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Full Paper

Anti-allergic effect of *Cyclopia* (honeybush) extracts via anti-degranulation activity in a murine allergy model for inhaled antigen

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The anti-allergic effects of extracts prepared from two species of honeybush, *Cyclopia genistoides* and *Cyclopia subternata*, were demonstrated *in vivo* in a murine allergy model for inhaled antigen induced with ovalbumin (OVA) inhalation to mimic pollen allergy. Intake of the extracts increased the production of OVA-specific immunoglobulin (Ig) E (IgE), IgG1, and IgG2a antibodies in serum and significantly suppressed anaphylactic reaction-induced body temperature decline. Moreover, the extracts significantly inhibited antigen–antibody-induced degranulation in RBL-2H3 cells. They also inhibited body temperature decline when the allergic mice were given them after antigen sensitization, indicating that anti-degranulation activity is the major mechanism underlying the anti-allergic effect of *Cyclopia* extracts. Despite their qualitative and quantitative differences in phenolic composition, the two extracts exhibited similar effects, suggesting that several active compounds might be involved in the activity. Therefore, oral administration of either *Cyclopia* extract potentially exerts a systemic anti-allergic effect, supporting the increased consumption of honeybush tea for general wellness and improved quality of life.

Key words : anti-allergic activity, *Cyclopia*, honeybush, respiratory allergy model, IgE, RBL-2H3 cell, degranulation

INTRODUCTION

In recent years, the number of individuals with allergies has increased in both developed and developing countries [1]. Sufferers of allergic rhinitis, an allergic reaction to pollen and molds, among others, fall into this category [2]. Pollen allergy is a typical type I allergic disease associated with respiratory sensitization to an inhaled antigen wherein pollen from certain plants that are otherwise harmless to humans are recognized as antigens, triggering allergic responses. Sneezing and other bothersome symptoms can disrupt daily life and considerably diminish the quality of life of patients [3]. Many people develop allergies to pollen from multiple plants, resulting in an increase in the duration of their symptoms within a year [4].

Currently, allergen-specific immune therapy is becoming more widespread, but its safety and efficacy have not been fully established. Many studies have reported that an anaphylactic reaction occurred during immune therapy, resulting in dropout from the treatment. Moreover, the final efficacy of the therapy has not reached the expected level at present. On the other hand, the risk for adverse effects cannot be denied when taking an anti-allergic drug for long time. Therefore, the use of functions of food factors with anti-allergic activity for allergic patients is attracting attention. Several studies have reported the anti-allergic effects of various foods and their components. The safety of them is considered to be advantageous when used for long-term treatment, especially for prevention. Several studies have investigated the modulating role of polyphenols against food allergies, asthma, and allergic rhinitis [5, 6]. Tea and herbal teas contain diverse phenolic compounds, including catechins and flavonols, with anti-allergic effects [7, 8]. It has been reported that epigallocatechin gallate, a type of catechin abundantly present in green tea, showed an anti-allergic effect by inhibiting mast cell degranulation [9]. In addition, luteolin and quercetin have also

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been reported to inhibit mast cell degranulation by inhibiting PKC signaling in mast cells [10].

A herbal tea made from *Cyclopia* species is called honeybush tea and commonly consumed in South Africa. Our previous study revealed that orally administered extracts (40% ethanol) of honeybush, in particular *Cyclopia genistoides* and *Cyclopia subternata,* significantly enhanced antibody production and regulatory T-cell induction in mice [11], exhibiting consistency with the results of a previous *in vitro* study [12]. Additionally, an aqueous extract of *C. subternata* was found to inhibit β-hexosaminidase release from immunoglobulin (Ig)E-sensitized RBL-2H3 cells [13]. The phenolic content of the extract, especially its mangiferin content, could be increased by using an aqueous–ethanolic mixture for extraction [12]. Mangiferin, a *C*-glycosyl xanthone, is not only a major phenolic constituent of *Cyclopia* extracts but also has been shown to be able to alleviate ovalbumin (OVA)-induced allergic rhinitis [14, 15]. In addition, hesperidin, which has also been shown to be present in *Cyclopia* extracts, has been reported to significantly suppress serum levels of IL-4, IL-5, IL-13, and OVA-specific IgE [16]. Investigation of the potential of *C. subternata* and *C. genistoides* aqueous–ethanolic extracts to alleviate pollen allergy is thus merited. Therefore, this study examined the anti-allergic effects of extracts prepared from *C. genistoides* and *C. subternata* using a murine respiratory allergy model mimicking pollen allergy, that is, an allergy model caused by the inhalation of an antigen without pre-sensitization by intraperitoneal immunization.

MATERIALS AND METHODS

Preparation of Cyclopia *extracts*

Cyclopia extracts that had previously been prepared by Murakami *et al*. [12] were used for both *in vitro* and *in vivo* testing. Briefly, dried shoots (leaves and stems) of *C. genistoides* and *C. subternata* were pulverized in a rotary mill (1-mm sieve) and extracted using 40% ethanol (v/v) in a 1:10 ratio (m/v) at 70°C, with stirring every 5 min. After 30 min, the extract was filtered through a Whatman No. 4 filter, the ethanol was removed under vacuum at 40°C using a rotary evaporator, and the remaining aqueous layer was lyophilized. The lyophilized extracts were stored in sealed glass containers under desiccation in the dark.

Mice

DO11.10 mice, that is, OVA-specific TCR transgenic mice, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in our laboratory. The T cells of these mice are I-A^d restricted and recognize the $OVA_{323–339}$ sequence. In this study, we used female heterozygous (+/–) mice obtained from male DO11.10 and female BALB/c (Clea Japan, Tokyo, Japan) mice. The mice were used for experiments at 7–28 weeks of age. They were handled according to the Tokyo University of Agriculture and Technology guidelines for the care and management of laboratory animals, and ethical approval for the experimental protocol was obtained before the study commenced.

Sensitization by inhaled antigens

The respiratory allergy model mimicking pollen allergy developed by Murakami *et al*. [12] was used. Briefly, DO11.10 mice (+/–) weighing 25–33 g were allowed to inhale 2 mg/mL of purified OVA (Wako Pure Chemical Industries, Tokyo, Japan)/saline solution in a box (22,000 cm^3) into which the antigen solution was sprayed using an ultrasonic atomizer (MUJI, Tokyo, Japan). The mice were exposed to the antigen for 1 hr twice a week over a period of 4 or 6 weeks depending on the experiment. Approximately 20 mL of OVA solution was atomized during each 1-hr treatment.

Treatment with Cyclopia *extracts*

The DO11.10 mice (+/–) were allowed to drink the *C. genistoides* and *C. subternata* extracts, which were suspended in sterile distilled water at 1 mg/mL, freely as their sole drinking fluid throughout the study period (6 weeks), beginning 1 week before the first OVA inhalation (–1 week). Since the *C. genistoides* and *C. subternata* extracts were not completely dissolved in water, a suspension was prepared by mixing them in sterile distilled water as much as possible to achieve the desired concentration. Because small precipitates gradually appeared in the suspension, it was changed once every 3 days.

In another experiment, the mice were treated with OVA inhalation for only 4 weeks to provide a sufficient IgE titer for the anaphylactic test. After confirming an adequate IgE titer, we discontinued the inhalation treatment, and the mice subsequently received extract treatment for only 3 days, starting 3 days after the last OVA inhalation. The control group mice drank sterile distilled water freely. Each experimental group comprised five mice.

Observation of anaphylactic reaction (decline in body temperature)

After 6 weeks of OVA inhalation to induce respiratory allergy in the DO11.10 mice, we monitored clinical symptoms, such as sneezing. Unfortunately, the frequency of sneezing was too unstable for us to obtain accurate results. Therefore, we adopted an alternative method of inducing allergic symptoms by which we could obtain more reliable data. Briefly, 500 µL of 25 mg/mL OVA/distilled water was injected into the abdominal cavity of the control and extract-treated mice 4 days after the last OVA inhalation. Anaphylactic reaction-induced alterations in the body temperatures of the mice were measured for 3 hr via a non-invasive method; that is, the surface temperature of each mouse's abdomen was measured using an infrared non-contact thermometer (A&D UT-701, Tokyo, Japan), according to previous studies [17, 18]. We previously confirmed the reproducibility of the results obtained from this method by taking several measurements from the same mouse (data not shown).

Blood collection and serum preparation

Blood was collected from the tail vein of each mouse once a week during the experimental period. The blood samples were stored at 4°C for 1 day and centrifuged (1,400 rpm, 4°C, 10 min) using a HITACHI 05PR-22 centrifuge (Tokyo, Japan). The supernatants were centrifuged again and collected as serum samples that were subsequently stored frozen at –30°C until used to determine antibody concentrations.

Measurement of OVA-specific serum antibody titer

OVA-specific IgE, IgG1, and IgG2a serum antibody titers were measured using an enzyme-linked immunosorbent assay, as previously described [19]. Briefly, 0.01% OVA/phosphate-buffered saline (PBS) solution was dispensed into 96-well plates

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at 100 µL/well (MaxiSorp™; Thermo Fisher Scientific, Roskilde, Denmark) and stored overnight at 4°C. The solution was removed, and the wells were washed three times with 0.05% PBS–Tween solution. Thereafter, 1% bovine serum albumin (Seikagaku Kogyo, Tokyo, Japan)/PBS solution was dispensed at 100 µL/well and incubated at room temperature for 2 hr. After washing the wells, samples were prepared for OVA-specific antibody titer measurement by diluting them appropriately in 0.05% PBS–Tween solution, adding them to the wells at 50 µL/well, and incubating them overnight at 4°C. The standard for IgG1 and IgG2a was prepared using a mixture of sera from several animals and used after appropriate dilution. However, the standard for IgE was purchased from Cayman Chemical (Ann Arbor, MI, USA). After washing the wells, biotin-conjugated rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA), biotin-conjugated rabbit anti-mouse IgG1 (BD Pharmingen), biotin-conjugated rat anti-mouse IgG1 (BD Pharmingen), or biotin-conjugated rabbit anti-mouse IgG2a (Invitrogen, Carlsbad, CA, USA) antibodies were each dispensed at 50 µL/well and incubated at room temperature for 2 hr. The wells were subsequently washed, and alkaline phosphatase-labeled streptavidin (Invitrogen) was dispensed at 50 µL/well and incubated at room temperature for 1 hr. Following the washing step, 1 mg/mL disodium *p*-nitrophenyl phosphate solution (Wako Pure Chemical Industries) was added to each well at 50 µL/well and allowed to react at room temperature. Absorbance was measured at 405 nm using an iMark™ microplate reader (Bio-Rad, Hercules, CA, USA) once a visible yellow color had developed. Sample antibody titers were expressed as relative concentrations to the standard.

Cell culture

RBL-2H3 cells were obtained from the Japanese Collection of Research Bioresources cell bank (Osaka, Japan) as a degranulation model. Minimum Essential Medium (MEM; Gibco, Waltham, MA, USA), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA) was used for cell culture. The cells were maintained in 24-well plates (Corning, NY, USA). They were seeded and cultured until confluent at 37°C and a CO_2 concentration of 5%. During passage, cells were detached using trypsin and ethylenediaminetetraacetic acid (EDTA) solution (Wako Pure Chemical Industries).

β-Hexosaminidase release inhibitory activity test

Cultured RBL-2H3 cells were seeded into 96-well plates (Corning) at 5×10^4 cells/well. An anti-2,4-dinitrophenylhydrazine (DNP)–IgE antibody (Sigma) was added to the cells at a final concentration of 500 ng/mL, and the cells were cultured for 24 hr to allow plate adherence. The medium was subsequently removed via aspiration, and the wells were washed twice with MEM. The *Cyclopia* extracts were dissolved in 40% dimethyl sulfoxide (DMSO), diluted to a DMSO concentration ≤0.4% using the MEM, and added to the cells. As the positive control, wortmannin was dissolved in DMSO and added to the cells at the final concentration of 100 ng/mL after dilution with MEM to a DMSO concentration ≤0.4%. The control group also included DMSO at a concentration ≤0.4%. The plates were incubated for 30 min, and 2,4-dinitrophenyl–human serum albumin (Sigma) was added at a final concentration of 5 µg/mL to each cell. After examining various antigen concentrations, we adopted 500 ng/mL as the antibody concentration and 5 mg/mL as the antigen concentration because the strongest level of degranulation was induced under those conditions. After 1 hr of incubation, the supernatant was collected from each well, and lysis buffer (1% Triton X-100 in 50 mM Tris and 20 mM EDTA, pH 7.5) was added to the remaining cells. The cell lysate was collected after cell sonication for 20 sec. The substrate solution (1.3 mg/mL of 4-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M citrate buffer; Sigma) was added to the cell supernatant and cell lysate and incubated at 37°C for 90 min. The reaction was terminated by adding 100 µL of 0.1 M Na_2CO_3/0.1 M Na_2HCO_3 solution (pH 9.8). Absorbance was measured at 405 nm, and the β-hexosaminidase release rate was calculated using the following formula, where A was the measured absorbance:

$$\beta\text{-hexosaminidase release rate (\%)} = 100 \times \left[\frac{(A_{\text{supernatant}} - A_{\text{blank of supernatant}})}{\{ (A_{\text{supernatant}} - A_{\text{blank of supernatant}}) + ((A_{\text{cell lysate}} - A_{\text{blank of cell lysate}}) \}} \right]$$

Statistical analysis

Statistical analysis was performed using Dunnett's test, and statistical significance was set at $p<0.05$. All experiments were performed at least twice.

Liquid chromatography–mass spectrometry analysis of extracts

The major phenolic compounds in the two extracts used in the present study were quantified and reported by Murakami *et al.* [12]. In the present study, the samples were subjected to liquid chromatography (LC) with high-resolution mass spectrometry (HRMS) analysis for the tentative identification of minor compounds and provision of a more comprehensive phenolic characterization of the extracts.

The high-performance liquid chromatography (HPLC) separation methods for *C. genistoides* and *C. subternata* described by Beelders *et al.* [20] and de Beer *et al.* [21] were slightly modified to suit the HRMS analysis. This entailed using 3-mm columns with the same stationary phases as originally used at a flow rate of 0.43 mL/min and replacing the aqueous mobile phases with 0.1% aqueous formic acid. All other separation parameters, namely, the stationary phase, column length, column temperature, and organic mobile phase, remained constant. LC–HRMS analysis was performed on an ACQUITY UPLC I-Class PLUS chromatography system comprising a binary pump, sample manager, thermostatted column oven, and diode-array detector (DAD) coupled with a SELECT SERIES cyclic ion mobility mass spectrometer (Waters Corporation, Milford, MA, USA). Electrospray ionization was performed in negative mode. Data were acquired from 120 to 1,200 amu in MSE mode. This entailed a low-collision energy scan (6 V) followed by a high-collision energy scan (ramped from 20 to 70 V) to simultaneously obtain molecular ion and fragment data. High mass accuracy was achieved via calibration with sodium formate and mass correction, with leucine enkephalin used as a lock spray. Nitrogen gas was used at the cone and desolvation gas flow rates of 50 and 1,100 L/hr, respectively. The source and desolvation temperatures were 120 and 550°C, respectively.

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RESULTS

Phenolic characterization of extracts

Table 1 summarizes the phenolic composition of the *C. subternata* and *C. genistoides* extracts. The extracts used in the current study were the same as the 40%-ethanol extracts employed by Murakami *et al.* [12], and the content of the major phenolic compounds has previously been discussed. Minor phenolic compounds that could not be quantified using HPLC–DAD were detected and tentatively identified using LC–HRMS in the present study and displayed along with the quantitative data. Most of the compounds were present in both extracts, with eriodictyol-*O*-hexose and isorhoifolin exclusively present in the *C. subternata* extract and maclurin-di-*O*,*C*-hexose, eriodictyol-*O*-(hexose-*O*-deoxyhexose), (2*S*)-5-*O*-neohesperidosylnaringenin, and narirutin exclusively present in the *C. genistoides* extract. The *C. genistoides* extract exhibited substantially high levels of the xanthones mangiferin (9.3%) and isomangiferin (2.5%). Moreover, the *C. subternata* extract had relatively high levels of mangiferin (3.0%), 3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone (2.3%), 3′,5′-di-β-D-glucopyranosylphloretin (1.6%), hesperidin (1.1%), and scolymoside (1.1%). A trace amount of scolymoside was present in the *C. genistoides* extract.

C. genistoides *and* C. subternata *extracts suppressed body temperature decline in the respiratory allergy mouse model*

We initially determined whether *C. genistoides* and *C. subternata* extract consumption affected body temperature decline, a symptom of the allergy mice, *in vivo*. The amount of consumption did not differ among the groups (data not shown). Sixty minutes after intraperitoneal antigen administration, the decrease in body surface temperature was significantly ($p<0.05$) smaller for the *C. subternata*-treated group than for the control group. Ninety minutes after intraperitoneal antigen administration, this protective effect was significant ($p<0.05$) for both the *C. genistoides*- and *C. subternata*-treated groups (Fig. 1).

C. genistoides *and* C. subternata *extracts increased OVA-specific IgE, IgG1, and IgG2a antibody production in the respiratory allergy mouse model*

To gain insight into the possible underlying mechanisms, the effects of *C. genistoides* and *C. subternata* consumption on the serum titers of OVA-specific IgE, IgG1, and IgG2a antibodies were examined. IgE and IgG1 are considered to be Th2-type antibodies, while IgG2a is Th1-type antibody. The OVA-specific IgE antibody titer increased significantly ($p<0.05$) in the *C. subternata*-treated group compared with that in the control group (Fig. 2a). The OVA-specific IgG1 titer in the *C. subternata*-treated group was also significantly ($p<0.05$) elevated, while

Table 1. Phenolic compounds detected in the *Cyclopia genistoides* (C. gen.) and *C. subternata* (C. sub.) extracts using LC-HRMS with quantitative data from Murakami *et al.* [12] given as g/100 g extract

Compound	Experimental $[M-H]^-$ (*m/z*)	Error (ppm)	Molecular formula	Fragment ions (*m/z*)	C. gen.	C. sub.
Benzophenones						
maclurin-di-*O*,*C*-hexose	585.1440	−2.7	$C_{25}H_{29}O_{16}$	495.08, 465.10*, 355.04, 303.04	+	−
3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone (IDG)	569.1517	1.9	$C_{25}H_{29}O_{15}$	479.12, 449.11*, 317.07, 287.06	0.9	2.3
3-β-D-glucopyranosylmaclurin	423.0939	2.8	$C_{19}H_{19}O_{11}$	356.97, 333.06, 303.05*	0.6	+
3-β-D-glucopyranosyliriflophenone	407.0985	1.7	$C_{19}H_{19}O_{10}$	317.06, 287.06*	1.2	0.7
Xanthones						
tetrahydroxyxanthone-di-*O*,*C*-hexose isomer A	583.1309	1.7	$C_{25}H_{27}O_{16}$	463.09, 421.08*, 331.05, 301.04	+	+
tetrahydroxyxanthone-di-*O*,*C*-hexose isomer B	583.1309	1.7	$C_{25}H_{27}O_{16}$	493.10*, 463.09, 437.06, 421.06, 331.04, 301.03	+	+
aspalathin derivative of (iso)mangiferin	871.1967	3.9	$C_{40}H_{39}O_{22}$	421.06	+	+
mangiferin	421.0779	1.9	$C_{19}H_{17}O_{11}$	331.05, 301.04*	9.3	3.0
isomangiferin	421.0778	1.7	$C_{19}H_{17}O_{11}$	331.05, 301.04*	2.5	0.7
Flavanones						
eriodictyol-*O*-hexose	449.1072	−2.7	$C_{21}H_{21}O_{11}$	287.05	−	+
eriodictyol-*O*-(hexose-*O*-deoxyhexose)	595.1680	2.9	$C_{27}H_{31}O_{15}$	459.11	0.2	−
eriocitrin	595.1666	0.5	$C_{27}H_{31}O_{15}$	287.06	+	0.5
(2*S*)-5-*O*-neohesperidosylnaringenin	579.1714	0.0	$C_{27}H_{31}O_{14}$	565.16*, 271.06	0.1	−
(2*R*)-5-*O*-neohesperidosylnaringenin	579.1722	1.4	$C_{27}H_{31}O_{14}$	459.12, 433.13, 271.06*	1.3	+
narirutin	579.1699	−2.6	$C_{27}H_{31}O_{14}$	271.06	+	−
hesperidin	609.1821	0.3	$C_{28}H_{33}O_{15}$	301.07	0.8	1.1
Dihydrochalcones						
3′,5′-di-β-D-glucopyranosyl-3-hydroxyphloretin	613.1780	1.8	$C_{27}H_{33}O_{16}$	493.13, 433.11, 403.10, 373.09*	+	0.5
3′,5′-di-β-D-glucopyranosylphloretin (PDG)	597.1823	0.7	$C_{27}H_{33}O_{15}$	477.14, 387.11, 357.10*	+	1.6
Flavones						
vicenin-2	593.1513	1.2	$C_{27}H_{29}O_{15}$	473.10, 383.07, 353.06*	0.4	0.2
scolymoside	593.1516	1.7	$C_{27}H_{29}O_{15}$	285.04	+	1.1
isorhoifolin	577.1565	1.4	$C_{27}H_{29}O_{14}$	269.04	−	+

*base peak ion; +, detected using LC-HRMS; −, not detected.

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that of IgG2a also tended to increase (Fig. 2b and 2c). A similar tendency was observed in the *C. genistoides* group.

C. subternata *and* C. genistoides *extracts inhibited RBL-2H3 cell degranulation in vitro*

While *C. genistoides* and *C. subternata* extract consumption suppressed body temperature decline in the OVA-treated mice, it was not effective in reducing OVA-specific IgE antibody production, suggesting an alternative mechanism; that is, *C. genistoides* and *C. subternata* directly inhibited mast cell degranulation. Both extracts were subsequently shown to significantly inhibit RBL-2H3 cell degranulation ($p<0.05$; Fig. 3).

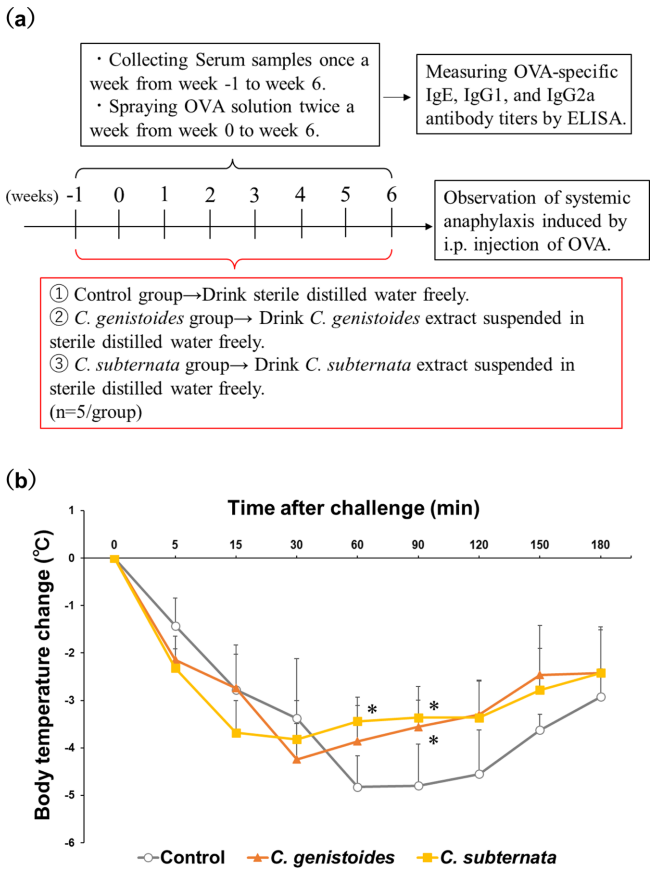

Fig. 1. Effects of *C. genistoides* and *C. subternata* extracts on antigen challenge-induced body surface temperature decline in a murine allergy model for inhaled antigen. (a) Schematic diagram of the animal model protocol. DO11.10 mice inhaled an atomized ovalbumin (OVA)-containing solution for 6 weeks and concomitantly consumed the honeybush extracts (1 mg/mL) as drinking fluid throughout the experimental period. Four days after the last OVA inhalation, the OVA solution was injected into the abdominal cavity of each mouse, and the change in body surface temperature of the mouse's abdomen was measured. The experiment was performed with five animals per group. (b) The results of the anaphylactic reaction were evaluated based on the decrease in body temperature. The results are expressed as the mean ± standard deviation. *Significant differences from the control group are indicated by $p<0.05$. The results are representative of two independent experiments.

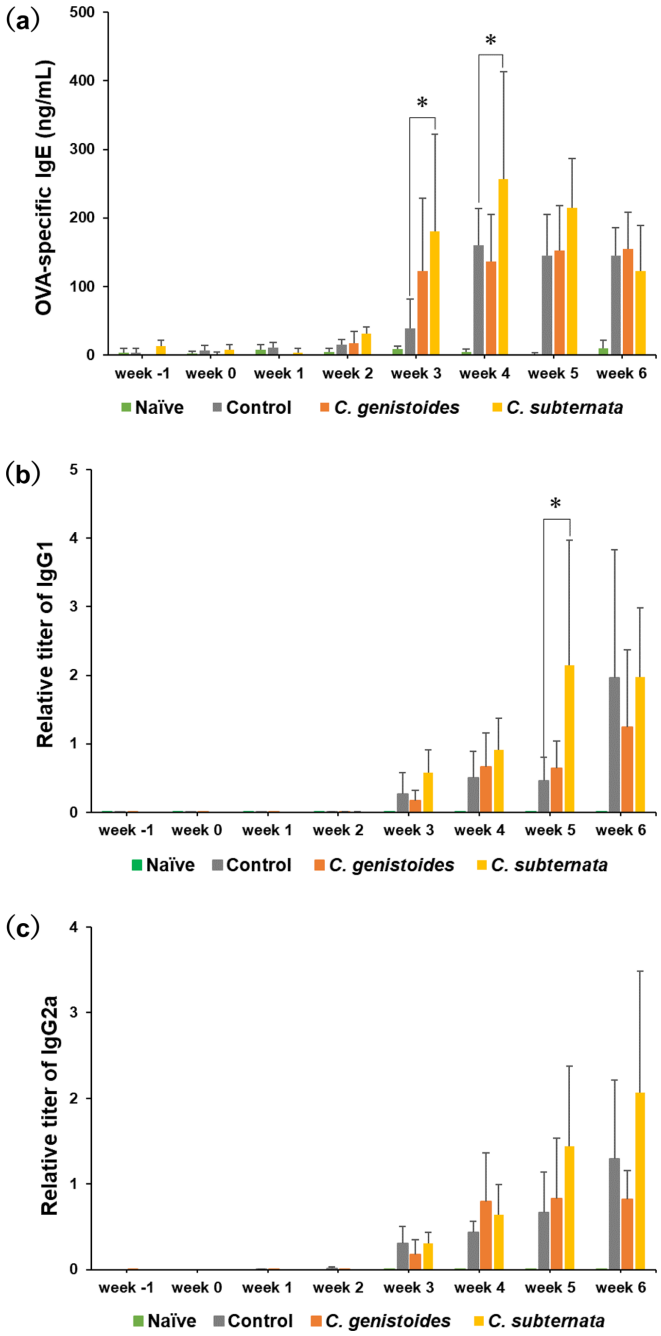

Fig. 2. Effects of *C. genistoides* and *C. subternata* extracts on ovalbumin (OVA)-specific antibody production in a murine allergy model for inhaled antigen. The DO11.10 mice were treated as described in Fig. 1. Briefly until the anaphylactic test, OVA-specific (a) IgE, (b) IgG1, and (c) IgG2a serum titers were measured for every week. The experiment was performed with five animals per group. The results are expressed as the mean ± standard deviation. *Significant differences from the control group are indicated by $p<0.05$. The results are representative of two independent experiments.

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C. genistoides *and* C. subternata *extract consumption after sensitization potentially prevented body temperature decline in the respiratory allergy mouse model*

Since *C. genistoides* and *C. subternata* extracts significantly suppressed RBL-2H3 cell degranulation *in vitro*, the subsequent step was to ascertain whether the extracts could inhibit body temperature decline in the allergy mice when the extracts were administered after sensitization. In this experiment, the mice were sensitized using antigen inhalation exclusively for 4 weeks; therefore, the timing of *C. genistoides* and *C. subternata* administration was shifted from week –1 to week 5; that is, the mice were exclusively administered the extracts only for 3 days. The results indicate that the decrease in body temperature of the *C. subternata* group was significantly ($p<0.05$) smaller than that of the control group at 15 and 120 min after intraperitoneal antigen administration. Similarly, *C. genistoides* treatment was significantly effective ($p<0.05$) in suppressing body temperature decline compared with the control at 15 min after antigen administration. In addition, the suppression tendency was observed throughout the testing period (Fig. 4). To compare the anti-allergic capacity of *C. genistoides* with that of *C. subternata*, the area bounded by the horizontal axis and the line of each group (Figs. 1 and 4, respectively) was calculated, and the result was expressed as the ratio of the area for the *C. genistoides* or *C. subternata* group to that for the control group (Fig. 5). Based on this ratio, the anti-allergic capacities were similar for *C. genistoides* and *C. subternata.* In addition, the ratios in both groups were similar for Figs. 1 and 4, indicating that the anti-allergic activities of the honeybush extracts observed in Fig. 1 were also predominantly enabled by anti-degranulation activity.

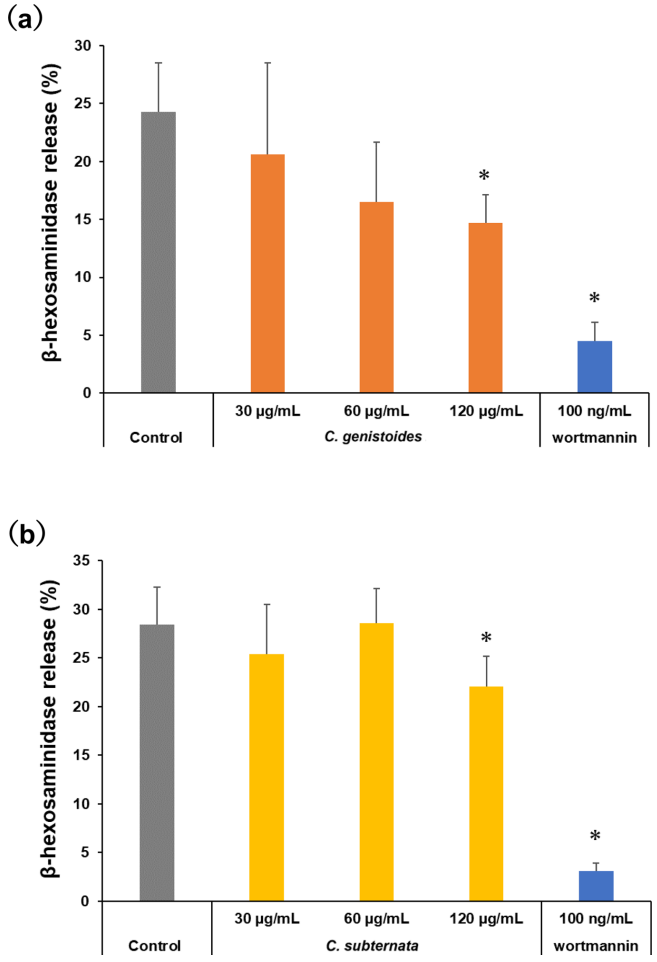

Fig. 3. Inhibition of antigen-induced degranulation of RBL-2H3 cells by *C. genistoides* and *C. subternata* extracts. RBL-2H3 cells were stimulated by an anti-dinitrophenylhydrazine (DNP)–IgE antibody and DNP–HSA in the presence of the (a) *C. subternata* and (b) *C. genistoides* extracts. The degranulation rate was calculated by measuring β-hexosaminidase release from the cells. The experiment was performed using five wells per group. The results are expressed as the mean ± standard deviation. *Significant differences from the control group are indicated by $p<0.05$. The results are representative of three independent experiments.

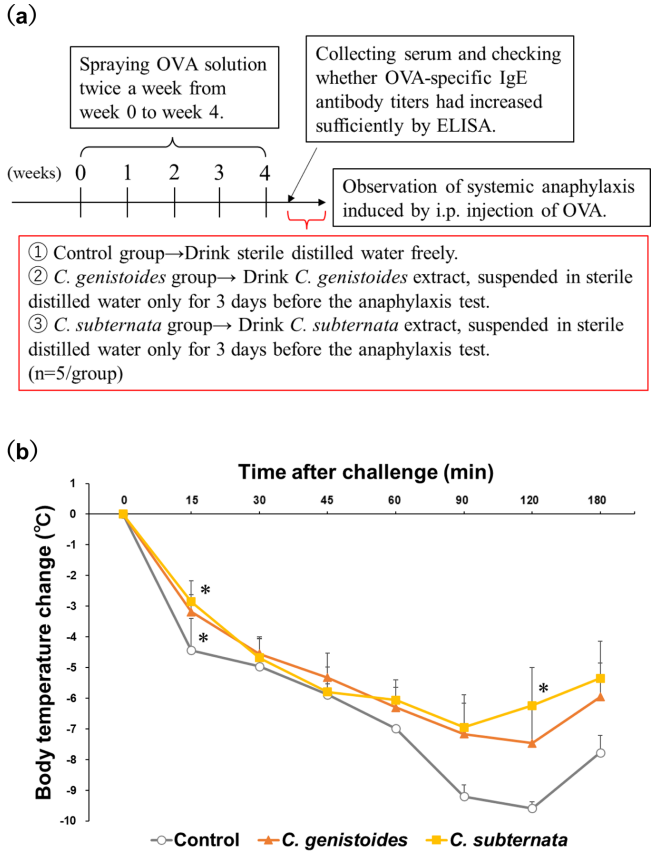

Fig. 4. Effects of *C. genistoides* and *C. subternata* extracts on antigen challenge-induced body surface temperature decline followed by extract treatment in a murine allergy model for inhaled antigen. (a) Schematic diagram of the animal model protocol. The DO11.10 mice inhaled an atomized ovalbumin (OVA)-containing solution for 4 weeks instead of 6 weeks, and after the last inhalation, the mice were treated with the extracts for 3 days. The mice were subsequently subjected to the anaphylactic test described in Fig. 1, and the changes in body surface temperature were measured. The experiment was performed with five animals per group. (b) The results of the anaphylactic reaction were evaluated based on the decrease in body temperature. The results are expressed as the mean ± standard deviation. *Significant differences from the control group are indicated by $p<0.05$. The results are representative of two independent experiments.

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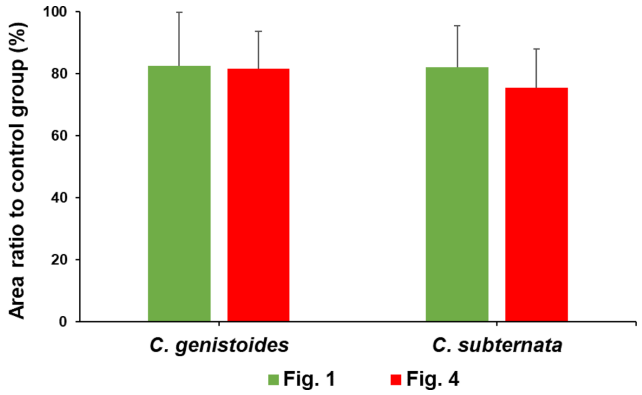

Fig. 5. Differences in body surface temperature suppression levels between Figs. 1b and 4b. The area bounded by the horizontal axis and the line of each mouse in Figs. 1b and 4b was calculated, and the area ratios for the *C. genistoides* and *C. subternata* groups relative to the control group were calculated. The results were shown as mean of each group ± standard deviation.

DISCUSSION

The present study aimed to determine whether 40% ethanol extracts of *C. genistoides* and *C. subternata*, which have been established to exhibit promising immunomodulatory activity by previous studies [11, 12], potentially alleviate allergic symptoms *in vivo*.

The present study focused on allergic rhinitis, which is characterized by nasal symptoms triggered by an IgE-mediated response to allergen exposure [22]. The murine respiratory allergy model used in this study relied on IgE production in response to antigen inhalation without pre-sensitization via intraperitoneal immunization, similar to that in human pollen allergy patients [19]. In contrast, the mice inhaled atomized OVA as a model allergen instead of pollen. Using this model, the effects of the extracts on respiratory allergy could be evaluated from the stage of sensitization with the allergen to the onset of symptoms.

The results revealed that *Cyclopia* extract consumption significantly suppressed anaphylactic reaction-induced body temperature decline, suggesting that honeybush tea consumption potentially alleviates allergic symptoms in humans. However, while the *Cyclopia* extracts suppressed allergic symptoms, they also increased the production of OVA-specific antibodies, including IgE, exhibiting consistency with the results of a previous study using a different experimental system [11]. On the other hand, several studies have shown that mangiferin, hesperidin, and pinitol, which were included in the honeybush extracts, suppressed OVA-induced IgE serum levels [14, 16, 23]. In this regard, the difference in the administered doses needs to be considered. In this study, the mice ingested a daily mangiferin dose equaling ca. 18 and 6 mg/kg body weight (BW) upon consuming the *C. genistoides* and *C. subternata* extracts, respectively, in a representative experiment. In a previous study by Piao *et al.* [14], mice with allergic rhinitis were treated with similar doses of mangiferin (5 or 20 mg/kg BW). Hesperidin was also shown to suppress serum OVA-specific IgE levels in a mouse allergy model for asthma at doses of 10 and 30 mg/kg BW, but not at 5 mg/kg BW [16]. Upon drinking the *C. subternata* and *C. genistoides* extracts, the mice ingested only 1.4 and 2.2 mg/kg BW of hesperidin, respectively. Furthermore, ingestion of the non-phenolic compound pinitol was revealed to reduce elevated IgE levels in mice with OVA-induced allergic rhinitis at doses of 10 and 20 mg/kg BW [23]. Both *Cyclopia* extracts would have contributed a daily dose of ca. 8 mg pinitol/kg BW, which was similar to the lower dose in the previous study. This evidence suggests that some of the compounds in the honeybush extracts could work to inhibit IgE production. Therefore, other factors evidently outweigh their IgE-suppression abilities.

Notably, despite differences in phenolic composition between the *C. subternata* and *C. genistoides* extracts, they exerted similar effects with comparatively equal efficacy. This implies that their effects are unlikely caused by a single compound, rendering extract standardization difficult.

Allergic symptoms are generally believed to worsen when IgE production increases, and indeed, high serum antigen-specific IgE levels have been reported in populations suffering from severe allergic symptoms [24]. Our results in Fig. 1, but not in Fig. 4, also showed that the symptom of anaphylactic reaction was slightly worse in the honeybush groups than in the control group at the early phase of the reaction, suggesting that the increased IgE production shown in Fig. 2 enhanced the allergic symptom. Several food factors, such as alginate oligosaccharide, an oligosaccharide obtained from a natural edible polysaccharide [25], and *Lactiplantibacillus plantarum*, a lactic acid bacterium involved in food fermentation [26], have been reported to suppress allergic symptoms by decreasing IgE levels. Although such foods can be expected to improve allergic conditions by modifying immune responses, Helin *et al.* [27] concluded that prolonged exposure would be required to observe their beneficial effects on the allergic response. In contrast, foods that inhibit mast cell degranulation can be expected to relieve allergic symptoms with certainty and immediacy. *Cyclopia* extracts can be expected to have such activity. In addition, since the *Cyclopia* extracts were also found to increase the production of antibodies other than IgE, in addition to alleviating allergies, they are expected to be effective in preventing infectious diseases by enhancing the body's defense ability.

The daily intake of the two extracts by the mice was ca. 200 mg/kg BW/day. This translates to a human equivalent dose of ca. 973 mg for an adult weighing 60 kg [28], an amount that can be consumed by drinking 2–3 cups of honeybush tea daily, irrespective of the *Cyclopia* species and state of oxidation of the plant material [29, 30]. If only a certain compound in the honeybush tea had the activity, considerably large volumes of the herbal tea, depending on the *Cyclopia* species and state of oxidation, would need to be ingested, which could be impractical. However, despite the differences in phenolic composition of the *C. subternata* and *C. genistoides* extracts, they exhibited relatively similar effects in promoting the production of OVA-specific IgE, IgG1, and IgG2a antibodies and suppressing the anaphylactic reaction. These results suggest that the effects were provided by different compounds and that they worked additively or synergistically to improve their efficiency. In addition, although we have not yet identified the components that have activity that promotes IgE antibody production and inhibits RBL-2H3 cell degranulation, we believe that we can prepare an extract with low IgE-promoting activity and a strong degranulation inhibitory effect by adjusting the extraction conditions.

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Although the mechanism by which the *Cyclopia* extracts inhibited RBL-2H3 cell degranulation has not been elucidated, the inhibition of FcεRI and/or IgE binding to the receptor might be rarely involved. The suppression level of the anaphylactic reaction in the honeybush extract group was stronger during the late phase of the reaction (60–180 min) than during the early phase (0–60 min), as shown in both Fig. 1 and Fig. 4, suggesting that the honeybush extracts did not suppress the expression of FcεRI and/or IgE binding but rather suppressed the subsequent signal transduction in the RBL-2H3 cells. Since it has been reported that luteolin, a type of polyphenol, inhibited signal transduction in mast cells [10], some polyphenols in the honeybush extracts could inhibit signal transduction in the RBL-2H3 cells. Another possible mechanism is that honeybush extract may have inhibited the production of reactive oxygen species (ROS) in the RBL-2H3 cells. Mast cell activation has been shown to be accompanied by ROS production. In both RBL-2H3 and bone marrow-derived mast cells, FcεRI cross-linking was reported to stimulate the rapid release of intracellular ROS, including hydrogen peroxide, that is, an oxidative burst [31]. Carnosic acid, a major polyphenol in rosemary, has been reported to reduce the production of ROS and inhibit the degranulation of bone marrow-derived mast cells [32]. Therefore, one possible mechanism is that antioxidants, such as the polyphenols in the *Cyclopia* extracts, scavenged ROS in the RBL-2H3 cells. The actual ROS-concentration changes and substances that act on ROS elimination in cells are important subjects of future research.

Previous studies have also demonstrated that *Cyclopia* extracts promote regulatory T cell (Treg cell) differentiation both *in vitro* and *in vivo* [11, 12]. They have also been revealed to enhance the production of interleukin 10 (IL-10), an inhibitory cytokine. Unfortunately, the present study could not determine whether these responses were involved in the alleviation of allergic symptoms in the allergy mice. In fact, Fig. 5 shows that the suppressive effects of the *Cyclopia* extracts on symptoms were similar between Figs. 1 and 4, indicating that anti-degranulation activity may be the major mechanism underlying the anti-allergic activity of honeybush. However, the IgE antibody titer increased in mice treated with the *Cyclopia* extracts during antigen sensitization compared with that in the control group, as shown in Fig. 2. Given that similar levels of anti-allergic effects were observed in both experiments shown in Figs. 1 and 4, the Treg cells and IL-10 induced by the *Cyclopia* extracts during sensitization might have exerted a part of the suppressive effect on symptoms in Fig. 1. Treg cells have been reported to directly inhibit mast cell degranulation through OX40-OX40L interactions [33]. IL-10 has been reported to inhibit the expression of FcεRI β chain, which is known to regulate the receptor expression and signaling in mast cells [34]. Therefore, it is possible that the Treg cells and IL-10 induced by the *Cyclopia* extracts in Fig. 1 could have suppressed allergic symptoms through these mechanisms.

In summary, oral administration of *C. genistoides* and *C. subternata* extracts suppressed RBL-2H3 cell degranulation and alleviated allergic symptoms elicited at sites distant from the gastrointestinal tract. These findings suggest that incorporating honeybush tea and food products containing honeybush extracts as functional ingredients into the daily diet could be an effective coping strategy for patients with various allergic diseases. However, further research is required to identify the physiologically active substances and determine the involvement of Treg cells.

ETHICS STATEMENT

The mice used in this study were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology. All animal experiments were approved by the Animal Ethics Committee of the Tokyo University of Agriculture and Technology (30-5, April 19, 2019; R03-9, March 18, 2021; R04-12, March 7, 2022).

AUTHOR CONTRIBUTIONS

HS, MH: Performing experiments, writing manuscript. AF, ON, SM: Performing experiments. DdB: Performing experiments, data curation. YM: Conceptualization, funding acquisition, writing manuscript. EJ, TY: Conceptualization, performing experiments, funding acquisition, writing manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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