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Glycyrrhiza uralensis flavonoids inhibit brain microglial cell TNF-α secretion, p-IκB expression, and increase brain-derived neurotropic factor (BDNF) secretion

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BDNF

Abstract *Objective:* Asthma sufferers exhibit high prevalence of anxiety/depression. Elevated tumor-necrosis factor-alpha (TNF-α) levels in peripheral system and central nervous system (CNS) are associated with anxiety/depression, whereas brain-derived neurotropic factor (BDNF) has anti-depressant effects. An anti-asthma herbal medicine intervention ASHMI inhibits peripheral TNF-α secretion in an animal model of asthma. We hypothesize that ASHMI and its compounds may have modulatory effects on CNS TNF-α and BDNF production. We sought to determine the effect of ASHMI and individual herb constituents on brain microglial cell TNF-α production, and identify the active compounds that suppress TNF-α and increase BDNF.
Methods: BV-2 mouse microglial cells were pre-treated with ASHMI or extracts of *Ganoderma lucidum* (*G. lucidum*), *Sophora flavescens Ait* (*S. flavescens*), and *Glycyrrhiza uralensis* Fischer (*G. uralensis*), the herbal constituents in ASHMI, or individual compounds isolated from *G. uralensis* at different concentrations and then stimulated with LPS. TNF-α levels in culture supernatants were measured by ELISA. The effect of active compounds on NFκB signaling pathway and on BDNF production were determined by western blotting and ELISA, respectively.
Results: ASHMI produced dose-dependent inhibition of TNF-α secretion by cultured-mouse microglia BV2 cells. Of the three herb extracts in ASHMI, only *G. uralensis* significantly and dose-dependently inhibited TNF-α production. Among the 5 flavonoids isolated from *G. uralensis*,

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isoliquiritigenin was the most effective. Isoliquiritigenin suppression of TNF-α production was associated with attenuation of p-NF-κB expression, and was accompanied by increased BDNF secretion. *Conclusion:* ASHMI and its effective flavonoid, isoliquiritigenin, inhibited TNF-α production by LPS stimulated microglial cells and elevated BDNF levels, which may prove to have anti-CNS inflammatory and anti-anxiety effects.

Introduction

Asthma, a chronic inflammatory airway disease, is the most common respiratory disease in the United States, affecting 25 million people.[1] Anxiety and depression are often associated with poor asthma control and quality of life.[2–7] The prevalence of serious psychological distress among adults with asthma (7.5%) is more than double that of the overall US population (3.0%) and adults without asthma (2.6%).[8] 20% to 40% of adolescents with asthma experience significant symptoms of anxiety.[9] Current asthma treatment involves the use of oral or inhaled corticosteroids and β_2-agonists and it has been suggested that these medications may be responsible for the association of asthma with depression and anxiety to some degree.[10,11] Higher levels of anxiety in asthmatic patients are positively associated with high concentrations and long-term use of inhaled corticosteroids.[12–14] These patients represent an important unmet clinical need. Chronic treatment of mice with corticosteroids is used as a model for depression.[15]

Traditional Chinese medicine (TCM) has a long history for the treatment of asthma, and is often used to simultaneously treat comorbid conditions. ASHMI, an extract of 3 herbs: *Ganoderma lucidum*, (*G. lucidum*), *Sophora flavescentis* (*S. flavescentis*), and *Glycyrrhiza uralensis* (*G. uralensis*), decreases inflammation in a murine asthma model. It was found to be safe and effective in clinical trials of adult asthmatics[16,17] and to inhibit macrophage TNF-α production in vitro.[18] It also was found to inhibit peripheral TNF-α production in animal model of asthma.[19] *G. uralensis* is the most common herb constituent both in TCM anti-asthma formulas and mood-disorder formulas.[20,21] We previously isolated 5 flavonoids from *G. uralensis*, and found that liquiritigenin (LQG), isoliquiritigenin (ILQG), 7,4′-dihydroxy flavones (7,4′-DHF) were more effective than liquiritin (LQ) and isoononin (ISO) in suppression of inflammation-associated chemokines and cytokines *in vitro*.[22] *G. uralensis* and its flavonoids have also been shown to have an anti-depressive effect in animal models.[23–25] However, precise mechanisms underlying these effects have not been elucidated.

Asthma is a Th2 cell-driven disease. However, pro-inflammatory cytokines such as TNF-α have been implicated in many aspects of the airway pathology in asthma and may play an important role in severe refractory asthma.[26] We previously showed that TNF-α increases airway mucus production and mucus gene expression.[27] TNF-α is a soluble, membrane-bound inflammatory cytokine produced by monocytes, macrophages, and T-cells. Although the mechanisms of development of depression and anxiety comorbid with asthma are not fully understood, inflammation contributes to development of depression.[28–31] TNF-α plays an important role in the peripheral system and central nervous system (CNS) function. TNF-α promotes neuroinflammatory cascades and also has direct toxic effects on oligodendroglia that lead to neuronal cell death and demyelination of oligiodentrites.[29,32,33] TNF-α is transcribed through, and activates, the Nuclear Factor Kappa B (NF-κB) signaling system.[34,35] NF-κB is normally sequestered in the cytoplasm of nonstimulated cells and consequently must be translocated into the nucleus to function. The subcellular location of NF-κB is controlled by a family of inhibitory proteins, IκBs, which bind NF-κB and mask its nuclear localization signal, thereby preventing nuclear uptake. In addition, increased depression has been reported to be associated with reduced levels of brain-derived neurotropic factor (BDNF), a growth factor produced by microglial cells.[36–38] BDNF induces neurogenesis, neuronal survival, and plasticity, thereby restoring the neurodegenerative damage caused by pro-inflammatory cytokines such as TNF-α. Although various studies have reported the biological effects of ASHMI herbal constituents and some of the isolated compounds,[39–43] no studies of ASHMI or compounds on CNS inflammation and BDNF production have been previously reported.

Microglial cells are the resident phagocytes and innate immune cells of the brain. Over the past few decades, there has been an increased interest in microglia, as many investigators have recognized the importance of this cell in the homeostasis, as well as various pathologies, of the central nervous system (CNS).[44] Primary microglia cultures are prevalent in neuroinflammatory research due to the similarities in phenotype to in vivo cells. Microglia cultures have been described as early as the 1930s; however, the use of cultures to study microglia function did not occur until after a method for obtaining and culturing large amounts of microglia was developed and improved upon.[44] BV-2 microglial cells derived from the cortex of mice early after birth have been used extensively in research related to neuroinflammatory neurodegenerative disorders.[45,46] In this study, we used BV-2 microglia cells and determined the effects of ASHMI, its three herbal constituents and purified flavonoids from *G. uralensis* on TNF-α secretion, NF-κB signaling pathway expression, and BDNF production.

Materials and methods

Herbal preparations and extractions and quality control

Extracts of ASHMI™ and individual herbs in ASHMI™ were manufactured by the Sino-Lion Pharmaceutical Company (a

Good Manufacturing Practice certified facility) in Weifang, China as described previously.[16]

Five flavonoids from *G. uralensis*, were isolated as before with modifications.[22] Briefly, dried aqueous extract of *G. uralensis* (200 g) was dissolved in distilled-water and loaded onto a macroporous resin column (Amberlite XAD-7 HP), and sequentially eluted with water, 20% aqueous ethanol, 70% aqueous ethanol, and 95% aqueous ethanol. 70% ethanol and 95% ethanol elutions were combined and concentrated under reduced pressure, then subjected to silica gel column chromatography, eluted with dichloromethane, dichloromethane-ethyl acetate step gradients. Sephadex LH20 and recrystallization methods were used to further purify the isolate compounds. By comparison of TLC, HPLC, and liquidchromatography—mass spectrometry (LC-MS; LCT-Premier micromass spectrometer, Waters Corp., Milford, MA) spectra with standards in our laboratory,[22] 5 flavonoids from *G. uralensis*, LQ LQG, ILQG, 7,4′-DHF, ISO were identified and Fig. 1A showed the structures of the compounds and Fig. 1B showed the presence of these compounds in 3-dimensional HPLC fingerprints of *G. uralensis*. The operating parameters of the mass spectrometer setting are optimized as: Positive ion mode; nebulizing gas at 50 L/h; Capillary and cone Voltage at 3200 V and 30 V respectively; Desolvation Temperature at 400 °C; Source Temperature at 120 °C; Desolvation gas: 500 L/h; High resolution mass acquisition Range set from *m/z* 50 to1000.

BV-2 microglial cell culture

BV-2 microglial cells were a gift from Dr. Badie Behnam, City of Hope Medical Center, CA. The cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 5% Fetal Bovine Serum (FBS) (Atlanta Biologics, Lawrenceville, GA), 1% L-glutamine (200 mM) and 1% penicillin-streptomycin at tissue culture condition. For the assay, the cells were scraped with a cell scraper, and plated in a 24-well plate at a concentration of 1×10^5 cells/ml in DMEM media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin. The plate was incubated at tissue culture condition for 48 h for

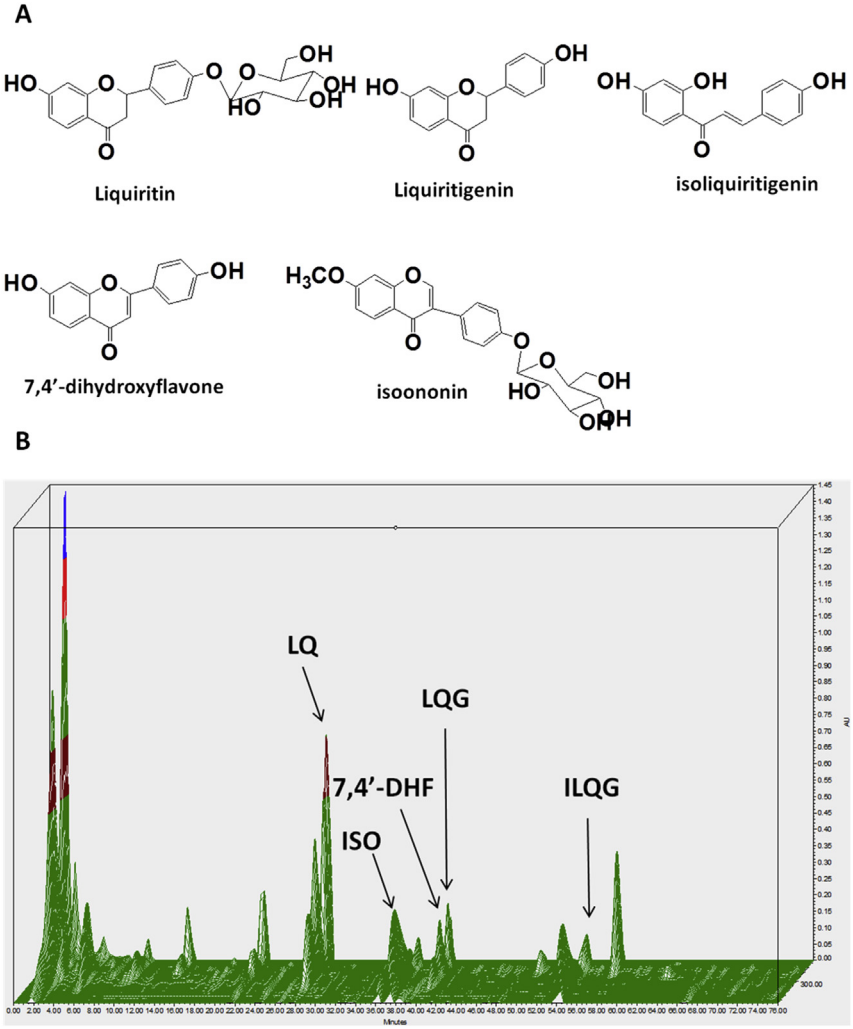

Figure 1 Compounds isolated from *G. uralensis*. **A.** Chemical structures, names of 5 compounds isolated from *G. uralensis*. **B.** 3D-HPLC-DAD fingerprint of *G. uralensis* with peaks identified corresponding to the compounds isolated.

the cells to reach confluence in the wells. The cells were either treated with ASHMI, *G. uralensis*, *G. lucidum*, or *S. flavescentis* (500—15 μg/mL). For initial screening of LQG, ILQG, 7,4′-DHF,LQ and ISO from *G. uralensis*, they were first cultured at 25 μg/ml, and LQG, ILQG, and 7,4′-DHF were then cultured (0.7—25 μg/mL), 0.1% DMSO or media (DMEM without FBS) alone and incubated at 37 °C for 8 h. After which 500 ng/mL Lipopolysaccharides (LPS) (Sigma—Aldrich, St. Louis, MO) was added to all wells including a positive control with LPS alone. Media alone wells without LPS or herb treatment served as negative control (with and without DMSO). The plates were further incubated at 37 °C for 18 h after which supernatants were collected and stored at −80 °C for cytokine ELISA.

ELISA

TNF-α and BDNF in the supernatants from the assay procedure were detected by using mouse TNF-α Mono/Mono ELISA kit (BD, San Jose, CA) and BDNF Emax® Immunol Assay System (Promega, Madison, WI) as per the protocol in the kits.

MTT assay

A 200 μl aliquot of BV2 microglial cell suspension at a concentration of 1×10^5 cells/ml was plated in each well in 96-well plate and similar concentration of herbs and LPS was maintained as described above, except 4 h prior to the end of 18 h incubation, 20 μl of MTT [3-94,5-dimethythiazol-2-yl0-2,5-diphenyltetrazolium bromide] solution (1 mg/mL) was added to each well. After 18 h the plate was removed from the incubator, the contents were aspirated from the wells and 200 μl of DMSO was added to each well. The plate was placed on a shaker for 1 h in the dark and the OD value was read at 595 nm. OD value of LPS alone was considered as 100%.

Western blot analysis

2×10^6 cells in DMEM media supplemented with 10% FBS, glutamine and 1% penicillin-streptomycin were seeded in 25 cm^2 flasks and incubated at 37 °C for 48 h. The cells were incubated with either media (DMEM without FBS), ILQG (6 μg/mL pre-treatment for 8 h) + LPS (500 ng/mL) or LPS alone for 30 and 60 min. Cytoplasmic extracts were prepared after the incubation using Nuclear Extract kit (Active Motif, Carlsbad, CA) and extracts were stored at−80 °C. Proteins from the cell extract were denatured with sample buffer (NuPage LDS sample buffer, Invitrogen) containing 10% DTT and heating for 10 min at 70 °C. Further, the denatured proteins were separated by loading 10 μg/lane on 10% SDS-gels and transferred to nitrocellulose membranes. Proteins were detected by anti-phospho-IκBalpha, anti-IκBalpha and anti-phospho-IκB-alpha, anti-IκB-alpha and anti-b-actin (loading control) mouse aniti-bodies (Cell Signaling) as primary antibody, goat anti-mouse IgG-HRP as the secondary antibody and ECL detection system (Thermo Scientific, Rockford, Illinois). The results of western blot were quantitative image analyzed by ICY software (Paris, France). IκB and p-IκB expression were modulated with β-actin expression and compared with non-stimulated, non-treated, medium alone group.

Statistics

A Students *t* test was used to compare the values between LPS stimulated and LPS stimulated plus treated culture supernatants. A *p* value <0.05 was considered to be statistically significant. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ the different levels of significance. All statistical analyses were performed using Sigma Stat software (Systat Software Inc. Point Richmond, CA, USA).

Results

ASHMI™ and herbal constituents suppress LPS-stimulated microglia cell TNF-α secretion

ASHMI™ at 125—500 μg/ml produced statistically significant ($p < 0.01$—0.001) and dose-dependent inhibition of TNF-α secretion by LPS stimulated-mouse BV-2 microglial cells when compared with LPS stimulated cells alone (Fig. 2A). IC 50 value was 116.8 μg/ml. Extracts of the individual ASHMI herbs were tested for potential inhibition of TNF-α production. Of these, *G. uralensis* produced significant and concentration-dependent inhibition of TNF-α production at all tested concentrations (15.6—500 μg/ml, $p < 0.001$, Fig. 2A). IC50 value of *G. uralensis* was 16.9 μg/mL. The other two herb constituents appeared to be less effective. *G. lucidum* only at 500 μg/ml and *S. flavescentis* at 250 μg/ml and 500 μg/ml showed significantly inhibition of TNF-α production ($p < 0.001$). Since there was no concentration-dependent data, IC50 value for *G. lucidum* and *S. flavescentis* at these tested doses were not generated. The IC 50 value of *G. uralensis* was approximately 7 fold less than ASHMI (IC50:116.8 μg/mL). Therefore *G. uralensis* might be the major contributor to ASHMI suppression of TNF-α production, although not the only one.

Cell viability was assessed by the MTT assay. Treatment with ASHMI at concentration up to 500 μg/mL did not cause any cell toxicity (Fig. 2B). There were no differences in cell viability of *S. flavescentis*- and *G. lucidum*-treated cells at any tested concentrations. *G. uralensis* at 125 μg/mL, the concentration well above its IC50 concentration (Fig. 2B), did not affect cell viability. Even at 500 μg/ml, there was only 25% of reduction of cell viability. These data suggested that ASHMI and three constituents have a high safety window.

ILQG from *G. uralensis* was a potent inhibitor of LPS-stimulated BV2 microglia cell TNF-α production

Since *G. uralensis* potently inhibited TNF-α production by BV-2 microglial cells, and a previous study showed that *G. uralensis* and the flavonoids LQ and ILQ showed clinical effects on depression-like behavioral changes,[24] we undertook identification of active flavonoids from *G. uralensis* on the TNF-α production. All five purified flavonoids, which were identified by comparison of TLC, HPLC, and LC-MC profiles with standards in our laboratory[22] as LQ, LQG, ILQG, 7,4′- DHF and ISO, at 25 μg/ml showed significant inhibition of TNF-α production (Fig. 3, $p < 0.001$).

LQG, ILQG almost eliminated TNF-α production and 7,4′-DHF suppressed TNF-α production by approximately 70%.

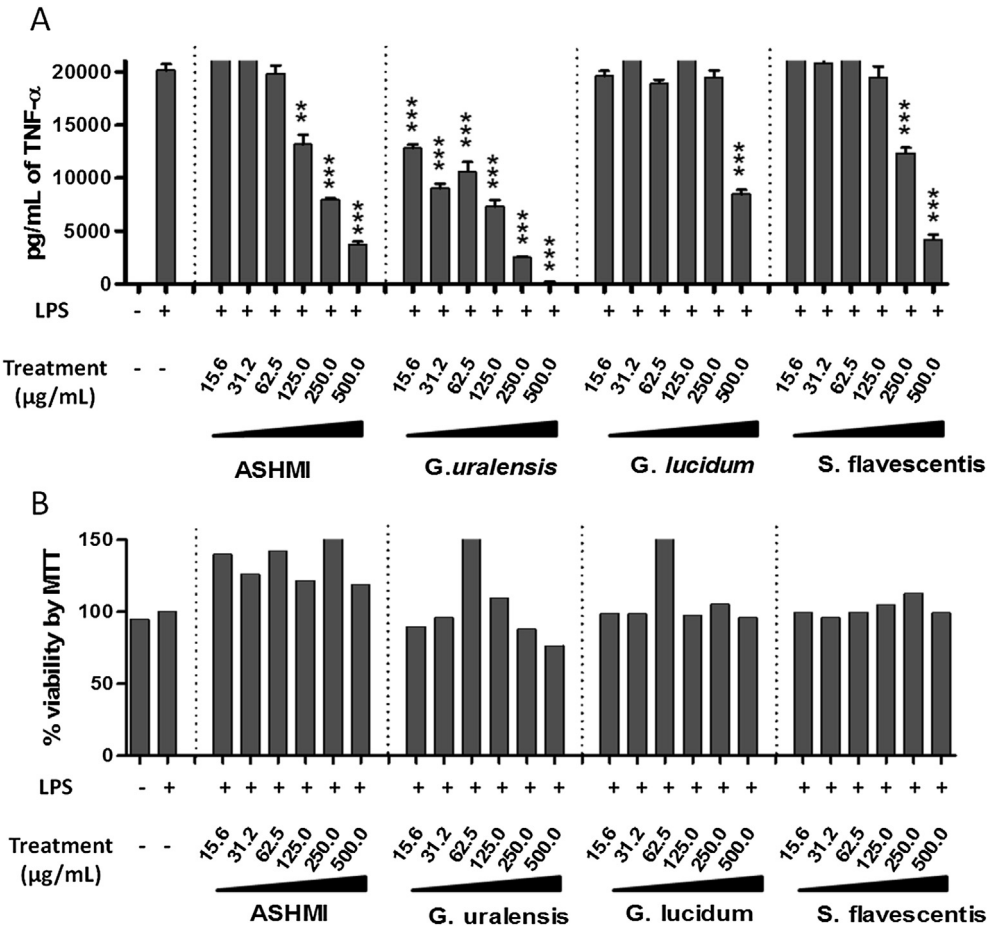

Figure 2 ASHMI™ and its 3 individual herbs inhibited TNF-α production by an LPS stimulated-mouse BV-2 microglial cells. **A**. BV-2 microglial cells were pre-incubated with or without herbal extract for 8 h, followed by addition of LPS (500 ng/mL) and culture for another 18 h. TNF-α production levels in cell culture supernatants were determined by ELISA. Data are expressed as mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ verses (Vs) LPS/No ASHMI or herb group. **B**. Cell viability was evaluated by MTT method versus stimulation alone group.

Because LQG, ILQG and 7,4′-DHF were the most effective *G. uralensis* flavonoids, we next conducted concentration-dependent study focusing on these 3 flavonoids. IC50 values of LQG, ILQG 7,4′-DHF were 4.86 μg/mL, 1.18 μg/mL and 2.21 μg/mL respectively, ILQG being most effective (Fig. 4A). ILQG at 6 μg/mL eliminated TNF-α production without cytotoxicity (Fig. 4B). Therefore, treatment concentrations of 6 μg/mL of ILQG were considered as optimal concentration for the following studies.

LQG from *G. uralensis* suppressed IkBα phosphorylation

In this study, we determined protein expression of both IκB and phosphorylation of IκB (pIκB) at different time points following LPS stimulation with or without ILQG pretreatment. p-IκB expression in the cytoplasmic extracts of BV-2 cells marked increased at 30 and 60 min after stimulation with LPS (positive control) compared to unstimulated cells (negative control) with optimal density ratio 10:1, and 250:1, respectively. Pre-treatment of cells with ILQG for 8 h resulted in reduction of p-IκB expression at 30 and 60 min compared to that in LPS stimulated, but untreated cells with optimal density ratio at 5:10 and 167:250 respectively. Consistently, ILQG pretreatment of BV2 cells also reduced LPS stimulation-induced reduction of IκB expression at 30 min capered to that in LPS stimulated, but untreated cells with optimal density 0.8:0.6 (Fig. 5).

ILQG from *G. uralensis* increased brain derived neurotrophic factor (BDNF) secretion by LPS-stimulated microglial cells

Microglial cells are also a source of BDNF secretion. However, this function can be suppressed by pro-inflammatory cytokines. It was shown that BDNF levels were negatively correlated with TNF-α level. Since ILQG potently inhibited TNF-α production, we hypothesized that it may increase BDNF production. We found significant and concentration-dependent enhancement of BDNF production by LPS stimulated microglial cells compared to untreated LPS stimulated microglial cells (Fig. 6, $p < 0.01$—0.001).

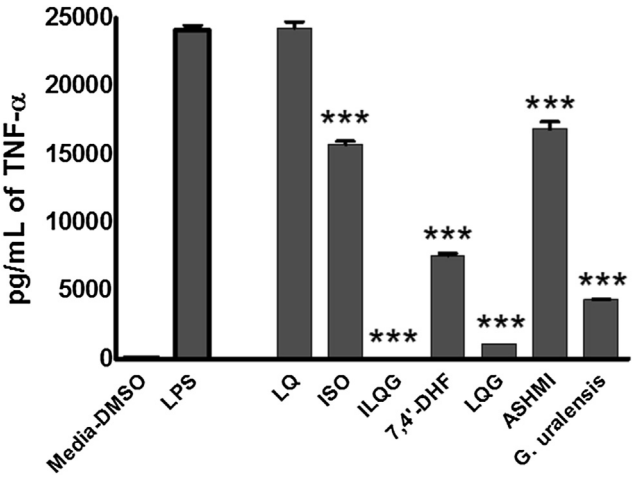

Figure 3 TNF-α production by an LPS stimulated-mouse BV-2 microglial cells treated with ASHMI™, *G. uralensis*, and 5 individual compounds isolated. BV-2 microglial cells were pre-incubated with or without treatment for 8 h, followed by addition of LPS (500 ng/mL) and culture for another 18 h. TNF-α production levels in cell culture supernatants were determined by ELISA. Data are expressed as mean ± SEM of three independent experiments. ***$p < 0.001$ verses (Vs) LPS/No treatment group.

Discussion

The prevalence of comorbid anxiety/depression symptoms with asthma is high.[9,47] The underlying mechanism for existence of comorbid stress in asthmatics is not fully understood but one theory is that stress induces upregulation of proinflammatory cytokines such as IL-1, IL-6 and TNF-α.[29,33,48,49] TNF-α is predominantly produced by monocytes and macrophages in the peripheral system. Microglial cells are the major source of TNF-α and other inflammatory cytokine production in the CNS. ASHMI, a mixture of three herb extracts has been shown to reduce airway hypersensitivity, eosinophil infiltration, goblet cell formation, and lung Th2 cytokine levels in a murine model of chronic asthma.[50] Its safety and effectiveness have been demonstrated in clinical trials. We previously showed that ASHMI and organic extract fractions inhibited TNF-α production by macrophages. In this study, we, for the first time, show that ASHMI™ reduces the production of TNF-α by LPS stimulated BV-2 mouse microglial cells in a dose-dependent manner. Consequently, we tested the 3 individual constituents in ASHMI™ and showed that *G. uralensis* showed greater suppression of TNF-α than *G. lucidum* and *S. flavescentis*. Concentrations which were effective were not toxic to the cells. *G. uralensis* might be responsible for ASHMI™ anti-TNF-α production by BV2 microglial cells.

G. uralensis, one of the most widely used Chinese herbs, has a wide range of anti-inflammatory properties. *G. uralensis* reduced circulating levels of TNF-α and IL-6, while increasing IL-10 in LPS treated mice.[51] *G. uralensis* has been shown to decrease IL-8 in lung fibroblast cells.[52] However, whether *G. uralensis* flavonoid compounds inhibit TNF-α proinflmamtory cytokine production by microglial cells have not been previously reported. Of the 5 flavonoids isolated and identified from *G. uralensis*, LQG, ILQG and 7,4′-DHF were more potent than LQ and ISO in suppression of TNF-α production (in a dose-dependent manner), ILQG being most effect with IC50 1.8 μg/ml (Table 1).

Activation of the NF-κB pathway is central to the expression of several pro-inflammatory cytokines including TNF-α. In this study we focused on ILQG and used optimal concentration to determine whether ILQG inhibits NF-κB activation by measuring p-IκB and IκB levels. Our data showed that pre-treatment of cells with ILQG resulted in reduction of p-IκB levels and IκB degradation. These results demonstrated that ILQG inhibited NF-κB signaling pathway activation. The upstream mechanisms are not investigated in this study. Toll-like receptors (TLRs) play a critical role in recognition of conserved pathogen-associated molecular patterns derived from various microbial pathogens. TLR signaling pathways trigger the activation of NF-κB mediated through both MyD88- and TRIF-dependent pathways. LPS (a TLR4 agonist) triggers both MyD88- and TRIF-dependent pathways leading to the activation of NF-κB. Se-Jeong Park et al.[53,54] demonstrated that ILQG inhibits both MyD88- and TRIF-dependent pathways and suppresses homodimerization of toll-like receptor 4 by RAW264.7 macrophages. Whether ILQG exhibits same regulatory effects on the TLR-mediated NF-κB signaling pathways on microglial cells requires further investigation in the future studies.

In addition to contributing to neurodegenerative process by inducing proinflammatory cytokines, microglia can also induce the release of neurotrophic factors such as BDNF. In our study, we observed that pre-treatment with ILQG induced BDNF production in a concentration-dependent manner in LPS-stimulated microglial cells. BDNF and its receptor tyrosine kinase receptor B (TrkB) play important roles in neurogenesis, cell survival, regulation of synaptic plasticity and maintenance of neurons throughout life.[55,56] Postmortem studies found that brains from depressed individuals contain reduced levels of hippocampal and cortical BDNF.[36,37] Direct infusion of BDNF into the brains of animals has been shown to produce an “antidepressant effect” in learned helplessness and forced swim models of depression.[38,57] Ours is the first study to show that treatment of LPS stimulated microglial cells with ILQG leads to beneficial production of BDNF.

ILQG is a chalcone compound and found in various medicinal herbs such as *G. uralensis* (licorice), *Allium ascalonicum*, *Sinofranchetia chinensis*, *Dalbergia odorifera*, and *Glycine max* L.[58] It shows various pharmacological properties including anti-cancer,[59,60] anti-inflammation, neuroprotective effects. We and others have reported ILQG showed anti-peripheral inflammatory effects. For examples ILQG inhibited LPS-stimulated pro-inflammatory cytokine(IL-6, IL12, and TNF-α) productions in bone marrow-derived dendritic cells[61]; LPS stimulated NO, IL-1 beta and IL-6 production by J774A.1 murine macrophages; LPS-induced iNOS and COX-2 expression via the attenuation of NF-κB in RAW 264.7 macrophages[62]; antigen-induced IL-4 and IL-5 production by memory Th2 cells in vitro[63]; and eotaxin-1

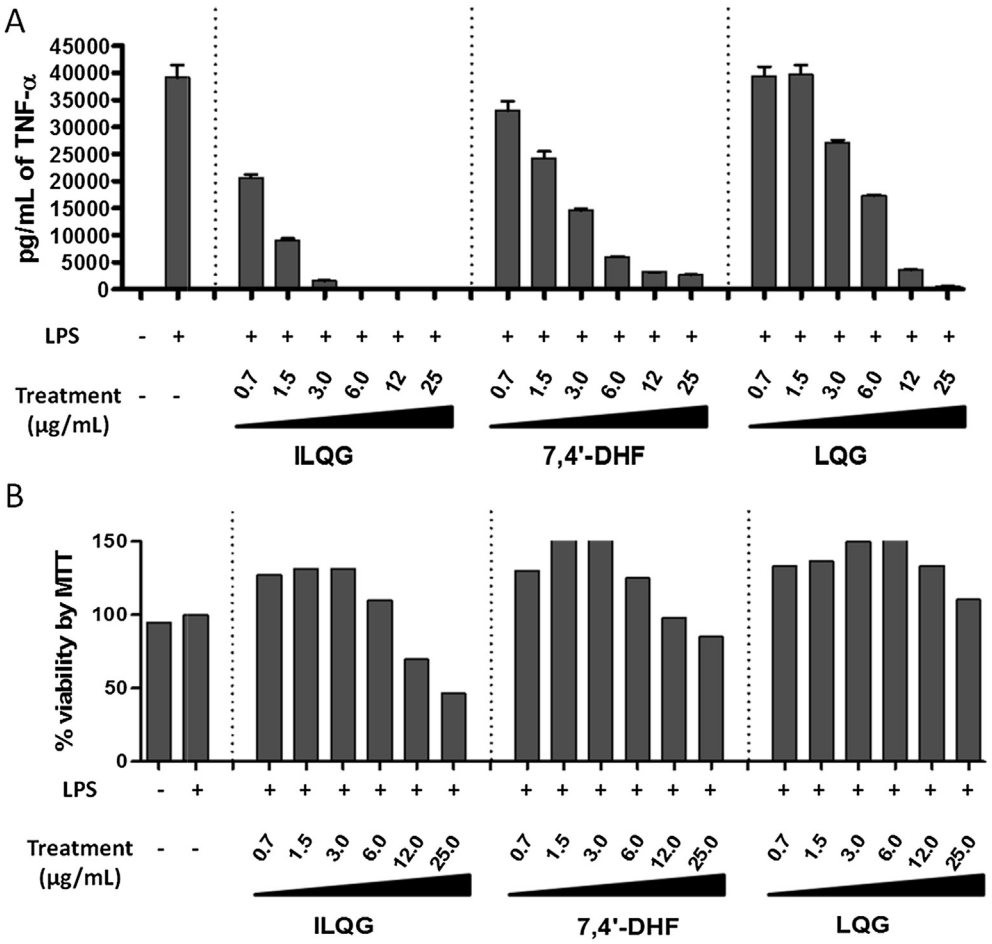

Figure 4 *G. uralensis* flavonoids LQG, ILQG 7,4′-DHF inhibited TNF-α production by an LPS stimulated-mouse BV-2 microglial cells. **A**. BV-2 microglial cells were pre-incubated with or without treatment for 8 h, followed by addition of LPS (500 ng/mL) and culture for another 18 h. TNF-α production levels in cell culture supernatants were determined by ELISA. Data are expressed as mean ± SEM of three independent experiments. **B**. Cell viability was evaluated by MTT method versus stimulation alone group.

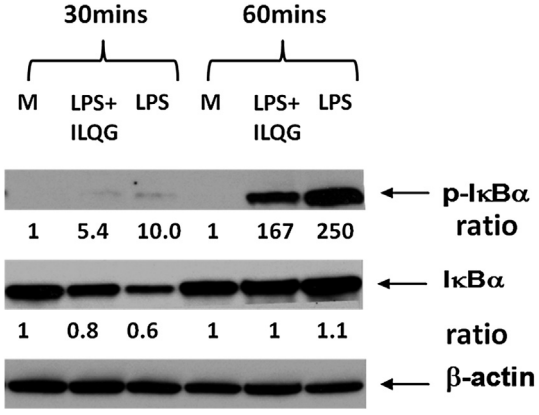

Figure 5 Regulatory effects of ILQG on p-IκBα, IκBα by LPS stimulated-mouse BV-2 microglial cells. Macrophage RAW 264.7 cells were cultured in medium or treated with ILQG (6 μg/mL) for 8 h, followed by stimulation of LPS (500 ng/mL) for 30 or 60 min. The expression of p-IκBα, IκBα and β-actin (as the control) in whole cell extracts were evaluated by western-blot analysis and quantitative ratio of p-IκBα, and IκBα expression was performed by modulated with β-actin expression and compared with medium alone group.

secretion by human fetal lung fibroblasts in vitro.[22] ILQG also was reported to have neuroprotective effect, for example, ILQG inhibited amyloid β protein-induced neurotoxicity in cultured rat cortical neurons by interfering with the increases of [Ca(2+)](i) and ROS[64]; protected against glutamate-induced mitochondrial damage and hippocampal neuronal cell death by limiting glutamate-induced oxidative stress in vitro[65]; protects against methamphetamine-induced neurotoxicity in mice.[66] These previous studies together with our new findings that ILQG suppressed TNF-α and NF-κB activation, and increased in BDNF suggest that ILQG may be a potential CNS anti-inflammatory compound, and ASHMI may have a potential for asthma patients with anxiety/depression comorbidities.

In conclusion, the prevalence of comorbid anxiety-depression symptoms is high in asthmatics, and corticosteroids for asthma treatment may enhance anxiety-depression. ASHMI has been shown to be safe and effective in patients with asthma with beneficial immunomodualtory effects. In this study, we showed that ASHMI and its herbal constituents *G. uralensis* flavonoids inhibit TNF-α production by LPS stimulated microglial cells and elevate BDNF levels. Further studies are required to fully elucidate the mechanisms of action underlying ASHMI and its

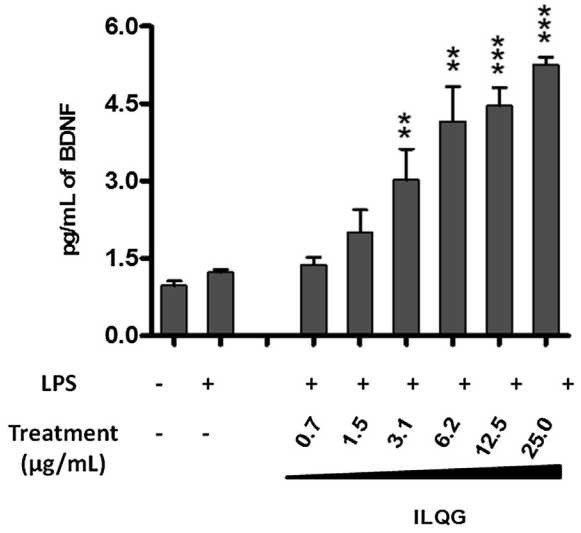

Figure 6 BDNF secretion by LPS-stimulated microglial cells treated with ILQG. BV-2 microglial cells were pre-incubated with or without ILQG for 8 h, followed by addition of LPS (500 ng/mL) and culture for another 18 h. BDNF production levels in cell culture supernatants were determined by ELISA. Data are expressed as mean ± SEM of three independent experiments.

Table 1 IC50 values of TNF-α Inhibition.

	IC_{50} (95% CI)
ASHMI	116.8 (116.8—116.8)
Gan-Cao	16.96 (15.27—18.84)
Isoliquiritigenin	1.18 (1.10—1.27)
7,4′ dihydroxyflavone	2.21 (1.89—2.58)
Liquiritigenin	4.86 (2.28—10.37)

compounds CNS anti-inflammatory effects, and to investigate clinical benefit in asthma patients with comorbid with anxiety/depression.

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Authors' disclosure

Dr. Xiu-Min Li has share on patent for the use of ASHMI (PCT/US05/08600 for ASHMI) and Herbal Spring LLC. The other authors have no financial interests to disclose.

Acknowledgment

We thank Mr. Henry Ehrlich for reading the manuscript and suggesting changes.

Abbreviations

G. lucidum *Ganoderma lucidum*
S. flavescentis *Sophora flavescentis Ait*
G. uralensis *Glycyrrhiza uralensis*
LQ liquiritin
LQG liquiritigenin
ILQG isoliquiritigenin
7,4′-DHF 7,4′-dihydroxyflavones
ISO isononin
BDNF brain derived nuerotropic
CNS central nerve system
TCM Traditional Chinese Medicine
ASHMI anti-asthma herbal medicine intervention

References

1. CDC. *National Center for Health Statistics: Asthma*; 2011. http://www.cdc.gov/nchs/fastats/asthma.htm. Accessed 18.01.12.
2. Deshmukh VM, Toelle BG, Usherwood T, et al. The association of comorbid anxiety and depression with asthma-related quality of life and symptom perception in adults. *Respirology*. 2008;13:695—702.
3. Di MF, Verga M, Santus P, et al. Close correlation between anxiety, depression, and asthma control. *Respir Med*. 2010; 104:22—28.
4. Eisner MD, Katz PP, Lactao G, et al. Impact of depressive symptoms on adult asthma outcomes. *Ann Allergy Asthma Immunol*. 2005;94:566—574.
5. Kovacs M, Stauder A, Szedmak S. Severity of allergic complaints: the importance of depressed mood. *J Psychosom Res*. 2003;54:549—557.
6. Lavoie KL, Bacon SL, Barone S, et al. What is worse for asthma control and quality of life: depressive disorders, anxiety disorders, or both? *Chest*. 2006;130:1039—1047.
7. Thomas M, Price D. Impact of comorbidities on asthma. *Expert Rev Clin Immunol*. 2008;4:731—742.
8. Oraka E, King ME, Callahan DB. Asthma and serious psychological distress: prevalence and risk factors among US adults, 2001—2007. *Chest*. 2010;137:609—616.
9. McGrady ME, Cotton S, Rosenthal SL, et al. Anxiety and asthma symptoms in urban adolescents with asthma: the mediating role of illness perceptions. *J Clin Psychol Med Settings*. 2010;17:349—356.
10. Brown ES, Khan DA, Nejtek VA. The psychiatric side effects of corticosteroids. *Ann Allergy Asthma Immunol*. 1999;83: 495—503.
11. Romero-Frais E, Vazquez MI, Sandez E, et al. Prescription of oral corticosteroids in near-fatal asthma patients: relationship with panic-fear, anxiety and depression. *Scand J Psychol*. 2005;46:459—465.
12. Cordina M, Fenech AG, Vassallo J, et al. Anxiety and the management of asthma in an adult outpatient population. *Ther Adv Respir Dis*. 2009;3:227—233.
13. Bolanos SH, Khan DA, Hanczyc M, et al. Assessment of mood states in patients receiving long-term corticosteroid therapy and in controls with patient-rated and clinician-rated scales. *Ann Allergy Asthma Immunol*. 2004;92:500—505.
14. Gift AG, Wood RM, Cahill CA. Depression, somatization and steroid use in chronic obstructive pulmonary disease. *Int J Nurs Stud*. 1989;26:281—286.
15. Zhao Y, Ma R, Shen J, et al. A mouse model of depression induced by repeated corticosterone injections. *Eur J Pharmacol*. 2008;581:113—120.

16. Kelly-Pieper K, Patil SP, Busse P, et al. Safety and tolerability of an antiasthma herbal formula (ASHMI™) in adult asthmatics: a randomized, double-blinded, placebo-controlled, dose escalation phase I study. *J Altern Complementary Med*. 2009;15:735—743.
17. Wen MC, Wei CH, Hu ZQ, et al. Efficacy and tolerability of anti-asthma herbal medicine intervention in adult patients with moderate-severe allergic asthma. *J Allergy Clin Immunol*. 2005;116:517—524.
18. Brown LL, Rusinova E, Li X. Development of an efficient fractionation procedure for the identification of active compounds in the complex ASHMI (anti-asthma herbal medicine intervention) formula. *J Allergy Clin Immunol*. 2009:S256 (Abstract).
19. Srivastava KD, Dunkin D, Liu C, et al. Effect of antiasthma simplified herbal medicine intervention on neutrophil predominant airway inflammation in a ragweed sensitized murine asthma model. *Ann Allergy Asthma Immunol*. 2014;112:339—347.
20. Li XM. Traditional Chinese herbal remedies for asthma and food allergy (current perspectives). *J Allergy Clin Immunol*. 2007;120:25—31.
21. Bensky D, Barolet R. *Chinese Herbal Medicine: Formulas & Strategies*. Seattle: Eastland Press; 1990.
22. Jayaprakasam B, Doddaga S, Wang R, et al. Licorice flavonoids inhibit eotaxin-1 secretion by human fetal lung fibroblasts in vitro. *J Agric Food Chem*. 2009;57:820—825.
23. Dhingra D, Sharma A. Antidepressant-like activity of *Glycyrrhiza glabra* L. in mouse models of immobility tests. *Prog Neuropsychopharmacol Biol Psychiatry*. 2006;30:449—454.
24. Wang W, Hu X, Zhao Z, et al. Antidepressant-like effects of liquiritin and isoliquiritin from *Glycyrrhiza uralensis* in the forced swimming test and tail suspension test in mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2008;32:1179—1184.
25. Zhao Z, Wang W, Guo H, et al. Antidepressant-like effect of liquiritin from *Glycyrrhiza uralensis* in chronic variable stress induced depression model rats. *Behav Brain Res*. 2008;194:108—113.
26. Brightling C, Berry M, Amrani Y. Targeting TNF-alpha: a novel therapeutic approach for asthma. *J Allergy Clin Immunol*. 2008;121:5—10.
27. Busse PJ, Zhang T, Termanini S, et al. Pathophysiology of late onset atopic asthma in a murine model. *J Allergy Clin Immunol*. 2006;117:S153 (Abstract).
28. Brustolim D, Ribeiro-dos-Santos R, Kast RE, et al. A new chapter opens in anti-inflammatory treatments: the antidepressant bupropion lowers production of tumor necrosis factor-alpha and interferon-gamma in mice. *Int Immunopharmacol*. 2006;6:903—907.
29. Hayley S, Poulter MO, Merali Z, et al. The pathogenesis of clinical depression: stressor- and cytokine-induced alterations of neuroplasticity. *Neuroscience*. 2005;135:659—678.
30. Dantzer R, O'Connor JC, Freund GG, et al. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci*. 2008;9:46—56.
31. Miller AH, Maletic V, Raison CL. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol Psychiatry*. 2009;65:732—741.
32. He BP, Wen W, Strong MJ. Activated microglia (BV-2) facilitation of TNF-alpha-mediated motor neuron death in vitro. *J Neuroimmunol*. 2002;128:31—38.
33. Himmerich H, Fulda S, Linseisen J, et al. Depression, comorbidities and the TNF-alpha system. *Eur Psychiatry*. 2008;23:421—429.
34. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*. 1996;274:782—784.
35. Collart MA, Baeuerle P, Vassalli P. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol Cell Biol*. 1990;10:1498—1506.
36. Dwivedi Y, Rizavi HS, Conley RR, et al. Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Arch Gen Psychiatry*. 2003;60:804—815.
37. Pandey GN, Dwivedi Y, Rizavi HS, et al. Brain-derived neurotrophic factor gene and protein expression in pediatric and adult depressed subjects. *Prog Neuropsychopharmacol Biol Psychiatry*. 2010;34:645—651.
38. Shirayama Y, Chen AC, Nakagawa S, et al. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J Neurosci*. 2002;22:3251—3261.
39. Zhang Q, Ye M. Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). *J Chromatogr A*. 2009;1216:1954—1969.
40. Bensky D, Gamble A. *Chinese Herbal Medicine: Materia Medica*. Revised ed. Seattle: Eastland Press; 1993.
41. Boh B, Berovic M, Zhang J, et al. *Ganoderma lucidum* and its pharmaceutically active compounds. *Biotechnol Annu Rev*. 2007;13:265—301.
42. Liu G, Dong J, Wang H, et al. Characterization of alkaloids in *Sophora flavescens Ait*. by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *J Pharm Biomed Anal*. 2011;54:1065—1072.
43. Wang SY, Hsu ML, Hsu HC, et al. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int J Cancer*. 1997;70:699—705.
44. Stansley B, Post J, Hensley K. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *J Neuroinflammation*. 2012;9:115.
45. Blasi E, Barluzzi R, Bocchini V, et al. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *J Neuroimmunol*. 1990;27:229—237.
46. Mazzolla R, Barluzzi R, Brozzetti A, et al. Enhanced resistance to *Cryptococcus neoformans* infection induced by chloroquine in a murine model of meningoencephalitis. *Antimicrob Agents Chemother*. 1997;41:802—807.
47. Strine TW, Mokdad AH, Balluz LS, et al. Impact of depression and anxiety on quality of life, health behaviors, and asthma control among adults in the United States with asthma, 2006. *J Asthma*. 2008;45:123—133.
48. Berthold-Losleben M, Himmerich H. The TNF-alpha system: functional aspects in depression, narcolepsy and psychopharmacology. *Curr Neuropharmacol*. 2008;6:193—202.
49. Joachim RA, Noga O, Sagach V, et al. Correlation between immune and neuronal parameters and stress perception in allergic asthmatics. *Clin Exp Allergy*. 2008;38:283—290.
50. Zhang T, Srivastava K, Wen MC, et al. Pharmacology and immunological actions of a herbal medicine ASHMI on allergic asthma. *Phytother Res*. 2010;24:1047—1055.
51. Kim JK, Oh SM, Kwon HS, et al. Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages. *Biochem Biophys Res Commun*. 2006;345:1215—1223.
52. Matsui S, Matsumoto H, Sonoda Y, et al. Glycyrrhizin and related compounds down-regulate production of inflammatory chemokines IL-8 and eotaxin 1 in a human lung fibroblast cell line. *Int Immunopharmacol*. 2004;4:1633—1644.
53. Park SJ, Youn HS. Suppression of homodimerization of toll-like receptor 4 by isoliquiritigenin. *Phytochemistry*. 2010;71:1736—1740.

54. Park SJ, Song HY, Youn HS. Suppression of the TRIF-dependent signaling pathway of toll-like receptors by isoliquiritigenin in RAW264.7 macrophages. *Mol Cells*. 2009;28:365—368.
55. Chen CC, Hsu LW, Huang LT, et al. Chronic administration of cyclosporine A changes expression of BDNF and TrkB in rat hippocampus and midbrain. *Neurochem Res*. 2010;35: 1098—1104.
56. Webster MJ, Herman MM, Kleinman JE, et al. BDNF and trkB mRNA expression in the hippocampus and temporal cortex during the human lifespan. *Gene Expr Patterns*. 2006;6: 941—951.
57. Siuciak JA, Lewis DR, Wiegand SJ, et al. Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). *Pharmacol Biochem Behav*. 1997;56:131—137.
58. Woo J, Cho S, Lee CJ. Isoliquiritigenin, a chalcone compound, enhances spontaneous inhibitory postsynaptic response. *Exp Neurobiol*. 2014;23:163—168.
59. Hirchaud F, Hermetet F, Ablise M, et al. Isoliquiritigenin induces caspase-dependent apoptosis via downregulation of HPV16 E6 expression in cervical cancer Ca Ski cells. *Planta Med*. 2013;79:1628—1635.
60. Wang Z, Wang N, Han S, et al. Dietary compound isoliquiritigenin inhibits breast cancer neoangiogenesis via VEGF/VEGFR-2 signaling pathway. *PLoS One*. 2013;8:e68566.
61. Li W, Sun YN, Yan XT, et al. Flavonoids from Astragalus membranaceus and their inhibitory effects on LPS-stimulated pro-inflammatory cytokine production in bone marrow-derived dendritic cells. *Arch Pharm Res*. 2014;37:186—192.
62. Kim JY, Park SJ, Yun KJ, et al. Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-kappaB in RAW 264.7 macrophages. *Eur J Pharmacol*. 2008;584:175—184.
63. Yang N, Patil S, Zhuge J, et al. *Glycyrrhiza uralensis* flavonoids present in anti-asthma formula, ASHMI, inhibit memory Th2 responses in vitro and in vivo. *Phytother Res*. 2013;27: 1381—1391.
64. Lee HK, Yang EJ, Kim JY, et al. Inhibitory effects of *Glycyrrhizae radix* and its active component, isoliquiritigenin, on Abeta(25-35)-induced neurotoxicity in cultured rat cortical neurons. *Arch Pharm Res*. 2012;35:897—904.
65. Yang EJ, Min JS, Ku HY, et al. Isoliquiritigenin isolated from *Glycyrrhiza uralensis* protects neuronal cells against glutamate-induced mitochondrial dysfunction. *Biochem Biophys Res Commun*. 2012;421:658—664.
66. Lee MJ, Yang CH, Jeon JP, et al. Protective effects of isoliquiritigenin against methamphetamine-induced neurotoxicity in mice. *J Pharmacol Sci*. 2009 Oct;111:216—220, 216 -20 2009; 111.