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Inhibitory activity of Brazilian green propolis components and their derivatives on the release of cys-leukotrienes

Hiroko Tani [a,*], Keiko Hasumi [a,b], Tomoki Tatefuji [a], Ken Hashimoto [a], Hiroyuki Koshino [b], Shunya Takahashi [b,*]

[a] Institute for Bee Products and Health Science, Yamada Apiculture Center, Inc., 194 Ichiba, Kagamino 708-0393, Japan
[b] Advanced Science Institute, RIKEN, 2-1 Wako 351-0198, Japan

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ABSTRACT

The effects of Brazilian green propolis ethanol extract on Cry j1-induced cys-leukotrienes and histamine release from peripheral leukocytes of patients with allergic rhinitis were investigated. One of the key mechanisms for the anti-allergic properties of the extract was revealed to be the suppression of cys-LTs release. Furthermore, a series of propolis components and their phenethyl esters were synthesized and evaluated as inhibitors of cys-LTs release. Artepillin C, baccharin, and kaempferide were the major active components of the ethanol extract. The inhibitory activity of artepillin C phenethyl ester was comparable to that of existing LT synthesis inhibitors.

1. Introduction

Propolis is a resinous substance collected by honeybees from leaf buds and cracks in the bark of various plants. Crude extracts of propolis have very complicated compositions, resulting from variations in geographical and botanical origin. These compounds can originate from plant exudates collected by bees, bee salivary gland secretions or substances, which are incorporated into propolis during its processing.[1] Brazilian green propolis produced in Southeast Brazil has been extensively consumed as a dietary supplement, particularly in Japan, and *Baccharis dracunculifolia* is the main botanical source.[2,3] It contains mainly prenylated derivatives of cinnamic acid, among which artepillin C (**1**) is known to be a major component.

In a previous study, we reported that the ethanol extract of Brazilian green propolis was able to alleviate symptoms in patients with seasonal allergic rhinitis by reducing therapeutic drug consumption and delaying the start of symptom onset.[4] Treatment with propolis also significantly relieved nasal obstruction.[5]

Seasonal allergic rhinitis is usually treated symptomatically with histamine H1 receptor antagonists, sympathomimetic vasoconstrictors, cysteinyl-leukotrienes (cys-LTs) synthesis inhibitors and cys-LTs receptor antagonists.[6–10] Leukotrienes are important mediators of allergic nasal obstruction, and cys-LTs synthesis inhibitors and receptor antagonists have been reported to be effective especially in the treatment of nasal obstruction.[9,10]

Caffeic acid (**2**), a representative constituent of Brazilian green propolis, has been reported to be an inhibitor of 5-lipoxygenase.[11] The enzyme 5-lipoxygenase plays a key role in regulating the production of leukotrienes. Therefore, other cinnamic acid derivatives may also suppress leukotriene synthesis and release. Furthermore, caffeic acid phenethyl ester (**9**), which is found in European and Chinese propolis is derived from poplar (*Populus*) bud is not found in Brazilian green propolis, and is more potent in several activities than caffeic acid.[12,13] Although **9** has been reported to cause allergic contact dermatitis,[14] it has received attention due to its potent pharmacological activities.[12–16]

In order to understand the possible mechanism of propolis ethanol extract, we investigated the effects of the ethanol extract on the release of proinflammatory cytokines from peripheral blood mononuclear cells, as well as histamine and cys-LTs release from peripheral leukocytes of allergic patients. Furthermore, the synthesis of a series of propolis components (**1** and **3–5**) and their phenethyl esters (**10–13**), and their inhibitory effects on the release of cys-LTs in differentiated HL-60, a human promyelocytic leukemia cell line are also discussed (Fig. 1).

* Corresponding authors. Fax: +81 86 854 3346 (H.T.); +81 48 462 4627 (S.T.).
E-mail addresses: ht0807@yamada-bee.com (H. Tani), shunyat@riken.jp (S. Takahashi).

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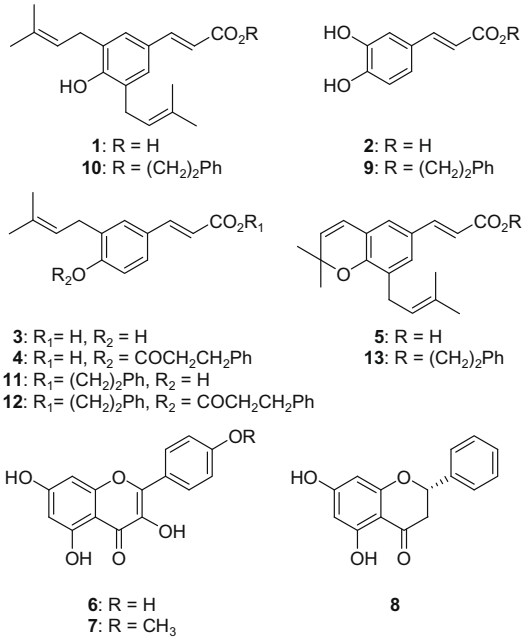


Figure 1. Structures of **1**–**13**.

2. Results and discussion

To investigate the influence of propolis ethanol extract, which contained 55% propolis extract as a solid, on cys-LTs, histamine, and proinflammatory cytokine release, peripheral leukocytes and peripheral blood mononuclear cells (PBMCs) were prepared from 10 allergic patients. After treatment with the propolis ethanol extract and Cry j1, which is a major antigen of Japanese cedar pollen, the amount of cys-LTs and histamine released from peripheral leukocytes, and IL-4, IL-5, and IL-13 released from PBMCs was measured.

The ethanol extract potently inhibited cys-LTs release in a concentration-dependent manner, with an IC_{50} value of 5.8 μg/ml (Table 1). On the other hand, the release of histamine was inhibited by the ethanol extract only at high concentration (100 μg/ml). In addition, the enhanced release of IL-5 and IL-13 seemed to be slightly inhibited by the addition of the ethanol extract, however, the values varied greatly among individual patients and were not statistically significant (Table 2). IL-4 was not detected even under extreme Cry j1 stimulation (10 μg/ml) (data not shown). These results suggested that the inhibition of cys-LTs release was one of the most important mechanisms of the anti-allergic properties of the ethanol extract. This was in agreement with our previous clinical observation, where the ethanol extract prevented nasal obstruction.

To evaluate the effect of propolis components on cys-LTs release, cinnamic acid derivatives **1** and **3–5** in Brazilian green propolis were prepared as follows: synthesis of artepillin C (**1**) was initiated with the Mizoroki–Heck reaction[17] of iodide **14**[18] with *t*-butyl acrylate in the presence of palladium acetate, tri-*o*-tolylphosphine and *n*-tetrabutylammonium chloride in aqueous *N,N*-dimethylformamide (DMF),[19,20] providing **15** in 81% yield. Treatment of the *t*-butyl ester with trimethylsilyl trifluoromethanesulfonate (TMSOTf) and 2,6-lutidine followed by acidic work-up gave **1** in 71% yield. Following the same procedure described above, the known phenol **16**[21] was transformed into *t*-butyl ester **18** and then drupanin (**3**) in 48% overall yield. Acylation of **16** with phenylpropionyl chloride was the first step in the synthesis of baccharin (**4**), giving **17** in 94% yield. The Mizoroki–Heck reaction of **17** with *t*-butyl acrylate was accomplished by the action of palladium acetate–triphenylphosphine in triethylamine and toluene to give **19** in 78% yield. Interestingly, the use of tri-*o*-tolyphosphine[18,21] instead of triphenylphosphine resulted in no reaction. Compound **19** underwent removal of *t*-butyl group to give baccharin (**4**) in 90% yield. Although DDQ oxidation of **1** was reported to produce culifolin (**5**), the yield was quite low (~25%).[22] Therefore, we searched a more efficient method for the preparation of **5**. A Pd-promoted etherification[23–25] of **15** gave a mixture of **20–22** in a variety of ratios (Table 3), while DDQ oxidation of **15** afforded **22** exclusively, albeit in a low yield. Selenocyclization[26] of **15** at −78 °C followed by H_2O_2 oxidation provided a mixture of **21** and **22** in 57% yield (**21**/**22** = 37/63 by ^{1}H NMR). The best results were obtained by the use of **16** as a starting material. Thus, propargylation[27] and thermal rearrangement[28] of **16** gave the 2,2-dimethylpyran derivative **24** in 70% yield. This reacted smoothly with *t*-butyl acrylate in toluene to give **22** in 78% yield. Synthesis of **22** from **18** via the corresponding propargyl ether was impractical due to the low yield. Upon treatment with TMSOTf-2,6-lutidine, **22** afforded culifolin (**5**) in 90% yield (Scheme 1).

Phenethyl ester **9** has been reported to be more potent in several activities than **2**.[12–16] Such results prompted us to prepare similar esters of **1** and **3–5** with the expectation of an increase in the activity. Compounds **1** and **3–5** each reacted with (2-bromoethyl)benzene in the presence of cesium carbonate in DMF to afford the corresponding phenethyl esters **10–13**, respectively, in high yield.

Table 1
Effects of propolis ethanol extract on Cry j1-induced cys-LTs and histamine release from peripheral leukocytes of patients with allergic rhinitis

Amount[a] (μg/ml)	Inhibition (%)	
	cys-LTs	Histamin
3	28.5 ± 13.4	7.2 ± 8.5
10	61.8 ± 12.1	7.5 ± 10.5
30	95.9 ± 2.9	10.5 ± 8.1
100	96.2 ± 3.6	77.2 ± 17.2

Each value represented the mean ± SD of 7 and 8 experiments.
[a] Propolis extract (solid).

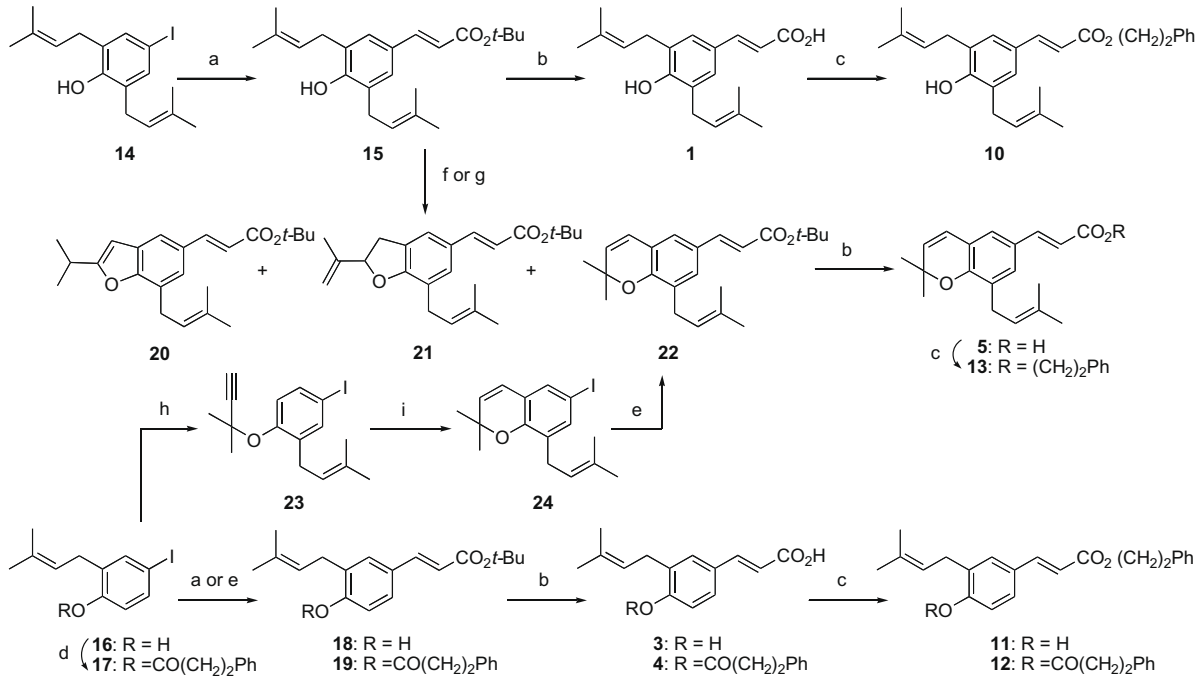
Table 2
Effects of propolis ethanol extract on Cry j1-induced cytokine release from PBMCs of patients with allergic rhinitis

Amount[a] (μg/ml)	Inhibition (%)	
	IL-5	IL-13
3	8.3 ± 19.5	10.7 ± 41.9
10	26.0 ± 15.3	29.6 ± 29.5
30	64.4 ± 27.5	75.2 ± 12.8

Each value represented the mean ± SD of 8 experiments.
[a] Propolis extract (solid).

Table 3
Cyclization reaction of **15**

Entry	Conditions	Products (%)		
		20	**21**	**22**
1	$PdCl_2$, AcONa, MeOH–H_2O, 35 °C	8	15	15
2	$PdCl_2$, O_2, AcONa, MeOH–H_2O, 55 °C	9	26	20
3	$PdCl_2(CH_3CN)_2$, NaOMe, benzene, 90 °C	7	—	6
4	DDQ, benzene, rt	—	—	19
5	PhSeBr, CH_2Cl_2, −20→0 °C, then 15% H_2O_2, pyridine, 0 °C→rt	—	7	23
6	PhSeCl, CH_2Cl_2, rt, then 15%H_2O_2, pyridine, 0 °C→rt	—	4	24
7	PhSeCl, CH_2Cl_2, −78 °C, then 15%H_2O_2, pyridine, 0 °C→rt	—	21	36



Scheme 1. Reagents and conditions: (a) *t*-butyl acrylate, $Pd(OAc)_2$, $(o\text{-}Tol)_3P$, $n\text{-}Bu_4NCl$, Et_3N, DMF–H_2O, 35 °C, 81% for **15**, 77% for **18** from **16**; (b) TMSOTf, 2,6-lutidine, dichloromethane, rt, and then dil HCl, rt, 71% for **1**, 62% for **3**, 90% for **4**, 98% for **5**; (c) (2-bromoethyl)benzene, Cs_2CO_3, DMF, rt, 72% for **10**, 78% for **11**, 83% for **12**, 90% for **13**; (d) 3-phenylpropionyl chloride, DMAP, Et_3N, dichloromethane, 0 °C, 94%; (e) *t*-butyl acrylate, $Pd(OAc)_2$, Ph_3P, Et_3N, toluene, 105 °C, 78% for **19** from **17**, 78% for **22** from **24**; (f) $PdCl_2$, O_2, AcONa, methanol-H_2O, 55 °C, 9% for **20**, 26% for **21**, 20% for **22**; (g) PhSeCl, dichloromethane, −78 °C, and then 15% H_2O_2, dichloromethane–pyridine, 0 °C, 57% (**21**/**22** = 37/63; ^1H NMR); (h) 3-chloro-3-methyl-1-butyne, DBU, CuCl, acetonitrile, 0 °C, 82% from **16**; (i) *N,N*-diethylaniline, 150 °C, 85%.

All synthesized compounds (**1**, **3–5**, and **10–13**) and some authentic samples (**2**, **6–8**, and **9**) were tested for their inhibitory activity with the ethanol extract on cys-LTs release using differentiated HL-60, a human promyelocytic leukemia cell line.[29] Two types of leukotriene synthesis inhibitors (nordihydroguaiaretic acid: NDGA and zileuton) were also tested in order to compare the activities. The ethanol extract and its components (**1–8**) moderately inhibited the release of cys-LTs (Table 4). The content of **1–8** in the ethanol extract, which contained 55% propolis extract as a solid, were approximately 20.7%, 0.1%, 1.9%, 7.5%, 0.3%, 0.2%, 3.6%, and 3.1% (w/w), respectively, as measured by HPLC analysis. When considering the contents of the ethanol extract, the main active components were artepillin C (**1**), baccharin (**4**), and kaempferide (**7**). It remains to be established whether minor components might have greater potency than these major components, since the potency of the ethanol extract was not totally explained by the potency of these three components.

Table 4
Inhibitory activity of cys-LTs release from differentiated HL-60 by DMSO

Compounds	IC_{50} values (μg/ml)
NDGA	0.08 ± 0.012
Zileuton	0.04 ± 0.0026
EtOH extract[a]	4.3 ± 1.5
Artepillin C (**1**)	7.0 ± 0.15
Caffeic acid (**2**)	44 ± 18
Drupanin (**3**)	10.3 ± 2.5
Baccharin (**4**)	4.4 ± 2.0
Culifolin (**5**)	1.6 ± 0.39
Kaempferol (**6**)	2.4 ± 0.9
Kaempferide (**7**)	2.0 ± 1.3
Pinocembrin (**8**)	6.1 ± 2.1
Caffeic acid phenethyl ester (**9**)	0.068 ± 0.0055
Artepillin C phenethyl ester (**10**)	0.094 ± 0.0027
Drupanin phenethyl ester (**11**)	0.20 ± 0.036
Baccharin phenethyl ester (**12**)	>10
Culifolin phenethyl ester (**13**)	>10

The values represent the mean ± SD ($n = 2$).
[a] Solid propolis extract.

Among the phenethyl esters, compounds **9**–**11** showed 667, 74, and 52 times more potent than each acid showed, and the potency of **10** was comparable to those of existing leukotriene synthesis inhibitors (NDGA and zileuton) and also to that of **9**, an active component of European and Chinese propolis. Structure–activity relationships of tested compounds indicated that the cys-LTs inhibitory activity increases with increasing lipophilicity. Further, the potencies of **12** and **13** were weaker than those of each acids showed that a phenolic hydroxyl group or an acid was essential for this activity.

Recently artepillin C was reported to have an inhibitory effect on prostaglandin E_2 and nitric oxide through NF-κB modulation using the macrophage cell line RAW 264.7.[30] In addition the inhibitor of nitric oxide synthase has been reported to prevent nasal obstruction by inhibiting venodilation of nasal mucosa induced by antigen challenge.[31] These findings suggested that preventing nasal obstruction using the Brazilian green propolis extract may be due to the inhibition of several inflammatory pathways.

Further study on the design and synthesis of a new class of cys-LTs release inhibitors with the cinnamic acid and/or flavanone scaffold with a favorable safety profile is in progress.

3. Experimental section

3.1. General procedures

All reactions were carried out under an argon atmosphere, unless otherwise noted. Melting points were determined using a Yanaco MP-500 apparatus and are uncorrected. IR spectra were

recorded with a JASCO VALOR-III spectrophotometer. ^1H and ^{13}C NMR spectra were obtained using Varian NMR system spectrometer at 500 MHz and 125 MHz, respectively. Chemical shifts were referenced to a residual signal of $CDCl_3$ (δ_H 7.26, δ_C 77.0) and CD_3OD (δ_H 3.30, δ_C 49.0). ESIMS were recorded on a Waters Micromass Quattro Premier XE mass spectrometer or JEOL JMS-T100LC mass spectrometer. Column chromatography was performed on Kanto Silica Gel 60N (spherical, neutral; 40–100 μm). Merck precoated Silica Gel 60 F254 plates, 0.25 mm thickness, was used for analytical thin-layer chromatography. The solvent extracts were dried with magnesium sulfate, and the solutions were evaporated under diminished pressure at 40–42 °C. Compounds on the TLC plates were detected under UV light (254 nm) and/or by spraying anisaldehyde–sulfuric acid reagent on the plate and heating it at 110 °C for 20 min. The spray reagent was prepared freshly before use by adding 1 ml of concentrated H_2SO_4 to a solution of 0.5 ml of *p*-anisaldehyde in 50 ml of acetic acid.

3.2. Chemicals

The authentic samples of caffeic acid, kaempferol, and kaempferide were purchased from Wako Co. (Osaka, Japan), and pinocembrin and caffeic acid phenethyl ester were from Sigma (St. Louis, MO).

3.3. Material

Brazilian green propolis was purchased from Apiários Floresta (Minas Gerais, Brazil).

3.4. Extraction

Brazilian green propolis (2 g) was pulverized with a homogenizer and extracted in 20 ml of ethanol. After stirring at room temperature for 12 h, the filtration was evaporated until the solid content reached 55%. The solid content was evaluated after evaporation in vacuo and further drying in an oven at 105 °C for 4 h.

3.5. Synthesis

3.5.1. *tert*-Butyl (*E*)-3-[4-hydroxy-3,5-bis(3-methylbut-2-enyl)phenyl]acrylate (15)

To a stirred solution of **14** (120 mg, 0.34 mmol), *t*-butyl acrylate (100 μl, 0.68 mmol), tri-*o*-tolylphosphine (32 mg, 0.10 mmol), and *n*-tetrabutylammonium chloride (29 mg, 0.10 mmol) in *N,N*-dimethylformamide–triethylamine–water (9:1:1; 1.2 ml) was added palladium acetate (15 mg, 0.07 mmol). The mixture was stirred at 35 °C for 4 h, cooled to rt, diluted with ether, washed successively with aqueous NH_4Cl, water, brine, dried, and concentrated. The residue was chromatographed on silica gel (*n*-hexane–ethyl acetate = 80:1→50:1) to give **15** (97.5 mg, 81%) as a faint yellow solid; IR (ATR) 3420, 2960, 1900, 1700, 1680, 1620, 1600, 1470, 1430, 1360, 1340, 1260, 1130, 1080, 980, 840 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.49 (d, J = 15.8 Hz, 1H), 7.15 (s, 2H), 6.20 (d, J = 15.8 Hz, 1H), 5.62 (s, 1H), 5.30 (m, 2H), 3.33 (d, J = 7.3 Hz, 4H), 1.78 (s, 6H), 1.77 (s, 6H), 1.53 (s, 9H);^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 154.7, 143.9, 134.8, 127.9, 127.5, 126.9, 121.5, 117.1, 80.1, 29.5, 28.2, 25.8, 17.9; HR MS (EI) calcd for $C_{23}H_{32}O_3$ (M^+) 356.2351, found 356.2349.

3.5.2. Artepillin C (1)

To a stirred solution of **15** (133 mg, 0.37 mmol), and 2,6-lutidine (850 μl, 7.3 mmol) in dichloromethane (4.0 ml) was added trimethylsilyl trifluoromethanesulfonate (642 μl, 3.7 mmol). The mixture was stirred at rt for 4.5 h, diluted with ether, washed successively with cold aqueous HCl, water, brine, dried, and concentrated to give a syrup (126 mg), which was dissolved in tetrahydrofuran (1.5 ml). To this solution was added 0.2 M HCl (1.5 ml), and the mixture was stirred at rt for 2 h, diluted with ether, washed successively with water, brine, dried, and concentrated to give a brown solid, which was recrystallized from *n*-hexane–ethyl acetate to give **1** (75 mg, 71%) as a colorless solid; mp 96–97 °C (*n*-hexane–ethyl acetate); IR (ATR) 3400, 1690, 1620, 1580, 1440, 1430, 1400, 1360, 1320, 1300, 1250, 1210, 1180, 1120, 1020, 970 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.71 (d, J = 16.0 Hz, 1H), 7.21 (s, 2H), 6.29 (d, J = 16.0 Hz, 1H), 5.32 (m, 2H), 3.35 (d, J = 7.5 Hz, 4H), 1.80 (s, 6H), 1.78 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.7, 155.4, 147.4, 135.2, 128.4, 127.7, 126.4, 121.3, 114.1, 29.5, 25.8, 17.9; HR MS (ESI) calcd for $C_{19}H_{24}O_3Na$ ($M+Na^+$) 323.1623, found 323.1621.

3.5.3. Artepillin C phenylethyl ester (10)

To a stirred solution of **1** (40.2 mg, 0.13 mmol) and cesium carbonate (46 mg, 0.14 mmol) in *N,N*-dimethylformamide (0.9 ml) was added (2-bromoethyl)benzene (30 μl, 0.20 mmol) at rt, and the mixture was stirred at rt for 18 h, then diluted with water. The resulting mixture was extracted with ether. The extracts were washed successively with water, brine, dried, and concentrated. The residue was chromatographed on silica gel (*n*-hexane–ethyl acetate = 30:1) to give **10** (38.0 mg, 72%) as a colorless solid; IR (ATR) 3400, 2950, 2840, 1680, 1620, 1590, 1470, 1450, 1420, 1370, 1340, 1260, 1160, 1130, 1080, 1110, 980, 880, 840, 800, 760, 690 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$):δ 7.58 (d, J = 16.0 Hz, 1H), 7.33–7.23 (m, 5H), 7.16 (s, 2H), 6.26 (d, J = 16.0 Hz, 1H), 5.66 (s, 1H), 5.31 (m, 2H), 4.41 (t, J = 7.0 Hz, 2H), 3.34 (d, J = 7.0 Hz, 4H), 3.01 (t, J = 7.0 Hz, 2H), 1.79 (s, 6H), 1.77 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.4, 155.0, 145.2, 138.0, 135.0, 128.9, 128.5, 128.0, 127.6, 126.6, 126.5, 121.4, 115.0, 64.8, 35.2, 29.5, 25.8, 17.9; HR MS (ESI) calcd for $C_{27}H_{32}O_3Na$ ($M+Na^+$) 427.2249, found 427.2250.

3.5.4. 4-Iodo-2-(3-methylbut-2-enyl)phenyl 3-phenylpropionate (17)

To a stirred solution of **16** (332 mg 1.15 mmol), *N,N*-dimethylaminopyridine (10 mg, 0.08 mmol), triethylamine (240 ml, 1.73 mmol) in dichloromethane (3.0 ml) was added dropwise 3-phenylpropionyl chloride (205 ml, 1.38 mmol) at 0 °C. The mixture was stitrred at 0 °C→rt for 1 h, diluted with ether, and treated successively with aqueous NH_4Cl, saturated aqueous $NaHCO_3$. The resulting mixture was washed with brine, dried, and concentrated. The residue was chromatographed on silica gel (*n*-hexane–ethyl acetate = 100:1→55:1) to give **17** (455 mg, 94%) as a colorless oil; IR (ATR) 2970, 2910, 1760, 1480, 1375, 1290, 1210, 1170, 1125, 1090, 865, 660 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.50 (m, 2H), 7.33–7.22 (m, 5H), 6.67 (d, J = 8.1 Hz, 1H), 5.14 (m, 1H), 3.09–3.06 (m, 4H), 2.91–2.88 (m, 2H), 1.74 (s, 3H), 1.65 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.9, 148.7, 139.9, 138.8, 136.2, 135.9, 133.9, 128.6, 128.3, 126.5, 124.2, 120.9, 90.6, 35.7, 30.9, 28.3, 25.7, 17.8; HR MS (EI) calcd for $C_{20}H_{21}O_2I$ (M^+) 420.0586, found 420.0571.

3.5.5. *tert*-Butyl (*E*)-3-[4-hydroxy-3-(3-methylbut-2-enyl)phenyl]acrylate (18)

Treatment of **16** (300 mg, 1.04 mmol) as described for preparation of **15** gave **18** (231 mg, 77%) as a colorless oil; IR (ATR) 3325, 2960, 2900, 1670, 1630, 1600, 1500, 1420, 1360, 1330, 1270, 1240, 1140, 1080, 980, 850, 820 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.52 (d, J = 16.0 Hz, 1H), 7.27–7.26 (m, 2H), 6.80 (d, J = 9.0 Hz, 1H), 6.23 (d, J = 16.0 Hz, 1H), 5.81 (br s, 1H), 5.31, (m, 1H), 3.35 (d, J = 7.0 Hz, 1H), 1.78 (s, 3H), 1.77 (s, 3H), 1.53 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.5, 156.5, 144.2, 134.2, 129.8, 127.5, 126.8, 121.4, 116.6, 115.8, 28.9, 28.2, 25.7, 17.7; HR MS (EI) calcd for $C_{18}H_{24}O_3$ (M^+) 288.1725, found 288.1717.

3.5.6. *tert*-Butyl (*E*)-3-[4-(3-phenylpropionyloxy)-3-(3-methylbut-2-enyl)phenyl]acrylate (19)

To a stirred solution of **17** (353 mg, 0.84 mmol), *t*-butyl acrylate (630 μl, 4.3 mmol), triphenylphosphine (25 mg, 0.095 mmol), and triethylamine (240 μl, 1.7 mmol) in toluene (5.7 ml) was added palladium acetate (10.4 mg, 0.046 mmol). The mixture was stirred at 105 °C for 6 h, cooled to rt, diluted with ether, washed successively with aqueous NH_4Cl, water, brine, dried, and concentrated. The residue was chromatographed on silica gel (*n*-hexane–ethyl acetate = 30:1→20:1) to give **19** (273 mg, 78%) as a colorless oil; IR (ATR) 2975, 2915, 1760, 1705, 1640, 1495, 1455, 1370, 1325, 1285, 1260, 1225, 1125 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.53 (d, J = 16.0 Hz, 1H), 7.36–7.22 (m, 7H), 6.94 (d, J = 7.2 Hz, 1H), 6.29 (d, J = 16.0 Hz, 1H), 5.17 (m, 1H), 3.14 (d, J = 5.6 Hz, 2H), 3.08 (t, J = 6.2 Hz, 2H), 2.91 (t, J = 6.2 Hz, 2H), 1.74 (s, 3H), 1.67 (s, 3H), 1.53 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 166.2, 150.1, 142.8, 139.9, 134.1, 133.7, 132.6, 129.7, 128.6, 128.3, 126.5, 122.6, 121.0, 120.0, 80.5, 35.8, 30.9, 28.5, 28.2, 25.7, 17.8; HR MS (ESI) calcd for $C_{27}H_{32}O_4Na$ (M+Na^+) 443.2198, found 443.2189.

3.5.7. Drupanin (3)

Treatment of **18** (100 mg, 0.35 mmol) as described for preparation of **1** gave **3** (50 mg, 62%) as a colorless solid; mp 143–144 °C (benzene); IR (ATR) 3300, 3125, 2975, 2925, 2850, 1675, 2525, 1640, 1590, 1510, 1430, 1360, 1310, 1260, 1220, 1150, 1100, 980, 940, 860, 820, 690 cm^{-1}; ^1H NMR (500 MHz, CD_3OD): δ 7.56 (d, J = 16.0 Hz, 1H), 7.27–7.23 (m, 2H), 6.76 (d, J = 9.0 Hz, 1H), 6.22 (d, J = 16.0 Hz, 1H), 5.30 (m, 1H), 3.28 (d, J = 7.4 Hz, 2H), 1.73 (s, 3H), 1.70 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 167.4, 156.5, 144.9, 137.9, 135.5, 130.1, 128.9, 128.5, 127.8, 127.4, 127.2, 126.5, 121.1, 116.1, 115.2, 64.9, 35.2, 29.6, 25.8, 17.9; HR MS (ESI) calcd for $C_{14}H_{16}O_3Na$ (M+Na^+) 255.0997, found 255.0997.

3.5.8. Baccharin (4)

Treatment of **19** (150 mg, 0.36 mmol) as described for preparation of **1** gave **4** (118 mg, 90%) as a colorless solid; mp 101–102 °C (methanol); IR (KBr) 2930, 1755, 1670, 1635, 1495, 1430, 1240, 1130, 690, 670 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.75 (d, J = 15.9 Hz, 1H), 7.41–7.23 (m, 7H), 6.98 (d, J = 8.8 Hz, 1H), 6.39 (d, J = 15.9 Hz, 1H), 5.19 (m, 1H), 3.16 (d, J = 7.0 Hz, 2H), 3.09 (t, J = 7.5 Hz, 2H), 2.92 (t, J = 7.5 Hz, 2H), 1.76 (s, 3H), 1.68 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.1, 170.9, 150.7, 146.4, 139.9, 134.3, 133.9, 131.9, 130.2, 128.6, 128.4, 126.9, 126.5, 122.8, 120.8, 117.1, 35.6, 30.9, 28.4, 25.7, 17.8; HR MS (ESI) calcd for $C_{23}H_{24}O_4Na$ (M+Na^+) 387.1572, found 387.1578.

3.5.9. Drupanin phenylethyl ester (11)

Treatment of **3** (30 mg, 0.13 mmol) as described for preparation of **10** gave **11** (34 mg, 78%) as a colorless oil; IR (ATR) 3350, 2900, 1680, 1620, 1600, 1500, 1420, 1380, 1320, 1300, 1260, 1230, 1170, 1160, 1080, 980, 880, 860, 820, 750, 700 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.60 (d, J = 16.0 Hz, 1H), 7.33–7.22 (m, 7H), 6.80 (d, J = 8.0 Hz, 1H), 6.27 (d, J = 16.0 Hz, 1H), 5.44 (s, 1H), 5.31 (m, 1H), 4.41 (t, J = 7.0 Hz, 2H), 3.36 (d, J = 7.0 Hz, 2H), 3.01 (t, J = 7.0 Hz, 2H), 1.79 (br s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.4, 156.5, 144.9, 137.9, 135.5, 130.1, 128.9, 128.5, 127.8, 127.4, 127.2, 126.5, 121.1, 116.1, 115.2, 64.9, 35.2, 29.6, 25.8, 17.9; HR MS (ESI) calcd for $C_{22}H_{24}O_3Na$ (M+Na^+) 359.1623, found 359.1622.

3.5.10. Baccharin phenylethyl ester (12)

Treatment of **4** (128 mg, 0.43 mmol) as described for preparation of **10** gave **12** (167 mg, 83%) as a colorless solid; IR (ATR) 2900, 1760, 1700, 1640, 1600, 1500, 1450, 1410, 1370, 1320, 1270, 1220, 1160, 1150, 1120, 1080, 980, 860, 750, 700 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.62 (d, J = 16.0 Hz, 1H), 7.36–7.24 (m, 12H), 6.82 (d, J = 8.5 Hz, 1H), 6.35 (d, J = 16.0 Hz, 1H), 5.17 (m, 1H), 4.42 (t, J = 7.0 Hz, 2H), 3.14 (d, J = 7.5 Hz, 2H), 3.08 (t, J = 7.5 Hz, 2H), 3.02 (t, J = 7.0 Hz, 2H), 2.91 (t, J = 7.5 Hz, 2H), 1.75 (s, 3H), 1.67 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 166.8, 150.3, 144.1, 139.9, 137.8, 134.2, 133.8, 132.3, 129.9, 129.0, 128.6, 128.5, 128.3, 126.5, 122.7, 120.9, 117.9, 65.0, 35.8, 35.2, 30.9, 28.5, 25.7, 17.8; HR MS (ESI) calcd for $C_{31}H_{32}O_4Na$ (M+Na^+) 491.2198, found 491.2186.

3.5.11. *tert*-Butyl (*E*)-3-[2-isopropyl-7-(3-methylbut-2-enyl)benzofuran-5-yl]acrylate (20), *tert*-butyl (*E*)-3-[2,3-dihydro-7-(3-methylbut-2-enyl)-2-(prop-1-en-2-yl)benzofuran-5-yl]acrylate (21), and *tert*-butyl (*E*)-3-[2,2-dimethyl-8-(3-methylbut-2-enyl)-2*H*-chromen-6-yl]acrylate (22)

(i) To a stirred solution of **15** (24.0 mg, 0.067 mmol) and sodium acetate (44 mg, 0.54 mmol) in methanol–water (17:1; 1.2 ml) was added palladium chloride (6.0 mg, 0.034 mmol). The mixture was stirred at 55 °C in the air for 1.5 h, diluted with ether, washed with water, brine, and concentrated. The residue was purified by preparative TLC (*n*-hexane–ethyl acetate = 12:1, three developments) to give **20** (2.1 mg, 9%), **21** (6.3 mg, 26%), and **22** (4.8 mg, 20%).

(ii) To a stirred solution of **15** (31.0 mg, 0.087 mmol) in dichloromethane (0.5 ml) was added phenylselenenyl chloride (16 mg, 0.083 mmol) at −78 °C. The mixture was stirred at −78 °C for 40 min, diluted with dichloromethane, washed with brine, concentrated to give a syrup (56.0 mg), which was dissolved in dichloromethane (0.5 ml) containing a trace amount of pyridine. To this solution was added 15% hydrogen peroxide (0.1 ml) at 0 °C, and the mixture was stirred for 45 min, washed with water, brine, and concentrated. The residue was chromatographed on silica gel (*n*-hexane–ethyl acetate = 44:1) to give a mixture of **21** and **22** (17.6 mg, 57%) as a colorless oil (**21**/**22** = 37/63 by ^1H NMR analysis).

(iii) Treatment of **24** (20.0 mg, 0.06 mmol) and *t*-butyl acrylate (40 μl, 0.29 mmol) as described for preparation of **19** gave **22** (15.6 mg, 78%).

Compound **20**. IR (ATR) 2960, 2910, 1700, 1630, 1600, 1460, 1360, 1280, 1150, 1120, 1080, 980, 850; ^1H NMR (500 MHz, $CDCl_3$):δ 7.65 (d, J = 16.0 Hz, 1H), 7.46 (d, J = 1.5 Hz, 1H), 7.20 (d, J = 1.5 Hz, 1H), 6.34 (s, 1H), 6.32 (d, J = 16.0 Hz, 1H), 5.39 (m, 1H), 3.57 (d, J = 7.5 Hz, 2H), 3.07 (m, 1H), 1.79 (s, 3H), 1.76 (s, 3H), 1.54 (s, 9H), 1.34 (d, J = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 165.7, 154.3, 144.7, 133.4, 129.4, 129.1, 125.2, 122.8, 121.3, 118.4, 118.1, 100.1, 80.2, 29.7, 28.2, 28.1, 25.8, 20.9, 17.8; HR MS (EI) calcd for $C_{23}H_{30}O_3$ (M^+) 354.2195, found 354.2184.

Compound **21**. IR (ATR) 2970, 2900, 1700, 1630, 1600, 1470, 1430, 1360, 1250, 1140, 1120, 1080, 980, 890, 850, 820 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.51 (d, J = 15.5 Hz, 1H), 7.18 (br s, 1H), 7.12 (br s, 1H), 6.18 (d, J = 15.5 Hz, 1H), 5.30 (m, 1H), 5.21 (dd, J = 9.5, 8.0 Hz, 1H), 5.07 (br, s, 1H), 4.90 (br s, 1H), 3.36 (dd, J = 15.7, 9.5 Hz, 1H), 3.31 (dd, J = 16.0, 7.5 Hz, 1H), 3.25 (dd, J = 16.0, 7.5 Hz, 1H), 3.02 (dd, J = 15.7, 8.0 Hz, 1H), 1.76 (s, 3H), 1.74 (s, 3H), 1.71 (s, 3H), 1.52 (s, 9H);^{13}C NMR (125 MHz, $CDCl_3$): δ 166.9, 159.7, 144.0, 143.9, 133.2, 128.9, 123.5, 122.0, 121.4, 116.6, 111.8, 85.9, 80.0, 34.6, 28.2, 28.1, 25.8, 17.8, 17.2; HR MS (ESI) calcd for $C_{23}H_{30}O_3Na$ (M+Na^+) 377.2093, found 377.2106.

Compound **22**. IR (ATR) 2960, 2910, 1700, 1630, 1600, 1480, 1410, 1385, 1365, 1360, 1330, 1265, 1140, 1080, 980, 845 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.48 (d, J = 15.9 Hz, 1H), 7.15 (d, J = 2.1 Hz, 1H), 6.99 (d, J = 2.1 Hz, 1H), 6.29 (d, J = 9.8 Hz, 1H), 6.19 (d, J = 15.9 Hz, 1H), 5.63 (d, J = 9.8 Hz, 1H), 5.26 (m, 1H), 3.26 (d, J = 7.3 Hz, 2H), 1.74 (s, 3H), 1.73 (s, 3H), 1.55 (s, 6H), 1.54 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 152.5, 143.7, 132.4,

130.9, 129.6, 129.3, 126.8, 124.0, 122.1, 120.9, 117.1, 80.0, 76.8, 28.2, 28.1, 25.8; HR MS (ESI) calcd for $C_{23}H_{30}O_3Na$ (M+Na^+) 377.2093, found 377.2085.

3.5.12. 1-(2-Methylbut-3-yn-2-yloxy)-4-iodo-2-(3-methylbut-2-enyl)benzene (23)

To a stirred solution of **16** (581 mg, 2.02 mmol), 3-chloro-3-methyl-1-butyne (0.226 ml, 2.02 mmol) and copper chloride (0.7 mg, 7 μmol) in acetonitrile (2.0 ml) was added dropwise DBU (0.33 ml, 2.21 mmol) at 0 °C, and the mixture was stirred at 0 °C for 1 h. After addition of aqueous NH_4Cl, the resulting mixture was extracted with ether. The extracts washed with water, brine, dried, and concentrated. The residue was chromatographed on silica gel (*n*-hexane–ethyl acetate = 1:0→20:1) to give **23** (584 mg, 82%) as a syrup; IR (ATR) 3290, 2985, 2915, 1580, 1480, 1235, 1140, 1090, 880, 860, 815 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.41 (br s, 1H), 7.40 (br d, J = 8.1 Hz, 1H), 7.27 (d, J = 8.1 Hz, 1H), 5.22 (m, 1H), 3.25 (d, J = 7.4 Hz, 2H), 2.57 (s, 1H), 1.74 (br s, 3H), 1.69 (br s, 3H), 1.66 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 153.7, 138.2, 136.3, 134.9, 132.9, 121.9, 120.7, 85.9, 85.5, 74.0, 72.2, 29.6, 28.5, 25.7, 17.8; HR MS (ESI) calcd for $C_{16}H_{19}OI$ (M^+) 354.0481, found 354.0480.

3.5.13. 6-Iodo-2,2-dimethyl-8-(3-methylbut-2-enyl)-2*H*-chromene (24)

A solution of **23** (233 mg, 0.66 mmol) in *N,N*-diethylaniline (10.0 ml) was heated at 150 °C with stirring for 3 h, cooled, diluted with hexane, and then poured into ice-water. The resulting mixture was extracted with hexane. The extracts were washed successively with cold aqueous HCl, water, saturated aqueous $NaHCO_3$, water, brine, dried, and concentrated. The residue was chromatographed on silica gel (*n*-hexane) to give **24** (198 mg, 85%) as a colorless oil; IR (ATR) 2967, 2920, 1735, 1640, 1445, 1380, 1360, 1255, 1200, 1165, 1090, 865, 720 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.23 (d, J = 2.2 Hz, 1H), 7.12 (d, J = 2.2 Hz, 1H), 6.21 (d, J = 9.8 Hz, 1H), 5.60 (d, J = 9.8 Hz, 1H), 5.23 (m, 1H), 3.20 (d, J = 7.6 Hz, 2H), 1.73 (br s, 3H), 1.71 (br s, 3H), 1.40 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 150.4, 137.6, 132.6, 132.4, 132.0, 131.4, 123.3, 121.9, 121.4, 82.4, 76.3, 31.6, 27.9, 27.8, 25.8, 22.6; HRMS (EI) calcd for $C_{16}H_{19}OI$ (M^+) 354.0481, found 354.0485.

3.5.14. Culifolin (5)

Treatment of **22** (238 mg, 0.67 mmol) as described for preparation of **1** gave **5** (178 mg, 89%) as a colorless solid; mp 130–131 °C (methanol); IR (ATR) 3000, 2950, 1670, 1620, 1590, 1460, 1440, 1370, 1360, 1330, 1265, 1245, 1200, 1140 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.68 (d, J = 15.5 Hz, 1H), 7.19 (d, J = 1.8 Hz, 1H), 7.05 (d, J = 1.8 Hz, 1H), 6.32 (d, J = 10.0 Hz, 1H), 6.27 (d, J = 15.5 Hz, 1H), 5.65 (d, J = 10.0 Hz, 1H), 5.27 (m, 1H), 3.27 (d, J = 7.0 Hz, 2H), 1.75 (s, 3H), 1.74 (s, 3H), 1.44 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.8, 153.3, 147.2, 132.7, 131.1, 129.9, 129.8, 126.3, 124.4, 122.0, 121.0, 114.1, 77.0, 28.2, 28.1, 25.8, 17.8; HR MS (ESI) calcd for $C_{19}H_{22}O_3Na$ (M+Na^+) 321.1467, found 321.1481.

3.5.15. Culifolin phenylethyl ester (13)

Treatment of **5** (22 mg, 0.07 mmol) as described for preparation of **10** gave **13** (27 mg, 90%) as a colorless solid; IR (ATR) 2950, 2900, 1700, 1620, 1590, 1460, 1420, 1360, 1320, 1290, 1250, 1220, 1200, 1160, 1130, 1070, 970, 940, 870, 840, 710, 690 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.57 (d, J = 16.0 Hz, 1H), 7.33–7.22 (m, 5H), 7.15 (d, J = 2.3 Hz, 1H), 7.01 (d, J = 2.3 Hz, 1H), 6.31 (d, J = 10.0 Hz, 1H), 6.25 (d, J = 16.0 Hz, 1H), 5.64 (d, J = 10.0 Hz, 1H), 5.26 (m, 1H), 4.41 (t, J = 7.3 Hz, 2H), 3.26 (d, J = 7.5 Hz, 2H), 3.01 (t, J = 7.3 Hz, 2H), 1.74 (s, 3H), 1.73 (s, 3H), 1.43 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.3, 152.8, 145.0, 137.9, 132.6, 131.0, 129.7, 129.6, 128.9, 128.5, 126.6, 126.5, 124.1, 122.1, 121.0, 114.9, 76.9, 64.8, 35.2, 28.2, 28.1, 25.8, 17.9; HR MS (ESI) calcd for $C_{27}H_{30}O_3Na$ (M+Na^+) 425.2093, found 425.2084.

3.6. HPLC analysis of extract

The analysis of the ethanol extract of Brazilian green propolis were conducted using HPLC and it was carried out with a Cosmosil 5C18-ARII column (Nacalai Tesque, 4.6 × 250 mm), with the gradient solvent system of CH_3CN (5%, 5 min hold, 50–30%, 5–50 min linear, and 30–60%, 50–160 min linear) in 0.1% TFA at a flow rate of 1.0 ml/min and detection at 260 nm..

3.7. Assays

The experiments with human blood were conducted at the National Sagamihara Hospital in accordance with the principle of the Declaration of Helsinki. Before withdrawing the blood, each subject signed an informed written consent form, which had been approved by the ethical committee of Yamada Apiculture Center, Inc.

3.7.1. Peripheral leukocytes and PBMCs

Thirty milliliters of blood were collected from 10 allergic patients who had a clinical history of pollinosis with positive specific IgE to Cry j1/2 antigen (CAP-RAST ⩾ class3). Peripheral blood leukocytes were isolated by dextran sedimentation. Briefly, heparinized venous blood was incubated with one-tenth volume of 4.5% dextran at room temperature for 45 min. The blood suspension was centrifuged and the pellet was washed with phosphate-buffered saline (PBS). The pellet is then washed in Tyrode's buffer containing human serum albumin (0.1%) and diluted into same buffer.

Peripheral blood mononuclear cells (PBMC) were obtained by sodium diatrizoate-Ficoll density gradient centrifugation (MP Biomedicals). PBMC were suspended in RPMI 1640 medium (Gibco) with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml) at 37 °C under 5% CO_2 for 6 days.

3.7.2. Differentiated HL-60 Cells

HL-60 cells obtained from Health Science Research Resources Bank (Tokyo, Japan) were grown in RPMI-1640 with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The density of HL-60 cells was maintained between 2.5×10^5 and 5×10^5/ml. The HL-60 cells were cultured in the same medium containing 1.25% DMSO for 6 days. The differentiation into myeloid cells resembling PMN leukocytes were confirmed by NBT reduction.

3.7.3. Analysis of cytokine production

PBMC were seeded in 96-well plates (2.0×10^5/well) and stimulated by 10 μg/ml Cry j1 in the presence or absence of propolis at 37 °C under 5% CO_2 for 6 days. The cytokines were measured by ELISA (IL-4, IL-5; BioSource, and IL-13; Beckman Coutler).

3.7.4. Analysis of histamine and cys-LTs release

Peripheral blood leukocytes were seeded in test tube ($1.5 \times 10^4 \sim 5.1 \times 10^4$/tube) and stimulated by Cry j1 (1 ng/ml) in the presence or absence of propolis at 37 °C under 5% CO_2 for 6 days. Medium from cultures peritoneal lavage fluids were analyzed quantitatively by histamine autoanalyzer and cys-LTs ELISA kit (Cayman 520501) according to the instructions.

Differentiated HL-60 cells were seeded in 96-well plates and stimulated by Ca^{2+} in the presence or absence of samples at 37 °C under 5% CO_2 for 6 days. The concentration of cys-LTs was determined using ELISA kit (Bühlmann CAST-2000).

3.7.5. Statistical analysis

The data were analyzed in JMP (CAS Institute) software. The IC_{50} values were estimated by non-linear fitting from individual experiments. *P* values less than 0.05 were considered significant.

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