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ORIGINAL ARTICLE

A New Highly Selective and Specific Anti-puerarin polyclonal Antibody for Determination of Puerarin Using a Mannich Reaction Hapten Conjugate

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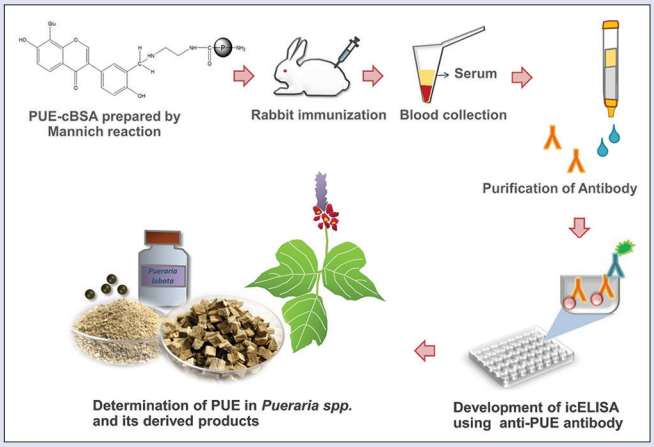
ABSTRACT

Background: Puerarin (PUE) is a phytoestrogen found in *Pueraria candollei* and *Pueraria lobata*. These plants are substantial for traditional medicine in various Asian countries. PUE is a key marker that can be found only in the *Pueraria* species. **Objective:** To establish the method for determination of PUE content which is required for quality control of pharmaceutical products. **Materials and Methods:** PUE-cationized bovine serum albumin conjugate was created via Mannich reaction. After the rabbit immunization, the obtain anti-PUE polyclonal antibody (PAb) was used to develop an enzyme-linked immunosorbent assay (ELISA). **Results:** An anti-PUE PAb possess a great sensitivity and specificity. The cross-reactivity analysis shows no cross-reaction of an established antibody against other substances. In addition, we successfully developed an indirect competitive ELISA (icELISA) for the quantitative analysis of PUE. The result of method validation conforms to acceptance criteria and correlates with high-performance liquid chromatography, the reference method. The icELISA was applied to determine PUE content in *Pueraria* spp. plant samples and its derived pharmaceutical products. **Conclusion:** This highly specific immunogen was created from the Mannich reaction. An icELISA can also be applied to other research propose in the further studies.

Key words: Enzyme-linked immunosorbent assay, polyclonal antibody, Pueraria spp., puerarin

SUMMARY

- The new immunogen conjugated (puerarin-cBSA) via Mannich reaction was successfully in rising of antibody against puerarin (PUE)
- The obtained anti-PUE polyclonal antibody (PAb) was high sensitivity and specificity to PUE
- An indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed and validated using anti-PUE PAb
- The established icELISA was applied to determine PUE content in various tuberous root of Pueraria spp
- Moreover, icELISA method can be applicable in Pueraria spp. derived products.



Abbreviations used: PUE: Puerarin; PAb: Polyclonal antibody; ELISA: Enzyme-linked immunosorbent assay; icELISA: Indirect competitive ELISA; cBSA: Cationized bovine serum albumin.

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INTRODUCTION

Puerarin (PUE) is a phytoestrogen found in *Pueraria candollei*, a famous Thai herbal medicine with rejuvenate properties.[1,2] PUE is also the main active compound that found in Kudzu (*Pueraria lobata*), the important traditional medicine in Chinese and Japan.[3] Its chemical structure is an isoflavonoid glycoside (8-C-glucosyl-4′, 7-dihydroxyisoflavone). Especially, PUE is a marker that can be found only in the *Pueraria* species. Over 17 species of *Pueraria* were distributed in Japan, Chinese, South Asia, South East Asia, and some country on Pacific Ocean.[3-5] PUE is a marker that has been used in most of the pharmacological studies. The activities of PUE show the effect on cardiovascular and cerebrovascular[6-10] endocrine systems and diabetes.[11] The anti-inflammatory[12] and anti-platelet aggregation activities of PUE were also reported.[13] Toxicity study of PUE in animal and human models shows its safety.[14,15] The efficacy and safety data of PUE result to the various formulations of PUE or crude extract of *Pueraria* spp. was most popular in China and Japan. The products from those countries have been imported to Thailand and others worldwide.

The variation of active compounds in *P. candollei* was depending on the plant species, cultivation, harvesting season and the industrial process.[16] As well as, the variation of active compounds in Kudzu

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that reported by Cherdshewasart *et al.*[17] and Jiang *et al.*[18] Therefore, the quality control and standardization of herbal material and herbal products have become important. The pharmacopeia and industrial manufacturing indicated PUE or daidzin as a marker for quality control of Kudzu or *P. candollei*. Many publication of analytical method for detection of PUE in herbal medicine and biological samples have been reported (thin layer chromatography, high-performance liquid chromatography [HPLC], LC-mass spectrometry and fluorescence) including the immunoassay methods.[19-22] However, there are some limitations of those methods.

An immunoassay method using the affinity and specificity of antibody-antigen binding reaction for antigen detection provides very beneficial methods for various researches proposes. Although anti-PUE polyclonal antibody (PAb) and anti-PUE monoclonal antibody MAb were reported, the antibodies characteristic still has cross-reactivity with structurally related chemicals.[21,22] Those publications, the antibodies were produced using the same immunogen conjugated method (sodium periodate reaction). The affinity and specificity of antibody depend on several factors; the preparation of immunogen conjugated method is the most important one. To obtain antibody against PUE with high specificity, the new immunogen through Mannich reaction has been prepared in the present study. Anti-PUE PAb was produced, developed, and validated methods as indirect competitive enzyme-linked immunosorbent assay (icELISA). Then, the characterized methods were applied to define PUE as a marker for quality control of herbal materials and herbal products containing PUE.

MATERIALS AND METHODS

Chemical and immunochemical reagents

All chemical reagents in our experiment were standard commercial products of analytical grade. The reference standard of PUE was obtained from Sigma-Aldrich (MO, USA). Daidzin, daidzein, genistin, and genistein were obtained from Fujicco Co., Ltd (Kobe, Japan). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants were provided by Sigma-Aldrich (MO, USA). Ethylenediamine (EDA) was obtained from Panreac AppliChem (Darmstadt, Germany). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was purchased from Fluka Chemical (Buchs, Switzerland). 0.1 M of 2-(N-morpholino) ethanesulfonic acid, 0.9% sodium chloride (NaCl), pH 4.7 (MES buffer saline) was obtained from Thermo Fisher Scientific (MA, USA). Peroxidase-labeled anti-rabbit IgG was obtained from MP Biomedicals (Ohio, USA). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animal

Two months of male New Zealand white rabbit was purchased from National Laboratory Animal Center, Mahidol University. The Animal Ethics Committee was approved animal handling and treatment protocol (Record No. AEKKU 43/2557).

Plant materials and samples preparation

P. candollei var. *mirifica* (PM) were collected from two sources in Thailand which are Suranaree University of Technology (Nakhon Ratchasima) and Ubon Ratchathani University (Ubon Ratchathani) (NI-PSKKU009-010). *Pueraria thomsonii* and *Pueraria phaseloides* were collected from Prachinburi and Surat Thani, Thailand (NI-PSKKU067-068), respectively. All reference specimens were deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. The dried tuber of *P. lobata* was obtained from the market in Fukuoka, Japan.

Cell suspension was obtained from stem-derived callus of PM which maintained on Murashige and Skoog (MS) liquid medium supplement with three combinations of hormones (0.1 mg/L thidiazuron, 1 mg/L α-naphthalene acetic acid and 0.5 mg/L 6-benzyladenine). Hairy root was obtained from the cotyledon leaves and stems part *in vitro* plantlets of PM which were infected with *Agrobacterium rhizogenes* ATCC 15834 and cultured in half-strength MS medium-free hormone.

Herbal products contain *Pueraria* spp. were purchased from the markets in Japan (Product No. 1–6) and Thailand (Product No. 7–13).

The dried powder of plant samples (200 mg), Product No. 1–6 (200 mg), and Product No. 7–13 (500 mg) were exactly weighted, extracted with ethanol (0.5 ml) by sonication for 15 min. Then, the extracts were centrifuged 10,000 rpm for 3 min; the supernatant was collected. The extraction was repeated for three times. The combined extract was evaporated and redissolved in 1 ml ethanol. All sample solutions were freshly prepared diluted into working concentration for PUE analysis.

Preparation of hapten-carrier immunogen conjugate

Preparation of cationized bovine serum albumin

Preparation of cationized BSA (cBSA) was followed the method of Muckerheide *et al.* (1987) with some modification. In brief, 5 mL of EDA was added to 5 ml of 0.1 M MES containing 0.9% NaCl buffer (pH 4.7) in an ice bath, and then adjust pH to 4.8 with 37% HCl. The mixture solution (2680 μL) was added to 1,000 μL of 100 mg native BSA and 60 mg of EDC, incubated at room temperature for 3 h. Then, the reaction was stopped by 2 M acetic acid and dialyzed five times against distilled water. Finally, the reaction was lyophilized and stored at −20°C. Preparation of cOVA was done as the same procedure.

Preparation of puerarin-cationized bovine serum albumin

Preparation of PUE-cBSA conjugated was based on Mannich reaction[23] with some modification. Briefly, 8 mg PUE was dissolved in 600 μL of absolute ethanol and then added 400 μL of 37% formaldehyde. The 8 mg cBSA was dissolved in 2 ml of 0.1 M MES containing 0.9% NaCl buffer (pH 4.7). Then, the hapten solution was drop wised into protein solution and stirred at 37°C for 24 h. The PUE-cBSA conjugate was dialyzed 5 times against distilled water. Finally, it was lyophilized and stored at −20°C. Preparation of PUE-cOVA conjugated was done as the same procedure.

Determination of the hapten number for the puerarin-cationized bovine serum albumin conjugate

The matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) autoflex III (Bruker Daltonics, Bremen, Germany) was used to determine the hapten number that contained in the PUE-cBSA conjugate. Briefly, 1–10 pmol of each native BSA, cBSA, and PUE-cBSA conjugated was mixed with a 1000 fold molar excess of Sinapinic acid in an aqueous acetonitrile solution containing 0.1% trifluoroacetic acid. Then, the mixtures were applied to a MALDI-TOF-MS system.

Immunization procedure

PUE-cBSA 5 mg was dissolved in 500 μL of 6 M urea to making 10 mg/ml conjugate stock solution. For the first immunization; 150 μL of conjugated stock solution was dissolved in 750 μL of sterile PBS and emulsified with an equal volume of Freund's complete adjuvant. Then, the emulsion was subcutaneously injected into 2 months of male New Zealand white rabbit. Four weeks later, the rabbits were immunized through intramuscular route (100 μL of conjugated stock solution in 800 μL of sterile PBS and emulsified with an equal volume

of Freund's incomplete adjuvant was used). Then, boosts were given at every 2 weeks interval. After 3–4 days of each boost, anti-PUE serum was taken from the marginal ear vein of immunized rabbit. Finally, anti-PUE serum was purified and further characterized.

Purification of anti-puerarin polyclonal antibody

The anti-PUE PAb from rabbit serum was purified using a protein G FF column (0.46 cm × 11 cm; Amersham Pharmacia Biotech, Uppsala, Sweden). The column was washed and equilibrium with 20 mM phosphate buffer (pH 7). Then, 2 mL of rabbit serum containing IgG was slowly loaded to the column. Adsorbed IgG was eluted with 100 mM citrate buffer (pH 2.7). The eluted IgG was neutralized with 1 M Tris-HCl buffer (pH 9), then dialyzed against distilled water for 5 times, and then lyophilized. Finally, powdered of PAb against PUE was obtained and used for further characterization.

Optimization and development of indirect enzyme-linked immunosorbent assay

The coating concentration of the capture antibody (PUE-cOVA) and the concentration of antibody (anti-PUE PAb) were investigated. Each PUE-cOVA at various concentrations (0.5, 1, 2.5 and 5 μg/mL) in carbonate buffer (50 mM, pH 9.6) was coated on 96 well immunoplate (100 μL/well) and incubated at 37°C for 1 h. Then, the plate was treated with 1% gelatin in PBS (300 μL/well) for 1 h to reduce nonspecific binding. After then, the plate was washed three times with PBS containing 0.05% (v/v) Tween20 (T-PBS). Later, the plate was reacted with 100 μL of anti-PUE PAb for 1 h and washed three times with T-PBS. Then 100 μL of 1:1000 dilutions of peroxidase-labeled anti-rabbit IgG was added and incubated for l h. After washing the plate three times with T-PBS, 100 μL of substrate solution (containing 0.1 M citrate buffer [pH 4.0], 0.003% [v/v] H_2O_2 and 0.3 mg/mL ABTS) was added and incubated for 15 min. Absorbance was measured by a microplate reader at 405 nm (Model 550 Microplate Reader BioRad Laboratories, CA, USA).

Optimization and development of an indirect competitive enzyme-linked immunosorbent assay

PUE-cOVA in carbonate buffer (50 mM, pH 9.6) was coated on 96 well immunoplate (100 μL/well) and incubated at 37°C for an hour. Then, the plate was blocked with 1% gelatin in PBS (300 μL/well) for 1 h. After the plate was washed three times with T-PBS, the mixture of free antigen and antibody (50 μL of various concentrations of PUE standards/samples contained PUE and 50 μL of anti-PUE PAb) was incubated for 1 h and washed three times with T-PBS. Then 100 μL of the secondary antibody (1:1000 dilutions of peroxidase-labeled anti-rabbit IgG) was added and incubated for an hour. After washing the plate three times with T-PBS, 100 μL of substrate solution was added and incubated for 15 min. After that, absorbance was measured by a microplate reader at 405 nm.

Evaluation of reactivity and specificity of anti-puerarin polyclonal antibody

The reactivity of anti-PUE PAb was determined by indirect ELISA as mentioned previously. The antibody specificity was performed in a cross-reactivity study of anti-PUE PAb with structure-related compounds and/or nonrelated compounds. Various structures over 17 compounds were tested by using an icELISA protocol as mentioned above. The antibody specificity was defined by % cross-reactivity which calculated based on the method of Weiler and Zeng[24] as follows.

% cross-reactivity = (IC_{50} of PUE/IC_{50} of tested compounds) × 100

Validation of indirect competitive enzyme-linked immunosorbent assay using anti-puerarin polyclonal antibody

The developed icELISA method was validated for sensitivity, specificity, precision, accuracy, and correlation with HPLC reference method.

Sensitivity

Different concentration of PUE standard was used as competitor in icELISA to investigate the sensitivity. The sensitivity of assay was defined as the limit of detection (LOD) which calculated at a concentration that gave 10% inhibition.

Specificity

The specificity of assay was defined as % cross-reactivity. Anti-PUE PAb was incubated with over 17 compounds by using an icELISA.

Precision

Intra- and inter-plate precision was investigated from the measurement range concentration of PUE. The intraplate precision was evaluated by the variation of the measured concentration of PUE from well-to-well ($n = 5$) within the same plate. Whereas, the interplate precision was obtained from plate to plate ($n = 5$). The precision was expressed as the standard deviation (SD) and relative standard deviation (%RSD).

Accuracy

The accuracy can be performed by the recovery study. The extract of *P. lobata* was spiked with a known quantity (0, 10, 25, and 50 μg/mL) of PUE standard and the spiked sample was analyzed by developed icELISA. Then, the percentage of recovery and the relative standard deviation was calculated.

% recovery = (measured amount − unspiked amount of sample)/spiked amount × 100

Correlation with reference high-performance liquid chromatography method

The HPLC method was developed and validated to compare with the icELISA for determination of PUE. The plant and product samples with different concentration of PUE (cover the concentration range of all analyzed samples) were analyzed by the assays. Then, a scatter graph of result from the analyzed concentration by two assays was constructed. The linear regression analysis between two assays with the degree of agreement indicates the correlation of the assays.

High-performance liquid chromatography conditions

The method was performed on an Agilent 1100 (Agilent Corp., Santa Clara, California). A reverse phase column (LiChroCart®, 125 mm × 4 mm, 5 μm particle size; Merck KGaA., Darmstadt, Germany) was selected. The mobile phase consisted of 15% acetonitrile containing 0.75% acetic acid, which flow rate was set at 0.6 mL/min. The detection wavelength was set at 254 nm. Approximately, 20 μL of each sample solution was injected. Then, the HPLC method was validated. The validation parameters were linearity, precision, and accuracy.

Application of an indirect competitive enzyme-linked immunosorbent assay using anti-puerarin polyclonal antibody to determine puerarin in *Pueraria* spp. and herbal products containing *Pueraria* spp.

The developed and validated icELISA method using anti-PUE PAb was applied to determine PUE in *Pueraria* spp. and herbal product containing *Pueraria* spp.

RESULTS AND DISCUSSION

Synthesis and characterization of hapten-carrier immunogen conjugate

Preparation of cBSA and PUE-cBSA was successfully confirmed by MALDI-TOF-MS analysis. The spectrum of native BSA, cBSA, and PUE-cBSA appeared approximately at m/z ratio 66348, 67013 and 68994, respectively [Figure 1]. The number of hapten molecules per molecule of protein of the conjugate was 4.8. The molecular weight of cBSA was higher than native BSA, indicated that after the reaction occurred, EDA molecules were added to form cBSA.[25] Then, the Mannich reaction was performed to generate PUE-cBSA [Figure 2]. The reaction occurred when compounds containing active hydrogen which can be linked to amine or amine group by formaldehyde in acidic condition.[23]

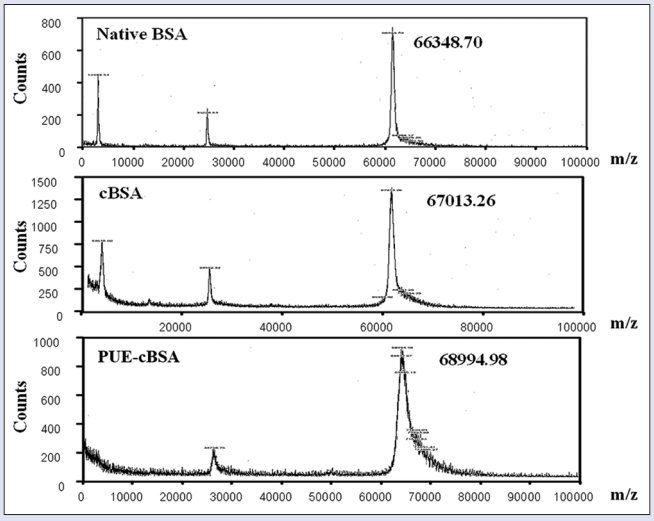

Figure 1: Matrix-assisted laser desorption/ionization time-of-flight Murashige and Skoog spectrum of native bovine serum albumin, cationized bovine serum albumin and puerarin-cationized bovine serum albumin

Optimization and development of indirect enzyme-linked immunosorbent assay and indirect competitive enzyme-linked immunosorbent assay

The coating of the capture antibody (PUE-cOVA) at various concentrations (0.5, 1, 2.5, and 5 μg/mL) and the concentration of antibody (anti-PUE PAb) were investigated in indirect ELISA [Figure 3]. 2.5 μg/mL of PUE-cOVA showed the appropriate reactivity of anti-PUE PAb. Therefore, the concentration of PUE-cOVA 2.5 μg/mL was used for icELISA.

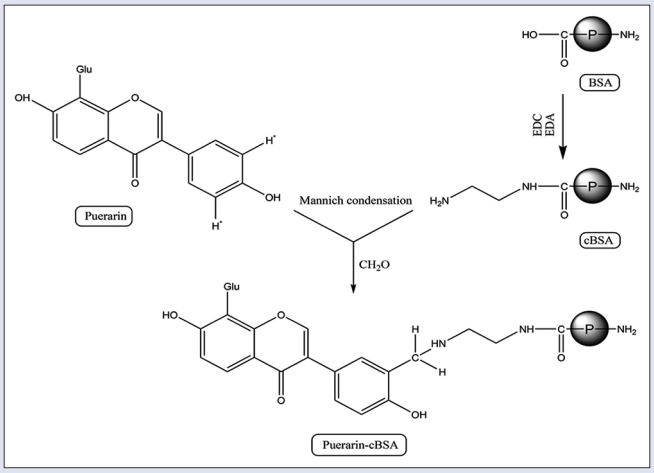

Figure 2: Scheme diagram shows preparation of cationized bovine serum albumin and puerarin-cationized bovine serum albumin conjugate

High-performance liquid chromatography validation

An HPLC method was set as a comparator to develop ELISA. The method was validated. The standard calibration curve of PUE exhibited linearity and a good correlation coefficient over the given range of 1.56–100 μg/mL. The linear regression equations from the calibration curve of PUE was y = 1.8165x + 1.5968 (R^2 = 0.9996). The intra- and inter-day precision is expressed in the range of 2.79–5.98 and 5.51–6.75%RSD, respectively. The percentage recoveries of PUE standard concentration from the spiked samples were in an acceptable range (94.66–98.38%). The validated results showed that the method was precise and accurate for determination of PUE in the crude extract of *Pueraria* spp.

Reactivity and specificity of anti-puerarin polyclonal antibody

In the indirect ELISA assay, the reactivity of antibody was determined by adding various concentration of anti-PUE PAb to the coated 96-well immunoplate with 2.5 μg/mL of PUE-cOVA. The reactivity of anti-PUE PAb as indicated in Figure 4. 8 μg/mL of anti-PUE PAb (the concentration at the absorbance was about 1) was selected for icELISA.

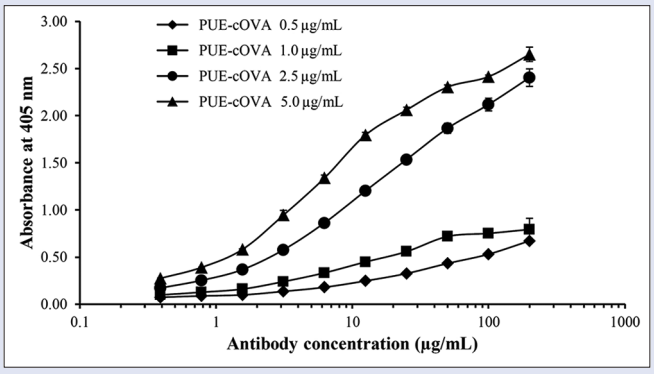

Figure 3: Effect of puerarin-cOVA coating concentration on reactivity of anti-puerarin polyclonal antibody

Various structures over 17 compounds (isoflavonoid, flavonoid, and stilbene) were tested for the specificity of antibody using an icELISA. Table 1 shows the % cross-reactivity of anti-PUE PAb with structure-related compounds. The result indicated that anti-PUE PAb did not have any cross-reaction with structure-related and/or nonrelated compounds. Especially, the compounds from isoflavonoid and flavonoid groups which have been reported the cross-reaction with PAb and MAb against PUE in a previous publication by Pongkitwitoon *et al.*[21] and Qu *et al.*[22] Pongkitwitoon *et al.*[21] reported that the characteristic of PAb has been shown to be cross-reactivity with its aglycone, daidzein (127.79%). Qu *et al.*[22] demonstrated the characteristic of MAb had cross-reactivity with baicalein (51.8%). Surprisingly, our obtained anti-PUE PAb was very successful with highly sensitivity and specificity for PUE. This might be due to the immunogenicity of new immunogen conjugate via Mannich reaction. The structure of PUE-protein conjugate has not been

modified during the reaction result in increasing of immunogenicity of the hapten conjugate.

Validation of indirect competitive enzyme-linked immunosorbent assay using anti-puerarin polyclonal antibody

The optimum concentration of the coating of the capture antibody (PUE-cOVA 2.5 μg/mL) and the antibody (anti-PUE PAb 8 μg/mL) were used in icELISA for construction the PUE standard competition curve. The PUE competition curve in Figure 5 shows the linearity range from 0.02–12.5 μg/mL. The calculated LOD was 0.02 μg/mL. The result indicated that icELISA using anti-PUE PAb had high sensitivity. The intra- and inter-plate precision on the reference standard concentration range of 0.31–5 μg/mL was determined by icELISA. The %RSD of intra- and inter-plate precision were <5%RSD which in acceptance criteria [Table 2]. The accuracy was determined by icELISA. Three concentration of PUE (10, 25 and 50 μg/mL) were spiked in the extract of *P. lobata*. The percentage recovery was presented in a range of 95.76%–105.13% with 3.42–8.93 of %RSD in Table 3. The plant and product samples with different concentration of PUE were analyzed by icELISA and reference HPLC assays in Table 4. The linear regression analysis between two assays was 0.9895 which indicated the high correlation of these two assays in Figure 6.

Table 1: Cross reactivity of anti-puerarin polyclonal antibody with structure-related compounds

Classification	Compound	Percentage cross reactivity
Isoflavonoids	PUE	100
	Daidzein	<0.01
	Daidzin	<0.01
	Genistein	<0.01
	Genistin	<0.01
	Kwakhurin	<0.01
	Glycitein	<0.01
Flavonoids	Rutin	<0.01
	Baicalein	<0.01
	Baicalin	<0.01
	Oroxylin A	<0.01
	Epicatechin	<0.01
	Quercetin	<0.01
	Kaempferol	<0.01
	Naringin	<0.01
Stilbenes	Resveratrol	<0.01
	Oxyresveratrol	<0.01

PUE: Puerarin

Table 2: Precision of the indirect competitive enzyme-linked immunosorbent assay using anti-puerarin polyclonal antibody

PUE (μg/mL)	Intraplate precision (%RSD)	Interplate precision (%RSD)
5.00	3.77	4.96
2.50	4.30	4.10
1.25	4.19	4.09
0.62	4.54	4.99
0.31	3.93	4.02

RSD: Relative standard deviation; PUE: Puerarin

Table 3: Accuracy of the indirect competitive enzyme-linked immunosorbent assay using anti-puerarin polyclonal antibody

Spiked PUE (μg/mL)	Mean±SD	%RSD	Percentage recovery
0	57.95±1.98	3.42	100.00
10.0	67.93±6.07	8.93	99.80
25.0	84.23±4.93	5.85	105.13
50.0	105.83±9.04	8.54	95.76

RSD: Relative standard deviation; SD: Standard deviation; PUE: Puerarin

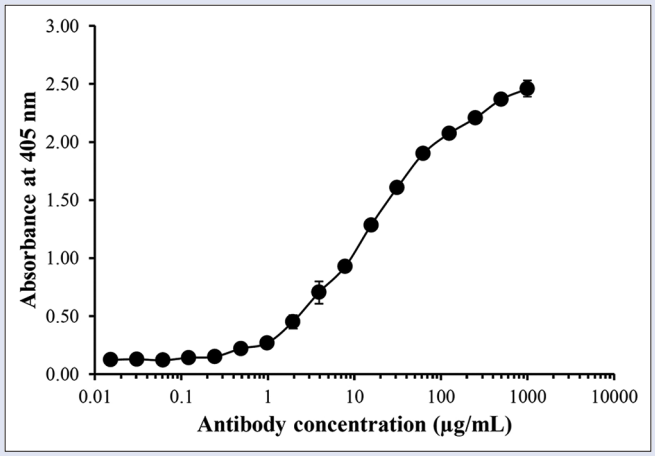

Figure 4: Reactivity of anti-puerarin polyclonal antibody (puerarin-cOVA coating 2.5 μg/mL)

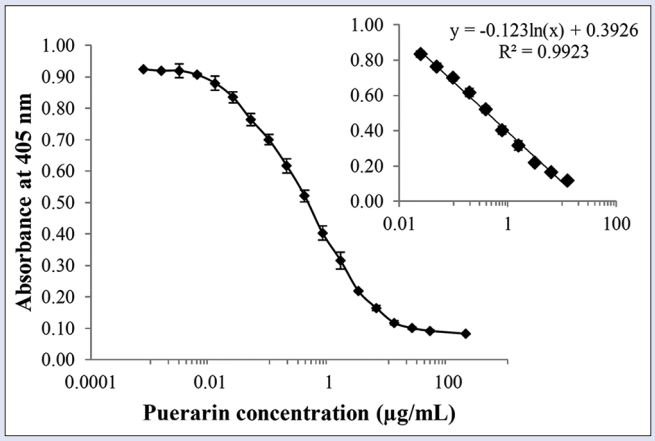

Figure 5: Puerarin standard competition curve

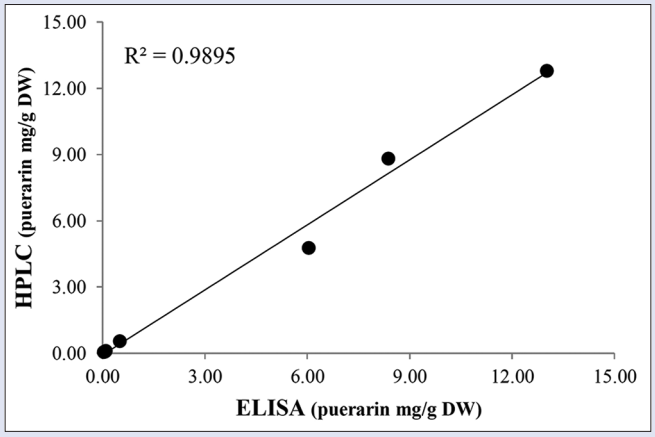

Figure 6: Correlation between enzyme-linked immunosorbent assay and high-performance liquid chromatography assay for puerarin determination

Application of an indirect competitive enzyme-linked immunosorbent assay using anti-puerarin polyclonal antibody to determine puerarin in *Pueraria* spp. and herbal products containing *Pueraria* spp.

The developed and characterized icELISA was applied to determine PUE content in various tuberous root of *Pueraria* spp. (PM, *P. thomsonii*, *P. phaseloides and P. lobata* including cell suspension and hairy root of PM). The PUE content was majorly found in *P. lobata* and *P. thomsonii*. Whereas the small amount of PUE was found in PM and *P. phaseloides* [Table 5]. In addition, herbal products contain *Pueraria* spp. from the markets in Japan (Product No. 1–6) and Thailand (Product No. 7–13) were also investigated the PUE content by icELISA. Table 6 shows PUE content in several types of marketed products derived from *Pueraria* spp. The high amount of PUE can be found in products containing *P. lobata* (4–12 mg/g dry weight). In contrast, the products contain PM has very low amount of PUE (0.02–0.2 mg/g dry weight).

Table 4: Correlation between enzyme-linked immunosorbent assay and high performance liquid chromatography assay for puerarin determination

Sample	PUE (mg/g DW)[a]	
	ELISA	HPLC
P. candollei var. *mirifica* hairy root	0.07±0.00	0.06±0.00
P. candollei var. *mirifica* (1)	0.51±0.05	0.54±0.04
P. lobata	13.02±0.76	12.78±0.73
P. thomsonii	6.04±0.12	4.77±0.52
Pueraria phaseoloides	0.05±0.00	0.05±0.00
Product number 2 (contained *P. lobata*)	8.38±0.17	8.81±0.40
Product number 9 (contained *P. candollei* var. *mirifica*)	0.10±0.00	0.11±0.01

[a]Mean±SD (*n*=3). HPLC: High performance liquid chromatography; ELISA: Enzyme-linked immunosorbent; *P. candollei*: *Pueraria candollei*; SD: Standard deviation; PUE: Puerarin; *P. lobata*: *Pueraria lobata*; *P. thomsonii*: *Pueraria thomsonii*

Table 5: Puerarin content in various *Pueraria* spp.

Sample name	Part used	PUE (mg/g DW)[a]
P. lobata	Root	13.02±0.76
P. candollei var. *mirifica* (1)	Root	0.51±0.06
P. candollei var. *mirifica* (2)	Root	0.04±0.00
P. thomsonii	Root	6.04±0.12
Pueraria phaseoloides	Root	0.05±0.00
P. candollei var. *mirifica* (hairy root)	Hairy root	0.07±0.00
P. candollei var. *mirifica* (cell suspension)	Cell suspension	0.02±0.00

[a]Mean±SD (*n*=3). SD: Standard deviation; *P. candollei*: *Pueraria candollei*; PUE: Puerarin; *P. lobata*: *Pueraria lobata*; *P. thomsonii*: *Pueraria thomsonii*

Table 6: Puerarin content in various herbal products containing *Pueraria* spp.

Product (*n*)	Dosage form	PUE (mg/g DW)[a]
Kakkonto (1)	Granules	12.66±0.71
Kakkonto (2)	Tablets	8.38±0.17
Kakkonto (3)	Tablets	8.78±0.16
Dokukatsu kakkonto (4)	Tablets	4.14±0.25
Kakkontokasenkyushini (5)	Tablets	7.31±0.24
Kakkontokajutsubuto (6)	Granules	5.96±0.52
Product (7)	Tablets	0.17±0.02
Product (8)	Powders	0.02±0.00
Product (9)	Capsules	0.10±0.00
Product (10)	Capsules	0.03±0.00
Product (11)	Capsules	0.02±0.00
Product (12)	Capsules	0.28±0.02
Product (13)	Capsules	0.05±0.01

[a]Mean±SD (*n*=3). PUE: Puerarin; SD: Standard deviation

CONCLUSION

The immunogenicity of new immunogen conjugated (PUE-cBSA) through Mannich reaction was successfully in rising of antibody against PUE. The anti-PUE PAb was produced and characterized. The obtained anti-PUE PAb was high sensitivity and specificity to PUE. Anti-PUE PAb did not have any cross-reaction with over 17 structure-related and/or nonrelated compounds. The icELISA was developed and validated using anti-PUE PAb. Validation parameters were in acceptance criteria.

The advantages of our developed and validated icELISA are high sensitivity, specificity, reproducibility, rapidity and simplicity for determination of PUE in various samples. The icELISA was applied to determine PUE content in various tuberous root of *Pueraria* spp. (PM, *P. thomsonii*, *P. phaseloides and P. lobata* including cell suspension, hairy root of PM and products containing *Pueraria* spp.). Moreover, icELISA method can be applicable in other research propose.

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Conflicts of interest

There are no conflicts of interest.

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