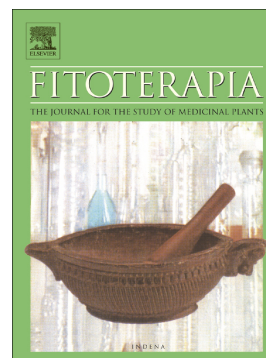


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## Anti-allergy activities of Kuji amber extract and kujigamberol

Miku Maruyama<sup>a,1</sup>, Miki Kobayashi<sup>a,1</sup>, Takeshi Uchida<sup>a</sup>, Eisaku Shimizu<sup>a</sup>, Hironori Higashio<sup>b</sup>, Misa Ohno<sup>c</sup>, Shota Uesugi<sup>c</sup>, Ken-ichi Kimura<sup>a,c,\*</sup>

<sup>a</sup>*Graduate School of Agriculture, Iwate University, Morioka 020-8550, Japan*

<sup>b</sup>*Center for Liberal Arts and Sciences, Iwate Medical University, Yahaba, 028-3694, Iwate*

<sup>c</sup>*Department of Biological Chemistry and Food Science, Iwate University, Morioka, 020-8550, Japan*

\* **Corresponding author.** Tel./fax: +81 19 621 6124. E-mail address: kimurak@iwate-u.ac.jp (K. Kimura)

<sup>1</sup> Authors with equal contributions.

**ABSTRACT**

Amber is fossilized tree resin and several biologically active compounds were isolated from ambers using the growth-restoring activity of the mutant yeast [*Saccharomyces cerevisiae* (*zds1Δ erg3Δ pdr1Δ pdr3Δ*)] involving  $\text{Ca}^{2+}$ -signal transduction. The aim of this study is to investigate the anti-allergic effect of both the methanol extract of Kuji amber (MEKA) and its main biologically active constituent, kujigamberol (15,20-dinor-5,7,9-labdatrien-18-ol) having activity against the mutant yeast. Both MEKA and kujigamberol inhibited the degranulation of RBL-2H3 cells by stimulation of thapsigargin (Tg) ( $\text{IC}_{50}$ = 15.0  $\mu\text{g/ml}$  and 29.1  $\mu\text{M}$ ) and A23187 ( $\text{IC}_{50}$ = 19.6  $\mu\text{g/ml}$  and 24.9  $\mu\text{M}$ ) without cytotoxicity, but not by stimulation of IgE+DNP-BSA (Ag) ( $\text{IC}_{50}$ > 50.0  $\mu\text{g/ml}$  and 50.0  $\mu\text{M}$ ). However, both inhibited  $\text{Ca}^{2+}$ -influx in RBL-2H3 cells by all three stimulations in a dose dependent manner. Leukotriene  $\text{C}_4$  production in RBL-2H3 cells stimulated by A23187 was also inhibited by both through the inhibition of ERK1/2 phosphorylation. In an ovalbumin-induced rhinitis model of guinea pigs, nasal administration of MEKA and kujigamberol inhibited nasal blockade in a dose-dependent manner and the effect was about 5 times potent than that of a steroid clinical drug, mometasone furoate. The growth-restoring activity of MEKA and kujigamberol against the mutant yeast is involved in the anti-allergic activities against cells and animals, and both are expected to be candidates for the development new anti-allergy agents.

**Keywords:** Kuji amber; kujigamberol (15,20-dinor-5,7,9-labdatrien-18-ol); *Saccharomyces cerevisiae*; RBL-2H3 cells; ERK; rhinitis guinea pig model

## 1. Introduction

$\text{Ca}^{2+}$  is one of the important second messengers in many cell types, including yeast and immune cells [1,2].  $\text{Ca}^{2+}$ -signal transduction is involved in many diseases and is critical for degranulation, generation of eicosanoids and production of cytokines in mast cells in response to antigen and other stimulants [3]. Type I allergy such as allergic rhinitis has been increasing especially in industrialized countries and the typical symptoms of nasal itching, sneezing, rhinorrhea and nasal congestion affect quality of life and work of patients [4]. Although current therapeutic drug such as corticosteroids for rhinitis and asthma is used in worldwide, natural products are expected to develop safe and effective therapeutic agents different from the steroids [5]. Budding yeast *Saccharomyces cerevisiae* is a simpler model organisms as eukaryote and used for drug screening [6,7].

Amber is fossilized tree resin constituted of organic polymers derived through complex maturation processes of the original plant resin and has been used mainly as ornament. Although it was used as medicine for muscle pain, headaches, skin allergies, etc. in the limited area, any biologically active compound from ambers has not been isolated until we have studied [8-11]. Our group focused on its biological activity for the first time and have already isolated several kinds of biologically active compounds involving  $\text{Ca}^{2+}$ -signal transduction inhibition from Kuji [*Araucaria* (Araucariaceae); 90-86 million years ago (Ma) (Prof. Hisao Ando, Ibaraki University, personal communication), Japan], Baltic (Pinaceae, Araucariaceae, Burseraceae; 56-34 Ma, Poland and Russia) and Dominican [*Hymenaea protera*, *Hymenaea verrucosa* (Fabaceae/Caesalpinaceae); 45-30 Ma and 20-15 Ma, Dominican Republic] ambers using a mutant *Saccharomyces cerevisiae* (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) (YNS17 strain)

[2,12-14]. There are three new compounds, 15,20-dinor-5,7,9-labdatrien-18-ol (kujigamberol), 13-methyl-8,11,13-podocarpatrien-19-ol (kujiol A) and 15,20-dinor-5,7,9-labdatrien-13-ol (kujigamberol B) from Kuji amber, three known compounds, agathic acid 15-monomethyl ester, dehydroabietic acid and pimaric acid from Baltic amber, and one new analogue compound, 5(10)-halimen-15-oic acid and two known compounds, 3-cleroden-15-oic acid and 8-labden-15-oic acid from Dominican amber [9,15-17]. The methanol extract of Kuji amber (MEKA) and its main biologically active compound, kujigamberol have growth-restoring activity that is a different phenotype from that of 3-cleroden-15-oic acid against the mutant yeast without cytotoxicity. Furthermore, 3-cleroden-15-oic acid prevented the degranulation of rat basophilic leukemia-2H3 (RBL-2H3) cells through the inhibition of  $\text{Ca}^{2+}$ -influx stimulated by thapsigargin (Tg) or the calcium ionophore A23187, not by immunoglobulin E+dinitrophenol-bovine serum albumin [IgE+DNP-BSA (Ag)] [16].

Mast cells are a key player in type I allergy and the activation of receptors with high affinity for IgE (FcεRI) on it is to liberate β-hexosaminidase (β-HEX), a general marker of degranulation, as well as allergic mediators such as leukotrienes, histamine, cytokines and prostaglandins. RBL-2H3 cells are suitable as a model for the mast cells and have been widely used to study IgE-FcεRI interactions, related to intracellular signaling pathways in degranulation and to evaluate anti-allergic agents. After crosslinking of their IgE-bound FcεRI by allergens, or  $\text{Ca}^{2+}$ -influx by Tg or A23187, mast cells release mediators which are histamine, prostaglandin  $\text{D}_2$  ( $\text{PGD}_2$ ), the leukotrienes  $\text{C}_4$  ( $\text{LTC}_4$ ) and  $\text{D}_4$  ( $\text{LTD}_4$ ) [18,19]. The  $\text{LTC}_4$  is a lipid mediator which is released by degranulation and causes allergy reaction such as nasal congestion [20,21].

The aim of this study is to investigate anti-allergic effect of MEKA and its main

biologically active constituent, kujigamberol having activity against the mutant yeast, on cells and animals. Surprisingly, MEKA showed potent biological activities against them and the activities did not match the content of kujigamberol in MEKA. The relationship between the activity of MEKA and the quantity of kujigamberol in MEKA was also clarified and discussed.

## 2. Materials and Methods

### 2.1. Chemicals and strains

Kuji amber was excavated from mines of Kuji Kohaku Co. Ltd. located in the upper part of the Tamagawa Formation of Kuji Group of Kuji city, Iwate Prefecture, northeastern Japan. Kuji amber (1029 g) was ground to a powder and extracted with MeOH. It was partitioned with EtOAc and subjected to silica gel column chromatography. Finally, kujigamberol was purified 75.7 mg as a colorless oil using HPLC [9,17]. Extracted samples of 100 mg/ml in MeOH (MEKA) were prepared for subsequent assays. HPLC analyses of MEKA and kujigamberol were carried out using the following conditions [Capcell pak 4.6 × 150 mm (Shiseido Co. Ltd., Tokyo, Japan), 75% MeOH, 1 ml/min, 220 nm]. The yeast strain used was a derivative of strain W303-1A and it was the YNS17 strain (*MATa zds1::TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) [7,8]. Difco<sup>®</sup> YPD broth and YPD agar were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). An immunosuppressive drug, FK506 (tacrolimus) was kindly provided by the Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc., Tokyo, Japan). Unless otherwise stated, chemicals used were of the best commercially available grade.

## 2.2. Animals

Male Hartley guinea pigs for the rhinitis model were obtained from Kyudo Co. Ltd., (Tosu, Japan) at the age of 8 weeks and housed in aluminum cages in a room controlled at a temperature of  $23\pm 2^{\circ}\text{C}$ , humidity of  $61\pm 8\%$  and with a 12 h lighting cycle (7:00-19:00). The experimental procedures employed in this study were in accordance with guidelines of the Industrial Animal Care and Use Committee at LSI Medience Co. (Kumamoto, Japan).

## 2.3. Growth restoring activity

Growth restoring activity was carried out using the YNS17 strain and 5  $\mu\text{l}$  of each sample were added on a plate as described previously [3]. The inhibitory activity of the  $\text{Ca}^{2+}$ -signal transduction was determined by way of the strength and/or distinction of the yeast growth zone. FK506 [2.5 ng/spot, calcineurin (protein phosphatase 2B) specific inhibitor] was used as a positive control [22].

## 2.4. Cell culture and cytotoxicity

RBL-2H3 cells (ATCC, Manassas, VA, USA) were maintained in DMEM supplemented with 10% heat-inactivated FBS (Biowest SAS, Nuaille, France) and antibiotics [penicillin (50 units/ml)-streptomycin (50  $\mu\text{g/ml}$ ), Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA] [23,24]. Cell viability was determined using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Dojindo Lab., Kumamoto, Japan). RBL-2H3 cells were plated in triplicate at a concentration of  $3 \times 10^4$  cells/well in a 96-well plate and incubated for overnight prior to treatment with various concentrations of MEKA or kujigamberol for 48 h. After 10

$\mu\text{l}$  of MTT (5 mg/ml) was added to each well and it was incubated for 4 h at 37 °C. Isopropanol (100  $\mu\text{l}$  in 0.04 N HCl) was added to dissolve the MTT formazan and the absorbance was measured at 560 nm with a microplate reader (Infinite F200 PRO, Tecan, Männedorf, Switzerland).

## 2.5. Measurement of the degranulation ( $\beta$ -HEX assay)

RBL-2H3 cells were grown overnight in 96-well plates ( $3 \times 10^4$  cells/well), 100  $\mu\text{l}$  DMEM was added and then sensitized with 0.5  $\mu\text{g/ml}$  IgE (Yamasa Co., Tokyo, Japan) for 2 h. After washing, cells were incubated in 98  $\mu\text{l}$  of Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.8 mM  $\text{CaCl}_2$ , 5.6 mM D-glucose, 20 mM HEPES, 1 mg/ml BSA, pH7.3) and 2  $\mu\text{l}$  of each sample for 30 min, then stimulated with 10  $\mu\text{l}$  DNP-BSA (1.0  $\mu\text{g/ml}$ , Merck Millipore Co., Billerica, MA, US) as an antigen for 10 min. RBL-2H3 cells were also stimulated with 10  $\mu\text{l}$  Tg (2  $\mu\text{M}$ , Sigma-Aldrich Co., St. Louis, MO, USA) for 20 min or 10  $\mu\text{l}$  A23187 (20  $\mu\text{M}$ , Sigma-Aldrich Co.) for 30 min without the sensitization by IgE. The supernatant (30  $\mu\text{l}$ ) was then transferred to a 96-well plate and incubated with 70  $\mu\text{l}$  substrate [1.3 mg/ml, PNAG (4-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide), Sigma-Aldrich Co.] in 0.1 M citrate buffer (pH 4.5) at 37 °C for 90 min. The reaction was stopped by adding 100  $\mu\text{l}$  of stop solution (0.4 M glycine-NaOH). The absorbance was measured at 405 nm by a microplate reader. The inhibition (%) of  $\beta$ -HEX release by the sample was calculated using the following equation, and  $\text{IC}_{50}$  values were determined graphically. Inhibition (%) =  $[1 - (\text{T} - \text{N}) / (\text{C} - \text{N})] \times 100\%$ : Control (C) was DNP-BSA (+), test sample (-); test (T) was DNP-BSA (+), test sample (+); test sample (+); and normal (N) was DNP-BSA (-),

test sample (-). Quercetin was used as a reference compound ( $IC_{50}=5.7 \mu M$ ) [16,23,24].

## 2.6. Measurement of $Ca^{2+}$ mobilization

RBL-2H3 cells were grown overnight in 96-well plates ( $3 \times 10^4$  cells/well), 100  $\mu l$  DMEM was added and then sensitized with 0.5  $\mu g/ml$  IgE (Yamasa Co., Tokyo, Japan) for 2 h. After washing, cells were incubated in 100  $\mu l$  the loading buffer [Fluo 4-AM (2.5  $\mu M$ , Dojindo Lab.) in Tyrode buffer with 1.25 mM probenecid, Wako] for 30 min at 37°C. After discarding the loading buffer, cells were incubated in 98  $\mu l$  of Tyrode buffer with 1.25 mM probenecid and 2  $\mu l$  of each sample in MeOH for 30 min. Fluo 4-AM loaded RBL-2H3 cells were excited at 485 nm, and the fluorescence emission was observed at 535 nm using a microplate reader. Each 10  $\mu l$  DNP-BSA (1.0  $\mu g/ml$ ), Tg (2  $\mu M$ ) or A23187 (20  $\mu M$ ) was added to each well at 60 sec and the fluorescence measured for 1000 sec [16,23,24].

## 2.7. Protein extraction

RBL-2H3 cells were seeded at  $4 \times 10^5$  cells, incubated for overnight. The cells were treated with MEKA or kujigamberol in a 60 mm dish for 30 min, and stimulated with A23187 (2  $\mu M$ ) for 10 min at 37°C under 5%  $CO_2$ . After washing with phosphate-buffered saline, total protein was extracted using RIPA buffer [10 mM Tris-HCl (pH 7.5), 1% NP-40, 0.01% sodium deoxycholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA] containing protease inhibitor cocktail Complete Mini (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor cocktail (Nacalai Tesque

Inc., Kyoto, Japan). After centrifugation ( $15,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ ), supernatant was collected as whole-cell lysate. The protein concentrations were determined using a BCA protein assay kit (Pierce, Waltham, MA, USA) [25].

### 2.8. Western blotting

The prepared samples containing  $10\mu\text{g}$  total protein were loaded on SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and blocking was performed in Tris-buffered saline containing 1% polyvinylpyrrolidone and 0.1% Tween 20. The membranes were then incubated with primary antibodies. After washing membranes with 0.1% Tween 20 in Tris-buffered saline, each blot was incubated with horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected using ECL (GE Healthcare, Little Chalfont, England) and ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA). Primary antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2, 1:5000) were purchased from Becton, Dickinson and Company, Sparks, USA, and those against phosphorylated ERK1/2 (1:2500) were purchased from Cell Signaling Technology, Danvers, MA, USA. Secondary antibodies, horseradish peroxidase-conjugated anti-mouse IgG (1:10000) and anti-rabbit IgG (1:2500) were purchased from Cell Signaling Technology [25].

### 2.9. Quantification of leukotriene production

RBL-2H3 cells were grown overnight in 96-well plates ( $3 \times 10^4$  cells/well). After washing, cells were incubated in  $98\mu\text{l}$  of Tyrode buffer and  $2\mu\text{l}$  of MEKA (0-25  $\mu\text{g}/\text{ml}$ ) or kujigamberol (0-25  $\mu\text{M}$ ) for 30 min, then stimulated with  $10\mu\text{l}$  A23187 (20  $\mu\text{M}$ ,

Sigma-Aldrich Co.) for 30 min and the generation of leukotriene C<sub>4</sub> (LTC<sub>4</sub>) was then quantified. The supernatant (50 µl) was transferred to a 96-well ELISA plate. The amount of LTC<sub>4</sub> was determined using ELISA kits according to the manufacture's instruction (Cayman Chemical, Co. Michigan, USA). LTC<sub>4</sub> release by MEKA and kujigamberol were calculated by the following equation, and IC<sub>50</sub> were determined graphically: inhibition (%) =  $[1 - (T - N) / (C - N)] \times 100$  %. Control (C) was A23187(+), test sample (-); test (T) was A23187 (+), test sample (+); blank (B) was A23187 (-), test sample (+); and normal (N) was A23187 (-), test sample (-).

#### 2.10. *Ovalbumin-induced rhinitis model*

The guinea pigs were systemically sensitized with 1 ml of 50 mg/ml ovalbumin solution and 1 mg of aluminum hydroxide gel. Two weeks after the sensitization, 20 µl of 50 mg/ml ovalbumin solution was nasally administered to induce allergic responses. The nasal blockade was evaluated by measuring the specific airway resistance (sRaw) using a two-chambered, double-flow plethysmograph system with a respiratory analyzer according to the method [26]. The sRaw measurements took place before the ovalbumin challenge, and 10 min and 1 h after the challenge. The changes in sRaw are expressed as “% increase in sRaw”, which are the percent relative increases in sRaw with respect to the baseline measured before the ovalbumin challenge. The value of “% increase in sRaw” at 10 min and 1 h after the ovalbumin challenge was taken as the value for the immediate airway response calculated by the trapezoidal method was taken as the value for the late airway response. Both MEKA and kujigamberol were suspended in 1% dipropylene glycol (DPG). Both were nasally administered 30 min before and 1 h after ovalbumin challenge.

### 2.11. Statistical analysis

The experimental results were independently in triplicate and expressed as the means  $\pm$  standard deviation (S.D.). Control groups and sample groups were compared by one-way analysis of variance (ANOVA). If there was a significant variation between the treatment groups, the Dunnett test was applied for RBL-2H3 cells. Also in rhinitis model, the Dunnett or Steel test was applied. Values of  $*P < 0.05$  and  $**P < 0.01$  were considered to indicate statistically significant differences. Control groups and a sample group were compared using unpaired Student t-tests for rhinitis model, and differences with  $^{##}P < 0.01$  were considered to indicate statistically significant differences.

## 3. Results

### 3.1. Quantification of kujigamberol in the methanol extract of Kuji amber

*(MEKA) and their activity against the mutant yeast.*

The content of kujigamberol in MEKA was calculated using a standard graph of the peak area of kujigamberol by HPLC (Fig. 1). MEKA contains kujigamberol at  $6.13 \pm 1.21$  mg/1 g of MEKA by HPLC. MEKA (100  $\mu$ g/ml) is equal to 0.6  $\mu$ g/ml (2.4  $\mu$ M) of kujigamberol. MEKA and kujigamberol showed a clear growth restored zone on the plate in a dose-dependent manner from 5 to 0.31  $\mu$ g/spot [Fig. 2(a)] and from 0.5 to 0.031  $\mu$ g/spot [Fig. 2(b)], respectively.

### 3.2. Inhibition activities of MEKA and kujigamberol against the degranulation and the $Ca^{2+}$ -influx in RBL-2H3 cells

$\text{Ca}^{2+}$ -signal transduction is involved in allergy [27], and MEKA and kujigamberol inhibited  $\text{Ca}^{2+}$ -signal transduction of the mutant yeast as shown by the growth-restoring activity (Fig. 2). Thus both were expected to have inhibition activity of degranulation and  $\text{Ca}^{2+}$ -influx in RBL-2H3 cells. MEKA showed potent inhibition activity against the degranulation of RBL-2H3 cells induced by Tg ( $\text{IC}_{50} = 15.0 \mu\text{g/ml}$ ) or A23187 ( $\text{IC}_{50} = 19.6 \mu\text{g/ml}$ ), not by Ag ( $\text{IC}_{50} > 50.0 \mu\text{g/ml}$ ) without cytotoxicity ( $\text{IC}_{50} > 50 \mu\text{g/ml}$ ), respectively [Fig. 3(a) and (b)]. Kujigamberol also showed potent inhibition activity against the degranulation of RBL-2H3 cells induced by Tg ( $\text{IC}_{50} = 29.1 \mu\text{M}$ ) or A23187 ( $\text{IC}_{50} = 24.9 \mu\text{M}$ ), not by Ag ( $\text{IC}_{50} > 50.0 \mu\text{M}$ ) without cytotoxicity ( $\text{IC}_{50} > 50 \mu\text{M}$ ), respectively [(Fig. 4(a) and (b)]

Additionally, MEKA inhibited  $\text{Ca}^{2+}$  mobilization in RBL-2H3 cells induced by Tg and A23187 stimulations about 100% at  $50 \mu\text{g/ml}$  without cytotoxicity. The inhibition activity by the Ag stimulation was weaker than them [Fig. 3(c)].  $\text{IC}_{50}$  values at 1000 sec were Ag ( $\text{IC}_{50} > 50.0 \mu\text{g/ml}$ ), Tg ( $\text{IC}_{50} = 13.6 \mu\text{g/ml}$ ) or A23187 ( $\text{IC}_{50} = 13.8 \mu\text{g/ml}$ ) and they were comparable to those of the inhibition activity of the degranulation. Kujigamberol inhibited  $\text{Ca}^{2+}$  mobilization in RBL-2H3 cells induced by Tg and A23187 stimulations about 100% at  $50 \mu\text{M}$  without cytotoxicity. The inhibition activity by the Ag stimulation was weaker than them [Fig. 4(c)].  $\text{IC}_{50}$  values at 1000 sec were Ag ( $\text{IC}_{50} > 50.0 \mu\text{M}$ ), Tg ( $\text{IC}_{50} = 10.6 \mu\text{M}$ ) or A23187 ( $\text{IC}_{50} = 6.0 \mu\text{M}$ ) and they were comparable to those of the inhibition activity of the degranulation.

### *3.3. Inhibition activities of MEKA and kujigamberol against the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and the $\text{LTC}_4$ production*

One of the important molecule in the signal transduction involving allergy is

ERK1/2 and the production of LTC<sub>4</sub> as a result [20]. MEKA and kujigamberol suppressed the phosphorylation of ERK1/2 in dose-dependent manners (IC<sub>50</sub> = 11.9 µg/ml and 19.5 µM, respectively) [Fig. 5(a)]. The activation of ERK1/2 related to generation of the LTC<sub>4</sub> and both inhibited the generation of LTC<sub>4</sub> (IC<sub>50</sub> = 7.9 µg/ml and 6.1 µM, respectively) [Fig. 5(b)]. These results suggested that MEKA and kujigamberol were expected to have anti-allergy activity against rhinitis model. The LTC<sub>4</sub> production was comparable to the inhibition of the phosphorylation of ERK1/2, Ca<sup>2+</sup>-influx and degranulation.

#### *3.4. Activities of MEKA and kujigamberol against the rhinitis model*

Nasal administration of ovalbumin solution to sensitized guinea pigs induced nasal blockade as an increase in sRaw. In the ovalbumin-exposed vehicle-treated group, the % increase in sRaw of immediate airway response at after 10 min was 574 ± 47% (Fig. 6a) and at after 1 h was 187 ± 26% (Fig. 6b), respectively. The sRaw values of MEKA (2 µg) and kujigamberol (2 µg) after 10 min were 152 ± 29% and 147 ± 31%, respectively (Fig. 6a). The sRaw values of MEKA and kujigamberol at after 1 h were 54 ± 15% and 72 ± 15%, respectively (Fig. 6b). Positive control, a steroid clinical drug, mometasone furoate (10 µg) showed sRaws as 165% ± 36% after 10 min (Fig. 6a) and 47 ± 16% after 1 h (Fig. 6b), respectively. Improvement activity of nasal blockade in guinea pigs showed almost the same activities between MEKA (2 µg), kujigamberol (2 µg) and mometasone furoate (10 µg). These results indicate that the activities of MEKA and kujigamberol are about 5 times stronger than that of a steroid clinical drug, mometasone furoate hydrate.

#### 4. Discussion

In the previous study, we isolated kujigamberol from Kuji amber by the screening using mutant yeast YNS17 strain. Since the YNS17 strain deleted a gene (*zds1*) involved in the  $\text{Ca}^{2+}$ -signal transduction, active compounds which show growth-restoring activity could be candidates for anti-diabetic or anti-allergic drugs, etc [2,3,12,14,16,28]. Kujigamberol has about 10 times more potent activity than that of MEKA against the mutant yeast and 5  $\mu\text{g}$  of MEKA contains only 0.031  $\mu\text{g}$  of kujigamberol by the HPLC analysis (Figs 1 and 2). Equal growth-restoring activity between 5  $\mu\text{g}$  of MEKA and 0.5  $\mu\text{g}$  of kujigamberol (Fig. 2) means that MEKA is about 15 times stronger than that of kujigamberol.

$\text{Ca}^{2+}$ -signal transduction is involved in allergy [3,27] and 3-cleroden-15-oic acid isolated from Dominican amber has growth restoring activity against the mutant yeast involving  $\text{Ca}^{2+}$ -signal transduction and prevented the degranulation of RBL-2H3 cells through the inhibition of  $\text{Ca}^{2+}$ -influx [16]. When an antigen forms crosslink of IgE-bound  $\text{Fc}\epsilon\text{RI}$ , tyrosine kinases such as Lyn or Syk are activated and then  $\text{Ca}^{2+}$ -influx is occurred.  $\text{Ca}^{2+}$ -influx induced by Ag, Tg or A23187 are caused degranulation and released inflammatory mediators such as histamine and leukotrienes [3,19]. Therefore, it was examined whether MEKA and kujigamberol also had an anti-allergic effect on RBL-2H3 cells which are used to study comprehensive events on mast cells induced by multivalent allergens. Both had inhibition activity against the degranulation in RBL-2H3 cells through the inhibition of  $\text{Ca}^{2+}$ -influx at microgram and micromole orders (Figs. 3 and 4). Inhibition activity of MEKA against degranulation by A23187 was about 50 times stronger than that of kujigamberol. As MEKA and kujigamberol suppressed degranulation induced by  $\text{Ca}^{2+}$ -influx stimulation,

their target(s) are likely to the  $\text{Ca}^{2+}$ -dependent pathway.

$\text{LTC}_4$  is one of the typical inflammatory mediators and is released by degranulation. Since  $\text{LTC}_4$  increases airway contraction and vascular permeability, it is closely related to asthma and nasal congestion [29].  $\text{LTC}_4$  is produced by phosphorylated ERK1/2 through the activation of the arachidonic acid cascade [30,31]. The  $\text{LTC}_4$  is a lipid mediator which is released by degranulation and causes allergy reaction, particularly nasal congestion and asthma through ERK1/2 phosphorylation [20,21,32-34]. MEKA and kujigamberol inhibited  $\text{LTC}_4$  production through the inhibition against ERK1/2 phosphorylation (Fig. 5). MEKA and kujigamberol potently suppressed  $\text{LTC}_4$  production, therefore, it is suggested that they have anti-allergy effect against the rhinitis model. Anti-allergic activity of MEKA and kujigamberol were measured using ovalbumin-induced rhinitis model of guinea pigs. As expected, both had potent anti-allergy effects against it and were about 5 times stronger than that of a steroidal clinical drug, mometasone furoate. The potency of MEKA is about 160 times higher than that of kujigamberol against the rhinitis model (Fig. 6). Those results suggest that MEKA includes minor potent biologically active compound(s) and/or other biologically active compound(s) having different mechanism of action (synergistic effect). MEKA and kujigamberol are expected to provide the basis for the development of a new types of anti-allergy drug. We will continue to isolate other biologically active compound(s) from MEKA and to clarify the mechanism of action of kujigamberol against the signal transduction of allergy.

## 5. Conclusion

In conclusion, the present study demonstrated that both MEKA and

kujigamberol that showed growth restoring activity against the mutant yeast involving  $\text{Ca}^{2+}$ -signal transduction inhibited degranulation and  $\text{Ca}^{2+}$ -influx in RBL-2H3 cells. They also suppressed the phosphorylation of ERK1/2 and generation of  $\text{LTC}_4$  in the cells and improved nasal congestion against the rhinitis model about 5 times stronger than that of mometasone furoate. Those results suggest that MEKA includes minor potent biologically active compound(s) and/or other biologically active compound(s) having different mechanism of action (synergistic effect). MEKA and kujigamberol are expected to provide the basis for the development of a new types of anti-allergy drug.

#### **Conflict of interest**

The authors declare that there are no conflicts of interest.

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**Figure legends**

**Figure 1.** Quantification of kujigamberol in the methanol extract of Kuji amber (MEKA) by HPLC analysis.

(a) Structure of kujigamberol (15,20-dinor-5,7,9-labdatrien-18-ol). (b) HPLC analytical pattern of kujigamberol (10 µg). (c) HPLC analytical pattern of MEKA (100 µg). Capcell pak C18 column (4.6 mm i.d. × 150 mm), MeOH:H<sub>2</sub>O = 80:20, 1 ml/min, PDA (205 nm). (d) Quantitative graph of kujigamberol. The quantity of kujigamberol in MEKA (c) was calculated by the area of the kujigamberol peak (a) on the HPLC chart.

**Figure 2.** The growth restored activity of the methanol extract of Kuji amber (MEKA) and kujigamberol against the mutant yeast (YNS17 strain) (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) in the presence of 0.3 M CaCl<sub>2</sub>.

(a) MEKA, 1: 5 µg/spot, 2: 2.5 µg/spot, 3: 1.25 µg/spot, 4: 0.63 µg/spot, 5: 0.31 µg/spot, 6: 0.16 µg/spot, 7: 2.5 ng/spot (FK506).

(b) Kujigamberol, 1: 0.5 µg/spot, 2: 0.25 µg/spot, 3: 0.125 µg/spot, 4: 0.063 µg/spot, 5: 0.031 µg/spot, 6: 0.016 µg/spot, 7: 2.5 ng/spot (FK506).

**Figure 3.** Anti-allergic effect of the methanol extract of Kuji amber (MEKA) against RBL-2H3 cells.

(a) Cell viability of RBL-2H3 cells by MEKA. Cell viability after 48 h was determined using the MTT assay.  $^{**}P < 0.01$ .

(b) Inhibition of degranulation against RBL-2H3 cells by the methanol extract of Kujigamber (MEKA). RBL-2H3 cells were sensitized with 0.5  $\mu\text{g/ml}$  IgE for 2 h, then added each sample for 30 min and stimulated with 10  $\mu\text{l}$  DNP-BSA (10  $\mu\text{g/ml}$ ) as an antigen for 10 min. RBL-2H3 cells were also stimulated with 10  $\mu\text{l}$  thapsigargin (2  $\mu\text{M}$ ) for 20 min or 10  $\mu\text{l}$  A23187 (20  $\mu\text{M}$ ) for 30 min without the sensitization by IgE. The inhibition (%) of  $\beta$ -hexosaminidase release by the sample was measured as an absorbance at 405 nm.

◆ : Ag, ■ : Tg, ▲ : A23187

(c) Inhibition of  $\text{Ca}^{2+}$  mobilization in RBL-2H3 cells by the methanol extract of Kujigamber (MEKA). The  $\text{Ca}^{2+}$  mobilization in RBL-2H3 cells was measured using Fluo 4-AM (2.5  $\mu\text{M}$ ) excited at 485 nm, and the fluorescence emission was observed at 535 nm in the presence of each stimulation.

— : 0  $\mu\text{g/ml}$  MEKA + stimulation,  
 — : 2.5  $\mu\text{g/ml}$  + stimulation, — : 10  $\mu\text{g/ml}$  + stimulation,  
 — : 50  $\mu\text{g/ml}$  + stimulation

**Figure 4.** Anti-allergic effect of kujigamberol against RBL-2H3 cells.

(a) Cell viability of RBL-2H3 cells by kujigamberol. Cell viability after 48 h was determined using the MTT assay.  $^{**}P < 0.01$ .

(b) Inhibition of degranulation against RBL-2H3 cells by kujigamberol. RBL-2H3 cells were sensitized with 0.5  $\mu\text{g/ml}$  IgE for 2 h, then added each sample for 30 min and stimulated with 10  $\mu\text{l}$  DNP-BSA (1.0  $\mu\text{g/ml}$ ) as an antigen for 10 min. RBL-2H3 cells were also stimulated with 10  $\mu\text{l}$  thapsigargin (2  $\mu\text{M}$ ) for 20 min or 10  $\mu\text{l}$  A23187 (20  $\mu\text{M}$ ) for 30 min without the sensitization by IgE. The inhibition (%) of  $\beta$ -hexosaminidase release by the sample was measure as an absorbance at 405 nm.

◆ : Ag, ■ : Tg, ▲ : A23187

(c) Inhibition of  $\text{Ca}^{2+}$  mobilization in RBL-2H3 cells by kujigamberol.

The  $\text{Ca}^{2+}$  mobilization in RBL-2H3 cells was measured using Fluo 4-AM (2.5  $\mu\text{M}$ ) excited at 485 nm, and the fluorescence emission was observed at 535 nm in the presence of each stimulation.

— : 0  $\mu\text{M}$  Kujigamberol + stimulation,  
— : 2.5  $\mu\text{M}$  + stimulation, — : 10  $\mu\text{M}$  + stimulation,  
— : 50  $\mu\text{M}$  + stimulation

**Figure 5.** The effects of the methanol extract of Kuji amber (MEKA) and kujigamberol on leukotriene production through the phosphorylation of ERK1/2 protein.

(a) Effects of MEKA and kujigamberol on the protein phosphorylation of ERK1/2.

RBL-2H3 cells were treated with MEKA and kujigamberol for 30 min at indicated doses and stimulated with 10  $\mu\text{l}$  A23187 (20  $\mu\text{M}$ ) for 30 min. Whole-cell lysates were subjected to western blot analysis. \*  $P < 0.05$ , \*\* $P < 0.01$ .

(b) Effects of MEKA or kujigamberol on the production of LTC<sub>4</sub>. RBL-2H3 cells were treated with MEKA or kujigamberol for 30 min and stimulated with 10 µl A23187 (20 µM) for 30 min. The inhibition (%) of LTC<sub>4</sub> production by the sample was measured as an absorbance at 405 nm. \*  $P < 0.05$ , \*\* $P < 0.01$ .

**Figure 6.** The effects of the methanol extract of Kuji amber (MEKA) and kujigamberol on an ovalbumin-induced rhinitis model.

Ovalbumin solution was nasally administered in sensitized guinea pigs to induce nasal blockade which was evaluated by measuring the specific airway resistance (sRaw). Immediate airway response is the value of “% increase in sRaw” at 10 min (a), and late airway response is the value of “% increase in sRaw” at 1 h (b). Each value represents the mean ± S.E.M. for six mice. \*  $P < 0.05$ , \*\* $P < 0.01$ .

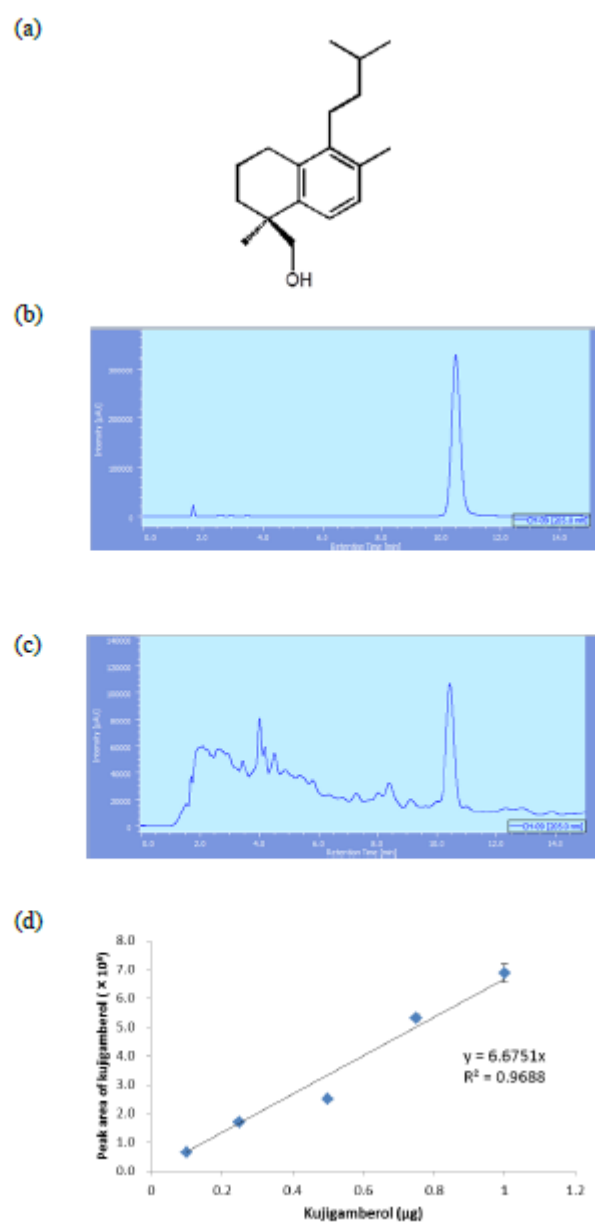


Figure 1.

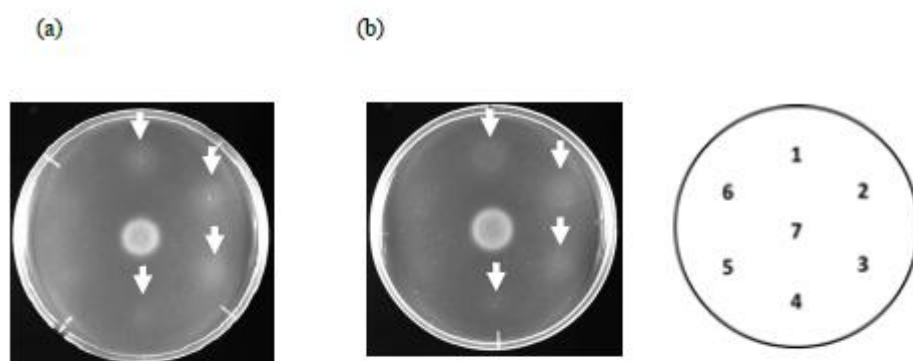
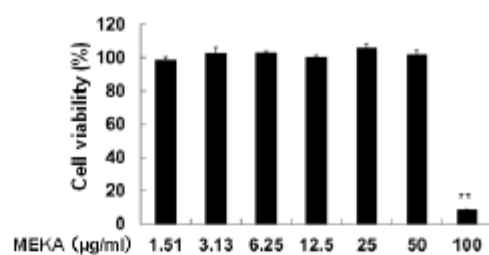
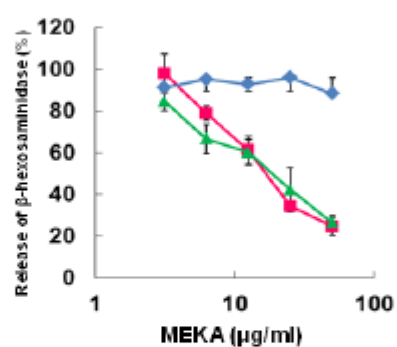


Figure 2.

(a)



(b)



(c)

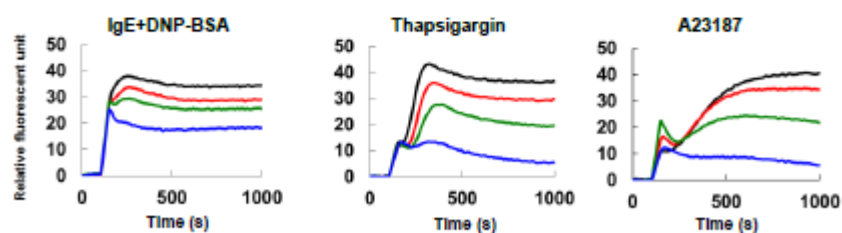
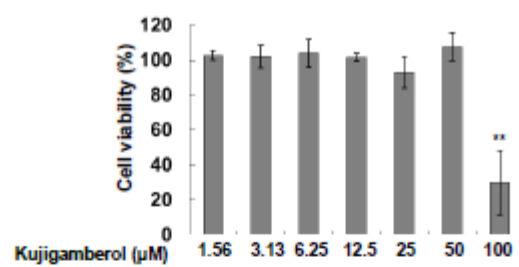
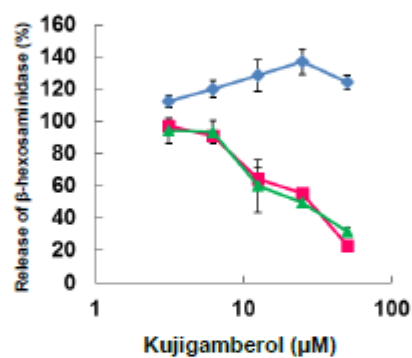


Figure 3.

(a)



(b)



(c)

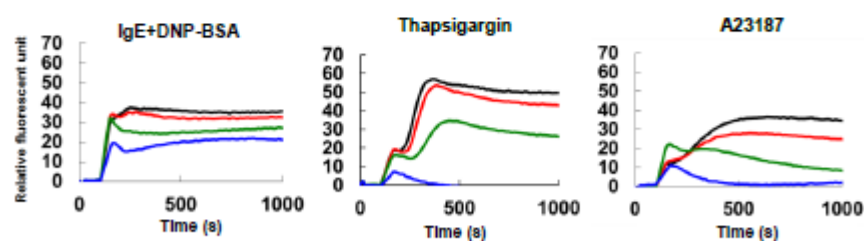
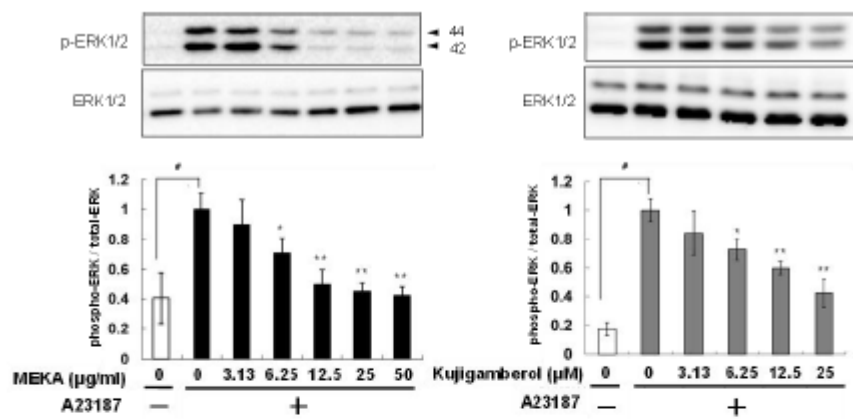


Figure 4.

(a)



(b)

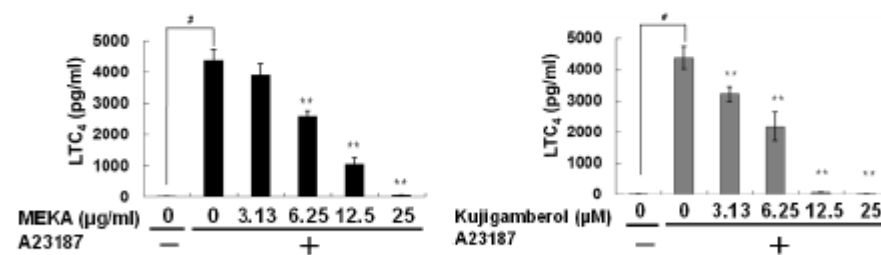


Figure 5.

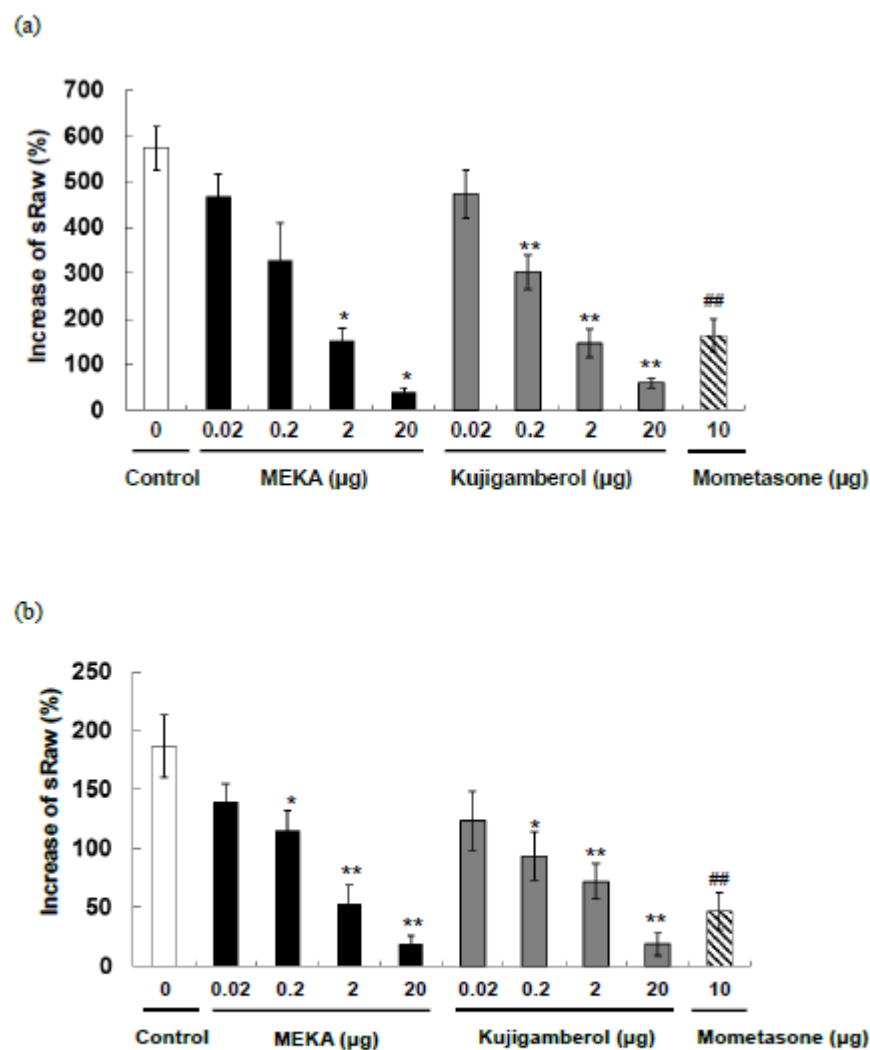
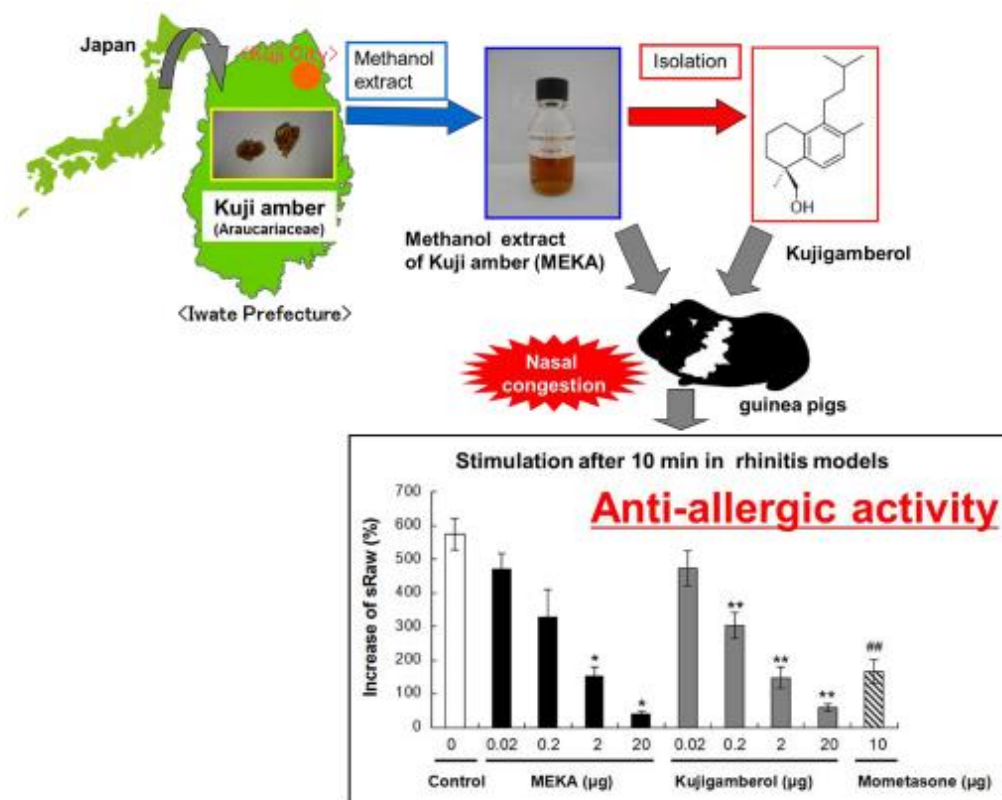


Figure 6.



Graphical abstract