxy-4-methylcoumarin, a phytochemical derivative, has been demonstrated to exhibit antioxidant, anti-apoptotic, and anti-aggregatory effects, its anti-allergic properties and underlying molecular mechanisms remain elusive. Therefore, this study was conducted to investigate the anti-allergic effects of 5,7-dihydroxy-4-methylcoumarin in two experimental models: rat basophilic leukemia-2H3 cells sensitized using dinitrophenyl-specific immunoglobulin E (IgE)/human serum albumin and a passive cutaneous anaphylaxis (PCA) murine model. Our findings demonstrated that 5,7-dihydroxy-4-methylcoumarin reduced the release of histamine and β-hexosaminidase and downregulated the mRNA expression of allergic-inflammatory cytokines, such as interleukin (IL)-4, IL-13, and tumor necrosis factor-alpha, as well as the inflammatory enzyme cyclooxygenase-2. Furthermore, 5,7-dihydroxy-4-methylcoumarin reduced the phosphorylation of mitogen-activated protein kinases such as extracellular signal-regulated kinase and p38, as well as protein kinase B. In vivo, 5,7-dihydroxy-4-methylcoumarin reduced PCA reaction, as evidenced by reduced Evans blue dye extravasation in IgE-mediated local allergic responses. Collectively, these results suggest that 5,7-dihydroxy-4-methylcoumarin holds promise as a novel candidate for the development of anti-allergic drugs.

Keywords 5,7-Dihydroxy-4-methylcoumarin, Anti-allergy, RBL-2H3 cell, Passive cutaneous anaphylaxis murine model

Introduction

Allergy is an immune-mediated disorder characterized by an exaggerated response of the immune system to otherwise harmless substances, such as pollen, animal hair, and specific food [1]. Common clinical manifestations include rash, itching, sneezing, swelling, skin redness, and rhinorrhea. The prevalence of allergies has been steadily increasing in tandem with industrialization and urbanization, attributed to elevated exposure to both non-hazardous and hazardous substances, such as metals, fine particulate matter, and air pollution [2–4]. Allergic diseases such as atopic dermatitis, asthma,

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allergic rhinitis, and anaphylaxis significantly impair the quality of life for affected individuals [5]. Among them, anaphylaxis is a severe and life-threatening allergic reaction that occurs rapidly after exposure to an allergen. It represents the most extreme form of an immediate IgE-mediated allergic reaction and can lead to shock if not treated promptly [6, 7]. The treatment of allergic diseases currently relies on the use of antihistamines and steroids [8, 9]. Antihistamines serve as the primary drugs for managing allergies. These drugs function as histamine H1 receptor antagonists, competitively binding to the histamine H1 receptor and thereby inhibiting the binding of histamine released by mast cells to the target receptor. Representative examples include chlorpheniramine and cetirizine [10], both of which act by this competitive antagonism mechanism [10]. Additionally, steroids are employed to alleviate or treat allergic inflammation. However, the use of both antihistamines and steroids is associated with several side effects, including but not limited to drowsiness, dry mouth, gastrointestinal disorders, arrhythmias, and tachycardia [11, 12]. Furthermore, the financial burden associated with the management and treatment of allergic diseases is significant [13]. Therefore, the development of novel anti-allergic drugs is imperative to enhance the quality of life for individuals affected by allergies and to alleviate the societal healthcare costs associated with allergy treatment. To address the limitations posed by antihistamines and steroids, considerable attention has been directed toward phytochemical derivatives. These compounds offer the potential for reduced side effects while maintaining pharmacological efficacy comparable to existing treatments.

Mast cells are central mediators in the pathogenesis of allergic diseases [14]. These immune cells are constitutively present in most tissues and play vital roles in both innate and acquired immunity. However, they also act as effector cells in allergic and autoimmune diseases [15]. Mast cells originate from hematopoietic progenitor cells. When mast cells are located within tissues, they undergo differentiation and maturation and release histamine, cytokines, and chemokines [16]. An allergic response is triggered upon the entry of an allergen into the body. Antigen-presenting cells, such as dendritic cells or macrophages, process and present the allergen-derived antigens to Th_2 cells [17]. In response, Th_2 cells secrete interleukin (IL)-4, which stimulates B cells to produce immunoglobulin E (IgE). The IgE subsequently binds to the allergen, forming an allergen-IgE complex, which cross-links with the high-affinity IgE receptor (FcεRI) on mast cells [14]. This cross-linking event leads to the phosphorylation of the Src family, initiating a cascade of downstream signaling pathways that culminate in the degranulation of histamine in mast cells [18–21]. Within hours of FcεRI stimulation, mitogen-activated protein kinases (MAPKs) and protein kinase B (AKT) are activated, driving the transcription of allergic-inflammatory cytokines, such as IL-4, IL-13, and tumor necrosis factor-alpha (TNF-α), as well as the inflammatory enzyme cyclooxygenase-2 (COX-2) [22, 23]. These molecular events sustain and amplify allergic inflammation, leading to its chronic persistence [17, 24].

Coumarins are a class of phytochemicals that are naturally synthesized in substantial quantities. Additionally, synthetic derivatives of coumarins have significantly expanded the catalog of known coumarin structures, presenting vast potential for discovering substances with diverse pharmacological effects [25]. 5,7-Dihydroxy-4-methylcoumarin, a specific derivative of coumarin, has been demonstrated to exhibit several bioactivities, including antioxidant [26], anti-apoptotic [27], and anti-aggregatory effects [28]. However, to date, there have been no reports investigating its anti-allergic effects or the underlying molecular mechanisms. Therefore, we aimed to investigate the anti-allergic effects of 5,7-dihydroxy-4-methylcoumarin using rat basophilic leukemia (RBL)-2H3 cells and passive cutaneous anaphylaxis (PCA) in vivo model.

Methods

Chemicals and reagents

5,7-Dihydroxy-4-methylcoumarin was purchased from Cayman. Alpha-minimum essential medium (α-MEM) and penicillin/streptomycin/glutamine (PSQ) were purchased from Corning and Gibco (Waltham, USA). Ketotifen fumarate salt (Cat. #K2628), anti-dinitrophenyl (DNP)-IgE (Cat. #D8406), DNP-human serum albumin (HSA, Cat. #A6661), toluidine blue (Cat. #89640), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat. #475989), trypan blue (Cat. #302643), dimethyl sulfoxide (DMSO), and all degranulation assay-related reagents were purchased from Sigma-Aldrich. Dulbecco's phosphate-buffered saline and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from WELGENE, Inc. (Gyeongsan, Korea), and Fluo-3 acetoxymethyl ester (Cat. #F1241) was purchased from Thermo Fisher Scientific (USA). Primary antibodies, including β-actin (Cat. #8457), phosphorylated (p)-p65 (Cat. #13346S), p65 (Cat. #8242), p-p38 (Cat. #9216), p-extracellular signal-regulated kinase (ERK) (Cat. #4370), p-AKT (Cat. #4060), and p-c-Jun N-terminal kinase (JNK) (Cat. #9255) were purchased from Cell Signaling Technology (Danvers, USA). Secondary antibodies, including goat anti-mouse IgG (Cat. #5220-0341) and goat anti-rabbit IgG (Cat. #5220-0036), were purchased from SeraCare (Gaithersburg, USA).

Evans blue (Cat. #E104208) was purchased from Aladdin Biochemical (Shanghai, China).

Cell culture

RBL-2H3 cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). RBL-2H3 cells were grown in α-MEM supplemented with 1% PSQ and 10% fetal bovine serum at 37 ℃ and 5% CO_2.

Cell viability assay

Cell viability was measured using the MTT assay. RBL-2H3 cells were seeded (3.8×10^4 cells/well) into a 96-well plate and allowed to stabilize for 24 h. Subsequently, the cells were incubated with various concentrations of 5,7-dihydroxy-4-methylcoumarin (25, 50, and 100 μM) for 7 h, followed by treatment with MTT (dissolved in α-MEM to 1 mg/ml) for 2 h. Next, the MTT solution was removed, and DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a Varioskan LUX microplate reader (Thermo Fisher Scientific, Waltham, USA).

Histamine release assay

RBL-2H3 cells were seeded (5×10^5 cells/well) into a 12-well plate and incubated with anti-DNP-IgE (0.2 μg/ml) in each well for 17 h. After incubation, the cells were washed with phosphate-buffered saline (PBS) and treated with 100 μM 5,7-dihydroxy-4-methylcoumarin or 40 μg/ml ketotifen for 1 h. Subsequently, DNP-HSA was added to the wells containing 5,7-dihydroxy-4-methylcoumarin or ketotifen for an additional 6 h. The supernatant was separated into Eppendorf tubes, and 0.1 N HCl and 60% perchloric acid were added. After 20 min of centrifugation, 5 N NaOH, 5 M NaCl, and n-butanol were added, and the mixture was vortexed. The organic phase was collected, mixed with 0.1 N HCl and m-heptane, and vortexed. The histamine in the aqueous phase was transferred to a 96-well plate and assayed using o-phthalaldehyde. The fluorescence intensity was measured using a Varioskan LUX microplate reader (Excitation: 360 nm, Emission: 440 nm).

β-hexosaminidase release assay

RBL-2H3 cells were seeded (5×10^5 cells/well) into a 12-well plate, and anti-DNP-IgE (0.2 μg/ml) was added to each well for 17 h. After incubation, the cells were washed with PBS and treated with 100 μM 5,7-dihydroxy-4-methylcoumarin or 40 μg/ml ketotifen for 1 h. Subsequently, DNP-HSA was added to the wells containing 5,7-dihydroxy-4-methylcoumarin or ketotifen for an additional 6 h. The supernatant was transferred to a 96-well plate, and 1 mM β-hexosaminidase substrate (4-nitrophenyl N-acetyl-β-D-glucosaminide dissolved in 0.2 M citrate buffer, pH 4.5) was added to each well. After incubation at 37 ℃ for 1 h 50 min, a stop solution (0.1 M Na_2CO_3/$NaHCO_3$ pH 10.0) was added to halt the reaction. Finally, the absorbance was measured at 570 nm using a Varioskan LUX microplate reader.

Toluidine blue staining assay

RBL-2H3 cells were seeded (5×10^5 cells/well) into a 12-well plate and sensitized with anti-DNP-IgE (0.2 μg/ml) to induce mast cell degranulation. After IgE stimulation, the cells were washed with PBS and stained with toluidine blue dye to visualize degranulation. First, the cells were washed three times with PBS, followed by fixation with absolute methanol for 30 min. After fixation, the cells were washed three times with PBS and stained with toluidine blue (0.1% w/v in 1% NaCl solution, pH 2.5) for 20 min. Subsequently, the cells were rewashed three times, and images of the stained cells were captured using an inverted microscope (IMT i-Solution, Inc.; 400× magnification).

RNA isolation and qRT-PCR analysis

Total RNA was isolated using TRIzol solution. RBL-2H3 cells (5×10^5 cells/well) were seeded into a 12-well plate and sensitized with IgE for 17 h. After sensitization, the cells were treated with 5,7-dihydroxy-4-methylcoumarin for 1 h and DNP-HSA for an additional 1 h. After treatment, the cells were washed 2–3 times with cold PBS, and TRIzol solution was added using up-and-down pipetting. After incubation for 5 min, chloroform was added, and the mixture was vortexed and incubated again for 2–3 min. Subsequently, the cells were centrifuged for 15 min at 12,000×*g* at 4 ℃. The aqueous phase was transferred to a new Eppendorf tube, and isopropanol was added. After incubation for 10 min, the samples were centrifuged for 10 min at 12,000×*g* at 4 ℃. The supernatant was discarded, and the pellet was resuspended with 75% ethanol. After brief vortexing, the samples were centrifuged for 5 min at 12,000×*g* at 4 ℃, and the supernatant was discarded. The pellet was air-dried for 5–10 min before being resuspended in RNase-free water. RNA concentration was determined using a NanoDrop (Thermo Fisher Scientific). For cDNA synthesis, RNA samples were prepared using the amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT). After RNA denaturation and synthesis using a PCR machine (SimpliAmp Thermal Cycler, Applied Biosystems), cDNA quantification was performed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Quantitative reverse transcription PCR (qRT-PCR) analysis was performed to measure mRNA expression. Samples of cDNA, deionized water, rat-specific qRT-PCR primers (Table 1), and PowerUp SYBR Green Master Mix (Applied Biosystems)

Table 1 Primers for qRT-PCR of RBL-2H3 cells

Gene name	Direction	Sequence (5′ → 3′)
Il-4	Forward	GTCACCCTGTTCTGCTTTCT
	Reverse	GACCTGGTTCAAAGTGTTGATG
Il-13	Forward	AACAGCAGCATGGTATGGAGCG
	Reverse	TGGGTCCTGTGGATGGCATTGC
Cox-2	Forward	CTGTACTACGCCTGAGTTTCTG
	Reverse	CTTGAAGTGGGTCAGGATGTAG
Tnf-a	Forward	GCAGATGGGCTGTACCTTATC
	Reverse	GAAATGGCAAATCGGCTGAC
β-actin	Forward	ACAGGATGCAGAAGGAGATTAC
	Reverse	ACAGTGAGGCCAGGATAGA

were added to the qRT-PCR plate. Subsequently, qRT-PCR was performed using the StepOnePlus Real-time PCR System (Applied Biosystems), and data were analyzed using the $2^{(-\Delta\Delta Ct)}$ method.

Western blot analysis

RBL-2H3 cells were seeded (2.7×10^6 cells/well) into a 60 mm dish and sensitized with anti-DNP-IgE (0.2 μg/ml) for 17 h. After sensitization, the cells were treated with 100 μM 5,7-dihydroxy-4-methylcoumarin for 1 h and exposed to DNP-HSA for 30 min. The cells were washed three times with cold PBS and collected into Eppendorf tubes using a cell scraper. To isolate total protein, cells were lysed with radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, leupeptin, and pepstatin) and phosphatase inhibitors (30 mM NaF, 1.5 mM Na_3VO_4). The protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking the membrane with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 30 min, the membrane was incubated overnight with primary antibodies at room temperature. Subsequently, the membrane was washed with TBS-T every 5 min for 30 min. Next, the membrane was incubated with secondary antibodies for 1 h and washed again with TBS-T every 5 min for 30 min. Finally, the membrane was treated with an ECL solution, and signal intensity was detected using a Chemidoc imaging system (iBright™ CL1500, Invitrogen).

Animal experiments

Eight-week-old male BALB/c mice were purchased and allowed to acclimate to their environment for 1 week before the experiment. The room temperature was maintained at 25 ± 2 ℃, with humidity controlled at 55 ± 5%. The mice were housed in a specific pathogen-free area under a 12 h light: dark cycle with ad libitum access to a standard chow diet and water. The experimental protocol was approved by the Animal Care and Use Committee of Gyeongsang National University Institute (GNU-221216-M0181).

Passive cutaneous anaphylaxis assay

A PCA assay was performed to assess extravasation caused by an allergic response. Male BALB/c mice (8 weeks old) were intradermally injected with anti-DNP-IgE in the ear, whereas the control group received an equal volume of PBS. After 24 h, the group treated with 5,7-dihydroxy-4-methylcoumarin received an intraperitoneal injection for 1 h. Before euthanasia, all mice were injected intravenously with DNP-HSA in PBS containing 2% Evans blue for 30 min. To extract the Evans blue dye, cropped ear tissue was soaked in 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13) overnight at 38 ℃ in the dark. After incubation, the supernatant was transferred to an Eppendorf tube and centrifuged at 3000 rpm at 4 ℃ for 15 min. Finally, the absorbance of the supernatant was measured at 620 nm using a Varioskan LUX microplate reader.

Statistical analysis

Data are presented as the mean ± standard deviation from three independent replicate experiments. Differences between groups were analyzed using the Student's t-test. Results with p-values < 0.05 were considered statistically significant.

Results

Effect of 5,7-dihydroxy-4-methylcoumarin on RBL-2H3 cell viability

Before evaluating the anti-allergic effects of 5,7-dihydroxy-4-methylcoumarin (Fig. 1A), its cytotoxicity was assessed using the MTT assay. The assay was conducted using various concentrations of 5,7-dihydroxy-4-methylcoumarin (25, 50, 100 μM). Notably, 5,7-dihydroxy-4-methylcoumarin did not induce dose-dependent cytotoxicity in RBL-2H3 cells (Fig. 1B). These results confirm that 5,7-dihydroxy-4-methylcoumarin is non-cytotoxic even at concentration up to 100 μM.

Inhibitory effect by 5,7-dihydroxy-4-methylcoumarin on β-hexosaminidase and histamine release in IgE-mediated RBL-2H3 cells

Upon allergen exposure, IgE-allergen complexes cross-link with FcεRI on mast cells, triggering degranulation [14, 21]. For our in vitro allergy model, we utilized RBL-2H3 cells, which, when sensitized with anti-DNP-IgE,

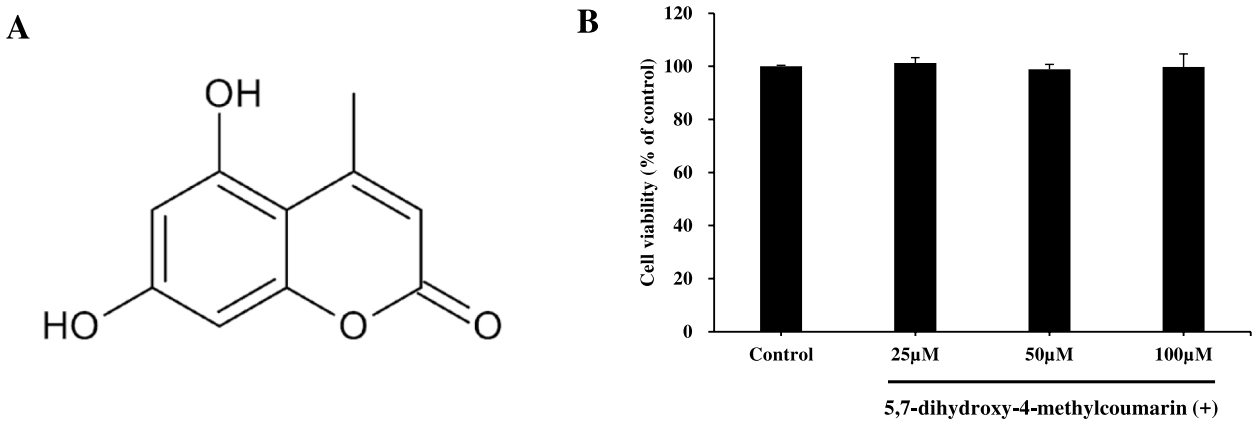

Fig. 1 Effect of 5,7-dihydroxy-4-methylcoumarin on RBL-2H3 cell viability. **A** Chemical structure of 5,7-dihydroxy-4-methylcoumarin. **B** Rat basophilic leukemia (RBL)-2H3 cells were incubated with the indicated concentrations of 5,7-dihydroxy-4-methylcoumarin, and cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data are presented as the mean ± standard deviation (SD); n = 3. *$p < 0.05$

also undergo degranulation upon exposure to the allergen DNP-HSA. β-hexosaminidase and histamine release assays were conducted to investigate whether 5,7-dihydroxy-4-methylcoumarin could inhibit degranulation. Results demonstrated that 5,7-dihydroxy-4-methylcoumarin significantly inhibited the release of β-hexosaminidase and histamine (Fig. 2A, B). Additionally, a toluidine blue staining assay was performed to visualize the extent of degranulation. The 5,7-dihydroxy-4-methylcoumarin-treated group exhibited a lower level of degranulation than the DNP-IgE/HSA-treated group (Fig. 2C). Furthermore, 5,7-dihydroxy-4-methylcoumarin (100 μM) showed more effective inhibition than the positive control, ketotifen, which is a histamine H1-receptor antagonist and mast cell stabilizer (Fig. 2A–C). These results suggest that 5,7-dihydroxy-4-methylcoumarin could effectively inhibit mast cell degranulation.

Inhibitory effect of 5,7-dihydroxy-4-methylcoumarin on the mRNA expression of allergic-inflammatory cytokines and inflammatory enzyme in IgE-sensitized RBL-2H3 cells

In allergic responses, mast cells produce allergic-inflammatory cytokines that exacerbate allergic inflammation. Specifically, the mRNA expression levels of allergic-inflammatory cytokines such as *Il-4*, *Il-13*, and *Tnf-α*, as well as the inflammatory enzyme *Cox-2*, are elevated [23, 24, 29, 30]. To investigate the potential inhibitory effect of 5,7-dihydroxy-4-methylcoumarin on the mRNA expression of these allergic-inflammatory mediators, we conducted qRT-PCR analysis. The results demonstrated that 5,7-dihydroxy-4-methylcoumarin significantly reduced the DNP-IgE/HSA-induced increase in the mRNA expression of *Il-4*, *Il-13*, and *Tnf-α* (Fig. 3A–C). Additionally, 5,7-dihydroxy-4-methylcoumarin reduced the DNP-IgE/HSA-induced increase in the mRNA expression of *Cox-2* (Fig. 3D). These results indicate that 5,7-dihydroxy-4-methylcoumarin has the potential to inhibit the expression of key allergic-inflammatory cytokines and enzymes.

Inhibitory effect of 5,7-dihydroxy-4-methylcoumarin on the phosphorylation of MAPKs and AKT in IgE-mediated RBL-2H3 cells

The MAPK pathway regulates the expression of cytokines, which can induce mast cell-meditated allergic inflammation. Furthermore, ERK, p38, and JNK, members of the MAPK family, are well known to be involved in allergic responses such as mast cell activation and mediator release [31, 32]. The AKT pathway also plays a critical role in the production of allergic-inflammatory cytokines and the process of degranulation [33, 34]. Studies have demonstrated that both MAPKs and AKT undergo phosphorylation during an allergic response [17, 24, 33]. Therefore, we investigated whether 5,7-dihydroxy-4-methylcoumairn can reduce the phosphorylation of MAPKs and AKT, which was increased following DNP-IgE/HSA treatment. As shown in Fig. 4, 5,7-dihydroxy-4-methylcoumarin reduced the phosphorylation of MAPKs (ERK and p38) (Fig. 4A, B); however, no significant effect was observed on JNK phosphorylation (Fig. 4C). Additionally, 5,7-dihydroxy-4-methylcoumarin reduced AKT phosphorylation (Fig. 4D). These results suggest that 5,7-dihydroxy-4-methylcoumarin could attenuate IgE-mediated activation of RBL-2H3 cells by regulating MAPK and AKT signaling [17, 24].

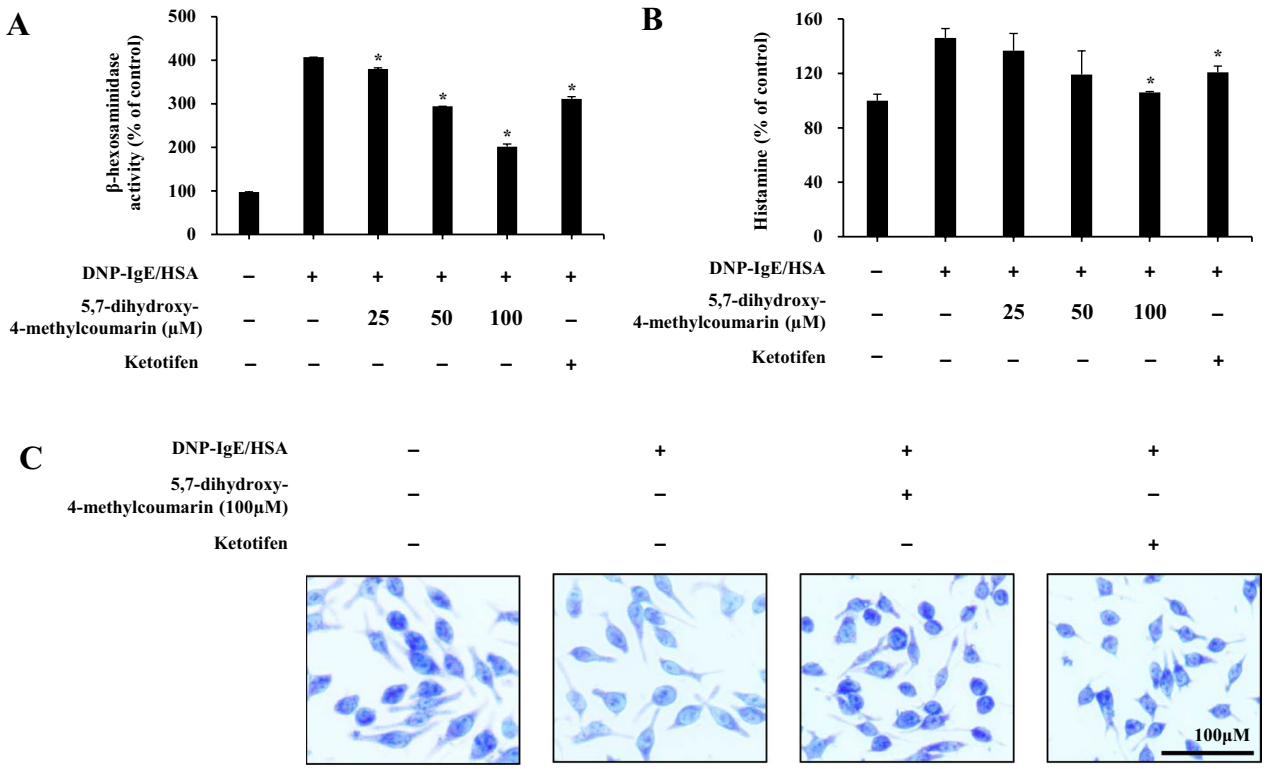

Fig. 2 Effect of 5,7-dihydroxy-4-methylcoumarin on IgE-mediated degranulation in RBL-2H3 cells. RBL-2H3 cells, sensitized with dinitrophenyl-immunoglobulin E (DNP-IgE), were treated with the indicated concentrations of 5,7-dihydroxy-4-methylcoumarin and subsequently activated with DNP-human serum albumin (HSA). The culture supernatants were collected to quantify the release of β-hexosaminidase (**A**) and histamine (**B**). In addition, the cells were stained with toluidine blue dye, and images were captured using inverted microscopy (**C**). Scale bar = 100 μm. The data are presented as the mean ± SD; n = 3. *$p < 0.05$ compared with the DNP-IgE/HSA-treated group

Anti-allergic effect of 5,7-dihydroxy-4-methylcoumarin in passive cutaneous anaphylaxis in vivo model

A PCA assay was conducted to confirm the anti-allergic effects of 5,7-dihydroxy-4-methylcoumarin in vivo. This model is based on a passively transferred anaphylactic response induced by antiserum. Upon interaction between the antigen and antibody fixed on the skin, histamine is released, which subsequently increases vascular permeability. This increased permeability allows the leakage of albumin-bound dye, resulting in the formation of a blue spot at the site of intradermal injection. The amount of extracted Evans blue dye and ear thickness were measured to determine the in vivo effects of 5,7-dihydroxy-4-methylcoumarin. As shown in Fig. 5, the amount of extracted Evans blue dye decreased in the 5,7-dihydroxy-4-methylcoumarin-treated group (Fig. 5B, C). Additionally, the ear thickness was notably thinner in the 5,7-dihydroxy-4-methylcoumarin-treated group than in the DNP-IgE/HSA-treated group (Fig. 5D). These results suggest that 5,7-dihydroxy-4-methylcoumarin reduced extravasation and alleviated allergic symptoms. Therefore, we propose that 5,7-dihydroxy-4-methylcoumarin can exert anti-allergic effects in vivo.

Discussion

Allergy is a condition characterized by an exaggerated immune response to typically harmless substances such as pollen and animal hair [1]. The prevalence of allergic diseases and the associated socioeconomic burden have been steadily increasing yearly [5, 13]. Currently, antihistamines or steroids are commonly used to treat allergic diseases; however, these treatments are often accompanied by significant side effects [8, 9, 11, 12]. Therefore, there is an urgent need to develop anti-allergic drugs with fewer side effects. Recently, phytochemical derivatives, known for their wide range of bioactivities, including anti-allergic effects, have garnered attention for their potential as novel drugs [35].

The results of this study revealed that 5,7-dihydroxy-4-methylcoumarin exerted anti-allergic effects both in RBL-2H3 cells and in a PCA murine model. Mast cell degranulation leads to the release of histamine, which contributes to common allergic symptoms such as

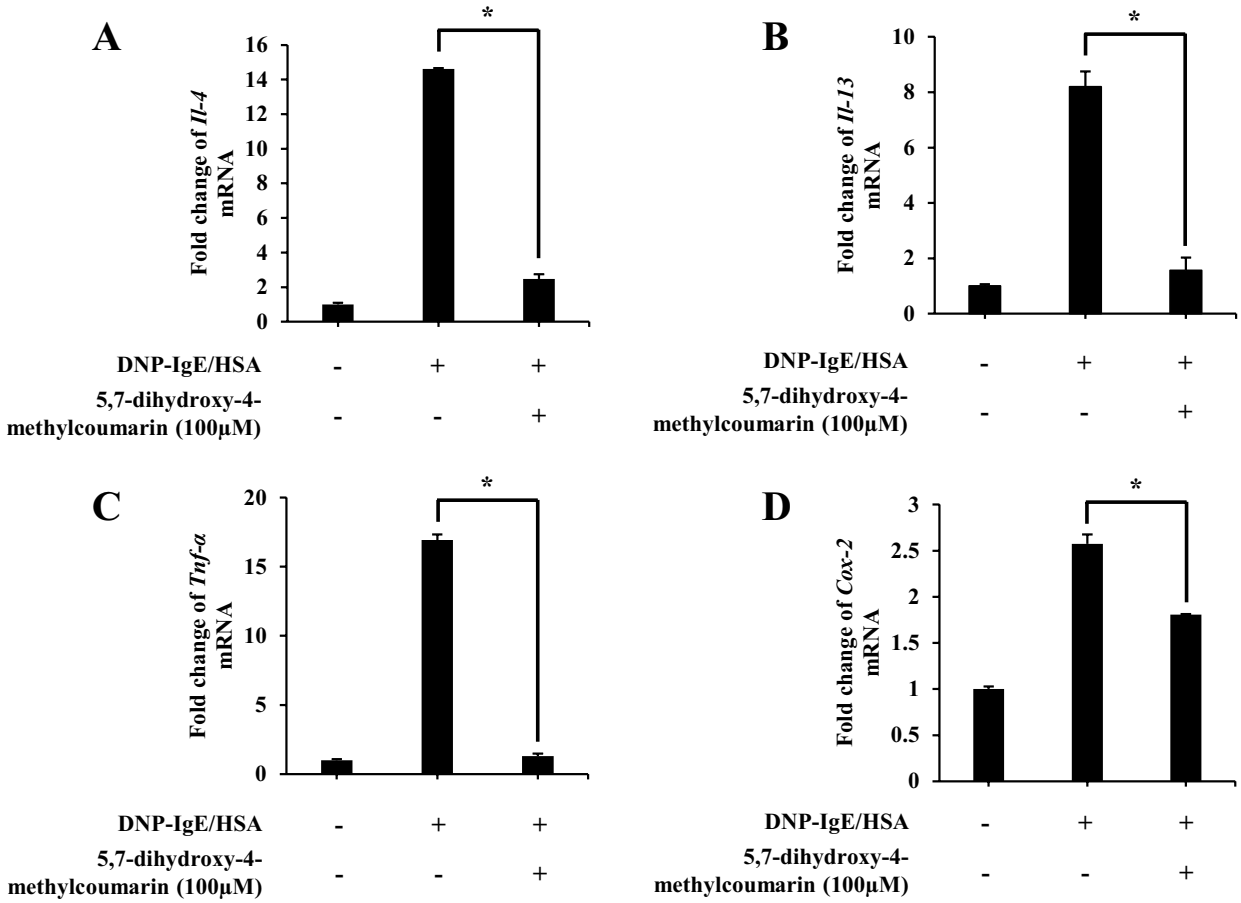

Fig. 3 Effect of 5,7-dihydroxy-4-methylcoumarin on the mRNA expression of pro-inflammatory cytokines and enzyme in RBL-2H3 cells. RBL-2H3 cells, sensitized with DNP-IgE, were treated with DNP-HSA following pretreatment with 5,7-dihydroxy-4-methylcoumarin (100 μM). The mRNA levels of pro-inflammatory cytokines and inflammatory enzyme were measured using quantitative reverse transcription PCR. *Il-4* (**A**); *Il-13* (**B**); *Tnf-α* (**C**); *Cox-2* (**D**). The data are presented as the mean ± SD; n = 3. *$p < 0.05$ compared with the DNP-IgE/HSA-treated group

sneezing, nasal congestion, and increased vascular permeability [36]. The histamine/β-hexosaminidase release experiment and toluidine blue staining revealed that 5,7-dihydroxy-4-methylcoumarin decreased mast cell degranulation. According to reported studies, reactive oxygen species (ROS) serve as key mediators in allergic diseases, including allergic and inflammatory skin conditions such as atopic dermatitis, urticaria, and psoriasis [37, 38]. Additionally, 5,7-dihydroxy-4-methylcoumarin has been reported to possess antioxidant properties by promoting ROS scavenging [26]. Given the role of ROS in promoting mast cell activation and degranulation, the anti-allergic effects of 5,7-dihydroxy-4-methylcoumarin observed in this study may be attributed to its potential ability to inhibit ROS production through its previously reported antioxidant effects. Moreover, 5,7-dihydroxy-4-methylcoumarin reduced vascular permeability in the PCA murine model. These findings suggest that 5,7-dihydroxy-4-methylcoumarin exerts anti-allergic effects by inhibiting mast cell degranulation in vitro and in vivo.

To investigate the molecular mechanisms underlying the anti-allergic effects of 5,7-dihydroxy-4-metylcoumarin, qRT-PCR and western blot analyses were performed. IL-4 mediates Th_2 cell differentiation and antibody-mediated immune response. Both IL-4 and IL-13 contribute to the enhancement of histamine release and promote mast cell-mediated allergic responses [39]. Additionally, TNF-α and COX-2 are key inflammation mediators that can induce inflammation [23, 24]. Our qRT-PCR assay revealed that 5,7-dihydroxy-4-methylcoumarin reduced allergic response by inhibiting the expression of allergic-inflammatory cytokines (*Il-4*, *Il-13*, and *Tnf-α*), as well as *Cox-2*. In the western blot assay, we assessed the phosphorylation level of MAPKs and AKT. Notably, p38 and ERK are known to regulate the production of inflammatory cytokines [40], whereas AKT is pivotal in the generation of these cytokines and the

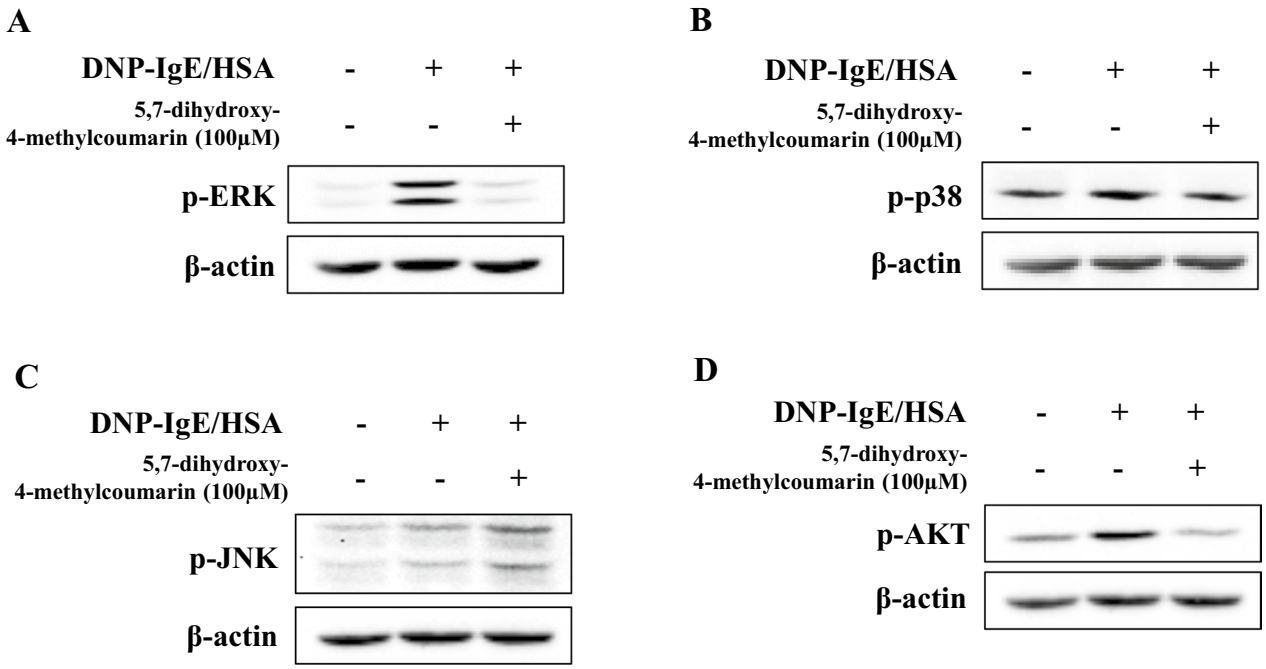

Fig. 4 Effect of 5,7-dihydroxy-4-methylcouamrin on the MAPK (ERK, p38, and JNK) and AKT pathways. RBL-2H3 cells, sensitized with DNP-IgE, were treated with DNP-HSA following pretreatment with 5,7-dihydroxy-4-methylcoumarin (100 μM). The phosphorylation level of each protein was determined via western blot analysis. Phosphorylated extracellular signal-regulated kinase (**A**); phosphorylated p38 (**B**); phosphorylated extracellular signal-regulated kinase (**C**); phosphorylated protein kinase B (**D**)

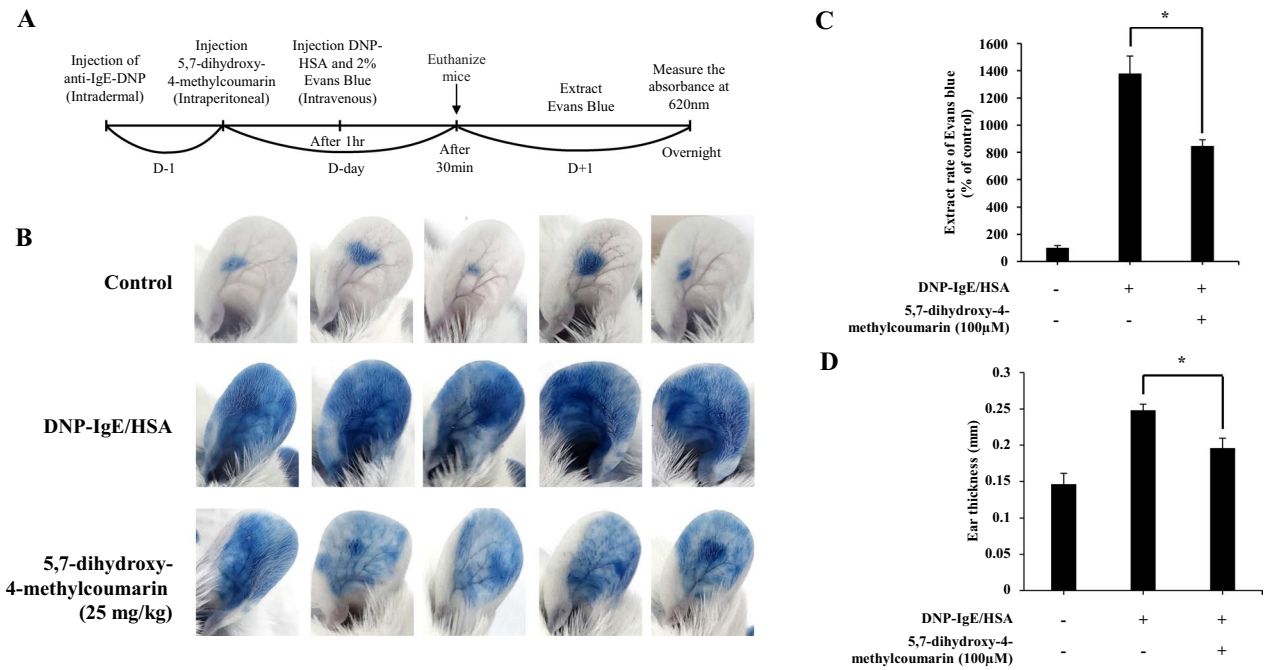

Fig. 5 Effect of 5,7-dihydroxy-4-methylcoumarin on IgE-mediated passive cutaneous anaphylaxis. **A** Overall schedule of the passive cutaneous anaphylaxis experiment. The ear skin of mice was sensitized with an intradermal injection of anti-DNP IgE (0.5 mg/site) for 24 h. 5,7-Dihydroxy-4-methylcoumarin was injected intraperitoneally at a dose of 25 mg/kg body weight before the intravenous injection of DNP-HSA and 2% Evans blue mixture. After 30 min, photographic images of the ears were taken (**B**). Subsequently, the ears were collected, and the dye was extracted as described in the Materials and Methods section, with quantification performed using a spectrophotometer (**C**). Ear thickness was measured (**D**). Graph data are represented as the mean ± SD (n = 5/group). *$p < 0.05$ compared with the DNP-IgE/HSA-treated group

enhancement of mast cell degranulation [34]. Our results demonstrated that 5,7-dihydroxy-4-methylcoumarin decreased the phosphorylation of ERK, p38, and AKT. These findings suggest that 5,7-dihydroxy-4-methylcouamrin exerts its anti-allergic effect by inhibiting ERK, p38, and AKT signaling. Consistent with our results, several compounds with inhibitory effects on antigen-induced allergic reactions have been reported to reduce the phosphorylation of MAPKs (p38 and ERK) but not JNK [41, 42]. Studies investigating Syk kinase and mast cell degranulation have underscored the predominant roles of p38 and ERK in cytokine regulation while demonstrating a limited involvement of JNK, which aligns with our findings regarding the regulatory roles of ERK and p38 in activated mast cells [43]. Our results indicate that 5,7-dihydroxy-4-methylcoumarin inhibited mast cell activation by suppressing the ERK and p38 signaling pathways.

In conclusion, our findings suggest that 5,7-dihydroxy-4-methylcoumarin exerts anti-allergic effects by inhibiting the production of allergic-inflammatory cytokines and suppressing the phosphorylation of MAPKs (ERK and p38) and AKT, which are critical mediators of cytokine expression and mast cell degranulation. Given that phytochemicals are well known for their diverse bioactivities, including anti-allergic properties, as evidenced by several studies [35], the identification of the anti-allergic effect of 5,7-dihydroxy-4-methylcoumarin underscores its potential as a novel candidate for the development of novel anti-allergy therapeutics. Future research is expected to further explore the efficacy of anti-allergy products derived from coumarin-based phytochemicals.

Abbreviations

IgE	Immunoglobulin E
FcεRI	High-affinity IgE receptor
MAPKs	Mitogen-activated protein kinases
AKT	Protein kinase B
TNF-α	Tumor necrosis factor-alpha
COX-2	Cyclooxygenase-2
RBL	Rat basophilic leukemia
PCA	Passive cutaneous anaphylaxis
α-MEM	Alpha-minimum essential medium
PSQ	Penicillin/streptomycin/glutamine
DNP	Dinitrophenyl
HSA	Human serum albumin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
PBS	Phosphate-buffered saline
qRT-PCR	Quantitative reverse transcription PCR
TBS-T	Tris-buffered saline containing 0.1% Tween-20
SD	Standard deviation
IL	Interleukin

Acknowledgements
Not applicable.

Author contributions
S. G., S-A. S., M. K., S. C., and M. K. performed the experiments. S. G. and C. S. L. wrote the manuscript under the guidance of C.S.L. S.Y.M., J.H.L., and H.H.P. contributed intellectually to the present study. All authors have read and approved the final manuscript.

Funding
This research was supported by the Korea Polar Research Institute (KOPRI) grant funded by the Ministry of Oceans and Fisheries (KOPRI Project No. * PE21900).

Availability of data and materials
The datasets that support the findings of this study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests. Chang Sup Lee is an Associate Editor of Applied Biological chemistry. Associate Editor status has no bearing on editorial consideration.

Received: 19 December 2024 Accepted: 2 January 2025
Published online: 14 January 2025

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