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Effects of propolis from different areas on mast cell degranulation and identification of the effective components in propolis

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ABSTRACT

Propolis is considered to down-regulate type I allergy, but the effective components of propolis remain unknown. In addition, propolis components vary depending on the area from which they are collected due to variations among wild plants in an area. Therefore, we compared the effects of water and ethanol extracts of propolis from Brazil and China on mast cell degranulation and cytokine production, thereby identifying effective components in propolis. The amount of released β -hexosaminidase via high-affinity IgE receptor I (Fc ϵ RI) from rat basophilic leukemia (RBL-2H3) cells was used as an index of degranulation. All propolis extracts inhibited degranulation from antigen-stimulated RBL-2H3 cells, but the effective doses differed according to collection areas. The ethanol extract of Chinese propolis, which was the strongest inhibitor of mast cell degranulation, was divided into compounds using normal- and reversed-phase liquid chromatography. The isolated anti-allergic components were identified as chrysin, kaempferol and its derivative, and chrysin was revealed to inhibit IL-4 and MCP-1 production from antigen-stimulated RBL-2H3 cells. HPLC quantification also revealed the Brazilian propolis extract to contain only small amounts of these flavonoids, which suggested that variation in propolis components could affect anti-allergic properties.

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1. Introduction

Propolis is a bee product which protects the hive from rain or bacterial invasion. Propolis consists of approximately 300 plant-derived compounds including flavonoids, phenolic acids, cinnamic acid derivatives, terpenoids, cellulose and amino acids [1]. Various bioactivities of propolis have been demonstrated, e.g., anti-bacterial, anti-tumor, anti-inflammatory, anti-oxidative, etc., effects [1]. The components of propolis depend on the area in which it was collected [2], for example, Brazilian propolis has abundant artepillin C which is one of the cinnamic acids and has anti-tumor activity [3]. On the other hand, Chinese propolis contains caffeic acid phenethyl ester (CAPE) which suppresses cell growth by regulating NF- κ B [4]. As components of propolis vary depending on the area from which samples are collected, the biological activities of propolis may differ according to production area. Indeed, Yoshizumi et al. compared the xanthin oxidase (XOD) inhibitory activities of Brazilian and Chinese propolis and found that Chinese propolis suppressed XOD to a greater extent than Brazilian propolis [5].

The anti-allergic activity of propolis is now well known, and propolis products have been marketed for their anticipated anti-allergic effects. Effects of propolis extracts on the immune system have been reported, e.g., immunoregulation in an OVA-sensitized airway inflammatory animal model [6] and various immune cells including mast cells [7–9]. CAPE, one of the Chinese propolis components, has been reported to significantly inhibit asthmatic reactions in a mouse model [10]. However, the components exerting these immunoregulatory effects and their amounts in propolis from different places have not yet been clarified.

We evaluated the effects of Brazilian and Chinese propolis extracts on chemical mediator release (degranulation) from rat basophilic leukemia (RBL-2H3) cells following antigen stimulation. Some components with anti-allergic activity were isolated from an ethanol extract of Chinese propolis and identified. We also compared the amounts of these anti-allergic components in Brazilian and Chinese propolis.

2. Materials and methods

2.1. Materials

Chemicals and sources were as follows: minimum essential medium (MEM) with Eagle's salt (Catalog No. M4655), chrysin (27214), penicillin-streptomycin solution (100 $\times$) from Sigma (St.

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Louis, MO, USA), fetal bovine serum (FBS) from SAFC Biosciences (Lenexa, Kansas, USA), 4-methylumbelliferyl 2-acetamide-2-deoxy- β -D-glucopyranoside from Toronto Research Chemicals Inc. (North York, Canada).

Four lots of propolis from Brazil (A, E, G, and G2) and one from China (C) were used. Each was extracted with ultrapure water (WEP) or with 95% ethanol (EEP).

2.2. Cells

Rat basophilic leukemia (RBL-2H3) cells were incubated in MEM with Eagle's salt supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin, at 37 °C with 5% CO₂.

2.3. β -hexosaminidase release via Fc ϵ RI

The β -hexosaminidase (β -hex) release assay was performed as reported previously, with slight modification [11]. RBL-2H3 cells (5×10^4 cells/well) were sensitized overnight with DNP-specific IgE (mouse ascites, 1:1000 diluted with medium) on a 96-well plate (costar, 3569). Cells were treated with propolis for 30 min, 3 h or 18 h before stimulation with 200 ng/mL DNP-BSA antigen for 30 min. After stimulation, amounts of β -hex released into the supernatant and remaining within the cells were determined by enzymatic assay with a fluorescent substrate, 0.1 M 4-methylumbelliferyl 2-acetamide-2-deoxy- β -D-glucopyranoside in 0.1 M sodium citrate (pH 4.5), for 30 min at 37 °C. The enzymatic reaction was then stopped by adding glycine buffer (pH 10.0), and fluorescence intensities were measured (Ex = 360 nm, Em = 480 nm).

β -hex release was determined as described below;

$$\text{Released } \beta\text{-hex}(\%) = 100 \times \beta\text{-hex}[\text{sup.}] / (\beta\text{-hex}[\text{sup.}] + \beta\text{-hex}[\text{cells}])$$

β -hex [sup.]; β -hex released into the supernatant after antigen stimulation.

β -hex [cells]; β -hex remaining within the cells after antigen stimulation.

Effects of propolis on β -hex release were expressed as relative released β -hex, calculated as below;

$$\text{Relative released } \beta\text{-hex}(\%) = 100 \times \{\text{released } \beta\text{-hex}[\text{propolis treated}](\%)\} / \{\text{released } \beta\text{-hex}[\text{untreated}](\%)\}$$

The data were obtained from four independent wells for each dose (n = 4).

2.4. Cytokine release assay

DNP-IgE sensitized cells were treated with chrysin for 30 min before stimulation with DNP-BSA. The supernatants of the cells were collected 3 h after the addition of antigen, and the amounts of released IL-4 and monocyte-chemoattractant protein-1 (MCP-1) were measured with Quantikine Rat IL-4 Immunoassay (R&D Systems, Inc., MN, USA) and Rat MCP-1 ELISA Kit (Thermo Fisher Scientific Inc., IL, USA), respectively, according to the manufacturer's protocol. The data were obtained from four independent wells for each dose (n = 4).

2.5. Identification of anti-allergic components

2.5.1. Fractionation of propolis extract by liquid chromatography

The ethanol extract of propolis from China (EEP-C) was evaporated and dissolved with CHCl₃, followed by normal-phase silica gel chromatography on BW-820MH (Fuji Silysia Chemical Ltd., Aichi, Japan) with CHCl₃/MeOH as follows; 1:100 (1L), 98:2 (1L), 90:10 (5L),

0:100 (3L). Fractions were collected around visible peaks, and each fraction was raised by thin layer chromatography (TLC) on a Silica gel 60 Plate F254 (Merck KGaA, Darmstadt, Germany) and developed with CHCl₃/MeOH 95:5. Similar TLC profiles were mixed together to obtain seven fractions designated Fr. A–G.

Fr. C and Fr. E were further submitted to reversed-phase column chromatography on a Chromatorex ODS DM1020T (Fuji Silysia Chemical Ltd.) and eluted with Ultrapure water/MeOH as follows; 50:50 (0.5L), 30:70 (1L), 0:100 (1L). Fractions were collected around visible peaks, and each fraction was added to the RBL-2H3 cells for testing of its anti-allergic effect based on the β -hexosaminidase release assay. The fractions which inhibited β -hexosaminidase release from RBL-2H3 cells were further fractionated by repeated silica gel column chromatography, and each effective component was thereby ultimately isolated.

2.5.2. Nuclear magnetic resonance (NMR)

The allergic components were dissolved in 7 mL of dimethyl sulfoxide (DMSO)-d₆ (Acros Organics, Belgium) and identified by comparing their spectra, 1D; ¹H- and ¹³C-NMR 2D; gCOSY, gHSQC, gHMBC obtained with a MERCURY plus 300NB (Varian, Inc., CA), using authentic samples.

2.6. Quantification of flavonoids in propolis

EEP-A was used as a representative Brazilian propolis, EEP-C as a representative Chinese propolis. The solid components were quantified using an Infrared Moisture Determination Balance (Kett Electric Laboratory, Tokyo, Japan). Then, each EEP was dissolved with 20% acetonitrile and submitted to HPLC on a Shiseido CAPCELL PAK C18 (Shiseido Co. Ltd., Tokyo, Japan). Chromatography was performed in stepwise gradient mode at 40 °C as follows; A: ultrapure water containing 0.1% Trifluoroacetic acid (TFA). B: acetonitrile containing 0.1% TFA [B concentration 10% (60 min), 10 → 60% (15 min), 60% hold]. The peak intensity was measured at a wavelength of 267.1 nm and RT 48.7 min for chrysin, at 365 nm and RT 37.9 min for kaempferol, and the flavonoid contents of EEP were calculated from a standard curve obtained with purified flavonoids.

2.7. Inhibition curve and statistics

Inhibition curve data were fit to the following dose–response logistic curve model for the best fit estimates of IC₅₀ values using KaleidaGraph version 3.6 (Synergy Software, Reading, PA).

$$y = a + (b - a) / (1 + (x/c)^d); a = E_0, b = E_{\max}, c = IC_{50}, d = \text{slope}$$

The results were expressed as means $\pm$ standard deviation (S.D.). The significance of differences was examined by Student's *t*-test, and *p* < 0.05 or *p* < 0.01 was considered to indicate a significant difference.

3. Results

3.1. Anti-allergic effects on mast cells of propolis extracts from different areas

We compared the effects of water (WEP) and ethanol (EEP) extracts of propolis, four from Brazil and one from China, on degranulation of antigen-stimulated rat basophilic leukemia (RBL-2H3) cells. RBL-2H3 cells were treated with propolis extracts for 30 min before antigen stimulation, and β -hexosaminidase release was used as the index of degranulation from activated RBL-2H3 cells. As shown in Fig. 1, both WEP and EEP of Chinese propolis extracts (C) inhibited degranulation more potently than those of Brazilian propolis (A, E, G and G2). Prolonging (3 h/18 h) the propolis treatment before antigen stimulation did not affect the β -hexosaminidase release results (Supplemental

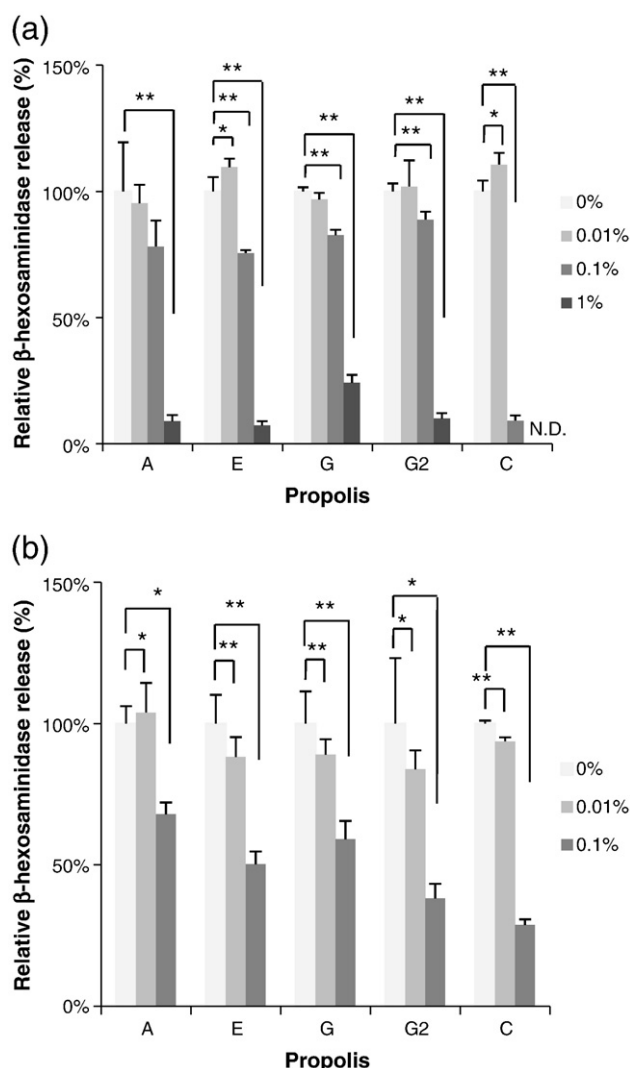


Fig. 1. Effects of propolis extracts from different areas on degranulation of antigen-stimulated mast cells. RBL-2H3 cells were treated with (a) water or (b) ethanol extracts of propolis from Brazil (A, E, G and G2) and China (C) and stimulated with specific antigens in propolis solution, and β -hexosaminidase release following activation of mast cells was quantified by enzymatic assay. The percentages of relative released β -hexosaminidase are shown on the ordinate. All data are the means $\pm$ S.D. of four independent experiments ($n=4$). The differences were examined by Student's *t*-test versus control cells (0%), and significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$). N. D. means "not determined". The cells treated with the highest dose of propolis extract were observed for cellular morphology after antigen stimulation, as described in Results.

Fig. 1). It is noteworthy that the solid contents of each EEP (w/w) were as follows; A 15.0%, C 11.6%, E 12.5%, G 11.5%, G2 14.6%. Therefore, EEP inhibited degranulation from RBL-2H3 cells with an almost 10-fold smaller quantity of WEP. Some extracts showed harmful effects on mast cells, such as disappearance or morphological changes with 1% (w/w) WEP-C. This dose of the Brazilian WEP exerted no such effects, while all cells treated with 1% (w/v) EEP, regardless of whether the propolis was Brazilian or Chinese, were affected.

As EEP-C showed the strongest inhibition of antigen-induced mast cell degranulation without harmful effects, we attempted to identify the anti-allergic components of EEP-C.

3.2. Identification of effective components in ethanol extracts of Chinese propolis

EEP-C was divided into 7 fractions by normal-phase chromatography (Fig. 2), and we used the β -hexosaminidase release assay to

investigate which fraction inhibited mast cell degranulation. All fractions but Fr. G inhibited mast cell degranulation, and Fr. B, C, D and E especially were inhibitory even at low doses (Table 1). Cells treated with Fr. B and Fr. D showed morphological changes at high doses. Based on these results, Fr. C and Fr. E which inhibited mast cell degranulation without damaging cells were further fractionated by reversed-phase chromatography (Fig. 2).

3.2.1. Identification of effective components in Fr. C

Fr. C was divided into 6 fractions by reversed-phase chromatography. The results of β -hexosaminidase release assay showed Fr. C-5 to strongly inhibit mast cell degranulation at low doses, while Fr. C-4 was slightly inhibitory at high doses (Table 2). As Fr. C-5 consisted of four peaks on HPLC profiles, each peak was isolated by performing reversed-phase chromatography once more. We found that Fr. C-5b and Fr. C-5c inhibited antigen-induced degranulation dose-dependently without cell damage even at 30 μ g/mL, while Fr. C-5d damaged the cells at 30 μ g/mL (Table 2). The NMR inductions suggested the main peak Fr. C-5b to represent chrysin (Fig. 3a) and the small peak Fr. C-5c galandol (Fig. 3b), both of which are flavonoids. In addition, purified chrysin inhibited degranulation from antigen-stimulated mast cells at almost the same dose as Fr. C-5b (Table 2).

To investigate the effects of chrysin on cytokine release from antigen-stimulated mast cells, the cells were treated with each propolis fraction for 30 min, followed by stimulation with antigen for 3 h, and the released IL-4 and MCP-1 were measured by ELISA. The IL-4 and MCP-1 releases from activated mast cells were mostly suppressed at 10 μ g/mL of propolis Fr. C-5, Fr. C-5b, and chrysin (Fig. 4).

3.2.2. Identification of effective components in Fr. E

Fr. E which inhibited degranulation as effectively as Fr. C was also fractionated by reversed-phase chromatography to isolate the anti-allergic components (Fig. 2). Four fractions were obtained, i.e. Frs. E-1 to E-4, and Fr. E-4 was revealed by β -hexosaminidase assay to inhibit mast cell degranulation (Table 3). Fr. E-4 consisted of three peaks in HPLC profiles, and the main peak Fr. E-4b weakly inhibited mast cell degranulation, while the small peak Fr. E-4a inhibited degranulation as potently as chrysin (Table 3). Fr. E-4c slightly inhibited degranulation but caused cell damage at 100 μ g/mL. NMR induction identified Fr. E-4a as kaempferol (Fig. 3c) and Fr. E-4b as 3-O-methylkaempferol (Fig. 3d). The main peak Fr. E-4b hardly suppressed of MCP-1 and IL-4 release from activated mast cells (Fig. 4).

3.3. Contents of chrysin and kaempferol in Brazilian and Chinese propolis extracts

The amounts of two flavonoids, chrysin and kaempferol, identified as anti-allergic components in the ethanol extract of Chinese propolis (EEP-C), were compared among Brazilian and Chinese EEP. EEP-A was used as a representative Brazilian propolis. HPLC quantification from a standard curve using purified flavonoids revealed EEP-C to contain small amounts of chrysin and kaempferol, while the amounts of these flavonoids in EEP-A were below detectable levels (Table 4).

4. Discussion

The components of propolis vary among plant sources within a collection area and seasonally [12,13]. In this study, we compared the effects of four lots of Brazilian and one lot of Chinese propolis on the degranulation of RBL-2H3 cells after specific antigen stimulation. The inhibitory effects on antigen-induced degranulation differed between Brazilian and Chinese propolis, with both water and ethanol extracts of Chinese propolis exerting more potent inhibitory effects than Brazilian propolis at the same concentration (Fig. 1). These results indicated that propolis components or their amounts differ according

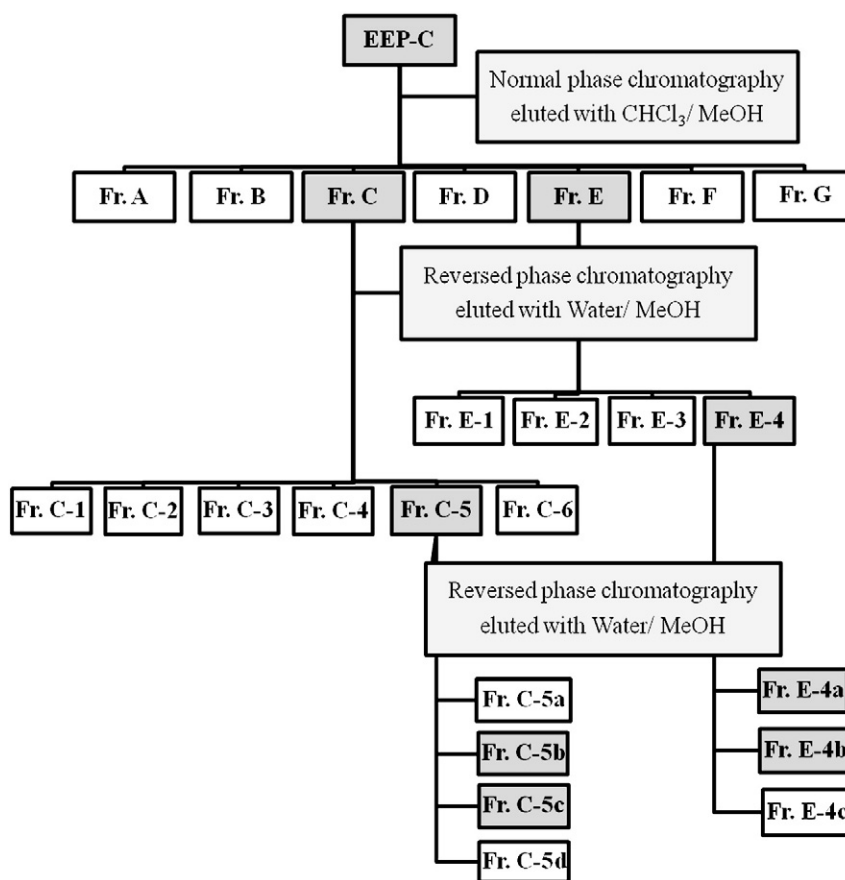


Fig. 2. Flow chart of fractionation of ethanol extract of propolis from China (EEP-C). EEP-C was divided into seven fractions by normal-phase liquid chromatography, and then Fr. C and Fr. E were further fractionated into a single peak by reversed-phase liquid chromatography to obtain the components exerting inhibitory effects on mast cell activation.

to where the propolis was collected. These results agree with Miyataka's study evaluating the effects of Brazilian/Chinese propolis on β -hexosaminidase release from rat peritoneal mast cells induced by Concanavalin A or Compound 48/80 [7]. Miyataka et al. also indicated that the type of stimulus affected the inhibition of degranulation by Brazilian and Chinese propolis and suggested that these effects of propolis depend on not only the amounts of certain effective components but the profile of these components in propolis.

In this study we identified chrysin and kaempferol as the major anti-allergic components in ethanol extracts of Chinese propolis which inhibited degranulation of antigen-stimulated RBL-2H3 cells

more potently than Brazilian propolis. Chrysin and kaempferol are the flavonoids previously reported to inhibit the release of chemical mediators [11] and/or cytokines [12] from mast cells, and BALB/c mice given flavonoids showed suppressed expressions of Th2 cytokines, such as IL-4 and IL-13 [13]. Kaempferol has also been reported to inhibit degranulation [14] and cytokine production [15] from activated mast cells, and we newly found that chrysin also suppresses cytokine production by mast cells following antigen stimulation (Fig. 4). The mechanisms for inhibition of mast cell activation by flavonoids have not been fully understood. Park and his co-workers showed that kaempferol suppressed elevation of intracellular calcium as well as histamine release from activated RBL-2H3 cells [16]. Our preliminary results also showed chrysin suppressed elevation of intracellular calcium of activated mast cells, mainly suppressed influx

Table 1
Results of β -hexosaminidase release assay with antigen-stimulated mast cells treated with EEP-C components.

Propolis	Cell morphological change ^a (0.5%)	Inhibition of β -hexosaminidase release (%)		
	0.05%	0.1%	0.5%	(v/v)
Fr. A	—	9.4	30.8	65.5
Fr. B	+	66.9	69.7	N.D. ^b
Fr. C	—	48.5	76.6	76.3
Fr. D	+	43.6	65.9	N.D.
Fr. E	—	31	62.9	88.4
Fr. F	—	15.5	34.4	72.7
Fr. G	—	—21.3	5.9	0.1
EEP-C	+	80.5	80.8	N.D.

^a Observed with a microscope 30 min after mast cell activation with antigens.

^b N. D. ; not detected.

Table 2
Results of β -hexosaminidase release assay with antigen-stimulated mast cells treated with Fr. C components.

Propolis	IC50 ^a (μ g/ml)	Cell morphological change ^b
Fr. C-4	152.2	—
Fr. C-5	1.73	—
Fr. C-5a	2.26E+08	—
Fr. C-5b	8.89	—
Fr. C-5c	27.78	—
Fr. C-5d	9.82E+15	+
Chrysin	5.89	—

^a Calculated from a dose–response logistic curve for 8 doses.

^b Observed with a microscope 30 min after mast cell activation with antigens.

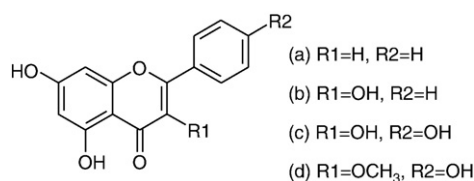


Fig. 3. Chemical structures of flavonoids identified as anti-allergic components of Chinese propolis. (a) chrysin, (b) galandin, (c) kaempferol, (d) 3-O-methylkaempferol.

from outside of the cells (unpublished results). Recently, Hirose and her co-workers made an interesting suggestion of the involvement of heme oxygenase-1 (HO-1) to inhibition of mast cell activation by kaempferol [17]. More investigations about the intracellular mechanisms of suppression of mast cell activations by each flavonoid are needed.

We previously evaluated the effects of 40 types of flavonoids on β -hexosaminidase release from antigen-stimulated mast cells and suggested that a free hydroxyl residue at C-5 plays an important role in this activity, and that a hydroxyl residue at C-4 or C-7 and also at C-4' might enhance the inhibition of activated mast cells [11]. The flavonoids identified in this study, chrysin, galandin and kaempferol, have hydroxyl groups at C-5 and C-7, and kaempferol furthermore has one at C-4' (Fig. 3). These are not the strongest inhibitors of degranulation among flavonoids [18] but cause little damage to cells, suggesting they might be beneficial as anti-

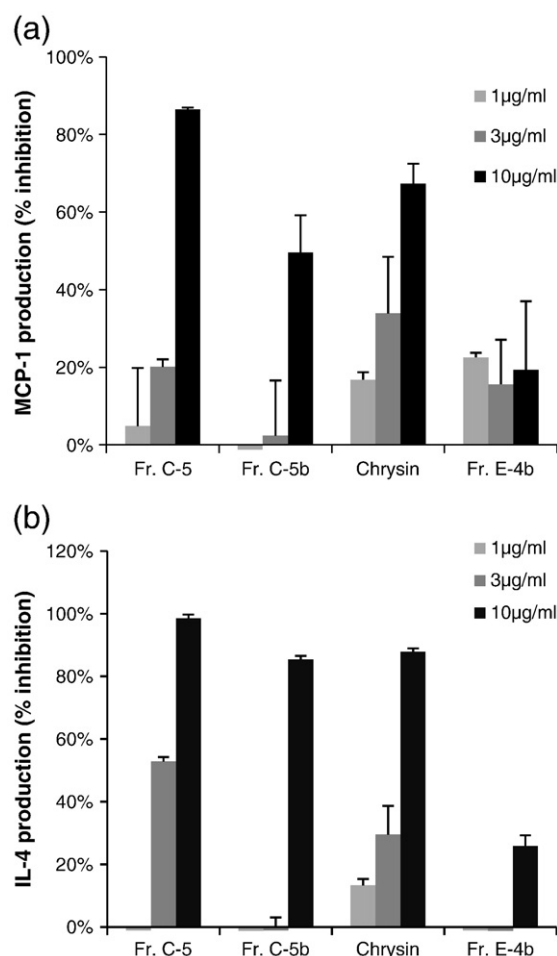


Fig. 4. The effects of propolis fractions on cytokine release from antigen-stimulated mast cells RBL-2H3 cells were treated with each propolis fraction for 30 min, followed by antigen stimulation for 3 h. Released (a) MCP-1 and (b) IL-4 were measured with ELISA and the inhibition % to the cytokine release from untreated cells were shown. All data are the means $\pm$ S.D. of four independent experiments.

Table 3

Results of β -hexosaminidase release assay with antigen-stimulated mast cells treated with Fr. E components.

	IC ₅₀ ^a (µg/ml)	Cell morphological change ^b
Fr. E-4a	3.52	—
Fr. E-4b	19.78	—
Fr. E-4c	31.75	+
Fr. E-4	11.83	+
Kaempferol	10.04	—

^a Calculated from a dose-response logistic curve for 6 doses.

^b Observed with a microscope 30 min after mast cell activation with antigens.

Table 4

Solid contents of flavonoids contained in EEP^a.

	Solid contents (w/w, %)	
	Kaempferol	Chrysin
EEP-A (Brazil)	N. D. ^b	N. D.
EEP-C (China)	0.26%	0.54%

^a Quantified from a standard curve of the intensities of HPLC peaks obtained from authentic samples.

^b N.D. Not detected.

allergic components of foods. In addition, we previously reported that O-methylation of flavonoids decreases their anti-allergic activities. In this study 3-O-methylkaempferol slightly decreased the inhibitory effect on degranulation exerted by kaempferol (Table 3), although the effects on cytokine release from activated RBL-2H3 cells seemed to be equal [16].

On the other hand, CAPE, a component of Chinese propolis extract, reportedly down-regulates NF- κ B activation and inhibits PAF release, thereby reducing anaphylaxis and anti-allergic activities in OVA-induced mice [19]. CAPE also inhibits mast cell degranulation [20]. We confirmed that purified CAPE dose-dependently inhibits antigen-induced degranulation, and its IC₅₀ was 83 μ M (data not shown).

Quantification of chrysin and kaempferol by HPLC showed these flavonoids to be undetectable in the Brazilian propolis extract (Table 4), though Brazilian propolis was reported to include small amounts of these flavonoids, i.e. less than those in Chinese propolis, by MS analysis [21,22]. Both the inhibitory effect on β -hexosaminidase release observed for fractions besides Fr. C or Fr. E in this study and the work of Miyatake's group indicate that compounds other than chrysin and kaempferol are the effective components of Brazilian propolis, and that Chinese propolis also has other effective components. Identification of these other anti-allergic components and quantification of their contents might facilitate investigating the anti-allergic activities of propolis.

In conclusion, we compared the effects of Brazilian and Chinese propolis extracts on mast cell degranulation and cytokine production. We identified effective components, chrysin and kaempferol, in the ethanol extract of Chinese propolis. Quantification by HPLC revealed that the Brazilian propolis extract had only small amounts of chrysin and kaempferol, suggesting that variation in the components of propolis could affect anti-allergic properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.intimp.2010.06.013.

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