

Clinical & Experimental Allergy / Early View

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Anti-IgE effect of small-molecule-compound arctigenin on food allergy in association with a distinct transcriptome profile

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Abstract

Background

Excessive production of IgE plays a major role in the pathology of food allergy. In an attempt to identify anti-IgE natural products, *Arctium Lappa* was one of the most effective herbs among approximately 300 screened medicinal herbs. However, little is known about its anti-IgE compounds.

Objective

To identify compounds from *Arctium Lappa* for targeted therapy on IgE production and explore their underlying mechanisms.

Methods

Liquid-liquid extraction and column chromatographic methods were used to purify the compounds. IgE inhibitory effects were determined on IgE-producing human myeloma

U266 cells, peanut-allergic murine model and PBMCs from food-allergic patients. Genes involved in IgE inhibition in PBMCs were studied by RNA sequencing.

Results

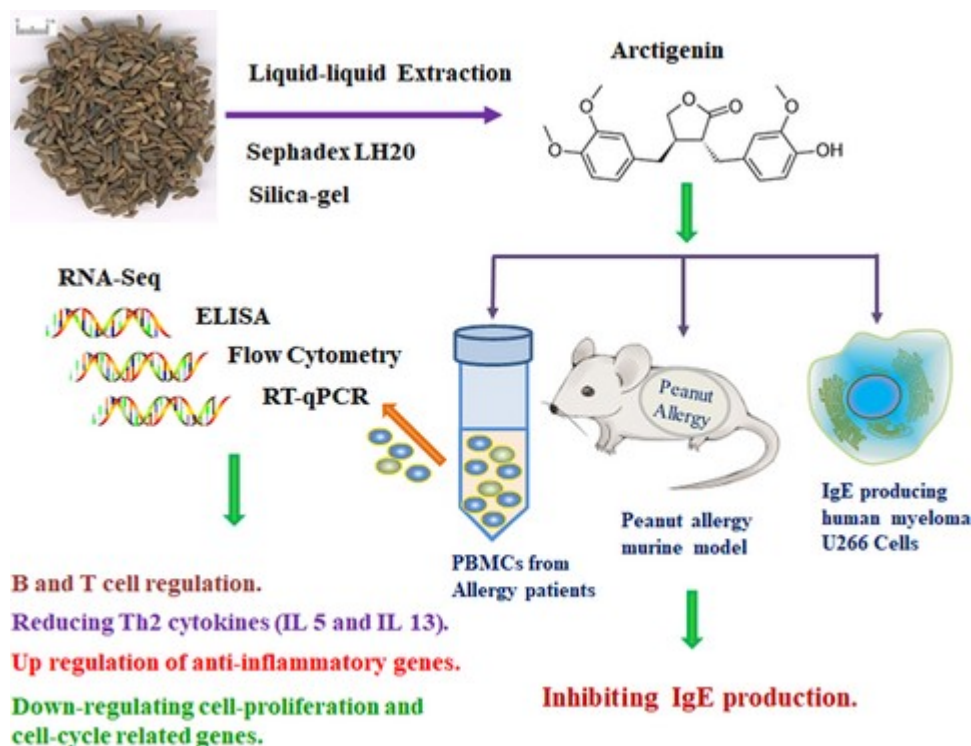
The main compounds isolated were identified as arctiin and arctigenin. Both compounds significantly inhibited IgE production in U266 cells, with arctigenin the most potent ($IC_{50}=5.09\mu\text{g/mL}$). Arctigenin (at a dose of 13 mg/kg) markedly reduced peanut-specific IgE levels, blocked hypothermia and histamine release in a peanut-allergic mouse model. Arctigenin also significantly reduced IgE production and Th2 cytokines (IL-5, IL-13) by PBMCs. We found 479 differentially expressed genes in PBMCs with arctigenin treatment ($p < .001$ and fold-change ≥ 1.5), involving 24 gene ontology terms ($p < .001$, FDR < 0.05); cell division was the most significant. Eleven genes including *UBE2C* and *BCL6* were validated by qPCR.

Conclusion

Arctigenin markedly inhibited IgE production in U266 cells, peanut-allergic murine model and PBMCs from allergic patients by down-regulating cell division, cell cycle-related genes and up-regulating anti-inflammatory factors.

Abstract

Arctigenin was separated and purified from Chinese herb *Arctium Lappa*. Arctigenin not only markedly inhibited IgE in allergic-mice and IgE-producing human myeloma cells and allergic patient PBMCs, and also significantly reduced Th2 cytokines (IL5, IL13) by PBMCs. Arctigenin effects were associated with down-regulated cell division and cell-cycle genes and up-regulated anti-inflammatory gene. Arctigenin may potentially be a novel treatment for IgE-mediated food allergies and other allergic disorders



Abbreviations

Ab

antibody

ATCC

American Type Collection Culture

CPE

crude peanut extract

CT

Cholera toxin

FA

Food allergy

FBS

fetal bovine serum

GO

gene ontology

IL-4/anti-CD40

hIL-4 and anti-CD40 antibody

PBMCs

peripheral blood mononuclear cells

PN-sIgE

peanut-specific IgE

RT-qPCR

real-time quantitative PCR

TCM

Traditional Chinese Medicine

Key messages

Arctigenin markedly inhibited IgE in allergic mice and IgE-producing human myeloma cells and allergic patient PBMCs.

Arctigenin effects were associated with down-regulated cell division and cell cycle genes and up-regulated anti-inflammatory gene.

Arctigenin may potentially be a novel treatment for IgE-mediated food allergies and other allergic disorders.

1 INTRODUCTION

Food allergy (FA) is a significant, worsening global public health issue. Over 2 decades, paediatric and adult incidence has increased dramatically in developed and some developing countries.¹⁻³ Food-induced reactions account for 81% of paediatric anaphylaxis cases.⁴ Despite worldwide prevalence, FA lacks sufficient therapies. Food avoidance and emergency injectable epinephrine remain the major standard of care, although the first FDA treatment, Palforzia[®], an oral immunotherapy for peanut allergy, was approved in January 2020. Food restriction, social isolation, fear and anxiety, particularly among teens,⁵⁻⁷ present complex familial and social challenges.⁸⁻¹¹

FA is primarily IgE-mediated.¹²⁻¹⁴ Specific (s) IgE production to food protein antigens in non-atopic individuals is very low (serum normal reference level <0.1 KU/L). FA patients may produce sIgE a thousand times norm (>100KU/L). Despite decades of awareness of the centrality of sIgE antibodies,¹² inhibiting excessive IgE production remains a challenge. Omalizumab, a humanized monoclonal anti-IgE antibody used as monotherapy¹⁵ or in combination with oral immunotherapy, also shows treatment promise,¹⁶⁻¹⁸ but it neutralizes secreted IgE without fully stopping production in human B cells.^{19, 20}

In the past few years, Traditional Chinese Medicine (TCM) formulas FAHF-2 (Food Allergy Herbal Formula-2) and ASHMI (Anti-Asthma Herbal Medicine Intervention) demonstrated therapeutic effects on food allergy and asthma, reducing allergic inflammation and serum antigen-specific IgE levels in murine models, displaying a comprehensive anti-allergic immune response.^{21, 22} Additionally, ASHMI has lowered IgE in human asthmatics,²³⁻²⁵ and more recently, butanol/ethyl acetate refined FAHF-2 (EB-FAHF-2) has received FDA approval for a phase II clinical trials, alongside multiple food oral immunotherapies and omalizumab.

We continue to advance TCM botanicals for food allergy and have established a library of over 300 Chinese herbal medicine crude extracts, collected from previous reports for their anti-inflammation, anti-cancer, anti-microbial and tested their anti-IgE effect in human B cell lines. Among these herbs, *Arctium Lappa* showed over 90% inhibition of IgE production at

500 µg/ml, making it particularly promising for identifying IgE inhibitory compounds.

This study's goal is to identify IgE inhibitory compounds from *Arctium Lappa* and clarify possible mechanisms. Arctiin and arctigenin were identified. Both showed strong inhibitory effect on IgE production in U266 cells, with arctigenin being the most potent. So, we further studied arctigenin's inhibition of IgE production in peanut-allergic mouse models and by PBMCs from food-allergic patients. Finally, RNA sequencing, flow cytometry and real-time quantitative PCR (RT-qPCR) revealed possible mechanism by which arctigenin inhibits IgE production.

2 METHODS

2.1 Identification of arctigenin and arctiin from *Arctium Lappa*

Dried *Arctium Lappa* aqueous extract was purchased from Guangdong Yifang Pharmaceutical Co., Ltd. (GMP certified, Guangdong, China). It was dissolved in water and extracted with dichloromethane. After a liquid-liquid extraction, D layer and water residue layer were concentrated and dried under pressure to obtain two fractions. The AL-D fraction was further fractionated and purified using repeated silica gel, and Sephadex LH-20 column chromatography methods (shown in Figure S1) yielding 2 pure compounds (Figure 1 a, b), identified using thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS; Agilent 1200 Series LC-MSD TOF system, Agilent Technologies, Danbury, CT) with standards (Sigma, USA).

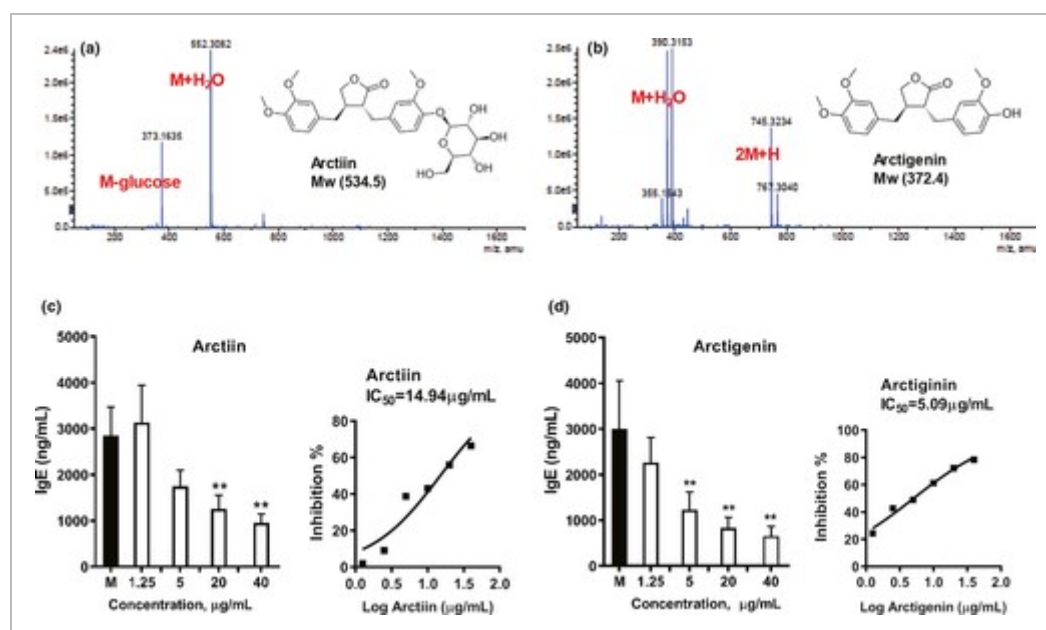


FIGURE 1

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Identification and dose-dependent inhibitory effect of arctiin and arctigenin. Mass spectrometry and chemical structure of arctiin (a) and arctigenin (b). Inhibitory effects and percentage of IgE inhibition vs log(x) concentration curve of arctiin (c) and arctigenin (d) on IgE production by U266 cells. U266 cells (2×10^5 cells/mL) were cultured with arctiin and

arctigenin at concentrations of 1.25, 5, 20 and 40 µg/mL for 6 days. The supernatants were harvested, and IgE levels were measured by ELISA. Results were expressed as mean ±SEM. ***p* < 0.01 (*n* = 4–8)

2.2 Human U266 cells culture and IgE measurement

U266 cells were purchased from American Type Culture Collection (ATCC) and cultured at 37°C under 5% CO₂ in RPMI-1640 medium containing 10% FBS, 1 mM sodium pyruvate, 10 µM β-mercaptoethanol and 0.5% penicillin streptomycin. In IgE inhibition studies, U266 cells were seeded in 24-well plates at a concentration of 2 × 10⁵ cells/well. Cells were treated with arctiin or arctigenin for 6 days. Supernatants were harvested for IgE measurement using an enzyme-linked immunosorbent assay (ELISA) kit (Mabtech, Inc, Cincinnati, Ohio).

2.3 Generation of peanut-allergic mouse model, and measurement of peanut-specific antibodies and histamine levels after arctigenin treatment

Five-week-old female C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free facilities at the Mount Sinai vivarium per standard guidelines for animal care and use.²⁶ Freshly ground, whole-roasted peanut (PN, White Rose Brand, Carteret, NJ, USA) were homogenized in phosphate-buffered saline (Mediatech, Manassas, VA, USA), and a crude peanut extract was prepared as previously described.²⁷ Cholera toxin (CT) was purchased from List Biological Laboratories (Campbell, CA, USA). Mice were subjected to weekly intragastric (i.g.) PN sensitization from week 0 through week 5 with ground peanut (10 mg) plus CT (20 µg) and boosted at week 6, 8 and 10. Peanut-allergic mice were treated with i.g. arctigenin at 0.4 mg/day/mouse, (equivalent to 13 mg/kg) or water (sham) beginning at week 11 and continued daily for 4 weeks. Age-matched naïve mice were included as controls. All mice were challenged at week 15 with ground peanut (200 mg). (Experimental design shown in Figure [S2](#).)

Anaphylactic symptoms were observed over 30 min and grades 0–5 used to score the reactions as previously described²⁷ with slight modifications; 0—no signs; 1—scratching and rubbing around the snout and head, diarrhoea without other systemic symptoms; 2—puffiness around the eyes and snout, redness around snout, pilar recti, reduced activity and/or decreased activity with increased respiratory rate; 3—laboured respiration, diarrhoea accompanied by drop in body temperature, and laboured respiration accompanied by drop in body temperature and sustained lack of voluntary motility, but activity after prodding, 4- laboured respiration, drop in body temperature, cyanosis around the mouth and the tail splaying of limbs with belly resting on cage floor, minimal or no activity after prodding, tremor and convulsions. 5—death. Reactions scored as 1 were defined as mild; 2 as moderate to severe; 3 and 4 as severe; 5 as very severe. Rectal temperatures were measured using a rectal probe, immediately after scoring (Harvard Apparatus, NJ).

2.4 Measurement of peanut-specific antibodies and histamine

Blood and fecal samples were collected one day before challenge. Sera were stored at -80°C until used. Peanut-specific IgE (PN-sIgE), IgG1, IgG2a and fecal PN-SIgA levels were determined by ELISA as previously described.²⁸ Plasma histamine (after challenge) was measured using an enzyme immunoassay kit (Immuno Tech Inc, Marseille, France) in accordance with manufacturer instructions.²⁹

2.5 Safety testing

8-week-old female Kunming mice purchased from the Shandong Laboratory Animal Center (Pengyue, Jinan, China). Safety testing experiments were performed according to the methods reported in previous study.³⁰ And the studies were approved by the Animal Care and Use Committee (Approval No. DWLL201804305) of Henan University of Chinese Medicine and were performed in strict accordance with the NIH guidelines for the care and use of laboratory animals. For acute toxicity analysis, naïve mice were fed with 200 mg/kg of arctigenin (~15 times the daily therapeutic dose) for 1 day. In sub-chronic toxicity assay, naïve mice were fed 100 mg/kg of arctigenin (~8 times the daily therapeutic dose) for 14 days. Water fed mice served as controls. Blood samples were collected after each experiment. Blood urea nitrogen (BUN), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurements for evaluation of kidney and liver functions, respectively, and routine blood test were performed by Lab Science Laboratory (Zhengzhou, Henan, China).

2.6 Study Participants, PBMC Isolation, Culture and IgE measurement

Informed consent was obtained from subjects with physician-diagnosed current food allergy (ages 2–26, $n = 26$) according to the Institutional Review Board, Icahn School of Medicine at Mount Sinai, Children's National Hospital, and New York Medical College. Patient characteristics are shown in Table 1. Per protocol, whole blood samples were collected and PBMCs isolated using a Ficoll gradient, via methods previously published in our laboratory.³¹ To assess the effect of arctigenin on IgE production, purified PBMCs (2×10^6 cells/mL) were cultured in 24-well plates in RPMI 1640 medium, PBMCs were stimulated with 100 ng/mL of recombinant human interleukin 4 (rIL-4) and 1 $\mu\text{g/mL}$ of anti-CD40 mAb in the presence or absence of arctigenin at different concentrations (0.1, 1 and 10 $\mu\text{g/mL}$) for 10 days, per previously established method.³² PBMCs cultured in the medium without stimulation or treatment, served as background control, and IgE levels were evaluated with ELISA kit.

TABLE 1. Characteristics of food-allergic patients. Pt: patient; F: female; M: male

Pt	Sex	Age	Total IgE	Peanut-specific	Other food allergies	Other atopic diseases
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No.			(KU/L)	IgE (KU/L)		
Pt 1	F	8	1439	>100	Eggs, nuts, soy	Seasonal allergy
Pt 2	F	11	1009	30.9	Eggs, nuts, fish and shellfish	Seasonal allergy, asthma
Pt 3	M	16	891	>100	Milk, coconut, fruit, nuts, soy, sesame seed	Seasonal allergy
Pt 4	F	6	474	24.3	Eggs, milk, flaxseeds, banana.	Seasonal allergy
Pt 5	M	22	181	24.9	Tree nut, cod fish	Seasonal allergy
Pt 6	M	10	657	565	Eggs, Soy, wheat, sesame oil	Seasonal allergy
Pt 7	M	5	2332	>100	Sesame oil, Lima beans and soy	Asthma, seasonal allergy
Pt 8	M	12	752	11.3	Eggs, milk	Asthma and seasonal allergy
Pt 9	M	16	891	>100	Nuts	Asthma and environmental allergy
Pt	F	4	100	0.14	Eggs, sweat potato,	Unknown

Note

Pt: patient; F: female; M: male; ND: not done; NA: not applicable

2.7 Flow Cytometry

To assess the effect of arctigenin on immunoglobulin production, PBMCs were cultured in Yssel's medium (Gemini Bio, West Sacramento, CA) with human recombinant IL-4 (100 ng/ml; PeproTech, Rocky Hill, NJ) and anti-CD40 (1 µg/ml; clone 82111; R&D Systems, Minneapolis, MN) in the presence or absence of arctigenin (1 µg/ml) for 7 days. Cells were harvested, then incubated with a fixable viability dye (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, Invitrogen/Thermo Fisher Scientific) and washed in PBS. Cells were then stained with fluorescently labelled antibodies specific for CD3 (clone OKT3), CD19 (clone SJ25C1), CD27 (clone LG.7F9), CD38 (clone HIT2), IgD (clone IA6-2) and FcεRI (clone AER-37; all antibodies from eBioscience/Thermo Fisher Scientific) in the presence of Human FcR Blocking Reagent (Miltenyi Biotec, Auburn, CA). Samples were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience/Thermo Fisher Scientific) according to the manufacturer's directions. Cells were then stained with

fluorescently labelled Abs specific for IgE (clone Ige21; eBioscience/Thermo Fisher Scientific), IgG1 (clone HP6001; SouthernBiotech, Birmingham, AL) and IgG4 (clone HP6025; SouthernBiotech). Subsequent washes were done with 1× Permeabilization Buffer (eBioscience/Thermo Fisher Scientific) according to the manufacturer's directions and resuspended in PBS plus 2% heat-inactivated FBS prior to data acquisition. Data were acquired on a Cytoflex S (Beckman Coulter, Indianapolis, IN) and analysed using FlowJo software (FlowJo, Ashland, OR).

2.8 RNA Sequencing (RNA-Seq)

Human PBMCs were cultured as described above and treated with arctigenin (10 µg/mL) at 37°C under 5% CO₂ for 10 days. Cells were harvested and total RNA isolated using TRIzol reagent (Gibco BRL, Rockville, Maryland) according to the manufacturer's instructions. RNA quality was examined by 4200 Tape Station (Agilent Technologies, Santa Clara, CA), and RNA concentration was determined by Qubit fluorometric quantitation (Life Technologies, Carlsbad, CA). For each sample, 200 ng of RNA was used to construct cDNA sequencing library using the TruSeq Stranded Total mRNA Library Preparation Kit (Illumina, San Diego, CA), in accordance with the manufacturer's protocol using poly-adenylated RNA isolation. Sequencing of paired-end reads (75 bp ×2) was performed in Illumina Next Seq 550 system.

2.9 RNA-Seq Data Analysis

Raw sequence reads were de-multiplexed and trimmed for adapters by using the Illumina bcl2fastq conversion (v2.19). Sequence reads of each sample were pseudo-aligned to human hg38 reference transcriptome, and gene transcript counts and abundance (TPM) were quantified by using Kallisto.³³ Differential expression of genes and transcripts was achieved in paired groups by using tximport³⁴ and DESeq2 packages³⁵ in R platform. Genes with adjusted *p*-value less than 0.001 were selected for gene-set enrichment assays in R (R package version 1.66.0.). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis results can be obtained by entering the genes list (according to the threshold of *p* < .001 and fold-change ≥1.5) into the David database³⁶ respectively. Genes coexpedia (www.coexpedia.org) and protein evolutionary relationships (STRING) were used to analyse the critical genes. The genes screened were used for network visualization using the Cytoscape software.³⁷ The RNA-Seq data are available at the GEO website under accession GSExxxx.

2.10 Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse transcription was performed to obtain the cDNA using ImProm-II Reverse Transcriptase (Promega Corporation, Madison, Wisconsin) according to manufacturer's instructions. The RT-qPCR amplification was performed using Maxima SYBR Green qPCR Master Mix kit (Fisher Scientific, Pittsburgh, Pennsylvania) on a real-time PCR machine

(Applied biosystems, America). The primer sequences are shown in Table [S1](#).

2.11 MTT assay for assessment of cell proliferation

Single cell suspensions were prepared from splenocytes of PN-allergic mice. B cells and CD4 T cells were purified by negative selection using magnetic bead-based cell isolation kits from Stemcell (Vancouver, Canada). B cells ($1 \times 10^6/\text{ml}$) were cultured in 200 μl of RPMI medium supplemented with 10% FBS, 1% penicillin Streptomycin and sodium pyruvate (all purchased from Gibco, CA) in 96-well round bottom plates and stimulated with 1 $\mu\text{g}/\text{ml}$ anti-mouse CD40 (BD biosciences, CA) +50 ng/ml recombinant mouse IL-4 (Biolegend, CA) in the presence and absence of indicated concentrations of arctigenin (Santacruz Biotechnology, CA) for 4 days under standard tissue culture conditions. Mouse CD4 T cells were cultured at $2 \times 10^6/\text{ml}$ and stimulated with 2.5 $\mu\text{g}/\text{ml}$ ConA (SigmaAldrich, MO) for 3 days. For MTT assay, plates were centrifuged and culture medium was replaced with 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Roche, IN) dissolved in PBS and cells were dispersed by gently vortexing the plate. After 4 h, plates were centrifuged and cells were washed twice with PBS. After final wash, 200 μl of DMSO (Fisher Scientific, NJ) was added to the wells. Plates were read on a Vmax spectrophotometer (Molecular Dynamics) at 590 nm, and OD values were recorded.

2.12 Measurement of cytokines in human PBMC and mouse CD4 T cell cultures

Human PBMCs were isolated from blood obtained from food-allergic patients enrolled in IRB-approved clinical study at New York Medical College. PBMCs were isolated by ficoll separation as described in our previous study.³⁸ Mouse CD4 T cells were isolated as described above for proliferation study. Human PBMCs ($2 \times 10^6/\text{ml}$) were cultured in 96-well plates and stimulated with Human T cell Activator CD3/CD28 Dynabeads (2 $\mu\text{l}/\text{ml}$, Invitrogen, CA) in the presence and absence of indicated concentrations of arctigenin 1a,b for 72 h. Murine CD4 T cells were cultured at $2 \times 10^6/\text{cell}/\text{ml}$ in 24-well plates. Cells were stimulated with ConA (2.5 $\mu\text{g}/\text{ml}$) in the presence and absence of indicated concentrations of arctigenin for 72 h. Human PBMC and Mouse CD4 T cell culture supernatants were harvested and stored at -20°C till use. Levels of human IL-5 and IL-13 were measured using ELISA kits from BD Biosciences, CA. Mouse IL-13 was measured using kit from R&D Systems, MN according to manufacturer's instructions.

2.13 Statistical analysis

Statistical analyses were performed using Prism 9 (GraphPad, La Jolla, CA). Differences between multiple groups were analysed by one-way ANOVA followed by the Dunnett' test, and the differences between two groups were analysed by paired t test. Correlation study was conducted using Pearson's method, in which a p -value ≤ 0.05 , based on a two-tailed test, was considered statistically significant.

3 RESULTS

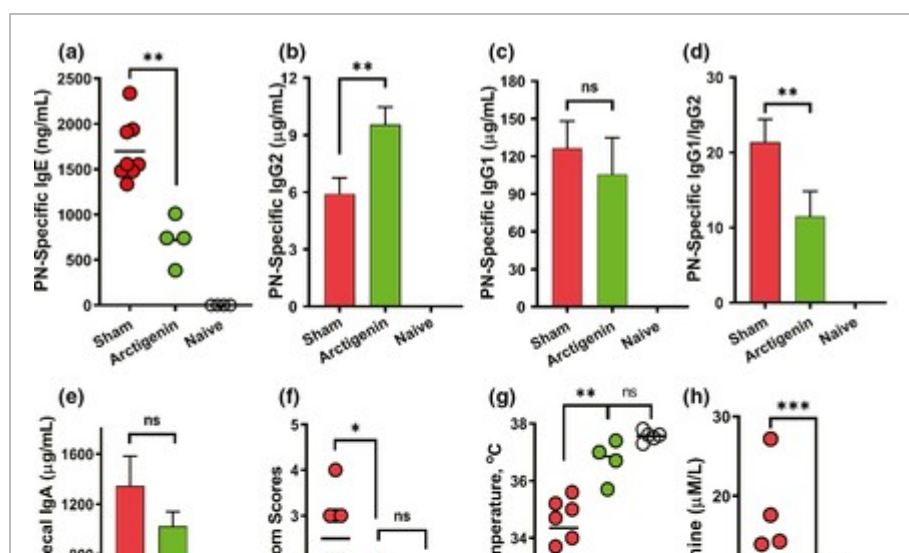
3.1 Isolation and identification of Arctiin and Arctigenin, and their inhibitory effects on IgE production

Two compounds were isolated and identified from *Arctium Lappa* by using liquid-liquid extraction, repeated silica gel and Sephadex LH20 chromatography. Compound 1 was identified as arctiin based on ions at m/z 373 (M-glucose) and m/z 552 (M+H₂O), and compound 2 was identified as arctigenin based on ions at m/z 390 (M+H₂O) and m/z 745 (2 M+H). The MS spectra and chemical structures of arctiin and arctigenin are shown in Figure [1a, b](#)

We tested inhibitory effects of arctiin and arctigenin at concentrations ranging from 1.25 to 40 $\mu\text{g/mL}$ on IgE production by U266 cells. As shown in Figure 1c, d, both dose-dependently reduced IgE production. Arctiin at 20 $\mu\text{g/mL}$ and arctigenin at 5 $\mu\text{g/mL}$ significantly suppressed IgE production. 50% inhibition concentration (IC₅₀) of arctiin and arctigenin was 14.94 $\mu\text{g/mL}$ and 5.04 $\mu\text{g/mL}$ (Figure [1c, d](#)) respectively. Arctigenin was more potent in suppressing IgE production by U266, so we subsequently focussed on arctigenin.

3.2 Effects of Arctigenin on IgE production and peanut anaphylaxis following oral peanut challenge in murine model

To evaluate arctigenin's *in vivo* effects, a peanut-allergic mouse model was adopted from previous studies.^{39, 40} Peanut-allergic mice received i.g. treatment with 400 $\mu\text{g}/\text{mouse}/\text{day}$ arctigenin for 4 weeks. Sham-treated peanut-allergic mice showed significantly elevated serum PN-sIgE levels compared to naïve (1698 vs. 0 ng/mL, $p < .001$, Figure [2a](#)). Arctigenin-treated mice showed significantly reduced serum PN-sIgE levels opposed to sham-treated (719 vs 1698 ng/mL, $p < .01$). Inhibitory effects on PN-sIgE production were consistent with those observed *in vitro* in U266 cells. Treatment significantly increased peanut-specific IgG2a levels ($p < 0.05$, Figure [2b](#)), but did not affect peanut-specific IgG1 (Figure [2c](#), with a significantly decreased IgG1/IgG2a ratio, Figure [2d](#)) or mucosal IgA levels (Figure [2e](#)).



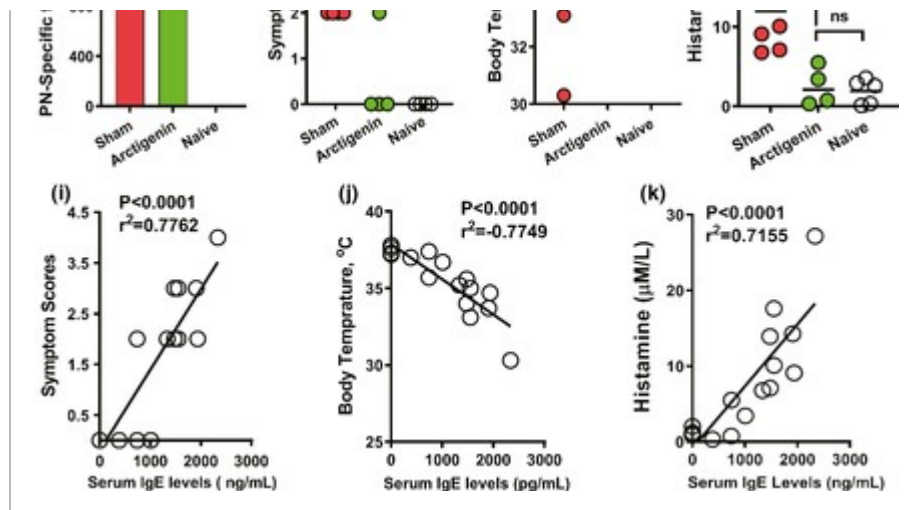


FIGURE 2

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Effects of arctigenin on peanut-specific antibodies and protection of anaphylactic in a peanut-allergic mouse murine model. Serum peanut-specific IgE (a), IgG2a (b), IgG1 (c), IgG1/IgG2a (d) and fecal peanut IgA (e) levels were measured by ELISA. Anaphylactic symptoms (f) were scored 25 to 30min after the challenge. Core body temperatures (g) were measured after scoring using a rectal thermometer. Serum histamine (h) levels were measured by ELISA. ns means no statistical difference, * $p < .05$, ** $p < .01$ or *** $p < .001$ ($n = 4-7$). Correlation between PN-IgE and anaphylactic symptom scores (i), body temperature (j) and histamine levels (k) were evaluated. Each circle represents an individual mouse

After week-15 peanut challenge (4 weeks post-treatment), all sham-treated mice developed anaphylactic reactions (symptoms scores ranging 2–4; median score=2.5). In contrast, arctigenin-treated mice significantly reduced anaphylaxis (symptom scores ranging 0–2; median score=0, Figure 2f). As shown in Figure 2g, sham-treated mice mean core body temperatures were significantly lower than naïve (33.95°C vs 37.48 °C, $p < .01$). Mean temperatures in the arctigenin-treated mice were significantly higher than sham-treated (36.7°C vs 33.95°C, $p < .01$); mean temperatures fluctuated minimally in naïve mice. Sham-treated mice consistently demonstrated significantly increased plasma histamine (Figure 2h) following challenge, whereas arctigenin treatment significantly reduced histamine by 81% (11989 vs 2084 nmol/L, $p < .001$).

Correlation analysis was conducted between serum IgE levels and symptoms scores, as well as core body temperature and histamine, following challenge. We found positive correlation between PN-sIgE and anaphylactic reaction scores (correlation coefficient $R^2 = 0.7762$, $p < .0001$, Figure 2i), negative correlation with core body temperature ($R^2 = 0.7749$, $p < .0001$, Figure 2j) and positive correlation with histamine levels ($R^2 = 0.7155$, $p < .0001$, Figure 2k) were indicated.

3.3 Safety testing of arctigenin treatment in vivo

Acute toxicity and sub-chronic toxicity were used to test the safety of arctigenin. Mice were fed 15 times the arctigenin therapeutic dose (1 day) or 8 times the therapeutic dose

(14 days). No deaths or abnormality occurred, and no significant changes in weight and food consumption were observed. All mice appeared healthy. Serum ALT, AST and BUN levels showed no significant difference compared to the control group, as they were within the normal range (Table S3). Upon conducting a haematology test on red blood cell (RBC), white blood cell (WBC), haemoglobin (HGB) and platelet (PLT) levels, both treatment groups did not differ significantly from the control group and were also within the normal range, indicating that arctigenin is highly safe.

3.4 Effects of arctigenin on human rIL-4 and anti-CD40 antibody stimulated IgE production by PBMCs of food-allergic patients

To translate animal studies to humans prior to clinical trials, we next examined arctigenin inhibition of IgE production by PBMCs from food-allergic patients with 0.1, 1 and 10 $\mu\text{g/mL}$. Our results demonstrated negligible IgE levels in medium-alone cultured cells, but significant elevation of IgE levels in IL-4 and anti-CD40-stimulated cells ($p < 0.05$ Figure 3a). Arctigenin dose-dependently and significantly inhibited IL-4 and anti-CD40-stimulated IgE production ($p < 0.05$ at 1, 10 $\mu\text{g/mL}$ of arctigenin, $n = 3\text{--}6$ Figure 3a). We evaluated cytotoxicity at the highest concentration of arctigenin tested (10 $\mu\text{g/mL}$) with none observed (Figure 3b $n = 5$). Next, we investigated whether arctigenin inhibition of IgE could be reproduced at relatively larger sample numbers at a single higher and safe concentration of 10 $\mu\text{g/mL}$. Arctigenin reproducibly and significantly inhibited IL-4 and anti-CD40 antibody stimulated IgE production ($p < 0.01$, Figure 3c, $n = 9$). There also was negative correlation between IgE and arctigenin concentration ($r = -0.7196$, $p < .001$, Figure 3d). Flow cytometry experiments also showed arctigenin ability to reduce in anti-CD40/IL-4- induced IgE, both with regard to the frequency of IgE+B cells (Figure 3f) and IgE expression on a per-cell basis (as measured by median fluorescence intensity (MFI; Figure 3g)). There was no significant effect on IgG1 or IgG4 production (data not shown). These results demonstrated that arctigenin exhibits a direct inhibitory effect on IgE production by human PBMCs from food-allergic subjects.

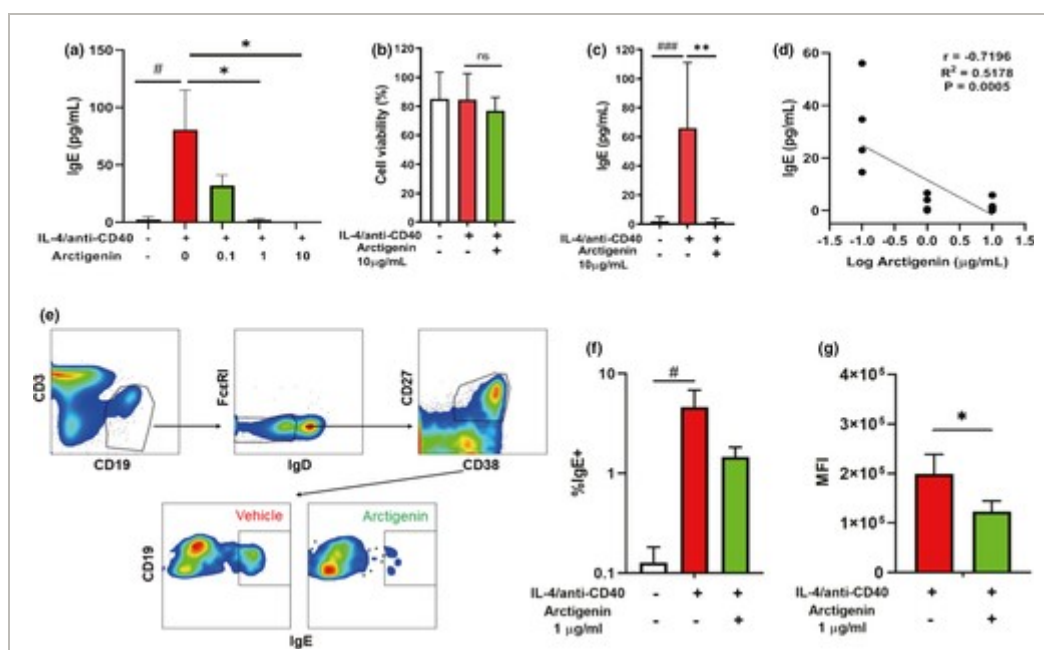
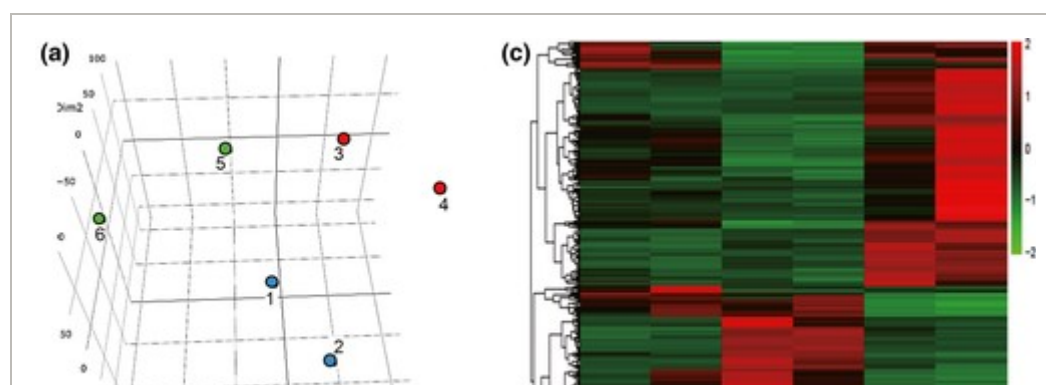


FIGURE 3
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Inhibitory effect of arctigenin on IgE production by human PBMCs. PBMCs (1.5×10^6 cells/mL) from food-allergic patients were collected and co-stimulated with human rIL-4 (100 ng/mL) and anti-CD40 mAb (1 μ g/mL) in the presence of different arctigenin for 10 days. IgE levels in supernatants were determined by ELISA. (a) Dose-dependent inhibitory effect of arctigenin on IgE production by human PBMCs at the concentrations of 0.1, 1 and 10 μ g/mL ($*p < .05$ vs 0 μ g/mL; $\#p < .05$ vs unstimulated ($n = 3-6$). (b) Cell viability of human PBMCs at 10 μ g/mL concentration of arctigenin ($p = 0.128$, $n = 6$). (c) Inhibitory effect of arctigenin on IgE production by PBMCs at 10 μ g/mL. $***p < .001$ vs 0 μ g/mL; $###p < .001$ vs unstimulated ($n = 9$). (d). Correlation study based on summarized data from Figure 3 A and C between various concentrations of arctigenin (0, 0.1, 1 and 10 μ g/mL) and IgE in response to rIL-4 anti-CD40 mAb stimulation. $r = -0.7169$, $***p < .001$. (e). Gating strategy for IgE⁺B cells. Dead cells and non-singlet events were first excluded. CD19⁺ cells were selected before gating on Fc ϵ RI and IgD cells. Of the remaining cells, CD38⁺ CD27⁺ cells were selected before examining staining for IgE. Gates were set using fluorescence minus one (FMO) control. Also shown are representative plots for stimulated, untreated cells and stimulated, arctigenin-treated cells. (f). Frequency of IgE⁺B cells among PBMCs stimulated with IL-4 and anti-CD40 in the presence (green) or absence (red) of arctigenin at 1 μ g/ml after 7 days of culture. $\#p < .05$ vs. unstimulated ($n = 6$). (g). Median fluorescence intensity (MFI) of IgE⁺B cells in Figure 3 e. $*p < .05$

3.5 Effect of arctigenin on transcriptomic profiles of human rIL-4/anti-CD40 stimulated food-allergic patient PBMCs

PBMCs collected from food-allergic patients were used to profile transcriptomic changes of arctigenin treatment. 23,000+ genes were identified in these gene expression profiles. They were grouped as medium control (blue), stimulated control (red) and stimulated +arctigenin (green) ($n = 2$), and the results of principal component analysis are shown in Figure 4a. We focussed on differentially expressed genes in stimulated vs stimulated +arctigenin group to explore the mechanism of arctigenin' inhibition of IgE production in response to stimulation. According to the threshold of $p < .001$ and fold-change of ≥ 1.5 , there were a total of 479 differentially expressed genes, including 171 up-regulated and 308 down-regulated, (Figure 4b, accounting for 2% of the total identified genes), listed in a heat map (Figure 4c) for further analysis.



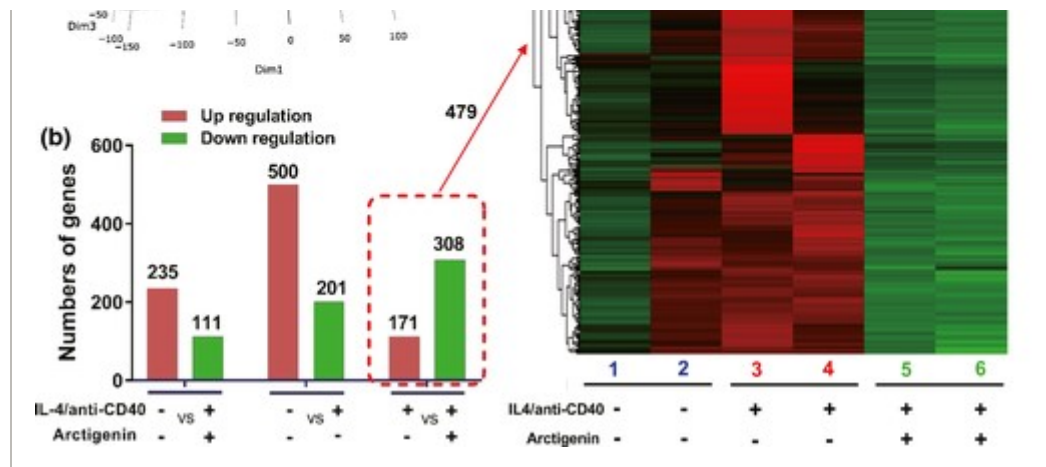
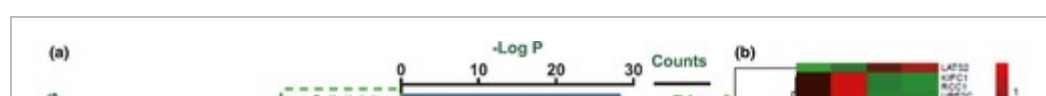


FIGURE 4

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Differential transcriptomic profiling. PBMCs from food-allergic patients in medium control, IL-4/anti-CD40 stimulation and IL-4/anti-CD40 stimulation treated with arctigenin were subjected to RNAseq analysis ($n = 2$ in each set). (a). Principal components analysis (PCA) was shown in three dimensions, 1 and 2: medium control; 3 and 4: IL-4/anti-CD40 stimulation; 5 and 6: IL-4/anti-CD40 stimulation treated with arctigenin. (b). Numbers of differentially expressed genes in pair-wise comparisons: medium vs stimulated, medium vs stimulated +arctigenin, and stimulated vs stimulated +arctigenin (at the threshold of $p < .001$ and fold-change ≥ 1.5). (c). The heat map of 479 differentially expressed genes, which were obtained by comparing stimulation with stimulation +arctigenin. Up-regulation is shown in red, and down-regulation is shown in green

According to systematic relationships of the 479 differentially expressed genes, the Web-based Gene Set Analysis Toolkit was used to identify GO terms and KEGG pathway. 24 significant GO terms were confirmed using David databases, with a $p < .001$ and false discovery rate (FDR) < 0.05 . The top 15 most significant GO terms are shown in Figure 5a, and cell division term (GO:0051301, $p = 4.62 \times 10^{-29}$), mitotic nuclear division term (GO:0007067, $p = 3.96 \times 10^{-25}$), DNA replication (GO:0006260, $p = 1.28 \times 10^{-23}$), sister chromatid cohesion term (GO:0007062, $p = 7.90 \times 10^{-19}$) and G1/S transition of mitotic cell cycle (GO:0000082, $p = 5.37 \times 10^{-17}$) were the top five GO annotated terms for biological process (BP). 74 genes closely associated with cell division are listed in Figure 5b. A majority of genes were down-regulated. To further explore which genes played the most important role in inhibiting IgE production after arctigenin treatment, these 74 genes were selected using the coexpedia databases to construct a gene co-expression network, and the top 10 co-expressed genes are listed in Figure 5c. MeSH heading (excluding Neoplasms) T (B) -Lymphocytes were further selected for improving network visibility (Figure 5d) using the Cytoscape software. In this network, the top five most interacting proteins, including UBE2C, CDC20, BIRC5, CCNB1 and MKI67 (marker of proliferation Ki-67), may play an important role in inhibiting PBMCs proliferation.



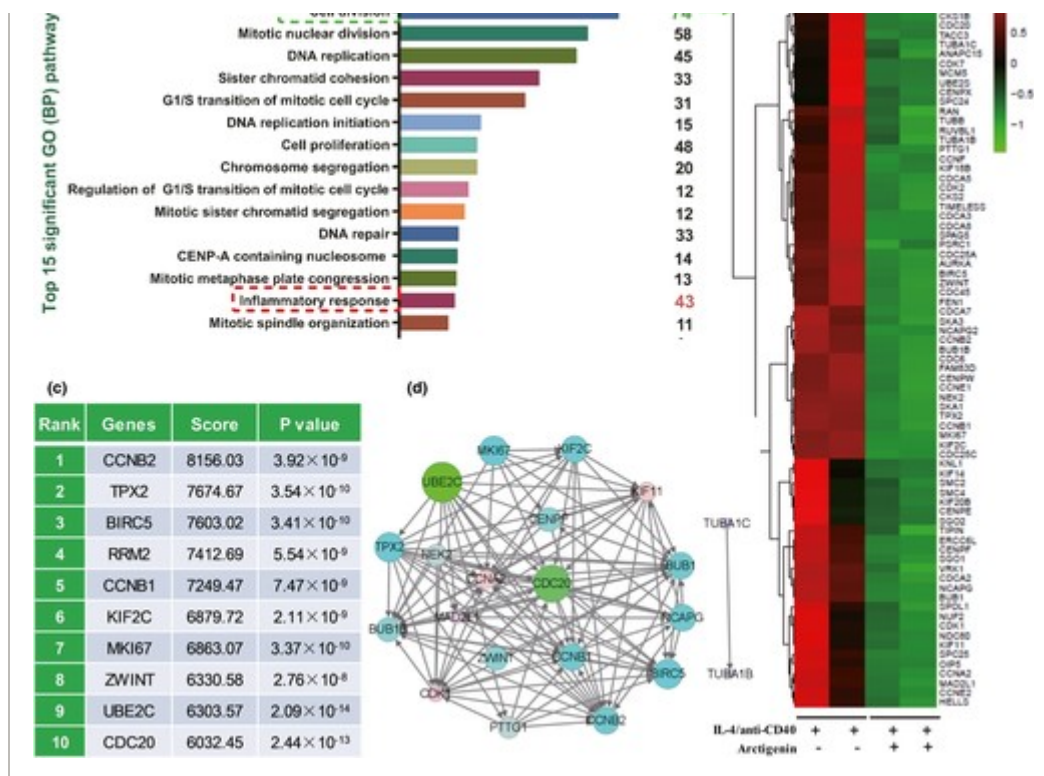


FIGURE 5

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Gene-set enrichment gene ontology analysis by 479 differentially expressed genes. (a) Top 15 significant GO terms in biological process with -log (P value) demonstrated in axis X. (b) Heat map of 74 differentially expressed genes in cell division GO term. (c). Co-expression scores of the top 10 genes in cell division (GO) counted by coexpedia databases. (d). Genes associated with the T (B) -lymphocytes were identified through co-expression net construction. Circled nodes represent the genes, and lines represent edges between genes. Bright green (or blue) represents key genes, whereas circle size is related to its – Log(p) of genes. More edges of a genes, more genes are connected with it, thus suggesting it has a more central role within the network. Arrow direction means from node 1 to node 2

In the inflammatory response term (GO:0006954, $p = 6.64 \times 10^{-8}$), 20 genes were down-regulated, including TNF receptor superfamily members (TNFRSF8, TNFRSF9, TNFRSF11A), C-C, X-C or C-X-C motif chemokines (CCL19, CCL17, CCL7, XCL2, CXCL9), which suggests many inflammatory factors were down-regulated after arctigenin treatment. Among up-regulated genes, BCL6 gene was particularly impressive, playing a unique anti-allergic role by regulating different proteins essential in allergy response signalling pathway.⁴¹⁻⁴³ Meanwhile, most genes from negative regulation of tumour necrosis factor production term (GO: 0032720, $p = 1.55 \times 10^{-4}$) and activation of cysteine-type endopeptidase activity involved in apoptotic process term (GO:0006919, $p = 4.40 \times 10^{-4}$) were up-regulated. These data suggest arctigenin suppression of IgE is associated with down-regulating cell cycle, DNA replication-related genes and up-regulating anti-inflammatory factors.

3.6 Validation of gene expression with RT-PCR

To confirm transcriptome profiling data, RT-PCR was performed to examine these representative genes, which were selected from the above most significant GO terms. Among them, we focussed on those genes with p value $< 1 \times 10^{-6}$ and fold change ≥ 2.5 , to show the contrary direction between stimulated vs. stimulated +treatment and medium vs. stimulated (shown in Table S2). A total of 11 differentially expressed genes were verified by real-time RT-PCR, as shown in Figure 6, including *UBE2C*, *KIF2C*, *MKI67*, *BIRC5*, *CDC45*, *CCNB1*, *FEN1*, *E2F1*, (Figure 6a) and *CCL19* gene, *BCL6* and *SPARC* (Figure 6b), which demonstrated similar trends among the normalized transcriptome data.

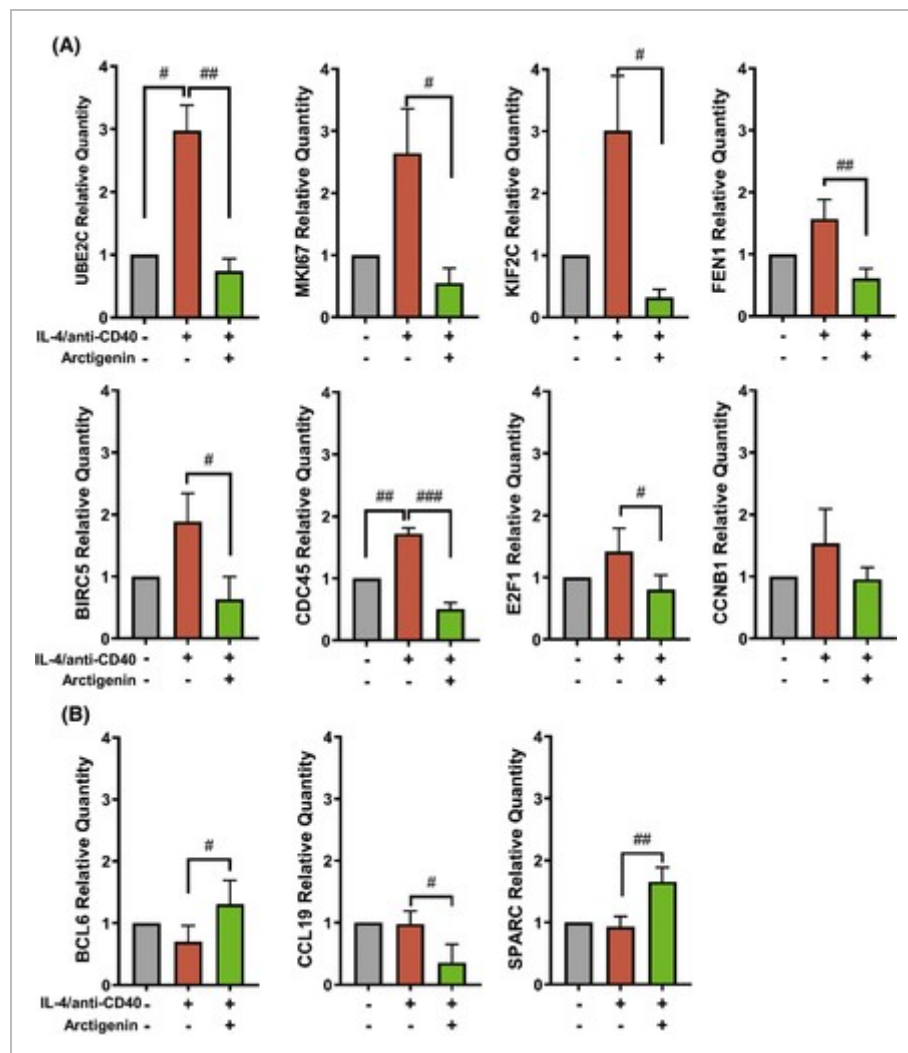


FIGURE 6

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Expression validation of representative genes with RT-qPCR. (a). Cell division and cell cycle-related genes. (b) Inflammatory response related genes. Data represent the mean $\pm$ SD ($n = 3$ or 4) of the relative quantification. # $p < .05$, ## $p < .01$ and ### $p < .001$

3.7 Evaluation of Arctigenin effects on T cells, B cells and dendritic cells

To determine whether arctigenin exerted effects on cells other than IgE-producing cells, we

examined arctigenin effects in T cells and dendritic cells using various cell-type specific stimuli. We stimulated human PBMCs from food-allergic patient with human T cell activator Dynabeads (α CD3/CD28 beads) for 3 days and assayed culture supernatants for the Th2 cytokines IL-5 and IL-13 by ELISA. To account for wide variations in responses to T cell stimulation across patients, cytokine production was normalized to stimulation control (α CD3/CD28 alone). As shown in Figure 7a, b, IL-5 and IL-13 levels in human PBMC cultures were significantly and dose-dependently decreased in the presence of arctigenin ($p < 0.05$ – 0.0001 vs α CD3/CD28 alone). Consistent with this, presence of arctigenin at 1, 5 or 10 μ g/ml in Con A (T cell mitogen) stimulated murine CD4 T cell cultures caused significant reductions in IL-13 levels (Figure 7c). In addition to cytokine responses, arctigenin was a potent suppressor of murine CD4 T cell proliferation in response to Con A as determined by MTT assay. Absorbance values in cultures treated with 1, 5 or 10 μ g/ml arctigenin showed reductions varying approximately between 50 and 85% (Figure 7d, $p < .0001$ for all concentrations vs Con A alone). We also evaluated the effects of arctigenin on the cell proliferation and apoptosis of IgE-producing B cells. When it was greater than 5 μ g/mL, arctigenin significantly inhibited the proliferation of U266 B cells and induced their apoptosis in a dose-dependent manner (Figure S4). Furthermore, arctigenin suppression of murine CD4⁺ T cell proliferation was similar to the same observed for purified murine B cells stimulated with α CD40/IL-4 (Figure 7e, $p < .001$ – 0.0001 vs α CD40/IL-4 alone for all concentrations tested) indicating that arctigenin targets both T and B cells. In contrast, we did not observe any effect of arctigenin on dendritic cell maturation (Figure S3) in response to 6-day stimulation with a mix of LPS, IL-4, GM-CSF, TNF- α , IL-6 and IL-1 β . Presence of arctigenin at 1 μ g/ml did not alter expression of costimulatory molecules CD80 or CD86.

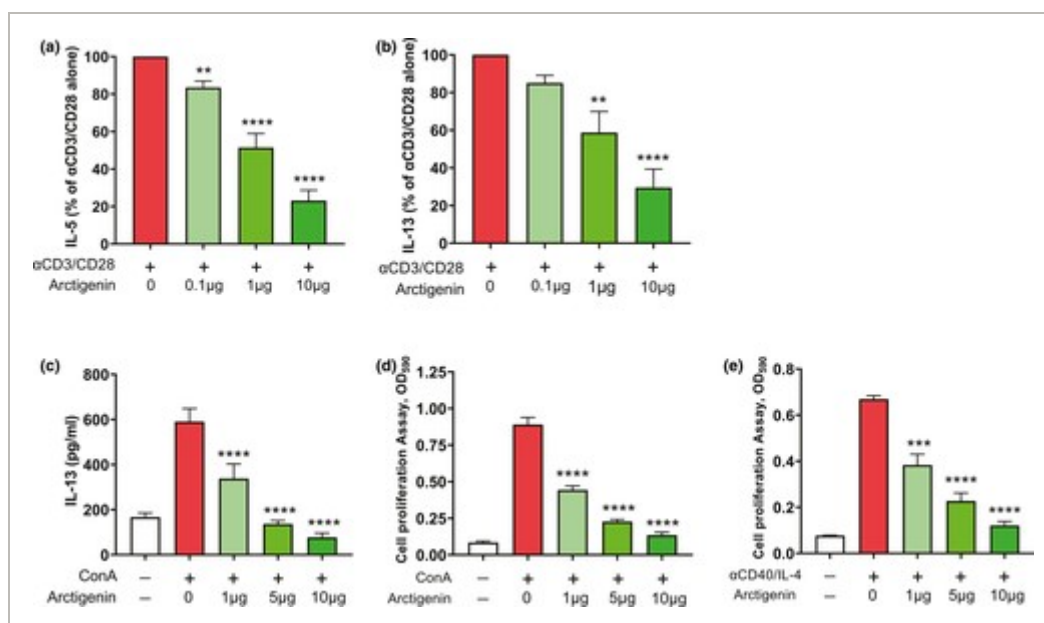


FIGURE 7

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Effects of arctigenin on Th2 cytokine production in human and murine T cells and proliferation of murine T and B cells.

PBMCs from food-allergic patients were stimulated with human T Cell Activator Dynabeads (α CD/CD28) for 3 days. IL-5

(A) and IL-13 (B) levels in culture supernatants were measured by commercial ELISA, and data were expressed as percent of stimulation control (α CD/CD28 alone). $N = 4\text{--}6$ patients/condition. Murine CD4 T cells isolated from spleens of PN-allergic and stimulated with ConA for 3 days. IL-13 in culture supernatants (Figure 7c) was measured by commercial ELISA. Effect of arctigenin on proliferation of murine CD4 T cells (Figure 7d) and purified B cells (Figure 7e) was evaluated by MTT assay. Data for Figure 7c–e are shown as group Means $\pm$ SEM. $N = 3\text{--}6$ mice/condition).

*= $p < .05$; ***= $p < .001$; ****= $p < .0001$ vs 0 $\mu\text{g/ml}$ arctigenin (red bars)

4 DISCUSSION

Arctium Lappa is a common Asian vegetable and deemed a general health tonic.^{44, 45} Arctigenin, a lignan found in its leaves, seeds and roots, is widely investigated as a potent anti-inflammatory compound,^{46–52} with some studies having also proven anti-tumour effects.⁵³ One study examined arctigenin and arctiin effects on the colonic tissue in mice with dextran sulphate sodium (DSS) and trinitrobenzene sulphonic acid (TNBS)-induced colitis, essentially replicating human inflammatory bowel disease (IBD). The same laboratory found arctigenin at 50 mg/kg, rather than arctiin, dramatically decreased elevated pro-inflammatory cytokines (TNF- α and IL-6) in rats with DSS-induced colitis.⁵⁴ However, studies of arctigenin and IgE-mediated food allergy are limited.

As mentioned, regulating IgE production is central to developing food allergy treatments. In this study, we demonstrated for the first time that arctigenin dose-dependently suppressed IgE production in vitro by U266 cells, upon observing a low 50% inhibition concentration (IC50) of arctigenin as low as 5.04 $\mu\text{g/mL}$. In vivo study using murine peanut-allergic model at 13 mg/kg also demonstrated that arctigenin significantly suppressed PN-sIgE, in which positive correlations between IgE and anaphylactic symptom scores, histamine levels and temperature negative correlation further supported the suppression of arctigenin on peanut-allergic reactions. Arctigenin also showed high safety: our in vivo toxicology study (Supplemental information) feeding mice daily dose of arctigenin 15x caused no abnormalities. Haematological parameters, AST, ALT and BUN, also remained within normal range (Table S3), consistent with the results reported by Tan et al.^{44, 45} In addition to efficacy in mice, arctigenin showed potent ability to reduce IgE production by PBMCs from food-allergic patients where 1 $\mu\text{g/mL}$ arctigenin resulted in 97.5% inhibition of IgE production.

Previous studies have shown safety even at very high doses. Our data provided acute and sub-chronic safety data, suggesting that the safety profile of the compound arctigenin is similar to its parent herb. Nevertheless, if it is registered as a product, we will conduct additional safety study for clinical use following FDA guidance. These findings suggest the anti-IgE effect observed in vitro was physiologically achievable *in vivo*—highly promising for human applications. Arctigenin is a minor compound in *Arctium Lappa*. It would be a major undertaking to isolate this compound from *Arctium Lappa* for the future clinical use. Studies are underway to generate synthetic versions of this molecule to make the product more reproducible, dependable and clinically applicable.

To clarify how arctigenin inhibits IgE production, gene expression profiling was developed to generate transcriptome profiles from food-allergic patient PBMCs. Transcriptome findings indicated that cell cycle and DNA replication likely play important roles in IgE-producing B cells. Recent studies have reported that IgE production in food-allergic patients was closely related to cell cycles of B cells or T cells. Ramadani et al.⁵⁵ reported that proliferative and cycling capacity of IgE-expressing B cell (IgE⁺ PB) and plasma cells (PCs) was greater than their IgG1⁺ cell counterparts. Their most recent study reports a number of positive regulating cell cycle genes were up-regulated in IgE⁺ PB and PCs, unlike in IgG1 cells, such as CDC25B, MYC, FOXM1, PLK4, CDC20, E2F2, MKI67 and MCM4.⁵⁶ In another study, David Martino and coauthors⁵⁷ observed that activated T cells are highly proliferative, leading to increased expression of cell cycle-related targets of the E2F transcription factor network, which controls cell division and DNA replication and MYC genes, which act as a molecular “cell division timer” to control metabolic switching upon antigen stimulation.

Our findings support those results. After treatment with 10 µg/mL arctigenin, the most significant genes differentially expressed, emerged in various cell division aspects including cell division mitotic nuclear division DNA replication sister chromatid cohesion and G1/S transition of mitotic cell cycle. Moreover, expression of these related genes was down-regulated, particularly among GDF10, KIF2C, UBE2C, KIF15, BUB1, BIRC5, ZWINT, BUB1B, TUBB, NCAPG2, NCAPG, FBXO5, CENPE, CDC20, BIRC5, SPDL1, CDC25C, SMC2, CDC25A, CCNB1, CCNB2, MKI67, TPX2, CDK2. Among these aforementioned genes, the top four most interacting genes included UBE2C, BIRC5, KIF2C and MKI67. Genes verified by RT-qPCR most involved in the cell division and cell cycle GO terms. UBE2C is essential for cell cycle progression, which along with APC/C (anaphase promoting complex/cyclosome) and CCNB1, are the key checkpoint controls of the ubiquitin pathway. BIRC5 plays a critical role in apoptosis, cell division and proliferation.⁵⁸ MKI67 triggered in differentiated cells after proliferative stimulation and disappeared after proliferating cell differentiation, which was used as a tumour proliferation marker.⁵⁹ Besides the above genes involved in the cell cycle-related pathways, we found several genes in the inflammatory pathway were closely related to inhibited IgE production. CCL19 and its chemokine receptor CCR7 play a role in development of allergic responses.^{60, 61} Liu et al. reported that baicalin attenuated inflammation in mice with OVA-induced asthma by suppressing CCR7/CCL19.⁶⁰ Interestingly, we confirmed 2 genes BCL6 and SPARC were significantly increased in the stimulated+arctigenin PBMCs than those stimulated alone. BCL6 contributes to negative regulation of various central molecules such as Th2 cytokines, CC chemokines. Available evidence has identified that BCL-6 is not only a repressor of IgE by repressing IL-4 function,^{27, 42, 43, 62} but also regulates IgE through generation of high-affinity memory B cells in germinal centres.⁴¹ Therefore, BCL6 may be a molecular target for IgE regulation in food allergy, for which there is limited report. SPARC is well-known for its counter-adhesive function, which causes cell rounding and apoptosis.⁶³ However, its role in IgE regulation or food allergy has yet to be explored.

In addition to evaluating arctigenin effects in PBMCs and purified murine B cells stimulated with α CD40/IL-4, we extended our studies to include investigation of effects on T cells and dendritic cells. Arctigenin markedly inhibited Th2 cytokine production from food allergy patient PBMCs and murine CD4⁺ T cells and suppressed proliferation. While cell cycle/DNA replication genes emerged as targets for arctigenin in B cells, we could not establish similar correlation for mouse CD4⁺ T cells (Data not shown). We believe more careful evaluation of additional T cell subsets and stimuli and inclusion of time points earlier than 72 h may be needed to clarify possible links. While 72-h post-stimulation time point is optimal for evaluation of cytokine production and proliferation, differential expression of cell cycle and DNA replication-related genes may be happening earlier. Further research is ongoing.

In conclusion, we, for the first time, uncover the unique action by which arctigenin, a lignin extracted from *Arctium Lappa*, potent inhibits IgE production in human myeloma B cell line, as well as in peanut-allergic mouse model and PMBCs from food-allergic patients, while simultaneously exhibiting a high safety profile. Arctigenin showed ability to regulate B and T cell responses. Transcriptome profiles revealed arctigenin down-regulates expression of genes in PBMCs of food-allergic patient associated with cell division such as *UBE2C* and up-regulates genes involved in transcriptional repressor of Th2 cytokines and immunoglobulins such as *BCL6*. This study suggests that arctigenin may have potential as novel intervention in treating prevalent IgE-mediated food and other allergies.

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Potential Conflicts of Interest

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Supporting Information



Filename	Description
cea14048-sup-0001-Supinfo.docx application/vnd.openxmlformats-officedocument.wordprocessingml.document: docx, 27.5 KB	Supplementary Material
cea14048-sup-0002-FigS1.pdf PDF document, 294.4 KB	Fig S1
cea14048-sup-0003-FigS2.pdf PDF document, 256.7 KB	Fig S2
cea14048-sup-0004-FigS3.pdf PDF document, 1.2 MB	Fig S3
cea14048-sup-0005-FigS4.pdf PDF document, 1.2 MB	Fig S4

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Supplemental information

Supplemental Methods

Dendritic Cell Culture and Flow Cytometry

To assess the effect of arctigenin on dendritic cell (DC) activation, purified PBMCs from food-allergic patients were cultured in 12-well plates at 2.5×10^6 cells/mL in GMP Dendritic Cell Medium (CellGenix, Portsmouth, NH) with 2 mM GlutaMax (Invitrogen/ThermoFisher Scientific, Waltham, MA). After 1 h, non-adherent cells were removed and frozen for T cell experiments (below). Adherent cells were cultured in DC medium with 1,000 U/mL IL-4 (R&D Systems, Minneapolis, MN) and 800 U/mL GM-CSF (sargramostim; Partner Therapeutics, Boston, MA) for 6 days, with feeding of additional IL-4 and GM-CSF at day 3. On day 6 of culture, DCs were matured and activated in the presence or absence of arctigenin (1 μ g/mL) in DC medium containing 30 ng/mL lipopolysaccharide (LPS; Millipore Sigma, St. Louis, MO), 1,000 U/mL IL-4, 800 U/mL GM-CSF, 10 ng/mL TNF- α (R&D Systems), 100 ng/mL IL-6 (CellGenix), and 10 ng/mL IL-1 β (R&D Systems). After 24 hours, cells were harvested and stained with a fixable viability dye (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, Invitrogen/ThermoFisher Scientific) and washed with phosphate-buffered saline (PBS). Cells were then stained with fluorescently labeled antibodies specific for HLA-DR (clone L243, BioLegend, San Diego, CA), CD45 (clone REA747, Miltenyi Biotec, Auburn, CA), CD80 (clone L307.4, BD Biosciences, San Jose, CA), CD86 (clone IT2.2, BioLegend), CD3 (clone SK7, BioLegend), CD19 (clone HIB19, BioLegend), CD56 (clone MEM-188, BioLegend), CD83 (clone REA714, Miltenyi Biotec), and CD14 (clone HCD14, BioLegend) in the presence of Human FcR Blocking Reagent (Miltenyi Biotec). Data were acquired on a Cytoflex S (Beckman Coulter, Indianapolis, IN) and analyzed using FlowJo software (FlowJo, Ashland, OR).

Apoptosis assay

U266 cells were seeded in 24-well plates at a concentration of 2.5×10^5 cells/well. Cells were cultured in the presence or absence of arctigenin at concentrations (0.1, 1, 5, and 10 μ g/mL) for 3 days. Then cells were harvested and stained with Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, China, Shanghai). Data were acquired on a CytoFLEX (Beckman Coulter, China, Shanghai) and analyzed using FlowJo software (FlowJo, China, Shanghai). U266 cells were seeded

in 96-well plates at a concentration of 2×10^4 cells/well. Cells were cultured in the presence or absence of arctigenin at concentrations (0.25, 1.25, 2.5, 5, and 10 $\mu\text{g/mL}$) for 3 days. Then cells were harvested (and counted by a cell counter (Countstar IC1000, China, Shanghai)). 10 μL CCK8 solution were added to each well. Cells were incubated for another 3 hours, and data was recorded at 450 nm with a microplate reader (SpectraMax i3, Molecule Device Inc., USA) (data not shown). Growth inhibition data were calculated by the following formula: Growth inhibition % = $(\text{Cell counts}_{\text{control}} - \text{Cell counts}_{\text{arctigenin}}) / \text{Cell counts}_{\text{control}} * 100\%$.

=Supplement Figure legends

Table S1. Primer sequences of identified genes.

Table S2. List of genes with the contrary direction between medium vs stimulated and stimulated vs stimulated + treatment.

Table S3. Acute and sub-chronic safety study. Mice (n=10 per group) were fed arctigenin at 15 times (1 day) or 8 times (14 days) of therapeutic doses. Morbidity and mortality were observed daily for 14 days. At the end, biochemistry and complete blood account were determined after mice were scarified. **BUN**, Blood urea nitrogen; **ALT**, Alanine aminotransferase; **AST**, Aspartate aminotransferase; **WBC**, white blood cells; **RBC**, red blood cells; **HGB**, Hemoglobin; **PLT**, platelet; **HCT**, Hematocrit; **MCV**, mean corpuscular volume; **MCHC**, mean corpuscular hemoglobin concentration; **MPV**, mean platelet volume.

Figure S1. Extraction and isolation protocol for the arctiin and arctigenin compounds.

Figure S2. Peanut-allergic mouse model protocol. C3H/HeJ mice were subjected to weekly intragastric peanut (PN) sensitization from week 0 through week 5 and boosted thereafter at weeks 6, 8 and 10. Peanut-allergic mice were treated with arctigenin (at a dose of 13.3 mg/kg), or water beginning at week 11 and continued daily for 4 weeks (till week 14). Mice were challenged at week 15 and then collected blood samples.

Figure S3. Effect of Arctigenin on apoptosis (A), growth inhibition (B) and cell viability (C) in human U266 cells. After culturing for 3 days in the presence or absence of arctigenin at different concentrations, cells were harvested and stained with Annexin V-FITC/PI and detected by flow cytometry. Cell counts and cell viability were acquired by a cell counter. Data shown are means + SEM. $n = 3$ or 4 . $*=p < 0.05$; $**=p < 0.01$; $***=p < 0.001$ vs $0 \mu\text{g/mL}$ arctigenin (red bars).

Figure S4. Dendritic cell (DC) maturation is not significantly affected by arctigenin. Adherent cells from PBMCs from food-allergic patients were cultured for 6 days in DC medium supplemented with IL-4 and GM-CSF. DCs were matured with LPS, IL-4, GM-CSF, TNF- α , IL-6, and IL-1 β in the presence or absence of $1 \mu\text{g/mL}$ arctigenin prior to staining. **A:** Gating strategy for DCs. Small cells and non-singlet events were first excluded. Live and CD45 $^{+}$ were selected before gating on HLA-DR $^{+}$ and CD3/CD19/CD56 $^{-}$ cells. Of the remaining cells, CD14 $^{-}$ cells were selected before examining staining for CD80 and CD86. Gates were set using Fluorescence Minus One (FMO) control. Shown are representative histograms for DCs matured in the presence (green) or absence (red) of arctigenin. **B:** Frequency of CD80 $^{+}$ and CD86 $^{+}$ DCs matured in the presence (green) or absence (red) of arctigenin. Data shown are means + SEM. $n = 5$.

Table S1. The primer sequences.

Genes	Primer sequence (5' to 3')	
	Forward	Reverse
UBE2C	5' -CATTGGCTGGATCAAACCCA-3'	5'-GGAGAACACGACTGCAACTG-3'
CDC20	5'- GACCACTCCTAGCAAACCTGG-3'	5'-GGGCGTCTGGCTGTTTTCA-3'
CDC45	5'-CTGCTCGTAGTCAAAGAGGATGT-3'	5'-AGAGGAGATAGTGGAGCAAACC-3'
KIF2C	5'-GTGTCTGTGTTAGGAAACGCCC-3'	5'-CACTTGCTAGGAATGGAAATCACAT-3'
BIRC5	5'-AGGAC CACCG CATCT CTACAT-3'	5'-AAGTC TGGCT CGTTCTCAGTG-3'
MKI67	5'-GCCCCAACCAAAAGAAAGTCT-3'	5'-AGCTTTGTGCCTTCACTTCCA-3'
CCNB1	5'-GACCTGTGTCAGGCTTTCTCTG-3'	5'-GGTATTTTGGTCTGACTGCTTGC-3'
E2F1	5'-GCTGGACCACCTGATGAATATCT-3'	5'-GGAGGGGCTTTGATCACCATAA-3'
FEN1	5'-GGG UCAAGAGGCUGAGUAATT-3'	5'-TTCCCAGUUCUCCGACUCAUU-3'
BCL6	5'-CTGGCTTTTGTGACGGAAAT-3'	5'-CTGGCTTTTGTGACGGAAAT-3'
SPARC	5'-GCAGCAATGACAACAAGACCT-3'	5'-ATTCGGTCAGCTCAGAGTCCA-3'
CCL19	5'GTGACCTGCATTA ACTCTTTACTTGC-3'	5'-TATGGCTCTGGCTCTACTGGTTG-3'
GAPDH	5'-GAGGCAGGGATGATGTTCTG-3'	5'-CAGCCTCAAGATCATCAGCA-3'

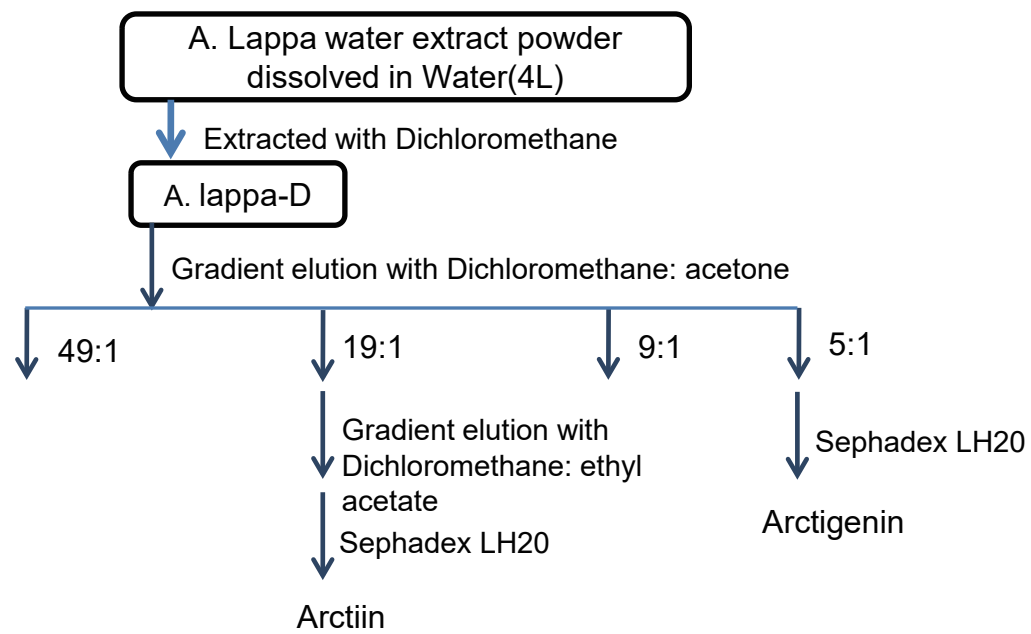
Table S2. The genes ($p < 0.05$) with the contrary direction between medium vs (rIL4 + anti CD40) stimulated and stimulated vs stimulated + arctigenin treatment.

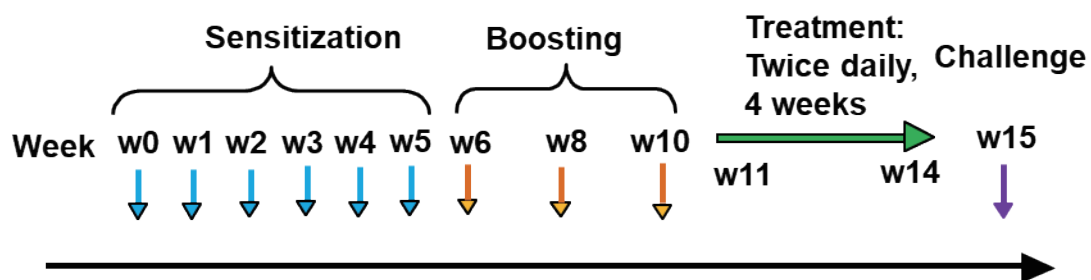
Items		Genes
Groups	Direction	
Medium vs Stimulated	Up	UBE2C , CCNB2, CCNB1, CDK1, CCNA2, CDKN3, BUB1, TPX2, RRM2, PTTG1, PCNA, NCAPG, CDC20 , CDCA5, CDCA8, CDC25A, NEK2, MKI67 , KIF2C , KIF20A, KIF11, FEN1, MCM2, MCM4, MCM10, RFC2, RFC3, PCNA, HDAC1, BIRC5 , ZWINT, RAB3C, GDF10, CCL19 , GTSE1, TNFRSF11A, TYMS, TLR5
Stimulated vs Stimulated +Treatment	Down	
Medium vs Stimulated	Down	SPARC , BCL6 , HDAC9, C5AR1, ITGAX, OLR1, FPR1, SLC7A11, GATA2, CUBN, JMJD7-PLA2G4B, LY86, CCR3, MS4A2, CD9, SERPINA1, MARCO, ABCA7, CXCL16, TGM2, MERTK, CHKB-CPT1B, CLN8
Stimulated vs Stimulated + Treatment	Up	

Table S3. Acute and sub-chronic safety study.

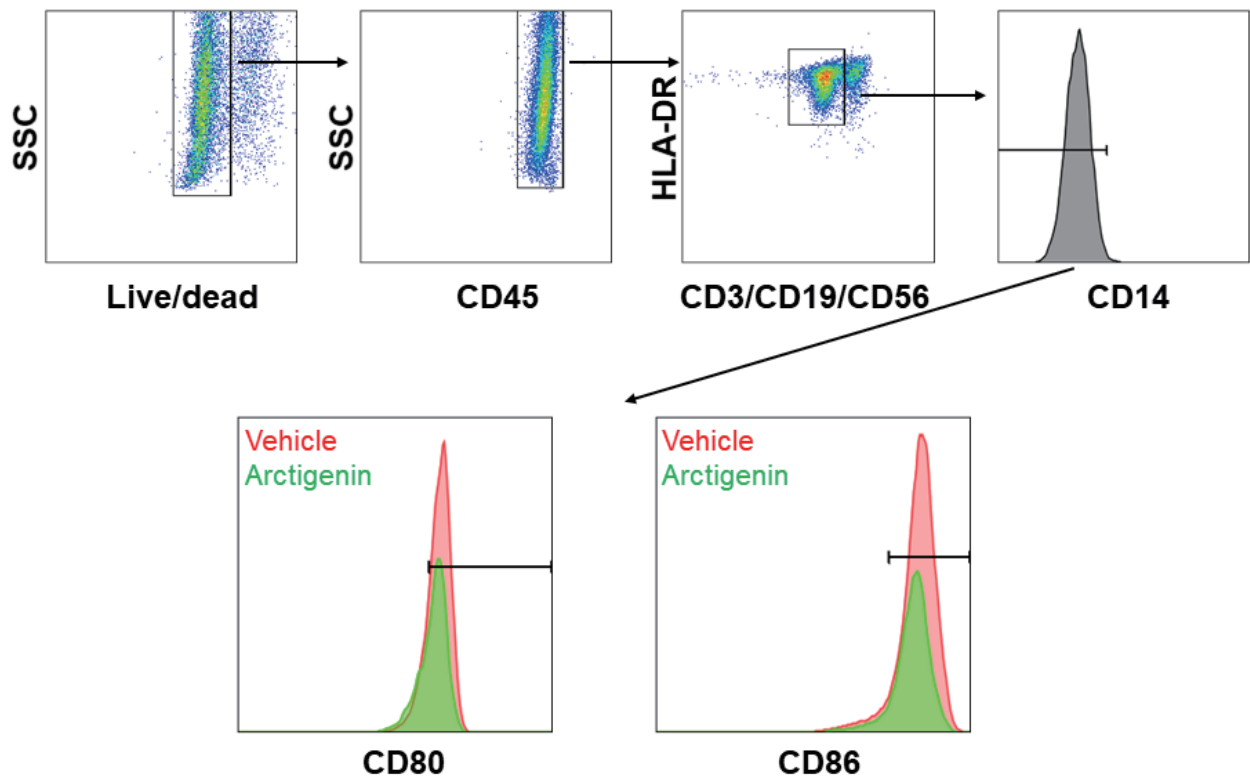
Items	Water	Arctigenin (Chronic)	Arctigenin (Acute)	Reference
Dose	5×	5×	10×	-
Time	14 days	14 days	1 day	-
Death	0/6	0/6	0/6	-
Morbidity Percent	0	0	0	-
BUN (mmol/L)	8.37 ± 1.32	8.52 ± 0.77	8.04 ± 1.31	8.45 ± 1.73
AST (u/L)	133.17 ± 10.23	138.67 ± 24.22	134.83 ± 28.64	147.40 ± 66.79
ALT (mmol/L)	45.83 ± 18.45	52.83 ± 14.32	54.50 ± 17.61	45.73 ± 37.32
WBC (x10 ⁹ /L)	8.23 ± 1.24	7.45 ± 0.81	8.61 ± 0.88	9.91 ± 3.29
RBC (x10 ⁹ /L)	9.49 ± 1.11	8.93 ± 0.99	9.17 ± 0.77	9.49 ± 2.79
HGB (g/L)	145.5 ± 19.13	129.3 ± 15.93	139.50 ± 10.01	136.50 ± 28.45
PLT (x10 ⁹ /L)	665.7 ± 183.52	589.7 ± 135.91	655.50±113.29	644.93 ± 197.15
HCT (%)	44.55 ± 5.62	37.80 ± 4.95	42.70 ± 3.43	41.35 ± 10.84
MCV (fL)	46.87 ± 0.83	41.49 ± 3.42	46.60 ± 1.73	44.93 ± 6.13
MCH (PG)	15.30 ± 0.45	15.27 ± 0.46	15.25 ± 0.55	14.91 ± 1.31
MCHC (g/L)	326.32 ± 5.01	300.17 ± 6.47	326.81 ± 4.71	321.20 ± 9.74

Note: BUN, Blood urea nitrogen; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; WBC, white blood cells; RBC, red blood cells; HGB, Hemoglobin; PLT, platelet; HCT, Hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MPV, Mean platelet volume.





A



B

