Preparation of a Monoclonal Antibody against Gintonin and Its Use in an Enzyme Immunoassay


Department of Physiology, College of Veterinary Medicine; Bio/Molecular Informatics Center, Konkuk University; Seoul 143–701, Korea: a Department of Veterinary Pharmacology and Toxicology, Konkuk University; Seoul 143–701, Korea: b Department of Physical Therapy, Cheongju University; Cheongju 363–764, Korea: c Department of Pharmaceutical Engineering, College of Health Sciences, Sangji University; Wonju 220–701, Korea: d Department of Pharmaceutical Engineering, College of Health Sciences, Sangji University; Wonju 220–701, Korea: and e Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University; Chunchon 200–701, Korea.

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Gintonin is a novel ginseng-derived G protein-coupled lysophosphatidic acid (LPA) receptor ligand. Gintonin elicits an \([\text{Ca}^{2+}]_i\) transient in animal cells via activation of LPA receptors. In vitro studies have shown that gintonin regulates various calcium-dependent ion channels and receptors. In in vivo studies, gintonin elicits anti-Alzheimer’s disease activity through the activation of the non-amyloidogenic pathway and anti-metastatic effects through the inhibition of autotaxin. However, a method for gintonin quantitation in ginseng has not been developed. In the present study, we developed an enzyme immunoassay (EIA) to measure gintonin. A monoclonal antibody was raised in a mouse using gintonin as the immunogen, and an indirect competitive EIA was used to measure gintonin. The working range was 0.01–10 \(\mu\)g per assay. The anti-gintonin monoclonal antibody did not cross-react with the ginsenosides Ra, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg3, or with LPAs such as LPA C16:0, LPA C18:0, LPA C18:1, and LPA C18:2. Using a standard curve, we measured the amount of gintonin in various ginseng extract fractions. Interestingly, we only detected a little amount of gintonin in conventional hot water extracts of Korean red ginseng. However, we can measure gintonin after ethanol extraction of Korean red ginseng marc. Thus, gintonin can be extracted from ginseng with ethanol but not water, and the remaining Korean red ginseng marc can be used to obtain gintonin. These results indicate that the EIA with the anti-gintonin monoclonal antibody can be used to quantify gintonin in various ginseng preparations, including commercial ginseng products.

Key words ginseng; gintonin; monoclonal antibody; enzyme immunoassay; gintonin quantitation

Ginseng is an herbal medicine with a variety of physiological and pharmacological effects. Ginseng is used worldwide as a tonic. Recent studies have shown that ginseng contains a novel G protein-coupled lysophosphatidic acid (LPA) receptor ligand, designated gintonin. Gintonin consists of LPAs and a ginseng protein complex. Gintonin exhibits several characteristics distinct from those other ginseng components such as ginsenosides, which are glycosides. Gintonin, but not ginsenosides, induces an intracellular free calcium concentration ([Ca\(^{2+}\)]_i) transient through its high-affinity interaction with LPA receptors. The gintonin-mediated [Ca\(^{2+}\)]_i transient is induced via the phospholipase C-IP_3 pathway. Gintonin also activates protein kinase C, mitogen-activated protein kinases, and phosphoinositide 4-kinase through multiple G proteins, such as Go\(_{11,2/3}\), Go\(_{14}\), and Go\(_{q/11}\).3,4,5,6 The gintonin-mediated [Ca\(^{2+}\)]_i transient is coupled to the regulation of Ca\(^{2+}\)-activated Cl\(^-\), Ca\(^{2+}\)-activated K\(^+\), KCNQ K\(^+\), and voltage-gated Kv1.2 channels and N-methyl-d-aspartate (NMDA) and P2X receptors.2,7 Oral administration of gintonin elicits anti-Alzheimer’s disease effects in vivo by activating the non-amyloidogenic pathway through LPA receptors and induces anti-metastatic effects by inhibiting autotaxin activity.7,8

Biochemical analysis has shown that gintonin consists of carbohydrates, lipids, and proteins. The protein component of gintonin consists of ginseng major latex-like protein 151 (GLP) and ginseng ribonuclease-like storage protein (GSP). GLP, which belongs to the Bet v 1 protein superfamily, contains a hydrophobic ligand-binding site and a glycine-rich loop called the P-loop, which might bind nucleotides.9 GSP is homologous to plant ribonuclease, but lacks ribonuclease activity.10 GSP expression in ginseng root varies seasonally. GSP is a storage protein that can be used during the vegetative stage.10 In a recent study, we investigated the crystal structure of GLP and demonstrated that GLP151 binds LPA.11 Gintonin induces the in vitro and in vivo pharmacological effects of ginseng by activating G protein-coupled LPA receptors, but a method for gintonin quantitation has not been developed.

We describe here the production of a monoclonal antibody against gintonin and the development of an enzyme immunoassay (EIA) to measure gintonin in various ginseng extract fractions. The working range was 0.01–10 \(\mu\)g/mL. The monoclonal antibody against gintonin did not cross-react with ginsenosides or LPAs. Our results indicate that the monoclonal antibody raised against gintonin can be used to measure gintonin from ginseng fractions and ginseng products, such as commercial ginseng products.

MATERIALS AND METHODS

Materials Gintonin, devoid of ginseng saponins, was prepared from Panax ginseng according to previously described methods. Gintonin was dissolved in deionized water and then diluted with phosphate buffered saline (PBS) before use. The commercial Korean Red ginseng extract products, which
are manufactured from whole Korean Red ginseng root using conventional hot water (products A, B) or from fermentation ethanol extraction (product C), were randomly purchased from local functional food market. We also purchased the dried and ground Korean Red ginseng marc from local market after confirming that the marc was remnant after hot water extraction of Korean Red ginseng root. We purchased standard ginsenosides used in this experiment from Ambo Laboratory (Daejeon, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Camarillo, CA, U.S.A.). L-lysophosphatidic acid (1-oleoyl-2-hydroxy-sn-glyce- ro-3-phosphate, 857130P) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). All other reagents used, including N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Immunization and Cell Fusion**

Eight Balb/c mice were immunized with gintonin. Pre-immune serum was collected from each mouse before immunization. For the first injection, 1 mg of gintonin was dissolved in 1 mL saline, and the solution was mixed and emulsified with 1 mL of Freund's complete adjuvant in the same ratio. The emulsion was administered to mice intraperitoneally. The same dose of the immunogen mixed in the same ratio with Freund's incomplete adjuvant was used as a booster once every 2 weeks for 2 months. For the final boost, 100 µg of gintonin in 100 µL saline was injected intravenously, and the remaining 100 µg of gintonin in 100 µL saline was emulsified with Freund's incomplete adjuvant in the same ratio and then injected intraperitoneally. On the third day after the final administration of the antigen, the mouse with the highest titer was sacrificed, and its spleen was isolated. The splenocyte suspension in serum-free DMEM medium was transferred to a 50-mL conical tube and centrifuged at 1500 rpm for 5 min. The splenocyte pellet was resuspended in 5 mL hemolyzing buffer and incubated at 0°C for 5 min. The suspension was then centrifuged at 1500 rpm for 5 min. The splenocyte pellet was resuspended in 5 mL serum-free DMEM, centrifuged again, and resuspended in 5 mL serum-free DMEM. Myeloma cells (SP2/O-Ag14 mouse myeloma cell line) were washed and added to the splenocyte suspension at a ratio of 1:2. The cell mixture was centrifuged at 1500 rpm for 5 min. The supernatant was removed completely, and the cell pellet was mixed gently. To induce fusion, 1 mL of 50% polyethylene glycol (PEG) 1500 solution was added with occasional gentle stirring at 37°C or 2 min, followed by 10 mL serum-free DMEM for 5 min. The fusion cell suspension was centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended in hpyoxanthine aminopterin thymidine (HAT) medium containing 15% FBS and seeded into 96-well culture plates with abdominal macrophages from ICR mice. The macrophages were cultured in HAT medium for 1 d before the addition of the fusion cells.

**Indirect Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Antibody-Producing Clones**

The plates were screened for the production of antibodies 2 weeks after cell fusion. A microplate was coated overnight at 4°C with 100 µL of gintonin or bovine serum albumin (BSA) dissolved in 50 µM carbonate buffer (pH 9.6). The plate was washed 4 times with PBS containing 0.05% Tween 20 (pH 7.4, T-PBS) and then blocked with 100 µL of 3% BSA in PBS (B-PBS) for 2 h at 37°C. The plate was washed again with PBS, and 100 µL of the supernatant from the cultured hybridoma cells was added to each well and incubated for 2 h at 37°C. The plates were washed 4 times with PBS, and 100 µL of diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) was added to each well and incubated for 2 h at 37°C. The plates were washed, and 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped by the addition of 50 µL of 0.5 M H2SO4. The activity of the enzyme bound to the solid phase was measured at 450 nm using an ELISA reader. Clones showing a positive response against gintonin and a negative response against BSA were selected and subcloned by limiting dilution. Finally, a single clone with a positive response against gintonin and a negative response against BSA was retrieved using the method described above.

**Ascites Production**

BALB/c mice were injected with 0.5 mL of pristine. Ten days later, individual hybridoma cell clones (1×107/mL) in PBS were administered to mice by intraperitoneal injection. Ascites were collected from the swollen mice and incubated for 1 h at 37°C and then overnight at 4°C. After centrifugation at 10000 rpm for 10 min, the supernatant was collected and kept at −70°C for later use. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and our protocol was approved by the Institutional Animal Care and Use Committee of Konkuk University.

**Antibody Purification and Isotyping**

The ascites was purified with protein A-conjugated agarose beads (KPL) equilibrated with PBS. The ascites was passed through a column containing the protein A resin, and 0.5-mL fractions of purified antibody were collected. Glycerine (pH 2.7) was used as the elution buffer, and the fractions were neutralized immediately with 50 µL of 1.5 M Tris base (pH 8.8). The purity of the mAb was confirmed using Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The immunoglobulin isotype of the selected mAb clone was determined using an Immuno-Type™ mouse mAb isotyping kit (BD Biosciences) according to the manufacturer's instructions.

**Indirect ELISA for the Measurement of Gintonin**

The anti-gintonin activity in ascites titer was determined using an indirect ELISA. Briefly, gintonin in coating buffer (50 mM carbonate buffer, pH 9.6) was added to each well of a 96-well microplate and incubated overnight at 4°C. After 4 washes with T-PBS, the plate was blocked with 100 µL of B-PBS for 2 h at 37°C. Once the plate was washed, mice ascites, diluted with B-PBS, was added to each well and incubated for 2 h at 37°C. The plate was washed with PBS 4 times, and 100 µL of diluted HRP-conjugated goat anti-mouse IgG was added to each well and incubated for 2 h at 37°C. The plate was washed again, and 100 µL of TMB substrate solution was added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped by the addition of 50 µL of 0.5 M H2SO4. The activity of the enzyme bound to the solid phase was measured at 450 nm using an ELISA reader.

**Optimization of the Anti-gintonin Antibody Concentration**

The optimal dilution with the highest P/N value was determined using serially diluted anti-gintonin antibody as the capture antibody and HRP-conjugated goat anti-mouse IgG as...
the detection antibody.

**Competitive Indirect EIA for the Detection of Gintonin** A microplate was coated overnight at 4°C with 50 µL of gintonin dissolved in 50 mM carbonate buffer (pH 9.6). The plate was washed 4 times with T-PBS and then blocked with 100 µL of B-PBS for 2 h at 37°C. The plate was then washed with PBS. To prepare a standard solution, gintonin was dissolved in PBS and diluted in the range of 10 ng–10 µg/50 µL of B-PBS per well. In a tube, 50 µL of diluted antibody in B-PBS was mixed with 50 µL of standard solution diluted in B-PBS. The mixture was then added to each microtiter well and incubated for 2 h at room temperature. The plate was washed as described above, and 100 µL of diluted HRP-conjugated goat anti-mouse IgG was added to each well and incubated for 2 h at 37°C. The plate was washed, and 100 µL of TMB substrate solution was added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped by the addition of 50 µL of 0.5 M H₂SO₄. The activity of the enzyme bound to the solid phase was measured at 450 nm using an ELISA reader.

**Preparations of Commercial Korean Red Ginseng Products and Korean Red Ginseng Marc and Competitive Indirect EIA for the Detection of Gintonin** The commercial Korean Red ginseng extract products (100 g each) were first lyophilized. The lyophilized commercial Korean Red ginseng extract (10 mg) was dissolved in PBS for competitive indirect EIA for the detection of gintonin. Korean Red ginseng marc (1 kg) was refluxed with 80% fermentation ethanol six times for 8 h at 80°C each. The ethanol extracts were concentrated with a yield of 80 g. Gintonin from Korean Red ginseng marc was obtained according to previously described Materials and Methods. Gintonin (1 mg) prepared from Korean Red ginseng marc was also dissolved in PBS for competitive indirect EIA for the detection of gintonin. A microplate was coated overnight at 4°C with 50 µL of gintonin dissolved in 50 mM carbonate buffer (pH 9.6). The plate was washed 4 times with T-PBS and then blocked with 100 µL of B-PBS for 2 h at 37°C. The plate was then washed with PBS. To prepare a standard solution, gintonin, commercial Korean Red ginseng product extracts with hot water or fermentation ethanol and ginseng marc were dissolved in B-PBS and diluted in the range of 30 ng–10 µg/50 µL of B-PBS per well. In a tube, 50 µL of diluted antibody in B-PBS was mixed with 50 µL of standard solution diluted in B-PBS. Other experimental procedures were performed according to above described competitive indirect EIA for the detection of gintonin.

**Preparation of LPAs and Ginsenosides and Cross-Reactivity Test** LPAs such as LPA C₁₆:₀, LPA C₁₈:₀, LPA C₁₈:₁, and LPA C₁₈:₂; and ginsenosides such as Ra, Rb₁, Rb₂, Rc, Rd, Re, Rf, Rg₁, and Rg₂ (1 mg/mL stock solution) were diluted to 0.2 µg/µL in 50 µL PBS, mixed with 50 µL of diluted antibody in a tube, and added to the well. The other procedures were the same as those described above for competitive indirect EIA.

**Data Analysis** All values are presented as the mean±standard error of the mean (S.E.M.). The differences between the means of control and experimental data were analyzed using an unpaired Student’s t-test. A value of p<0.05 was considered statistically significant.

**RESULTS**

**Isolation of Clones That Secreted a Monoclonal Antibody against Gintonin** In the present study, we first screened clones that secreted a monoclonal antibody against gintonin according to the protocol shown in Fig. 1A. We selected 7 clones and injected them into mice to produce ascites (Fig. 1B). Finally, we selected the clone that showed the highest titer against gintonin. Next, we purified the antibody against gintonin from mouse ascites and isotyped the antibody. We found that the titer of the monoclonal antibody was 10-fold higher than that of mouse ascites. In isotyping experiments, the monoclonal antibody against gintonin was IgG₁ with a kappa chain. In experiments using purified antibody, we obtained optimal results when we coated wells with 0.1 µg gintonin, prepared the purified antibody at a concentration of 0.1 µg/mL, and used the rabbit anti-mouse IgG secondary antibody-HRP conjugate at a 1:1000 dilution (Figs. 2A, B). The results showed that we had produced a monoclonal antibody against gintonin.

**Standard Curve for Gintonin** Once the assay procedure was optimized, we obtained a standard curve for the gintonin concentration. When the absorbance of each standard was plotted versus its gintonin concentration on a linear–log scale, a sigmoidal curve was obtained (Fig. 3). Plotting logit (B/Bo)
versus log standard gintonin concentration yielded a linear response ($r^2 = 0.97$). The assay range for gintonin using this EIA was 30 ng–10 µg/well.

Assay Specificity The specificity of the monoclonal antibody against gintonin was evaluated in cross-reactivity assays using ginseng components such as ginsenosides and LPAs. As shown in Fig. 4A, the monoclonal antibody against gintonin did not cross-react with ginsenosides such as Ra, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg3. In addition, the monoclonal antibody against gintonin did not cross-react with LPAs such as LPA C16:0, LPA C18:0, LPA C18:1, and LPA C18:2, which are bioactive ingredients in gintonin (Fig. 4B). The accuracy of the assay method was assessed by spiking samples with known concentrations of samples and comparing the recovered amounts with the input amounts. In the spiking experiments with known quantities of gintonin, we obtained a mean analytical recovery of 99% for gintonin (Table 1). The results showed that the monoclonal antibody against gintonin reacted with gintonin but not with ginsenosides and LPAs. We performed inter-assay and intra-assay validation experiments with gintonin in crude ethanol ginseng extract. As shown in Table 1, the intra-assay recovery ranged from 96 to 103%, and the inter-assay recovery ranged from 101 to 103%. The data showed that the EIA method produced accurate and repeatable results.

Detection of Gintonin in Ginseng Products We analyzed the gintonin content in commercial ginseng products using the optimized EIA. We were able to measure gintonin in Korean Red ginseng total extract manufactured through ethanol extraction (product C), but only detected a little amount of gintonin in commercial Korean Red ginseng products, which are manufactured using hot water extraction procedure (products A, B). The results suggest that gintonin is not fully extracted with hot water (Fig. 4C). We found that most commercial Korean Red ginseng extract preparations generate Korean Red ginseng marc as a by-product of hot water extraction. Therefore, we further examined whether gintonin is present after ethanol extraction of Korean Red ginseng marc. We found that Korean Red ginseng marc contained about 0.10–0.15% gintonin, and we were able to measure the gintonin obtained from Korean Red ginseng marc using the EIA (Fig. 4C, Marc). Next, we performed an additional competitive indirect EIA for gintonin, commercial Korean Red ginseng products and Korean Red ginseng marc. We could obtain a standard curve for the gintonin concentration (Fig. 4D). Hot water extract of Korean Red ginseng products (products A, B) showed G/G0 ratio close to 1, even if the concentrations of products were high as much as 10 µg/well. In ethanol extract of Korean Red ginseng (product C) and Korean Red ginseng marc, G/G0 ratio decreased when their concentrations were increased. These results indicate that hot water extract of Korean Red ginseng commercial product (products A, B) contain much less amount of gintonin, compared to ethanol extract of Korean Red ginseng commercial product (product C) and Korean Red ginseng marc (Marc). Taken together, the results indicate that ethanol but not water is a useful agent for gintonin preparation from ginseng and show that Korean Red ginseng marc can be recycled after the manufacture of commercial Korean Red ginseng.

DISCUSSION

Gintonin, a ginseng-derived LPA receptor ligand, induces a $[Ca^{2+}]_i$ transient in animal cells that express LPA receptors. Studies have shown that the gintonin-mediated $[Ca^{2+}]_i$ tran-
sient is coupled to important pharmacological effects, such as the attenuation of amyloid plaque formation in the brain.\(^7,13\) In addition, gintonin inhibits autotaxin activity to suppress metastasis.\(^8\) However, a method for quantifying the amount of gintonin in ginseng has not been developed. In the present study, we produced a monoclonal antibody against gintonin and developed an EIA for gintonin quantitation in ginseng and ginseng products.

The monoclonal antibody against gintonin produced in this study has 3 characteristics. First, the monoclonal antibody had a high titer, similar to that of other monoclonal antibodies against plant components.\(^{14}\) Second, the monoclonal antibody against gintonin does not cross-react with ginsenosides (Fig. 4A), thus enabling quantitation of gintonin in ginseng extracts containing ginsenosides. Third, the monoclonal antibody does not cross-react with LPAs (Fig. 4B), which might make it possible to measure gintonin without interference from endogenous LPAs. The results indicate that the antigenicity derived from gintonin and that the monoclonal antibody specifically interacts with gintonin. Thus, the antibody can be used to

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**Table 1. Recovery of Gintonin Measured with Indirect Competitive EIA Using the Anti-gintonin mAb**

<table>
<thead>
<tr>
<th>Spiked level (µg/mL)</th>
<th>Mean±S.E. (µg/mL)</th>
<th>Intra-assay (n=5)</th>
<th>Inter-assay (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recoveries (%)</td>
<td></td>
<td>Recoveries (%)</td>
</tr>
<tr>
<td>1</td>
<td>0.96±0.06</td>
<td>96.12±5.12</td>
<td>1.02±0.01</td>
</tr>
<tr>
<td>2</td>
<td>2.06±0.08</td>
<td>103.12±4.04</td>
<td>2.03±0.04</td>
</tr>
<tr>
<td>4</td>
<td>3.90±0.12</td>
<td>97.51±3.09</td>
<td>4.12±0.17</td>
</tr>
</tbody>
</table>

All values are the mean±S.E. of triplicate samples for each level.
measure gintonin in various ginseng products.

In a previous study, we showed that ginseng contains about 0.2% gintonin. We also showed through biochemical analysis that the components of gintonin are similar to the components of ginseng. For example, the LPA content of gintonin is about 9.5%. Gintonin comprises about 26, 23, and 26% protein, lipid, and carbohydrate, respectively. Glucose is a major component, constituting 95% of the carbohydrate portion. Gintonin contains fatty acids such as palmitic, stearic, and linoleic acid in ester form, as well as other minor fatty acids, that are endogenous in animal plasma. The proportion of linoleic and palmitic acid is higher than that of other fatty acids.

It is unlikely that the carbohydrate and lipid components of gintonin act as immunogens for antibody production against gintonin because these components are also present in animals. On the other hand, the protein components of gintonin differ from those of animals. Gintonin contains GLP and GSP as major proteins, which have been detected only in ginseng. Thus, the protein components of gintonin might contribute to antigenicity and antibody production.

The monoclonal antibody against gintonin did not recognize LPAs, which are the main bioactive components of gintonin. This might be due to the presence of endogenous LPAs in animal plasma, most of which bind to plasma proteins such as albumin and gelsolin for transport to target organs. In addition, the monoclonal antibody against gintonin did not cross-react with ginsenosides. The physico-chemical properties of gintonin and ginsenosides differ. Ginsenosides are ginseng-derived glycosides, whereas gintonin consists of carbohydrates, proteins, and lipids and acts as a ligand for G protein-coupled LPA receptors. Ginsenosides separate from gintonin during anion exchange chromatography because only gintonin binds the anion exchange resin. Thus, the monoclonal antibody against gintonin can be used to measure gintonin in mixtures containing ginsenosides, such as crude ginseng extracts.

In previous studies, we developed a method to quantitate ginseng saponins such as ginsenoside Rf. We produced monoclonal antibodies against ginsenoside Rf with high titers and without cross-reactivity with other ginsenosides, other than ginsenoside Rg1. Ginsenoside Rf was conjugated as a hapten to albumin because ginsenoside Rf was not enough large to induce an antigenic reaction in the animal. In the present study, we produced a monoclonal antibody without gintonin–albumin conjugation. The results showed that gintonin was large enough to induce antibody production.

Although we were able to quantify gintonin in ginseng extracts prepared with ethanol, we only detected a small amount of gintonin in conventional hot water extracts of ginseng. The results suggest that hot water is not a suitable agent for gintonin extraction and that gintonin extraction from ginseng requires an organic solvent. Gintonin contains a lipid moiety, which is hydrophobic and insoluble in water. Its presence might explain the poor yield of gintonin in water extracts. Consistent with this explanation, we were able to quantify gintonin in Korean red ginseng marc by water extraction (Fig. 4C), although the yield of gintonin was about two-thirds the yield obtained from ginseng. Korean red ginseng marc is a by-product of ginseng extract production. In the future, the use of Korean red ginseng marc for gintonin preparation with ethanol extraction could yield a large amount of gintonin at a very low cost.

In conclusion, we produced a monoclonal antibody against gintonin and used the monoclonal antibody in EIAs to measure the amount of gintonin present in various ginseng extract fractions and commercial ginseng products. We propose that the monoclonal antibody against gintonin could be used to understand the molecular mechanisms underlying gintonin’s physiological and pharmacological effects.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


