



Berberine and limonin suppress IgE production by human B cells and peripheral blood mononuclear cells from food-allergic patients

Nan Yang, PhD^{*}; Julie Wang, MD^{*}; Changda Liu, PhD^{*}; Ying Song, MD^{*}; Shuwei Zhang, PhD[†]; Jiachen Zi, PhD[†]; Jixun Zhan, PhD[†]; Madhan Masilamani, PhD^{*}; Amanda Cox, MD^{*}; Anna Nowak-Wegrzyn, MD^{*}; Hugh Sampson, MD^{*}; and Xiu-Min Li, MD, MS^{*}

^{*} Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, New York

[†] Department of Biological Engineering, Utah State University, Logan, Utah

ARTICLE INFO

Article history:

Received for publication December 19, 2013.

Received in revised form July 16, 2014.

Accepted for publication July 21, 2014.

ABSTRACT

Background: Currently, there is no satisfactory treatment for IgE-mediated food allergy. Food Allergy Herbal Formula 2 (FAHF-2) and butanol-purified FAHF-2 (B-FAHF-2) have been shown to protect against peanut-induced anaphylaxis and inhibit IgE synthesis in a murine model.

Objective: To determine which herbs and compounds in FAHF-2 and B-FAHF-2 suppress IgE production.

Methods: The effect of FAHF-2 and B-FAHF-2 on IgE production was determined using a human B-cell line (U266). Individual compounds were isolated and identified using column chromatography, liquid chromatographic mass spectrometry, and nuclear magnetic resonance techniques. The potency of compounds on IgE suppression were investigated using U266 cells and verified using human peripheral blood mononuclear cells (n = 25) from peanut-allergic patients. Epsilon germline transcript expression was determined. Phosphorylated I κ B α level was analyzed using the In-Cell Western assay. The mRNA expression of signal transducer and activator of transcription-3, T-box transcription factor TBX21, interferon- γ , forkhead box P3, GATA-binding protein 3, interleukin-10, and interleukin-5 also were analyzed using real-time polymerase chain reaction.

Results: FAHF-2 and B-FAHF-2 inhibited IgE production by U266 cells. B-FAHF-2 was 9 times more effective than FAHF-2. Two compounds that inhibited IgE production were isolated from *Philodendron chinensis* and identified as berberine and limonin. Berberine was more potent and inhibited IgE production by peripheral blood mononuclear cells by 80% at 0.62 μ g/mL. Berberine significantly inhibited ϵ -germline transcript expression by peripheral blood mononuclear cells. Phosphorylated I κ B α level was significantly suppressed and mRNA expressions of T-box transcription factor TBX21 and signal transducer and activator of transcription-3 were significantly increased by berberine.

Conclusion: Berberine and limonin mediated IgE suppression. The mechanism by which berberine modulates ϵ -germline transcript expression might be through regulating the phosphorylated I κ B α level and the expressions of signal transducer and activator of transcription-3 and T-box transcription factor TBX21.

Trial Registration: ClinicalTrials.gov identifier NCT00602160.

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Reprints: Xiu-Min Li, MD, MS, Pediatric Allergy and Immunology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029-6574; E-mail: xiu-min.li@mssm.edu.

Disclosure: Drs Li and Sampson hold US patent PCT/US05/08600 for FAHF-2 and are shareholders of Herbs Springs, LLC. Dr Sampson has received a grant from and served on the medical advisory board of FARE.

Funding Sources: This study was supported by the National Institutes of Health/National Center for Complementary and Alternative Medicine (grants 1R01AT001495-01A1 and 2R01 AT001495-05A1), the Food Allergy Initiative, and the Winston Wolkoff Fund for Integrative Medicine for Allergies and Wellness (to Dr Li). Dr Wang is supported in part by a grant from the National Institutes of Health/National Institute of Allergy and Infectious Diseases (K23 AI083883). This study also was supported in part by a Utah State University faculty startup fund to Dr Zhan.

<http://dx.doi.org/10.1016/j.anai.2014.07.021>

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Introduction

The prevalence of food allergies has continued to increase, with 8% of children and 4% of adults in the United States now suffering from food allergies.¹ Most food allergies are the result of food protein specific IgE antibody production; exposure to the triggering antigen can lead to potentially life-threatening reactions, including anaphylaxis. Efforts to decrease IgE levels are being explored as a therapeutic approach for food allergies. In an early multicenter trial of the anti-IgE antibody TNX-901, peanut-allergic patients who received TNX-901 had a significant decrease in symptoms after peanut challenge compared with the placebo group.² Furthermore, the median threshold of sensitivity to peanut increased from 178 mg of peanut protein (the equivalent to one half a peanut) to 2.8 g

(almost 9 peanuts). Subsequently, another anti-IgE antibody, omalizumab (Xolair, Genentech, San Francisco, California), was investigated in a randomized, double-blinded, parallel-group, placebo-controlled study for peanut allergy.³ Although the trial was suspended early owing to safety concerns related to 2 anaphylactic reactions during screening oral food challenges performed before administration of any study drug, 14 subjects completed the post-therapy (24-week) oral food challenge. Based on the limited data, there appeared to be a greater shift in peanut tolerability in subjects treated with omalizumab compared with placebo that was accompanied by a decrease of serum peanut specific IgE.³ In 2011, Nadeau et al⁴ performed a phase I study using anti-IgE in conjunction with oral immunotherapy in 11 milk-allergic children. Nine of these subjects were successfully desensitized within 7 to 11 weeks. Although cotreatment with anti-IgE allowed for rapid desensitization, adverse reactions still occurred in all subjects, and 3 required epinephrine injections. Recently, Schneider et al⁵ performed a pilot study using omalizumab to facilitate rapid oral desensitization in peanut-allergic patients with high levels of IgE. All 13 subjects tolerated a cumulative dose of 992 mg of peanut flour on the first day. Twelve of 13 subjects tolerated 4,000 mg of peanut flour after approximately 7 to 12 weeks. Although the safety data showed improvement compared with previous studies, 50% of subjects developed grade 1 or grade 2 reactions and 2 patients required epinephrine. Omalizumab therapy is expensive, is not indicated for patients with high total serum IgE levels, is associated with risk of anaphylactoid reactions, and must be administered in a physician's office. Therefore, it is time consuming and not practical for many patients. Furthermore, omalizumab binds circulating IgE but does not directly suppress B-cell IgE production. An alternative approach that inhibits IgE production would be a novel therapeutic strategy for food allergy.

The Chinese herbal medication, Food Allergy Herbal Formula 2 (FAHF-2), consisting of 9 Chinese herb extracts,⁶ completely protects peanut-allergic mice against peanut-induced anaphylaxis and lowers peanut specific IgE levels.^{6,7} B-FAHF-2 is a refined FAHF-2 product using butanol purification.⁸ B-FAHF-2 is equally effective in the murine model of peanut anaphylaxis and significantly suppresses peanut specific IgE production at only 20% of the FAHF-2 dose.⁸ Given the significant role of IgE in mediating food-allergic reactions, the aims of this study were to determine the direct effect of FAHF-2 and B-FAHF-2 in suppressing IgE production and identify active components in constituent herbs using in vitro B-cell lines and human peripheral blood mononuclear cells (PBMCs) from peanut-allergic patients.

Methods

FAHF-2, B-FAHF-2, and Individual Herb Extracts

Food Allergy Herbal Formula 2 is a dried aqueous extract of 9 herbs purified with ethanol. It is produced in a Good Manufacturing Practice—certified facility (Xiyuan Chinese Medicine Research and Pharmaceutical Manufacturer, Beijing China) and stored at room temperature.^{9,10} All herbs used for manufacture were of Chinese origin. The quality of the raw herbs was established according to the standards of the *Pharmacopoeia of the People's Republic of China*¹¹ as previously described.^{10,12} Based on organoleptic and microscopic examinations, raw herbs used in FAHF-2 were identified as follows: fruits of *Prunus mume*, skin of the fruits of *Zanthoxylum schinifolium*, roots of *Angelica sinensis*, rhizome of *Zingiber officinalis*, twigs of *Cinnamomum cassia*, bark of *Philodendron chinensis*, rhizome of *Coptis chinensis*, roots of *Panax ginseng*, and fruiting body of *Ganoderma lucidum*. The botanical information for the individual herbs, including geographic location, harvest season, preprocessing, heavy metal and pesticide residues, and quality control methods, have been published previously.¹² The manufacturing process has been

described in the authors' previous publication.¹⁰ Briefly, the raw herbs were cut into small pieces, soaked in water for 30 minutes, and then boiled for 2 hours. Decoctions were collected and concentrated under vacuum.

Butanol-purified FAHF-2 is a concentrated form of FAHF-2 that was produced as previously described.⁸ High-performance liquid chromatographic (HPLC) fingerprints of FAHF-2, B-FAHF-2, and individual herb extracts were generated (eMethods and eFig 1) for product quality control according to guidelines issued by the US Food and Drug Administration¹³ as previously described.¹⁰

Isolation, Purification, Identification and Quantification of Active Compounds from *P chinensis*

In testing the IgE inhibitory property of the individual herb constituents, 3 were found to exhibit significant anti-IgE effects (*P chinensis*, rhizome of *C chinensis*, and *G lucidum*; eFig 2). As a first step in isolating and identifying active compounds from the effective herbs in FAHF-2, the authors focused on *P chinensis* for cost effectiveness (it is 7.5 and 3.8 times less expensive than the rhizomes of *C chinensis* and *G lucidum*, respectively). The authors used their previously established methods to isolate and purify the active compounds.¹⁴ In brief, 200 g of dried aqueous extract of *P chinensis* was dissolved in 4 L of distilled water and extracted with an equal volume of chloroform (4 L; eFig 3). The chloroform extract was separated further based on the polarity of components using silica gel (Merck 230–400 mesh; Sigma-Aldrich, St. Louis, Missouri) and eluted with a dichloromethane-methanol (DM) mixture at ratios of 49:1, 19:1, 9:1, and 1:1 sequentially. Based on thin-layer chromatographic and HPLC profiles, collections that contained the same peaks were combined to yield 4 major subfractions designated DMF-A, DMF-B, DMF-C, and DMF-D. DMF-A and DMF-C contain simple major peaks compared with DMF-B and DMF-D and were the focus of further isolation and purification in this study. DMF-A (1.2 g) was further separated using silica gel chromatography with a mobile phase of dichloromethane-ethyl acetate mixture at different ratios of 19:1, 9:1, and 5:1. One compound was recrystallized from 19:1 dichloromethane-ethyl acetate eluent and named DMF-A1 (45 mg). DMF-C (3.6 g) was further separated and purified using a Sephadex LH20 column with methanol as the solvent, resulting in a pure compound designated DMF-C1 (232 mg). The 2 compounds were characterized by liquid chromatographic mass spectrometry and compared with standard compounds and ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy (eMethods).⁸

Human U266 Myeloma Cell Culture

The IgE-producing human U266 myeloma cell line (American Type Culture Collection, Rockville, Maryland) has been used for screening pharmaceutical agents for IgE inhibitory effects¹⁵; thus, this cell line was used to test the potential IgE inhibitory effects of herb extracts and purified compounds. Cells were cultured at 37°C under 5% CO₂ in complete media containing RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mmol/L of sodium pyruvate, 1 × 10^{−5} mol/L of β-mercaptoethanol, and 0.5% penicillin-streptomycin. Cells were grown at an initial concentration of 2 × 10⁵ cells/mL. FAHF-2 and B-FAHF-2, individual herb extracts, and isolated compounds at different concentrations were added at day 0. Supernatants were harvested after 6 days in culture, and IgE levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Mabtech, Inc, Cincinnati, Ohio). In each experiment, the authors ran duplicate ELISAs. In vitro experiments were repeated at least 3 times.

Study Participants and PBMC Isolation and Culture

Blood samples were obtained from 25 pediatric patients with physician-diagnosed food allergy (6–17 years old, 20 boys and 5 girls). Patients with physician-diagnosed food allergy as documented by physician-determined history of allergic reactions to peanut and/or tree nut and/or fish and by a positive skin test result (mean wheal diameter ≥ 3 mm than the mean of the saline control) and/or food allergen specific IgE level (peanut, tree nut or fish specific IgE ≥ 0.7 kU/L) were eligible for the study. This study was approved by the Mount Sinai institutional review board. Written informed consent was obtained before enrollment.

The PBMCs were isolated by Ficoll Hypaque (Pharmacia, Piscataway, New Jersey) using density gradient centrifugation at 1,800 rpm for 30 minutes and washed 3 times in phosphate buffered saline. To determine the optimal stimulation of IgE production by PBMCs, PBMCs at 1.5×10^6 cells/mL were suspended in complete RPMI medium (200 μ mol/L of L-glutamine, 1% penicillin-streptomycin, 25 mmol/L of HEPES, and 10% heat-inactivated fetal calf serum) and costimulated with human recombinant interleukin (rIL)-4 at 0.01 and 0.1 μ g/mL (R&D Systems, Minneapolis, Minnesota) and anti-CD40 monoclonal antibody (mAb) at 10 and 100 μ g/mL (R&D) for 10 and 14 days. At the end of incubation, the supernatants were collected and IgE levels were analyzed using an ELISA kit (Mabtech, Inc). Because PBMCs stimulated with 100 ng/mL of rIL-4 and 1 μ g/mL of anti-CD40 mAb produced the highest level of IgE, this culture condition was used for subsequent studies. PBMCs were stimulated with 100 ng/mL of rIL-4 and 1 μ g/mL of anti-CD40 mAb and cultured in the presence or absence of 20 μ g/mL of the 2 purified compounds for 10 days for screening active compounds and at different concentrations (0.15, 0.31, 0.62, 1.25, 2.5, 5, and 10 μ g/mL) to examine dose responses for the effective compounds. IgE level was evaluated using ELISA. The percentage of IgE inhibition was calculated as: inhibition (%) = (IgE concentration in treated sample)/(IgE concentration in untreated sample) $\times$ 100. In some cases, if enough PBMCs were obtained, aliquots of PBMCs prepared from the same patients were used for different experiments.

Cell Viability

Cell viability was evaluated using trypan blue exclusion as previously described.¹⁶ Briefly, 10 μ L of cell suspension from each culture was mixed with an equal volume of trypan blue dye. The mixture was loaded into a hemocytometer and cells were counted under a microscope. The percentage of viable cells was calculated as: viable cells (%) = (total number of viable cells)/(total number of cells) $\times$ 100.

Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was used to detect the ϵ -germline transcription (ϵ GLT). Human PBMCs (1.5×10^6 cells/mL) were co-incubated with DMF-C1 compound (10 μ g/mL) at 37°C under 5% CO₂ for 4 days. Cells were harvested and total RNA was isolated using Trizol reagent (Gibco BRL, Rockville, Maryland) according to the manufacturer's instructions. The RNA concentrations were quantified by triplicate optical density readings (Bio-Rad SmartSpect 3000; Bio-Rad, Hercules, California). Reverse transcription was performed to obtain the cDNA using ImProm-II Reverse Transcriptase (Promega Corporation, Madison, Wisconsin) as described by the manufacturer's instructions. The RT-PCR amplification was performed using Maxima SYBR Green qPCR Master Mix (2 $\times$) kit (Fisher Scientific, Pittsburgh, Pennsylvania) with ϵ GLT primers or glyceraldehyde 3-phosphate dehydrogenase primers. The primer sequences were listed as ϵ GLT forward: 5'-CACATCCACAGGCACCA AAT-3'; ϵ GLT reverse: 5'-ATCACCGGCTCCGGGAAGTA-3'; glyceraldehyde 3-phosphate dehydrogenase forward: 5'-GAGGCAGGGATGATGT TCTG-3'; glyceraldehyde 3-phosphate dehydrogenase reverse: 5'-

CAGCCTCAAGATCATCAGCA-3'; signal transducer and activator of transcription-3 (STAT3) forward: 5'-ACCTGCAGCAATACCATGAC-3'; STAT3 reverse: 5'-AAGGTGAGGGACTCAAAGTGC-3'; T-box transcription factor (T-bet) forward: 5'-GATGCGCCAGGAAGTTTCAT-3'; T-bet reverse: 5'-GCACAATCATCTGGGTACATT-3'; interferon- γ (IFN- γ) forward: 5'-AAAGATGACCAGAGCATCCA-3'; IFN- γ reverse: 5'-TTGC GTTGACATTCAAGTC-3'; forkhead box protein 3 (Foxp3) forward: 5'-GTGGCCCGGATGTGAGAA-3'; Foxp3 reverse: 5'-GCTGTCCAG AGACTGTACCATCT-3'; GATA-binding protein 3 (GATA-3) forward: 5'-GCGGGTCTATACAAAATGA-3'; GATA-3 reverse: 5'-GCTCTCCT GGCTGCAGACAGC-3'; IL-10 forward: 5'-CTGCCTAACATGCTTCGAGA-3'; IL-10 reverse: 5'-TGGCAACCCAGGTAACCTTA-3'. IL-5 forward: 5'-GCCATGAGGATGCTTC TGCA-3'; IL-5 reverse: 5'-GAATCCTCAGAGTCT CATTGGCTATC-3'. All primers were synthesized by Sigma-Aldrich Corporation (St Louis, Missouri).

In-Cell Western Blot

The PBMCs (1×10^5 cells/mL) were cultured with or without DMF-C1 (10 μ g/mL) and stimulated with or without rIL-4 (100 ng/mL) and anti-CD40 (1 μ g/mL) mAb for 10 days. In-Cell Western Assay (Li-Cor, Lincoln, Nebraska) was performed according to the manufacturer's instructions. In brief, cells were incubated with antibodies against phosphorylated I κ B α (p-I κ B α ; Cell Signaling Technology, Beverly, Massachusetts) and β -actin (Santa Cruz Biotechnology, Santa Cruz, California) overnight. After washing with 0.1% Tween phosphate buffered saline buffer, secondary antibodies IRDye800CW donkey anti-goat and IRDye680 donkey anti-rabbit (Li-Cor) were added and incubated at room temperature for

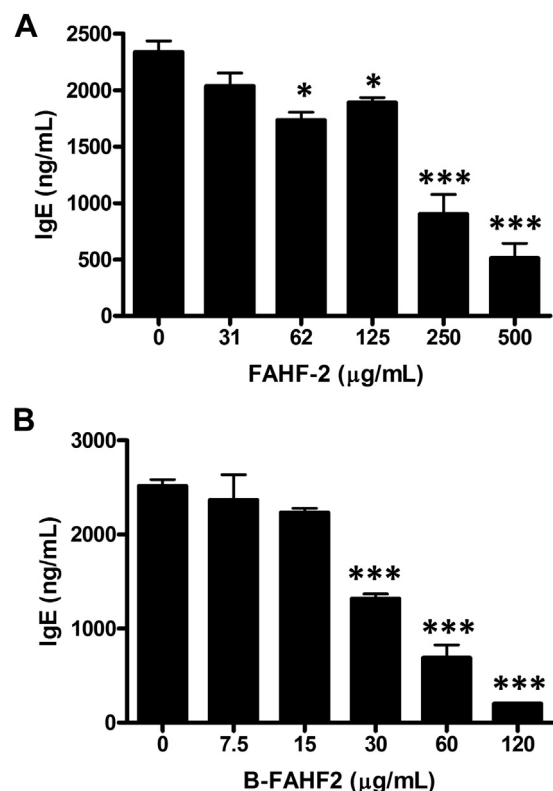


Figure 1. Dose-dependent inhibitory effect of herbal formulas Food Allergy Herbal Formula 2 (FAHF-2) and butanol-purified FAHF-2 (B-FAHF-2) on IgE production by U266 cells. (A) Inhibitory effect of FAHF-2 on IgE production by U266 cells. (B) Inhibitory effect of B-FAHF-2 on IgE production by U266 cells. U266 cells (2×10^5 cells/mL) were cultured with FAHF-2 and B-FAHF-2 at equivalent concentrations as indicated for 6 days. The supernatants were harvested and IgE levels were measured by enzyme-linked immunosorbent assay. Results were expressed as mean $\pm$ SE. * $P < .05$; *** $P < .001$ ($n \geq 3$).

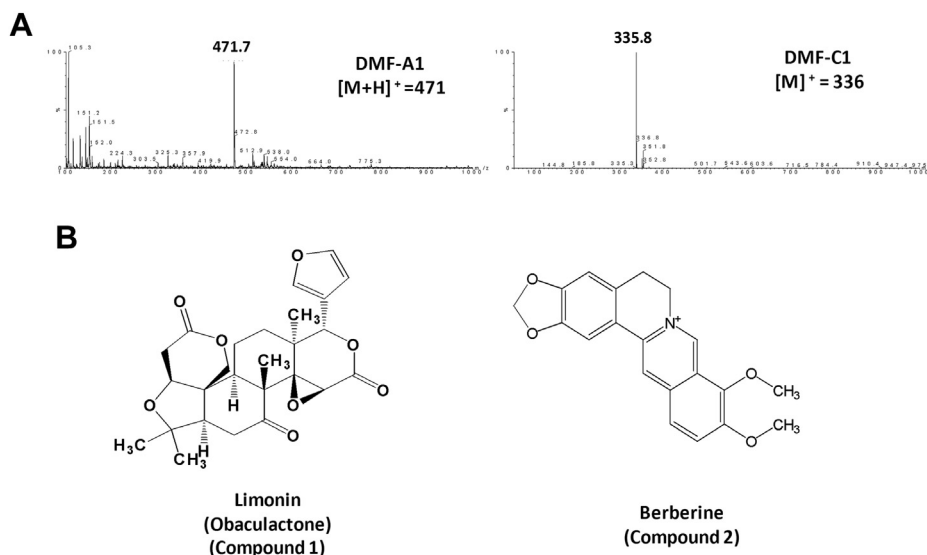


Figure 2. Compounds isolated from *Philodendron chinensis*. (A) Mass spectra of DMF-A1 (limonin) and DMF-C1 (berberine). (B) Chemical structures of compounds 1 (limonin) and 2 (berberine).

1 hour in the dark. Plates were scanned and analyzed with an Odyssey CLx Infrared Imaging System (Li-Cor).

Statistics

One-way analysis of variance was performed followed by Bonferroni correction for all pairwise comparisons. For skewed data, differences between groups were performed by 1-way analysis of variance on rank followed by the Donne method for all pairwise comparisons. *P* values were determined by a 2-sided calculation. A

P value less than or equal to .05 was considered statistically significant. All statistical analyses were performed using Sigma Stat 3.5 (Systat Software, Inc, Chicago, Illinois).

Results

FAHF-2 and B-FAHF-2 Inhibited IgE Production by U266 Cells

To evaluate the effects of FAHF-2 on IgE production, U266 cells were incubated with FAHF-2 at different concentrations (0, 31, 62, 125, 250, and 500 $\mu\text{g}/\text{mL}$). FAHF-2 inhibited IgE production in a

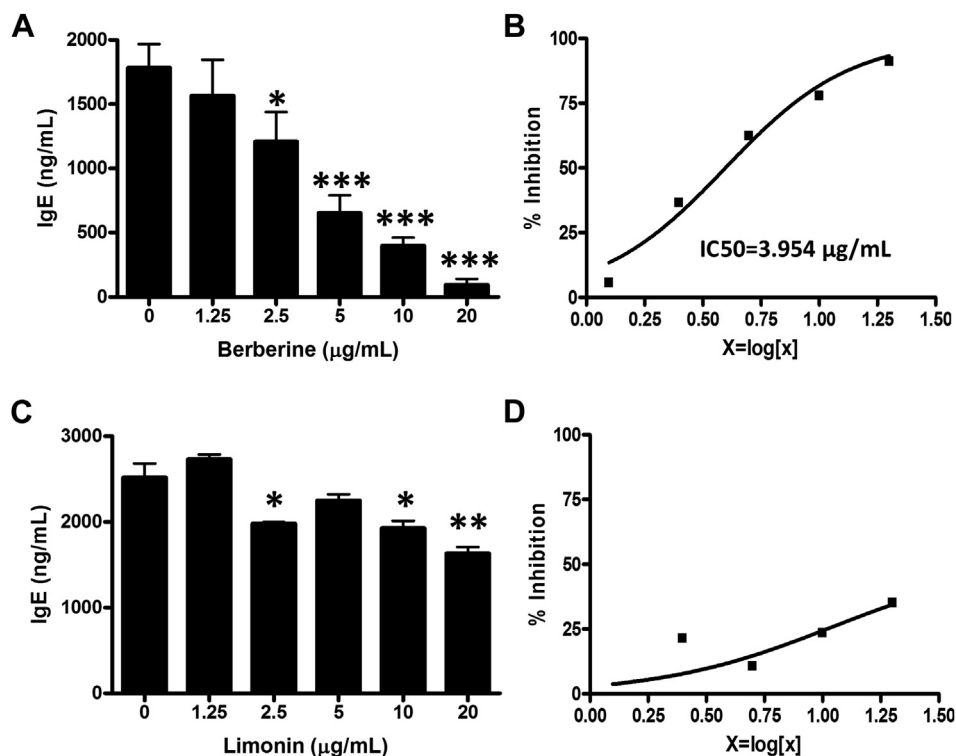


Figure 3. Dose-dependent inhibitory effect of berberine and limonin on IgE production by U266 cells. (A) Inhibitory effect of berberine on IgE production by U266 cells. (B) Percentage of IgE inhibition vs $\log(x)$ berberine concentration curve. (C) Inhibitory effect of limonin on IgE production by U266 cells. (D) Percentage of IgE inhibition vs $\log(x)$ limonin concentration curve. **P* < .05; ***P* < .01; ****P* < .001 (*n* $\geq$ 3). IC₅₀, half-maximal inhibitory concentration.

dose-dependent manner (Fig 1A), with 125 and 500 $\mu\text{g/mL}$ of FAHF-2 significantly inhibiting IgE production ($P < .001$). At the highest concentration (500 $\mu\text{g/mL}$), inhibition reached 78.1%.

Previous results have shown that the yield of butanol extraction of FAHF-2 is $22.37 \pm 1.55\%$.⁸ Therefore, B-FAHF-2 was tested on U266 cells at concentrations of 0, 7.5, 15, 30, 60, and 120 $\mu\text{g/mL}$ equivalent to that of FAHF-2 based on yield value. Similar to FAHF-2, B-FAHF-2 inhibited IgE production by U266 cells in a dose-dependent manner (Fig 1B). Suppression of IgE by B-FAHF-2 reached statistical significance at 30, 60, and 120 $\mu\text{g/mL}$ ($P < .001$) and maximally inhibited IgE production (92.0%) at the highest concentration (120 $\mu\text{g/mL}$). The half-maximal inhibitory concentration (IC_{50}) was 9.2-fold lower for B-FAHF-2 than for FAHF-2 (34.44 vs 313.6 $\mu\text{g/mL}$). Trypan blue exclusion assays showed that FAHF-2 and B-FAHF-2 did not affect cell viability at the concentrations used (eFig 4A and B).

These data show that B-FAHF-2, comprised of less-polar components, is more potent than FAHF-2 at inhibiting IgE production. This finding is consistent with HPLC fingerprint features of B-FAHF-2 and FAHF-2. Although 36 peaks were detected in FAHF-2 and B-FAHF-2 (eFig 1), B-FAHF-2 showed stronger intensity for most less-polar and nonpolar peaks even at an approximately 5-fold lower HPLC loading concentration (40 mg/mL) than FAHF-2 (198 mg/mL). Peak 1 corresponds to polar constituent. Its intensity in the B-FAHF-2 extract was decreased by 94% compared with that in the FAHF-2 extract. B-FAHF-2 was more potent than FAHF-2 in suppressing IgE production owing to the more concentrated less-to-nonpolar compounds.

Two Compounds from *P. chinensis* Were Isolated and Characterized as Limonin and Berberine

Philodendron chinensis aqueous extracts were extracted further and fractionated, and 2 compounds, DMF-A1 (compound 1) and DMF-C1 (compound 2), were purified. The purity of each compound was greater than 98% as determined by analytical HPLC. The structures of compounds 1 and 2 were identified using liquid chromatographic mass spectrometry (Fig 2A–B) and ^1H and ^{13}C NMR spectroscopy (eTable 1). Mass spectra data of compound 1 showed a significant $[\text{M} + \text{H}]^+$ ion peak at m/z 471. Data of compound 2 showed a $[\text{M} + \text{H}]^+$ peak at m/z 336 (Fig 2A). The molecular weights of compounds 1 and 2 were determined to be 470 and 336 g/mol, respectively. The ^1H NMR and ^{13}C NMR data of compounds 1 and 2 are consistent with previously reported data of limonin^{17,18} and berberine chloride.¹⁸ Thus, based on the mass spectrometric and NMR data, compounds 1 and 2 were identified as limonin and berberine, respectively.

Berberine Was a More Effective Inhibitor of IgE Production by U266 Cells than Limonin

The bioactivities were analyzed in vitro on U266 cells in the presence of berberine or limonin at different concentrations. As shown in Figure 3A, berberine inhibited IgE production in a dose-dependent manner, reaching 94.6% inhibition at 20 $\mu\text{g/mL}$. The IC_{50} value was 3.95 $\mu\text{g/mL}$ (Fig 3B). Limonin showed a mild decrease of IgE production by U266 cells. The greatest inhibitory effect of limonin was 35.1%, observed at a concentration of 20 $\mu\text{g/mL}$ ($P < .01$; Fig 3C). Unlike berberine, no dose-dependent effect was observed for limonin. Cell viability assays showed no toxicity at any concentration tested (eFig 4C and D).

Berberine Was More Effective than Limonin in Suppressing IgE Production by PBMCs from Patients with Food Allergies

To further examine the effect of berberine and limonin on IgE production, PBMCs from food-allergic patients were used. To optimize culture conditions, the authors tested different concentrations of anti-CD40 antibodies (0.1 and 1 $\mu\text{g/mL}$) and rIL-4

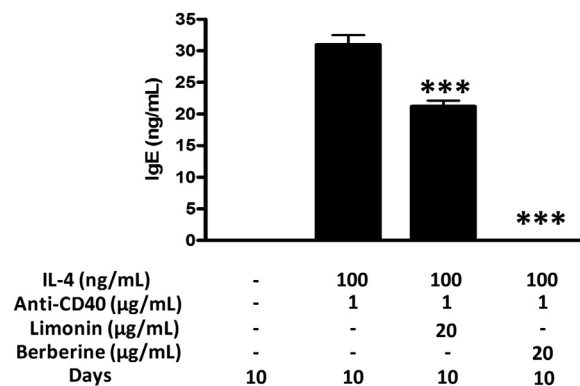


Figure 4. Inhibitory effect of berberine and limonin on IgE production by human peripheral blood mononuclear cells. To identify active compounds, PBMCs (1.5×10^6 cells/mL) from food allergic patients were collected and co-stimulated with human recombinant interleukin-4 (IL-4; 100 ng/mL) and anti-CD40 monoclonal antibody (1 $\mu\text{g/mL}$) in the presence of different compounds at 20 $\mu\text{g/mL}$ for 10 days. IgE levels were determined by enzyme-linked immunosorbent assay. *** $P < .001$ ($n = 4$).

(10 and 100 ng/mL) and culture durations (10 and 14 days).^{19,20} The results showed that human PBMCs generated the highest concentration of IgE when co-incubated with 100 ng/mL of rIL-4 and 1 $\mu\text{g/mL}$ of anti-CD40 mAb for 10 days (eFig 5). Using these parameters, the authors assessed the inhibitory effects of berberine and limonin. Berberine completely inhibited the IgE

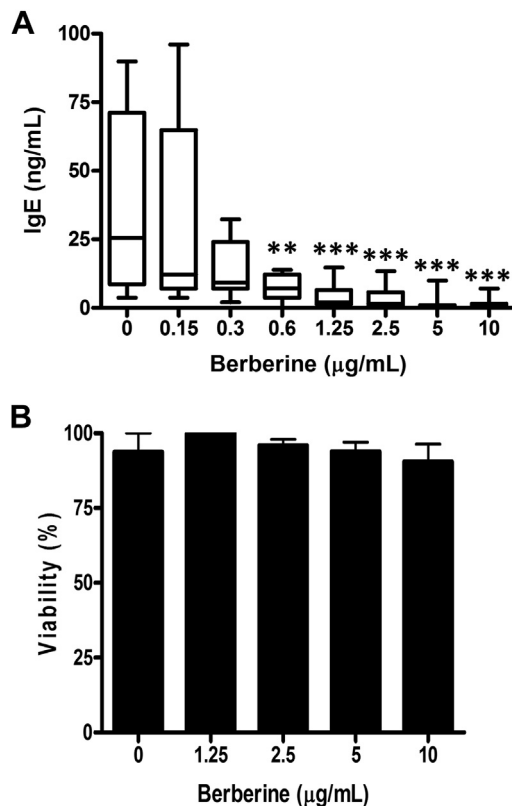


Figure 5. Dose-dependent inhibitory effect of berberine on IgE production by human peripheral blood mononuclear cells. (A) Inhibitory effect of berberine on IgE production by peripheral blood mononuclear cells at different concentrations. Peripheral blood mononuclear cells from food-allergic patients were isolated. Cells were costimulated with human recombinant interleukin-4 (100 ng/mL) and anti-CD40 monoclonal antibody (1 $\mu\text{g/mL}$) in the presence or absence of different concentrations of berberine as indicated for 10 days. IgE levels in supernatants were determined by enzyme-linked immunosorbent assay. (B) Cell viability of human peripheral blood mononuclear cells at different concentrations of berberine. ** $P < .005$; *** $P < .001$ ($n = 6$).

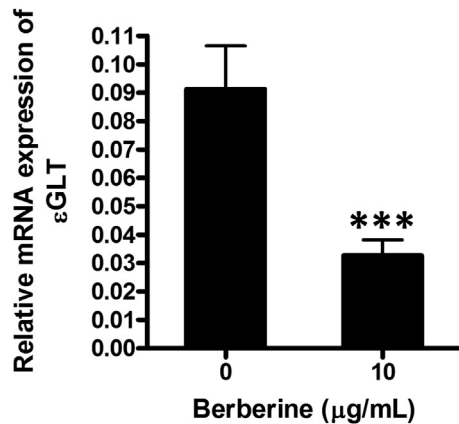


Figure 6. Berberine suppressed the expression of ϵ -germline transcript (ϵ GLT) by peripheral blood mononuclear cells from food-allergic patients. Peripheral blood mononuclear cells were isolated from food-allergic patients and costimulated with human interleukin-4 (1 μ g/mL) anti-CD40 monoclonal antibody (1 μ g/mL) in the presence of berberine at 10 μ g/mL for 4 days. RNA was extracted using Trizol. The relative expression level of ϵ GLT was determined by comparison with glyceraldehyde 3-phosphate dehydrogenase mRNA expression. *** $P < .001$ ($n = 7$).

production by human PBMCs at 20 μ g/mL, whereas limonin produced 33.9% inhibition at the same concentration (Fig 4). Dose-dependent effects of berberine on IgE production by PBMCs were investigated further (Fig 5A). Significant inhibition starting at 0.62 μ g/mL ($80.67 \pm 9.24\%$ inhibition) was observed. The IC_{50} value was calculated to be 0.20 μ g/mL. Cell viability was not compromised at any dose of berberine tested (Fig 5B). The authors assessed potential apoptosis using flow cytometry and annexin V. After 2 days of incubation with 10 μ g/mL of berberine, PBMCs were incubated with CD19 antibody and stained with annexin V. The result showed approximately 16% stained B cells in berberine-treated and untreated cells. No significant difference was observed. These data are included in the supplemental information (eFig 6).

Berberine Inhibited ϵ GLT by PBMCs

Epsilon germline transcription is a critical step for IgE isotype switching.²¹ To test whether the mechanism of the inhibition effect of berberine on IgE production is through regulation of the ϵ -germline, the authors analyzed the mRNA expression of ϵ GLT by

RT-PCR. The mRNA expression of ϵ GLT was detected in 7 patients' PBMCs. The RT-PCR results showed that the relative mRNA expression of ϵ GLT was significantly inhibited when co-incubated with berberine (Fig 6) at a concentration of 10 μ g/mL compared with untreated cultures ($P < .001$).

Berberine Inhibited Phosphorylated $I\kappa B\alpha$

Nuclear factor- κ B (NF- κ B) signaling pathways are involved in B-cell activation.²² The NF- κ B signaling pathway may be involved in upstream mechanisms underlying berberine inhibition of IgE synthesis. NF- κ B is inactive when reacted with the inhibitory $I\kappa$ B protein. Phosphorylation of $I\kappa$ B results in the exposure of the nuclear localization signals on NF- κ B. This eventually leads to the translocation of NF- κ B to the nucleus, activation of the $I\epsilon$ promoter, and enhancements of ϵ GLT transcripts. Therefore, the authors determined p- $I\kappa B\alpha$ levels in berberine-treated human PBMCs. The p- $I\kappa B\alpha$ levels were significantly increased after stimulation with IL-4 and anti-CD40 antibody ($P < .005$). Berberine treatment at 10 μ g/mL significantly suppressed p- $I\kappa B\alpha$ levels compared with the untreated cells ($P < .05$; Fig 7).

Berberine Increased STAT3 and T-bet Expression

To explore the mechanism of berberine's effects on PBMCs, the authors performed RT-PCR to analyze gene expression levels of STAT3, T-bet, IFN- γ , forkhead box P3 (Foxp3), GATA-3, IL-10, and IL-5 (Fig 8). Cells treated with 10 μ g/mL of berberine showed significantly increased mRNA expression of T-bet ($P < .001$) and STAT-3 ($P < .005$) in stimulated PBMCs. The mRNA levels of IFN- γ , Foxp3, and IL-10 showed a trend of increase. No significant change in GATA-3 expression was observed. The mRNA level of IL-5 was significantly decreased ($P < .05$).

Discussion

Food Allergy Herbal Formula 2 has shown remarkable efficacy at protecting against peanut-induced anaphylaxis in a murine model of peanut allergy.^{8,23} However, the mechanisms underlying these immune changes are unclear, as are the relative contributions of each herb or compound within the herbs to these observed findings. In this study, the authors aimed to identify the herbs that mediate suppression of IgE production and identify the relevant active compounds.

Medicinal herbals contain medicinal and nonmedicinal ingredients. Previous studies have shown that several classes of

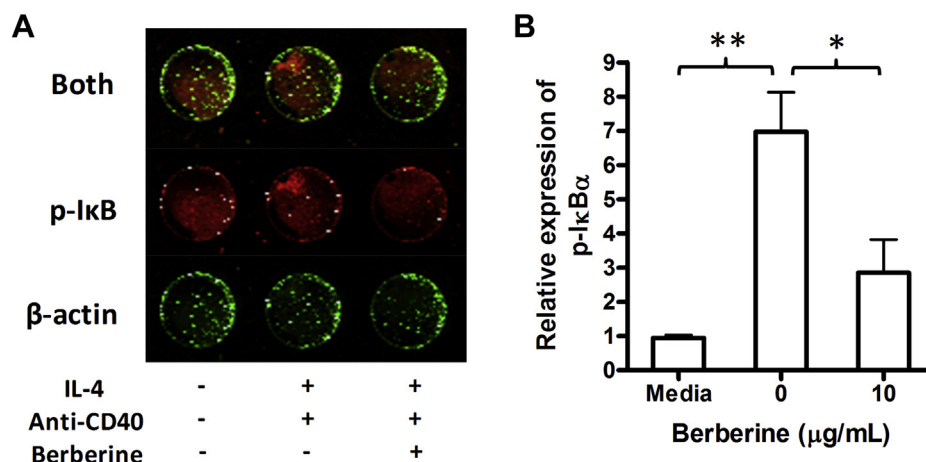


Figure 7. Berberine suppressed the phosphorylated $I\kappa B\alpha$ (p- $I\kappa B\alpha$) in peripheral blood mononuclear cells from food-allergic patients. Peripheral blood mononuclear cells were isolated and cultured as described in Figure 5. In-Cell Western Assay was conducted as described in the Methods section. (A) Representative In-Cell Western Assay with 700-nm and 800-nm channels detecting p- $I\kappa B\alpha$ and β -actin. (B) Relative expression of p- $I\kappa B\alpha$ measured by In-Cell Western Assay ($n = 6$). * $P < .05$; ** $P < .005$. IL-4, interleukin-4.

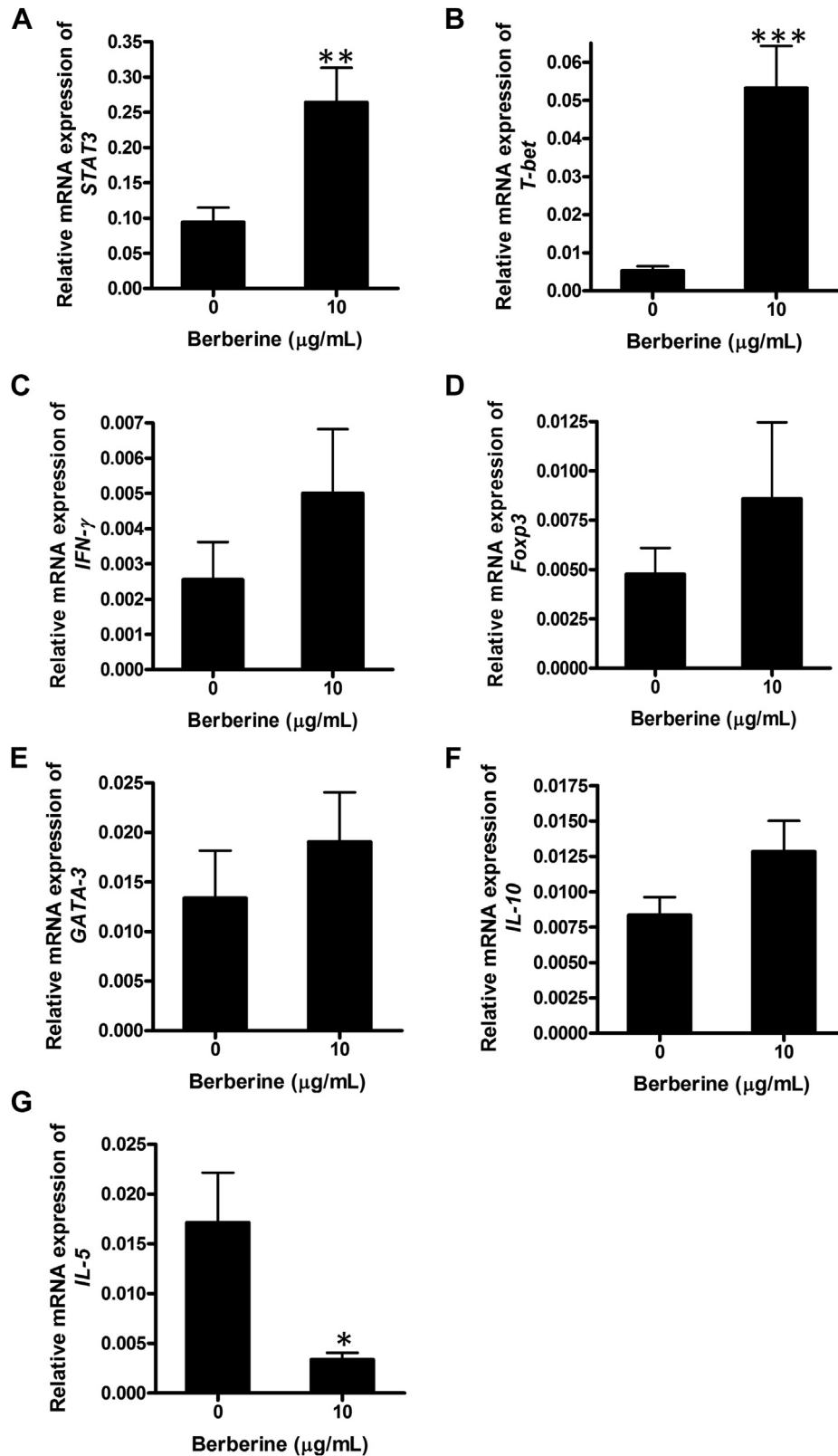


Figure 8. Berberine enhanced the expression of signal transducer and activator of transcription-3 (STAT3) and T-box transcription factor TBX21 (T-bet) transcripts by peripheral blood mononuclear cells from food-allergic patients. Peripheral blood mononuclear cells were isolated and cultured as described in Figure 6. RNA was extracted using Trizol. The expression of (A) STAT3, (B) T-bet, (C) interferon- γ (IFN- γ), (D) forkhead box P3 (Foxp3), (E) GATA-3, (F) interleukin-10 (IL-10), and (G) interleukin-5 (IL-5) were determined by comparison with glyceraldehyde 3-phosphate dehydrogenase mRNA expression. * $P < .05$; ** $P < .01$; *** $P < .001$ ($n = 7$).

natural compounds, including alkaloids, flavonoids, and triterpenoids, may have antiallergic effects.^{24,25} These compounds are primarily nonpolar or less-polar small molecules (<1,000 Da). The

authors previously showed that butanol purification of FAHF-2 concentrates the nonpolar and less-polar compounds, increasing the potency of the formula.

Because the clinical effects of FAHF-2 and B-FAHF-2 are associated with significantly decreased allergen-specific IgE levels, the authors explored the inhibitory effects of FAHF-2, B-FAHF-2, and individual herbal constituents on IgE production using a human B-cell line. B-FAHF-2 was 9 times more potent than FAHF-2, suggesting that the less-polar compounds that are concentrated in B-FAHF-2 contain IgE inhibitory properties. In this first attempt to isolate active compounds in B-FAHF-2, the authors focused on *P. chinensis*, 1 of the 3 individual herbs that showed significant anti-IgE effects. After chloroform fractionation and column chromatographic isolation and purification, 2 compounds were isolated and were identified as berberine and limonin by liquid chromatographic mass spectrometry and NMR.

Berberine is a plant alkaloid that has been widely used in Chinese and Ayurvedic medicine primarily to treat intestinal symptoms (diarrhea) given its antimicrobial and antiprotozoal effects.²⁶ Additional pharmacologic actions reported include anticancer activity, cardioprotective effects, and anti-inflammatory effects, specifically suppression of proinflammatory cytokines such as tumor necrosis factor- α .^{27,28} However, to the authors' knowledge, this is the first study to assess its effects on IgE production directly. The authors found that berberine is effective in suppressing IgE production by human U266 cells without any signs of cytotoxicity with an IC₅₀ value of 3.95 μ g/mL. The IgE inhibitory effect of berberine was examined further using PBMCs from pediatric patients with food allergies. B-cell activation requires at least 2 signals, CD40L from T cells (signal 1) and T-helper cell type 2 cytokines (signal 2).²² Previous studies have shown that anti-CD40 antibodies and IL-4 can induce IgE production by PBMCs from patients with house dust mite allergies.^{19,20} However, the concentration of anti-CD40 and that of IL-4 varied between the studies. In this study, the authors optimized the culture conditions and found that anti-CD40 (1 μ g/mL) and IL-4 (100 ng/mL) induced IgE production by PBMCs after 10 days in culture, and berberine markedly inhibited this process. Another dose-dependent study showed that berberine exhibits a potent IgE inhibitory effect; the inhibition reached statistical significance at as low as 0.62 μ g/mL. The IC₅₀ value was calculated as 0.20 μ g/mL. Importantly, the authors found that berberine also exhibits a high safety profile in PBMCs because no signs of cytotoxicity (cell death or apoptosis) were observed at the concentrations up to 10 μ g/mL, well above the IC₅₀ concentration.

Immunoglobulin E production requires class-switch recombination. In general, isotype switching is regulated at the level of germline transcription of the constant heavy-chain genes.²² Because berberine suppression of IgE production is not due to cytotoxicity, as a first attempt to understand the mechanisms underlying berberine inhibition of IgE production, the authors assessed the relative expression of ϵ GLT in berberine-treated and control cells. The RT-PCR results showed that berberine significantly inhibited ϵ GLT transcription. This suggests that berberine inhibition of anti-CD40 and IL-4–induced IgE production is due, at least in part, to alteration of ϵ GLT. Although there are no direct studies on whether and how berberine suppresses IgE synthesis, some studies have shown that berberine suppresses NF- κ B signaling pathways, which is associated with its anticancer, anti-inflammatory, and antidiabetic actions.^{29,30} Activation of NF- κ B signaling pathways is one of the upstream mechanisms underlying B-cell activation and ϵ GLT expression.^{22,31,32} Therefore, the authors performed the In-Cell Western experiment to analyze the level of p-I κ B α , the marker of activation of NF- κ B signaling, expressed in PBMCs. The results showed that berberine treatment significantly inhibited p-I κ B α levels.

In contrast to ϵ GLT, STAT3, Foxp3, and T-bet are transcription factors that negatively regulate IgE synthesis. Deficiency in any of these genes has been reported to be associated with strikingly

elevated IgE levels in patients.^{33,34} The authors found that STAT3 and T-bet gene expression was significantly increased, and Foxp3 gene expression showed a trend toward increase. In addition, they found a trend of upregulation of IL-10 and IFN- γ and a significant suppression of IL-5 gene expression (Figure 8). It has been suggested that berberine affects poly A RNA in cancer cells,³⁵ but another study showed that berberine stabilizes poly A RNA to assist in neuron survival during cerebral ischemia injury.³⁶ These different findings seem to depend on different cell types. For the first time, the authors investigated the anti-IgE production effect of berberine on human PBMCs. These findings suggest that berberine suppression of IgE synthesis by PBMCs is not due to overall suppression of gene transcription and is involved in multiple regulatory mechanisms. However, further studies are needed to understand the precise mechanisms.

The authors also found that limonin, a tetranortriterpenoid, has some inhibitory effects on IgE production by a human B-cell line and by PBMCs, but it was less potent than berberine. Limonin is responsible for the bitterness of citrus fruits, such as lemon, grapefruit, and orange,³⁷ and is often removed during the production of fruit juices to improve the taste.³⁸ The present study is the first to show that limonin suppressed IgE production in vitro and suggests that this compound may be beneficial for allergies.

In addition to *P. chinensis*, several other plants, including *C. chinensis*, contain berberine. Therefore, berberine also may be an important active compound in *C. chinensis* that is responsible for the IgE inhibitory effect of *C. chinensis*. Although berberine and limonin isolated from *P. chinensis* were identified as IgE inhibitory compounds, with berberine being most effective, other compounds from *P. chinensis*, *C. chinensis*, and *G. lucidum* also may contribute to suppression of IgE production by FAHF-2 and B-FAHF-2. Further investigation is required.

In summary, for the first time, the authors have shown that berberine and limonin exhibit suppressive effects on IgE production by a human B-cell line and PBMCs from food-allergic pediatric patients in a nontoxic manner. Berberine also exhibits inhibitory effects on ϵ GLT expression by PBMCs. This study identifies some of the active molecules contained in FAHF-2 and B-FAHF-2 and provides insights into potential mechanisms underlying the effects of these formulas in treating IgE-mediated food allergy. Further studies are needed to identify additional active molecules that are mediating the favorable immune-modulatory effects of FAHF-2 and B-FAHF-2 and to understand the mechanisms by which these molecules are exerting their effects.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2014.07.021>.

References

- [1] Branum AM, Lukacs SL. Food allergy among children in the United States. *Pediatrics*. 2009;124:1549–1555.
- [2] Leung DY, Sampson HA, Yunginger JW, et al. Effect of anti-IgE therapy in patients with peanut allergy. *N Engl J Med*. 2003;348:986–993.
- [3] Sampson HA, Leung DY, Burks AW, et al. A phase II, randomized, double-blind, parallel-group, placebo-controlled oral food challenge trial of Xolair (omalizumab) in peanut allergy. *J Allergy Clin Immunol*. 2011;127:1309–1310.
- [4] Nadeau KC, Schneider LC, Hoyte L, Borras I, Umetsu DT. Rapid oral desensitization in combination with omalizumab therapy in patients with cow's milk allergy. *J Allergy Clin Immunol*. 2011;127:1622–1624.
- [5] Schneider LC, Rachid R, LeBovidge J, et al. A pilot study of omalizumab to facilitate rapid oral desensitization in high-risk peanut-allergic patients. *J Allergy Clin Immunol*. 2013;132:1368–1374.
- [6] Srivastava KD, Kattan JD, Zou ZM, et al. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *J Allergy Clin Immunol*. 2005;115:171–178.
- [7] Song Y, Qu C, Srivastava K, et al. Food allergy herbal formula 2 protection against peanut anaphylactic reaction is via inhibition of mast cells and basophils. *J Allergy Clin Immunol*. 2010;126:1208–1217.

- [8] Srivastava K, Yang N, Chen Y, et al. Efficacy, safety and immunological actions of butanol-extracted Food Allergy Herbal Formula-2 on peanut anaphylaxis. *Clin Exp Allergy*. 2010;41:582–591.
- [9] Patil SP, Wang J, Song Y, et al. Clinical safety of Food Allergy Herbal Formula-2 (FAHF-2) and inhibitory effect on basophils from patients with food allergy: extended phase I study. *J Allergy Clin Immunol*. 2011;128:1259–1265.e2.
- [10] Wang J, Patil S, Yang N, et al. Safety, tolerability, and immunologic effects of a food allergy herbal formula (FAHF-2) in food allergic individuals: a randomized, double-blinded, placebo-controlled, dose escalation phase I study. *Ann Allergy Asthma Immunol*. 2010;105:75–84.
- [11] The State Pharmacopoeia Commission of The People's Republic of China. *Beijing, China: People's Republic of China*; 2005:1–791.
- [12] Kattan JD, Srivastava KD, Zou ZM, et al. Pharmacological and immunological effects of individual herbs in the Food Allergy Herbal Formula-2 (FAHF-2) on peanut allergy. *Phytother Res*. 2008;22:651–659.
- [13] US Food and Drug Administration (FDA); Center for Drug Evaluation and Research. *Guidance for Industry Botanical Drug Products*. Revised; 2004.
- [14] Jayaprakasam B, Doddaga S, Wang R, et al. Licorice flavonoids inhibit eotaxin-1 secretion by human fetal lung fibroblasts in vitro. *J Agric Food Chem*. 2009;57:820–825.
- [15] Lopez-Exposito I, Castillo A, Yang N, Liang B, Li XM. Chinese herbal extracts of *Rubia cordifolia* and *Dianthus superbus* suppress IgE production and prevent peanut-induced anaphylaxis. *Chin Med*. 2011;6:35.
- [16] Yang N, Patil S, Zhuge J, et al. Glycyrrhiza uralensis flavonoids present in anti-asthma formula, ASHMI, inhibit memory Th2 responses in vitro and in vivo. *Phytother Res*. 2013;27:1381–1391.
- [17] Breksa AP III, Dragull K, Wong RY. Isolation and identification of the first C-17 limonin epimer, epilimonin. *J Agric Food Chem*. 2008;56:5595–5598.
- [18] Hasada K, Yoshida T, Yamazaki T, et al. Application of 1H-NMR spectroscopy to validation of berberine alkaloid reagents and to chemical evaluation of *Coptidis rhizoma*. *J Nat Med*. 2011;65:262–267.
- [19] Chalubinski M, Grzegorzczak J, Kowalski ML. Glucocorticoid-induced immunoglobulin E synthesis by peripheral blood mononuclear cells from allergic and nonallergic subjects. *Ann Allergy Asthma Immunol*. 2011;107:251–257.
- [20] Iwamoto K, Hiragun T, Takahagi S, et al. Fucoidan suppresses IgE production in peripheral blood mononuclear cells from patients with atopic dermatitis. *Arch Dermatol Res*. 2011;303:425–431.
- [21] Oettgen HC. Regulation of the IgE isotype switch: new insights on cytokine signals and the functions of epsilon germline transcripts. *Curr Opin Immunol*. 2000;12:618–623.
- [22] Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol*. 2003;3:721–732.
- [23] Srivastava KD, Bardina L, Sampson HA, Li XM. Efficacy and immunological actions of FAHF-2 in a murine model of multiple food allergies. *Ann Allergy Asthma Immunol*. 2012;108:351–358.
- [24] Ehrman TM, Barlow DJ, Hylands PJ. Phytochemical databases of Chinese herbal constituents and bioactive plant compounds with known target specificities. *J Chem Inf Model*. 2007;47:254–263.
- [25] Ehrman TM, Barlow DJ, Hylands PJ. Phytochemical informatics of traditional Chinese medicine and therapeutic relevance. *J Chem Inf Model*. 2007;47:2316–2334.
- [26] Vuddanda PR, Chakraborty S, Singh S. Berberine: a potential phytochemical with multispectrum therapeutic activities. *Expert Opin Investig Drugs*. 2010;19:1297–1307.
- [27] Lou T, Zhang Z, Xi Z, et al. Berberine inhibits inflammatory response and ameliorates insulin resistance in hepatocytes. *Inflammation*. 2011;34:659–667.
- [28] Shang W, Liu J, Yu X, Zhao J. [Effects of berberine on serum levels of inflammatory factors and inflammatory signaling pathway in obese mice induced by high fat diet]. *Zhongguo Zhong Yao Za Zhi*. 2010;35:1474–1477.
- [29] Hamsa TP, Kuttan G. Antiangiogenic activity of berberine is mediated through the downregulation of hypoxia-inducible factor-1, VEGF, and proinflammatory mediators. *Drug Chem Toxicol*. 2012;35:57–70.
- [30] Lan T, Wu T, Chen C, et al. Berberine attenuates high glucose-induced proliferation and extracellular matrix accumulation in mesangial cells: Involvement of suppression of cell cycle progression and NF-kappaB/AP-1 pathways. *Mol Cell Endocrinol*. 2014;384:109–116.
- [31] Bacharier LB, Geha RS. Molecular mechanisms of IgE regulation. *J Allergy Clin Immunol*. 2000;105:S547–S558.
- [32] Altin J, Shen C, Liston A. Understanding the genetic regulation of IgE production. *Blood Rev*. 2010;24:163–169.
- [33] Torgerson TR, Linane A, Moes N, et al. Severe food allergy as a variant of IPEX syndrome caused by a deletion in a noncoding region of the FOXP3 gene. *Gastroenterology*. 2007;132:1705–1717.
- [34] Vale-Pereira S, Todo-Bom A, Gerales L, et al. FoxP3, GATA-3 and T-bet expression in elderly asthma. *Clin Exp Allergy*. 2011;41:490–496.
- [35] Mantena SK, Sharma SD, Katiyar SK. Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. *Mol Cancer Ther*. 2006;5:296–308.
- [36] Chai YS, Yuan ZY, Lei F, et al. Inhibition of retinoblastoma mRNA degradation through poly (A) involved in the neuroprotective effect of berberine against cerebral ischemia. *PLoS One*. 2014;9:e90850.
- [37] Roy A, Saraf S. Limonoids: overview of significant bioactive triterpenes distributed in plants kingdom. *Biol Pharm Bull*. 2006;29:191–201.
- [38] Manners GD. Citrus limonoids: analysis, bioactivity, and biomedical prospects. *J Agric Food Chem*. 2007;55:8285–8294.

eMethods

Generation of HPLC Fingerprint of FAHF-2, B-FAHF-2, and Individual Herb Extracts

The separation was performed on a ZORBAX SB-C18 (4.6 × 150 mm, 5 μm) column (Agilent, Santa Clara, California) using a gradient method as previously described.¹ In brief, 10 μL of mobile phase (1:1 mixture) dissolved extracts or compounds was injected in a high-performance liquid chromatograph with the ultraviolet detector set at 254 nm. The mobile phases consisted of 0.1% aqueous formic acid and acetonitrile at a constant flow rate of 1 mL/min. The linear gradient program started at 2% to 25% acetonitrile in 0 to 45 minutes, 25% to 35% acetonitrile in 45 to 70 minutes, 35% to 55% in 70 to 85 minutes, and 55% to 75% in 85 to 95 minutes and was maintained at 75% for 5 minutes. Data were collected and processed with Waters Empower software (Waters Corporation, Milford, Massachusetts).¹

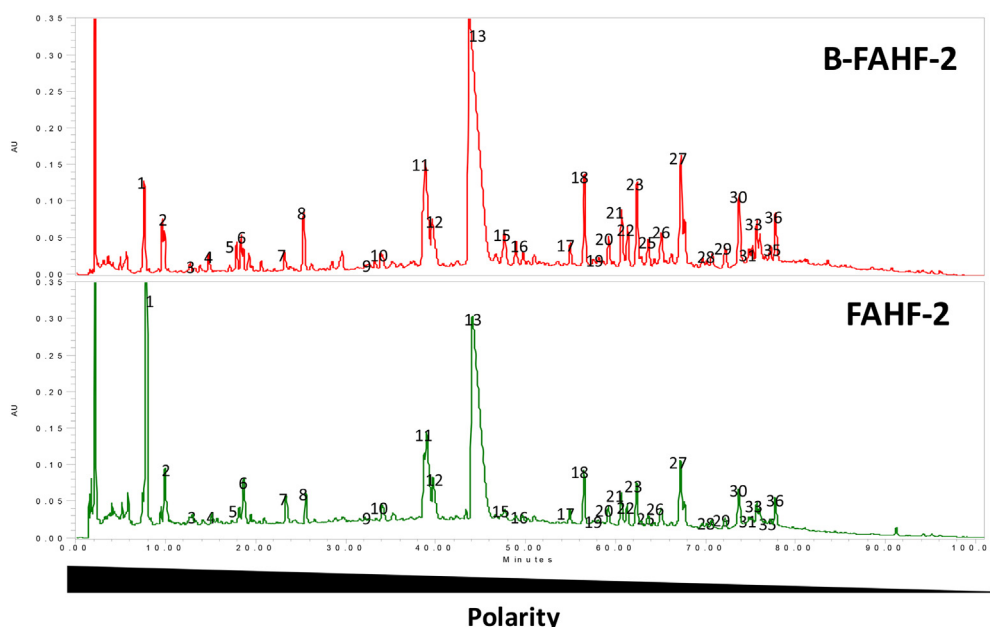
Liquid Chromatographic Mass Spectrometry and NMR for Identification of Compounds

Molecular weight was identified by liquid chromatographic mass spectrometric time-of-flight analysis in electrospray (ESI

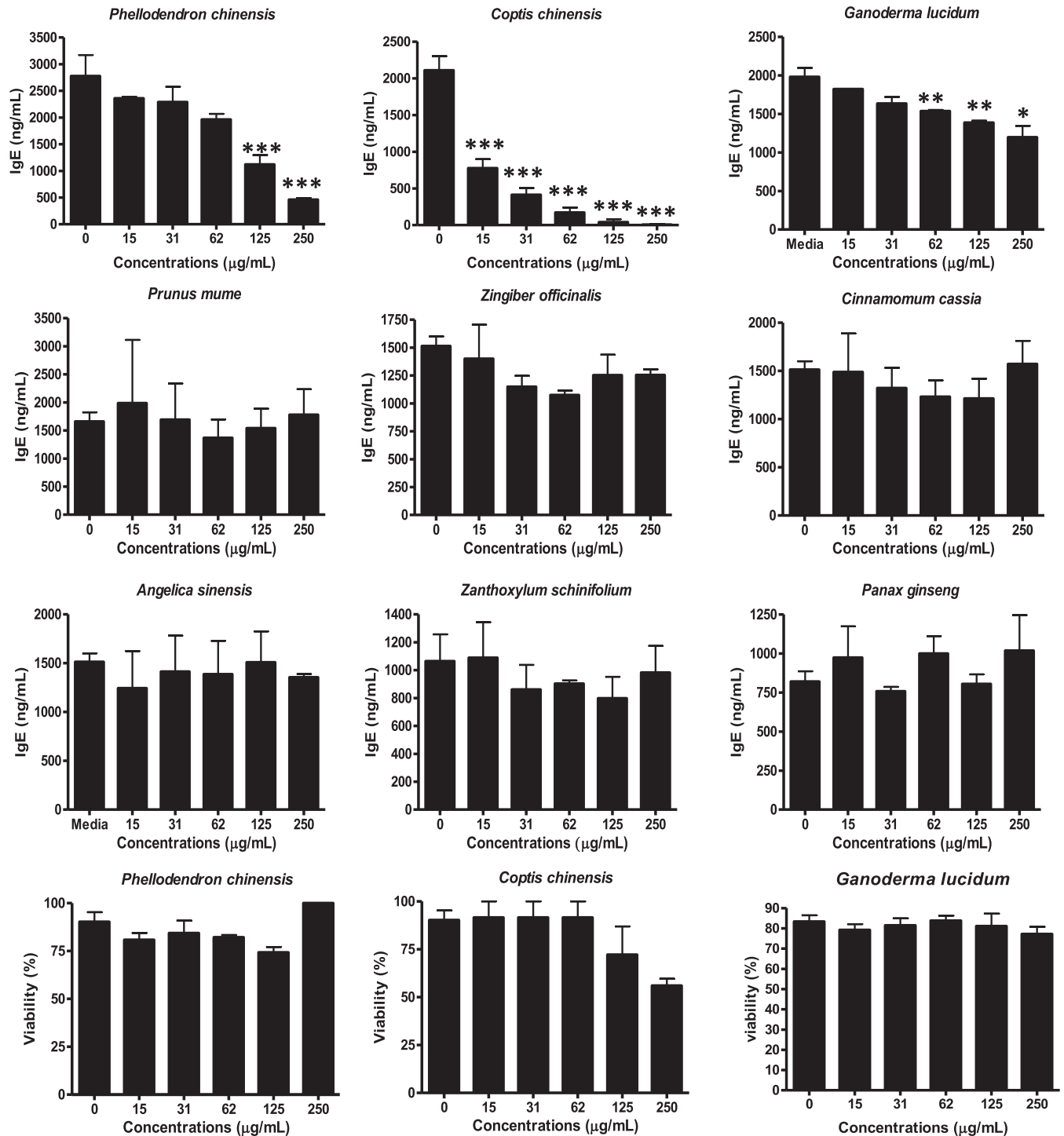
International, Arlington, Virginia) positive mode. The LCT premier time-of-flight mass spectrometer (Waters Corporation) was coupled to a Waters Alliance 2695 HPLC system. A 10-μL sample was analyzed under the same gradient condition described earlier. The mass range was m/z 50 to 1000 in W optics mode. Nitrogen gas was used as a nebulizer and desolvation gas with flow rates of 40 and 500 L/h, respectively. The source temperature was set to 110°C. The capillary voltage and cone voltage were set to 3,200 and 25 V, respectively. ¹H and ¹³C NMR spectra were obtained on a JOEL instrument (at 300 MHz and 75 MHz, Peabody, Massachusetts) using dimethyl sulfoxide-d6 as the solvent (eTable 1).

eReference

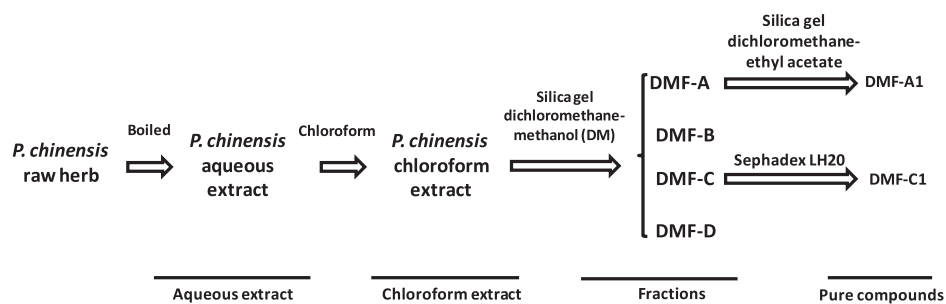
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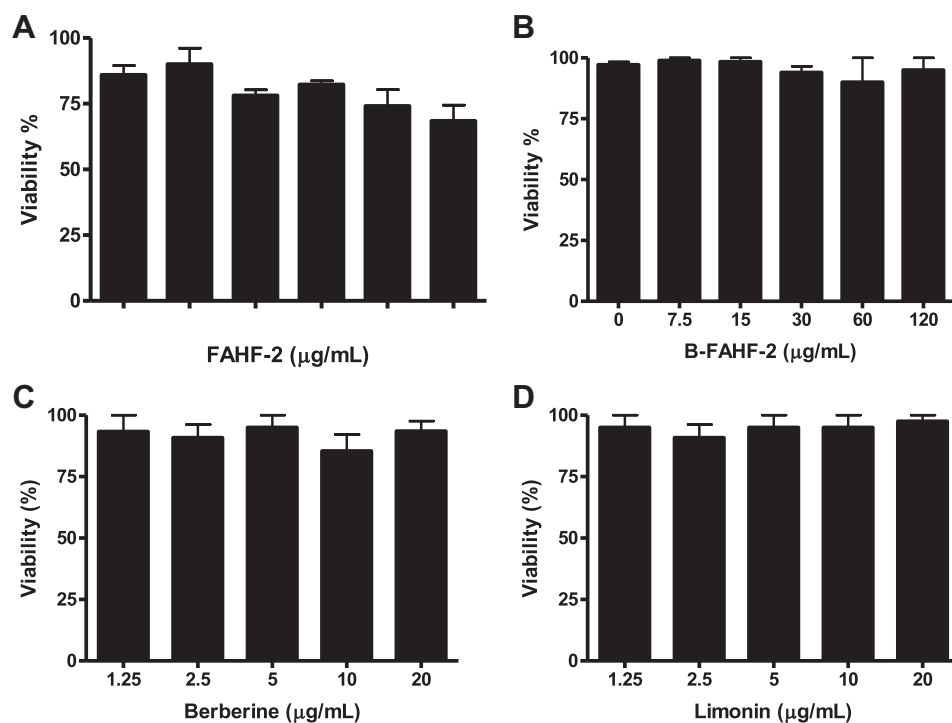
eFigure 1. High-performance liquid chromatographic fingerprints of Food Allergy Herbal Formula 2 (FAHF-2) and butanol-purified FAHF-2 (B-FAHF-2). Thirty-six peaks were detected.



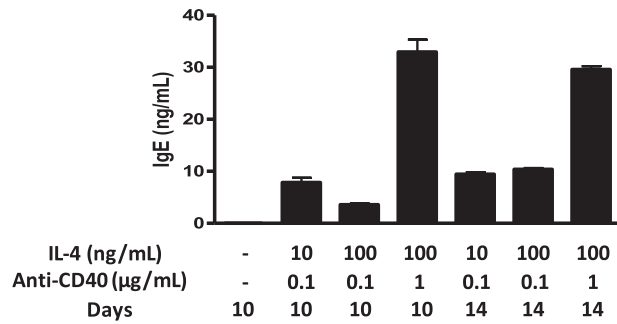
eFigure 2. Dose-dependent studies of individual herbal constituents in Food Allergy Herbal Formula 2 on IgE production by U266 cells. * $P < .05$; ** $P < .01$; *** $P < .001$ ($n \geq 3$).



eFigure 3. Flow chart of isolation and purification process of compounds from *Philodendron chinensis*.



eFigure 4. Cell viability test for (A) Food Allergy Herbal Formula 2 (FAHF-2), (B) butanol-purified FAHF-2 (B-FAHF-2), (C) berberine, and (D) limonin.

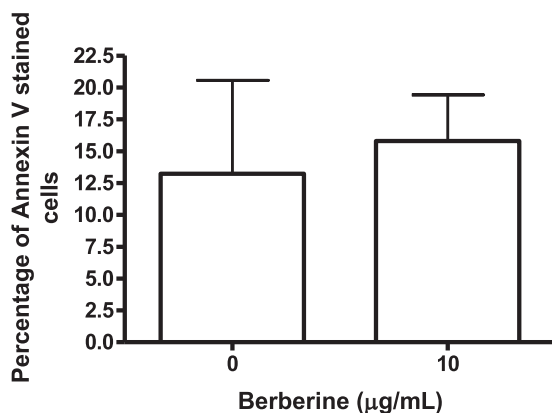


eFigure 5. Dose and timing effects of interleukin-4 (IL-4) and anti-CD40 monoclonal antibody stimulation on IgE production by human peripheral blood mononuclear cells.

eTable 1

¹H and ¹³C nuclear magnetic resonance spectra data of DMF-A1 (compound 1)

Position	δ H (deuterated dimethyl sulfoxide)	δ C (chloroform-D)
1	4.11 (¹ H, dd, $J = 16.8, 3$ Hz)	79.4
2	2.61 (¹ H, dd, $J = 17.1, 4.2$ Hz), 3.10 (¹ H, dd, $J = 15, 5.4$ Hz)	35.9
3		169.3
4		80.5
5	2.26 (¹ H, dd, $J = 14.7, 3$ Hz)	60.7
6	2.42 (¹ H, dd, $J = 14.7, 3$ Hz), 2.76 (¹ H, d, $J = 16.2$ Hz)	36.6
7		206.3
8		51.5
9	2.51 (¹ H, m)	48.3
10		46.1
11	1.90 (¹ H, m)	19.1
12	1.22 (¹ H, m), 1.73 (¹ H, m)	31.0
13		38.2
14		65.9
15	4.08 (¹ H, s)	54.1
16		166.8
17	5.46 (¹ H, s)	79.4
18	1.01 (³ H, s)	20.9
19	4.47 (¹ H, d, $J = 12.9$ Hz), 4.90 (¹ H, d, $J = 13.1$ Hz)	65.6
20	6.49 (¹ H, d, $J = 1.02$ Hz)	109.9
21		120.2
22	7.70 (¹ H, br.s)	141.3
23	7.64 (¹ H, t, $J = 1.74$ Hz)	143.4
24	1.09 (³ H, s)	21.6
25	1.17 (³ H, s)	30.4
26	0.98 (³ H, s)	17.8



eFigure 6. Percentage of annexin V–stained B cells in human peripheral blood mononuclear cells.