

Analysis of Immunogenicity of *Acanthopanax senticosus* Injection

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Abstract

The cause of the immunogenicity of *Acanthopanax senticosus* injection has been studied. The method used for the preparation of the injection includes boiling the plant material in water, precipitation with ethanol, and filtration to produce the extract. In the current study, this extract was treated with saturated ammonium sulfate to collect the protein from the extract. Indicators of systemic allergy, including total immunoglobulin (IgE), interleukin -4 (IL-4), and interferon-gamma (IFN- γ) levels in the antiserum of Guinea pigs sensitized with this protein extract were used to determine the immunogenicity of the protein extract. Testing was performed using Western blotting to identify IgE in antiserum binding in Guinea pigs sensitized by injection. The target protein band was further identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The protein extract from *A. senticosus* caused positive reactions and significantly increased the levels of total IgE, IL-4, and IFN- γ in the serum of sensitized Guinea pigs. Western blotting showed that a group of proteins with a molecular weight of 60 000 in the *A. senticosus* protein extract acted as the major immunogenic protein by binding to serum IgE of Guinea pigs sensitized with *A. senticosus* injection. The protein was a pyruvate kinase homologous protein with high sequence similarity to the pyruvate kinase protein of *Vitis vinifera*. It was concluded that the allergic reaction caused by the injection of *A. senticosus* may be related to the interaction of these macromolecular proteins within the body.

Keywords

immunogenicity, *Acanthopanax senticosus* injection, Western blotting, MALDI-TOF-MS, allergic reaction

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Acanthopanax senticosus (Rupr. et Maxim.) Harms (Family: Araliaceae) injection is a traditional Chinese medicine, utilizing extracts from the roots, rhizomes, and stems of the species.¹ It has the functions of strengthening the liver and kidneys, strengthening bone, and can improve and regulate the body's immune function.^{2,5} Since the publication of the first case report in 1988, reports of its adverse reactions have gradually increased.⁶ Of the 33 types of traditional Chinese medicine injections (TCMI) listed in the 2004 National Essential Drugs List, the number of adverse drug reaction (ADR) reports for *A. senticosus* ranked fourth.⁷ The recorded ADR of *A. senticosus* injection includes rash, dizziness, headache, and fever. According to the World Health Organization (WHO), ADR severity grading standard, *A. senticosus* injection can be life-threatening grade I and can lead to pathophysiological changes in various organs.⁸ As a result, serious complications from the use of the injection cannot be ignored and the allergic compounds of *A. senticosus* injection urgently require identification and quality improvement.

The production of *A. senticosus* injection is by water extraction and alcohol precipitation. The main disadvantage of this method is that it is impossible to completely remove

macromolecular substances such as plant proteins. Indirect enzyme-linked immunosorbent assay (ELISA) and ultrafiltration electrophoresis experiments have been used to detect the presence of certain macromolecular proteins in *A. senticosus* injection, and the results suggest that these proteins have strong immunogenicity and the ability to trigger type I allergic reactions.⁹ Different methods, including chemical reaction, molecular weight distribution, and ELISA, have been used to identify the immunogenicity of *A. senticosus* injection. However, due to the different sensitivities of these methods, each has its own advantages and disadvantages.¹⁰⁻¹² It has, therefore, been difficult to obtain comprehensive and reliable information on

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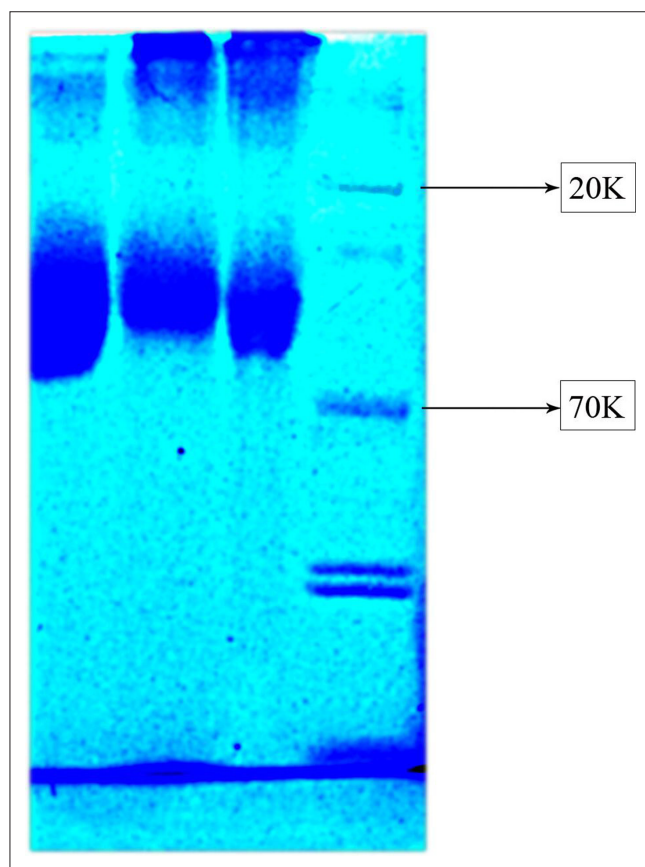


Figure 1. Results of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins extracted from *Acanthopanax senticosus*.

macromolecular substances in trace residual impurities in TCMI by only 1 method.

Drawing on the concept of proteomics, this study used Western blotting coupled with mass spectrometry to identify immunologic proteins in *A. senticosus* injection, to provide a methodological reference for the basic screening of allergens in TCMI.

Materials and Methods

Animals and Materials

Specific pathogen-free (SPF) male and female white Guinea pigs ranging from 250 to 350 g body weight and certified SCXK (Hei) 2008004 were provided by the Safety Evaluation Center of Heilongjiang University of Chinese Medicine and maintained under SPF conditions for 5 days prior to experimentation.

Acanthopanax senticosus was bought from Harbin Sankeshu Medicine Market (Hulin, Heilongjiang, China); *A. senticosus* extract was prepared by boiling the plant material in water and precipitation with ethanol, according to the Chinese

Pharmacopeia Commission Prescription of *Chinese Medicine* (Volume 18) of *Drug Standards*.¹

Other materials used in this study included protein molecular weight standard reagent markers (Beijing Kangrun Chengye Biotechnology Co., Ltd.), Guinea pig total immunoglobulin E (IgE) kit, horseradish peroxidase (HRP)-anti-Guinea IgE, Guinea pig interferon (IFN)- γ kit (RD company, USA), *A. senticosus* injection (batch number 20160702), and HRP-labeled IgE secondary antibody (batch number 160024, Shanghai Yiji Industrial Co., Ltd., China) and an Autoflex speed matrix-assisted laser desorption (MALDI)/ionization time of flight (TOF)/TOF-mass spectrometer (Bruker Dalton, Germany).

The animal experimental protocol was reviewed by the Animal Ethics Committee of Heilongjiang University of Traditional Chinese Medicine, license number: SN14073-D.

Extraction of Protein From A. senticosus Extract by the Saturated Ammonium Sulfate Method

According to the production process of *A. senticosus* injection,¹ *A. senticosus* powder was decocted twice, for 2 hours each time. The combined filtrates were concentrated, filtered, and alcohol added with stirring to reduce the volume to 50%. After standing for 12 hours and being centrifuged at $5000\times g$ for 20 minutes, the supernatant was collected to obtain *A. senticosus* extract. One hundred milliliters of the extract was placed on a magnetic stirrer, and 80% saturated ammonium sulfate was slowly added to the ice bath as it was stirred. The solution was allowed to completely precipitate and was then centrifuged at $2100\times g$ for 30 minutes to obtain a precipitate. The protein precipitate was dissolved in a small amount of pure water and then placed in a dialysis bag (molecular weight 3500) and continuously dialyzed in distilled water. The distilled water was continuously changed until the ammonium sulfate was completely removed. After dialysis, the membrane liquid was freeze-dried and the lyophilized product was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis detection and content determination.

PAGE of Protein Products

For SDS-PAGE electrophoresis, a 15% polyacrylamide separation gel and 5% concentrated gel SDS-PAGE were used. Electrophoresis was carried out using a BG-verMINI vertical electrophoresis apparatus (Thermo Fisher, USA). Fifteen milligrams of protein extract was dissolved in 10 μ L of loading buffer consisting of Tris-hydrochloric acid, pH 6.8 (60 mM), SDS (2%), bromophenol blue (0.1%), glycerin (25%), and β -mercaptoethanol (14.4 mM). This was heated in boiling water for 3 minutes before the protein solution was loaded onto the gel. The voltage used was set in a range of 60-120 V to assess the electrophoretic performance. After the electrophoresis was finished, the gel was dyed for 30-60 minutes and

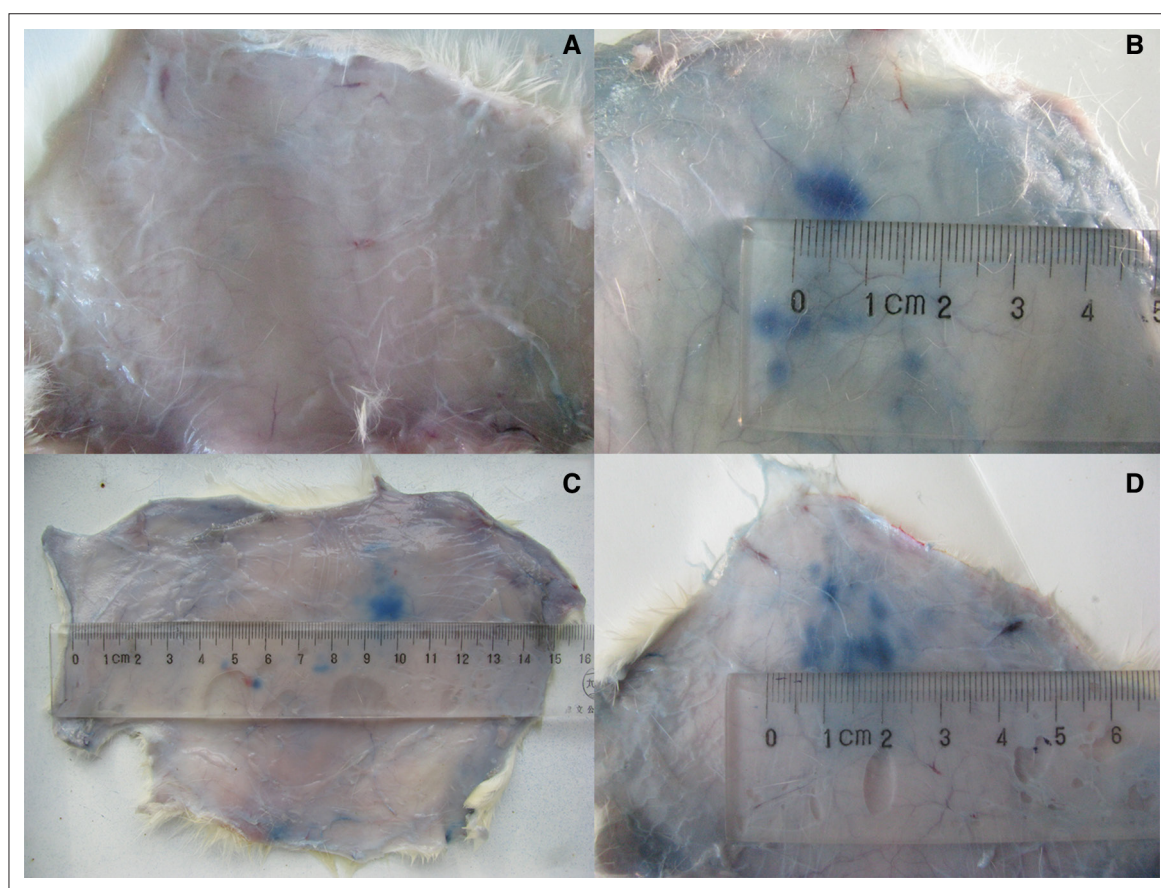


Figure 2. Passive cutaneous anaphylaxis results of (a) control group, (b) ovalbumin group (1 mg:1 mL) and (c, d) *Acanthopanax senticosus* protein precipitate group (13.5 mg:1 mL)

then rinsed with distilled water several times. The gel was then decolorized for 24 hours until the protein band was clear.

Determination of Protein Content in *A. senticosus*

Extracts

The *A. senticosus* protein precipitate was dissolved in 2 mL of distilled water, and a 0.05 mL sample was placed in a test tube and diluted to 1 mL with distilled water. The solution (40 μ L) was taken from each tube and placed in a colorimetric well, and 180

μ L of Coomassie Brilliant Blue was added. The colored solution was mixed, allowed to stand for 2 minutes, and the absorbance measured using a colorimetric instrument at 595 nm.

Passive Cutaneous Anaphylaxis Test

Serum samples (100 μ L) from Guinea pigs sensitized with *A. senticosus* protein precipitate were diluted 2-fold in physiological saline and then injected intradermally into the shaved sides of a Guinea pig. After 48 hours, sera transferred Guinea pigs were

Table 1. Identification and Bioinformatic Analysis of Pyruvate Kinase-Related Sequence of *Vitis vinifera* by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry.

1	Mqsthlllee	PIRMASILEP	Skrsfppamt	Kivgtlgprs	Rsvgisgct
51	TAGMCVARFD	FSWGDAEFHQ	ETVENLKVAV	KSTKKLCAVM	LDTVGPQLQV
101	LNRSEHPISL	QVDSLVLVTP	NQDKEATSNL	LPLNFGGLSK	AVKKGDTIFI
151	GQYLFTGSET	TSVWLEVTEV	DGEDVVCCLK	NSATLAGSLY	TLHVSQIRID
201	LPTLTDKDKKE	VISTWGVQNK	IDFLSLSYTR	HAEDVRHARE	FLSKLGDLNQ
251	TQIFAKIENI	EGLNHFDEIL	QEADGIILSR	GNLGIDLPEE	KVFLFQKAAV
301	YKCNMAGKPA	VITRVDSMT	DNLRPTRAEA	TDVANAVLDG	SDAILLGAET
351	LRGLYPVETI	STVNKICAEA	EKFVNQDFYF	KKAVKHVGEP	MTHLESIASS
401	AVRAAISVKA	SVIVCFTSTG	KAARLIGKYR	PTMPVISVVI	PRLKTNQLRW
451	TFSGAFEARQ	SVIVRGIFPM	LADPRHPAES	TNATNESILK	VALDHGKAFG
501	VIKSHDRIVV	COKVGDAVV	KIIELED		

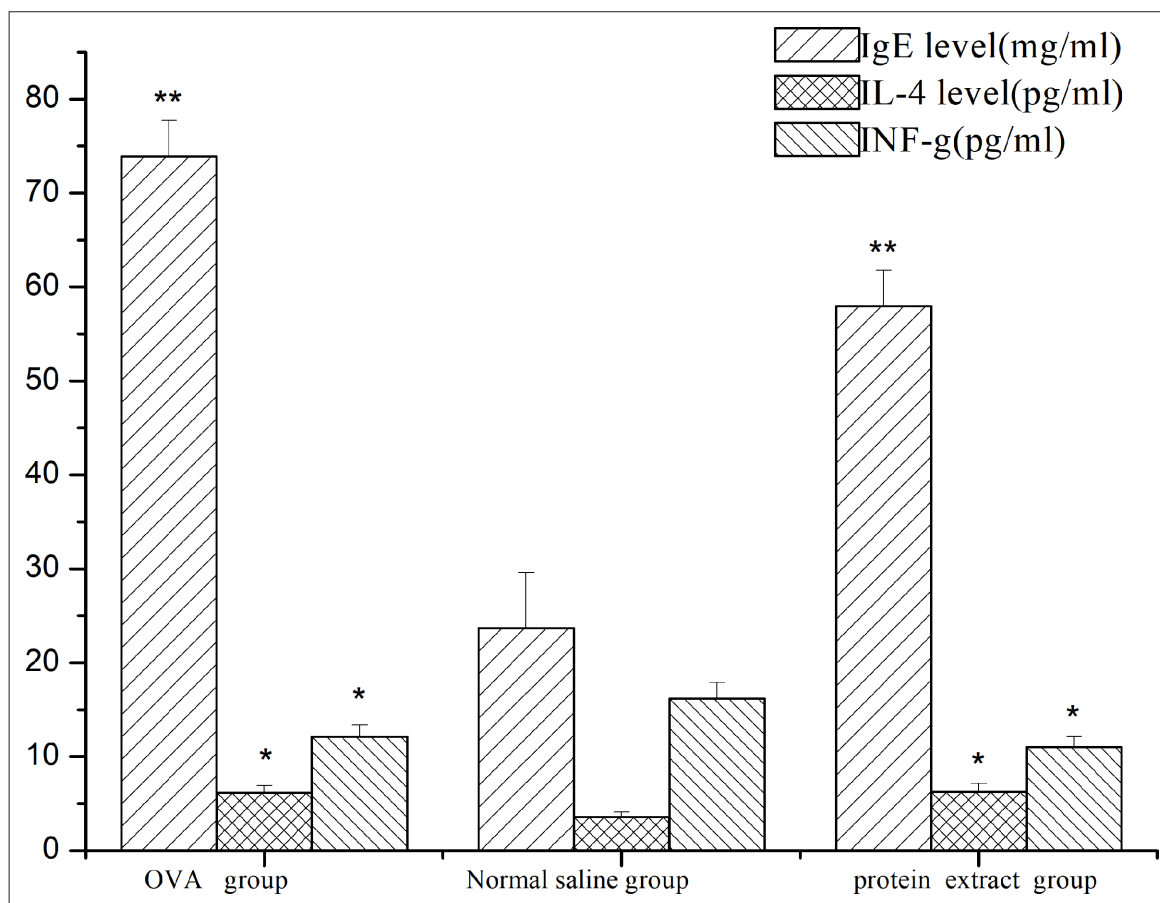


Figure 3. Levels of immunoglobulin E (IgE), interleukin-4 (IL-4), and interferon-gamma (IFN- γ) in guinea pig serum after sensitization of protein extract from *Acanthopanax senticosus* ($\pm$ SD, $n = 6$, $*P \leq 0.05$, $**P \leq 0.01$).

stimulated by injection of 1% Evan's Blue containing *A. senticosus* protein precipitate. The animals were euthanized, and the diameter of the blue spot under the skin was recorded 30 minutes later.

Determination of IgE, IL-4, and IFN- γ in Guinea Pig Serum After Sensitization with Protein

Extract from *A. senticosus*. Eighteen Guinea pigs were randomly divided into 3 treatment groups: an *A. senticosus* protein extract group treated with 13.5 mg lyophilized protein extract powder dissolved in 1 mL water for injection; an ovalbumin (OVA) positive group treated with 1 mg ovalbumin dissolved in 1 mL water for injection; and a negative control group injected with saline. The experimental drugs were injected into the skin on the back of a Guinea pig at 4 points, once every other day for 3 treatments. Fourteen days after the last sensitization, the Guinea pigs were anesthetized with ether and blood was taken from the heart, allowed to coagulate at room temperature, centrifuged at $2100 \times g$ for 20 minutes to separate the serum, and placed in a refrigerator at -20°C for later use. Guinea pig, total IgE, interleukin (IL)-4, and IFN- γ assay kits were used to measure the total IgE, IL-4, and IFN- γ levels in Guinea pig antiserum by the

double-antibody sandwich method, and the optical density (OD) values of each group were recorded.

Systemic Allergy

Fourteen days after the last sensitization, the same doses of drugs in each group were injected into the ear vein to challenge the sensitized Guinea pigs. Symptoms of systemic allergy were observed 30 minutes after injection and assessed as follows: 0 = no symptoms, 1 = scratching and rubbing around the nose, 2 = puffiness around the mouth and eyes, 3 = difficulty breathing, mouth and tail bun, 4 = convulsions, and 5 = death.¹³

Immunological Characteristics and Protein Identification of *A. senticosus* Injection as Identified by Western Blotting

Guinea pigs were sensitized with *A. senticosus* injection (batch number 20160702, 5.6 mL/kg) to obtain antiserum, with the sensitization protocol being the same as for the protein extract.

The immunological characteristics of *A. senticosus* proteins were analyzed using the Western blotting method. The protein bands of the extract in the gel were transferred to the nitrocellulose membrane by using a Bio-Rad (Bio-Rad, USA) transfer tank, and then the nitrocellulose membrane was washed for 5

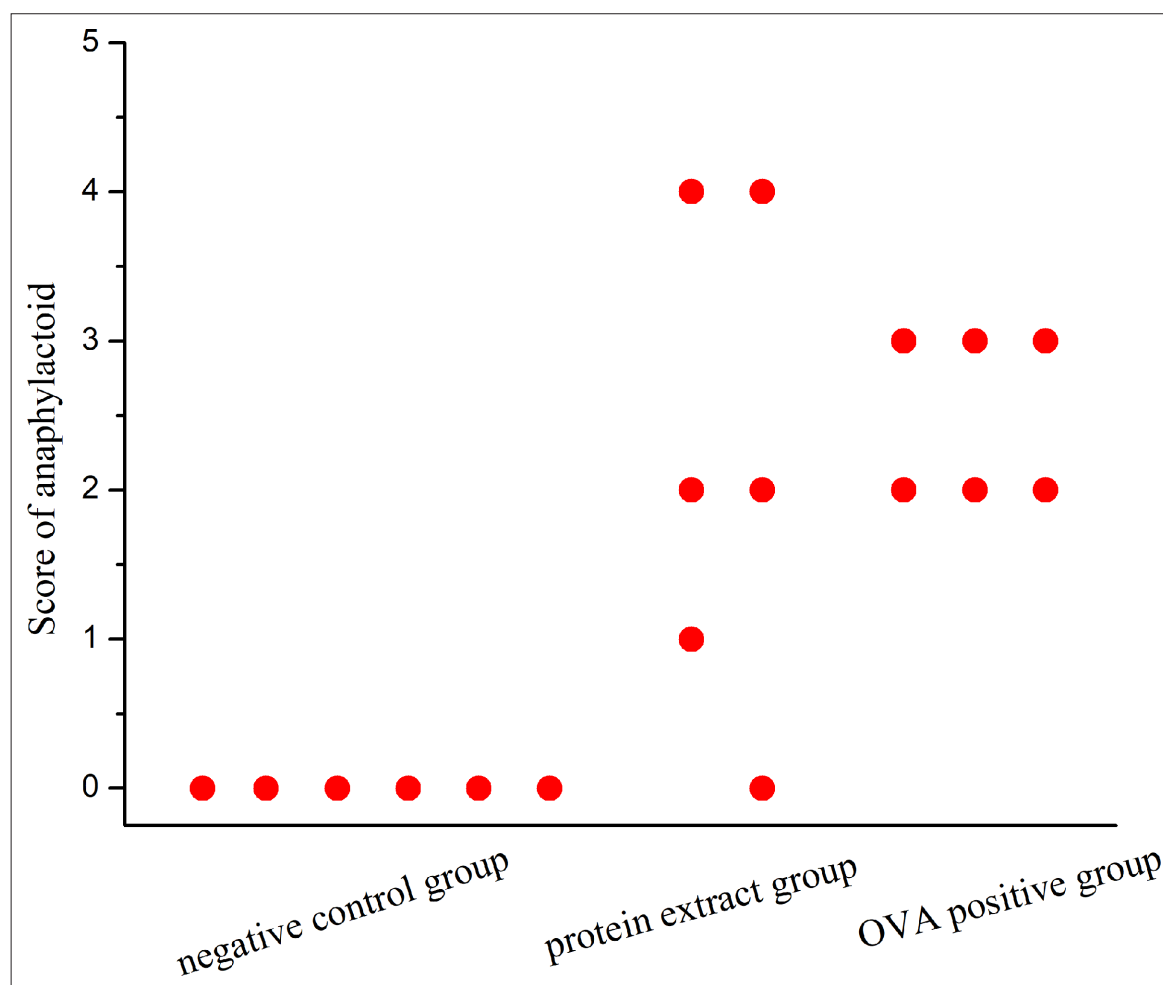


Figure 4. System allergy symptom scores in guinea pigs. OVA, ovalbumin.

minutes twice with Tris-buffered saline (TBS). The membrane was blocked with 1% bovine serum albumin (BSA) blocking solution overnight at 4°C, and the membrane was washed 3 times for 5 minutes each time. Sensitized Guinea pig serum was added at a 1:2 dilution with 1% BSA-TBS, and the solution was incubated at 37°C. After incubation for 2 hours, the membrane was washed 3 times, and a 1:100-fold diluted secondary HRP-anti-Guinea IgE was added. The membrane was washed 5 times at room temperature for 2 hours and the color was revealed by exposure. The relative molecular mass was analyzed after imaging.

Mass Spectrometric Detection of Target Bands

Target strips in the SDS-PAGE gel underwent enzyme digestion and were then analyzed by MALDI-TOF/TOF-mass spectrometry. The ultraviolet (UV) wavelength was set to 355 nm, the repetition rate was 200 Hz, the acceleration voltage was 20 000 V, and the optimal mass resolution was 1500 Da. The scan quality range was 700-3200 Da. The mass spectra of all experimental samples were obtained using the default mode. Flex Analysis (Germany, Bruker Dalton Co.,

Ltd.) software was used to filter peaks and identify signal peaks. The NCBI database was searched using BioTools (Germany, Bruker Dalton Co., Ltd.) software to find related proteins with matching peptide masses ranging from 800 to 4000 Da.

Results

SDS-PAGE Results

The gel protein electrophoresis after SDS-PAGE is shown in Figure 1, where the molecular weights of the proteins in *A. senticosus* were mainly between 20 kDa and 70 kDa, with 4 distinct bands clearly visible.

Determination of Protein Content by the Saturated Ammonium Sulfate Method

The total protein was quantitatively determined by the Coomassie Brilliant Blue method as 360.8 mg/kg.

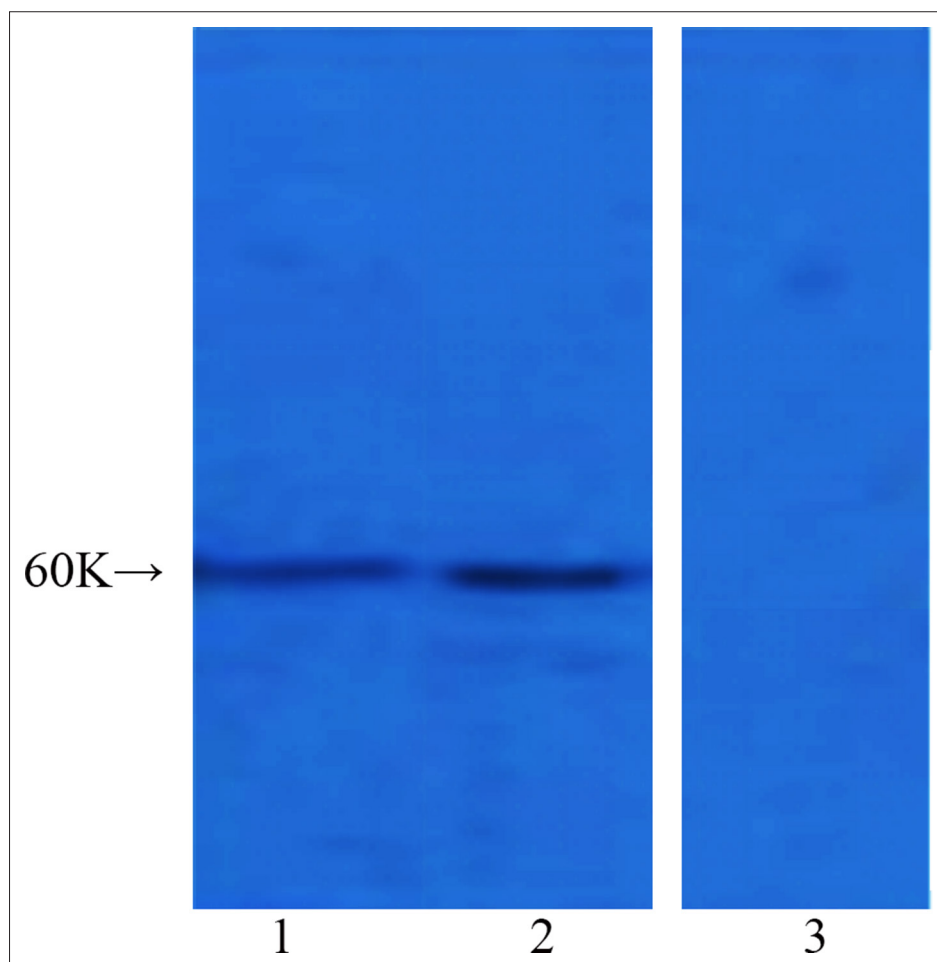


Figure 5. Western-blotting results of protein from *Acanthopanax senticosus* injection. 1 and 2: Serum sensitized with *A. senticosus* injection (20160702); 3: negative serum.

Passive Cutaneous Anaphylaxis Test Results After Immunization

The serum of *A. senticosus* protein precipitate sensitized Guinea pigs caused positive reactions, with the mean diameter of the blue spot being 4.18 ± 0.19 mm and the positive reaction rate 67%. After passive injection of *A. senticosus* protein precipitate antiserum and stimulation by injection of 1% Evan's Blue containing *A. senticosus* protein precipitate, the dye exudated into the skin to form a clear blue spot, as shown in Figure 2.

Total IgE, IL-4, and IFN- γ Levels of Protein-Sensitized Guinea Pigs

The total IgE, IL-4, and IFN- γ contents of the protein extracts in sensitized Guinea pigs are shown in Figure 3. The levels of total IgE, IL-4, and IFN- γ of the positive drug albumin group and the protein extract group were significantly different from those of the blank group ($P < 0.05$).

Systemic Allergy Results

Obvious systemic reactions in the protein extract sensitized Guinea pigs were observed within 30 minutes after stimulation, while the saline negative control group Guinea pigs showed no reactions. In the animals sensitized with 13.5 mg protein extract, a number had puffiness around the mouth and eyes, while a number had convulsions (Figure 4).

*Identification of Immunogenic Protein in *A. senticosus* Injection*

The immunogenic protein from *A. senticosus* protein extract was identified from the immunological characteristics of the Guinea pig mixed serum sensitized with *A. senticosus* injection (20160702). The results showed that a protein with a molecular weight of about 60 000 from the protein extract of *A. senticosus* could bind to IgE in Guinea pig serum sensitized with *A. senticosus* injection as the immunogenic protein (Figure 5).

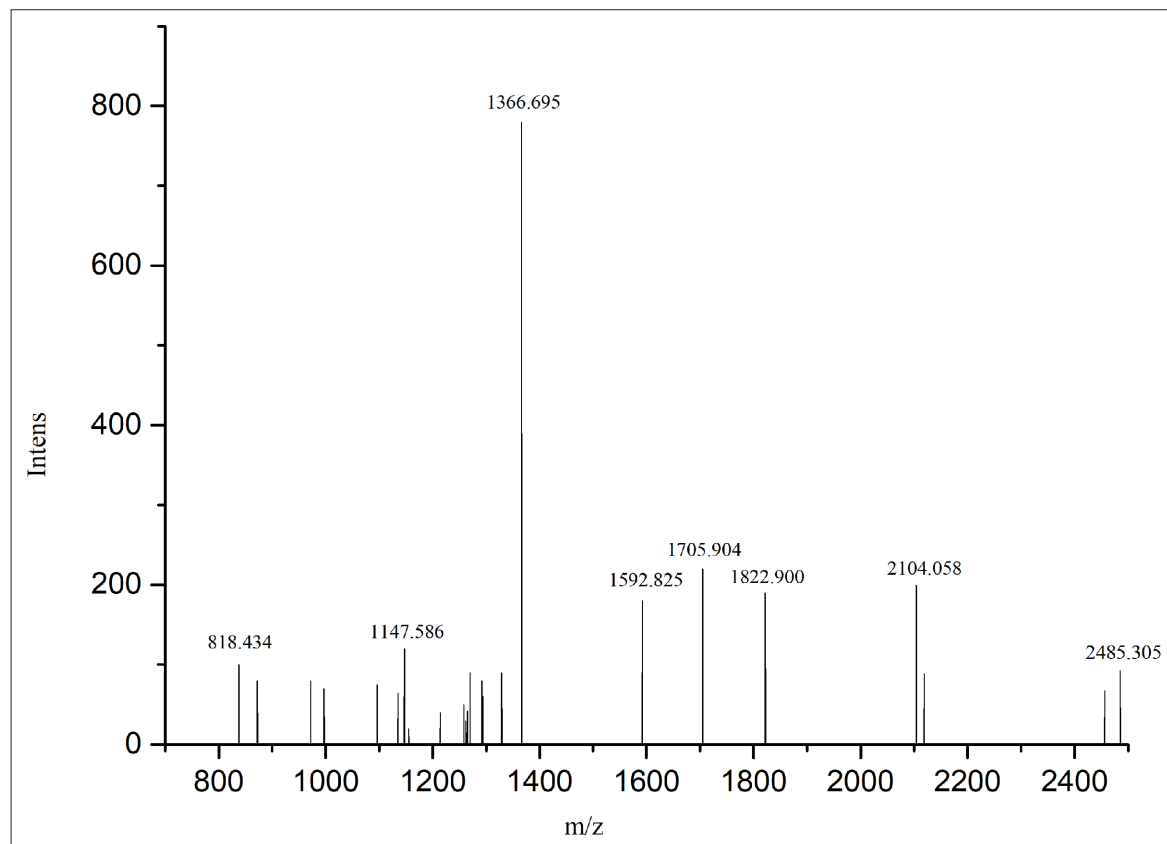


Figure 6. First-order mass spectrometry detection results of the target protein band.

Mass Spectrometric Identification of Target Proteins

The target protein band selected from the SDS-PAGE gel was digested and identified by mass spectrometry to obtain a complete peptide fingerprint (Figure 6). The molecular weights ranged from 818.434 to 2485.305, of which 1366.695, 1592.825, 1705.904, and 1822.900 had a high signal to noise ratio. Mass spectrometric analysis of the abundance of the peptides was performed (Figure 7), followed by a Mascot search for the peptide quality information in the NCBI plant protein library. The nucleotide molecular weight information of the peptide fingerprint significantly matched the pyruvate kinase gi|225449659 peptides of *Vitis vinifera* (Tables 1 and 2), and, therefore, the target protein could be a pyruvate kinase homologous protein of *A. senticosus*.

Conclusions

A passive cutaneous anaphylaxis test is suitable for most model animals because it is a highly sensitive drug allergic reaction detection method that can be used for the qualitative evaluation of a drug-specific immune response and also for quantitative evaluation of responses such as intensity or specificity or the amounts of IgE and IgG produced.¹⁴ This study also examined the allergenic properties involving specific IgE binding of the protein extract, as well as more classic immunogenicity of specific IgG binding.

The results suggest that *A. senticosus* injection could significantly increase total IgE levels in the serum of sensitized Guinea pigs and could cause positive results in a passive cutaneous anaphylaxis test in these animals, which are associated with type I allergic reactions, consistent with the reported literature.¹⁵⁻¹⁷ In this study, due to the low content of protein in *A. senticosus* injection, proteins were extracted from *A. senticosus* extract, to increase the macromolecular protein substance levels. Pharmacological experiments indicated that the immunogenicity of *A. senticosus* injection may be residual protein impurities from the preparation process. To identify the immunogenicity of *A. senticosus* injection, the Western blotting analysis was used to identify allergic proteins and mass spectrometry to detect target bands obtained by Western blotting. This, and the mass spectrometry results, showed that the target protein, with a molecular weight of approximately 60 000 Da, should be a homologous pyruvate kinase protein of *A. senticosus*. This protein may be one that causes an allergic reaction to *A. senticosus* injection.

By clarifying the basis of the immunogenicity of *A. senticosus* injection, the causes can be either removed or reduced in the production process to establish a new quality standard for this TCMI. In addition, depending on the characteristics of the immunogenicity, a specific detection method for it in *A. senticosus* injection can be formulated.

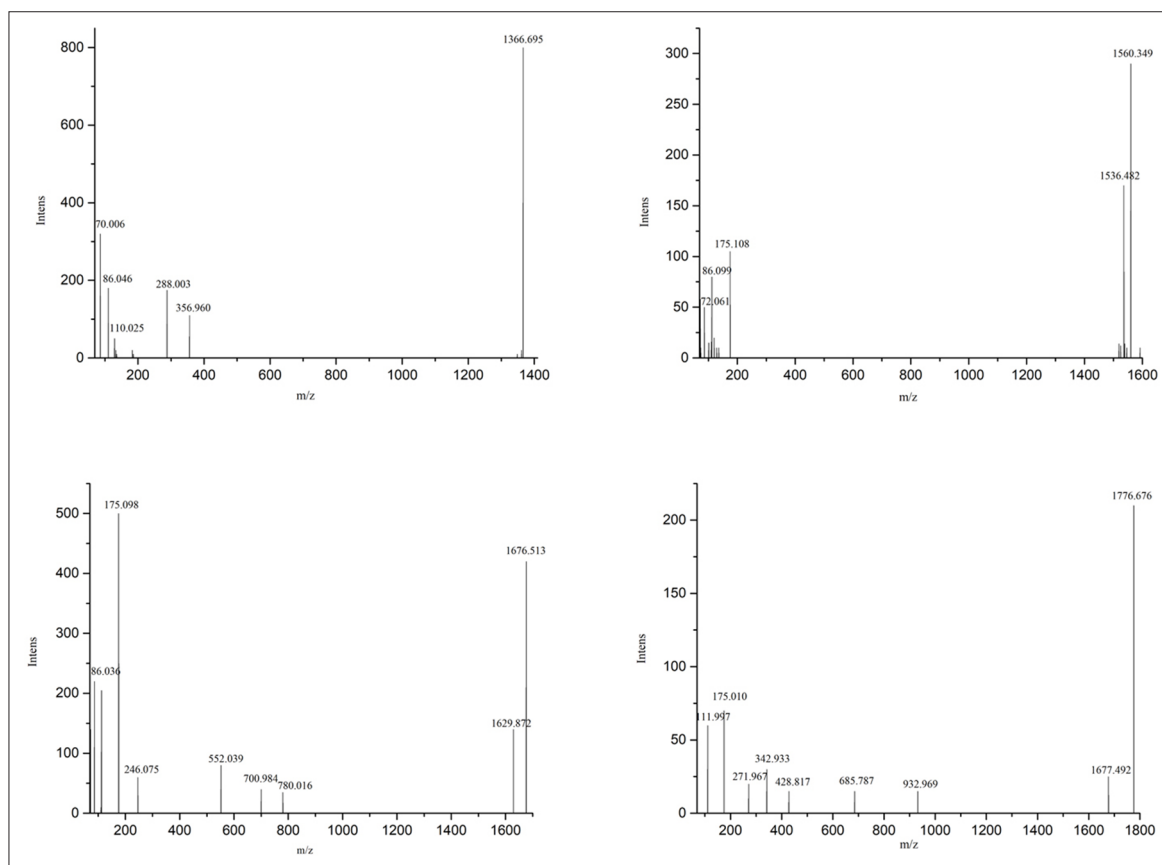


Figure 7. Second-order mass spectrometry detection results of the target protein band.

In the subjects' allergic reaction to a drug, the nature of the general antigen is directly related to the molecular weight. A small molecular weight substance is easily removed by the body, but when the molecular weight of an antigenic substance is more than 4000 Da, there is the capability to cause an allergic reaction.^{18,19} In TCMI, proteins are the compounds that most commonly cause adverse sensitive reactions.²⁰⁻²² Proteomics technology has been widely used in the study of allergic reactions and has become the main method for isolation, identification, and removal of immunogenicity.²³⁻²⁶ The strategy was

to separate the prepared protein compounds by either SDS-PAGE or 2-dimensional PAGE and then electrotransfer to a cellulose acetate film (polyvinylidene difluoride); then the hybridization of the immunogenic experimental animal serum and the IgE antibody can be conducted. The immunogenicity can next be detected by DAB color development or enhanced chemiluminescence and the target band can be subjected to mass spectrometry.^{27,28} In this study, Western blotting coupled with MALDI-TOF-mass spectrometry was used to offer a prospective method to analyze the immunogenicity of *A. senticosus*

Table 2. Bioinformatic Analysis on the Purified 60 kDa Protein.

	Accession	Mass	Score	Description
1	gi 225449659	58098	68	PREDICTED: pyruvate kinase, cytosolic isozyme-like (<i>Vitis vinifera</i>)
2	gi 118445193	27526	67	Hypothetical protein BALH_p0014 (<i>Bacillus thuringiensis</i> str. Al Hakam)
3	gi 62317934	14770	66	Cytoplasmic protein (<i>Brucella abortus</i> bv. one str. 9-941)
4	gi 229162721	9319	64	Hypothetical protein bcere0009_34910 (<i>Bacillus cereus</i> R309803)
5	gi 332815802	119618	64	PREDICTED: RNA-binding protein 44 (<i>Pan troglodytes</i>)
6	gi 296090381	56551	63	Unnamed protein product (<i>Vitis vinifera</i>)
7	gi 15892575	107956	63	Hypothetical protein RC0652 (<i>Rickettsia conorii</i> str. Malish 7)
8	gi 288561468	74250	62	Doxyribonucleic acid polymerase small subunit DP1 PolD1 (<i>Methanobrevibacter ruminantium</i> M1)
9	gi 104782251	38236	60	AraC family transcriptional regulator (<i>Pseudomonas entomophila</i> L48)
10	gi 225157638	146019	60	Hypothetical protein ObacDRAFT_8028 (<i>Opitutaceae bacterium</i> TAV2)

injections, which could provide guidance for identifying immunogenicity in other TCMI.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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