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Original paper

Enhancement of Anti-inflammatory and Anti-allergic Activities with Combination of Luteolin and Quercetin in *in vitro* Co-culture System

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Polyphenols are well known to possess diverse physiological functions. We have reported that luteolin suppressed tumor necrosis factor (TNF)-α and interleukin (IL)-8 secretions in an *in vitro* co-culture system composed of RAW264.7 cells stimulated with lipopolysaccharide and Caco-2 cells, respectively. It was also reported that luteolin possessed anti-allergic activity with *in vitro* co-culture of Caco-2/RBL-2H3 cells. In this work, we investigated the activity of luteolin in combination with other polyphenols using both *in vitro* co-culture systems. Caco-2 cells were treated with the maximum concentration of luteolin (75 μM) that did not produce anti-inflammatory activity, in combination with other polyphenols such as quercetin, kaempferol, curcumin, galangin and chrysin (each 75μM). All combinations of the tested polyphenols except chrysin significantly increased luteolin hyperpermeability to the basolateral side, curcumin in particular. The co-culture system analysis demonstrated that curcumin or quercetin in combination with luteolin significantly suppressed TNF-α production and IL-8 mRNA expression. Moreover, the combination of luteolin and quercetin suppressed β-hexosaminidase activity as an index of anti-allergic activity. Combination of quercetin and luteolin was demonstrated to increase luteolin permeability to the basolateral compartment of the *in vitro* co-culture systems, resulting in the observed anti-inflammatory and anti-allergic activities.

Introduction

Flavonoids have a variety of biological effects such as anti-tumor, anti-oxidant, anti-allergic, anti-microbial, and anti-angiogenic properties (Shimada *et al*., 2006). Some flavonoids are known for their anti-inflammatory effects, which may be attributable to their ability to inhibit the production of pro-inflammatory genes and mediators (Yuan *et al*., 2006). Luteolin (3',4',5,7-tetra-hydroxyl-flavone), a flavonoid contained in celery, green pepper, perilla leaf and chamomile, is also well known for its anti-inflammatory and anti-allergic activities (Ueda *et al*., 2002). Luteolin has been reported to inhibit lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α, interleukin (IL)-6, and inducible nitric oxide (NO) production *in vitro* (Chen *et al*., 2007; Xagorari *et al*., 2001). Recently, it was reported that luteolin (20 and 50 mg/kg body weight) significantly ameliorated shortening of colon length and histological score in a dextran sulfate sodium (DSS)-induced colitis model, and inhibited NF-κB nuclear translocation (Nishitani *et al*., 2013). Moreover, it was

Abbreviations

DNP, dinitrophenol; DSS, dextran sulfate sodium; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; IgE, immunoglobulin E; IL-8, interleukin-8; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NO, nitric oxide; PCR, polymerase chain reaction; RT, reverse transcription; SB, Siraganian buffer; SULTs, sulfotransferases; TNF-α, tumor necrosis factor-α; UGTs, UDP-glucuronosyltransferases

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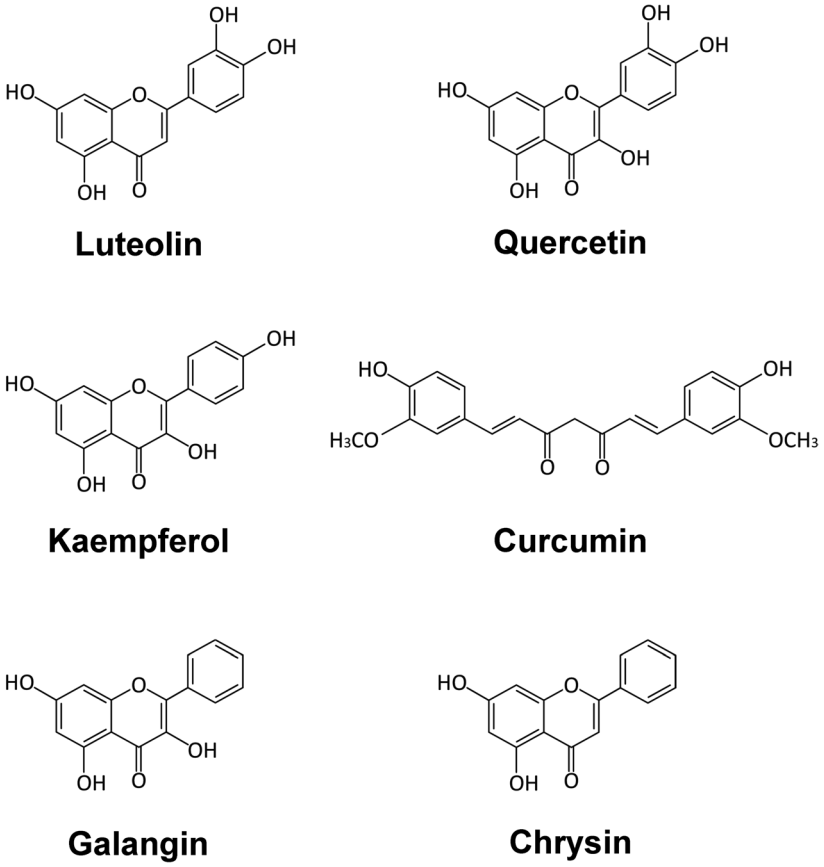

Fig. 1. Chemical structure of polyphenols tested in the present study.

demonstrated that concentrations of luteolin aglycone (not its glycoside) in the body are important for its anti-inflammatory activity. However, it is well known that the amount of luteolin in blood is low (Shimoi *et al.*, 1998), since the majority of flavonoid aglycones are conjugated with glucuronic acid or sulfuric acid by epithelial cells during intestinal absorption (Murota *et al*., 2002). The small intestine is thought to be responsible for the first-pass metabolism of flavonoids. Glucuronidation mediated by various UDP-glucuronosyltransferases (UGTs) and sulfation mediated by various sulfotransferases (SULTs) are suggested to be important metabolic pathways of flavonoids in the intestine. It has been demonstrated that luteolin is also conjugated by UGTs and SULTs (Li *et al*., 2007), indicating the difficulty for the effects by luteolin to be exerted in the human body. Hence, it is essential to increase the absorption of luteolin in order to enhance its physiological activities.

It was previously reported that the interactive metabolism of flavonoids occurred by their combined administration to rat (Hashimoto *et al*., 2005). In addition, Chakrabarti and Ray (2015) reported that the combination of luteolin and silibinin showed synergistic anti-tumor action. The synergistic mechanism of luteolin and chicoric acid was reported, in which luteolin played a central role in ameliorating LPS-induced inflammatory cascades via inactivation of NF-κB and Akt pathways, and chicoric acid was reported to strengthen the anti-inflammatory activity of luteolin through NF-κB attenuation (Park *et al*., 2011). Thus, it was predicted that combined use of luteolin and flavonoids would synergistically affect inflammatory and allergic activities. In this study, we evaluated the activities of combinations of luteolin and other polyphenols including quercetin, kaempferol, curcumin, galangin and chrysin (Fig. 1) using co-culture systems composed of RAW264.7/Caco-2 cells or RBL-2H3/Caco-2 cells, which are effective methods to estimate anti-inflammatory (Tanoue *et al*., 2008) and anti-allergic activities (Yamashita *et al*., 2016), respectively.

Materials and Methods

Reagents Luteolin was purchased from Sarsynthese (Genay, France). Dulbecco's Modified Eagle's Medium (DMEM) mixed with glutamine containing 1.0 g/L glucose, LPS from *Escherichia coli* O127, and recombinant murine TNF-α were from Wako Pure Chemical Industries (Osaka, Japan). Minimum essential medium (MEM) was from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) for L929 cells, RPMI 1640 medium, and MEM nonessential amino acids (NEAA) were from Gibco BRL (Grand Island, NY). DMEM with glutamine containing 4.5 g/L glucose and budesonide was from Sigma (St. Louis, MO). FBS for Caco-2 and RAW264.7 cells was from Biological Industries (Beit, Israel). β-Glucuronidase/aryl-sulfatase and β-glucuronidase were from Roche (Basel, Switzerland). Anti-dinitrophenyl (DNP) IgE, DNP-albumin, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were purchased from Sigma. Other chemicals and reagents were

ordinary commercial and guaranteed products.

Cell culture The human intestinal epithelial cell line, Caco-2, was cultured in DMEM (glutamine, high glucose) supplemented with 1% MEM–NEAA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% decomplemented FBS (56℃, 30 min). The murine macrophage cell line, RAW 264.7, was cultured in DMEM (glutamine, low glucose) supplemented with 10% (v/v) decomplemented FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The murine fibrosarcoma cell line, L929, was cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were incubated in a humidified 5% CO_2 incubator at 37℃. The rat basophilic leukemia cell line, RBL-2H3, was cultured in dishes in Eagle's MEM supplemented with 10% (v/v) decomplemented FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine. Cell cultures were incubated at 37℃ in a 5% CO_2 incubator. Passage numbers 17–32 were used. When cells reached 80% confluence, they were recovered from the culture dish or flask by trypsin digestion after washing with PBS. After centrifuging, the cells were suspended in cell media and cultured in a new dish or flask. *In vitro* assay using co-culture systems composed of RAW264.7/Caco-2 and RBL-2H3/Caco-2 was performed according to our previous studies for anti-inflammatory activity (Tanoue *et al*., 2008) and anti-allergic activity (Yamashita *et al*., 2016), respectively.

RAW264.7/Caco-2 co-culture system Caco-2 cells were seeded at 3.75×10^5 cells/well onto Transwell insert plates (4.67 cm^2, 0.4 μm pore size, Corning CoStar Corp., Cambridge, MA). The cell culture medium was changed every 3 day until the cells were fully differentiated (TER value >1200 $\Omega \cdot cm^2$), and the cells were used at passage numbers 48–62. RAW264.7 cells were seeded at 8.5×10^5 cells/well into 6-well tissue culture plates and incubated overnight to completely adhere to the well, and the cells were used at passage numbers 10–30. After replacing all media with RPMI1640, the Transwell insert on which Caco-2 cells had been cultured were added to multiple plate wells preloaded with RAW264.7 cells. In an experiment to evaluate anti-inflammatory activity, 1.5 mL of test sample was applied to the apical side for 3 h, and then LPS was added to the basolateral side in this model. After additional incubation of 3 h, the culture supernatants from the basolateral side were collected for TNF-α measurement. Caco-2 cells were harvested for total RNA isolation and applied to subsequent real-time polymerase chain reaction (PCR).

TNF-α measurement The amount of TNF-α in the culture medium was quantified by cytolytic assay in L929 cells using murine recombinant TNF-α as the standard (Takada *et al*., 1994). L929 cells were plated in 96-well microplates in MEM including 10% FBS and cultured for 4 h. The medium was replaced with 50 μL of fresh RPMI 1640 medium (supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) containing 6 μg/mL actinomycin D (Wako Pure Chemical) and 50 μL of supernatant. Samples were cultured again for 20 h in a 5% CO_2 incubator at 37℃. After the medium was removed, cell lysates were stained with 0.1% crystal violet in ethanol/formaldehyde for 15 min at room temperature and washed with water and dried, then dissolved in 100 μL of ethanol-PBS (1:1, v/v). Absorbance of the stained solution in wells was measured using a microplate reader at an analysis wavelength of 570 nm and a reference wavelength of 630 nm. The concentration of TNF-α was calculated using a standard curve.

RNA isolation and real-time PCR Total RNA was isolated from cells using Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The reverse transcription (RT) of RNA for quantitative PCR was performed using a High capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The RT reactions were performed in a thermal cycler (Gene Amp® PCR System 9700, Thermo Fisher Scientific) at 25℃ for 10 min, 37℃ for 120 min and 85℃ for 5 min. Real-time PCR was conducted using the 7500 Fast Real Time PCR system and Taqman Fast universal PCR master mix kit (Thermo Fisher Scientific) according to the manufacturer's instructions. A TaqMan probe was used in the real-time PCR with the following product number: Human IL-8 Assay ID: Hs00174103_m1. Human GAPDH Assay ID: Hs99999905_m1 was used as an endogenous control (Thermo Fisher Scientific). For all panels, the bars represent the relative values of the target gene to endogenous gene expression, as determined by the relative quantification method (ΔΔCT).

Quantitative analysis of luteolin content Caco-2 cells in the co-culture system were incubated with luteolin for 3 h and subsequently incubated with LPS for an additional 3 h to activate RAW254.7 cells; the Caco-2 cells were incubated with luteolin for a total of 6 h. After 6 h incubation of the Caco-2 cells monolayer with luteolin on the apical side, the basolateral and apical solutions were collected and divided into two aliquots. Half of the basolateral solution was mixed with β-glucuronidase/aryl-sulfatase in 1 M acetate buffer (pH 5.0) at 37℃ for 4 h or β-glucuronidase in 400 mM phosphate buffer (pH 6.2) at 37℃ for 1 h. The mixture was extracted with ethyl acetate after the addition of hesperetin (1 μmol) as an internal standard. The ethyl acetate layer was dried under a nitrogen stream and dissolved in 250 μL methanol followed by 0.22 μm filtration. The resultant supernatant solutions were used as a glucuronidase with or without aryl-sulfatase-treated and an untreated sample. The amount of luteolin metabolites (glucuronides with or without sulfates) was calculated as the difference between the amounts of luteolin from glucuronidase with or without aryl-sulfatase-treated and untreated samples. Luteolin was identified by HPLC as described previously (Nishitani *et al*., 2013).

RBL-2H3/Caco-2 co-culture system Caco-2 cells were seeded at a concentration of 0.6×10^5 cells/well onto 24-well Transwell insert plates (0.33 cm^2, 0.4 μm pore size, Corning Costar Corp.). The cell culture medium was changed every 3 days until the cells

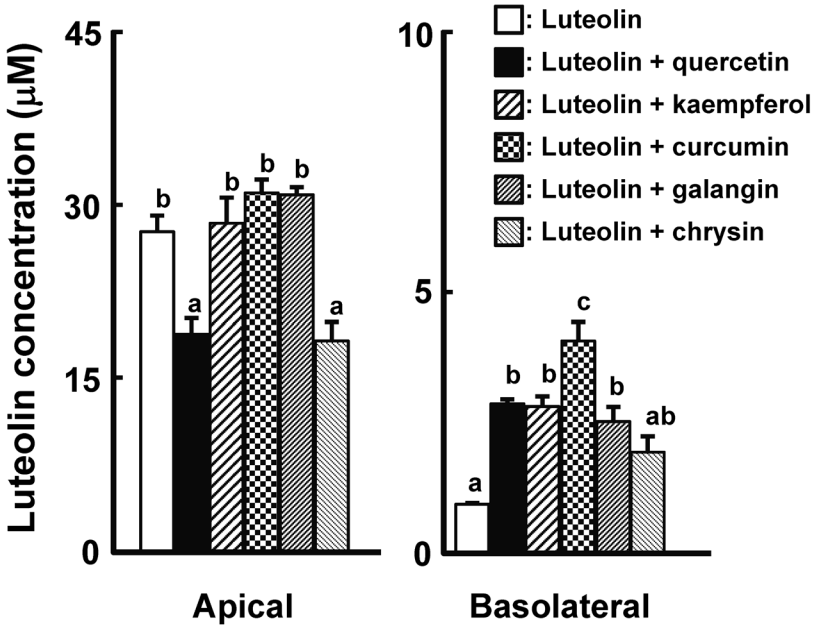

Fig. 2. Luteolin contents in apical and basolateral side of Caco-2 cell mono-culture in combination of luteolin with some polyphenols.
Luteolin (75 μM) was added to the apical compartment of Caco-2 mono-culture on transwell for 3 h. Luteolin aglycone in the basolateral supernatant was extracted with ethyl acetate and measured by HPLC. Values represent the means ± SE (n=3). Items with different letter were significantly different (P, 0.05).

were fully differentiated (TER value>300 $\Omega \cdot cm^2$). RBL-2H3 cells were seeded at 2.0×10^5 cells/500 μL/well onto 24-well tissue culture plates in Eagle's MEM and incubated overnight with anti-DNP IgE at a final concentration of 1 μg/mL. After replacing all media with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2), the Transwell inserts (with cultured Caco-2 cells) were added into the plate wells preloaded with RBL-2H3 cells. In an experiment to evaluate the anti-allergic activity of the test samples, 0.2 mL of SB or test sample solution was applied into the apical side. After incubation for 6 h, the cells were challenged with DNP-albumin at a final concentration of 10 ng/mL for 10 min at 37℃. The plate was cooled in an ice bath for 10 min to stop the degranulation response. The supernatant in the basolateral side was applied to the β-hexosaminidase assay.

β-Hexosaminidase assay To evaluate anti-allergy effects, an *in vitro* assay using the RBL-2H3 mono-culture system was performed in accordance with a previous study (Yamada *et al*., 2007). RBL-2H3 cells (4.0×10^5 cells/mL) were plated at 500 μL/well in a 24-well tissue culture plate in Eagle's MEM, and were sensitized overnight with 1 μg/mL anti-DNP IgE in a 5% CO_2 incubator at 37℃. The cells were washed three times with SB and exposed to 200 μL of test sample solutions for 6 h at 37℃. After incubation, the cells were challenged with 10 ng/mL DNP-albumin for 10 min at 37℃. The plate was cooled in an ice bath for 10 min to stop the degranulation response. The supernatant prepared above (50 μL) was transferred to a 96-well plate, and incubated with an equal volume of substrate solution (5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.2 M citrate buffer at pH (4.5) for 1 h at 37℃. After adding 100 μL/well of stop buffer (0.2 M Tris, pH 8.0), the absorbance at 405 nm was measured using a microplate reader. The percentage of β-hexosaminidase released into the supernatants was calculated as a percentage of the degranulation group.

Statistical analysis Each result was expressed as the mean ± standard error. Statistical significance between more than two groups was analyzed by a one-way ANOVA and the Tukey–Kramer test. Statistical significance was defined as $p < 0.05$.

Results and discussion

Influence of flavonoids on luteolin content in the basolateral supernatant Since the physiological activity of luteolin is highly dependent on its concentration (Nishitani *et al*., 2013), luteolin (75 μM) and individual flavonoids (quercetin, kaempferol, curcumin, galangin and chrysin: 75 μM) were added to the apical side of the Transwell insert containing the Caco-2 cell mono-culture, and the contents were measured by HPLC. As shown in Fig. 2, the combination of all tested flavonoids except for chrysin significantly increased the permeability of luteolin to the basolateral side; specifically, curcumin showed the greatest effect. When Caco-2 cells were treated simultaneously with luteolin and quercetin or kaempferol, luteolin contents in the basolateral side were increased to almost 2-fold (approximately 2.5–3.5 μM). In the apical side, treatment with quercetin and chrysin decreased the luteolin contents to almost 18.5 μM. It was reported that the concentration of luteolin in rat plasma reached 1.79 μM for 6 h after oral administration of luteolin (22.8 μmol/kg body weight) to

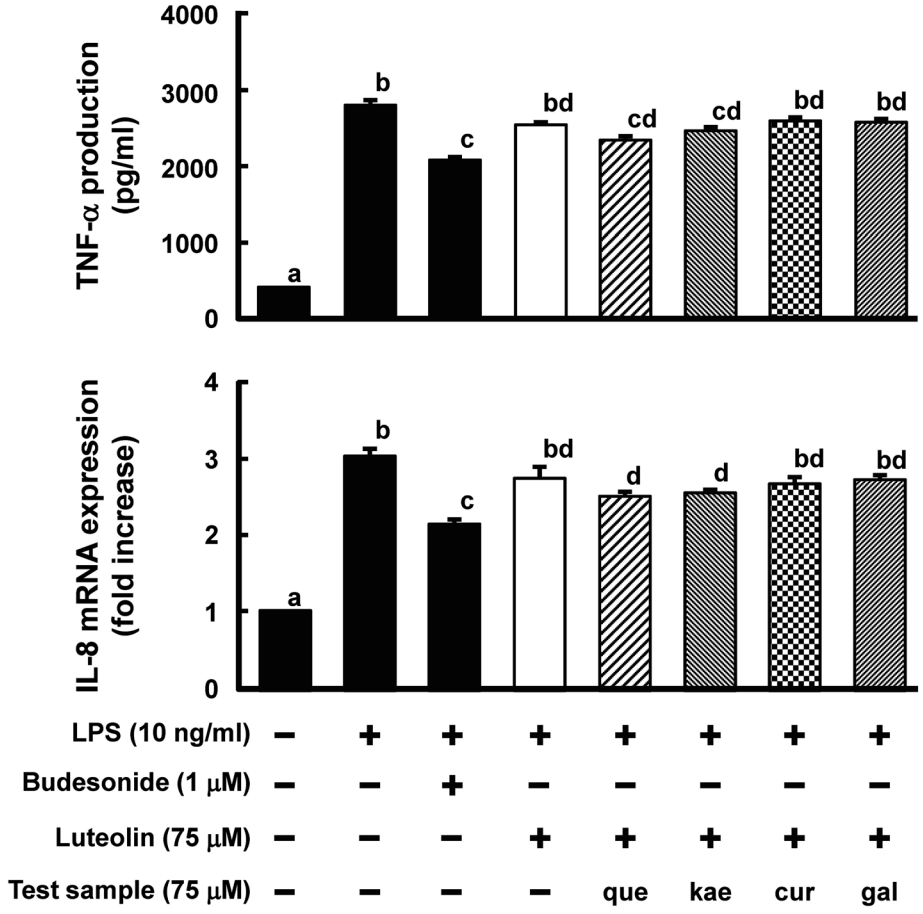

Fig. 3. Anti-inflammatory activity of combination of luteolin with some polyphenols in RAW264.7/Caco-2 co-culture system.
Luteolin (75 μM) was simultaneously added with some polyphenols (75 μM) to the apical compartment of the Caco-2/RAW264.7 co-culture model for 3 h. Subsequently, LPS was added to the basolateral compartment to a final concentration of 10 ng/mL, followed by incubation for an additional 3 h. Values represent the means ± SE (n=3). Items with different letter were significantly different (P, 0.05).

rats (Torii-Yasuda *et al*., 2015). It was ascertained that the concentration of luteolin in the basolateral side of the co-culture system was physiologically relevant. Thus, the combination of luteolin and certain polyphenols enhanced luteolin contents in the basolateral side. It is well known that luteolin is metabolized to glucuronides and sulfates of luteolin (Torii-Yasuda *et al*., 2015). Among the polyphenols tested here, kaempferol, curcumin, and galangin might inhibit conjugation with glucuronic acid or sulfuric acid, resulting in the increase of luteolin aglycone, although quercetin would promote the permeability of luteolin. The mechanism of permeability promotion might be related to the catechol structure in the B-ring of flavonoids. In the future, it is necessary to clarify the structure-activity relationship by comparing more molecules including flavonoids in order to understand the absorption and metabolism of luteolin.

Anti-inflammatory activity of combinations of luteolin and flavonoids in the RAW264.7/Caco-2 co-culture system Budesonide was used as a positive control (Tanoue *et al*., 2008). It was demonstrated that the combination of quercetin or kaempferol (75 μM each) with luteolin (75 μM) significantly suppressed TNF-α production and IL-8 mRNA expression, even though 75 μM luteolin was added to the apical side of the co-culture system, a concentration that did not produce anti-inflammatory activity (Fig. 3). As shown in Fig. 4, RAW264.7 cells when directly treated with 1.5 μM luteolin showed significantly suppressed TNF-α production, whereas 1 and 0.5 μM luteolin did not. As the luteolin concentration in the basolateral side was approximately 1.5 μM when luteolin was added in combination with quercetin or kaempferol in the apical side of the Caco-2 cells mono-culture (Fig. 2), these results indicated that combinations of luteolin with quercetin or kaempferol had additive anti-inflammatory activity. However, it remains unclear why curcumin did not exert anti-inflammatory activity, since the increase in curcumin contents in the basolateral side was greater than the combination of quercetin or kaempferol with luteolin.

Contents of each luteolin metabolite at different quercetin concentrations We measured the contents of luteolin and its metabolites. After 6 h incubation with luteolin (75 μM) and quercetin (37.5, 75 and 150 μM) using the RAW264.7/Caco-2 co-culture system, the amounts of luteolin and metabolites in the basolateral supernatant were measured by HPLC analysis.

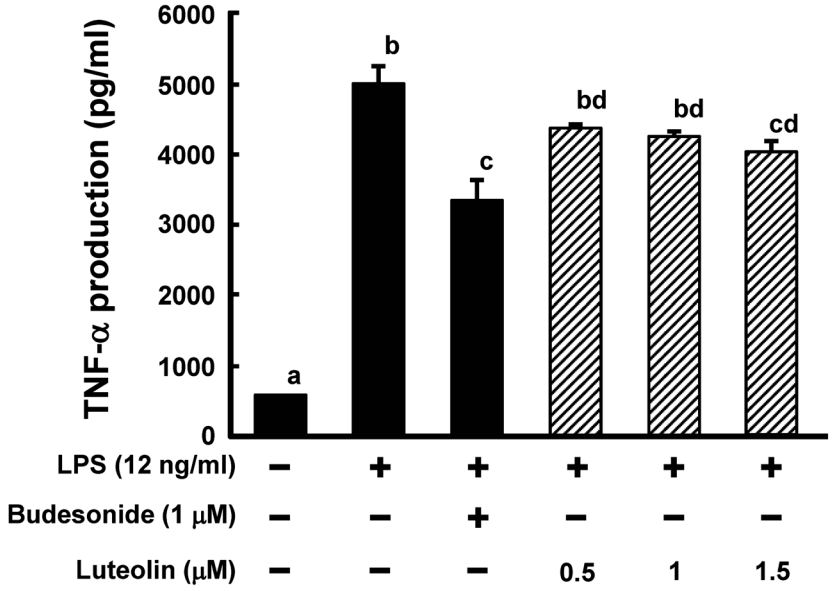

Fig. 4. Suppression of TNF-α production from RAW264.7 cells stimulated with LPS by luteolin. Luteolin was added directly to RAW264.7 cells and incubated for 3 h. LPS was added to the basolateral compartment to a final concentration of 12 ng/mL, followed by incubation for an additional 3 h. Values represent the means ± SE (n=3). Items with different letter were significantly different (P, 0.05)

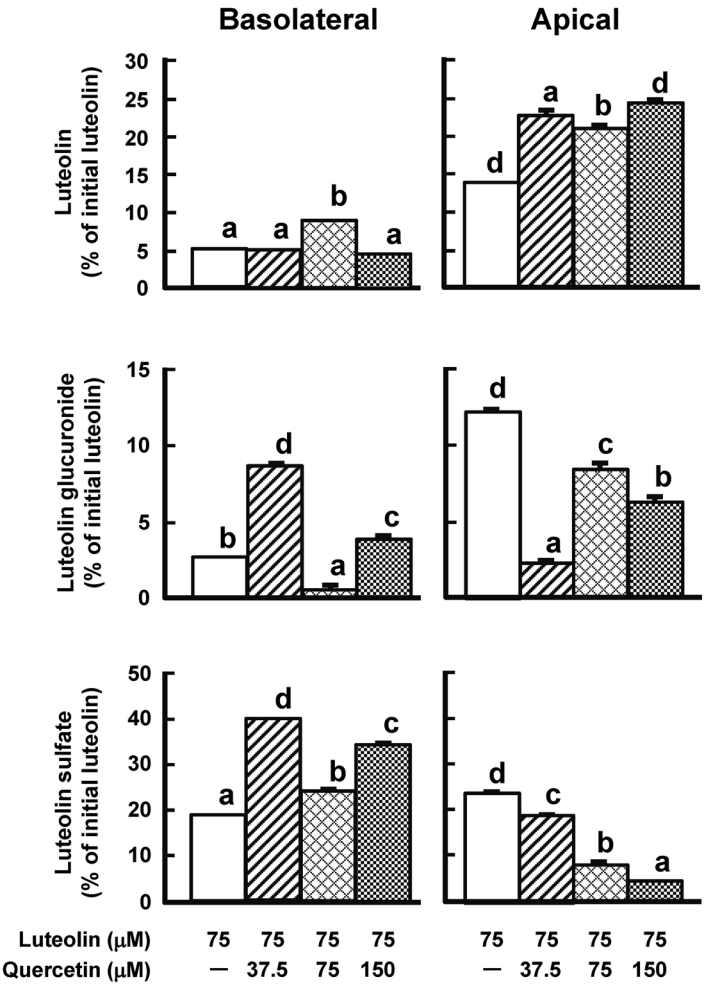

Fig. 5. Change of luteolin, luteolin glucronide and luteolin sulfate in the combination of luteolin and quercetin. Each concentration of luteolin and quercetin was added into basolateral side in RAW264.7/Caco-2 co-culture system and incubated for 6 h. The basolateral supernatant was used to measure the amount of luteolin and its metabolites by HPLC analysis. Items with different letter were significantly different (P, 0.05).

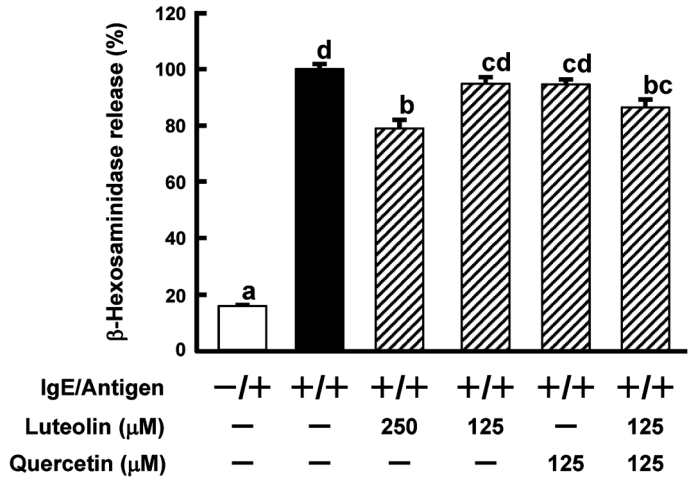

Fig. 6. Anti-allergic activity of the combination of luteolin and quercetin in RBL-2H3/Caco-2 co-culture system.
Luteolin with or without quercetin were added into apical side in RBL-2H3/Caco-2 co-culture system for 6 h. RBL-2H3 cells were challenged with 10 ng/mL final concentration of DNP albumin for 10 min at 37℃. Items with different letter were significantly different (P, 0.05).

Interestingly, only treatment with equal amounts of luteolin and quercetin increased luteolin contents in the basolateral side by 2-fold (Fig. 5). However, the luteolin content was maintained at similar levels with the other quercetin concentrations. It was demonstrated that the content of luteolin sulfate in the apical side was decreased with increasing quercetin concentrations. On the other hand, combination with quercetin at 37.5 or 150 μM increased the contents of luteolin glucuronide and sulfate in the basolateral side. Because polyphenols are generally known to lose biological activity by conjugation, the combination with quercetin at these concentrations, *i.e.,* different concentrations from that of luteolin, would not increase the biological activities, including anti-inflammatory activity, of luteolin. Further study is needed to clarify why an equal concentration of quercetin is able to increase luteolin aglycones.

Anti-allergic activity of combinations of luteolin and quercetin in the RBL-2H3/Caco-2 co-culture system It was reported that luteolin is the best inhibitor of histamine and β-hexosaminidase release, indicating that luteolin might possess anti-allergic activity (Yang *et al*., 2013). The effect of combinations of luteolin and quercetin on anti-allergic activity was determined using the RBL-2H3/Caco-2 co-culture system (Yamashita *et al*., 2016). As shown in Fig. 6, luteolin at 250 μM significantly suppressed β-hexosaminidase release. Moreover, the combination of luteolin (125 μM) and quercetin (125 μM), which individually did not show anti-allergic activity, exhibited activity. When 125 μM of luteolin and quercetin each were added to the apical side of Caco-2 cells, the luteolin concentration in the basolateral side was estimated to be approximately 3 μM based on the results in Fig. 5. This concentration was equal to a concentration of 250 μM luteolin added to the apical side. In conclusion, it was demonstrated that the combination of quercetin and luteolin increased the permeability of luteolin to the basolateral compartment, and anti-inflammatory and anti-allergic activities were exerted in co-culture systems composed of RAW264.7/Caco-2 and RBL-2H3/Caco-2 cells, respectively. However, it is necessary to investigate these activities using *in vivo* experiments.

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