Journal of Ginseng Research 48 (2024) 474–480

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: www.sciencedirect.com/journal/journal-of-ginseng-research

Research Article

Monoclonal antibody-based enzyme-linked immunosorbent assay for quantification of majonoside R2 as an authentication marker for Nngoc Linh and Lai Chau ginsengs

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ARTICLE INFO

Keywords:
Enzyme-linked immunosorbent assay
Majonoside R2
Monoclonal antibody
Ngoc Linh ginseng
Vietnamese ginseng

ABSTRACT

Background: Recent years have witnessed increasing interest in the high amount of ocotillol-type saponin in *Panax vietnamensis*, particularly in relation to majonoside R2 (MR2). This unique 3%–5% MR2 content impart Ngoc Linh and Lai Chau ginsengs with unique pharmacological activities. However, in the commercial domain, unauthentic species have infiltrated and significantly hindered access to the authentic, efficacious variety. Thus, suitable analytical techniques for distinguishing authentic Vietnamese ginseng species from others is becoming increasingly crucial. Therefore, MR2 is attracting considerable attention as a target requiring effective management measures.

Methods: An enzyme-linked immunosorbent assay (ELISA) was developed by producing monoclonal antibodies against MR2 (mAb 16E11). The method was thoroughly validated, and the potential of the immunoassay was confirmed by high-performance liquid chromatography with ultraviolet spectroscopy. Furthermore, ELISA was applied to the assessment of the MR2 concentrations of various *Panax* spp., including Korean, American, and Japanese ginsengs.

Results and conclusions: An icELISA using mAb 16E11 exhibited linearity between 3.91 and 250 ng/mL of MR2, with detection and quantification limits of 1.53 and 2.50 – 46.6 ng/mL, respectively. Based on this study, the developed icELISA using mAb 16E11 could be a valuable tool for analyzing MR2 level to distinguish authentic Ngoc Linh and Lai Chau ginsengs from unauthentic ones. Furthermore, the analysis of the samples demonstrated that Ngoc Linh and Lai Chau ginsengs exhibit a notably higher MR2 value than all other *Panax* spp. Thus, MR2 might be their ideal marker compound, and various bioactivities of this species should be explored.

1. Introduction

Panax species, commonly known as ginseng, is a member of the Araliaceae family. *Panax* spp. has been the focus of scientific research for over a century owing to its immunomodulatory properties [1] and a broad range of physiological functions, including its potential as a cardiovascular and central nervous system stabilizer [2–5]. More than 14 species have been identified worldwide with various medicinal applications [6]. Among six known species, common species are known as Korean ginseng (*P. ginseng*; PG); the others are American ginseng

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https://doi.org/10.1016/j.jgr.2024.05.004
Received 5 February 2024; Received in revised form 5 May 2024; Accepted 20 May 2024
Available online 23 May 2024

(*P. quinquefolius*); Chinese ginseng (*P. notoginseng*); Japanese ginseng, also known as bamboo ginseng (*P. japonicum*); Vietnamese ginseng (*P. vietnamensis*); and Himalayan ginseng (*P. pseudoginseng*) [1].

Among the three recognized ginseng species in Vietnam [7], *Panax vietnamensis* Ha et Grushv. represents an invaluable and endemic species [8]. This ginseng was discovered in the Ngoc Linh Mountain in 1973, well known as "Ngoc Linh ginseng" [8], and the authenticity of its root was officially protected by the Geographical Indication "Ngoc Linh" as Registration Certificate No. 00049 [9–11]. The ginseng species was further enriched with the discovery of the two subspecies, *P. vietnamensis* var. *fuscidiscus* from southern Yunnan, China in 2003 [12] and *P. vietnamensis* var. *langbianensis* from Lang Bian Mountain, Vietnam in 2016 [13]. Put differently, the species are divided into three types, namely *P. vietnamensis* Ha et Grushv. or "Ngoc Linh ginseng," *P. vietnamensis* var. *fuscidiscus*, and *P. vietnamensis* var. *langbianensis* [7]. Additionally, *P. vietnamensis* var. *fuscidiscus* was also found to be naturally distributed in Lai Chau Province, Vietnam and became well known as Lai Chau ginseng [7,14]. The fresh root of Lai Chau ginseng produced in Lai Chau Province was ensured its authenticity by the trademark "Sam Lai Chau or Lai Chau ginseng" in 2022 [15].

These two authentic ginsengs share high similarities regarding their molecular and morphological characteristics, invaluable bioactive constituents, precious biological activities, and intensive cultivation stages to meet the market demand [7,8,14,16]. Both authentic ginsengs were especially characterized by their extremely high concentrations of invaluable majonoside R2 (MR2), which is a tetracyclic triterpenoid saponin with a furan ring linked to aglycones (Fig. 1). The MR2 contents of authentic ginsengs are often >5 %, whereas those of other adulterants are small or zero [17–24]. More attractively, studies have demonstrated that MR2 exhibits chemoprotective, anticancer, or hepatocytoprotective activities [17–22]; thus, most extant studies on both ginsengs have intensively determined MR2 as a key active component. The investigation of saponin accumulation in *P. vietnamensis* Ha et Grushv. during its cultivation also focused on MR2 as a major component, and MR2 reached a maximum of ~5 % at the age of 5 years, indicating an appropriate harvest age [25].

As characterized with essential chemical and biological properties, "Ngoc Linh ginseng" was recognized as a national product in 2017 and the price of 1 kg of the best fresh root of genuine "Ngoc Linh ginseng" was 3000 US dollars (2017) [21]. However, the commercialization of the two authentic ginsengs in Vietnam is hindered by less valuable adulterants, such as *P. stipulealatus* Tsai & K.m. Feng, *P. vietnamensis* var. *langbianesis*, and *P. bipinnatifidus* Seem., which are extremely challenging to distinguish with the naked eye.

The two genuine ginsengs can be distinguished from adulterants using their molecular and morphological characteristics [[26–28]] or by chemical analysis. Biological tools do not evaluate the concentrations of essential chemical compounds, which are low at early ages and accumulate at the later stage of development in the Ngoc Linh ginseng [25]. Thus, quantitative analysis was performed to authenticate the root of authentic Ngoc Linh ginseng based on the total saponin content and three major compounds, including MR2, ginsenoside Rg1, and ginsenoside Rb1 [7,8]. The biological activity and high concentration (>5 %) of MR2 make it unique; thus, it is the most important compound in quantitative analyses for authenticating Ngoc Linh ginseng, with an approximate requirement of 3.6 % MR2 in authentic root at the age of 6 years [9–11]. Additionally, the trademark of the fresh root of Lai Chau ginseng was recently granted in 2022, and the quantitative authentication was achieved by the determination of total saponin content and the unique characterization of MR2, with a minimum requirement of 3 % MR2 in absolute dried material [15]. Hence, MR2 was highlighted as the most crucial molecule and even a unique compound used for the quantitative authentication of the Vietnamese ginseng roots, as well as the presence of prominent biological benefits therein.

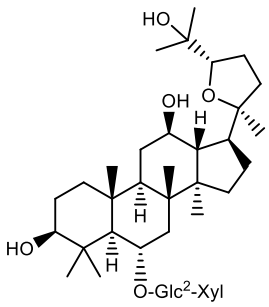

Fig. 1. Chemical structure of MR2.

The existing general method for detecting MR2 involved employing analytical techniques that leverage chromatographic properties in addition to various detection methods [29–33]. Further, the absence of chromophore characteristics limits the capability of detecting MR2 via simple UV spectroscopy [19]. Therefore, developing a more sensitive approach for distinguishing Ngoc Linh ginseng and Lai Chau ginseng from other ginseng types, as well as ensuring accurate quality control in pharmaceutical and therapeutic applications, are of utmost significance.

An immunoassay is a highly specific technique that is based on antigen–antibody complex reactions. Many small molecules can be determined by immune-specific reactions [34]. Enzyme-linked immunosorbent assay (ELISA) has become increasingly prevalent in the measurement of plant secondary metabolites [34–38] owing to its economical nature, notable effectiveness, and environmentally sustainable process [34].

A MR2-targeting monoclonal antibody was generated in this study. The generated monoclonal antibody 16E11 (mAb 16E11) was characterized by determining its cross-reaction with structure-related compounds and an ELISA using mAb 16E11 was validated for its sensitivity and precision. Thereafter, the developed method was employed to establish ELISA for quantifying the MR2 content as well as distinguishing Ngoc Linh ginseng and Lai Chau ginseng from other species. Moreover, the accuracy of the newly devised method was confirmed through traditional detection using high-performance liquid chromatography using ultraviolet–visible spectroscopy (HPLC/UV–Vis).

2. Materials and methods

2.1. Chemicals and reagents

MR2 and related compounds (≥98 % purity), including majonoside R1 (MR1), pseudoginsenoside RT4 (PRT4), vinaginsenoside R1 (VR1), vinaginsenoside R2 (VR2), and vinaginsenoside R11 (VR11), were isolated and identified by nuclear magnetic resonance by Dr. Le Van Huy of the Research Institute for Biotechnology and Environment (Nong Lam University, Ho Chi Minh City, Vietnam). Enriched RPMI-1640 Delbecco's-Ham's F12 (eRDF) and RD-1 were acquired from Kyokuto Pharmaceutical Industrial (Tokyo, Japan). Bovine serum albumin (BSA), ovalbumin (OVA), aminopterin, and polyethylene glycol (PEG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The employed fetal bovine serum (FBS) was acquired from Gibco (Carlsbad, CA, USA). The chemicals employed in this study, i.e., 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hypoxanthine, were acquired from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The goat anti-mouse IgG H&L (HRP) (ab6789) was acquired from Abcam (Cambridge, Massachusetts, USA). The additional chemicals and reagents employed in the experiments were of analytical grades.

2.2. Animals

Male BALB/c mice weighing 17–22 g were obtained from Charles River Laboratories (Kanagawa, Japan) and were aged 5 weeks before distributed. The mice were subjected to standard treatment, following

the guidelines established by the Committee on the Ethics of Animal Experiments at Kyushu University's Graduate School of Pharmaceutical Sciences (reference no. A20-002-3)

2.3. Preparation of Panax spp. root samples

This study comprised the analyses of six *Panax* roots and one *Randia siamensis* (*R. siamensis*) fruit **(14)**, which reportedly exhibit incredibly high triterpenoid saponin contents [39]. Five *Panax* root samples, namely *P. vietnamensis* var. *fuscidiscus* or Lai Chau ginseng **(1)**, *P. vietnamensis* var. *vietnamensis* or Ngoc Linh ginseng **(2)**, *P. vietnamensis* var. *langbianesis* N.V. Duy et al. **(3)**, *P. bipinnatifidus* **(4)**, and *P. stipuleanatus* **(5)**, were collected and identified by Dr. Le Van Huy. Further, *P. japonicus* **(6)** and *P. ginsengs* collected in Japan (PG - 1 **(7)** root powder were purchased from Uchidawakanyaku Ltd. (Tokyo, Japan). Furthermore, four additional *P. ginsengs* were acquired from China (PG - 2, **(8)**); prescribed by a doctor from Korea, Korean white ginseng (PG - 3, **(9)**); company product from Korea (PG - 4, **(10)**), and Korean red ginseng (PG - 5, **(11)**). Two *P. quinquefolius* species, which originated from Canada (PQ - 1, **(12)**) and China (PQ - 2, **(13)**), were utilized. Voucher specimens are at the Herbarium of the Faculty of Pharmaceutical Sciences, Kyushu University (Voucher specimen Nos. P0001–P0013 and R0001). All the plant samples were ground into fine particles and weighed accurately (50 mg) for extraction. Thereafter, they were extracted four times with methanol using an ultrasonic sonicator (500 μL/time). The supernatant solutions were collected after centrifugation for 10 min at 9200 g, transferred into a 2 mL tube, and dried completely at 42 °C. Further, each sample was redissolved with 1 mL of methanol. After complete dissolution, the sample extracts were diluted to suitable concentrations for detection by ELISA and HPLC methods.

2.4. Preparation of the MR2 conjugates

The MR2–BSA immunogen and MR2–OVA immobilized antigen were produced by the reaction of sodium periodate ($NaIO_4$), as detailed in several extant studies [35,38]. In summary, in the context of immunogenic conjugates, a solution of distilled water (1 mL) and $NaIO_4$ (3.02 mg) was added gradually to a solution of MR2 (3.01 mg) in methanol (1 mL), after which the reaction proceeded for 3 h at 25 °C. Subsequently, BSA (6.35 mg) was added to a 50 mM carbonate buffer solution (pH 9.6; 2 mL). Next, the resultant reaction mixture was subjected to continuous stirring at 25 °C for 14 h. The product reaction required dialysis for three days to remove the excess reagents before lyophilization. The conjugates were verified by matrix-assisted laser desorption/ionization combined with time-of-flight mass spectrometry (MALDI-TOF MS) to quantify the hapten quantity [34]. To obtain the MR2–OVA conjugates, similar steps based on the $NaIO_4$ reaction were employed to prepare the coating agent using MR2 (3.03 mg) and OVA (6.13 mg).

2.5. Production and purification of MR2–targeting monoclonal antibody

The initial immunization emulsion was prepared by thoroughly mixing a solution of the MR2–BSA conjugates dissolved in phosphate-buffered saline (PBS) using Freund's complete adjuvant (Becton Dickinson Company, MD, USA) in a 1:1 ratio. The subsequent dose was prepared in the same manner as the first dose using Freund's incomplete adjuvant (Becton Dickinson Company, MD, USA) in the same ratio, and an emulsion containing 50 μg of immunogen was delivered intraperitoneally to individual animals 14 days after initial immunization. The next immunization (100 μg of immunogen) was administered without an adjuvant. After the second dose, mouse serum was collected from the tail vein after 5 days of administration to test for the antibody titer and activity against MR2 using indirect ELISA (iELISA) and indirect competitive ELISA (icELISA). Following the fifth immunization, the mice exhibiting the most evident activities and displaying the lowest cross-reactivity (CR; %) toward the structurally related compounds to MR2 were subjected to splenectomy. The splenocyte with SP2/0 (a mouse myeloma cell line) was fused using the well-established PEG technique [40]. Subsequently, the hybridoma cells were grown in an eRDF medium comprising RD-1 and 10 % FBS. Hypoxanthine, aminopterin, and thymidine were included as additives to facilitate the separation of the hybridoma cells from the nonhybridized ones. After two weeks of the culturing process, the presence of antibodies against MR2 in the culture media was assessed by iELISA. The clone-selection process was performed using limiting dilution, followed by antibody titer and activity screening via iELISA and icELISA [41], for the identification of positive clones. Subsequently, the 16E11 clone exhibiting minimal CR (%) was expanded in a nonaminopterin additive to enhance the production of anti-MR2 mAb until a volume of 1 L was achieved. Thereafter, the colony 16E11 cells were transferred to an FBS-devoid medium and cultured for three days before purification.

To purify mAb 16E11 from the hybridoma (16E11)-secreted supernatant, a Protein G Sepharose 4 Fast Flow column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was equilibrated with 10 mM phosphate buffer (PB, pH 7.0) . The supernatant was applied to the column after filtration and pH adjustment (pH 7.0) with a 1 M Tris-HCl (pH 9.0) solution, followed by a washing buffer (10 mM PB, pH 7.0) and an elution buffer (100 mM citrate buffer, pH 3.0). The eluted portion was promptly neutralized using a solution of 1 M Tris-HCl (pH 9.0). Each eluted fraction was determined by iELISA. Subsequently, the fractions containing mAb 16E11 were combined, dialyzed, and allowed to dry to yield the powder.

2.6. Indirect and indirect competitive enzyme-linked immunosorbent assay procedures

Next, mAb 16E11 was characterized using iELISA and icELISA. These methods were deployed to assess the reactivity of the antibody as well as its ability to competitively bind to free MR2. The experimental protocol for iELISA followed previously established methods [35–38] with slight adjustments. Briefly, the MR2–OVA conjugates were immobilized by adding the MR2–OVA conjugates in a 50 mM carbonate buffer (pH 9.6) (2 μg/mL; 100 μL/well) on a 96-well immunoplate. The reaction plate was incubated at 37 °C for 1 h. Next, PBS containing skim milk (5 % (w/v); 300 μL/well) was employed to mitigate non-specific binding to other substances. After 1 h of incubation, 5 % (v/v) methanol (50 μL/well) and various mAb 16E11concentrations in phosphate-buffered saline containing 0.005 % (v/v) Tween 20 (TPBS) (50 μL/well) were mixed and incubated for another 1 h (37 °C), followed by 10,000 times dilution of goat anti-mouse IgG H&L (HRP) (100 μL/well) for 1 h (37 °C) and a substrate solution (0.3 mg/mL ABTS in 100 mM citrate buffer (pH 4.0) supplemented with 0.003 % (v/v) H_2O_2; 100 μL/well). After 20 min of incubation, the UV absorbance was measured at 405 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Inc., Waltham, MA, USA). At every step after incubation, the 96-well plate was washed three times with TPBS.

In icELISA, competitive inhibition using a free antigen was evaluated. After the blocking step, various concentrations of MR2 in 5 % (v/v) methanol (50 μL/well) and mAb 16E11 (50 μL/well; 6.25 μg/mL) were mixed well and incubated for 1 h before proceeding to the following step, as previously described.

2.7. Development of indirect competitive enzyme-linked immunosorbent assay for MR2 detection and evaluation to verify antibody performance

To validate the immunoassay, CR (%) of mAb 16E11 was determined by comparing it with those of the other secondary metabolites present in *P. vietnamensis* var. *vietnamensis*. By calculating the half-maximal inhibitory concentration (IC50) of MR2 competing with the investigated compounds, CRs (%) was evaluated using the equation below, and the results confirmed the specificity of the method toward MR2 rather

than other similar chemicals.

$$\text{CRs (\%)} = \frac{\text{IC50 of MR2}}{\text{IC50 of candidate compounds}} \times 100.$$

The spiked recovery assay was performed to investigate the matrix effect on the assay by spiking 6.25 %, 12.50 %, 25 %, 50 %, and 75 % (w/w) of free MR2 standard compounds into a *P. vietnamensis* var. *vietnamensis* extract exhibiting a MR2 content of 36.9 ± 4.82 mg/g dry weight. The recovery percentage was calculated from the detected and spiked amounts of MR2 at different concentrations using the equation below. The acceptable range of the recovery rate was 80%–120 %, as previously described [42].

$$\textit{Recovery}\ (\%) = \frac{\textit{Detected amount} - 36.9}{\textit{spiked amount}} \times 100$$

2.8. Determination of the MR2 content of plant samples using icELISA

The plant samples were analyzed by icELISA. Antigen coating, i.e., the MR2–OVA conjugates (4 μg/mL), was applied to a 96-well plate (100 μL/well) and allowed to adhere for 1 h. Thereafter, nonspecific binding was prevented by blocking with PBS containing 5 % (w/v) skim milk. Subsequently, the diluted sample extracts or standard MR2 (50 μL/well) were incubated in the presence of 6.25 μg/mL of mAb 16E11 (50 μL/well). After an incubation period of 1 h, anti-mouse IgG with horseradish peroxidase (HRP), 1:10000, was introduced (100 μL/well). Subsequently, ABTS in a 100 mM citrate buffer, 0.003 % (v/v) H_2O_2, was added (100 μL/well). After further incubation for 20 min, the UV absorbance was measured at a wavelength of 405 nm. Thereafter, a standard curve was generated for MR2, and the concentrations of MR2 in the extracts were determined from the standard curve–derived range.

2.9. Determination of the MR2 content of plant samples using HPLC-UV

To confirm the MR2 immunoassay-detection approach, HPLC-UV using the SPD-20A UV–Vis detector (Shimadzu Co., Kyoto Japan) was employed to quantify the concentrations of MR2 in the samples. HPLC pump was PU-4580 with DG-4580 as degassing unit (Jasco Co., Tokyo, Japan). The chromatographic analysis was performed using a COSMOSIL Packed column 5C18-AR-II with an inner diameter and length of 4.6 × 150 mm, respectively. The adopted HPLC-UV condition was slightly modified, following extant research findings [19]. In summary, the mobile phase was 27 % (v/v) acetonitrile isocratic, and MR2 detection was performed using a UV detector set at 196 nm. The flow rate was left constant at 1.0 mL/min, whereas the injection volume was 20 μL. The analyses were performed in triplicate. All samples were filtered through a 0.45 μM Millipore® filter, and all mobile phases were degassed before use.

3. Results and discussion

3.1. Production of MR2–targeting monoclonal antibodies

Conjugation was achieved through the $NaIO_4$ reaction between MR2 and the carrier protein, BSA. The immunogenic probability of the MR2–BSA conjugates was investigated by assessing the hapten number. The molecular weights of BSA and the MR2–BSA conjugates, as determined from the MALDI-TOF MS (BRUKER Autoflex III) spectra, were 66,382 and 68,943 Da, respectively (Supplementary Fig. 1). Based on the molecular weight of MR2 (~787 Da), the analysis revealed that the MR2–BSA conjugates contained approximately three MR2 molecules. The extant studies revealed that a higher number of hapten molecules (ranging from 15 to 30) corresponds to an increased potential to function as an effective immunogen [34]. Conversely, a reduced number of hapten molecules corresponds to eliciting a more specific immunological response. Sakamoto et al. [35] studied the efficacy of mAb against harringtonine (HT), observing that the hapten number of the HT–BSA conjugates exhibited a hapten number of two molecules, as prepared by the $NaIO_4$ reaction. This finding demonstrated a notable level of specificity and sensitivity in relation to the low hapten number. Krittanai et al. [36] and Kitisripanya et al. [38] developed conjugates that gained seven and three hapten molecules, respectively, resulting in a highly specific antibody production.

After the fourth immunization using the MR2–BSA conjugates, the antibody from the mouse serum attained 80 % inhibition. Following the administration of the fifth boost dose, the mouse splenocyte was prepared and fused with the SP2/0 cell to produce a hybridoma. The limiting-dilution technique was employed to obtain a pure, single clone. Based on its superior sensitivity and selectivity toward MR2, the 16E11 clone was selected, and the antibody produced by 16E11 (mAb 16E11) was further purified after being derived from the second clone by limiting dilution. The fraction with a >1.0 absorbance was collected, mixed, and dialyzed against distilled water for 10 days. Subsequently, the resulting solution was lyophilized, resulting in a powdered product (216 mg).

Next, the isotype of the purified mAb 16E11 was identified using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics, Mannheim, Germany). Based on the result, mAb 16E11 was classified as an IgG1 subclass with a kappa light chain antibody.

3.2. Optimization and validation of indirect competitive ELISA

The reactivity of mAb 16E11 was investigated by iELISA and graphically represented as a sigmoidal curve (Fig. 2A). The graph revealed the optimal concentration required for the implementation of icELISA. Thus, the concentration of mAb 16E11 was established at 6.25 μg/mL based on an absorbance spectrum of ~1.0.

Quantitative icELISA was performed under optimized conditions, as previously described [34–38], and Fig. 2B shows the linearity curve. The range of the detectable concentration of MR2 was 3.91–250 ng/mL, with a determination coefficient (R^2) of 0.9922. Further, IC50 was 10.8 ng/mL. The limit of detection (LOD) and limit of quantitation (LOQ) were 1.53 and 2.50–46.6 ng/mL, respectively.

Furthermore, CR (%) was determined. The concentrations of the triterpenoid saponins, i.e., ginsenoside Rb1, ginsenoside Rd, ginsenoside Re, and ginsenoside Rg1, were <0.005 % each, and PRT4 exhibited the highest CR (%), followed by MR1 and VR1, with corresponding percentages of 70.7 %, 68.1 %, and 39.3 %, respectively (Table 1). This high CR (%)can be accounted for by the conjugate reaction using the $NaIO_4$ oxidative method. This method involves the oxidative cleavage of the adjacent 1,2-diols present in the sugar moiety, resulting in the formation of imides with the amino group of lysine residues found in the carrier protein [34]. Notably, mAb 16E11 could bind to the monoglucoside structure after the cleavage of the second glucose molecule due to the $NaIO_4$ reaction. This indicated that mAb 16E11 could react with PRT4 and MR1 containing those aforementioned monoglucoside structures. The analysis revealed that the MR2 content was significantly higher than those of PRT4, MR1, and VR1 by ~80, 40, and 160 times [18,21–24], respectively, despite the presence of CR (%) with structurally similar compounds. Derived from the root sample with the greatest concentration of 69.2 mg/g, the estimated quantities of PRT4, MR1, and VR1 from %estimate were 0.85, 1.83, and 0.43 mg, respectively. Compared with HPLC, which yielded 66.8 mg/g of MR2 (Table 2), such an outcome is plausible. Based on its precision, it can be concluded that icELISA using mAb 16E11 would be a good option for MR2 detection in *Panax* spp., particularly *P. vietnamensis*.

icELISA exhibited high repeatability and reproducibility, as indicated by the low percentages of relative standard deviation (%RSD). Specifically, the %RSD values for the repeatability (intraday) and reproducibility (interday) were in the 0.92%–3.01 % and 4.25%–7.28 % ranges, respectively (Table 3). Furthermore, when assessing the accuracy, the root extract that contained MR2 was used as the plant matrix.

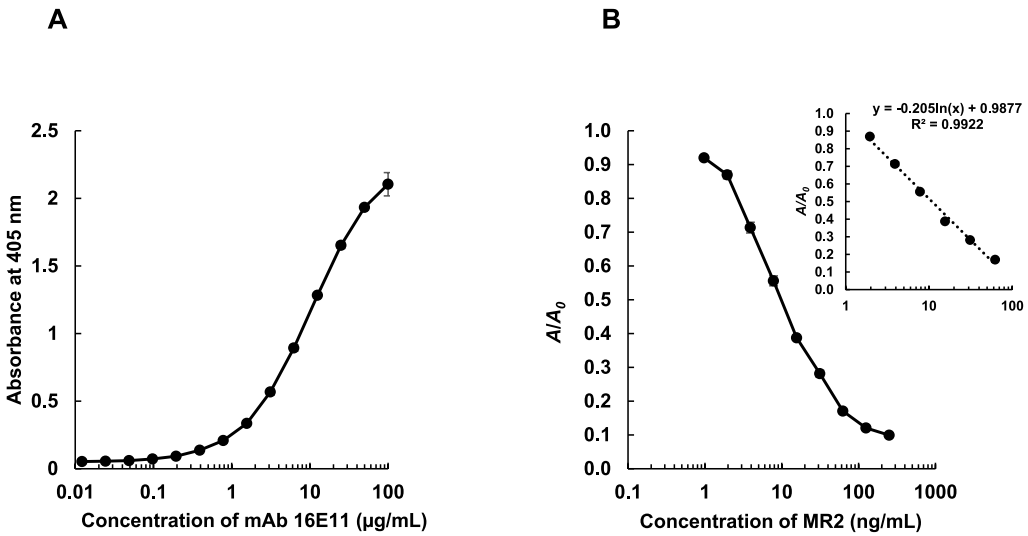

Fig. 2. Reactivity of mAb 16E11 in ELISA. **A**: The reactivity of mAb 16E11 against MR-2 OVA (4 μg/mL) determined by iELISA. **B**: The reactivity of mAb 16E11 (6.25 μg/mL) against free MR2 (0.39–250 μg/mL) determined by icELISA. *A*: the absorbance at 405 nm from the well with free MR2 A_0: the absorbance at 405 nm from the control well where control was 5 % (v/v) methanol.

Table 1
Cross-reactivity (CR, %) of mAb 16E11 against structured related compounds.

Compounds, amount in *P. vietnamensis* (estimated %w/w)	CR (%)
MR2	100.00
MR1	68.06
PRT4	70.67
VR1	39.26
VR2	12.24
VR11	4.21
Ginsenoside Rb1	<0.005
Ginsenoside Rd	<0.005
Ginsenoside Re	<0.005
Ginsenoside Rg1	<0.005

The measured amount of MR2 in the extract was 36.9 ± 4.82 mg/g dry weight. To evaluate the accuracy, the extract was supplemented with MR2 at concentrations of 6.25%–75.0 % of MR2 in the extract. The expected amounts of added MR2 were 39.2, 41.5, 46.1, 55.4, and 64.6 mg, respectively. As presented in Table 4, the outcomes exhibited recovery ranging from 85.3 % to 109 %, which is within the acceptable range of 80–120 % [43].

3.3. Determination of MR2 in actual plant samples by icELISA

To assess the efficacy of the proposed methodology, the MR2 contents of 13 root samples of *Panax* spp. **(1 - 13)** and one *R. siamensis* **(14)** fruit sample were analyzed. The icELISA results revealed that *P. vietnamensis* var. *fuscidiscus* **(1)** and *P. vietnamensis* var. *vietnamensis* **(2)** exhibited high MR2 concentrations, measuring 6.92 ± 0.463 and 3.83 ± 0.434 % (w/w), respectively. Conversely, *P. vietnamensis* var. *langbianesis* **(3)**, commonly referred to as unauthentic Vietnamese ginseng, displayed significantly lower MR2 concentrations, measuring $3.48 \times 10^{-3} \pm 1.91 \times 10^{-4}$ % (w/w). Further, PQ - 1 **(12)** and PQ - 2 **(13)** from Canada and China exhibited MR2 contents of approximately $1.13 \times 10^{-2} \pm 7.70 \times 10^{-4}$ % (w/w) and $8.04 \times 10^{-3} \pm 8.04 \times 10^{-4}$ % (w/w), respectively, and they were ranked second among other *Panax* spp. However, the MR2 quantities were significantly lower (400–600 times) than those of *P. vietnamensis* var. *fuscidiscus* **(1)** and *P. vietnamensis* var. *vietnamensis* **(2)**. The other *Panax* spp. exhibited a relatively diminished MR2 quantity. Most of the *P. japonicus* **(6)** as well as PG - 1 **(7)**, PG - 2 **(8)**, PG - 3 **(9)**, PG - 4 **(10)**, and PG - 5 **(11)** samples

Table 2
Determination of MR2 in plant samples including 13 samples with 6 different types of *Panax* spp. root and 1 *R. siamensis*'s fruit.

Sample	Total MR2 Amount (%w/w)	
	icELISA	HPLC-UV
(1) *P. vietnamensis* var. *fuscidiscus*	6.92 ± 0.463	6.68 ± 0.274
(2) *P. vietnamensis* var. *vietnamensis*	3.83 ± 0.434	3.80 ± 0.142
(3) *P. vietnamensis* var. *langbianesis*	$3.48 \times 10^{-3} \pm 1.91 \times 10^{-4}$	N.D.
(4) *P. pseudoginseng* var. *bipinnatifidus*	$3.47 \times 10^{-4} \pm 2.87 \times 10^{-5}$	N.D.
(5) *P. stipuleanatus*	$3.06 \times 10^{-4} \pm 2.68 \times 10^{-5}$	N.D.
(6) *P. japonicus*	$2.17 \times 10^{-3} \pm 1.41 \times 10^{-4}$	N.D.
(7) PG - 1 (*P. ginseng* cultivated in Japan)	$2.07 \times 10^{-3} \pm 1.43 \times 10^{-4}$	N.D.
(8) PG - 2 (*P. ginseng* cultivated in China)	$3.60 \times 10^{-4} \pm 2.22 \times 10^{-5}$	N.D.
(9) PG - 3 (Korea White Ginseng prescribed by doctor)	$3.88 \times 10^{-4} \pm 1.77 \times 10^{-5}$	N.D.
(10) PG - 4 (Korea White Ginseng from company)	$1.84 \times 10^{-3} \pm 1.15 \times 10^{-4}$	N.D.
(11) PG - 5 (Korea Red Ginseng)	$1.02 \times 10^{-3} \pm 1.69 \times 10^{-5}$	N.D.
(12) PQ - 1 (*P. quinquefolius* cultivated in Canada)	$1.13 \times 10^{-2} \pm 7.70 \times 10^{-4}$	N.D.
(13) PQ - 2 (*P. quinquefolius* cultivated in China)	$8.04 \times 10^{-3} \pm 8.04 \times 10^{-4}$	N.D.
(14) *R. siamensis*'s fruit	N.D.	N.D.

N.D. = Not detected.

exhibited comparable MR2 levels with *P. pseudoginseng* **(4)** and *P. stipuleanatus* **(5)**. An examination of the different origins of PG revealed that the source from Japan **(7)** exhibited comparable or greater MR2 concentration among the other PGs. Furthermore, a comparison between white and red ginseng from Korea sources (PG - 3 **(9)**, PG - 4 **(10)** and PG - 5 **(11)**) revealed that no substantial difference existed between their MR2 contents. In the case of *R. siamensis*, the plant with high triterpenoid saponins content [39], exhibited undetectable amount of MR2. To the best of the authors' knowledge, this study could be the first publication that identifies the MR2 concentrations of over 6 *Panax*

Table 3
Intra-day and inter-day validation to determine precision of ELISA method using mAb 16E11.

Concentration of MR2 (ng/mL)	RSD%	
	Intra-day (n = 6)	Inter-day (n = 3)
62.5	3.01	5.13
31.3	2.49	7.28
15.6	2.65	6.26
7.81	0.923	5.67
3.91	2.07	5.53
1.95	1.83	4.25

Table 4
Recovery determination using 6.25, 12.5, 25.0 50.0 and 75.0 % spike-level level of root sample of *P. vietnamensis* containing 36.9 ± 4.82 mg/g.

Spike-level of MR2% (w/w)	Spike-amount of MR2 (mg/g)	Detected MR2 content (mg/g)	Expected amount (mg/g)	Recovery (%)
0 %	0	36.9 ± 4.82	–	–
6.25 %	2.30	39.2 ± 3.04	39.2	99.1
12.5 %	4.60	41.9 ± 5.67	41.5	109
25.0 %	9.20	44.7 ± 2.53	46.1	85.3
50.0 %	18.5	54.4 ± 1.08	55.4	95.1
75.0 %	27.7	64.3 ± 4.60	64.6	99.4

Note: The standard MR2 were spiked with the amount of 0, 2.30, 4.60, 9.20, 18.5 and 27.7 mg/g to the plant sample with previously observed for MR2 content. Recovery (%) was calculated from detected MR2 content to determine the correlation between the expected and the analyzed amount of samples from ELISA.

spp. Furthermore, compared with the MR2 chemical detected in different ginseng types, it can be implied that MR2 may serve as a marker compound for separating *P. vietnamensis* (Ngoc Linh and Lai Chau ginsengs) from other varieties. According to the Vietnamese government document for *P. vietnamensis* authenticity, quantitative examination of fresh Ngoc Linh ginseng root requires 3 compounds: MR2, ginsenoside Rg1, and ginsenoside Rb1. The concentration of each saponin rises with root age, as documented from 4 to 15-year-old [10] (Supplementary Table 1). Thus, MR2 was dominating at 4-, 5-, and 6-year-olds, with concentrations of 2.0 %, 3.0 %, and 3.6 %, respectively, and proportionate to ginsenoside Rg1 and ginsenoside Rb1 by increasing with age in each root [11]. While quantitative examination of fresh Lai Chau ginseng roots requires at least 3 % MR2 in absolute dried material at 5-year-old and total saponin content [15]. Thus, the situation emphasizes importance of MR2 in quantitative analysis. To ensure Vietnamese ginseng quality, quantitative MR2 analysis is the most important official authentication step that classifies the specific specie. The quantitative measurement of unique MR2 using icELISA meets official genuine standards and might be utilized as a quality marker for Ngoc Linh and Lai Chau ginsengs. This analytical development can considerably enhance Vietnamese ginseng production and quality control.

3.4. Determination of MR2 in actual plant samples by HPLC-UV

MR2 was previously isolated from *P. japonicus* **(6)**, although only 0.1 % was detected [30]. The findings reported here indicate that MR2 was present in the additional *Panax* spp. in addition to *P. vietnamesis*. Various types of liquid chromatography with different detection methods have been employed to identify and measure MR2. MR2 has been determined using HPLC-UV. Zhu [19] designed an HPLC system to analyze 11 triterpenoid saponins, including MR2; it exhibited an LOD of 3 μg/mL. However, an assessment of the MR2 concentrations of 3 different types of *Panax* spp., MR2 was detected in *P. vietnamensis* at 58.80 mg/g dry weight, whereas it was undetectable in the other two *Panax* spp. High-resolution MS and multiple-stage tandem MS analyses were employed to identify 5 distinct ginseng varieties. The relative abundance (%) revealed that only the MR2 signal was observed exclusively in the root sample of *P. vietnamensis*, indicating the significantly high presence of MR2 therein. Tran et al. [18] used LC-electrospray ionization tandem mass spectrometry to measure the presence of MR2 in *P. vietnamensis* samples obtained from Quang Nam Province, Vietnam. The results revealed that the *P. vietnamensis* sample contained approximately ~1.85 % of MR2. HPLC-UV detection and charged aerosol detection (CAD) were employed for the analyses of MR2 and other ocotillol saponins. MR2 could be detected at LOD of 0.0312 and 0.0076 mg/mL using UV detection and CAD, respectively [31]. The assessment of other chromatography procedures confirmed that the developed icELISA approach outperformed others in terms of cost-effectiveness and the absence of any specific skill requirement. The specificity of the antigen–antibody combination as well as the increased sensitivity of this method make it more efficient in MR2 detection for differentiating authentic Ngoc Linh and Lai Chau ginsengs from other *Panax* spp.

To assess the reliability of the developed method, a modified version of HPLC-UV was employed for MR2 determination. The analysis of MR2 presented a challenge owing to its non-chromophore nature for UV detection. Nonetheless, Zhu et al. [19] established an HPLC approach that employed a UV-detection wavelength of 196 nm to effectively quantify ocotillol saponin, including MR2. After a few adjustments, MR2 was examined with LOD and LOQ of 6.52 and 20.0 μg/mL, respectively. The retention time was between the interval of 3.5 and 3.8 min (Supplementary Fig. 2). Further, calibration curves were established for MR2 at a concentration range of 15.6–500 μg/mL. Regarding the comparison between the existing methods and the newly developed ones, icELISA demonstrated the potential to detect MR2 at the nanogram level compared with HPLC-UV at the microgram level. The antigen–antibody complex approach employed in this study demonstrated an outstanding sensitivity level, enabling the detection of low quantities of MR2 observed in *Panax* spp. other than Ngoc Linh and Lai Chau ginsengs.

4. Conclusions

Here, an MR2-targeting mAb was generated. Further, mAb 16E11 can be employed for icELISA development, achieving a LOD that is as low as 1.53 ng/mL while maintaining satisfactory accuracy and precision levels. This methodology can be employed for the detection of MR2 in *Panax* spp. The approach reported herein can be used as a quantitative screening tool for Ngoc Linh and Lai Chau ginsengs, which are officially protected by the Geographical Indication “Ngoc Linh” and the trademark “Lai Chau ginseng”, respectively. This will greatly assist the Vietnamese ginseng industry by facilitating the application potential of MR2-rich products. Advanced ELISA can be employed to distinguish the raw materials of the MR2-rich Ngoc Linh and Lai Chau ginsengs from those derived from different species with low content of MR2, which is necessary for quality control in pharmaceutical and medicinal product applications.

Declaration of competing interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgements

Dr. Le Van Huy would like to thank the financial support from the SAM VIET NHAT company for his work in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.05.004.

References

[1] Hu Y, He Y, Niu Z, Shen T, Zhang J, Wang X, Hu W, Cho JY. A review of the immunomodulatory activities of polysaccharides isolated from *Panax* species. J Ginseng Res 2022;46:23–32.

[2] Zhou W, Chai H, Lin PH, Lumsden AB, Yao Q, Chen CJ. Molecular mechanisms and clinical applications of ginseng root for cardiovascular disease. Med Sci Monit 2004;10:187–92.

[3] Kim JH. Cardiovascular diseases and *Panax* ginseng: a review on molecular mechanisms and medical applications. J Ginseng Res 2012;36:16–26.

[4] Hou W, Wang Y, Zheng P, Cui R. Effects of ginseng on neurological disorders. Front Cell Neurosci 2020;14:55.

[5] Ong WY, Farooqui T, Koh HL, Farooqui AA, Ling EA. Protective effects of ginseng on neurological disorders. Front Aging Neurosci 2015;7:129.

[6] Christensen LP. Chapter 1 ginsenosides: chemistry, biosynthesis, analysis, and potential health effects. Adv Food Nutr Res 2008:1–99. Academic Press.

[7] Quang TP, Thi KHT, Ngoc BT, Dinh TP, Thanh TB, Thi HAN, Son NT, Son HT, Hung TT, Khuong NV. The ecology and saponins of Vietnamese ginseng–*Panax vietnamensis* var. *fuscidiscus* in north Vietnam. Asian J Agric Biol 2019;7:334–43.

[8] Thanh N, Van T, Lai H, Khiem N, Mai L, Xuyen D, Oanh PT, Hai DV, Dien ND, Tan ND. Bioactive compounds and biological activities of Vietnamese ginseng (*Panax Vietnamensis* Ha et Grushv.). In: Murthy HN, Paek KY, Park SY, editors. Bioactive compounds in the storage organs of plants. reference series in Phytochemistry. Cham: Springer; 2023. p. 1–25. https://doi.org/10.1007/978-3-031-29006-0_23-1.

[9] Decision number: 1247/QĐ-UBND. Author: The People's Committee of Kon Tum Province. Title of a government document: Decision for the management and use regulations of geographical indication Ngoc Linh for Ngoc Linh ginseng of Kon Tum Province. 2021. p. 1–11. Kon Tum Province, Vietnam. Year.

[10] Decision number: 3957/QĐ-UBND. Author: The People's Committee of Quang Nam Province. Title of a government document: Decision for the management and use regulations of the geographical indication Ngoc Linh. 2017. p. 1–19. Quang Nam Province, Vietnam. Year.

[11] Registration Certificate No. 00049. Name of government department: national Office of intellectual property. Title of a government document: Decision No. 2465/QĐ-SHTT on the amendment of geographical indication registration No. 00049 for Ngoc Linh ginseng. Place of publication: Industrial Property Official Gazette of Vietnam. 2018. p. 1135–9. ISSN 0868-2534. Number 366, Volume B (09.2018). Part VI: geographical indication registrations.

[12] Zhu S, Fushimi H, Cai S, Chen H, Komatsu K. A new variety of the genus *Panax* from southern Yunnan, China and its nucleotide sequences of 18S ribosomal RNA gene and matK gene. J Jap Bot 2003;78:86–94.

[13] Duy NV, Trieu LN, Chinh ND, Tran VT. A new variety of *Panax* (Araliaceae) from Lam vien plateau, Vietnam and its molecular evidence. Phytotaxa 2016;277:47–58.

[14] Phan K, Le T, Phan K, Vo D, Phan V. In Lai Chau ginseng Panax vietnamensis var. fuscidiscus K. Komatsu, S. Zhu & SQ Cai, I. morphology, ecology, distribution and conservation status, Proceedings of the 2nd VAST-KAST workshop on biodiversity and bioactive compounds, Hanoi, Vietnam, 2013; pp 65–73.

[15] Decision number: 1183/QĐ-UBND. Author: The People's Committee of Lai Chau Province. Title of government document: Decision for the management and use regulations of the trademark Sam Lai Chau. Edited by the People's Committee of Lai Chau Province, Vietnam. Edited place: Lai Chau Province. 2022. p. 1–17. Vietnam. Year.

[16] Trang NT, Mai NT, Zhuravlev YN. Application of DNA barcoding to authentic *Panax vietnamensis*. Am Sci Res J Eng Techno Sci 2017;29:60–7.

[17] Liu J, Xu Y, Yang J, Wang W, Zhang J, Zhang R, Meng Q. Discovery, semisynthesis, biological activities, and metabolism of ocotillol-type saponins. J Ginseng Res 2017;41:373–8.

[18] Tran QL, Adnyana IK, Tezuka Y, Nagaoka T, Tran QK, Kadota S. Triterpene saponins from Vietnamese ginseng (*Panax vietnamensis*) and their hepatocytoprotective activity. J Nat Prod 2001;64:456–61.

[19] Zhu S, Zou K, Cai S, Meselhy MR, Komatsu K. Simultaneous determination of triterpene saponins in ginseng drugs by high-performance liquid chromatography. Chem Pharm Bull 2004;52:995–8.

[20] Cao Y, Wang K, Xu S, Kong L, Bi Y, Li X. Recent advances in the semisynthesis, modifications and biological activities of ocotillol-type triterpenoids. Molecules 2020;25:55–62.

[21] Le QU, Lay HL, Wu MC, Thi HN, Duy-Lam N. Phytoconstituents and biological activities of *Panax vietnamensis* (Vietnamese ginseng): a precious ginseng and call for further research - a systematic review. Nat Prod Commun 2018;13:1381–4.

[22] Quan LT, Ketut AI, Yasuhiro T, Yuko H, Ikuo S, Youichi K, Qui KT, Shigetoshi K. Hepatoprotective effect of majonoside R2, the major saponin from Vietnamese ginseng (*Panax vietnamensis*). Planta Med 2002;68:402–6.

[23] Yamasaki K. Bioactive saponins in Vietnamese ginseng, *Panax vietnamensis*. Pharm Biol 2000;38:16–24.

[24] Duc NM, Nham NT, Kasai R, Ito A, Yamasaki K, Tanaka O. Saponins from Vietnamese ginseng, *Panax vietnamensis* HA et GRUSHV. collected in Central Vietnam. I. Chem Pharm Bull 1993;41:2010–4.

[25] Vu-Huynh KL, Nguyen HT, Van LTH, Ma CT, Lee GJ, Kwon SW, Park JH, Nguyen MD. Accumulation of saponins in underground parts of *Panax vietnamensis* at different ages analyzed by HPLC-UV/ELSD. Molecules 2020;25:3086.

[26] Zhu S, Zou K, Fushimi H, Cai S, Komatsu K. Comparative study on triterpene saponins of ginseng drugs. Planta Med 2004;70:666–77.

[27] Trieu LN, Duy NV, Tien TV. Genetic diversity of *Panax vietnamensis* var. *langbianensis* populations in Lamvien Plateau -Vietnam detected by inter simple sequence repeat (issr) markers. J Biotechnol 2019;17:651–61.

[28] Giang VNL, Waminal NE, Park HS, Kim NH, Jang W, Lee J, Yang TJ. Comprehensive comparative analysis of chloroplast genomes from seven *Panax* species and development of an authentication system based on species-unique single nucleotide polymorphism markers. J Ginseng Res 2020;44:135–44.

[29] Lü JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293–302.

[30] Morita T, Kasai R, Tanaka O, Zhou J, Yang T, Shoji J. Saponins of Zu-Tziseng, rhizomes of Panax japonicus C. A. Meyer var. major (Burk.) C.Y. Wu et K. M. Fens collected in Yunnan, China. Chem Pham Bull 1982;30:4341–6.

[31] Tanaka K, Kubota M, Zhu S, Sankawa U, Komatsu K. Analysis of ginsenosides in ginseng drugs using liquid chromatography-fourier transform ion cyclotron resonance mass spectrometry. Nat Prod Commun 2007;2:625–32. gui.

[32] Nguyen HT, Vu-Huynh KL, Nguyen HM, Le HT, Le THV, Park JH, Nguyen MD. Evaluation of the saponin content in *Panax vietnamensis* acclimatized to Lam Dong province by HPLC–UV/CAD. Molecules 2021;26:5373.

[33] Van LTH, Lee SY, Kim TR, Kim JY, Kwon SW, Nguyen NK, Park JH, Nguyen MD. Processed Vietnamese ginseng: preliminary results in chemistry and biological activity. J Ginseng Res 2014;38:154–9.

[34] Sakamoto S, Putalun W, Vimolmangkang S, Phoolcharoen W, Shoyama Y, Tanaka H, Morimoto S. Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. J Nat Med 2018;72:32–42.

[35] Sakamoto S, Yusakul G, Tsuneura Y, Putalun W, Usui K, Miyamoto T, Tanaka H, Morimoto S. Sodium periodate-mediated conjugation of harringtonine enabling the production of a highly specific monoclonal antibody, and the development of a sensitive quantitative analysis method. Analyst 2017;142:1140–8.

[36] Krittanai S, Pichetpongtorn P, Sakamoto S, Putalun W. Monoclonal antibody-based immunoassay for the specific quantification of licochalcone A: an active chalcone in licorice. Food Agric Immunol 2022;33:220–34.

[37] Noguchi K, Imahori D, Kido Y, Nuntawong P, Tanaka H, Morimoto S, Sakamoto S. Quality assessment method for Chinpi, dried *Citrus* spp. peel and its derived Kampo medicines using specific monoclonal antibody against hesperidin. Phytochem Anal 2023;34:652–60.

[38] Kitisripanya T, Krittanai S, Udomsin O, Jutathis K, Komaikul J, Juengwatanatrakul T, Sakamoto S, Tanaka H, Morimoto S, Putalun W. Development of an enzyme-linked immunosorbent assay for determination of miroestrol using an anti-miroestrol monoclonal antibody. Planta Med 2017;83:855–61.

[39] Khwanchuea R, Queiroz EF, Marston A, Jansakul C, Hostettmann K. Saponins from fresh fruits of *Randia siamensis* (lour) roem. & schult. (Rubiaceae). Nat Prod Commun 2008;3:163–70.

[40] Fuller S.A., Takahashi M., Hurrell J.G. Fusion of myeloma cells with immune spleen cells. Curr Protoc Biol 2001. Chapter 11; Unit 11.7.11.7.1–11.7.4.

[41] Fuller S.A., Takahashi M., Hurrell J.G. Cloning of hybridoma cell lines by limiting dilution. Curr Protoc Mol Biol 2001. [Chapter 11]: Unit 11.8.11.8.1–11.8.2.

[42] Vdovenko MM, Gribas AV, Vylegzhanina AV, Sakharov IY. Development of a chemiluminescent enzyme immunoassay for the determination of dexamethasone in milk. Anal Methods 2012;4:2550–4.

[43] Andreasson U, Perret-Liaudet A, Van LC, Blennow K, Chiasserini D, Engelborghs S, Fladby T, Genc S, Kluse N, Kuiperij HB, et al. A practical guide to immunoassay method validation. Front Neurol 2015;6:179–86.