



Berberine as a chemical and pharmacokinetic marker of the butanol-extracted Food Allergy Herbal Formula-2

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ABSTRACT

Rationale: Food Allergy Herbal Formula 2 (FAHF-2) provided protection against peanut anaphylaxis in a murine model and induced beneficial immune-modulation in humans. Butanol-refined FAHF-2, B-FAHF-2, retained safety and efficacy in the peanut allergic murine model at only 1/5 of FAHF-2 dosage. One compound, berberine, was isolated and identified *in vitro* as a bioactive component present in FAHF-2 and B-FAHF-2. The aim of this study was to investigate berberine as a chemical and pharmacokinetic marker of B-FAHF-2.

Methods: The consistency of constituents between B-FAHF-2 and FAHF-2 was tested. Peanut allergic C3H/HeJ mice were orally administered with 1 mg of berberine or B-FAHF-2 containing an equivalent amount of berberine, and the ability to protect against peanut anaphylaxis and pharmacokinetic parameters were determined. Human intestinal epithelial cells (Caco-2) were cultured with berberine with or without the nine individual herbal constituents in B-FAHF-2, and the absorbed berberine levels were determined.

Results: Berberine is one of the major components in B-FAHF-2 and FAHF-2 formula. In a peanut allergic mouse model, B-FAHF-2, but not berberine, protected mice from anaphylaxis reactions. Pharmacokinetic profiles showed that the C_{max} of B-FAHF-2 fed mice was 289.30 ± 185.40 ng/mL; whereas berberine alone showed very low bioavailability with C_{max} value of 35.13 ± 47.90 ng/mL. Caco-2 cells influx assay showed that 7 of 9 herbal constituents in B-FAHF-2 increased berberine absorption at rates ranging from 18 to 205%.

Conclusions: B-FAHF-2 remarkably increased the bioavailability of berberine. Berberine can be used as chemical and pharmacokinetic marker of B-FAHF-2. Other herbal components in B-FAHF-2 may facilitate the absorption of berberine.

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1. Introduction

Food allergy has been a growing and serious public health concern in the US [1]. No cure for food allergies has been developed, and no effective prophylaxis exists other than specific food avoidance. Parenteral administration of epinephrine is the only approved rescue therapy for an anaphylactic event [2]. The combination of avoidance and emergency treatment substantially impairs the quality of life in food allergic patients and their families. Consequently, research into developing food allergy treatment is a priority. More researchers have begun to investigate traditional Chinese medicine and use it as a source of alternative or complementary therapy for food allergies because of its demonstrated safety, low cost, and reputed effectiveness [3,4].

An herbal formula consisting of nine individual herbal extracts, called Food Allergy Herbal Formula 2 (FAHF-2), completely blocked peanut-induced anaphylaxis in a peanut allergic mouse model [3]. Also, FAHF-2 treatment produced at least 6 months protection against peanut specific anaphylaxis after discontinuing the therapy [5]. In an acute phase I study, FAHF-2 showed an immunomodulatory effect following 1 week treatment [6]. However, the large amount of FAHF-2 tablets required for daily usage (30 tablets per day) made the medication adherence very difficult. In a double-blind, 6 months, phase II clinical trial of FAHF-2, 44% of the subjects had <80% adherence for 2 months during the treatment period, and 30% did not adhere in the last 2 months of treatment [7]. This poor adherence rate severely affected the evaluation of the clinical trial. In order to reduce the dosage burden for patients, a butanol-refined FAHF-2 product, B-FAHF-2, was generated. It showed the same effective therapeutic protection against peanut anaphylaxis as FAHF-2 treatment at only 1/5 of the FAHF-2 dosage [8]. *In vitro* experiments showed that B-FAHF-2 was 9 times more potent than FAHF-2 on the inhibition of IgE production [9]. Toxicity experiments in mice showed that refined B-FAHF-2 formula was also as safe

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as FAHF-2; no mortality or morbidity were observed after the mice were fed 12 times the effective dose [8].

Berberine is a natural isoquinoline alkaloid common in the rhizome, roots, and stem bark of a number of Chinese herbs of the *Berberis* genus [10]. It is frequently used for its antibacterial, antioxidant, anticancer, cholesterol-lowering, and anti-inflammatory activities [11–13]. Two herbal constituents, *Phellodendron chinense* (*P. chinense*) and *Coptis chinensis* (*C. chinensis*), in both FAHF-2 and B-FAHF-2 formula contain berberine. Previously, we have successfully isolated and identified berberine from *P. chinense*. Bioactive experimental results showed that berberine significantly inhibited mast cell activation via suppression of Syk signaling pathway [14]. It also potently inhibited IgE production by a human B cell line and peripheral blood mononuclear cells (PBMCs) from pediatric food allergic subjects in a non-toxic manner [9]. Berberine might have potential to be used as therapy for IgE-mediated food allergy. However, the *in vivo* effect of berberine alone on IgE mediated food allergy had not been tested in our peanut food allergy murine model and pharmacokinetics of berberine in B-FAHF-2 had not been investigated.

The aims of this present study were to investigate the percentage of berberine present in B-FAHF-2 formula, compare the effect of berberine alone to that of B-FAHF-2 in an animal model, and investigate the comparative pharmacokinetics of berberine to berberine in the B-FAHF-2 formula. In addition, herbal constituents present in FAHF-2/B-FAHF-2 formulas that may affect berberine bioavailability were determined using human intestinal epithelial cells.

2. Materials and methods

2.1. Formula preparation

The berberine standard (purity > 98%) was purchased from Sigma Aldrich (St Louis, MO). FAHF-2 and B-FAHF-2 products were in the form of tablets produced by Xiyuan Chinese Medicine Research and Pharmaceutical Manufacturer, Chinese Academy of Traditional Chinese Medicine Sciences (Beijing, China), and obtained from Beijing Sheng Hua Times Medical Technology Development Ltd. Co., Beijing China, and stored in a dry place at room temperature. The herbal constituents have been described in details previously [6].

2.2. High-performance liquid chromatography (HPLC) and mass spectrometry (MS)

Liquid chromatography was performed using a Waters 2690 HPLC system coupled to a 2996 PDA detector (Waters, Milford, MA). Chromatographic separation was carried out on a ZORBAX SB-C18 (4.6 × 150 mm, 5 μm) column (Agilent, Santa Clara, CA). The mobile phases consisted of 0.1% formic acid and acetonitrile. The flow rate was set as 1 mL/min. The separation gradient program was: 2% to 25% mobile phase B in 45 min, 25% to 35% B in the following 25 min, 35% to 55% in the next 15 min, 55% to 75% in another 10 min, at 75% B for 5 min. 10 μL of FAHF-2 and B-FAHF-2 samples, which were dissolved in the mixture of mobile phases at 1:1 ratio, were injected for analysis. Data was collected and processed with Waters' Empower software.

LC-MS operation and analysis of data were performed using a Waters 2695 HPLC system coupled with Water's LCT premier micromass mass spectrometer. A 13 min linear elution program was used as follows: from 5% to 90% mobile phase B in 9 min, 90% to 90% mobile phase B for 3 min, 90% to 5% in 1 min. The flow rate was set as 1 mL/min. The mass spectrometer parameters were: Capillary Voltage: 3000 V; Cone Voltage: 15 V; Aperture I: 25 V; Desolvation Temperature: 400 °C; Source Temperature: 120 °C; Desolvation gas: 500 L/h; Nebulizing gas: 40 L/h; Electrospray ionization, Acquisition Range: *m/z* 50–1000. Data was analyzed by MassLynx software.

2.3. Quantitative analysis of berberine in B-FAHF-2

B-FAHF-2 powder was dissolved into acetonitrile to make the final concentration to be 1 mg/mL. B-FAHF-2 (1 mg/mL) mixed with berberine standard (final concentration, 100 μg/mL) in acetonitrile was also prepared. 10 μL of each sample was injected into the LC-MS system and analyzed for their peak intensities. Each sample was analyzed four times.

2.4. Animals

Female C3H/HeJ mice were purchased from Jackson laboratory (Bar Harbor, ME) and maintained in the animal facility at Icahn School of Medicine at Mount Sinai. Standard guidelines for the care and use of animals were followed [15]. All mice animal experiments were approved and performed according the instruction and guidelines of the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at Mount Sinai.

2.5. Peanut sensitization/challenge, berberine and B-FAHF-2 formula treatment of the murine model

To generate the peanut allergy model, female C3H/HeJ mice were sensitized weekly intragastrically with 10 mg ground peanut with 20 μg of cholera toxin (CT) for 5 weeks (Fig. 1). After the last sensitization, 50 mg of ground peanut with 20 μg CT per mouse was used to boost the peanut specific hypersensitivity [14]. Mice were randomly split into 3 groups. Berberine at 1 mg/mouse/day and B-FAHF-2 at 12 mg/mouse/day (formula containing equivalent 1 mg of berberine) were administered 24 h after the week 6 boost. The treatment period was 6 weeks. The daily dose of B-FAHF-2 was based on our previously published publication [8]. The daily dose of berberine is based on the percentage of berberine present in B-FAHF-2 formula. The Sham group was orally fed with water. Non-sensitized mice served as naïve controls. After the 6 weeks of treatment, mice were challenged with peanut (200 mg/mouse) in week 12.

Anaphylactic symptoms were evaluated 30 min after the oral challenge by following our scoring system described previously [3]. Rectal temperatures were also measured immediately after scoring using a rectal probe (Harvard Apparatus, Holliston, MA). Blood samples were collected and the peanut specific IgE level in serum was measured using ELISA [16].

2.6. Pharmacokinetics study

2.6.1. Administration

C3H/HeJ mice were randomly separated into two groups. Each group contained 10 mice. Mice were fasted for 12 h before the oral administration of berberine or B-FAHF-2. Blood samples (100 μL) were collected from the facial vein at 0.25, 0.5, 1, 2, 4, 6, 16, 24, and 48 h after the administration. Samples were saved in untreated test tubes and left in standing position for 30 min to coagulate. After the clotting, samples were centrifuged at 5000 rpm for 15 min at room temperature. The clear supernatant serum samples were collected and stored at −80 °C for further usage.

2.6.2. Prepare of standard solution

The standard stock solution was prepared by dissolved 2 mg of berberine in 20 mL of acetonitrile to yield a concentration of 1 mg/mL. Clozapine was used as the internal standard (IS). Stocks of clozapine were prepared by dissolving pure clozapine in acetonitrile to make the final concentration of 5 mg/mL. All stock solutions were kept at 4 °C before use. The samples for standard calibration curves were prepared by spiking the blank mouse serum (50 μL) with the appropriate IS and berberine standard solutions to yield the following concentrations: 15, 31, 62, 125, 250, 500, and 1000 ng/mL. The IS concentration was 5 μg/mL.

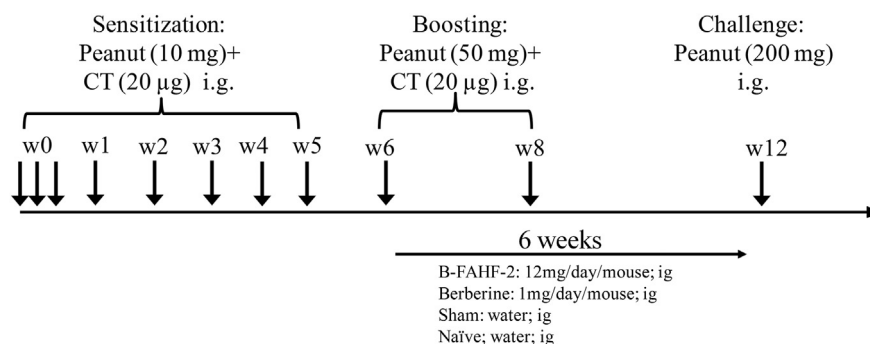


Fig. 1. Peanut allergy model and the experimental protocol. C3H/HeJ mice were orally sensitized with 10 mg of peanut with cholera toxin (CT) weekly for 5 weeks, and boosted at weeks 6 and 8 with 50 mg of peanut with CT at 20 µg. Daily oral treatments of B-FAHF-2 ($n = 5$) or berberine ($n = 5$) were started at week 6, 24 h after the boosting. Sham group ($n = 7$) of mice received water. Total treatment period was 6 weeks. Mice were then challenged with 200 mg of peanut at week 12.

2.6.3. Blood sample preparation

Serum samples and calibration standards were extracted using a liquid-liquid extraction technique. Thawed serum samples (50 µL) were added with 300 µL of acetonitrile containing IS. The mixtures were vigorously mixed by vortexing for 1 min at the highest speed. The mixtures were then left on ice for 15 min. After protein precipitation, the mixtures were centrifuged at 10,000 rpm for 15 min. The supernatants were transferred to a new tube and dried under vacuum. The samples were then reconstituted in 50 µL of acetonitrile and analyzed using a Waters LC-MS system (Waters corp. MA)

2.6.4. Recovery

Berberine and clozapine at 250 ng/mL were prepared using mouse blank serum samples. After protein precipitation and processing, the concentrations of recovered berberine and clozapine were determined.

2.6.5. Accuracy

Three concentrations (0.25, 1, and 5 µg/mL) of berberine standard solutions were prepared using mouse blank serum samples and measured five times on the same day for intraday and on different days for the interday accuracy tests.

2.6.6. Pharmacokinetic analysis

The concentrations of berberine in the serum of berberine treated and B-FAHF-2 treated mice were evaluated using the equation from the standard curves. Pharmacokinetic analysis was performed using the PK-solver program [17].

2.7. Caco-2 cell uptake assay

Caco-2 human epithelial (ATCC, Rockville, MD) monolayer cell assay has been used as an intestinal *in vitro* model to evaluate chemicals absorption levels [18]. Caco-2 cells were seeded at 1×10^5 cells/well in 24 well plates and maintained in high glucose DMEM medium with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), sodium pyruvate, and 1% non-essential amino acids, for 2 weeks to grow to confluence and differentiate. On the experiment day, cells were washed with pre-warmed Hank's balanced salt solution (HBSS) (Sigma-Aldrich, St. Louis, MO) and incubated with 1 mL of pre-warmed HBSS for 30 min. After conditioning with HBSS, cells were incubated at 37 °C with 50 µg/mL berberine in the presence or absence of 250 µg/mL of each of the 9 individual herbal constituents in B-FAHF-2 formula. The control wells received 0, 50, 100 and 200 µg of berberine alone. After 1 h of incubation, cells were washed 3 times with ice-cold HBSS. Cells were then scraped in 500 µL of ice-cold HBSS and centrifuged. Collected cells were re-suspended in 100 µL of distilled water and stored at -80 °C for further processing. Cells were lysed by 3 cycles of freeze/thaw. Protein concentrations were analyzed by Bradford assay.

75 µL of the lysed solution was mixed with 500 µL of acetonitrile for protein precipitation. The mixture was then vortexed strongly for 3 min with 5 min interval. The mixture was then centrifuged at 10000 rpm for 15 min. Supernatants were dried under vacuum and re-suspended in 50 µL of acetonitrile. The berberine level in each sample was analyzed using LC-MS.

2.8. Statistical analysis

Statistical analyses were performed using one-way ANOVA, pairwise and multiple-comparison testing between groups, as well as the Mann-Whitney rank sum test. A P -value ≤ 0.05 was considered to be statistically significant. All statistical analyses were performed using Sigma Stat software (Systat Software Inc., Point Richmond, CA, USA).

3. Results

3.1. Percentage of berberine present in FAHF-2 and B-FAHF-2

10 µL of 98.07 mg/mL of FAHF-2 and 10 µL of 21.18 mg of B-FAHF-2 were analyzed using HPLC. A total of 31 peaks were detected (Fig. 2A). The HPLC of B-FAHF-2 showed the similar fingerprint pattern as that of the FAHF-2 formula. The peaks of several polar constituents present in FAHF-2 were not detected in B-FAHF-2. By comparing with the berberine standard, it was found that the peak 13 at retention time of 43 min was berberine. This result confirmed that B-FAHF-2 contained berberine. The relative peak intensities of the major peaks presented in both formulas were also measured. The peak area ratio of detected peaks were analyzed and listed in Table 1. Most of the less polar components showed a higher peak area ratio in B-FAHF-2 than in FAHF-2. The peak area ratio of berberine in B-FAHF-2 was $42.71 \pm 6.54\%$ vs. $35.79 \pm 3.14\%$ in FAHF-2 formula. Overall, the B-FAHF-2 formula was a refined and concentrated FAHF-2 formula. To further investigate the quantity of berberine present in B-FAHF-2 formula, 1 mg/mL of B-FAHF-2 and the same concentration of B-FAHF-2 spiked with 100 µg/mL of berberine were analyzed using LC-MS. The berberine levels were detected at M^+ of 336 (Fig. 2B). Quantitative analysis of berberine in B-FAHF-2 was performed by calculating the ratio of peak area and the amount of standard berberine injected using the following equation:

$$\text{Berberine \%} = 1 / \{10 * [\text{Peak intensity of (B-FAHF-2 + Ber)} / \text{Peak intensity of B-FAHF-2} - 1]\}.$$

The berberine level in B-FAHF-2 formula was calculated as $9.1 \pm 1.8\%$.

3.2. B-FAHF-2 protected PN allergic mice against anaphylaxis after oral challenge while berberine failed

We next compared the ability of B-FAHF-2 and berberine alone to suppress peanut allergy in a murine model. The results showed that

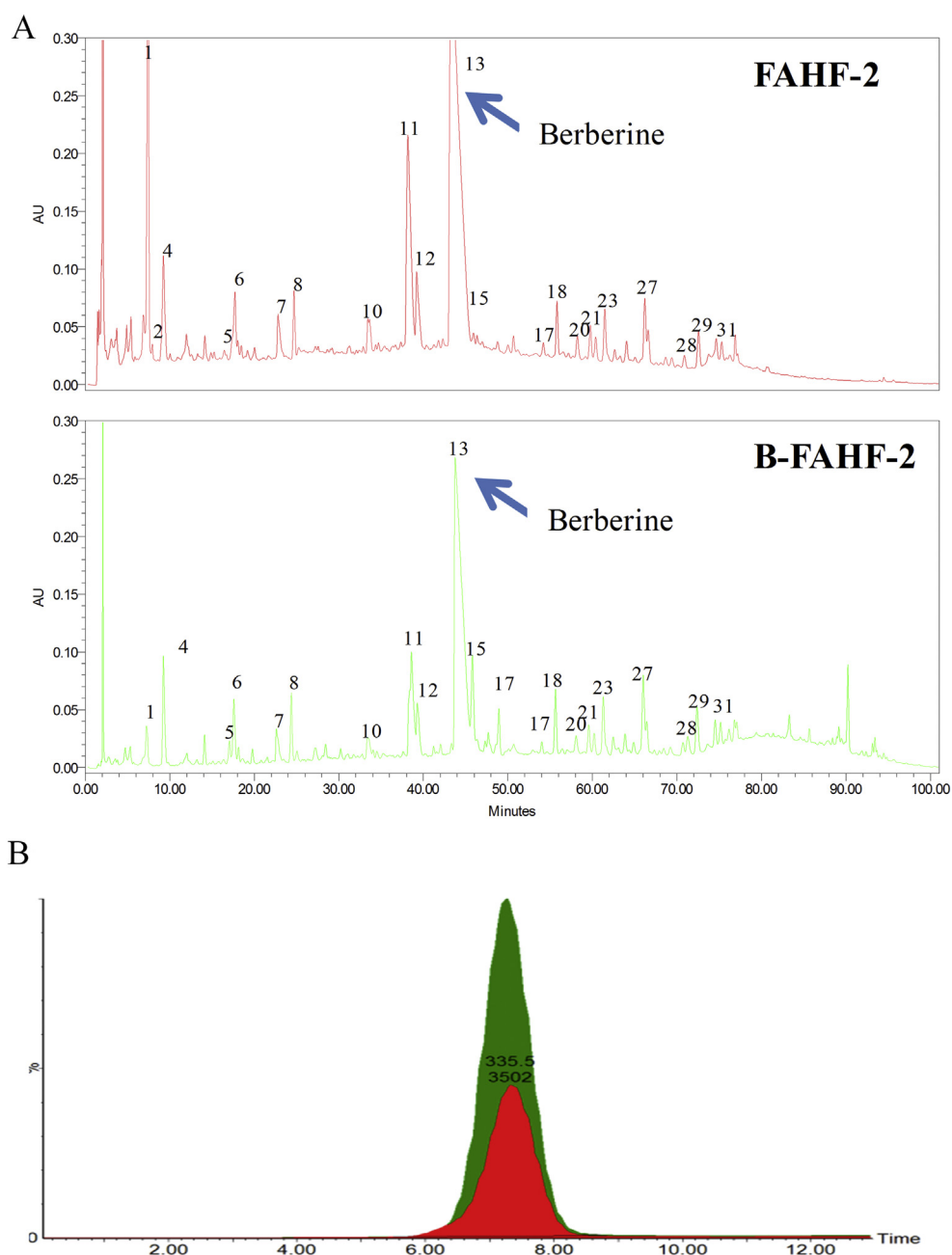


Fig. 2. The presence of berberine in FAHF-2/B-FAHF-2 formula. A. HPLC fingerprint of B-FAHF-2 and FAHF-2 formulas. The peak of berberine was labeled. B. Chromatogram of the peak intensities of berberine presented in B-FAHF-2 (1 mg/mL; Red color) and in B-FAHF-2 (1 mg/mL) mixed with 100 µg/mL of berberine (Green color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

all sham-treated mice developed symptoms of anaphylaxis (Fig. 3A) and significant drops in core body temperatures (hypothermia, $P < 0.01$, Fig. 3B) following challenge. In contrast, 6 weeks of B-FAHF-2 treatment largely protected the mice from anaphylaxis and prevented hypothermia following the final challenge (Fig. 3A and B, $P < 0.001$ vs. Sham). Surprisingly, the berberine-alone-fed mice showed severe anaphylaxis symptoms, and the median symptom scores were essentially the same as sham-treated mice ($P > 0.01$, Fig. 3A). The body temperatures of berberine-treated mice dropped significantly following challenge ($P < 0.05$, Fig. 3B). Fig. 3C illustrates severely swollen lips in berberine treated mice whereas B-FAHF-2 treated mice showed normal appearance of lips. Three independent PN-specific IgE ELISA results from pooled samples of each group ($n = 5-7$) were expressed as mean $\pm$ SD. The PN specific serum IgE levels in B-FAHF-2 treated mice were significantly reduced compared to the Sham group ($P < 0.01$,

Fig. 3D) and berberine treated group ($P < 0.001$, Fig. 3D). No significant difference was found between the berberine-treated mice and Sham group.

3.3. Pharmacological study of berberine in the murine model

3.3.1. Method validation

3.3.1.1. Selectivity. The retention times of berberine and the internal standard, clozapine, were 7.2 min and 5.4 min respectively. Mass spectra showing molecular masses of 336 (berberine) and 327 (clozapine) with their structures are shown in Fig. 4A. The selectivity assay was evaluated by analyzing blank mouse serum. Results showed that the blank mouse serum had no interference at the retention time and the detection windows using molecular masses of berberine or clozapine.

Table 1

The retention time and peak area ratio of detected peaks in FAHF-2 and B-FAHF-2 formulas. Yellow color: peaks in FAHF-2 with higher area ratio than that of in B-FAHF-2; green color: peaks in FAHF-2 with lower area ratio than that of in B-FAHF-2. N/D: not detected.

	FAHF-2		B-FAHF-2	
Name	Retention Time	% Area	Retention Time	% Area
Peak 1	7.427	7.64	7.247	1.96
Peak 2	7.935	0.86	8.161	N/D
Peak 3	9.251	3.35	9.251	3.8
Peak 4	10.038	0.74	9.641	N/D
Peak 5	17.71	1.97	17.58	2.56
Peak 6	18.093	N/D	18.093	N/D
Peak 7	22.802	1.22	22.61	1.73
Peak 8	24.698	1.05	24.381	2.18
Peak 9	33.423	0.44	33.394	1.37
Peak 10	33.623	0.67	34.467	N/D
Peak 11	38.164	8.61	38.407	2.13
Peak 12	39.198	2.47	39.277	2.77
Peak 13	43.241	34.5	43.762	39.21
Peak 15	45.921	0.48	45.801	4.52
Peak 16	46.354	0.43	47.67	0.89
Peak 18	55.805	1.02	55.628	2.31
Peak 20	58.216	0.51	58.062	0.95
Peak 21	59.742	0.7	60.199	0.86
Peak 22	60.379	0.49	61.281	2.6
Peak 23	61.463	1.14	62.457	0.84
Peak 25	66.186	1.32	63.856	0.9
Peak 27	66.59	0.67	66.416	1.13
Peak 29	72.546	0.8	72.371	2.06
Peak 30	74.637	0.9	74.514	1.09
Peak 31	75.285	0.56	75.154	0.97

3.3.1.2. Calibration curve. The calibration curve was prepared by plotting the peak area ratios of berberine to clozapine versus the nominal concentrations of berberine. The calibration curve showed good linearity

over the range 30 ng/mL–1000 ng/mL. The equation was calculated as: $y = 1.5105x + 0.0189$, $R^2 = 0.9907$.

3.3.1.3. Recovery. The extraction recovery was estimated by comparing berberine and clozapine at 250 ng/mL. The extraction recovery was calculated by comparing the peak intensity of berberine and clozapine extracted from serum samples with these two compounds directly injected to LC-MS. The extraction recovery of berberine was determined as 83.04%.

3.3.1.4. Accuracy. The accuracy of the assay was determined by analyzing both berberine and clozapine at three different concentrations at 0.25, 1, and 5 μ g/mL ($n = 4$). The intraday and interday accuracy results are summarized in Table 2. Results of both compounds showed that the accuracy of this assay were acceptable.

3.3.2. Pharmacokinetic study

After the oral administration of B-FAHF-2 and berberine, serum samples at different time points were collected. The serum berberine concentration-time profiles of the berberine group and B-FAHF-2 group are shown in Fig. 4B. The main pharmacokinetic parameters are shown in Table 3. After orally feeding with B-FAHF-2, the berberine was absorbed and reached to highest concentration (C_{max}) at 30 min (T_{max}) with the value of 289.30 ± 185.40 ng/mL. After oral administration of berberine compound, the C_{max} of berberine was calculated as 35.13 ± 47.90 ng/mL with the T_{max} at 60 min. The results showed that berberine has a very low bioavailability properties as other publication described [19,20]. The C_{max} of berberine in B-FAHF-2 treated mice was >8 times higher than that of berberine alone treated mice. It was also found that two peaks presented in the berberine-serum-concentration-time curves after the administration. The T_{max} of berberine in B-FAHF-2 treated mice was at 30 min after the oral administration. The

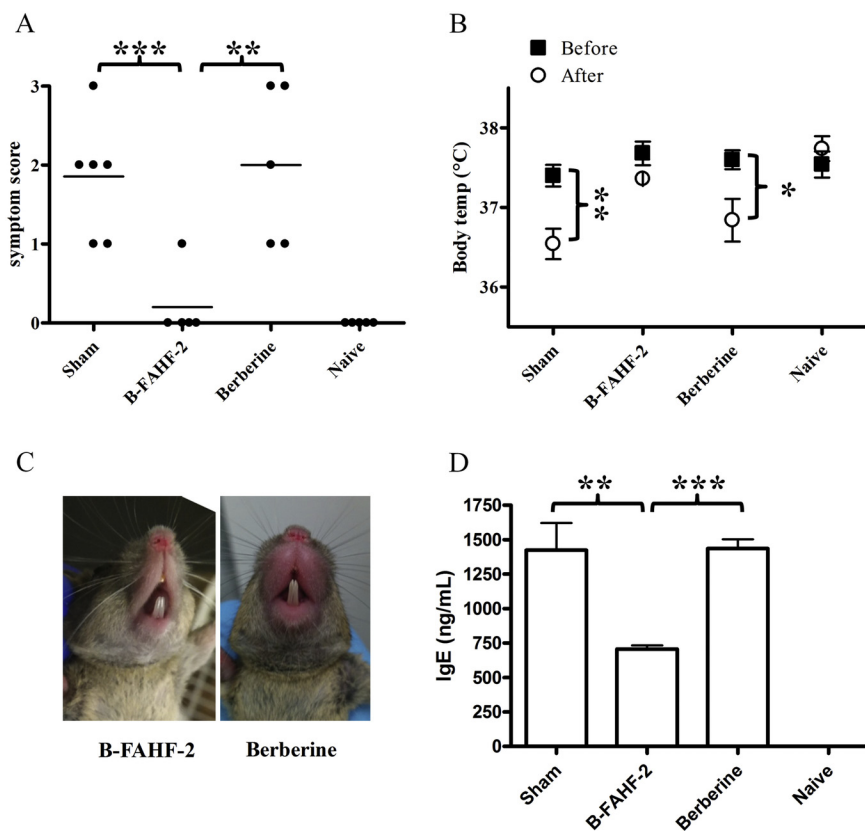


Fig. 3. Effect of berberine and B-FAHF-2 on the peanut specific anaphylaxis reaction. A. Anaphylactic symptom score; B. Post-challenge body temperatures; C. Photograph showing oral region of treated mice; The swollen mouth observed was assigned as a score of 2. D. Peanut specific IgE level measured by ELISA. Three independent ELISA results from pooled samples of each group ($n = 5-7$) were expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

berberine level in the blood was decreased by 16 h after the oral feeding. But, interestingly, it started increasing slowly and was still detectable even 24 h after the drug treatment. After 48 h, berberine was not detectable. The elimination half life $T_{1/2}$ of B-FAHF-2 was calculated as 10.48 h. Two small peaks were also observed in the berberine alone fed mice. Berberine level peaked at 1 h, then decreased to 0 at 4 h and increased again at lower level at 16 h. After 24 h, berberine was not detectable. The $T_{1/2}$ of berberine was 11.09 h.

3.4. Berberine uptake by Caco-2 cells in the presence and absence of 9 B-FAHF2 herbs

The uptake of berberine by Caco-2 cells was measured using LC-MS and adjusted for protein concentration in collected cells. The berberine-absorption curve was generated by analyzing the absorption of berberine alone by Caco-2 cells at different concentrations (50, 100, and 200 $\mu\text{g/mL}$). The polynomial equation of the berberine-absorption curve is $y = 0.0003x^2 + 0.0596x$ with R^2 as 0.9996 (Fig. 5A). When incubated with 50 $\mu\text{g/mL}$ of berberine alone, the final concentration of berberine absorbed by Caco-2 cells was calculated as 4.06 $\mu\text{g/mL}$. The absorption percentage is $9.75 \pm 2.21\%$. When co-incubated with other constituents (at 250 $\mu\text{g/mL}$) in B-FAHF-2, *Angelica sinensis* (*A. sinensis*), *Fructus prunimune* (*F. prunimune*), *Zanthoxylum bungeanum* (*Z. bungeanum*), and *Panax ginseng* (*P. ginseng*), the final detected berberine levels were detected at 12.39 $\mu\text{g/mL}$, 9.45 $\mu\text{g/mL}$, 9.20 $\mu\text{g/mL}$, and 7.91 $\mu\text{g/mL}$ respectively. These data show that berberine-uptake levels were markedly increased. The percentages of the enhancement of berberine absorption by *A. sinensis*, *F. prunimune*, *Z. bungeanum*, and *P. ginseng* were 205.40%, 132.78%, 126.80%, and 94.84% respectively.

The berberine levels in *Cinnamomum cassia* (*C. cassia*), *Zingiber officinale* (*Z. officinale*) and *Ganoderma lucidum* (*G. lucidum*) cultured cells were detected as 6.76 $\mu\text{g/mL}$, 5.20 $\mu\text{g/mL}$ and 4.77 $\mu\text{g/mL}$. These three herbal constituents enhanced the berberine absorption by 66.66%, 26.95% and 17.57%. Both *P. chinense* and *C. chinensis* contain berberine. The absorption of berberine by Caco-2 cells in the presence of these two herbal medicines alone showed berberine levels as 0.99 $\mu\text{g/mL}$ and 7.45 $\mu\text{g/mL}$. When cultured with berberine (50 $\mu\text{g/mL}$), the berberine levels in *P. chinense* and *C. chinensis* cultured cells were 5.29 $\mu\text{g/mL}$ and 11.31 $\mu\text{g/mL}$. The increased absorption in the presence of *P. chinense* and *C. chinensis* were 6.13% and –4.86% respectively.

4. Discussion

In this study, we demonstrated that, as seen in FAHF-2, berberine can be used as the chemical marker of B-FAHF-2 formula. Butanol extraction process removed most of the polar constituents in the FAHF-2 formula and kept the less polar and non-polar parts. Our HPLC data also showed that the B-FAHF-2 showed a similar fingerprint pattern and major peak intensities as those of FAHF-2 even at only 1/5 of the FAHF-2 amount. This indicated that the B-FAHF-2 formula is a concentrated FAHF-2 formula. Among the nine constituents of B-FAHF-2 formula, only *P. chinense* and *C. chinensis* contain the berberine compound. Our data showed that berberine is $9.1 \pm 1.8\%$ of the whole B-FAHF-2. This result showed that berberine is one of the major components present in B-FAHF-2 formula. By comparing the peak area ratio values, B-FAHF-2 contained a higher percentage of berberine than FAHF-2. These data support the previous finding that B-FAHF-2 is more potent than FAHF-2 in suppression of IgE production *in vitro*, [9]

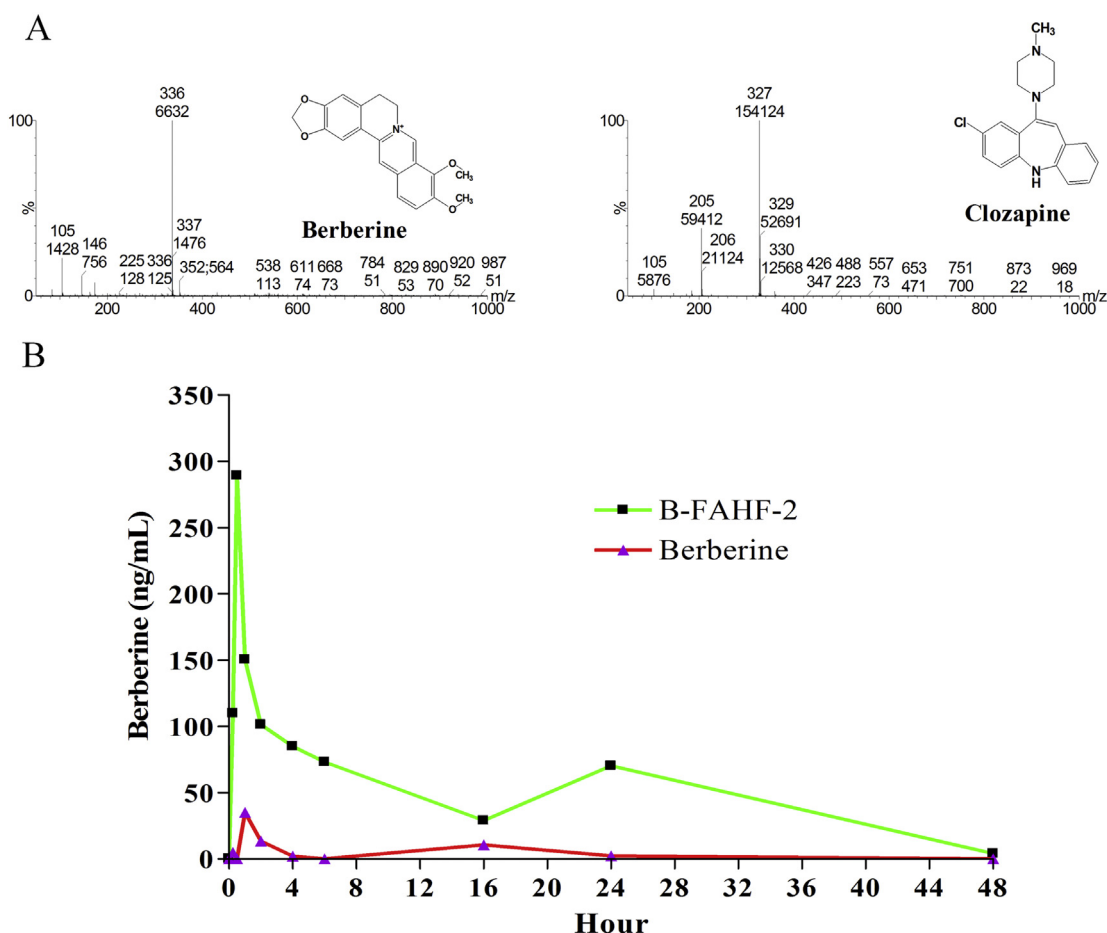


Fig. 4. Pharmacokinetic study of berberine and B-FAHF-2. A. Structures of berberine and internal standard (IS) clozapine; B. Concentration-time curves of berberine in mouse serum after oral administration of berberine alone (1 mg) and B-FAHF-2 formula (12 mg, containing 1 mg of berberine).

Table 2
Accuracy of the analysis of berberine and clozapine.

	Added ($\mu\text{g/mL}$)	Intraday Measured ($\mu\text{g/mL}$)	Accuracy	Added ($\mu\text{g/mL}$)	Inter day Measured ($\mu\text{g/mL}$)	Accuracy
Clozapine	0.25	0.26	105.80%	0.25	0.26	103.39%
	1	1.01	100.97%	1	0.96	96.43%
	5	4.81	96.20%	5	5.27	105.52%
BBR	0.25	0.26	104.00%	0.25	0.26	105.45%
	1	0.98	98.10%	1	0.98	98.04%
	5	4.76	95.29%	5	4.86	97.29%

and also completely prevents peanut anaphylaxis at only 1/5 of the FAHF-2 dose [8]. This indicated that the butanol extraction process mainly maintained the effective constituents and removed the less- or non-effective components. Also, it was found that berberine significantly inhibited the IgE production by human B cell line *in vitro* and by human PBMCs *in vitro* [9]. These findings led us to hypothesize that berberine, in addition to its role as chemical marker of B-FAHF-2, might be also a bioactive/pharmacological marker of B-FAHF-2 formula *in vivo*.

To test this hypothesis, we evaluated effects of the berberine compounds alone on the peanut allergic mouse model as compared to B-FAHF-2. Surprisingly, berberine-alone treatment failed to suppress peanut allergic reactions, whereas B-FAHF-2 treated mice were significantly protected against anaphylaxis and showed significantly reduced PN specific IgE levels. To understand why berberine suppressed IgE production *in vitro* but not *in vivo*, we performed pharmacokinetic studies of berberine and B-FAHF-2 in a mouse model. Our results showed that berberine alone had a very low bioavailability property as described in other publications [19,20]. The $C_{\max}$ of berberine alone fed mice was significantly lower than that of berberine in B-FAHF-2 formula. Also, the AUC value of B-FAHF-2 is 2441.10 ng/mL·h, which is about 13 times higher than that of the berberine (176.87 ng/mL·h). These results suggest that mice absorbed more berberine when fed with B-FAHF-2 formula than when fed with berberine alone, implying that certain herbal constituents in B-FAHF-2 improved the pharmacokinetics parameters of berberine. By screening the total ion chromatography (TIC) data, we didn't detect jatrorrhizine, which is one of the metabolites of berberine, in the serum of berberine or B-FAHF-2 treated mice [21]. This suggests that the low berberine level in the serum might not be due to the fast metabolism processing, but due to poor absorption. Studying tissue distribution of berberine may help explain the low blood level of berberine. Two peaks were found in the serum-concentration-time curves of B-FAHF-2 and berberine alone. This might be due to the distribution and re-absorption properties of berberine. Further study to determine the berberine tissue distribution is required. In addition, the smaller $T_{\max}$ of B-FAHF-2 than that of the berberine alone also suggested that the faster absorption rate of B-FAHF-2 than berberine, while the $T_{1/2}$ retained the similar values between B-FAHF-2 and berberine.

It has been reported that berberine has poor bioavailability through intestine absorption. The maximum concentration of berberine was detected as 9.48 ng/mL after oral administration of 100 mg/kg of pure berberine in rats [19]. Hua *et al.* also reported that only 0.4 ng/mL of berberine was detected in human plasma after an oral dose of 400 mg of berberine [22]. Previously, our data showed that the IC50 value of berberine on human PBMC IgE production was 0.2 $\mu\text{g/mL}$ *in vitro* [9], which is about 500 times higher than the detected concentration in

Table 3
The mean main pharmacokinetic parameters of berberine alone and in B-FAHF-2 after oral administration to mice.

Group	$C_{\max}$ (ng/mL)	$T_{\max}$ (h)	AUC 0–t (ng/mL·h)	$T_{1/2}$
Berberine	35.13 $\pm$ 47.90	1	176.87	11.09
B-FAHF-2	289.30 $\pm$ 185.40	0.5	2441.10	10.48

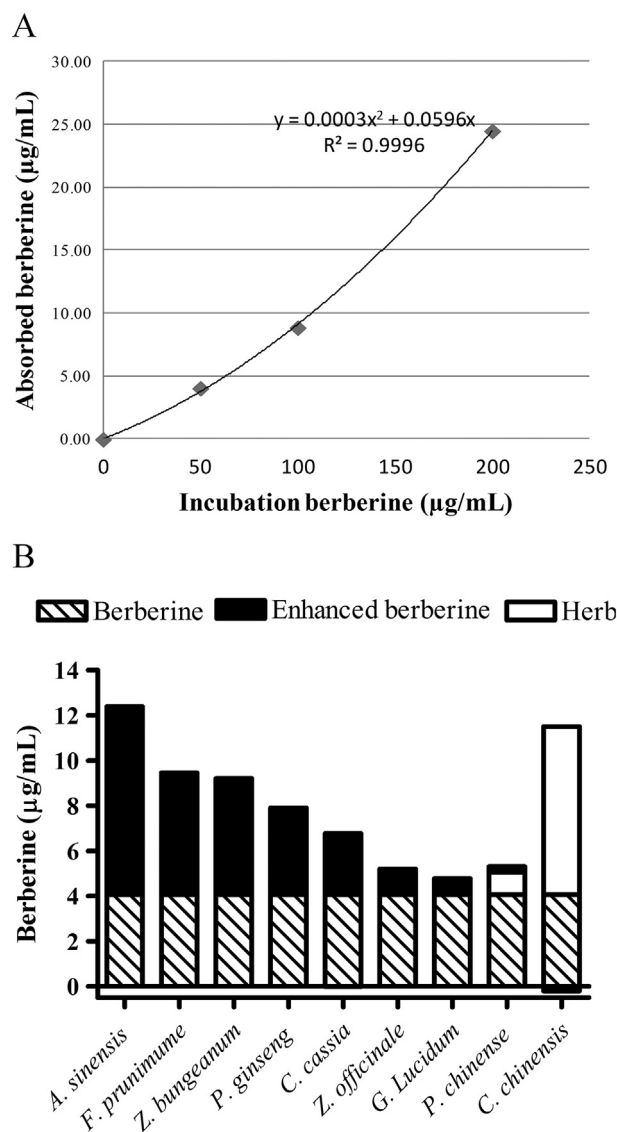


Fig. 5. Berberine uptake assay in Caco-2 cells. A. Absorption curve of berberine alone taken up by Caco-cells; B. Absorbed berberine level in the presence of other nine individual herbal constituents in B-FAHF-2 formula. Slanted striped bar: berberine level when incubated with berberine alone at 50 $\mu\text{g/mL}$; Solid bar: enhanced berberine level when incubated with berberine (50 $\mu\text{g/mL}$) and individual herbal constituent (250 $\mu\text{g/mL}$); Open bar: berberine level in herbal constituents alone at 250 $\mu\text{g/mL}$.

human plasma (0.4 ng/mL) and is about 8 times higher than the berberine $C_{\max}$ value (35.13 $\pm$ 47.90 ng/mL) found in this study. This may explain the poor protection of berberine alone in our peanut allergic murine model. Taken together, single usage of pure berberine compound is not a feasible therapy for the treatment of food allergy, but it may be a potential pharmacological maker of B-FAHF-2.

As the first step to determine whether herbal constituents present in the whole formula might help intestinal absorption of berberine, we used Caco-2 cells for the berberine permeability experiments. When incubated with berberine alone at 100 $\mu\text{g/mL}$, there was a linear relationship ($y = 0.0873x$, $R^2 = 0.9971$) between the incubation concentration and the absorbed berberine by Caco-2 cells. When the incubation concentration went to 200 $\mu\text{g/mL}$, a polynomial equation curve fit the relationship of the incubation concentration and absorbed berberine ($y = 0.0003x^2 + 0.0596x$, $R^2 = 0.9996$). This data suggested that increasing the incubation berberine concentration might increase the absorbed berberine rate by Caco-2 cell. Caco-2 cells were incubated with berberine in the presence or absence of other 9 individual herbal medicines. Our results showed that four herbal constituents, *A. sinensis*,

F. prunimune, *Z. bungeanum*, and *P. ginseng* remarkably enhanced the berberine absorption >100% or near 100%. Three herbs, *C. cassia*, *Z. officinale* and *G. lucidum*, moderately increased the berberine absorption by 66.66%, 26.95% and 17.57%. Two herbal constituents containing berberine, *P. chinense* and *C. chinensis*, only had minimal effect on berberine absorption. *P. chinense* only increased the berberine absorption by 6.13%, and *C. chinensis* even decreased the berberine absorption by 4.86%. In 2013, Chen *et al.* found that two herbal formulas, SGMX (Samgiumgagambang) and WHW (Wen-Pi-Tang-Hab-Wu-Ling-San), increased the berberine absorption rate in rats [23]. SGMX consists of 15 different herbal medicines, including *Z. officinale* and *C. cassia*, which overlap with B-FAHF-2 formula. WHW is a formula of 14 herbal medicines, in which 5 herbal constituents, *A. sinensis*, *P. ginseng*, *Z. officinale*, *C. cassia*, and *C. chinensis* are also present in B-FAHF-2 formula. These observations are consistent with our finding that these 4 herbal medicines facilitate the absorption of berberine. Considering the pharmacokinetics parameters we found in our mouse study, other components present in B-FAHF-2 formula may be acting to alter the behavior of the intestinal barrier in the mice, hence increasing the maximum serum concentration of berberine. However, Chen *et al.* didn't find these SGMX and WHW formulas increased the C_{max} of berberine absorption [23], which is different from our data.

In summary, we demonstrated that berberine is the most abundant major ingredient in B-FAHF-2. It can be quantitatively detected in B-FAHF-2 product and in blood samples following oral ingestion of B-FAHF-2. Therefore, berberine can be used as a chemical marker in B-FAHF-2 for quantitatively monitoring its product quality, and as a pharmacokinetic marker in animal models. The findings may be valuable to develop methodologies to monitor adherence during clinical studies of B-FAHF-2 by detecting berberine levels in the peripheral blood and urine samples. Additionally, the poor bioavailability of berberine alone limits the direct usage of this active compound on food allergy or other diseases. Patients would need to orally take substantially higher dose of berberine compound than that in B-FAHF-2 formula to reach a therapeutic effect. On the other hand, B-FAHF-2 formula improved the absorption of berberine. We found that 7 out of 9 individual constituents present in B-FAHF-2 formula enhanced intestinal epithelial cell absorption of berberine *in vitro*. This finding might provide a potential approach to increase the potency of berberine therapy with a low oral dose.

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A US patent application regarding FAHF-2 (reference No. 60554775) has been issued and Dr. Xiu-Min Li shares this patent and shares the membership of Herbal Springs LLC.

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