

Chinese sweet tea (*Rubus suavissimus*) polyphenols attenuate the allergic responses in a Balb/c mouse model of egg allergy

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ABSTRACT

The aim of this study was to evaluate the effects of *Rubus suavissimus* S. Lee extract against hen egg ovalbumin (OVA)-induced allergic response in mice. BALB/c mice were sensitized with OVA via intraperitoneal injection for four weeks and subsequently administered the different doses of *Rubus suavissimus* S. Lee extract (0.1, 0.5 and 1.0% w/v) in drinking water. We demonstrated that *Rubus suavissimus* S. Lee extract can attenuate OVA allergic response in mice by inducing immune desensitization through Th1/Th2 immunological modulation, a regulatory response involving the transcription factor FOXP3, induction of a suppressed IL-4, but increased IL-10, IL12a and INF- γ , enhanced immunoglobulin isotype IgA production, and suppression of histamine and MCPT-1. These results suggest that *Rubus suavissimus* S. Lee extract may be a potentially useful candidate for the design of a functional food component in targeting management of food allergy.

1. Background:

Food allergy is one of the growing health concerns that affects 3% to 4% of adults and up to 6% of children (Loh, Tang, Loh, & Tang, 2018; Sampson, 2016). Both the prevalence and severity of food allergies have increased in past few decades (Sampson, 2016; Sicherer & Sampson, 2010). Earlier studies suggest that Immunoglobulin E (IgE)-mediated immune response to proteins in food can be life-threatening, leading to responses ranging from repeated scratching, diarrhea, vomiting to the closure of the airway (Colgan & Hankel, 2010; Shridhar, Changqi, & Valerie, 2016). Among the major food allergens, egg is one of the most common ones, especially in young children (Caubet & Wang, 2011; Savage, Matsui, & Wood, 2007). Mainly glycoproteins from egg white have identified as food allergens. Among all the glycoproteins in egg white ovalbumin (OVA) and ovomucoid (OVM) play the most crucial role in the egg-induced allergenic response (Honma, Aoyagi, & Kohno, 1991). OVM is heat resistant and also resistant to digestive enzymes (Caubet & Wang, 2011). On the other hand, OVA is not heat resistant and digested and absorbed in the gut (Caubet & Wang, 2011; Martos, Contreras, Molina, & López-Fandiño, 2010).

Rubus suavissimus S. Lee is a perennial shrub widely distributed in southwestern China. *R. Suavissimus* leaf is also called *tiancha*, or Chinese sweet tea due to its sweet flavor. The leaf of *R. Suavissimus* is consumed

as a beverage tea, natural sweetener, and herbal medicine in southern China, particularly Guangxi Zhuang Autonomous region (Chou et al., 2009; Ezure & Amano, 2011; Koh et al., 2010). The presence of diterpene glycosides, such as rubusoside and steviol monoside, contributes to the sweetness of Chinese sweet tea. *R. Suavissimus* leaf extract contains several plant polyphenols, such as glucose oxidase (GOD)-type ellagitannins (EGA) and gallic acid (GA), which are bioactive natural products with potential pharmacological benefits in long-term health protection, such as lower blood pressure, relieve diabetic symptoms, reduce oxidative stress-related chronic diseases and age-related disorders (Hsu & Yen, 2007). A large body of emerging evidences supports the observation that increased consumption of plant-based beverages with high polyphenol content exerts significant health-promoting effects such as reduced risk of developing metabolic syndromes (e.g. high blood pressure, glucose level and excess body fat) (Potenza et al., 2007; Wu et al., 2003). Recent studies have indicated their anti-inflammatory effects using a LPS-induced low grade inflammatory C57BL/6J mouse model and contributes to restoring immune and metabolic homeostasis, thereby preventing early onset of metabolic disorders such as obesity and type 2 diabetes. Thus, these compounds support the use of Chinese sweet tea as a complementary medicine to help reduce the occurrence of low-grade inflammation diseases (Zhang, Qi, Zeng, Tsao, & Mine, 2020). However, the effect of

Abbreviations: OVA, egg ovalbumin; IP, intraperitoneal injection; GOD, glucose oxidase; EGA, ellagitannins; GA, gallic acid; PBS, phosphate buffered saline; MCPT-1, Mast Cell Protease-1; IgE, immunoglobulin-E; FoxP3, forkhead box P3

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Rubus suavisissimus S. Lee extract on allergic response has not elucidated yet. Therefore, the primary objective of the current project is to evaluate the effect of *Rubus suavisissimus* S. Lee extract on OVA-induced allergic response in a Balb/c mouse model.

2. Materials and methods

2.1. Materials

Rubus suavisissimus S. Lee leaf extract powder was purchased from Marugen Pharmacy Ltd (Okayama, Japan). This *Rubus suavisissimus* S. Lee leaf hot water extract contains 30.1% polyphenol and 7.0% GOD type polyphenol (data not shown). We previously reported that was identified three major components as gallic acid (GA) (1659.0 ± 142.5 mg/100 g dry weight), ellagic acid (EGA) (4622.7 ± 142.0 mg/100 g dry weight) and rubusoside (RUB) (333.0 ± 67.7 mg/100 g dry weight) (Zhang et al., 2020).

2.2. Experimental design and sensitization protocol

All experimental protocols were in compliance with the Canadian Council for Animal Care guidelines and all animal use protocols were based on and approved by University of Guelph Animal Care Committee (AUP 1567). SBALB/c female mice (6–8 weeks old; 16–18 g) were divided into 5 groups (n = 12 per group). Ovalbumin (OVA: 50 µg/mouse) was given by interperitoneal injection (IP) (Dissolved in 50 µl of saline and 50 µl of alumni adjuvant) once a week for four weeks. The specific IgE levels were checked on 5th and 11th week from randomly selected animals blood plasma for each group to ensure the allergenic sensitization. On the final day, allergic reactions were induced by administering 20 mg of OVA orally (week 14) and all mice were humanely euthanized after 30 min of monitoring, blood and tissues were collected subsequently. Complete experimental plan and other details are illustrated in Fig. 1. Clinical scores were evaluated in a blinded fashion by three experienced independent observers for twelve different clinical symptoms. Adding scores for each individual sign and averaging the symptoms value total clinical scores were obtained for each animal (Rupa & Mine, 2006). Briefly, mice were challenged orally with 20 mg of OVA, observed for 30 min and clinical scores were assigned in a blinded manner by four experienced observers. Scores were as follows: 0 = no signs; 1 = repeated scratching, rubbing around the snout and

head; 2 = puffiness around eyes and snout, diarrhoea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; 3 = wheezing, laboured respiration, cyanosis around the mouth and tail; 4 = tremor and convulsions and 5 = death. Total scores for each animal were obtained by adding average scores for individual Clinical signs (Mine et al., 2019). Blood was collected for various biomarker assays such as measurement of histamine, antibody activity and flow cytometry etc. All samples were stored at -80°C until further analysis. Spleen was collected for measuring cytokines.

2.3. Cytokine analysis from mouse spleen

Individual spleens were collected at the end point post challenge aseptically from each mouse. Single cell suspensions were prepared and splenocyte cell viability was assessed by trypan blue exclusion. Cells were cultured in 24 well plates in triplicate at a density of 2.5×10^6 cells/ml as unstimulated (control) or stimulated with 50 µg/well of OVA. Culture supernatants were collected after 72 h incubation at 37°C and stored in aliquots at -20°C . Concentrations of cytokines were measured in culture supernatants using ELISA cytokine kits following manufacturer's instructions (ebiosciences, CA, USA for IL-4, IFN- γ , IL-10, IL-12, IL-17A, and TGF- β).

2.4. Antibodies analysis from mouse blood plasma and feces

OVA-specific immunoglobulin-E (IgE), immunoglobulin-G1, and IgG2a concentrations were measured from plasma and IgA concentration were measured from feces using indirect sandwich-type ELISA according to manufactures protocol (ebiosciences, CA, USA). Determination of individual subclasses of serum allergen-specific IgE, IgG1, IgG2a, and feces sp-IgA were examined. Briefly, 96-well microtiter plates (Costar, Corning Inc., NY, USA) were coated with 2 µg/well of OVA (in 100 µl of 50 mM carbonate buffer; $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 9.6) for 24 h at 4°C . Rabbit anti-mouse IgE conjugated to alkaline phosphatase was used for the detection of IgE, and rat monoclonal anti-mouse IgA was used for IgA followed by streptavidin–HRP conjugate. For IgG1 and IgG2 rat monoclonal anti-mouse IgG1 and IgG2a were used followed by streptavidin–HRP conjugate. Plates were washed 3 times with 200 µl of 0.05% Tween PBS (PBST 0.01 M, pH 7.4) per well after coating with 2% bovine serum albumin /phosphate buffered saline (PBS) at 37°C for 1 h. Washing was repeated and sera diluted (1:10 for

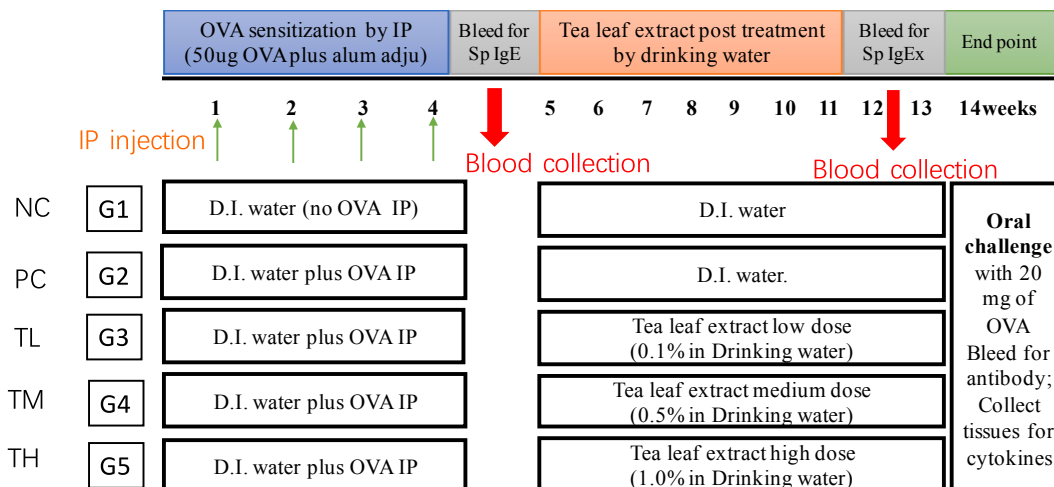


Fig. 1. Experimental study design. Five groups (n = 12/group) of female BALB/C mice, 6–8 week old were used in this study. During the first 4 weeks, mice were sensitized with ovalbumin [OVA: 50 µg/mice dissolved in 100 µl of saline:aluminum adjuvant (1:1)] given intraperitoneally (IP) once a week. The animals in the negative control group did not receive an injection only de-ionized (DI) water with regular chow. From 5th week, *Rubus suavisissimus* S. Lee extract was administered in drinking water with three dosages (Low: 0.01%; Medium: 0.5%; and High: 1.0%) up to 13th week. On the 14th week, mice were orally challenged with OVA (20 mg/mouse), scored for clinical allergic reactions and then humanely euthanized by cervical dislocation. Blood and other tissues samples were then collected for further analysis.

IgE measurement, 1:100 for IgG1 and IgG2a, no dilution of IgA) in 0.05% PBST were added at 100 μ l per well and incubated for overnight at room temperature with gentle shaking. For IgA, proteins were extracted using appropriate inhibitor cocktail from feces and protein concentrations were measured according to BCA assay. Plates were washed again and the bound antibodies were detected by adding respective detection antibodies at room temperature for 1 h. Specific binding activity was detected by addition of 100 μ l/well of 3, 3', 5'-tetramethylbenzidine substrate (Sigma). The reaction was terminated with 1 N H₂SO₄ after 30 min and absorbance at 450 nm was measured by a micro titer reader (Bio-Rad 550, Hercules, USA). For total antibody same sandwich ELISA method was used but the plates were coated by capture antibody as per the manufacturers instruction.

2.5. Flow cytometric analysis

Antibody labelling techniques for flow cytometry was performed using standard techniques, adapted from BD Biosciences (Mississauga, Canada). Blood was collected post challenge from individual mouse and pooled (n = 6/group). Flow cytometry staining protocol was carried out as recommended by the manufacturer (BD bioscience) and the sample was stained with 100 μ l of FITC conjugated anti-mouse CD25 antibody (558642; BD Biosciences, California, USA) and PE conjugated anti-mouse-FOXP3 (eBioscience, CA, USA) and analysis of labeled cells was performed using a Accuri Flow Cytometer (BD). Live cells were gated on the basis of forward and 90° light scatter characteristics. At least 10,000 events were acquired from each sample and all data sets were analyzed using Accuri Express software (BD Biosciences).

2.6. Statistics and data analysis

Results are expressed as mean values $\pm$ SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's multiply comparison test or a one-tailed, unpaired Student's *t*-test (GraphPad Prism version 5.0). ANOVA with the Tukey's post hoc test was used when analyzing the means of more than two groups. The Student's *t*-test was used when analyzing the means of two specific groups.

3. Results

3.1. Clinical scores and allergenic response of mice

On the final experimental day allergy were induced into the mice by oral gavage of OVA and then the mice were observed for next 30 mins for clinical scoring. Fig. 2 illustrated the clinical signs observed in different groups of mice. After challenge, animals in the positive control group (2.83 ± 0.19) developed statistically significant clinical scores of food allergy compared to the negative control (0.59 ± 0.15) ($p < 0.001$). But *Rubus suavisissimus* S. Lee extract treatment (Low, Medium, and High) did not reach any statistical significance compared to positive control group, however a trend towards a decrease in clinical scores was observed with the treatment groups compared to the positive control.

3.2. Histamine and MCPT-1 concentration

Histamine and MCPT-1 (Mast Cell Protease-1) was evaluated as a measure for the mast cell activation and degranulation. The concentration of Histamine and MCPT-1 was measured. The MCPT-1 level went up and positive control (747.80 ± 98.74 ng/mL) significantly difference with negative control (50.05 ± 4.70 ng/mL) (Fig. 3B), but showed significantly decreased with high dose *Rubus suavisissimus* S. Lee extract high dose treatment group (369.30 ± 41.72 ng/mL) only ($p < 0.05$). The histamine level did significantly decreased with *Rubus suavisissimus* S. Lee extract treatment in all doses ($p < 0.001$) (Fig. 3A).

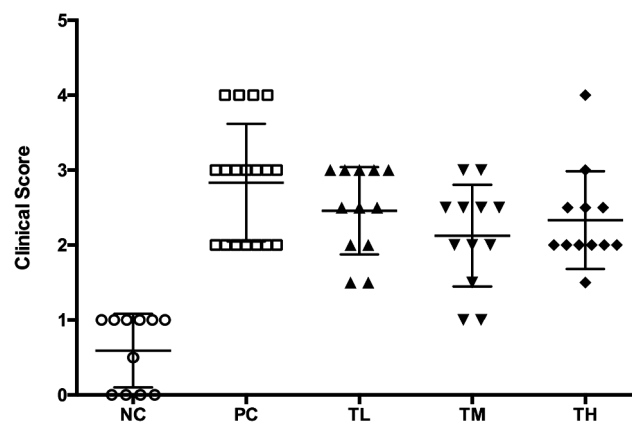


Fig. 2. Clinical scoring of allergic symptoms. On the 14th week of experimental period, clinical scores for each mouse post-OVA challenge were calculated. Average scores assigned by three independent observers in a blinded fashion are represented. The statistical differences between groups were determined, ** and *** indicate $P < 0.01$ and $P < 0.001$ respectively as compared to the positive-control (PC) group.

3.3. Antibody concentrations in plasma and feces

Specific and total antibody levels were measured. Specific IgE and total IgE levels measured in the blood plasma. Specific IgE levels increased in positive control group (1.50 ± 0.09) compared to negative control (0.18 ± 0.05), but after *Rubus suavisissimus* S. Lee extract intervention a decreasing trend was not observed (Fig. 4B). The total IgE levels decreased significantly after *Rubus suavisissimus* S. Lee extract low-high dose treatments (L: 24.10 ± 3.79 μ g/mL; M: 22.96 ± 3.87 μ g/mL; H: 19.92 ± 2.84 μ g/mL, respectively) compare to Positive control group (43.71 ± 5.11 μ g/mL) (Fig. 4A).

The total IgG levels (Fig. 4C) increased compared to negative control (97.33 ± 21.68 μ g/mL) in positive control (948.30 ± 28.47 μ g/mL) but treatment groups decreased total IgG1 level in low (461.50 ± 22.89 μ g/mL), medium (492.60 ± 21.45 μ g/mL) and high dose (375.20 ± 26.01 μ g/mL) ($p < 0.001$). *Rubus suavisissimus* S. Lee extract intervention decreased specific IgG levels in medium group (1.59 ± 0.03) compare to positive control (1.79 ± 0.03) (Fig. 4D). The IgG1 and IgG2a (specific and total) measured from blood plasma as well. The specific IgG1 levels did not show significant difference after *Rubus suavisissimus* S. Lee extract intervention (Fig. 5B), while it decreased total IgG1 in medium (3866.00 ± 361.20 ng/mL) and high dose (3592.00 ± 375.30 ng/mL) compared to positive control (5537.00 ± 129.40 ng/mL) ($p < 0.001$) (Fig. 5A). On the other hands, total IgG2a showed a significantly decreased in high dose (149.40 ± 34.56 ng/mL) only compared to positive control (368.40 ± 39.10 ng/mL) ($p < 0.001$) after *Rubus suavisissimus* S. Lee extract treatment (Fig. 5C). But the specific IgG2a level showed a down regulation in all treatment groups compare to positive control ($p < 0.001$) (Fig. 5D).

The IgA levels (specific and total) measured from the total protein extracted from the mice feces. The specific IgA in *Rubus suavisissimus* S. Lee extract treatment medium and high dose groups showed a significantly increased (Medium: 2.21 ± 0.35 ; high: 2.13 ± 0.45 , respectively) compared to positive control (0.478 ± 0.05) (Fig. 6B) and the total IgA showed an increase trend (low: 506.60 ± 24.01 ng/mL, and high: 902.60 ± 42.91 ng/mL) compare to positive control group (315.70 ± 48.14 ng/mL) (Fig. 6A).

The Flow cytometry analysis showed that the FOXP3+ and CD25+ cells number decreased as allergenic response in positive control (10.41 ± 1.22) compared to negative control (50.47 ± 9.00) ($p < 0.05$). But after *Rubus suavisissimus* S. Lee extract intervention showed the FOXP3+ cells significantly increased in medium and high

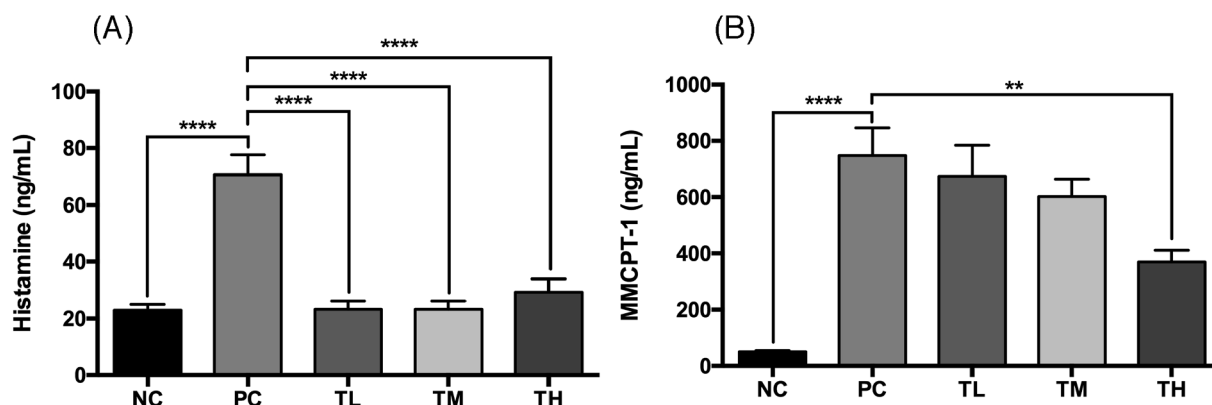


Fig. 3. Serum histamine and mast cell protease levels. (A) Serum histamine concentration and (B) Mouse mast cell protease (MMCPT). The histamine and MMCPT concentrations were measured by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, **, and **** indicate $P < 0.01$, and $P < 0.001$ respectively, as compared to the positive-control (PC) group.

dose treatment groups (M: 40.05 ± 13.46 , H: 52.67 ± 12.84), but not in low dose group (Fig. 7)

Cytokine expression in spleenocyte was measured from the cultured spleen cells. The IL-4 levels was decreased compare to positive control (56.88 ± 5.01 pg/mL) in medium (15.81 ± 0.22 pg/mL) and high (33.24 ± 9.01 pg/mL) groups after *Rubus suavisissimus* S. Lee extract treatment (Fig. 8A). IL-12a level showed a trend of increased after *Rubus suavisissimus* S. Lee extract low dose intervention, but failed significant change (Fig. 8B). IL-10 was observed to increase at low (324.80 ± 24.22 pg/mL) dose only, but decreased in high dose compared to positive control (209.90 ± 19.36 pg/mL) (Fig. 8C). There was no difference of IL-17A with intervention (Fig. 8D), but *Rubus suavisissimus* S. Lee extract decreased TGF- β in all interventions (Fig. 8F). IFN- γ level was significantly increased after *Rubus suavisissimus* S. Lee extract medium dose intervention (218.10 ± 48.92 ng/mL),

compared to positive control group (82.17 ± 8.45 ng/mL) (Fig. 8E).

4. Discussion:

Food allergy is an immunologically adverse reaction caused by foods. It is an IgE-dependent type I hypersensitivity reaction due to the imbalance of Th1/Th2. The prevalence of allergic diseases remains on the rise and has become a significant global health problem. Different contributing factors have been reported such as changing dietary habits and decreased microbial exposure (commonly described as the hygiene hypothesis) (Özdemir, 2010). Preventive and therapeutic approaches including anti-IgE monoclonal antibodies, oral tolerance induction, peptide-based and DNA-based immunotherapies, genetically modified allergens and probiotics or prebiotics showed promising results but still require further investigations (Wood, 2016). Due to the complex nature

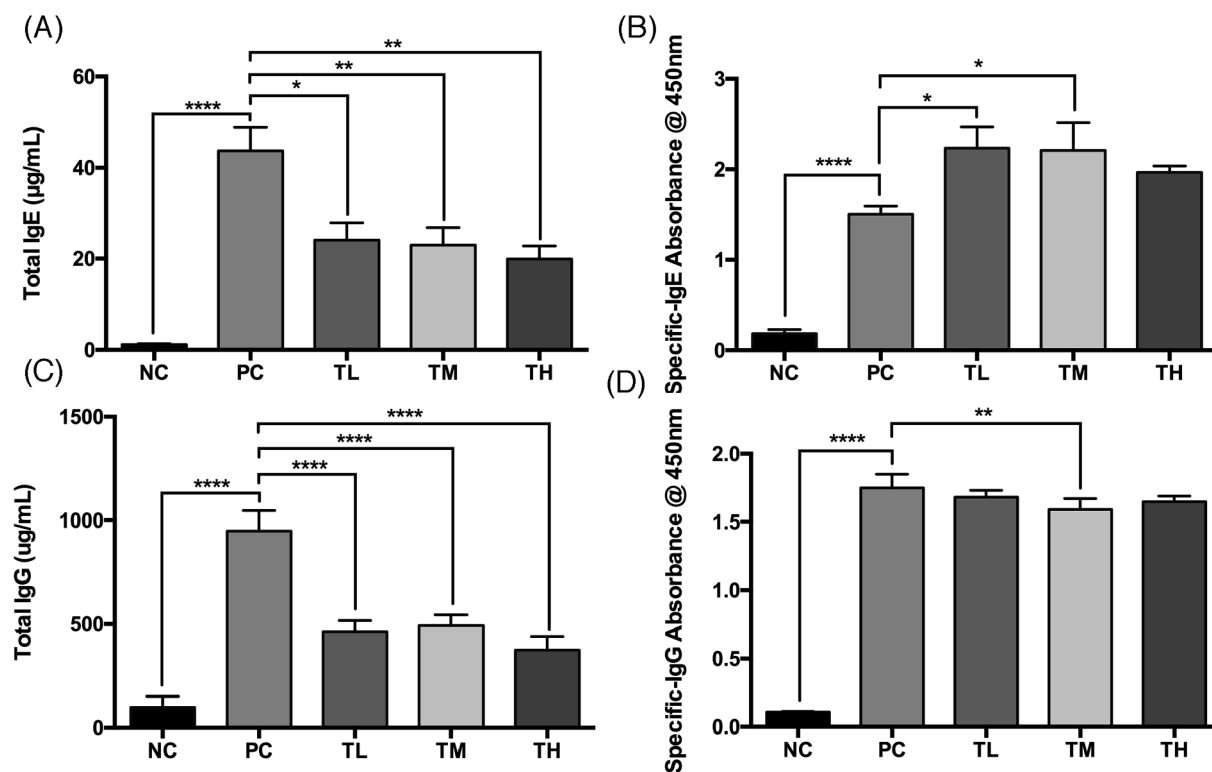


Fig. 4. Serum total and OVA-specific IgE and IgG levels. (A) Total IgE and (B) OVA-Specific IgE, (C) Total IgG, and (D) OVA-specific IgG concentration in serum. Total and OVA-sp-IgE and IgG concentrations were measured by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, *, **, and **** indicate, $p < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, as compared to the positive-control (PC) group.

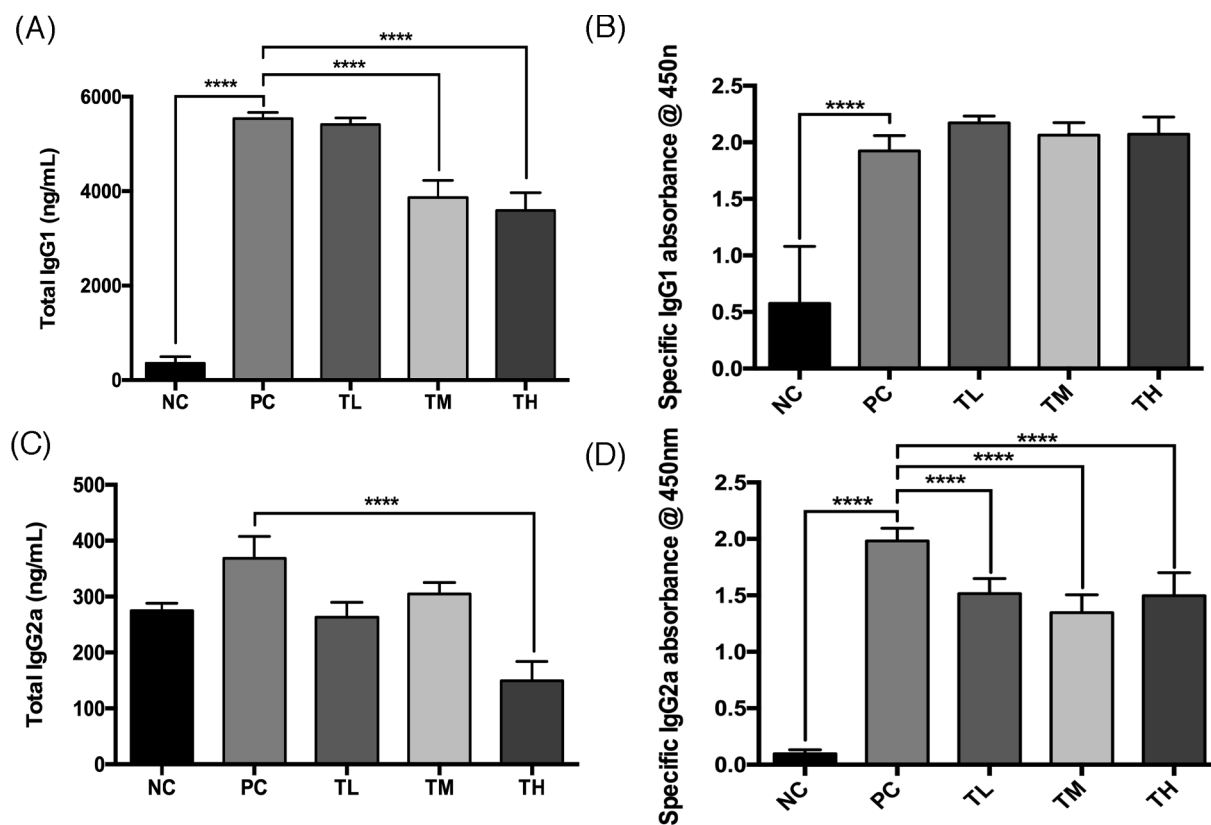


Fig. 5. Serum total and OVA-specific IgG1 and IgG2a levels. (A) Total IgG1, (B) OVA-specific IgG1, (C) Total IgG2a, and (D) OVA-specific IgG2a concentration in serum. Total and OVA-sp-IgG1 and IgG2a concentrations were measured by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, **** indicate $P < 0.001$ as compared to the positive-control (PC) group.

of allergic mechanisms and still not completely unlocked, standard practices are limited to allergen avoidance, nutritional advice, and immediate access to emergency medication. A food allergic murine model sensitized by i.p. injection followed by the orally challenge of anti-allergic bioactive compounds was established in our past study (Rupa, Nakamura, Katayama, & Mine, 2014).

There are supportive evidences that supplementation of infant formulas with medicinal herb based polyphenols may prevent eczema and asthma in infants (Magrone & Jirillo, 2012). Polyphenols found in foods and plant sources and have been investigated for their anti-allergic effect in different disease models and in human clinical trials, and were reported polyphenols modulate the process of allergic sensitization and

their direct effect on allergic effector cells such as mast cells inhibit mediator release, resulting in the alleviation of symptoms (Singh, Holvoet, & Mercenier, 2011). However, there is concern over the reliability of the evidence due to not all trials reporting beneficial outcomes for all infants (Ozdemir, 2010).

This study aimed to study the therapeutic effects of *R. Suavissimus* leaf extract against OVA-induced allergy in mice. The responses to the various treatments were measured by allergic clinical symptoms, histamine, and mast cell protease concentration, serum total and specific anti-OVA antibody as IgE, IgG, IgG1, and IgG2a, as well as cytokine production levels in spleen, and percentage of T-reg cells in the blood. We found that 9 weeks of oral administration of *Rubus suavissimus* leaf

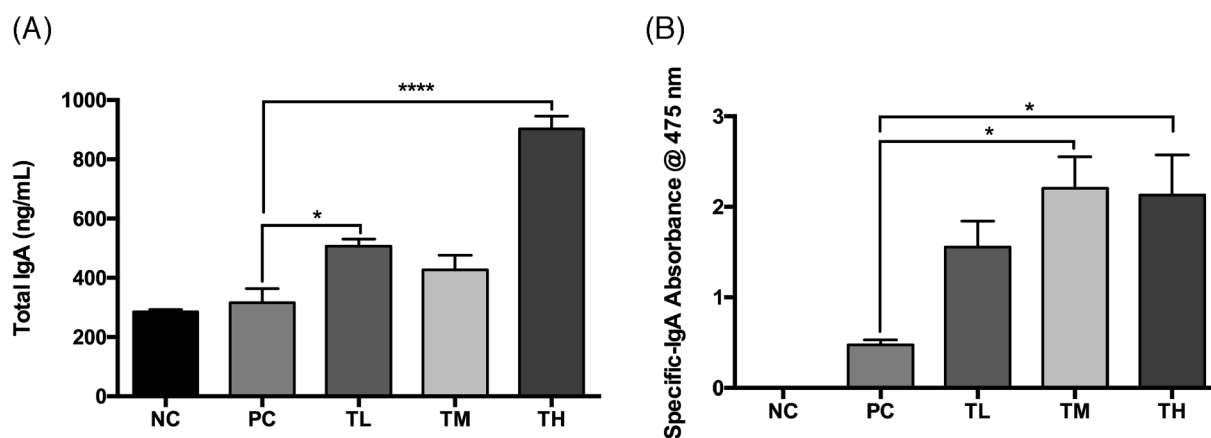


Fig. 6. Total and OVA-specific IgA were detected from pooled fecal samples collected from weeks 13 using biotinylated rat monoclonal anti-mouse IgA antibody followed by avidin-HRP conjugate. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, *, and **** indicate $P < 0.05$, and $P < 0.0001$ respectively as compared to the positive-control (PC) group.

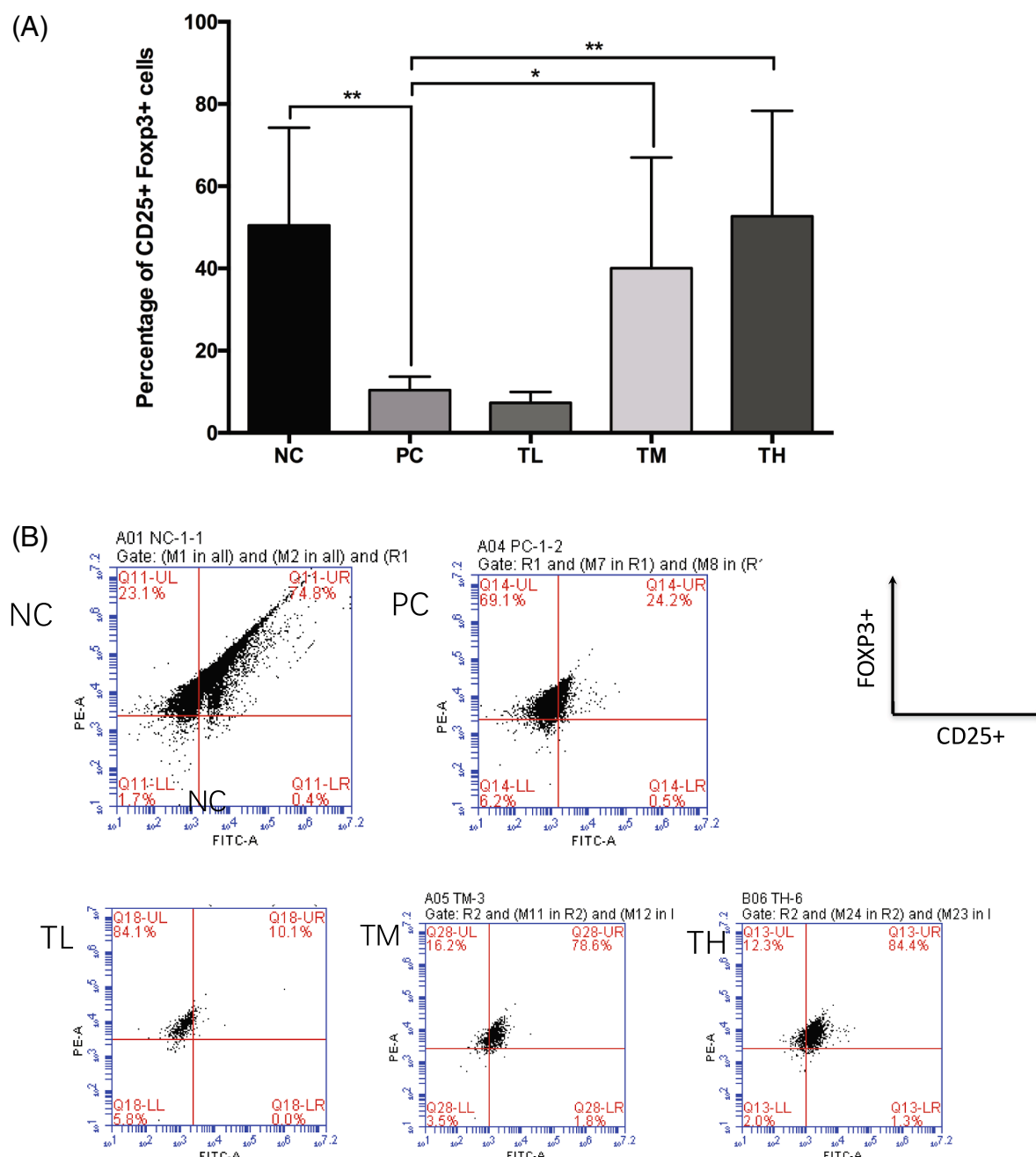


Fig. 7. Percentage of CD25 + and Foxp3 + cells in serum. (A) Percentage of CD25 + Foxp3 + cells were determined by flow cytometry from whole blood of mice collected post oral challenge. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ animals per treatment group, * and ** indicate, $p < 0.05$ and $P < 0.01$ respectively, as compared to the positive-control (PC) group. (B) A representative image of CD25 + and Foxp3 + cells for each group and respective percentage of cells for each quadrant are shown.

extract in drinking water showed a trend of decreasing clinical signs such as scratching, lethargy, gastrointestinal signs and respiratory difficulty, but failed to observe significance of clinical sign at both low and high doses as shown in Fig. 2.

Histamine has been well recognized as a significant mediator of the allergic diseases (Xie & He, 2005). Histamine levels were significantly reduced after *R. Suavissimus* leaf extract intervention. We demonstrated earlier that *Quillaga* saponin exerted inhibitory effects on histamine release and on IgE-mediated responses in rat basophil cells (Zhang, Yang, & Mine, 2012). This is in a good agreement with previous studies conducted in a BALB/c mouse model using a genetically modified allergen, T-cell epitope peptides or supplemental beta-1.4. mannobiose (Rupa and Mine, 2006, 2012; Yang, Yang, & Mine, 2010). Mast cells are

an important mediator-secreting cell in allergic diseases, MMCP-1 predominantly expressed in the mucosal mast cells. The degranulation of mast cell leads to the increased levels of MMCP-1 indicating the increase in mucosal permeability in allergic reactions (Benedé & Berin, 2018; Galli & Tsai, 2012; Rupa et al., 2014). *R. Suavissimus* leaf extract significantly reduced the MMCP-1 levels in high dose group. Our study demonstrated that *R. Suavissimus* leaf extract could potentially attenuate the development of allergic reactions.

As previously reported that the major polyphenol compounds in *Rubus suavissimus* leaf extract are GA, EGA and RUB (Zhang et al., 2020). GA is known to have diverse biological effects such as anti-oxidation, anti-inflammation, and anti-cancer (Hsu & Yen, 2007) and recently it has been reported that GA can inhibit histamine release and

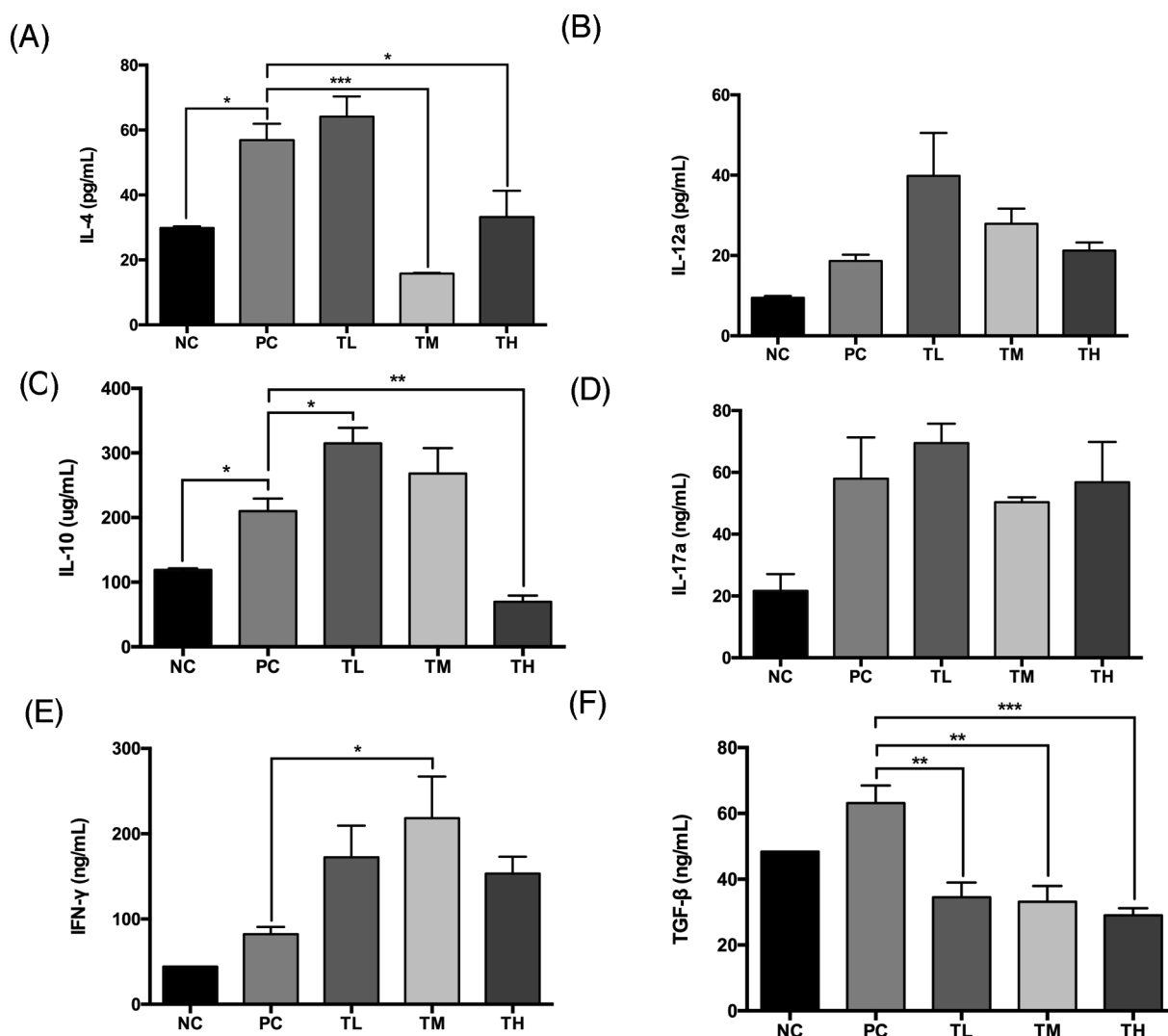


Fig. 8. Cytokine production cultured splenocytes. Spleens were collected from mice and pooled within each group ($n = 6$ per group). Single cell splenocyte cultures were prepared and plated at 2.5×10^6 cells/mL in triplicate wells in a 24-well plate and were either stimulated (PC, TL, TM, and TH) with $50 \mu\text{g}/\text{well}$ of OVA for 72 hrs or unstimulated (NC). The cytokine concentration of cell culture supernatants for (A) IL-4, (B) IL-12a, (C) IL-10 (D) IL-17a (E) IFN- γ , and (F) TGF- β were determined by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 12$ per treatment group, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively, as compared to the positive-control (PC) group.

pro-inflammatory cytokine in mast cells (Kim, Jun, & Suk, 2006). Therefore, the current anti-allergic activity of *R. Suavissimus* leaf extract could be attributed with a rich of GA in the extract, however, the role of other compound is still remained unclear.

In this study, we observed that administration of *R. Suavissimus* leaf extract did not significantly decrease serum OVA-specific IgE concentration, but led to significantly lower total IgE production compared to positive group. This suggest that the therapeutic effect of *R. Suavissimus* leaf extract may be due to different regulatory mechanisms inducing immune tolerance to OVA allergen. An earlier study reported similar findings in that specific immunotherapy did not significantly change specific IgE levels post-treatment; but led to suppression of allergic responses in human study (Jutel et al., 2003; Möbs et al., 2010). Previous studies from our laboratory also observed the same phenomena (Mine et al., 2019; Yang et al., 2013). In the present study, measurement of serum IgE titers was not a robust predictor of an allergic reaction. A possible explanation for this may be that quantification of allergen-specific serum IgE may not reflect allergy-mediating activity of leucocyte Fc ϵ R1-bound antibody. This type of tolerance may be induced by several other key mechanisms that include clonal deletion and induction of tolerogenic Treg cells that produce IL-10 and/or

TGF- β (du Pré & Samsom, 2011; Akdis & Akdis, 2011).

The measurement of serum OVA-specific IgG, and sub-isotypes IgG1 and IgG2a exhibited an interesting phenomenon. The OVA-specific IgG levels did not differ in the treatment groups with only exception to the medium dose group (Fig. 4D). On the contrary, the serum OVA- total and specific IgG2a (Th1) levels were significantly reduced after the intervention of *R. Suavissimus* leaf extract indicating the effect of treatment in reducing antibody isotypes (Fig. 5C, D). The OVA-specific IgG1 (Th2) levels did not differ in the treatment groups. Additionally, the significant reduction of total IgG and IgG1 levels further suggests that it is most likely that allergic suppression induced by *R. Suavissimus* leaf extract indicated possible immunological tolerance by clonal anergy or it may be possible that there may be other IgG sub-isotypes that may have contributed to the induction of tolerance, which were not tested here. (Rupa & Mine, 2019)

IgA is the predominant antibody found in mucus membranes and mucosal IgA acts as the first line of defence helping to protect against the entrance of foreign substances (Mathias et al., 2011). Studies showed that elevated concentrations of intestinal IgA played an important role in tolerance induction and reduced risk of IgE-associated allergic diseases (Kukkonen, Kuitunen, & Savilahti, 2010). Moreover,

intestinal IgA synthesis is necessary for the maintenance of intestinal immunity and homeostasis (Fagarasan, Kawamoto, & Suzuki, 2009). Our study demonstrated that *R. Suavissimus* leaf extract up regulated the mucosal IgA secretion and therefore, the therapeutic effect of *R. Suavissimus* leaf extract on allergy may partially be attributed to enhanced intestinal IgA secretions.

The transcription factor Foxp3 appears to be critical in the suppressive abilities of Treg cells. The decrease in Foxp3 + CD25 + Treg cells, resulting in an increase of Th2-derived immune response by increasing the production of cytokines (Nouri-Aria, 2009). Our present study demonstrated that the intervention of *R. Suavissimus* leaf extract significantly increases the percentage of Foxp3 + CD25 + Treg cells. IL-4 is produced by Th2 cells and promotes the production of antigen-specific IgE by B cells (Mathias et al., 2011). Following the increase in the percentage of Foxp3 + CD25 + T-Reg cells, we found that oral administration of *R. Suavissimus* leaf extract lowered the production of IL-4 at medium and high dose (i.e., a Th2-biased cytokine) suggesting that *R. Suavissimus* leaf extract may be able to modulate the Th1/Th2 balance towards alleviation of allergic responses. Previous studies reported that heat denaturation of OVA led to significantly decreased levels of IL-4 in mice (Sicherer & Sampson, 2010).

5. Conclusions

In conclusion, these data provide evidence for role of *Rubus suavissimus* S. Lee extract in attenuation of OVA allergic response in mice by inducing immune tolerance through Th1/Th2 modulation, a regulatory response involving the transcription factor FOXP3, induction of a suppressed IL-4, but increased IL-10, IL12a and INF- γ , enhanced immunoglobulin isotype IgA production, and influencing mast cell functionality (suppression of histamine and MMCPT-1). This may suggest that *Rubus suavissimus* S. Lee extract may be a potentially useful candidate for the design of a functional food component in targeting management of food allergy.

Ethics

All experimental protocols were carried out in accordance with the Canadian Council for Animal Care guidelines and all animal use protocols were approved by University of Guelph Animal Care Committee (AUP07R113).

CRedit authorship contribution statement

Yoshinori Mine: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing. **Kaustav Majumder:** Data curation, Investigation, Methodology, Software, Validation, Visualization. **Yan Jin:** Data curation. **Yuhan Zeng:** Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contribution

YM designed the study and wrote the manuscript. YJ, KM and YZ contributed to data collection. KM and YZ performed the statistical analysis and interpretation of the results. YM and KM performed proof reading. All authors read and approved the final manuscript.

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