

Identification of Possibility of *Glycyrrhiza uralensis* as an Allergen by Protein Analysis

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Abstract Various secondary metabolites derived from plants are useful for humans to preserve their health. For this reason, medical herbs have been consumed universally without deep research about the dangerousness of these substance. However, they can produce unexpected adverse reactions in humans. Among these side effects, allergic reactions involving human immunoglobulin E can occur due to antimicrobial or antibacterial substances in plants. *Glycyrrhiza uralensis* has been prescribed for the treatment of numerous diseases given its multiple types of pharmaceutical efficacy, including its antimicrobial and antibacterial activities. Therefore, we researched whether *Glycyrrhiza uralensis*, which has antimicrobial ability, can cause allergic reaction in humans. To test the hypothesis devised here, we conducted proteomic level experiments. We utilized SDS-PAGE using a protein extract of *Glycyrrhiza uralensis*, with Coomassie blue staining to confirm the protein pattern and western blotting to identify Human IgE binding proteins in *Glycyrrhiza uralensis*. Among the many candidate sera, these methods detected three positive sera. Moreover, an identified protein section with reactivity associated with Human IgE was analyzed by LC-MS/

MS. Our experimental data provide a basis for testing the possibility of *Glycyrrhiza uralensis* as an allergen by identifying the reactivity between human Immunoglobulin E and proteins of *Glycyrrhiza uralensis*.

Keywords: IgE, Allergy-Q, Herbal medicine, Plant protein

Introduction

Plants have provided the motivation to produce new drugs throughout history. Medicines originating from plant have had positive effects on the maintenance of health and welfare of mankind¹ as plants natural sources. In addition, diverse bioactive compounds used to create medicines have been found in plants. There is an infinite amount of potential in plants². This explains why natural products have long been a favorite for those who create medicines, especially given their significant efficacy, selectivity and safety. Natural therapies using herbal medicines have been developed as a means increasing immunity and boosting resistance against influenza and diabetes. Until recently, diverse new pharmacological actions of plants are reported such as anti-mutagenicity of purple rice husk and liver injury alleviation of Korean red ginseng^{3,4}.

These benefits commonly come from a diverse range of botanical secondary metabolites. Secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids have been derived from plants. Specifically, with these metabolites, it is feasible to create antimicrobial products in vitro⁵. For example, flavonoids which originate from plants which act as pigments reportedly

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offer many benefits to humans, such as antioxidant and anti-proliferative effects, as well as the inhibition of inflammatory cells and guarding against breast cancer. Therefore, foods including natural compounds attract public attention⁶. However, at the same time, certain hydroxy-flavans, a type of flavonoid, can be allergenic, and those who ingest such substances, especially flavonoids and phenolic compounds associated with flavonoids, can experience toxic effects⁷.

We refer to an agent that causes the death of microscopic organisms or that arrests the development of microorganisms an 'antimicrobials' or 'antimicrobial substance'. Among antimicrobials, antibiotics are especially defined as commonly in contact with fungi and bacteria. Antimicrobials are commonly used as a remedy for bacterial infections by repressing the synthesis of the cell wall, interrupting the bacterial membrane, or blocking the bacterial metabolic process. Antibiotics, which are also antimicrobials, protect the body from infectious diseases caused by microbes as a first line of defense⁴. They kill bacteria or fungi using many mechanisms, including the targeted suppression of the synthesis of the cell wall, restraining the synthesis of membranes or disturbing the action of bacterial enzymes. Though for most antibiotics, the usual dose is regarded as safe given the reduced concern over impurities in the preparations, some antimicrobials can cause severe side effects that damage humans health⁸. Antibiotics are well known as a common allergen causing factor, akin to pollen, mold, dust mites, and pet dander. However, the prevalence rates have not yet been precisely calibrated. Plant-derived antimicrobials can alleviate many unexpected adverse effects, such as erythema, fever, and eczema which may be caused by antibiotics which were synthesized artificially. They may also be an effective means of curing those who suffer from infectious diseases at the same time⁹. For example, penicillin from *Penicillium* can causes allergic reactions or allergic shock in the human body. Even plant-derived allicin, an antimicrobials found in garlic (*Allium sativum*), has been reported as an antigen associated with different forms of allergic contact dermatitis, including eczema and irritant contact dermatitis. For these reasons, plants are recognized as one of the main allergen inducers which react with Human IgE, and many advanced countries are aware of the potential of Type 1 allergies from various factors, including plants, as a serious matter, as Type 1 allergies results in serious disease and even death from anaphylaxis¹⁰. It is also on record that anaphylactic reactions from food or plants are associated with numerous cases of death and emergency situations¹¹.

Glycyrrhiza species are among the most commonly

used herbal medicines internationally. They are also recognized as a treatment for many diseases, particularly peptic ulcer, hepatitis C, and pulmonary and skin conditions. In addition, *Glycyrrhiza* species have numerous pharmacological effects, such as antimicrobial, anti-inflammatory, anticancer, anti-oxidative, and antiviral properties¹². For instance, Iso-flavonoids and coumarins from *Glycyrrhiza uralensis* extract have the potential to suppress pathogenic bacteria¹³. In addition, liquiritin of *Glycyrrhiza uralensis* shows antidepressant-like activity in a mouse model. Many studies have identified the beneficial aspects of *Glycyrrhiza uralensis* but data focusing on the disadvantages or risks of *Glycyrrhiza uralensis* are rare¹⁴. Although only a small percentage of reported opposite drug effects indicated allergic reactions to antibiotics, growing health care costs and higher death rates are clearly related to these outcomes¹⁵. Furthermore, herbal medicines, which are already used widely for prescriptions (such as *Astragalus membranaceus* and *Prunus armeniaca*) reportedly contain proteins containing allergy-causing factors associated with Human IgE^{16,17}.

We started this research based on an idea about the allergy-causing potential of *Glycyrrhiza*, which can arise due to the antimicrobial properties of *Glycyrrhiza*. Our research is based on the Allergy-Q test system, which is a diagnostic kit for the detection of allergens through measurements of the IgE reaction intensity of responses to IgE-specific allergens.

Results

Propose the Possibility of *Glycyrrhiza uralensis* as an Allergen

Diverse secondary metabolites, such as tannins, flavonoids, alkaloids or organic acid in plants, contribute to human health as they are used to create medicines given their broad range of pharmacological properties². However, specific secondary metabolites such as antimicrobial and antibiotic substances can trigger allergic reactions⁷. To evaluate whether *Glycyrrhiza uralensis* can cause allergic reactions, whether derived from secondary metabolites or not, we studied the existence of proteins and measured the IgE intensity in isolated proteins of *Glycyrrhiza uralensis* in 400 volunteers who provided human sera using Allergy-Q diagnostic kit. Through the SDS-PAGE and Coomassie blue staining of the *Glycyrrhiza uralensis* extract, we confirmed the extract has several proteins (Figure 1A). We observed a significant increase in the IgE intensity in three sera among the 400 sera tested and selected positive sera using Allergy-Q test (Figure 1B). The Allergy-Q test

is diagnostic kit for allergen detection which works by measuring IgE intensity levels^{16,17}. The results here suggest that the allergic potential of *Glycyrrhiza uralensis* means that it can act as an allergen.

Glycyrrhiza uralensis Extract Reacts with Human Immunoglobulin E on Positive Sera

To examine whether *Glycyrrhiza uralensis* has the potential to induce allergic reactions by binding with human IgE, we observed the human IgE intensity between isolated proteins of *Glycyrrhiza uralensis* and selected three positive sera and three negative sera using western blots.

We confirmed the reactivity of *Glycyrrhiza uralensis* extract against human IgE on different membranes

with the selected positive sera using western blotting as well as reactions in a TMB solution. On the three selected positive sera, we observed protein bands, binding with human IgE using western-blotting (Figure 2A). Additionally, we attempted to observe the protein bands of *Glycyrrhiza uralensis* in the three negative human sera using western blotting. However, None were detected other than faint IgE-binding bands (Figure 2C). The TMB solution was used to distinguish unspecific reactions from specific reactions. In the positive sera, we secured two out of three membranes showing clear IgE-binding bands. On the other hand, there were no detected bands on the negative candidates when tested with the TMB solution. Accordingly, the faint bands detected on the negative sera are evidence of indirect, unspecific IgE binding. As a result, we realized that Among the proteins of *Glycyrrhiza uralensis*, some proteins bind to human IgE (Figure 2B and 2D).

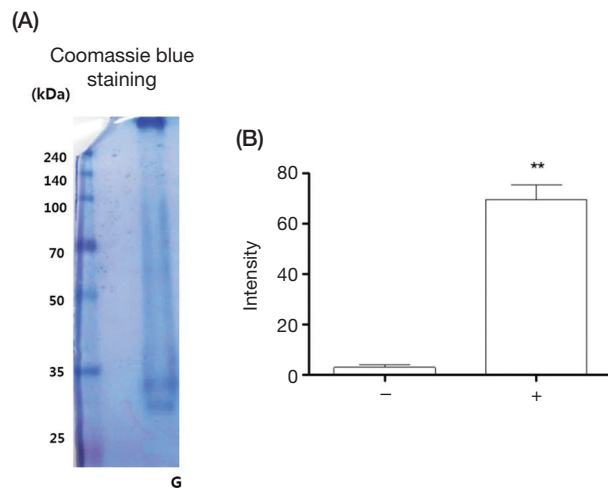


Figure 1. (A) *Glycyrrhiza uralensis* proteins were analyzed by SDS-PAGE and Coomassie blue staining. In this study, we selected three positive candidate sera shown marked intensity with immunoglobulin E and by contrast, sera that displayed few reactions with IgE were set as negative candidates among the 400 candidate sera. G; *Glycyrrhiza uralensis* extract. (B) Reactivity of *Glycyrrhiza uralensis* with human immunoglobulin E was detected by Allergy-Q. With a paired t-test, Allergy-Q system was detected. (-); negative sera, (+); positive sera, ** $p < 0.01$.

Identification of the Candidate Protein of the *Glycyrrhiza uralensis* Kernel with Human IgE Binding Protein

To identify the proteins of *Glycyrrhiza uralensis* which were human IgE-binding proteins, we undertook the in-gel digestion of the indicated number of detected human IgE antibodies (see the arrows in Figure 2B) and analyzed the human IgE detected proteins at the same locations on digested SDS-PAGE gels by LC-MS/MS (Table 1).

Discussion

In this experiment, we utilized SDS-PAGE with Coomassie blue staining, western blotting, and membranes which react to a TMB solution using *Glycyrrhiza uralensis* extract based on Allergy-Q tests, which is an allergen diagnostic method for detecting the intensity of IgE-binding proteins against any materials containing allergens. Using the Allergy-Q tests, we se-

Table 1. Identified *Astragalus membranaceus* IgE binding proteins.

No.	NCBI BLAST	Protein name	Score	Mass
1	KOM56729.1	hypothetical protein LR48_Vigan10g262100 [Vigna angularis]	48	31239
2	KRH05635.1	hypothetical protein GLYMA_17G239000 [Glycine max]	43	49948
3	GAQ89059.1	hypothetical protein KFL_004830100 [Klebsormidium flaccidum]	43	285819
4	AOV62774.1	1-deoxy-D-xylulose-5-phosphate reductoisomerase [Morus alba]	45	51580
5	AAD27874.2	class III chitinase, partial [Sphenostylis stenocarpa]	45	29318
6	XP_008379190.1	PREDICTED: probable methyltransferase PMT9 [Malus domestica]	43	71293
7	XP_016477507.1	PREDICTED: golgin subfamily A member 8H-like [Nicotiana tabacum]	43	41111

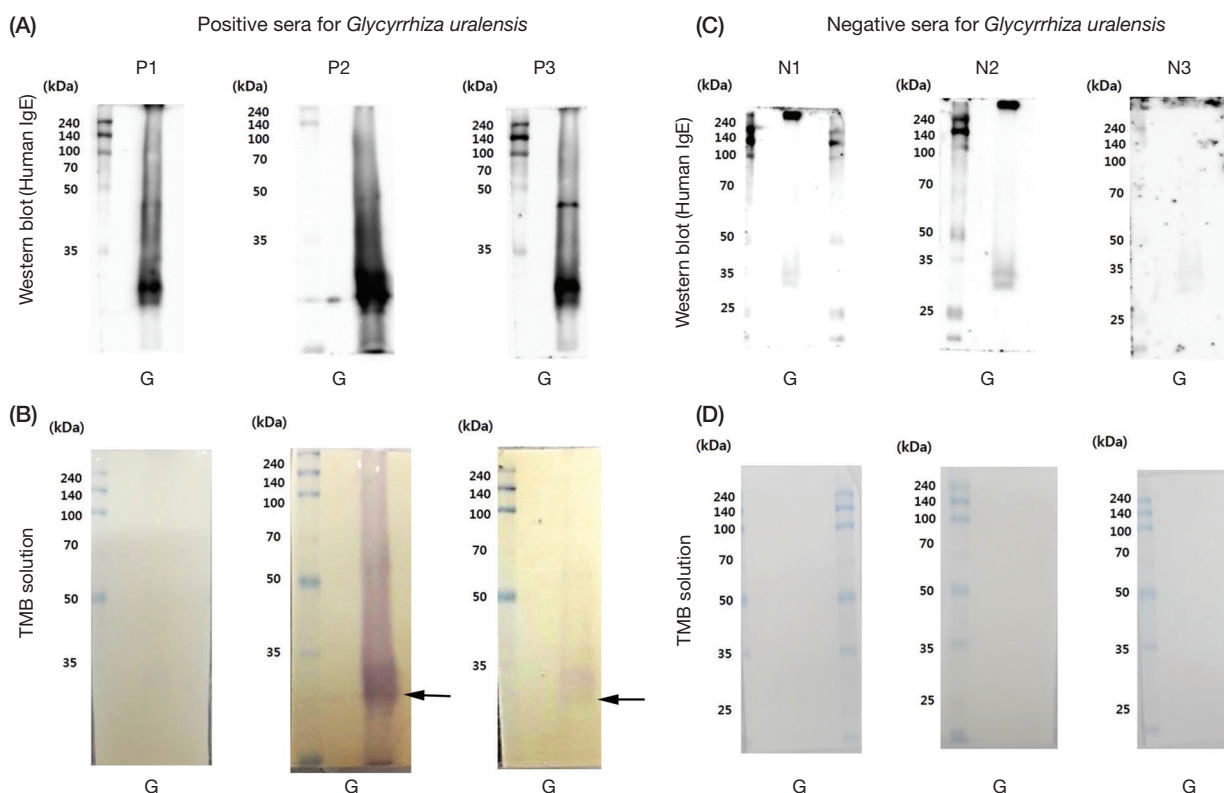


Figure 2. Western-blot analysis and TMB reaction on the selected candidate sera was identified on the nitrocellulose membrane (A and B). The human IgE reactivity was observed on the positive and negative candidate sera. The specific IgE-binding on positive candidate sera (arrow around 35 kDa) were analyzed by LC-MS/MS, and, the sera of negative candidate of IgE reactivity were indicated (C and D). Each membrane was verified by TMB solution to prevent unspecific binding proteins under each nitrocellulose membrane. G; *Glycyrrhiza uralensis* extract.

lected three positive sera and three negative sera from the original randomized 400 human sera. As a result, we determined the patterns of the protein bands in the *Glycyrrhiza uralensis* extract. In addition, high intensity bands from the strong combination with IgE were detected via western blotting of the three positive sera, but only two sera maintained the color reaction with the TMB substrate.

Similarly, in the case of the negative sera, the bands related to the IgE reactivity were dimly visible, but they did not show a color reaction in the TMB solution, signifying that the weak intensity band detected was an unspecific response. It is known that *Glycyrrhiza uralensis* is well known for its pharmacological actions, especially for ulcers, hepatitis C, and pulmonary and skin disease, and that some protein of *Glycyrrhiza uralensis* are used for the production of medication. High-intensity bands showed strong reactivity with human IgE according to western blotting, and the TMB reactions on the positive human sera. And that band were isolated and analyzed by liquid chromatog-

raphy coupled to tandem mass spectrometry (LC-MS/MS) to identify the existing constituent components which can bind with human immunoglobulin E. Table 1 presents a list of the analyzed proteins which bind to human IgE in the isolated proteins of *Glycyrrhiza uralensis*. Through an analysis of the results from LC-MS/MS, we enumerated the names of the identified proteins of *Glycyrrhiza uralensis* (Table 1). Hypothetical proteins have functional roles in reaction to magnesium ion binding, zinc ion binding, and manganese ions in plants¹⁸. While the functional roles of the hypothetical proteins (Locus: KOM56729.1, KRH05635.1, and GAQ89059.1) of *Glycyrrhiza uralensis* remain unknown, several identified hypothetical proteins which have been used to find allergens with consensus sequences of *N*-glycosylation in almond kernels and *Aspergillus fumigatus*^{19,20}. Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. Specifically, plant chitinases attack pathogens directly, conferring disease resistance by degrading chitin, a component of fungal cell walls²¹. However, class III

chitinase reportedly acts as an allergen without a hev-ein domain in latex^{22,23}. Even in the absence of reports of other proteins regarding the possibility of allergic activities apart from hypothetical proteins and chitinase, further study is warranted to examine whether or not the IgE binding domain exists in these substances. For instance, the probable methyltransferase PMT9 and golgin subfamily A member 8H-like are transmembrane bitopic proteins localized in the golgi apparatus membrane. 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) is an enzyme which converts 1-deoxy-D-xylulose 5-phosphate (DXP) and 2-C-methyl-D-erythritol 4-phosphate (MEP)²⁴. This enzyme is responsible for the terpenoid biosynthetic pathway in *E. coli*²⁵.

Our group assessed whether an IgE reaction tested on western blotting using human IgE could be caused by *Glycyrrhiza uralensis*. Although we identified IgE-binding proteins using human sera which showed positive candidates from an isolated protein of *Glycyrrhiza uralensis*, 2D gel electrophoresis, associated with spot analyses, and direct tests of the binding regions in terms of the protein structure are needed for a specific and precise evaluation of IgE binding proteins. As noted in the introduction, numerous and diverse plants have been used in traditional herbal medicines. For example, it has been reported that more than 400 herbal remedies are used traditionally only for diabetes mellitus²⁶. In fact, many developing countries depend on plant-derived traditional medicines, as various plant extracts can be used for one or more diseases and can create strong active components. For these reasons, globally, the importance of plants as a new medicines source is expected to increase. *Glycyrrhiza uralensis*, the experimental material here, which is indispensable for the creation of medicinal herbs, has been used to develop new drugs. For instance, some components of *Glycyrrhiza uralensis* extract can reportedly control neuronal cell death. Moreover, it is likely that an extract of *Glycyrrhiza uralensis* will be used to develop unprecedented medicines for Parkinson's disease given their ability to control the death of dopaminergic neuronal cells²⁷. When plants are used for the development of new pharmaceuticals to the extent they are at present, it is necessary to reconsider the dangerousness of using plants. Our results showed that some proteins in *Glycyrrhiza uralensis* extract can respond to human IgE in relation to allergic reactions. Thus, they can be an allergen to humans. Clearly, hot water extracts of plants may have only a slight potential to cause allergies in humans due to protein denaturation. However, great cautiousness is clearly needed when using medicinal plants, especially *Glycyrrhiza uralensis*, as a

treatment or novel drug candidate. Therefore, we suggest some risks or the likelihood of side effects given the possibility of causing allergies, particularly when using *Glycyrrhiza uralensis* as a prescription and using it for the development of medications or therapeutic agents.

Materials and Methods

Herbal Protein Isolation

Herbal medicine was purchased from Omniherb (Yeoncheon, Republic of Korea)²⁸. The herbal tissue was shock frozen in liquid nitrogen, and then ground using a mortar and pestle until a smooth paste was achieved. Herbals were vortexed (Scientific Industries, USA) and soaked overnight in 20 mM Tris buffer (pH 8.5) at 4°C. After incubated at 4°C, the buffer were centrifuged on 13,000 rpm. Supernatant derived from the buffer was isolated and measured in protein quantitative analysis. Isolated supernatant concentration was then determined using Bradford reagent (Bio-Rad, USA) and a microplate reader (Bio-Rad, USA). All herbal extracts were stored at -80°C¹⁶.

SDS-PAGE and Western Blot Analysis

Prunus armeniaca kernel were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The represented positive allergen about house dust mite, *Dermatophagoides farinae* was purchased by Diagnostische Systeme & Technologien GmbH (DST, Schwerin, Germany). DokDo-MARK Broad-Range Protein Ladder (Elpis bio, Daejeon, Republic of Korea) was used as molecular weight markers to estimate protein size. To staining the separated proteins on SDS-PAGE, Coomassie G-250 Stain (Bio rad) was used²⁹. The separated proteins were transferred from the gel to a nitrocellulose membrane (NC) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio rad). And the NC membranes were then blocked with 3% Bovine Serum Albumin (BSA) for 1 hour at room temperature, and then incubated overnight with the patient sera, which was diluted 1 : 5 in 3% BSA. After washing with PBS-Tween (PBS-T), the membrane was incubated with HRP-labeled goat IgG-anti-human IgE (Thermo Scientific, Rockford, IL 61105 USA), which was diluted 1 : 5,000 in 3% BSA, for 1 hour at room temperature. After rinsing with PBS-T, the membrane was incubated with Luminata Crescendo Western HRP substrate (EMD Millipore, Billerica, MA, USA) for 5 minutes at room temperature³⁰. In case of TMB substrate reaction, NC membranes detected secondary an-

ti-body of HRP-labeled human IgE was incubated on TMB solution (Sigma) during 5-15 minutes.

Study Participants

Between June and December of 2014, participants who volunteered the blood donation at Kyung Hee Medical Center for the diagnosis of their allergic diseases were enrolled in our study. All patients' sera sampling for Allergy-Q test was performed. Informed consent was obtained from all patients. The Institutional Review Board of the Kyung Hee University College of Korean Medicine approved this study (IRB no. KOMCIRB-140821-HR-003).

In vitro Allergen sIgE Measurements

Serum aliquots were sent to the laboratory without any patient identification. We performed allergen sIgE detection using 2 different systems simultaneously (PROTIA™ Allergy-Q; Proteometech, Seoul, Republic of Korea). The Allergy-Q test was operated according to the manufacturers' instructions.

In-gel Digestion with Trypsin and Extraction of Peptides

Protein bands from SDS-PAGE gels were excised and in-gel digested with trypsin according to established procedures³¹. In brief, protein bands were excised from stained gels and cut into pieces. The gel pieces were washed for 1 h at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile (ACN). Following the dehydration of gel pieces in a centrifugal vacuum concentrator (Biotron, Inc., Incheon, Korea) for 10 min, gel pieces were rehydrated in 50 ng of sequencing grade trypsin solution (Promega, Madison, WI, USA). After incubation in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight, the tryptic peptides were extracted with 5 µL of 0.5% TFA containing 50% (v/v) ACN for 40 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator. Prior to mass spectrometric analysis, the peptides solution was subjected to a desalting process using a reversed-phase column³². In brief, after an equilibration step with 10 µL of 5% (v/v) formic acid, the peptides solution was loaded on the column and washed with 10 µL of 5% (v/v) formic acid. The bound peptides were eluted with 5 µL of 70% ACN with 5% (v/v) formic acid.

Identification of Proteins by LC-MS/MS

After desalting, eluted tryptic peptides were separated

and analyzed using a nano ACQUITY UPLC (Waters, Milford, MA, USA) directly coupled to a Finnigan LCQ DECA ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA). In brief, the peptides were bound to the ACQUITYUPLC peptide BEH C18 column (1.7 µm size, 130Å in pore size, 100 µm × 100 mm) with distilled water containing 0.1% (v/v) formic acid and the bound peptides were eluted with a 40 min gradient of 0-90% (v/v) acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 0.4 µL/min. For tandem mass spectrometry, a full mass scan range mode was $m/z = 400 - 2000$ Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with relative collision energy of 55%. The individual spectra from MS/MS were processed using the SEQUEST software (Thermo Quest, San Jose, CA, USA) and the generated peak lists were used to query NCBI database using the MASCOT program (Matrix Science Ltd., London, UK). We set the modifications of methionine and cysteine for MS analysis and tolerance of peptide mass was 2 Da. MS/MS ion mass tolerance was 1 Da, allowance of missed cleavage was 1, and charge states (+1, +2, and +3) were taken into account for data analysis. We took only significant hits as defined by MASCOT probability analysis³³.

Statistical Analysis

The significant data was analyzed using Prism 5 software with paired t-test. Bars exhibit SEMs of means for triplicate assays. Statistical significance of IgE detection using Allergy-Q was analyzed by paired t-test (P cutoff value = 0.05).

Author Contribution

Eun-Jin An and Kang-Hoon Kim equally contributed to overall study in this article. In-Seung Lee isolates herbal protein. Ji Young Park and Yumi Kim performed western blot assay. Daeho Kwon and Hyeung-Jin Jang designed all academic study.

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Human and Animal Rights All human blood samples were approved by the Institutional Review Board of the Kyung Hee University College of Korean Medicine (IRB

no. KOMCIRB-140821-HR-003).

Conflict of Interest Eun-Jin An declares no conflict of interest. Kang-Hoon Kim declares no conflict of interest. In-Seung Lee declares no conflict of interest. Ji Young Park declares no conflict of interest. Yumi Kim declares no conflict of interest. Woo Sang Jung declares no conflict of interest. Daeho Kwon declares no conflict of interest. Hyeung-Jin Jang declares no conflict of interest.

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