

• Research Article

Constituents of the anti-asthma herbal formula ASHMI™ synergistically inhibit IL-4 and IL-5 secretion by murine Th2 memory cells, and eotaxin by human lung fibroblasts *in vitro*

Bolleddula Jayaprakasam¹, Nan Yang¹, Ming-Chun Wen², Rong Wang³, Joseph Goldfarb⁴, Hugh Sampson¹, Xiu-Min Li¹

1. Center for Chinese Herbal Therapy for Asthma and Allergy, Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
2. Department of Pneumology, Weifang Asthma Hospital, Weifang 261041, Shandong Province, China
3. Department of Human Genetics, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
4. Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

OBJECTIVE: Anti-asthma herbal medicine intervention (ASHMI™), a combination of three traditional Chinese medicinal herbs developed in our laboratory, has demonstrated efficacy in both mouse models of allergic asthma, and a double-blind placebo-controlled clinical trial in patients with asthma. This study was designed to determine if the anti-inflammatory effects of individual herbal constituents of ASHMI™ exhibited synergy.

METHODS: Effects of ASHMI and its components aqueous extracts of Lingzhi (*Ganoderma lucidum*), Kushen (*Sophora flavescens*) and Gancao (*Glycyrrhiza uralensis*), on Th2 cytokine secretion by murine memory Th2 cells (D10.G4.1) and eotaxin-1 secretion by human lung fibroblast (HLF-1) cells were determined by measuring levels in culture supernatants by enzyme-linked immunosorbent assay. Potential synergistic effects were determined by computing interaction indices from concentration-effect curve parameters.

RESULTS: Individual Lingzhi, Kushen and Gancao extracts and ASHMI (the combination of individual extracts) inhibited production of interleukin (IL)-4 and IL-5 by murine memory Th2 cells and eotaxin-1 production by HLF-1 cells. The mean 25%-inhibitory-concentration (IC₂₅) values (mg/mL) for ASHMI, Lingzhi, Kushen and Gancao for IL-4 production were 30.9, 79.4, 123, and 64.6, respectively; for IL-5 production were 30.2, 263, 123.2 and 100, respectively; for eotaxin-1 were 13.2, 16.2, 30.2, and 25.1, respectively. The IC₅₀ values (mg/mL) for ASHMI, Lingzhi, Kushen and Gancao for IL-4 production were 158.5, 239.9, 446.7, and 281.8, respectively; for eotaxin-1 were 38.1, 33.1, 100, and 158.5, respectively. The interaction indices of ASHMI constituents at IC₂₅ were 0.35 for IL-4, 0.21 for IL-5 and 0.59 for eotaxin-1. The interaction indices at IC₅₀ values were 0.50 for IL-4 and 0.62 for eotaxin-1 inhibition. Inhibition of IL-5 did not reach IC₅₀ values. All interaction indices were below 1 which indicated synergy.

CONCLUSION: By comparing the interaction index values, we find that constituents in ASHMI™ synergistically inhibited eotaxin-1 production as well as Th2 cytokine production.

KEYWORDS: medicine, Chinese traditional; medicine, herbal; plant extracts; anti-asthma herbal medicine intervention (ASHMI); anti-asthmatic agents; chemokine CCL11; interleukin-4; interleukin -5; *in vitro*

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Correspondence: Xiu-Min Li, MD, MS, Professor; Tel: +1-212-241-4661; E-mail: xiu-min.li@mssm.edu

1 Introduction

Asthma is a growing major public health problem worldwide, especially in Western countries, affecting 18.9 million adults and 8.2 million children in the USA^[1]. Potent anti-inflammatory corticosteroids are the cornerstone of asthma treatment. However, these medications are not fully satisfactory, and there are concerns regarding known side effects of systemic corticosteroids, such as disturbance of adrenal function and overall immune suppression^[2-4]. The chronic nature of this disease and the lack of definitive preventive and curative therapies lead up to 60% of patients to seek alternative medicine treatments^[5]. Traditional Chinese medicine (TCM) formulas have been used to treat asthma for centuries. A number of well controlled clinical studies on several TCM formulas including Modified Mai Men Dong Tang (mMMDT, 5 herbs)^[6], Ding Chuan Tang (DCT, 9 herbs)^[7] and AST1 (the combination of Mai Men Dong Tang and Liu Wei Di Huang Wan, 10 herbs)^[8] and anti-asthma herbal medicine intervention (ASHMITM, 3 herbs)^[4] provided evidence of clinical efficacy and safety and immunomodulatory effects. ASHMITM is the first herbal medicine to receive approval for phase I and II clinical trials as a US Food and Drug Administration (USFDA) investigational new drug (IND) (IND No. 71526) for treating asthma. ASHMITM is composed of aqueous extracts of Lingzhi (*Ganoderma lucidum*), Kushen (*Sophora flavescens*) and Gancao (*Glycyrrhiza uralensis*)^[4]. ASHMITM was shown to be safe and tolerable with no detectable adverse effects on liver and kidney function in a controlled phase I study of adult patients with asthma. It increased lung function (forced expiratory volume in one second), reduced symptom scores and decreased β_2 -agonist use, to a degree similar to prednisone in adults with moderate to severe asthma, but without prednisone's adverse effect on adrenal function and no overall immune suppression^[4]. ASHMITM also produced clinical and immunologic benefits in children aged 5 to 14 years with persistent asthma when used as an add-on therapy^[9]. Studies using a murine model of asthma found that ASHMITM abrogated airway hyperreactivity, which was associated with suppression of airway eosinophilic inflammation and airway remodeling, including lung collagen deposition, goblet cell mucus production, and Th2 cytokine production^[10,11]. The mechanisms of ASHMITM's potent anti-inflammatory action are unknown.

The concept of synergy between individual herbs in a formula is basic to TCM formulations^[12] and is believed to be responsible for their clinical efficacy and safety. The importance of synergy research in developing a new generation of phytopharmaceuticals has been emphasized by Wagner and Ulrich-Merzenich^[13]. However, synergistic

activities between components of anti-asthma TCM formulas are largely unexplored. The widely used anti-asthma like formula TJ-19 (also named Sho Seiryu To in Japanese, and Xiao Qing Long Tang in TCM), was shown to be more effective than any individual herbal constituent in inhibiting histamine-induced reactions in an early study^[14]. However, there is no proof that this phenomenon is due to synergism among the constituents in TJ-19. We previously found that no individual herb in ASHMITM was as effective as ASHMITM formula in suppressing airway inflammation in an animal model of asthma^[15]. Our preliminary study also showed that removing any single herb from ASHMITM reduced its inhibitory effect on airway eosinophilic inflammation (unpublished data). However, whether the herbal constituents in ASHMITM act synergistically or additively is unknown.

Allergic asthma is a chronic airway inflammatory disease in which memory (polarized) Th2 cells that secrete Th2 cytokines interleukin (IL)-4 (IL-13) and IL-5 following antigen encounter are the major driving force. D10.G4.1 (D10) cells are well characterized memory Th2 cells^[16-18]. Eotaxin, a cysteine-cysteine (CC) chemokine, induces the migration of eosinophils from blood into the lungs by acting on the CC chemokine receptor CCR3^[19]. Lung fibroblasts are a major cellular source of eotaxin-1^[20]. Human lung fibroblast (HFL-1) cells are commonly used to investigate eotaxin-1 secretion and mechanisms of action of eotaxin-inhibitory agents^[21-23]. In this study, we employed D10 cells and HFL-1 cells and pharmacological isobole methods^[13] with the purpose of demonstrating that the combination of individual Lingzhi, Kushen and Gancao extracts in ratios equivalent to that in ASHMITM act synergistically to suppress Th2 cytokines and eotaxin-1 production.

2 Materials and methods

2.1 Materials and reagents

High pressure liquid chromatography (HPLC) grade acetonitrile (ACN), methanol (MeOH), and phosphoric acid (H_3PO_4) were purchased from Fisher Scientific (Pittsburgh, PA, USA). HFL-1 cells and D10 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2 Preparation of herb extracts

All herbs used in this study were of Chinese origin and inspected for identity and quality by certified TCM pharmacists. Only herbs meeting the quality and safety standards of the *Pharmacopoeia of the People's Republic of China*^[24] were used in product preparation. Standard ASHMITM was prepared by aqueous extraction of all three herbs together as described previously^[25]. The quality and consistency of raw herbs, extraction and final product all meet the requirement of the USFDA based on the *Guidance for Industry: Botanical Drug Products* issued

by the USFDA^[26], and described in our previous publication^[11,25,27]. Individual herb extracts of Lingzhi, Kushen, and Gancao were produced by the Sino-Lion Pharmaceutical Company (Weifang, China)^[4,25]. Briefly, herbs were soaked in 10 times volume of water for 1 h, boiled for 2 h, and the decoction was collected. The residue was boiled again with 10 times volume of water for 2 h. The decoctions were combined, concentrated under reduced pressure (60 °C, 0.08 Mpa), and dried. Based on the yield and the ratio of individual raw herbs in the formula, ASHMI for this study was made by combining the individual extracts in the percentages of 35, 45 and 20 of Lingzhi, Kushen and Gancao (Table 1) (experimental ASHMI). The levels of heavy metal, pesticide and microbial residues all met the standards for botanical products^[28-31]. In this paper, ASHMITM was used to indicate the standard ASHMI product, and ASHMI was used to indicate the mixture of individual herb extracts used in the synergy study.

2.3 Endotoxin testing of the ASHMI product

Endotoxin in ASHMI was measured using the Pyrogen Plus assay kit (Lonza, MA, USA). A total of 250 µL of ASHMI solution was added to tubes containing reconstituted *Limulus* amoebocyte lysate and incubated for 60 min at 37 °C. Endotoxin at concentrations from 0.5 to 0.01 EU/mL was used as controls. No clot formation was found in ASHMI-containing tubes, indicating endotoxin level was below 0.03 EU/mL or 0.02 ng/mL, the limit of sensitivity of this kit. Gel-clot formation occurred in endotoxin control tubes at and above 0.032 5 EU/mL.

2.4 HPLC fingerprints

HPLC fingerprints of individual herb extracts, standard ASHMITM, and the combination of individual herbal extracts (ASHMI) were generated to monitor the presence or absence of individual herbs.

A total of 50 mg of ASHMI (equivalent to 437 mg of raw herbs in the proportions listed above) was dissolved in 10 mL of H₂O, and then extracted with 5 mL of *n*-butanol five times. The combined extracts were dried and re-dissolved in 2 mL of 50% methanol. A total of 10 µL of the resulting solution was injected into the HPLC system. For analysis of each single herb extract, an amount of extract of that herb equivalent to the amount in 50 mg of ASHMI extract was prepared based on its yield and analyzed using the method described above. All samples were analyzed in triplicate.

HPLC analysis was performed on a Waters Alliance system coupled with a 996 photodiode-array detector (scan from 200 to 400 nm). The separation was carried out on a Waters SunfireTM C₁₈ column (5 µm, 4.6 mm×150 mm i.d., Waters Corporation, Milford, MA, USA) with a Zorbax ODS guard column (5 µm, 4.6 mm×12.5 mm i.d., Agilent Technologies, Santa Clara, CA, USA) at 30 °C. A mixture of 0.1% phosphoric acid (A) and acetonitrile (B) was used as the mobile phase. Gradient started in linear gradient mode (2%-48 % of B for 0 to 75 min) with a flow-rate of 1 mL/min. Data were acquired and processed with the Empower[®] software system.

The HPLC method was validated by building calibration curves, repeatability, precision, stability and recovery. In brief, samples were processed using the same preparation method and analyzed at different time points. The standard deviation of the individual peak area and the retention time was calculated and less than 2%. Intra-day and inter-day analyses were also performed and the peak areas and retention time were analyzed for evaluation of precision and stability. Known compounds were added into samples as standards and processed using the same method to determine the recovery. The validation results showed that the sample preparation method and the HPLC method were reliable.

2.5 Cell culture

2.5.1 Th2 cell culture

D10 cells, a classic Th2 clone derived from AKR mice, produce Th2 cytokines upon conalbumin (CA) stimulation^[17,32]. D10 cells were cultured in equal amounts of Roswell Park Memorial Institute medium and EHAA (Click's) medium containing 10% rat growth factor and conalbumin (100 µg/mL)^[17]. Freshly isolated spleen cells from naive AKR mice were irradiated and used as antigen-presenting cells (APCs). To expand the cells, D10 cells were mixed with APCs in the ratio of 1:5. After one week, D10 cells were collected and rested for 2 d in fresh medium without CA and then used in experiments. D10 cells (0.5×10^6) and irradiated-APCs (2.5×10^6) were plated in 24-well plates, stimulated with CA (50 µg/mL) and cultured with medium alone or with herbal extracts at 4, 20, 100, and 500 µg/mL. Each culture condition was done in triplicate. After 72 h, supernatants were collected and IL-4 and IL-5 levels were determined by enzyme linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA). Our preliminary study showed that cytokine production

Table 1 Components of herbal medicines in ASHMI

Name	Latin name	Plant part	Amount (% of total)	Yield (%)	Percentage of experimental ASHMI
Lingzhi (L)	<i>Ganoderma lucidum</i>	Fruiting body	62.5	6	35
Kushen (K)	<i>Sophora flavescens</i>	Roots	28.1	17	45
Gancao (G)	<i>Glycyrrhiza uralensis</i>	Roots	9	20	20

by D10 cells peaked at 72 h following antigen stimulation, and *in vitro* treatment with ASHMI for 72 h significantly reduced Th2 cytokine production without causing cytotoxicity. We therefore used a 72 h culture protocol.

2.5.2 HFL-1 cell culture

HFL-1 cells were grown in F-12K medium (ATCC, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), and 1% penicillin-streptomycin (100 units/mL for penicillin and 100 mg/mL for streptomycin) at 37 °C in a 5% CO₂ humidified incubator, as previously described^[21]. The medium was changed every 3 d. Cells were sub-cultured by detaching cells with 0.05% trypsin-ethylenediaminetetraacetic acid (Gibco BRL, Grand Island, NY, USA). The cells were counted and 5×10^4 cells were plated per well onto a 24-well culture plate and incubated for 48 h in growth medium. Cells were then washed with phosphate-buffered saline (PBS) and herbal extracts dissolved in culture medium were added to the wells. Medium alone served as the control. Each concentration was tested in triplicate wells in three independent experiments. Cells were cultured for 96 h (The culture duration selected is based on our preliminary study). The supernatant was then collected and eotaxin-1 level was determined by ELISA (R&D Systems, Minneapolis, MN, USA).

2.6 ELISA

ELISA was conducted according to the manufacturer's directions. Briefly, 96-well plates (Nalge Nunc Int., Rochester, NY, USA) were coated with the required concentration of coating antibody and kept overnight at 4 °C. Plates were then washed with washing buffer (0.05% Tween20-PBS) and blocked for 1 h at room temperature. Samples and standards (100 µL) were added to the plates and incubated for 2 h. Then, plates were washed and incubated with detection antibody and then horseradish peroxidase (HRP), using a commercial kit as per instruction by the manufacturer (R&D Systems). Substrate solution was added, incubated (30 min), and the reaction was stopped using stop solution (2N H₂SO₄). The resulting solution was read at 450 nm using a microplate reader.

2.7 Cell viability assay

D10 cells were grown in suspension, and their viability following culture was determined by trypan blue exclusion assays. Cell suspensions were mixed with trypan blue (1:1 ratio) and inserted into a hemocytometer. Stained (dead) cells and non-stained (viable) cells were counted under a microscope. The viability of adherent HFL-1 cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described^[33]. In brief, cells were counted and seeded in a 96-well plate. After 48 h, herbal extracts in growth medium were added to the wells and cells were incubated for 48 h. Then, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated

at 37 °C for 3 h. The medium was aspirated and an aliquot of 200 µL of dimethyl sulfoxide was then added to each well. The resulting colored solution was read using an optical-density reader at 595 nm. The percent viability was calculated with respect to medium alone: the number of viable cells in ASHMI and individual herb extract cultures was divided by the number of viable cells in the medium alone culture and then multiplied by 100.

2.8 Calculation of IC₅₀, IC₂₅ and interaction indices

The percentage of inhibition was calculated by comparing values obtained by ELISA to respective controls. Data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA, USA) and expressed as percent inhibition. The 50% and 25% inhibitory concentrations (IC₅₀ and IC₂₅) for IL-4 and eotaxin-1 were calculated with 95% confidence intervals by nonlinear regression analysis performed on sigmoidal concentration-response (variable slope) curves. Goodness of fit was documented by R² values of 0.95 to 0.99 for all of the curves generated. The interaction index (II) was calculated for ASHMI by using the equation: $dL/DL + dK/DK + dG/DG = II$, where, dL, dK and dG are the concentrations of Lingzhi, Kushen, and Gancao in ASHMI; DL, DK and DG are the concentrations of the herbs alone that produce equivalent responses^[13,34]; dL/DL, dK/DK and dG/DG are concentration ratios. The sum of the concentration ratios is the II. An II < 1, indicates synergism; II = 1 indicates additivity; and II > 1 indicates antagonism^[13,34].

3 Results

3.1 Characterization of individual and combined herb extracts

HPLC fingerprints showed that the combination of individual extracts (experimental ASHMI) had a pattern consistent with that of the standard ASHMITM product. By comparing the on-line ultraviolet spectra and retention times (t_R), peaks in the standard ASHMITM and experimental ASHMI with those of the individual herb extracts, it showed that peaks 16, 21, 30 and 32 originate from Gancao, peaks 3, 7, 8, 9, 10, 11, 12, 13, 14, 17, 18, 19, 20 and 22 from Kushen, and peaks 5, 23, 24, 25, 26, 27, 28, 29, 31, 33 and 34 from Lingzhi (Figure 1A). Furthermore, using purified compounds isolated and identified in our lab, we determined the identity of the major peaks, including liquiritin (peak 16) and glycyrrhizin (peak 32) from Gancao^[21], and ganoderic acid D (peak 26) and ganoderic acid A (peak 27) from Lingzhi^[35]. By using standard compound purchased from Sigma (St. Louis, MO, USA) and Chroma-Dex (Santa Ana, CA, USA), we identified the matrine (peak 7) and kushenol O (peak 19) from Kushen (Figure 1B). Experimental ASHMI and the individual herbal extracts were employed for the following studies.

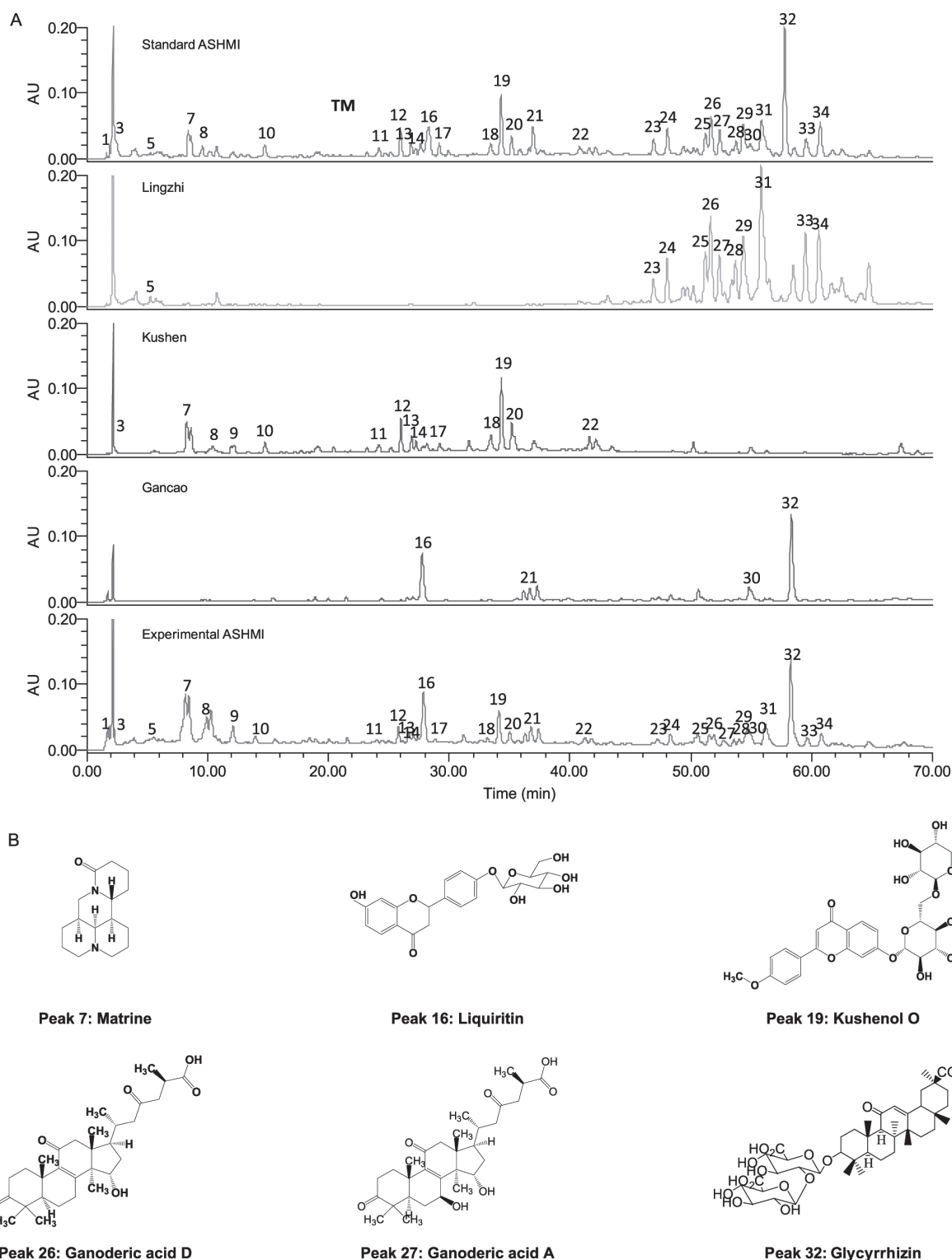


Figure 1 Consistency of HPLC fingerprints and major chemical constituents in different preparations of ASHMI

A: HPLC fingerprints generated from standard ASHMITM, experimental ASHMI, and individual herb extracts. B: Characterization of chemical constituents of major corresponding peaks between standard ASHMITM, experimental ASHMI and individual herb extracts. Peaks 16 and 32 correspond to liquiritin and glycyrrhizin from Gancao; peaks 7 and 19 correspond to matrine and kushenol O from Kushen; peaks 26 and 27 correspond to ganoderic acid D and ganoderic acid A from Lingzhi.

HPLC: High pressure liquid chromatography.

3.2 ASHMI constituents exhibited synergy in suppressing memory Th2 cell IL-4 production

To determine the synergistic effect of ASHMI constituents on Th2 cytokine inhibition, we cultured D10 cells with ASHMI and individual herb extracts of Lingzhi, Kushen and Gancao at different concentrations. ASHMI produced concentration-dependent inhibition of production of IL-4, a key Th2 cytokine involved in allergic airway inflammation in asthma. At the highest concentration tested (500 $\mu\text{g/mL}$), ASHMI produced 75% inhibition (Figure 2A). The individual extracts also produced concentration-dependent inhibition of IL-4 production (Figure 2B, C and D). To provide evidence of synergy between constituents of ASHMI on IL-4 production we calculated IC_{25} and IC_{50} values (Table 2). The mean IC_{25} value of ASHMI was 30.9 $\mu\text{g/mL}$ while IC_{25} for each of the individual extracts Lingzhi, Kushen, and Gancao

were 79.4, 123.0 and 64.6 $\mu\text{g/mL}$, respectively (Table 2). The concentrations of the individual extracts at the ASHMI IC_{25} value were only 10.8 for dL, 13.9 for dK and 6.2 for dG, which were markedly lower than the IC_{25} of the individual herbal extracts. The II, the sum of the ratios of concentrations in the mixture at ASHMI IC_{25} value (dL, dK, and dG) to IC_{25} for the individual extracts (DL, DK, and DG), $\text{dL}/\text{DL}+\text{dK}/\text{DK}+\text{dG}/\text{DG}$ ($0.14+0.11+0.10$) was 0.35. Similarly, the II for IC_{50} values was 0.50 (Table 2). Both II values of IC_{25} and IC_{50} were less than 1, demonstrating synergistic inhibition of IL-4 production. Synergy was even more pronounced at the lower concentrations.

3.3 ASHMI constituents exhibited synergy in suppressing IL-5 production by Th2 cells

ASHMI constituents also exhibit synergy in suppressing production of IL-5, a cytokine involved in eosinophil

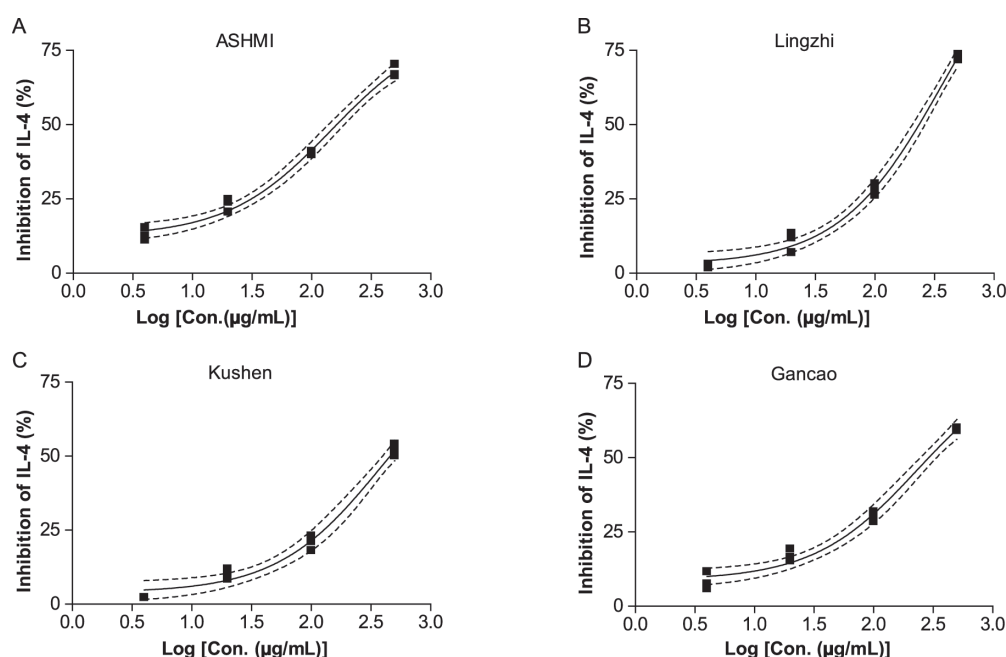


Figure 2 Effects of A) ASHMI; B) Lingzhi; C) Kushen; and D) Gancao on conalbumin-induced interleukin-4

D10 cells were cultured for 72 h with or without herbal extracts in the presence of antigen conalbumin and antigen-presenting cells. Interleukin-4 level was measured from the supernatant by enzyme-linked immunosorbent assay and the percentage of inhibition was calculated with respect to untreated cells. Three replications were run at each concentration. Concentration-response curves were constructed using Prism 4.0 (GraphPad Software, San Diego, CA, USA). The dotted lines indicate the 95% confidence intervals.

Table 2 IC_{25} and IC_{50} values and concentration ratios of ASHMI, Lingzhi, Kushen and Gancao on interleukin-4 inhibition

($\mu\text{g/mL}$)

Constituent	IC_{25} (95% CI)	IC_{50} (95% CI)	Concentration in ASHMI at IC_{25}	Concentration in ASHMI at IC_{50}	Concentration ratio at IC_{25}	Concentration ratio at IC_{50}
ASHMI	30.9 (25.1-38.9)	158.5 (131.8-190.5)	—	—	—	—
Lingzhi (L)	79.4 (67.6-95.5)	239.9 (199.5-263.0)	10.8	55.8	0.14	0.23
Kushen (K)	123.0 (100.0-158.5)	446.7 (398.1-524.8)	13.9	71.3	0.11	0.16
Gancao (G)	64.6 (52.5-79.4)	281.8 (239.9-331.1)	6.2	31.7	0.10	0.11

activation. ASHMI and each individual extract produced concentration-dependent inhibition of IL-5 production by D10 cells (Figure 3A, B, C and D). Because the maximum inhibition produced by ASHMI was approximately 40%, concentration ratios were calculated only at the IC₂₅ for ASHMI.

The mean IC₂₅ of ASHMI was 30.2 µg/mL, and IC₂₅ for the individual extracts Lingzhi, Kushen, and Gancao, were 263, 123.2 and 100 µg/mL, respectively. The concentrations of individual extracts in ASHMI at its IC₂₅, dL, dK and dG were only 10.6, 13.6, and 6 µg/mL, markedly lower than the IC₂₅ for the individual herbal extracts. The II (the sum of the concentration ratios at IC₂₅) was 0.21, which was considerably lower than 1 (Table 3), demonstrating synergy in inhibition of IL-5 production.

3.4 ASHMI constituents exhibited synergy in suppressing eotaxin-1 production by HFL-1 cells

Eotaxin, together with Th2 cytokines, plays an important role, in mediating airway eosinophilic inflammation by recruiting eosinophils^[36]. Lung fibroblasts are a major cellular source of eotaxin^[20]. HFL-1 cell has been widely used to investigate inhibition of eotaxin production^[21,22]. ASHMI and its constituents, Lingzhi, Kushen, and Gancao showed concentration-dependent inhibition of eotaxin-1 production (Figure 4A, B, C and D). ASHMI inhibited eotaxin-1 production almost completely at the highest concentration tested (500 µg/mL), as did Lingzhi.

We investigated the possibility of synergy at both IC₂₅ and IC₅₀ values for ASHMI constituents. The mean IC₂₅ of ASHMI was 13.2 µg/mL and IC₂₅ values for Lingzhi,

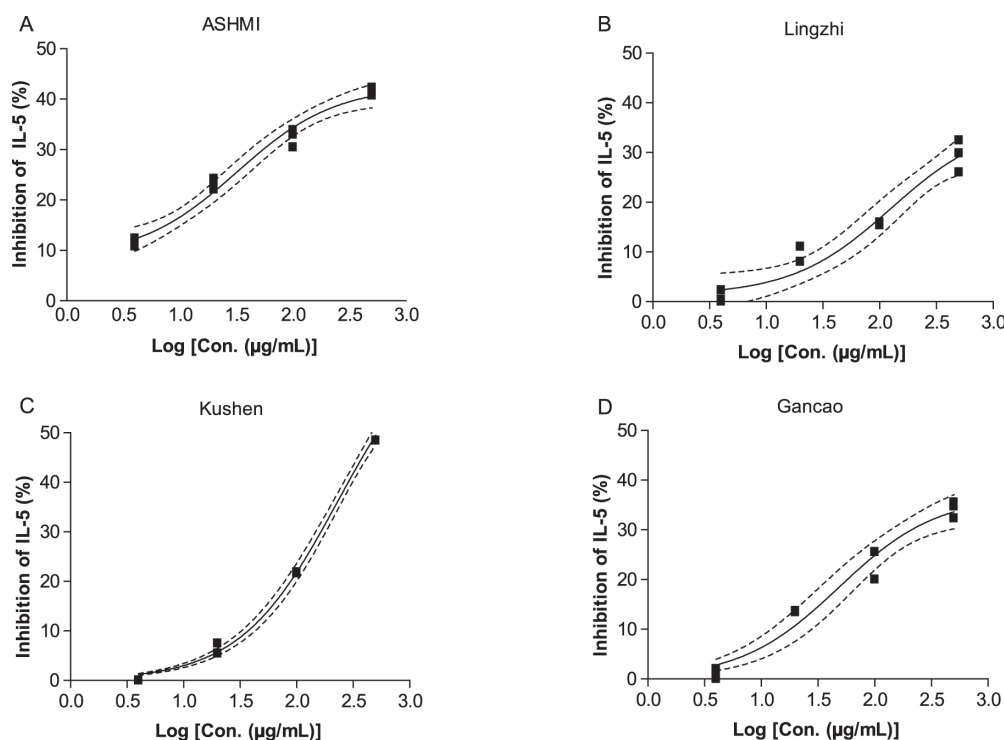


Figure 3 Effects of A) ASHMI; B) Lingzhi; C) Kushen; and D) Gancao on conalbumin-induced interleukin-5. D10 cells were cultured for 72 h with or without herbal extracts in the presence of antigen conalbumin-presenting cells. Interleukin-5 level was measured from the supernatant by enzyme-linked immunosorbent assay and percent inhibition was calculated with respect to negative control. Three replicates were run at each concentration. Concentration-response curves were constructed using Prism 4.0 (GraphPad Software, San Diego, CA, USA). The dotted lines indicate the 95% confidence intervals.

Table 3 IC₂₅ values and concentration ratios of ASHMI, Lingzhi, Kushen and Gancao on interleukin-5 inhibition

Constituent	IC ₂₅ (95% CI)	Concentration in ASHMI at ASHMI IC ₂₅	Concentration ratio at IC ₂₅
ASHMI	30.2 (23.4-38.9)	—	—
Lingzhi (L)	263.0 (173.8-416.9)	10.6	0.04
Kushen (K)	123.2 (107.2-141.3)	13.6	0.11
Gancao (G)	100.0 (66.1-147.9)	6.0	0.06

Kushen and Gancao were 16.2, 30.2 and 25.1 $\mu\text{g/mL}$, respectively. The concentrations of constituent herbal extracts in ASHMI at its IC_{25} (dL, dK and dG) were 4.6, 5.9, and 2.7 $\mu\text{g/mL}$, respectively, markedly lower than the individual herb IC_{25} . Thus the II at IC_{25} was 0.59, indicating synergy (Table 4). At the ASHMI IC_{50} , the II of 0.62 similarly indicates synergistic actions of the three herbal constituents.

3.5 Cell viability

No cytotoxicity was detected in ASHMI- or any individual herb-containing D10 cell culture at any concentration (data not shown). ASHMI, Kushen and Gancao produced no toxicity in HFL-1 cultures. Lingzhi reduced HFL-1 cell viability by 20% at the highest concentration tested (500 $\mu\text{g/mL}$).

4 Discussion

TCM is becoming increasingly popular in Western countries due to its reputed safety and clinical efficacy for chronic conditions, including allergy and asthma^[5]. In TCM practice, the most widely used type of herbal treatment is “formulas” comprised of specific mixtures of several herbs. It is believed that complex interactions produce synergistic effects and a reduction of side effects and toxicity^[12]. However, few studies have demonstrated synergistic actions between herbal constituents in TCM formulas. Kan *et al*^[37] showed that compounds in *Angilica*

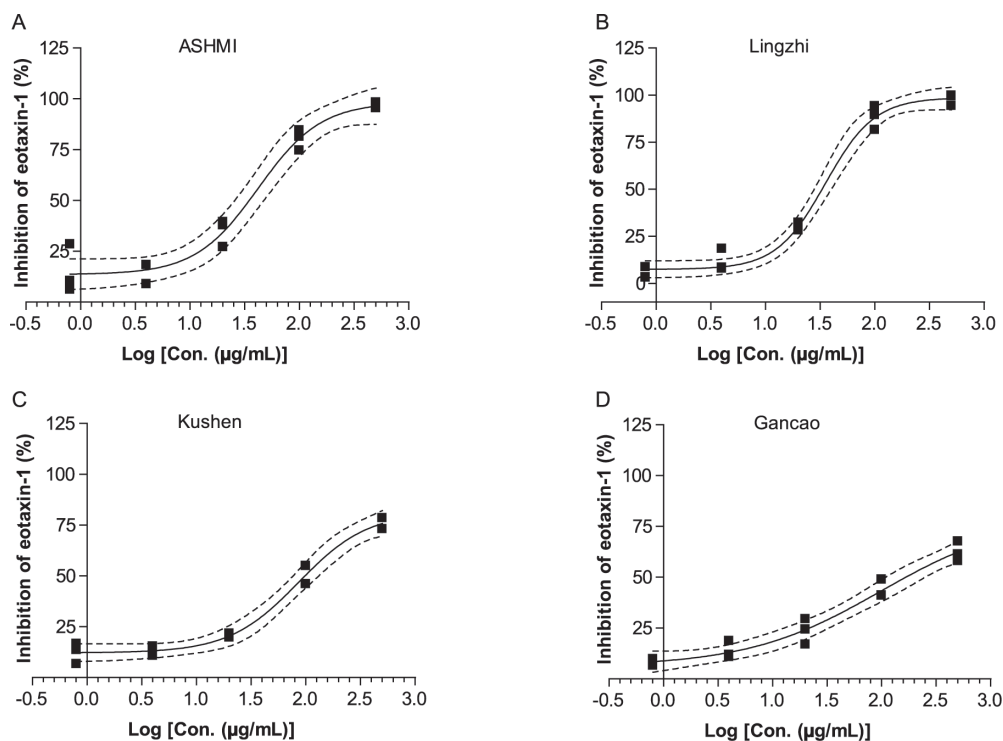


Figure 4 Effects of A) ASHMI; B) Lingzhi; C) Kushen; and D) Gancao on eotaxin-1 production by human lung fibroblasts

Human lung fibroblasts were cultured with or without herbal extracts for 48 h. Eotaxin-1 level in the culture supernatants was measured by enzyme-linked immunosorbent assay. The percentage of inhibition was calculated with respect to untreated cells. Three replicates were run at each concentration. Concentration-response curves were constructed using Prism 4.0 (GraphPad Software, San Diego, CA, USA). The dotted lines indicate the 95% confidence intervals.

Table 4 IC_{25} and IC_{50} values and concentration ratios of ASHMI, Lingzhi, Kushen and Gancao on eotaxin-1 inhibition

($\mu\text{g/mL}$)

Constituent	IC_{25} (95% CI)	IC_{50} (95% CI)	Concentration in ASHMI at IC_{25}	Concentration in ASHMI at IC_{50}	Concentration ratio at IC_{25}	Concentration ratio at IC_{50}
ASHMI	13.2 (7.4-18.2)	38.1 (26.9-44.7)	—	—	—	—
Lingzhi (L)	16.2 (13.2-20.0)	33.1 (26.9-39.8)	4.6	13.3	0.28	0.40
Kushen (K)	30.2 (20.0-39.8)	100.0 (83.2-120.2)	5.9	17.2	0.20	0.17
Gancao (G)	25.1 (13.2-33.1)	158.5 (107.2-239.8)	2.7	7.6	0.11	0.05

sinensis synergistically inhibited proliferation of colon cancer cells. A TCM formulation consisting of *Rhizoma Corydalis* and *Rhizoma Curcumae* also exhibits synergistic antitumor activity^[38]. To date, there has been no demonstration that constituents in anti-asthma TCM formulas act synergistically. One of the challenges might be that the formulas contain too many herbs to perform a standard pharmacological study. As cited previously, among all anti-asthma formulas previously studied, ASHMITM contains the fewest number of herbs which simplifies the determination of the roles of individual constituents on inflammatory processes involved in asthma. ASHMITM is the dried powder of extracts of three herbs (standard ASHMI)^[25]. In order to study the role of the three herb constituents of ASHMITM, we extracted the individual herbs and combined them together to make experimental ASHMI. HPLC fingerprints demonstrated consistency of the major peaks present in experimental ASHMI and standard ASHMITM and in individual extracts. In this study, we initially established concentration-effect curves for each individual herb extract and the combined extracts (ASHMI) on IL-4 and IL-5 production by murine Th2 memory cells, and eotaxin-1 production by HFL-1 cells. Lingzhi showed greater inhibition of IL-4 and eotaxin-1 production than Kushen and Gancao. Kushen produced greater inhibition of the production of IL-5 than Lingzhi and Gancao. However, a combination of individual herb extracts (ASHMI) showed the most potent inhibition of IL-4 and IL-5 production. However, this finding may be due to additive effects of constituents in the formula.

One commonly used method to determine whether multiple components of a mixture are acting additively, synergistically or antagonistically is the computation of the II. This index, an extension of the classical isobologram, sums the ratios for a specified effect level of the concentration of the component in the mixture, to that of the component alone. An $II < 1$ indicates synergy, an $II = 1$ indicates additivity, and an $II > 1$ indicates antagonism^[13,34]. The results reported above based on mean IC_{25} and IC_{50} values support the conclusion that the three herbal extracts in ASHMI work synergistically to reduce production of IL-4, IL-5 and eotaxin-1 in cell culture. II values for all effects tested were below 1 (range 0.21 to 0.62). To our knowledge, this is the first demonstration of Chinese herbal constituents of an anti-asthma formula exhibiting synergism in inhibiting production of endogenous substances critical to the inflammatory response.

In this study, neither ASHMI nor individual herb extracts were cytotoxic to D10 Th2 memory cells. However, Lingzhi, but not ASHMI, Kushen or Gancao, reduced the percent of viable HFL-1 cells by 20% at the highest dose tested. These results demonstrated that a high concentration of ASHMI that produced almost maximal inhibition of eotaxin-1 was not toxic. Wager and Ulrich-Merzenich

noted that synergism between natural products may, in addition to greater than additive therapeutic effects, also include elimination or lessening of adverse effects^[13]. The lack of cytotoxicity of ASHMI (500 μ g/mL) on HFL-1 cells while producing maximal suppression of eotaxin-1 production reflects additional example of synergy of herbal constituents in ASHMI.

In summary, we for the first time, showed that ASHMI is significantly more potent than any of its constituent herbs in direct suppression of IL-4 and IL-5 production by Th2 cells (no overlap in the 95% confidence intervals for IC_{25} or IC_{50}), and that this is due to synergism among the ASHMI constituents. None of the herbal extracts were cytotoxic to Th2 cells at any concentration tested. With respect to eotaxin-1 production by HFL-1 cells, ASHMI had a concentration-response curve similar to that of Lingzhi but without the cellular toxicity at concentrations producing maximal inhibition, and with the strong possibility of synergism among the three herbal constituents. Future research to investigate synergistic effects of ASHMI constituents and active compounds on major asthma mechanisms *in vivo* in an animal model of asthma is underway.

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6 Competing interests

Drs. Xiu-Min Li, Hugh Sampson and Ming-Chun Wen are named inventors of a patent for the use of ASHMITM (PCT/US05/08600 for ASHMITM) and have shares of Herbal Spring LLC., which has exclusive rights to the patent. The other authors have no financial interests to disclose.

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