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Inhibitory effects of sesquiterpene lactones isolated from *Eupatorium chinense* L. on IgE-mediated degranulation in rat basophilic leukemia RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice

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ABSTRACT

Sesquiterpene lactones (SQTLs) have been shown to suppress the degranulation as inferred by histamine release in rat basophilic leukemia RBL-2H3 cells. In this study, we isolated the 9 kinds of SQTLs from *Eupatorium chinense* L. and examined the effects of these SQTLs on the degranulation in RBL-2H3 cells. The chemical structures of two novel compounds (SQTL-3 and 8) were determined. All the SQTLs suppressed the degranulation from Ag-stimulated RBL-2H3 cells. To disclose the inhibitory mechanism of degranulation by SQTLs, we examined the activation of intracellular signaling molecules such as Lyn, Syk, and PLCγs and intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$). None of these SQTLs showed the activation of Syk and PLCγs. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) was elevated by FcεRI activation, but SQTLs treatment reduced the elevation of $[Ca^{2+}]i$ by suppressing Ca^{2+} influx. Thus, it was suggested that the suppression of Ag-stimulated degranulation by these SQTLs is mainly due to the decreased Ca^{2+} influx.

Furthermore, in order to clarify the in vivo effect of SQTL-rich extract, we administered SQTL-rich extract to the type I allergic model mice and measured the passive cutaneous anaphylaxis (PCA) reaction induced by IgE-antigen complex. The SQTLs remarkably suppressed PCA reaction in a dose-dependent manner. Thus, it was suggested that SQTLs would be a candidate as an anti-allergic agent.

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1. Introduction

Allergy is classified into five types: type I (anaphylactic type, e.g., Anaphylactic shock), type II (antibody-mediated cytotoxic type, e.g., Autoimmune hemolytic anemia), type III (immune complex type, e.g., lomerulonephritis), type IV (cellular immunity type, e.g., Tuberculin reaction), type V (stimulative type, e.g., Graves' disease). In type I allergy, binding of antigen (Ag) to the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils induces the release of intragranular mediators such as histamine, arachidonic acid metabolites, proteases, serotonin, heparin.[1–3] Thus, mast cell is plays a crucial role in the allergic reaction.

Eupatorium (syn. *Ayapana* Spach) is a genus of flowering plants containing 36–60 species, most of which are herbaceous perennial plants growing to 0.5–3 m tall, but a few are shrubs. Species of *Eupatorium* have been used for folk medicine, for instance, to excrete uric acid which causes gout, but they also contain toxic compounds that can cause liver damage. Recently, it has been reported that some species of *Eupatorium* have anti-bacterial,[4] anti-inflammatory,[5] anti-oxidant,[5] or anti-tumor activity.[6,7] We have also shown in previous reports that eupalinin A, a natural phytoalexin included in *Eupatorium chinense* L., induced cell growth inhibition and type II programmed cell death induction (autophagy) in HL60 cells.[8,9] Recently, it was also shown that sesquiterpenes suppressed the degranulation from mast cells.[10,11] In the present study, we have isolated 9 kinds of sesquiterpene lactones (SQTLs) from *Eupatorium chinense* L. and determined the chemical structures of two novel SQTLs. Moreover, we investigated the effect of these SQTLs on intracellular signaling pathways

Abbreviations: Ag, antigen; A23187, calcimycin calcium ionophore; Btk, Bruton's tyrosine kinase; CRAC channels, Ca^{2+} release-activated conducting Ca^{2+}; ERK, extracellular signal-regulated kinase; EGCG, (−)-epigallocatechin gallate; EGTA, *O,O′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IgE, immunoglobulin E; SAPK/JNK, stress-activated protein kinase/c-jun-N-terminal kinase; LTs, leukotriens; Lyn, Src family protein kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PVDF, polyvinylidene fluoride; Syk, Syk/Zap-70 family protein kinase, spleen tyrosine kinase.

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leading to degranulation in Ag-mediated rat basophilic leukemia RBL-2H3 cells. SQTLs significantly suppressed the elevation of intracellular free Ca^{2+} concentration ([Ca^{2+}]i). Furthermore, we demonstrated that administration of the SQTLs-rich extract to mice markedly suppressed the passive cutaneous anaphylaxis (PCA) reaction. Thus, these compounds would be candidates for the anti-allergic agents.

2. Results

2.1. Determination of chemical structures of novel sesquiterpene lactones (SQTL-3 and -8)

In this study, we have isolated nine kinds of SQTLs from *Eupatorium chinense* (Fig. 1A). Interestingly, SQTL-3 and -8 are newly defined compounds in this study. SQTL-1, 2, 4–7, and 9 were identified as follows, respectively; SQTL-1: eupalinin A, SQTL-2: eupalinin C, SQTL-4: (3*S*,6*R*,7*R*,8*R*)-3-hydroxy-8-sarracenyloxygermacra-1(10),4,11(13)-trien-6,12-olide, SQTL-5: deacetyleupaserrin,

SQTL-6: (3*R*,6*R*,7*R*,8*R*)-3-acetoxy-8-sarracenyloxyhelianga-1(10),4,11(13)-trien-6,12-olide, SQTL-7: (3*S*,6*R*,7*R*,8*R*)-3-hydroxy-8-acetoxysarracenyloxygermacra-1(10),4,11(13)-trien-6,12-olide, and SQTL-9: eupaformosanin.

SQTL-3 was isolated as a colorless oil and its specific rotation $[\alpha]_D^{25}$ was −116.0. The HR-EI-MS showed an M^+ ion peak at *m/z* 406.2005, indicating the molecular formula of $C_{22}H_{30}O_7$ (calcd 406.1992). The ^1H NMR spectrum (Table 1) showed a methyl [δ_H 1.83 (s, H_3-15)], a hydroxymethylene [δ_H 4.10, 4.62 (each m, H_2-14)], an *exo*-methylene [δ_H 5.97, 6.21 (each d, *J* = 2.0 Hz, H_2-13)], two methylenes [δ_H 2.06 and 2.44 (each m, H_2-2); 2.33 and 2.78 (each m, H_2-9)], three oxymethines [δ_H 5.20 (t, *J* = 3.2 Hz, H-3), 5.80 (d, *J* = 9.6 Hz, H-6), 5.27 (m, H-8)], a methine [δ_H 3.21 (m, H-7)], two olefins [δ_H 5.39 (m, H-1), 5.23 (d, *J* = 9.6 Hz, H-5)] together with an acetyl methyl [δ_H 2.07 (s)] and four protons assignable to a 2-methylbutyroyl group [δ_H 2.33 (m, H-2′), 1.42, 1.60 (each m, H_2-3′), 0.85 (t, *J* = 6.4 Hz, H_3-4′), 1.12 (d, *J* = 6.4 Hz, H_3-5′)]. Comparison of the ^1H and ^{13}C NMR data of SQTL-3 with those of schkuhrin I[12] and hiyodorilactone C acetate[13] suggested that SQTL-3 should be a heliangolide-type sesquiterpene lactone (Table 2). In the HMBC spectrum (Fig. 1B), H_3-15 was correlated with C-3, C-4 and C-5; whereas H_2-14 was correlated with C-1 and C-10. Therefore, the methyl and hydroxymethylene groups were substituted at C-4 and C-10, respectively. The acetoxyl group adhering at C-3 was determined by the HMBC correlation between H-3 (δ_H 5.20) and an acetyl carbonyl carbon at δ_C 169.9. There was no correlation between H-8 and C-1′ in the HMBC spectrum, however, the substitution of the 2-methylbutyroyloxy group at C-8 was confirmed by the downfield shift of C-8 resonated at δ_C 79.6. The relative stereochemistry was fixed by extensive analysis of NOESY correlations (Fig. 1B). The NOE correlations between H-7/H-5, H-5/H_3-15 and H_3-15/H-3 indicated that those protons were in α-orientation when H-7 was defined in the α-configuration, and *Z* geometry of the 4, 5-double bond. As a consequence, H-8 correlating with H-13 ($\Delta\delta_H$ 5.97) was assigned as α-orientation. The H-6 correlating with one of the H_2-14 (Δ_H 4.10) revealed 6β-orientation and *Z* geometry of 1,10-double bond. The structure of SQTL-3 was thus elucidated as (3*S*,6*R*,7*R*,8*R*)-3,14-dihydroxy-8-(2′-methylbutyroyloxy)-helianga-1(10),4,11(13)-trien-6,12-olide.

SQTL-8 was obtained as a colorless oil and gave a negative optical rotation ($[\alpha]_D^{25}$ −28.0). The protonated molecular ion was observed at *m/z* 363.1800 in the HR-FAB-MS, which suggested the quasimolecular formula to be $C_{20}H_{27}O_6$ (calcd 363.1795 for $[M+H]^+$). The ^1H NMR spectrum measured in $CDCl_3$ (Table 1) showed a methyl [δ_H 1.95 (3H, s)] and a set of *exo*-methylene [δ_H 5.59 and 6.24 (1H each, d, *J* = 2.9 Hz)], suggesting SQTL-8 should be a sesquiterpene lactone. The presence of an aldehyde group and a 2-methylbutyroyl ester was also indicated by the following signals; δ_H 9.42 (s, C*H*O), 0.87 (t, *J* = 7.8 Hz, H_3-4′), 1.11 (d, *J* = 7.2 Hz, H_3-5′), 1.47, 1.63 (each m, H_2-3′) and 2.33 (m, H-2'). The ^1H–^1H COSY and HMBC spectra (Fig. 1C) inferred that the methyl (δ_H 1.95) correlating with C-3, C-4 and C-5 was assignable to H_3-15, and the aldehyde proton (δ_H 9.42) correlating with C-1, C-9 and C-10 was adopted as H-14. Moreover, a carbonyl carbon (δ_C 175.3, C-1′) in the 2-methylbutyroyl group was correlated with an oxygenated methine proton (Δ_H 6.35, H-8), indicating that the ester moiety adhered to C-8. Consequently, the planar structure of SQTL-8 was deduced to be the same as eupachifolin A[14] isolated from *Eupatorium chinense* var. *simplicifolium*, although the NMR assignments did not resemble each other. Since the chemical shifts due to H-5 and H-6 of SQTL-8 were unfortunately overlapping in $CDCl_3$, SQTL-8 was alternatively measured in C_6D_6 and fully assigned by means of 2D-NMR techniques (Tables 1 and 2). In the NOESY spectrum (Fig. 1C), essential NOE correlations were observed between H_3-15/H-6, H-3/H-5 and H-1/H-14, inferring *E* geometries on both 1,10- and 4,5-double bonds for SQTL-8 opposite to 1(10)*E* and 4*Z* geometries for eupachifolin A. Hence, the structure of SQTL-8 was determined as (3*S*,6*R*,7*R*,8*R*)-3-hydroxy-8-(2'-methylbutyroyloxy)-14-oxomelampa-1(10),4-dien-6,12-olide.

2.2. Inhibition of Ag-stimulated β-hexosamonidase and histamine release by sesquiterpene lactones

To investigate the effects of the SQTLs on degranulation, we measured the release of β-hexosamonidase from Ag-stimulated RBL-2H3 cells. Release of β-hexosamonidase by DNP–BSA stimulation was inhibited to various extents by SQTLs (Fig. 2A). Of these compounds, SQTL-5, -7, -8, and -9 showed strong suppression of β-hexosamonidase and histamine release (Fig. 2A and B). A calcium ionophore A23187-induced β-hexosamonidase release was also inhibited by SQTL No. 5, -7, -8, and -9 (Fig. 2C). Thus, the inhibitory effects of degranulation by SQTLs were considered to be mainly due to the inhibition of Ca^{2+}-dependent degranulation process.

2.3. Inhibition of [Ca^{2+}]i elevation by sesquiterpene lactones

In order to investigate the mechanism underlying inhibition of degranulation by the SQTLs, we examined involvement of Ca^{2+}. Upon Ag-stimulation of FcεRI-activated of mast cells, the [Ca^{2+}]i level was rapidly increased and retained sustained levels thereafter (Fig. 3). However, SQTLs in the presence of the elevation of [Ca^{2+}]i was significantly suppressed. Especially, SQTL-5 exerted the strongest prevention of [Ca^{2+}]i elevation. As for SQTL-8 and -9, there were some significant inhibitory effects. In contrast, inhibitory effect of SQTL-7 was marginal.

2.4. Effect of sesquiterpene lactones on intracellular signaling pathways in Ag-stimulated RBL-2H3 cells

To gain further insight into the mechanism underlying inhibitory effect of degranulation by SQTLs, we examined the early intracellular signaling pathway. FcεRI cross-linking on mast cells has been reported to activate non-receptor-associated protein tyrosine kinase such as Lyn and Syk.[15,16] Firstly, we examined phosphorylation of Lyn, Syk, and PLC-γs which play important roles for degranulation response. As shown in Figure 4A, phosphorylation of Syk and PLCγ1/2 was not significantly affected by the SQTLs.

Next, we examined their effects of SQTLs on three major mitogen-activated protein kinases (MAPKs; ERK, JNK, p38). As shown in Figure 4B, phosphorylation of ERK1/2 and JNK1/2 induced by Ag-stimulation was not affected by SQTLs. On the other hand,

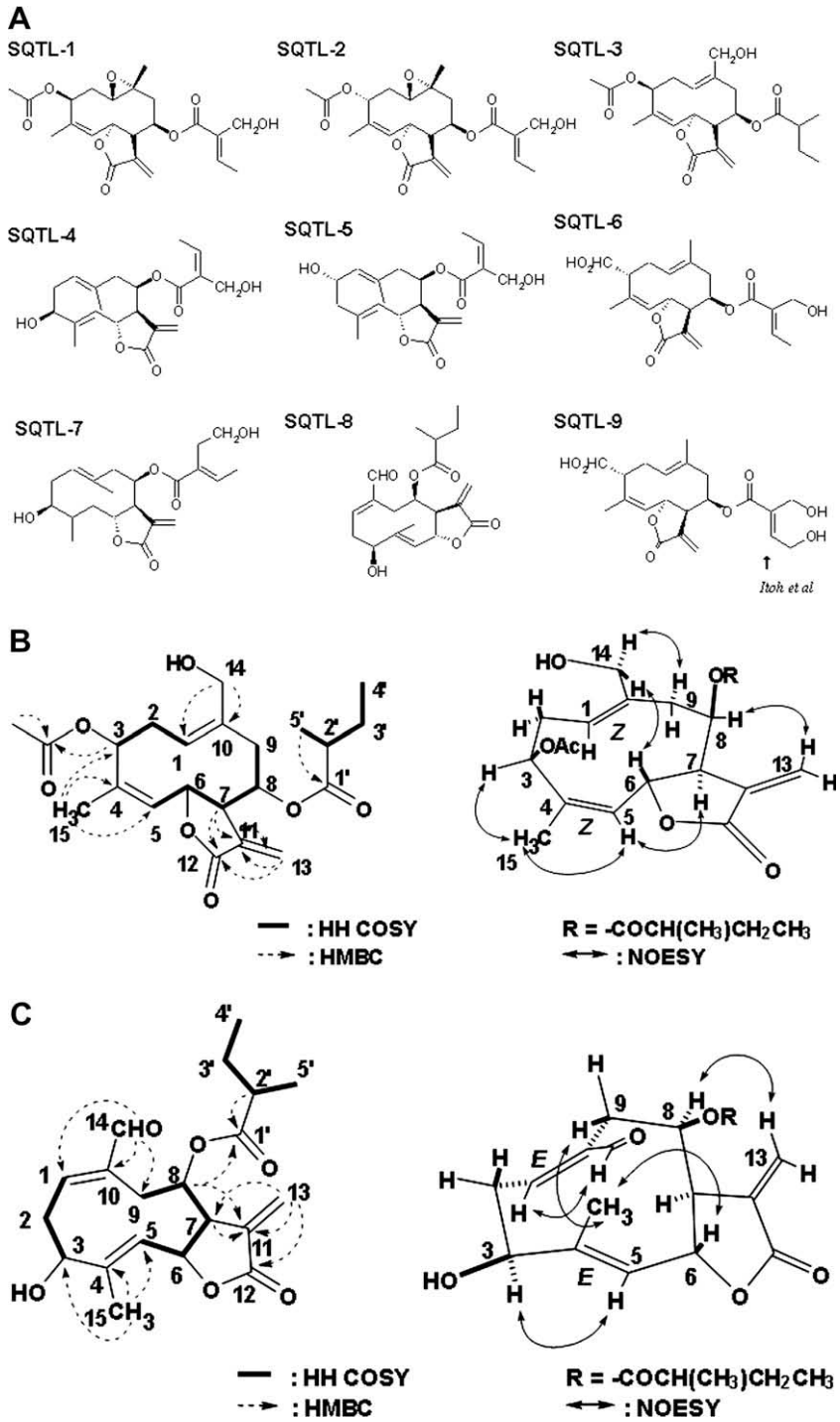

Figure 1. Chemical structures of sesquiterpene lactones which isolated from *Eupatorium chinense* L. (B) HMBC and NOESY correlations observed in SQTL-3. (C) HMBC and NOESY correlations observed in SQTL-8.

phosphorylation of p38 MAP kinase was moderately decreased by addition of SQTLs. Phospho-Akt/total-Akt ratio was suppressed by the SQTLs treatment.

2.5. Suppression of mouse passive cutaneous anaphylaxis (PCA) reaction by SQTL-rich extract from *Eupatorium chinense* L.

SQTL-rich extract from *Eupatorium chinense* L., which was extracted with acetone and concentrated, inhibited Ag-stimulated degranulation (IC 50 value: 22 μg/ml). To disclose the effect of the extract on type I allergy, we examined PCA reaction. SQTL-rich extract significantly suppressed mouse PCA reaction in a dose-dependent manner (Fig. 5A and B). As shown in Figure 5, PCA reaction was completely suppressed by the SQTL-administration of 200 mg and 300 mg/kg mouse body weight.

3. Discussion

In this study, we have shown the inhibitory effects on degranulation by SQTLs isolated from *Eupatorium chinense* L. in RBL-2H3 cells.

SQTLs suppressed the elevation of $[Ca^{2+}]i$ mediated by its influx from extracellular medium rather than the IP_3-mediated release of

Table 1
^{1}H NMR chemical shifts (δ) for SQTL-3 and -8

H	SQTL-3 ($CDCl_3$)	SQTL-8 ($CDCl_3$)	SQTL-8 (C_6D_6)
1	5.39 (m)	6.68 (t, 9.2)	5.61 (t, 7.7)
2	2.44 (m)	2.57 (2H, m)	2.61 (2H, m)
	2.06 (m)		
3	5.20 (t, 3.2)	4.30 (t, 6.8)	3.53 (dd, 11.1, 2.4)
5	5.23 (d, 9.6)	5.12 (m)[b]	4.52 (d, 10.8)
6	5.80 (d, 9.6)	5.12 (m)[b]	5.03 (t, 9.7)
7	3.21 (m)	2.41 (m)	2.00 (m)
8	5.27 (m)	6.35 (t, 8.0)	6.54 (t, 8.2)
9	2.78 (m)	2.78 (dd, 14.0, 8.0)	2.64 (dd, 16.4, 8.2)
	2.33 (m)[a]	1.92 (m)	2.04 (m)
13	6.21 (d, 2.0)	6.24 (d, 2.9)	6.14 (d, 3.3)
	5.97 (d, 2.0)	5.59 (d, 2.9)	5.34 (d, 3.3)
14	4.10 (m)	9.42 (s)	8.98 (s)
15	1.83 (3H, s)	1.95 (3H, s)	1.59 (3H, s)
2′	2.33 (m)[a]	2.33 (m)	2.09 (m)
3′	1.60 (m)	1.63 (m)	1.51 9m)
	1.42	1.47 (m)	1.26 (m)
4′	0.85 (3H, t, 6.4)	0.87 (3H, t, 7.8)	0.73 (3H, t, 7.7)
5′	1.12 (3H, d, 6.4)	1.11 (3H, d, 7.2)	0.91 (3H, d, 7.2)
Ac	2.07 (3H, s)		

[a,b] Overlapping signals.

Table 2
^{13}C NMR data for SQTL-3 and -8

C	SQTL-3 ($CDCl_3$)	SQTL-8 ($CDCl_3$)	SQTL-8 (C_6D_6)
1	127.3	150.9	150.2
2	29.8	35.1	34.8
	77.5	74.7	75.9[a]
3	137.1	139.8	139.2
5	127.8	123.6	124.1
6	76.4	75.1	75.9[a]
7	48.9	49.2	49.3
8	79.6	65.3	65.6
9	38.1	28.6	28.7
10	139.8	143.3	143.1
11	141.2	134.8	135.9
12	170.4	169.5	169.1
13	124.4	121.2	120.2
14	61.5	195.3	194.6
15	23.4	10.8	10.4
1′	175.4	175.3	174.7
2′	42.7	41.1	41.2
3′	27.5	26.7	27.1
4′	12.2	11.5	11.6
5′	17.1	16.8	16.9
Ac	169.9		
	21.5		

[a] Overlapping signals.

Ca^{2+} from endoplasmic reticulum. As for the elevation of $[Ca^{2+}]i$ regulator, Yoshimaru et al. have been reported that endogenous ROS plays as a Ca^{2+} regulator.[17–20] The intracellular ROS production was observed immediately after Ag-stimulation and was most likely due to an NADPH oxidase.[21,22] In order to reveal the production of intracellular ROS, we measured it by using CM-H_2DCF-DA fluorescent probe. DCF oxidation was gradually increased by Ag-treatment. SQTLs were also found to show similar results as shown in Ag-treatment (data not shown). We also measured the radical-scavenging activity of SQTLs by DPPH radical-scavenging method. These SQTLs did not exhibit radical-scavenging activity. Accordingly, the intracellular ROS was thought not to be associated with the suppression of Ca^{2+} influx by SQTLs. Therefore, it was thought that SQTLs suppress Ca^{2+} influx through the other mechanism.

Recent studies reported that Syk-deficient mast cells completely abrogated the degranulation, elevation of $[Ca^{2+}]i$, and activation of the ERK and JNK MAP kinase pathways.[23,24] Akt is regulated by Lyn/Syk/Btk.[25] These observations indicated that Syk is essential for the degranulation signal transduction. However, SQTLs did not suppress the Ag-mediated Syk activation. Therefore,

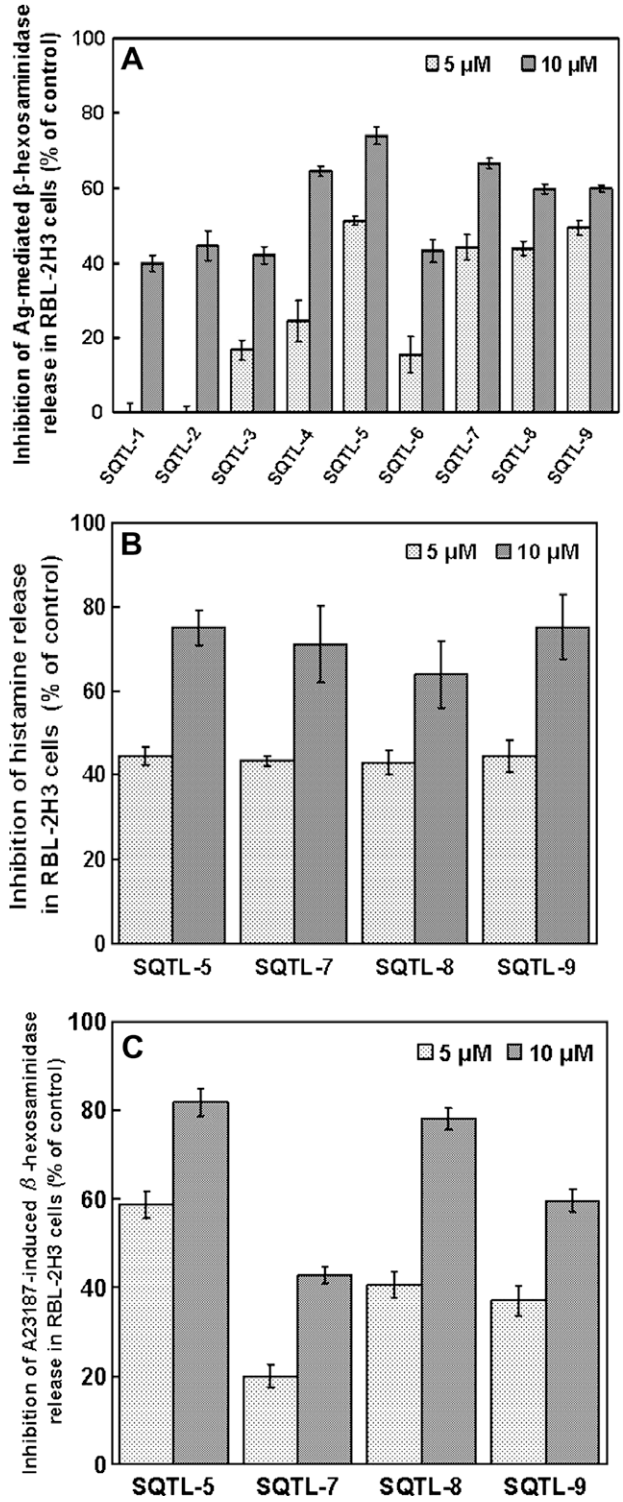

Figure 2. Inhibitory effects of the sesquiterpene lactones on Ag-stimulated degranulation from rat basophilic leukemia RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were stimulated with DNP–BSA in the presence of the each sesquiterpene lactone (SQTL). As a marker of degranulation, we measured the release of β-hexosaminidase (A) and histamine (B). (C) RBL-2H3 cells were stimulated with A23187 is in the presence of each sesquiterpene lactone (SQTL). Values are mean ± SEM ($n = 10$) of the inhibition of degranulation release. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance followed by Fisher's multiple range test).

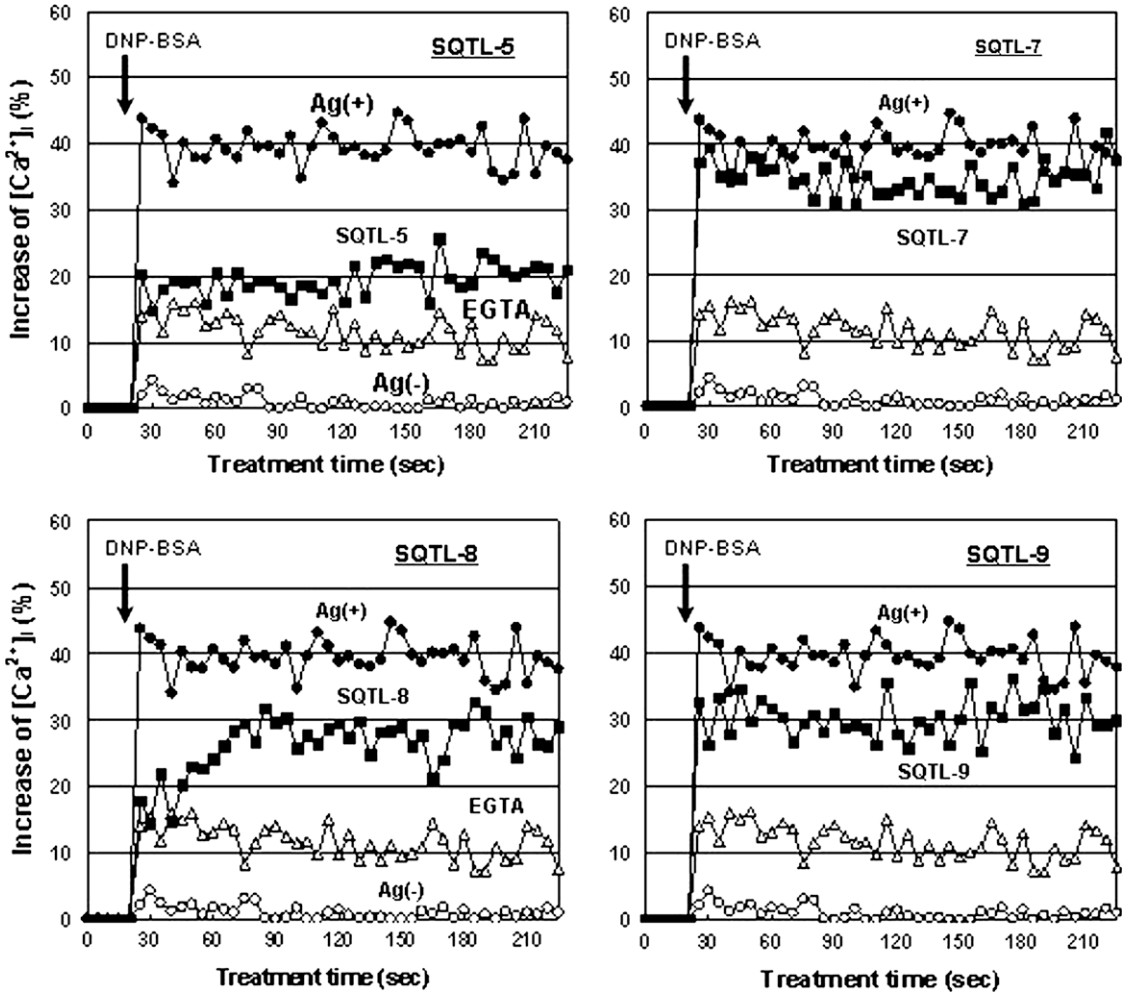

Figure 3. Suppression of $[Ca^{2+}]i$ elevation by SQTLs in Ag-stimulated RBL-2H3 cells. Ag-stimulated RBL-2H3 cells were stimulated with either DNP–BSA in loading buffer, DNP–BSA in loading buffer including SQTLs, or DNP–BSA in calcium-free loading buffer including EGTA (1 mM; selective Ca^{2+} chelating reagent) for indicated periods. Intracellular calcium was measured as described in Section 4. Each value represents the mean with SEM (n = 32). ●: Ag-stimulated, ○: Ag-untreated, □: Ag plus EGTA-treated, ■: Ag plus each SQTL-treated.

SQTLs do not affect Lyn/Syk/Btk pathway. Furthermore, SQTLs significantly suppressed Akt activation. It has been reported that PLCγ1 is regulated by PI3K.[26,27] We also indicated in our previous study that LY294002, a PI3K specific inhibitor, blocked the degranulation, but MAP kinase pathway had no effect.[28] In our present study, activation of PLCγ1 was not seen by SQTLs.[28] Thus, it seems likely that SQTLs may directly inhibit Akt activation.

Taken together, although we need further study to clarify the mechanism underlying inhibition of degranulation by the SQTLs, it was considered that the suppression of Ca^{2+} influx play as a major mechanism of the inhibition of degranulation (Fig. 6). Moreover, it was to be noted that SQTL-rich extract suppressed type I allergy reaction in mouse model, suggesting that SQTL-rich extract would be a potential candidate as an effective agent for type I allergy.

4. Materials and methods

4.1. Extraction and isolation of sesquiterpene lactones

The dried aerial parts (1.8 kg) of *Eupatorium chinense* were extracted with acetone at room temperature. The filtered solution was concentrated in vacuo to yield an extract (94.1 g: SQTL-rich extract), which was subjected to CC (Chromatorex DMS; MeOH–H_2O 3:7→5:1) to afford nine main fractions (Fr. 1–9). Fr. 4 (9.7 g) was separated by CC (Silica Gel 60; $CHCl_3$–acetone 40:1→1:1) to yield, after further purification by CC (Sephadex LH-20; MeOH), compounds **1** (56.6 mg),[29] **2** (97.9 mg),[29] **3** (35.0 mg), **4** (14.0 mg)[30] and **5** (26.8 mg).[31] Fr. 6 (10.5 g) was applied to CC (Silica Gel 60; *n*-hexane–acetone 10:1→1:1) to obtain compounds **6** (6.0 mg),[32] **7** (21.2 mg),[32] **8** (29.6 mg) and **9** (20.1 mg).[33]

4.2. Reagents and materials

Monoclonal mouse IgE anti-dinitrophenol (DNP) was purchased from Yamasa Co. Ltd (Tokyo, Japan). The 25 × Complete®, a mixture of protease inhibitors mixture was from Roche (Penzberg, Germany). The phosphatase Inhibitor Cocktail® 1 and 2 was from Sigma (St. Louis, Mo, USA). The antibodies to anti-rat p44/42 MAP Kinase (ERK), anti-rat phospho-p44/42 MAPK (Thr202/Tyr204) (*p*-ERK), anti-rat SAPK/JNK (JNK), anti-rat phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), anti-rat p38 MAP kinase (p38), anti-rat phospho-p38 MAP Kinase (Thr180/Tyr182) (p-p38), anti-rat Lyn, anti-rat phospho-Lyn, anti-rat $cPLA_2$, anti-rat phosphor-$cPLA_2$, anti-rat phosphor-PLCγ1, anti-rat phosphor-PLCγ2, and anti-phospho-tyrosine (pTyr) were from Cell Signaling Technology (MA, USA). The antibodies to anti-rat Syk were from Santa Cruz Biotechnology (CA, USA). The antibodies to anti-rat β-actin were

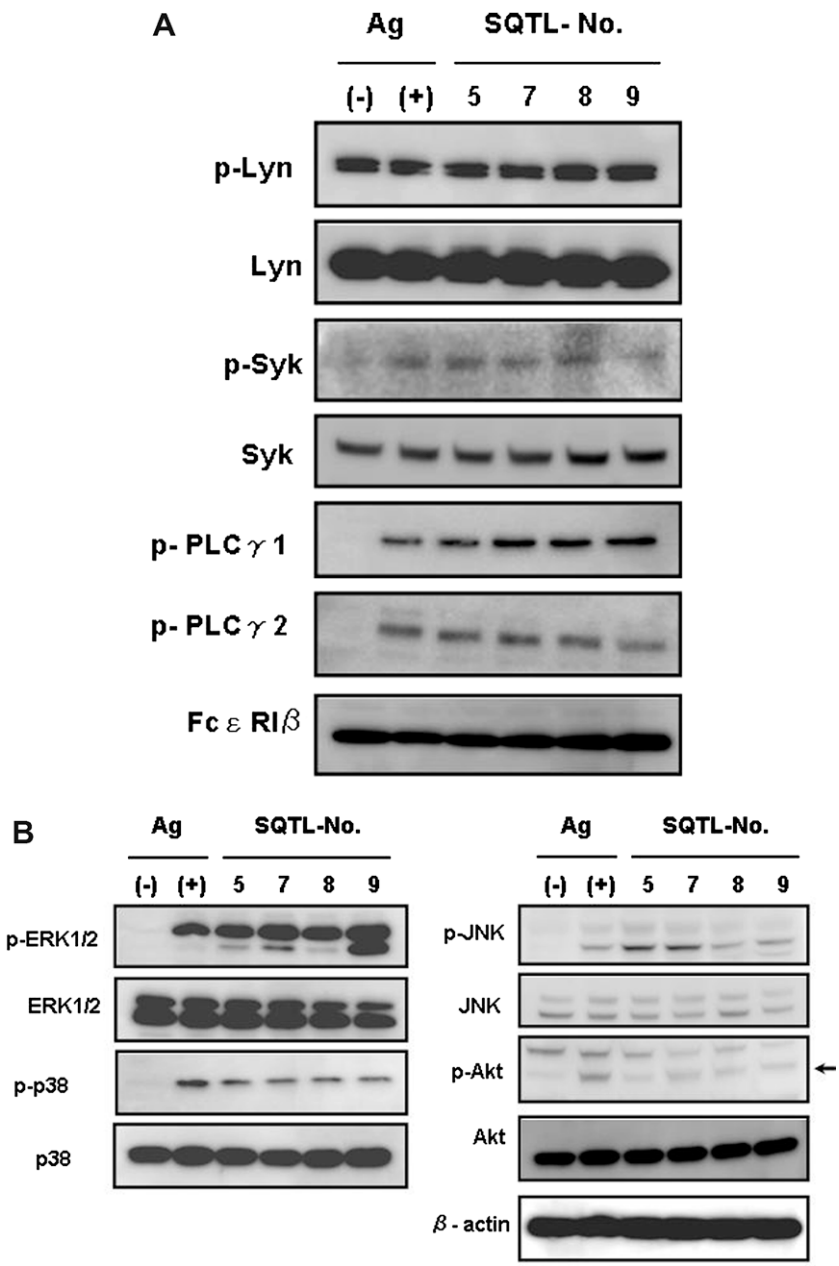

Figure 4. Effect of SQTLs on the signaling pathway in Ag-stimulated RBL-2H3 cells. SQTLs-treated cells were stimulated with DNP–BSA for 10 min and were resuspended in RIPA buffer. Twenty micrograms of protein of each cell lysate was separated by SDS–PAGE and electroblotted onto a PVDF membrane for Western blotting. (A) Phosphorylation of Syk, Lyn, and PLCγs. (B) Phosphorylation of ERK1/2, JNK1/2, p38 MAPkinase, PI3 K/Akt, and $cPLA_2$ in Ag-mediated activation.

from Sigma. FcεRIβ antibody was kindly provided by Dr. J. Rivera (NIH, molecular immunology and inflammation branch). Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from GE Healthcare Sci. (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, England). The other reagents were purchased from standard sources and were of molecular biology grade or higher.

4.3. Cell culture

RBL-2H3 cells were obtained from Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum essential medium (Gibco, MD, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

4.4. β-Hexosaminidase and histamine release assay

As a marker of degranulation, we measured the release of β-hexosaminidase and histamine.[34] RBL-2H3 cells were inoculated into 24-well plates (5×10^5 cells/ml, 400 μl/well; Nunc, Roskilde Denmark) and cultured for 1 h. Then, monoclonal mouse IgE anti DNP was added to cultured medium at a concentration of 0.45 μg/ml and incubated for 24 h. Treated cells were washed twice by Siraganian buffer (in mM: NaCl 119, KCl 5, $MgCl_2$ 0.4, PIPES 25, NaOH 40, pH 7.2) and them added 160 μl of Siraganian buffer containing 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA. After incubation at 37 °C for 10 min, the IgE-sensitized cells were treated with 5 μM or 10 μM of SQTLs (20 μl) at 37 °C for 30 min. Subsequently, 20 μl of DNP-labeled bovine serum albumin (DNP–BSA) was added to cultured medium at a concentration of 10 μg/ml

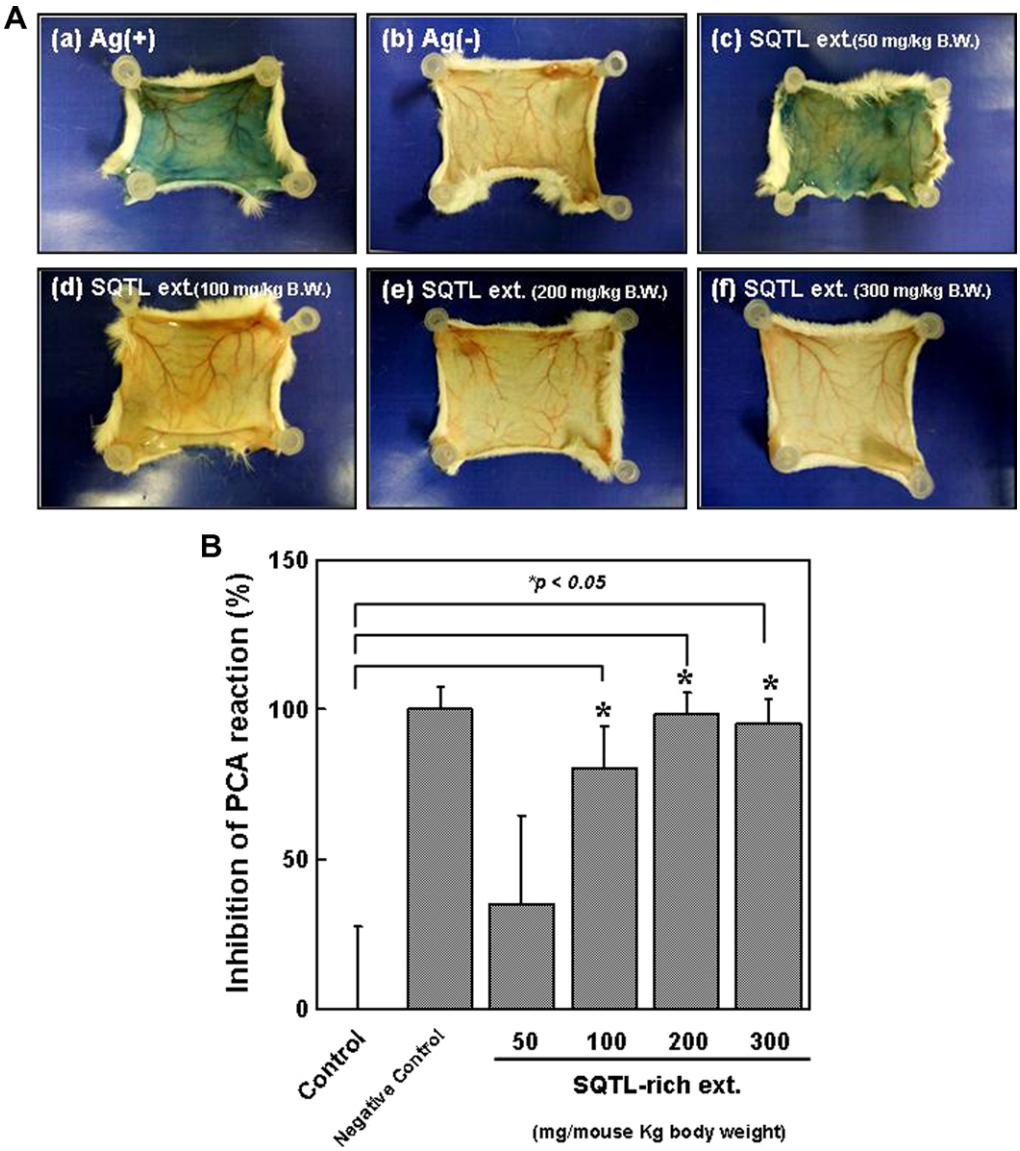

Figure 5. Effect of SQTL-rich extract on mouse passive cutaneous anaphylaxis reaction induced by IgE-antigen complex. (A) Imaging of extravasated Evans blue on dorsal skin. (a) antigen-stimulated, (b) antigen-untreated, (c) antigen plus SQTL-rich extract (50 mg/kg mouse body weight (B. W.)), (d) antigen plus SQTL-rich extract (100 mg/kg mouse B. W.), (e) antigen plus SQTL-rich extract (200 mg/kg mouse B. W.), (f) antigen plus SQTL-rich extract (300 mg/kg mouse B. W.). (B) The amount of extravasated Evans blue dye from dorsal skin by SQTL-rich extract administration. To treated skin solution was added 4 ml of a mixture of acetone and 0.2 M phosphoric acid (13:5) and extracted the extravasated Evans blue dye. The absorbance was measured with a colorimetric microplate reader at 620 nm.

and was incubated for 10 min. To terminate the reaction, the treated cells were stayed on ice for 10 min and then centrifuged at 300×g at 4 °C for 10 min.

To measure β-hexosaminidase release, the Ag-stimulated Siraganian buffer (50 μl) was transferred into a 96-well multimicroplate, and then incubated with 50 μl of 0.1 M citrate buffer (pH 4.5) including 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide at 37 °C for 1 h. One hour later the reaction denaturized by stopped buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a colorimetric microplate reader at 405 nm.

To measure histamine release, 75 mg of NaCl and 50 μl of 1 N NaOH, 500 μl of a mixture 3:2 (v/v) of n-butanol and chloroform was added to 200 μl of Ag-stimulated Siraganian buffer and mixed for 5 min. The organic layer was recovered and mixed with 15 μl of 1 N NaOH and 10 μl of 0.2% *o*-phthalaldehyde, and stayed for 5 min at room temperature. This reaction terminated by adding 15 μl of 0.5 N H_2SO_4, and then the fluorescence intensity was measured by fluorocount microplate reader (MTP-600F, CORONA ELECTRIC Co. Ltd, Hitachinaka, Japan, excitation wavelength 360 nm, emission wavelength 450 nm). The percentage inhibition of histamine release was calculated as follows: inhibition of histamine release (%) = [1 − (test − negative control)/(positive control − negative control)] × 100.

4.5. Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} level was determined with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells (5×10^4 cells/well) were seeded into 94-black walled-bottom clear micro plates and incubated for 1 h. After incubation, monoclonal mouse IgE anti DNP was added to cultured medium at a concentration of 0.45 μg/ml and incubated for 24 h. The IgE-sensitized cells were washed twice with PBS and incubated with 100 μl of loading buffer containing Fluo-3AM (Calcium Kit-Fluo 3™) for 1 h. The treated cells were washed with PBS and incubated with 90 μl of loading buffer (Calcium Kit-Fluo 3™) including 10 μM of SQTLs for

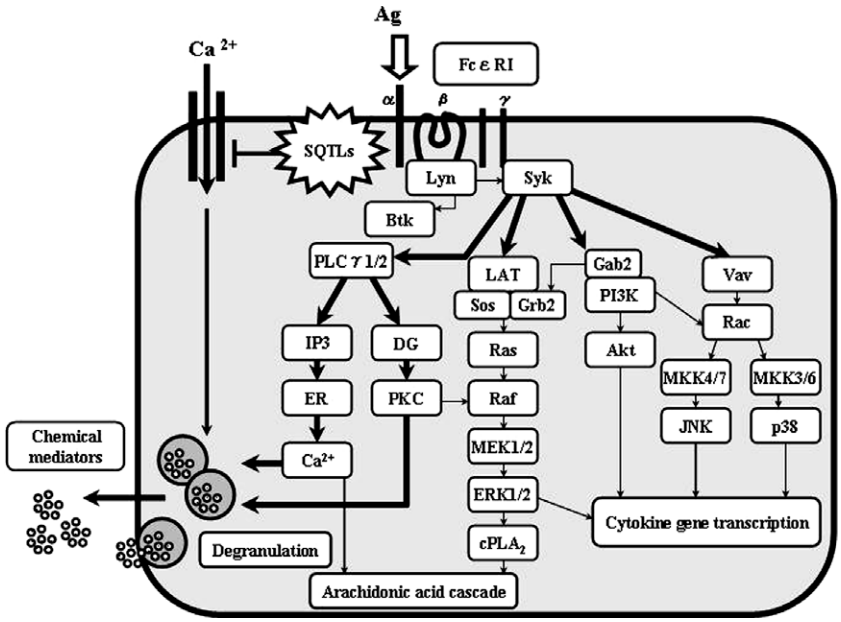

Figure 6. A scheme showing the inhibitory effect on degranulation by the sesquiterpene lactones in RBL-2H3 The inhibition of Ag-stimulated degranulation by SQTLs was mainly due to suppress the $[Ca^{2+}]i$ elevation.

30 min. Changes in intracellular Ca^{2+} concentration induced by DNP–BSA (10 μg/ml) were measured with excitation at 490 nm and emission at 530 nm a fluorometric imaging plate reader.

4.6. Measurement of intracellular ROS level by CM-H_2DCF-DA fluorescent probe

Amount of intracellular ROS was measured by using 5-(and-6-)-carboxy-2′, 7′-dichlorodihidrofluorescein diacetate (CM-H_2DCF-DA). CM-H_2DCF-DA is a fluorogenic freely permeable tracer specific for ROS assessment. It is deacetylated by intracellular esterases to the non-fluorescent 2′,7′-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by ROS. The IgE-sensitized RBL-2H3 cells (5×10^4 cells/well) were incubated with 10 μM CM-H_2DCF-DA for 30 min at 37 °C. Cells were washed twice with PBS to remove the excess of CM-H_2DCF-DA. CM-H_2DCF-DA-loaded cells were incubated with 10 μM SQTLs for 30 min. CM-H_2DCF-DA-SQTL treated cells were stimulated by DNP–BSA (10 μg/ml) and then measured with excitation at 490 nm and emission at 530 nm with a fluorometric imaging plate reader.

4.7. Measurement of DPPH radical-scavenging activity

To measure antioxidant activity, a DPPH radical-scavenging assay was carried out according to the previous method with a slight modification.[35] Briefly, the DPPH radical-scavenging activity was measured in a reaction mixture containing 0.5 mM DPPH radical solution 0.1 ml, 99% ethanol 0.8 ml, and 0.1 ml of SQTL solutions (10 μM). The solution was rapidly mixed and the scavenging capacity was measured by monitoring the decrease in absorbance at 517 nm. Vitamin C (l-ascorbic acid) and vitamin E (α-tocopherol), and EGCG were used as positive controls.

4.8. Immunoprecipitation and immunoblot analysis

For preparation of cell lysate, RBL-2H3 cells were washed twice with PBS and harvested. The cell pellet was resuspended in RIPA buffer containing 25 × Complete®, and Phosphatase Inhibitor Cocktail® (Roche). Protein content was measured with a DC Protein assay kit (BIO RAD, Hercules, CA). Whole cell lysates were incubated with Protein A agarose beads (Roche) over night at 4 °C. After centrifugation, cell lysate were incubated with protein A agarose beads bound with the pTyr antibody for 3 h at 4 °C. The beads were washed 3 times with lysis buffer, and then resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer containing 2% 2-mercaptoethanol, and boiled for 5 min. The beads were removed by centrifugation, and supernatants were subjected to Western blot analysis. After blockage of nonspecific binding sites for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4 °C with Syk antibody. Moreover, other proteins (20 μg of protein of each cell lysate) were separated by SDS–PAGE and electroblotted onto a PVDF membrane (Du Pont, Boston, MA). The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody or anti-rabbit antibody at room temperature, and then washed three times with TPBS. Proteins were detected with enhanced ECL kit and chemiluminescence detector (LAS-1000, Fuji, Japan).

4.9. Animals and diets

Four-week-old ICR male mice (Japan SLC, Ltd, Hamamatsu, Japan) were housed in plastic cages in an air-conditioned room (22 ± 2 °C) with a 12-h light and dark cycle (lighting from 07:00 to 19:00). All mice were fed commercial CE-2 pellets (Clea Japan, Inc., Tokyo) and water ad libitum for one week to accustom them to the surroundings. This study was approved by the Gifu International Institute of Biotechnology Animal Use Committee, and the animals were maintained according to the guidelines of Gifu International Institute of Biotechnology for the care of laboratory animals.

4.10. Measurement of mouse passive cutaneous anaphylaxis (PCA) reaction

An IgE-induced passive cutaneous anaphylaxis reaction was measured according to the previous method of Choo et al.[36] with

a slight modification. The ICR mice were shaved back hair, and then injected 10 μg of anti-DNP–BSA into each two dorsal skin sites. The sites were outlined with a water-insoluble marker. After 24 h, each mouse was treated with 100 μl of corn oil or acetone extract in 100 μl (100, 200, 300 mg/kg body weight) by oral administration. One hour after the corn oil or SQTL-rich extract administration, each mouse received an injection of 200 μl of DNP–BSA of 1% Evans blue (SIGMA) in PBS, containing 200 μg of DNP–BSA. One hour later treated mice were sacrificed and removed their dorsal skin. The skins (1 cm × 1 cm) were dissolved with 1 N KOH at 37 °C for 24 h and the extravasated Evans blue dye extracted with mixture of acetone and 0.2 M phosphoric acid (13:5) from treated skin solution. The amount of dye was determined colorimetrically at 620 nm.

4.11. Statistical analysis

All data were analyzed first by one-way ANOVA, and subsequently by Fisher's-multiple range test. The differences among the means were considered significant at $p < 0.05$.

5. Conclusions

In this study, we have demonstrated that sesquiterpene lactones (SQTLs) isolated from *Eupatorium chinense* L., significantly suppressed the degranulation in Ag-mediated activation of FcεRI in RBL-2H3 cells. It was thus suggested that inhibition of degranulation by the SQTLs was mainly due to inhibition of the $[Ca^{2+}]i$ elevation. To be of great interest to note, the administration of SQTLs significantly inhibited the passive cutaneous anaphylaxis (PCA) reaction induced by IgE-antigen complex. Thus, SQTLs

would be an effective agent for anti-allergy.

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References and notes

1. Beaven, M. A.; Metzger, H. *Immunol. Today* **1993**, *14*, 222.
2. Turner, H.; Kinet, J. P. *Nature* **1999**, *402*, 24.
3. Mekori, Y. A.; Metcalfe, D. D. *Immunol. Rev.* **2000**, *173*, 131.
4. Sasikumar, J. M.; Doss, A. P.; Doss, A. *Fitoterapi* **2005**, *76*, 240.
5. de las Heras, B.; Slowing, K.; Bendi, J.; Catretero, E.; Ortega, T.; Toledo, C.; Bremejo, P.; Iglesias, I.; Abad, M. J.; Gomez-Serranillos, P.; Liso, P. A.; Villar, A.; Chiriboga, X. *Ethnopharmacology* **1998**, *61*, 161.
6. Yan, S. P.; Huo, J.; Wang, Y.; Lou, L. G.; Yue, J. M. *J. Nat. Prod.* **2004**, *67*, 638.
7. Shen, Y. C.; Lo, K. L.; Kuo, Y. H.; Khalil, A. T. *J. Nat. Prod.* **2005**, *68*, 745.
8. Itoh, T.; Ito, Y.; Ohguchi, K.; Ohyama, M.; Iinuma, M.; Otsuki, M.; Nozawa, Y.; Akao, Y. *Bioorg. Med. Chem.* **2008**, *16*, 721.
9. Itoh, T.; Ohguchi, K.; Nozawa, Y.; Akao, Y. *Anticancer Res.* **2009**, in press.
10. Morikawa, K.; Matsuda, H.; Toguchida, I.; Ueda, K.; Yoshikawa, M. *J. Nat. Prod.* **2002**, *65*, 1468.
11. Miyata, N.; Gon, Y.; Nonomura, S.; Endo, D.; Yamashita, K.; Matsumoto, K.; Hashimoto, S.; Ra, C. *Int. Immunopharmacol.* **2008**, *8*, 874.
12. Pettei, M. J.; Miura, I.; Kubo, I.; Nakanishi, K. *Heterocycles* **1978**, *11*, 471.
13. Fang, N.; Mabry, T. J. *Phytochemistry* **1988**, *27*, 283.
14. Ito, K.; Sakakibara, Y.; Haruna, M. *Chem. Lett.* **1979**, *12*, 1473.
15. Siraganian, R. P.; Zhang, J.; Kimura, T. Regulation and function of protein tyrosine kinase Syk in FcεRI-mediated signaling. In *Signal Transduction in Mast Cells and Basophils*; Razin, E., Rivera, J., Eds.; Springer: Berlin, Germany, 1999; pp 115–133.
16. Kawakami, Y.; Kitaura, J.; Satterthwaite, A. B.; Kato, R. M.; Asai, K.; Hartman, S. E.; Maeda-Yamamoto, M.; Lowell, C. A.; Rawlings, D. J.; Witte, O. N.; Kawakami, T. *J. Immunol.* **2000**, *165*, 1210.
17. Yoshimaru, T.; Suzuki, Y.; Matsui, T.; Yamashita, K.; Ochiai, T.; Yamaki, M.; Shimizu, K. *Clin. Exp. Allergy* **2002**, *32*, 612.
18. Suzuki, Y.; Yoshimaru, T.; Inoue, T.; Niide, O.; Ra, C. *Chem. Immunol. Allergy* **2005**, *87*, 32.
19. Heneberg, P.; Draber, P. *Curr. Med. Chem.* **2005**, *12*, 1859.
20. Inoue, T.; Suzuki, Y.; Yoshimaru, T.; Ra, C. *Biochim. Biophys. Acta* **2008**, *1783*, 789.
21. Suzuki, Y.; Yoshimaru, T.; Matsui, T.; Inoue, T.; Niide, O.; Nunomora, S.; Ra, C. *J. Immunol.* **2003**, *171*, 6116.
22. Swindle, E. J.; Metcalfe, D. D. *Immunol. Rev.* **2007**, *217*, 186.
23. Costello, P. S.; Turner, M.; Walters, A. E.; Cunningham, C. N.; Bauer, P. H.; Downward, J.; Tybulewics, V. L. *Oncogene* **1996**, *13*, 2595.
24. Zhang, J.; Berenstein, E. H.; Evans, R. L.; Siraganian, R. P. *J. Exp. Med.* **1996**, *184*, 71.
25. Kitaura, J.; Asai, K.; Maeda-Yamamoto, M.; Kawakami, Y.; Kikkawa, U.; Kawakami, T. *J. Exp. Med.* **2000**, *192*, 729.
26. Barker, S. A.; Caldwell, K. K.; Hall, A.; Martinez, A. M.; Pfeiffer, J. R.; Oliver, J. M.; Wilson, B. S. *Mol. Cell Biol.* **1995**, *6*, 1145.
27. Barker, S. A.; Caldwell, K. K.; Pfeiffer, J. R.; Wilson, B. S. *Mol. Cell Biol.* **1998**, *9*, 483.
28. Ito, T.; Ohguchi, K.; Iinuma, M.; Nozawa, Y.; Akao, Y. *Bioorg. Med. Chem.* **2008**, *16*, 4500.
29. Ito, K.; Sakakibara, Y.; Haruna, M.; Lee, K. H. *Chem. Lett* **1979**, *12*, 1469.
30. Gershenzon, J.; Mabry, T. J. *Phytochemistry* **1984**, *23*, 1959.
31. Kupchan, M. S.; Fujita, T.; Maruyama, M.; Britton, R. W. *J. Org. Chem.* **1973**, *38*, 1260.
32. Gil, R. R.; Pacciaroni, A. D. V.; Oberti, J. C.; Diaz, J. G.; Herz, W. *Phytochemistry* **1992**, *31*, 593.
33. Lee, K. H.; Kimura, T.; Haruna, M.; McPhail, A. T.; Onan, K. D.; Huang, H. C. *Phytochemistry* **1977**, *16*, 1068.
34. Tachibana, H.; Sunada, Y.; Miyase, T.; Sano, M.; Maeda-Yamamoto, M.; Yamada, K. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 452.
35. Itoh, T.; Umekawa, H.; Furuichi, Y. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 448.
36. Choo, M. K.; Park, E. K.; Han, M. J.; Kim, D. H. *Planta Med.* **2003**, *69*, 518.