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RESEARCH ARTICLE

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Immunochromatographic assay for the detection of kwakhurin and its application for the identification of *Pueraria candollei* var. *mirifica* (Airy Shaw & Suvat.) Niyomdham

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Abstract

Introduction: The plant *Pueraria candollei* var. *mirifica* (Airy Shaw & Suvat.) Niyomdham (PM), known by its common Thai name as *white Kwao Krua*, is sometimes misidentified because it presents similar botanical characteristics to those of *Butea superba* (*red Kwao Krua*). The phytochemicals in PM are phytoestrogens in the class of isoflavonoids, but *Butea superba* contains flavonoids that exhibit androgenic and antiestrogen effects.

Objectives: This research aims to develop a simple analytical method for identification and to differentiate PM from *red Kwao Krua* and other *Pueraria* species.

Methods: A gold nanoparticle-based immunochromatographic assay (ICA) was developed for the detection of kwakhurin (Kwa), a unique compound found in PM. The parameters, including sensitivity, accuracy, precision, and specificity, were validated. All samples were analyzed using ICA and high-performance liquid chromatography with UV detector (HPLC-UV). The results of the two methods were compared for consistency checking.

Results: The cutoff limit of Kwa detection was 160 ng/mL, which was lower than in the HPLC-UV method. The repeatability and reproducibility of the ICA preparation and assembly showed high precision. The cross-reactivity to related isoflavonoids was less than 0.32%, which implied high specificity of the ICA for Kwa. Moreover, false-positive and false-negative results from other plant extracts were not observed.

Conclusion: The developed ICA is applicable for distinguishing PM from *red Kwao Krua* and other *Pueraria* species. This simple analytical method can be applied for the identification of raw PM materials in the industrial and agricultural sectors.

KEYWORDS

Butea superba, identification, immunochromatographic assay, kwakhurin, *Pueraria mirifica*, *white Kwao Krua*

1 | INTRODUCTION

The plant *Pueraria candollei* var. *mirifica* (Airy Shaw & Suvat.) Niyomdham (PM) is known by its common Thai name as *white Kwao Krua*. PM benefits the health of menopausal women because of the estrogenic activity of its chemical compounds. *Pueraria* species such as PM, *P. lobata*, *P. phaseoloides*, and *Glycine max*. contain common isoflavonoids, such as puerarin, daidzin, daidzein, genistin, and genistein.

Moreover, other isoflavonoids, including kwakhurin (Kwa), kwakhurin hydrate, tuberosin, mirificin, and puemiricarpene, and other chemicals, such as coumestrol, miroestrol, deoxymiroestrol, and isomiroestrol, were also found in PM. Although the amount of Kwa and its estrogenic activity are not high, it is an identity marker of PM.[1] Therefore, the analysis of Kwa benefits the quality control of PM. However, *Butea superba* Roxb. or *red Kwao Krua* in common Thai name, which belongs to Fabaceae along with PM, is a popular herb as well. The characteristics of the species are similar because they belong to the same family. They are climbing plants with trifoliate leaf, and the roots are the parts that are used of both species. The identification by microscopic tests is difficult, especially in powder form, because their roots exhibit similar characteristics to starch granules. Although their appearance is similar, they are genetically different. Therefore, the polymerase chain reaction (PCR) method can be used for their identification.[2–4] In addition, the chemical and pharmacological activities of these two herbs are different. *Red Kwao Krua* is composed of flavonoids such as 3,7,3′-trihydroxy-4′-methoxyflavone, 3,3′-dihydroxy-4′-methoxy-flavone-7-O-β-D-glucopyranoside, 7-hydroxy-6,4′-dimethoxyisoflavone, medicarpin, formononetin, prunetin, and 7,4′-dimethoxyisoflavone.[5] A clinical investigation of *red Kwao Krua* indicated its potential use as an agent against erectile dysfunction.[6] Moreover, *red Kwao Krua* showed an androgenic effect and exhibited antiestrogenic activity in female rats[7] and cell assays.[8]

The misidentification of PM may lead to undesirable effects because the consumption of *red Kwao Krua* and PM has been shown to induce opposite effects. Previously, cycleave PCR was successfully developed for the differentiation of *Pueraria candollei* (*white Kwao Krua*), *Butea superba* (*red Kwao Krua*), and *Mucuna macrocarpa* (*black Kwao Krua*).[3] However, this method requires technical skills and equipment for DNA preparation and analysis. Therefore, the method is not convenient for a large number of samples or in-field investigations. Thus, a chemical approach regarding their constituents may be a simple analytical technique for differentiating the species. Among isoflavonoids, Kwa is a unique compound found in PM roots only.[9,10] Therefore, the analysis of Kwa can distinguish PM from *red Kwao Krua*, other *Pueraria* spp., and *G. max*. Consequently, a simple analytical method would be useful for identifying PM and differentiating it from *red Kwao Krua* and other *Pueraria* species. The identification of PM is essential for research, the quality control of raw materials in herbal product industries, and the safe consumption of PM. Regarding the potent estrogenicity of PM, the possibility of side effects and reproductive toxicity occur due to improper usage. The detection of Kwa can facilitate quality and safety control.

Previous studies showed many methods for Kwa determination. The recombinant antigen-binding fragment (Fab) antibody-based indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed, which showed selectivity and sensitivity for Kwa [limit of detection (LOD) 8.16 ng/mL].[11] The monoclonal antibody (IgG)-based icELISA was developed for the standardization of *P. candollei* materials, in which the sensitivity was higher than in the previous report (LOD 1.13 ng/mL). Recently, a new format of icELISA was invented using Kwa magnetic particle conjugates, which also provided high sensitivity (LOD 1.90 ng/mL) and a faster time of analysis than the conventional icELISA.[12] All icELISA methods mentioned above showed higher sensitivity than the high-performance liquid chromatography method (HPLC), in which the LOD was 0.93 μg/mL.[13] However, both ELISA and HPLC face limitations, including complicated procedures and lengthy processes for determination. HPLC analysis notably exhibited not only low sensitivity but also chemical interference from other phytochemicals.

The development of a simple and rapid method for PM identification is a challenge. The immunochromatographic assay (ICA) is one of the interesting alternative methods for development. In previous studies, this method was approved for the detection of many substances such as danofloxacin[14] and methyl-3-quinoxaline-2-carboxylic acid.[15] The present study aimed to develop an ICA for Kwa detection that is an effective method for the identification of PM and its differentiation from *red Kwao Krua* and other *Pueraria* spp. Moreover, the developed ICA exhibited a high sensitivity with a cutoff limit of 160 ng/mL, and this analytical procedure boasts advantages such as being simple and rapid. The details of the development and characterization of this method are thoroughly described in this research article.

2 | EXPERIMENTAL SETUP

2.1 | Chemical and immunological reagents

A Kwa standard (≥96%) was obtained by isolation from the tuberous root of PM using column chromatography, as described in a previous study.[13] Puerarin (99.2%), daidzin (97.3%), daidzein (97.3%), genistin (99%), and genistein (99.3%) were purchased from LKT Laboratories, Inc. (MN, USA), and coumestrol (95.5%) was purchased from Sigma Aldrich (MO, USA). Colloidal gold nanoparticles (AuNPs, 1 OD, 20-nm particle size) were purchased from Expedeon Ltd. (CA, USA). Bovine serum albumin (BSA) of molecular biology grade was purchased from Himedia (Mumbai, India). For preparing Kwa–carrier protein conjugates, BSA (≥97%) and human serum albumin (HSA; ≥99%) were purchased from Sigma-Aldrich (MO, USA). Monoclonal antibodies against Kwa (mAb 11F) and Kwa–carrier protein conjugates (Kwa–HSA and Kwa–BSA) were prepared as described in a previous study.[13] Goat anti-mouse IgG H&L antibodies were purchased from Abcam (Cambridge, UK). Other reagents, such as sucrose (Ajax Finechem Pty Limited, New Zealand), polyethylene glycol 6000 (PEG6000, Loba Chemie, India), K_2CO_3 (Ajax Finechem Pty Limited, New Zealand), Tween-20 (Ajax Finechem Pty Limited, New Zealand), sodium lauryl sulfate (SDS, KEMAUS, Australia), absolute ethanol (RCI Labscan, Thailand), and deionized (DI) water (RCI Labscan, Thailand), were high-purity products of analytical grade. Nitrocellulose membrane, cellulose fiber pads, and glass fiber pads were purchased from EMD Millipore (CA, USA). All other chemical reagents were analytical reagents obtained from commercial sources.

2.2 | Conjugation of colloidal AuNPs and mAb 11F

The anti-Kwa monoclonal antibody (mAb 11F) was generated using hybridoma technology, as described in our previous study.[13] The antibody mAb 11F was conjugated with AuNPs, and the resultant mAb 11F–AuNP conjugate functioned as a detection agent in the ICA. Preparation of the mAb 11F–AuNP conjugate was performed according to a previous method[16] with some modifications. Briefly, an aqueous K_2CO_3 solution [2% (*w*/*v*), 20 μL] was added to the AuNP suspension (1 mL). After mixing well, the mAb 11F solution (2.5 mg/mL in phosphate-buffered saline (PBS) containing 10% (*v*/*v*) glycerol, 20 μL) was added. Then, the mixture was incubated for 10 min at room temperature. A BSA solution [10% (*w*/*v*) in 0.1 M Tris–HCl pH 8, 99.9 μL] was added to fill the unoccupied surface space of the AuNPs. A solution of PEG6000 [10% (*w*/*v*) in 0.1 M Tris–HCl pH 8, 11.1 μL] was incorporated to stabilize the mAb 11F–AuNP conjugate. The mixture was then incubated at room temperature for an additional hour. After that, the mAb 11F–AuNP conjugate was collected via centrifugation at 7000 rpm for 30 min at 4°C. The supernatant was discarded, and the mAb 11F–AuNP conjugate pellet was suspended in 0.1 M Tris–HCl pH 8 buffer containing 1% (*w*/*v*) BSA and 0.1% (*w*/*v*) PEG6000. Centrifugation was performed again under the same conditions. After discarding the supernatant, a mixture of 1% (*w*/*v*) BSA solution in 0.1 M Tris–HCl pH 8 (40 μL), 1% (*w*/*v*) sucrose in 0.1 M Tris–HCl pH 8 (8 μL), 1% (*v*/*v*) Tween-20 in 0.1 M Tris–HCl pH 8 (4 μL), and water (4 μL) were added to the mAb 11F–AuNP conjugate pellet for resuspension. The obtained suspension of the mAb 11F–AuNP conjugate was kept at 4°C and used within 1 week. The mAb 11F–AuNP conjugate (10 μL) was applied to a glass fiber pad (6 × 6 mm^2), which was then dried at room temperature for 1 h. The pad containing the mAb 11F–AuNP conjugate was then ready for subsequent preparation for use in the ICA.

2.3 | Preparation of the ICA

Test and control zones on a nitrocellulose membrane pad (6 × 60 mm^2) were applied with 2.5 mg/mL Kwa–BSA conjugate (1 μL) and 1 mg/mL goat anti-mouse IgG H&L (1 μL), respectively. A stock solution (4 mg/mL) of Kwa–BSA conjugate was prepared in 4 M urea and 50mM Tris–HCl at pH 8, and then it was diluted to a final concentration of 2.5 mg/mL in 1% (*w*/*v*) SDS in PBS. The goat anti-mouse IgG H&L antibody stock solution (2 mg/mL) was diluted twice in 1% SDS. The nitrocellulose membrane pad was dried at room temperature for 1 h after having been spotted with the reagents of the test and control zones. Then, the membrane was immersed and gently shaken in 1% (*w*/*v*) BSA dissolved in PBS for 2 h to avoid nonspecific binding between the antibody and the membrane during ICA analysis. After that, the membrane was washed three times using 0.05% Tween-20 in PBS (T-PBS) for 5 min. The membrane was dried at room temperature for 1 h and was then ready to be assembled with the other parts. The prepared ICA was kept at 4°C and used for analysis within 1 week. The nitrocellulose membrane pad was equipped with the mAb 11F–AuNP-conjugate-loaded glass fiber pad (6 × 6 mm^2), a cellulose fiber sample pad (6 mm width×15 mm length), and a cellulose adsorbent pad (6 mm width×15 mm length) (Figure 1A). During the analytical procedure, a sample solution (400 μL) was added to a small test tube. Then, an assembled strip was immersed in the solution. The bottom edge of the mAb 11F–AuNP-conjugate-loaded glass fiber pad was maintained above the sample solution. The sample migrated upward via capillary action through the mAb 11F–AuNP conjugate pad, test zone, and control zones. The flow-through sample solution was deposited on the cellulose adsorbent pad, which maintained a continuous sample flow. The results were visually observed after 15 min of ICA development.

2.4 | Characterization of the ICA

The sensitivity of the ICA was evaluated; several concentrations of the Kwa standard (1.64 ng/mL – 2.5 μg/mL) were prepared in 5% (*v*/*v*) ethanol. Therefore, ethanol 5% (*v*/*v*) was used as a negative control. The sensitivity of analysis was defined as the cutoff limit. The lowest concentration of the tested Kwa solutions that completely inhibited binding between the mAb 11F–AuNP conjugate and the test zone (Kwa–BSA conjugates) was defined as the cutoff limit. At this concentration, the test zone disappeared. The ICA results were evaluated visually. If the concentration of Kwa was equal to or higher than the cutoff limit, the test zone disappeared because free Kwa in the sample completely interacted with mAb 11F.

The specificity of the ICA was evaluated and expressed in terms of the percentage of cross-reactivity (%CR), which was calculated as the reactivity of the mAb 11F–AuNP conjugate toward Kwa in comparison with that toward other investigated compounds. Compounds that have been found in *Pueraria* species and *G. max* (including puerarin, daidzin, genistin, daidzein, genistein, and coumestrol) were tested. In addition, other plant extracts, including those of *Boesenbergia rotunda*, *Butea superba*, *Kaempferia parviflora*, *Zingiber cassumunar*, *Angelica sinensis*, *Zingiber officinale*, *Derris scandens*, *Pueraria phaseoloides*, *Pueraria lobata*, and *G. max*, which are usually included in PM formulations or contain phytochemicals similar to those of PM, were used for the CR experiment. Overall, the specificity test determined the possibility of false-positive detections caused by other components of the sample extracts. The following equation was utilized for calculating the percentage of CR:

$$\mathrm{CR}\,(\%) = \frac{\text{The cutoff limit of Kwa detection}}{\text{The cutoff LOD of an investigated compound}} \times 100\%$$

The precision of the ICA method was evaluated by repeatability and reproducibility tests. The repeatability was evaluated using the same production batch of ICA reagents (the mAb 11F–AuNP conjugate and the membrane). The experiment was performed in triplicate (n = 3) using a 160 ng/mL Kwa solution as a positive control and 5% (*v*/*v*) ethanol as a negative control. Then, the reproducibility of the ICA method was determined using ICA reagents from different batches of

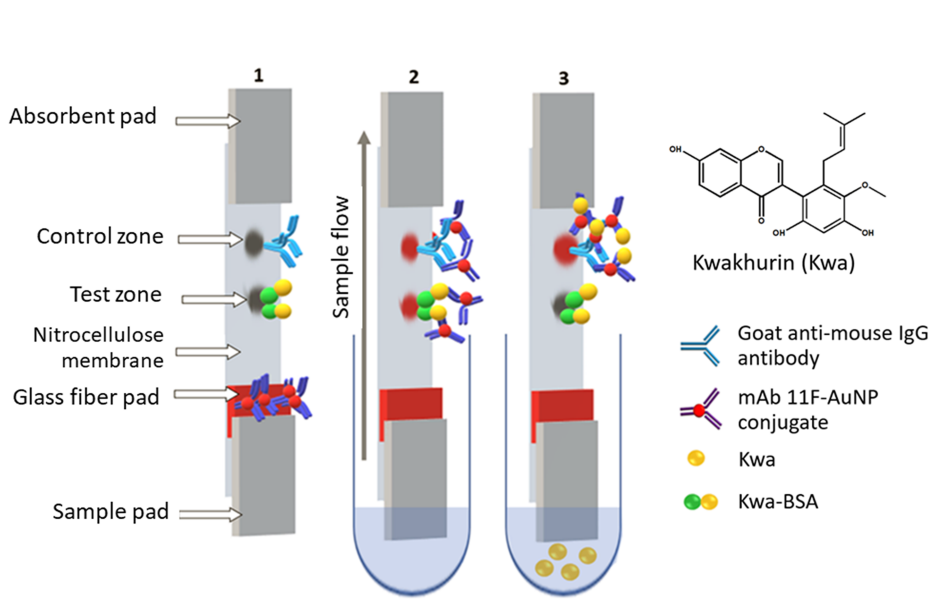

FIGURE 1 An illustration of the components in the immunochromatographic strip (A-1), immunochromatographic assay (ICA) analysis in the absence of kwakhurin (Kwa) (A-2), and ICA analysis in the presence of Kwa (A-3). Concentration-dependent characteristics of the immunochromatographic assay were observed in Kwa detection (B), in which various concentrations of Kwa were used for the experiment, 2500, 1000, 400, 160, 64, 25.6, 10.24, 4.096, and 1.64 ng/mL, and occupied lanes 1–9, respectively. Lane 10 contained 5% ethanol (*v*/*v*) as negative control [Colour figure can be viewed at wileyonlinelibrary.com]

production (n = 3). This test indicated if the ICA preparation process was precise for Kwa analysis even if the reagents were from different batches of production. The accuracy and reliability of the ICA analysis were evaluated for the possibility of false-negative results, which may be caused by other components in the sample solutions. A Kwa solution at a concentration of 160 ng/mL was spiked into the samples that had yielded negative results for Kwa detection, and then these samples were reanalyzed.

2.5 | Sample preparation

The plant sample of *P. phaseoloides* was identified and collected by Dr. Yusakul, School of Pharmacy, Walailak University, Thailand. The dry roots of *P. lobata* were supplied by Tochimoto (Tenkaido Co., Osaka, Japan). The other samples, including rhizomes of *B. rotunda*, stems of *B. superba*, rhizomes of *K. parviflora*, rhizomes of *Z. cassumunar*, roots of *A. sinensis*, rhizomes of *Z. officinale*, and stems of *D. scandens*, were purchased from Thaprachan Herb Co., Ltd. (Bangkok, Thailand). These plant samples were ground to a fine powder. An amount (200 mg) of each sample was accurately weighed. Absolute ethanol (1 mL) was added to the samples and vortexed. These plants were extracted using ethanol, which provided a high extraction efficiency of PM isoflavonoids.[17] Extraction was performed using sonication (37 kHz) for 30 min at room temperature. The supernatant (extract) was collected after centrifugation at 8000 rpm for 5 min. The sample residue was re-extracted for two additional times using the same procedure. All extracts of each sample were combined and placed at room temperature until the solvent ran out; then the remaining residue was dissolved in absolute ethanol (1 mL). Before

analysis using the developed ICA, the samples were diluted (20-fold dilution) with water, and the solvent of the sample solution was adjusted to 5% (*v*/*v*) ethanol. For the HPLC analysis, the sample solutions were diluted five times with absolute ethanol before analysis.

2.6 | HPLC analysis

The analytical performance of the ICA was compared with that of the HPLC analysis. The HPLC-UV analysis was performed on a Thermo Scientific Dionex Ultimate 3000 (Thermo Scientific, MA, USA) chromatograph consisting of a variable wavelength detector (VWD-3100), an autosampler (WPS-3000SL), a tertiary pump (LPG-4300SD), and a column compartment (TCC-3000SL). A total of 10 μL of a sample or standard solution was injected into a C18 analytical column (VertiSep™ HPLC columns, 250 mm × 4.6 mm, 5 μm particle size; Vertical Chromatography Co., Ltd., Nontaburi, Thailand). The utilized mobile phases were 60% (*v*/*v*) acetonitrile in water (solvent B) and 1% (*v*/*v*) acetic acid in water (solvent A). The flow rate was l mL/min, and the column was eluted with a linear gradient program of 30–40% solvent B over 0–7 min, 40–70% solvent B over 7–10 min, 70% B over 10–25 min, and 70–100% solvent B over 25–28 min. Then, the mobile phase was returned to 30% solvent B and maintained for 3 min to equilibrate the column for the next injection. The column compartment temperature was set at 30°C, and the eluted compounds were monitored at 254 nm.

3 | RESULTS AND DISCUSSION

3.1 | Immunoassay strip invention and characterization

The results showed that a concentration of 2.5 mg/mL Kwa-BSA (1 μL/spot) produced sufficient color intensity of the test zone. The color of the control zone was also intense and clear. The color intensity of the test zone not only depended on the type and concentration of the Kwa–carrier protein spotted at the zone but also influenced the availability of the antibody on the surface of the mAb 11F–AuNP conjugate. Therefore, the preparation of the mAb 11F–AuNP conjugate was optimized using 1 mL of AuNPs and varying volumes (20, 40, and 80 μL) of the mAb 11F solution (2.5 mg/mL). The results indicated that the ICA sensitivity decreased with increasing mAb 11F solution volumes (Figure S1, electronic supplementary material, ESM). When the antibody mAb 11F was more available on the surface of the mAb 11F–AuNP conjugate, more free Kwa was required to completely occupy the antibody such that it could not bind to the test zone. Therefore, the optimal amount of antibody on the surface of the AuNPs was needed for a clear test zone and sufficient detection sensitivity. The results indicated that 20 μL of the mAb 11F solution was optimal and provided a sufficient color intensity of the test zone and high detection sensitivity for Kwa. Therefore, the optimal conjugation procedure, which consisted of 1 mL AuNPs and 20 μL (40 μg) of the mAb 11F solution, was selected for further validation of the ICA for Kwa detection.

Sensitivity is a critical performance factor of the ICA for Kwa detection because Kwa is often found in small amounts in PM roots. The Kwa content was in the range of $6.4–6.7 \times 10^{-3}$% (*w*/*w*) in PM tuberous roots,[1] $3.07 \times 10^{-3}–2.39 \times 10^{-2}$% (*w*/*w*) in the root bark, and 2.59×10^{-4} in PM roots without bark.[13] Thus, the high sensitivity of a method for Kwa detection is required for a variety of PM-related samples. In this experiment, authentic Kwa solutions (400 μL) were prepared in the concentration range of 1.64 ng/mL–2.50 μg/mL in 5% (*v*/*v*) ethanol and subjected to the ICA. The color intensity of the test zone inversely correlated with the Kwa concentration after 15 min of applying the ICA strips. That is, the higher the concentration of Kwa was, the lower the visually observed color intensity of the test zone was (Figure 1B). In the competitive ICA format, the test zone disappears when the concentration of the analyte (Kwa, free antigen) exceeds the cutoff limit. In this study, the test zone completely disappeared when the 160 ng/mL Kwa solution was applied. Therefore, the cutoff limit of Kwa detection was defined as the concentration of 160 ng/mL (Figure 1B). In previous studies, the LOD of Kwa analysis using HPLC was 0.93 μg/mL.[13] Thus, the LOD of the ICA developed for Kwa was approximately 5.8 times higher than that of HPLC. The determination range of Kwa via icELISA was from 1.53 to 48.8 ng/mL. Thus, the concentration of 48.8 ng/mL was the concentration that almost completely inhibited binding between the mAb 11F and Kwa–HSA conjugate in the icELISA. Therefore, the sensitivity of the ICA was three times less than that of the icELISA when the same binding phenomenon was considered between both assays. In view of convenience, however, the icELISA required at least 4.5 h. The developed ICA in this study is simple in terms of analysis, the procedure is rapid, and the sensitivity is high. In addition, the method does not require large volumes of organic solvent, unlike the HPLC method.

The precision of the developed ICA for Kwa detection was evaluated in terms of repeatability and reproducibility. The ICA was tested in triplicate ($n = 3$) using a Kwa solution (160 ng/mL) as a positive control and 5% (*v*/*v*) ethanol as a negative control. The ICAs presented positive results for all samples that contained Kwa, and negative results were observed for the negative control. The repeatability and reproducibility results are shown in Figure 2, respectively. The tests indicated the precise Kwa detection and robustness of the production procedure of the developed ICA.

The specificity of the ICA is presented in terms of %CR. The CR of the ICA toward other PM phytochemicals, such as puerarin, daidzin, genistin, daidzein, genistein, and coumestrol, was investigated. The %CR was less than 0.32% for puerarin, daidzin, genistin, daidzein, and genistein, and less than 1.6% for coumestrol (Figure 3). These results indicated the specificity of the ICA for Kwa. The binding specificity of pure mAb 11F was highly specific to Kwa as well, as investigated using the icELISA procedure.[13] Moreover, the binding specificity of mAb 11F was not altered via its conjugation with the AuNPs. In a specific manner, the ICA could detect Kwa in the PM samples. Usually, PM is utilized in traditional medicines containing other medicinal

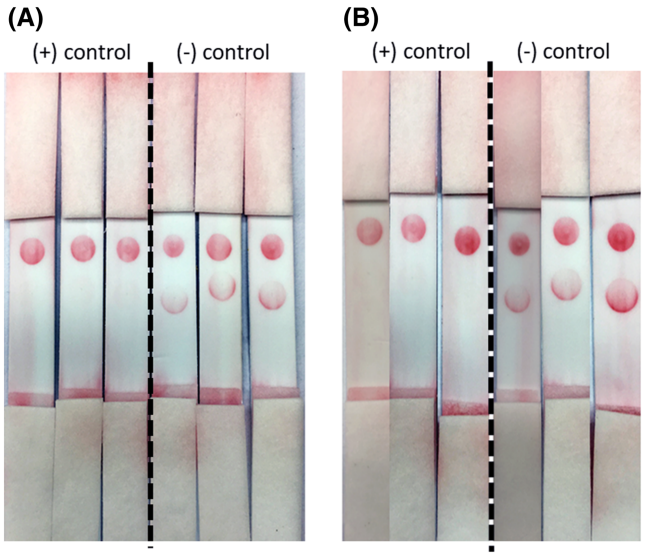

FIGURE 2 The repeatability (A) and reproducibility (B) of the developed immunochromatographic assay (ICA), in which 5% (*v*/*v*) ethanol was used as a negative control, and 160 ng/mL kwakhurin (Kwa) was used as a positive control

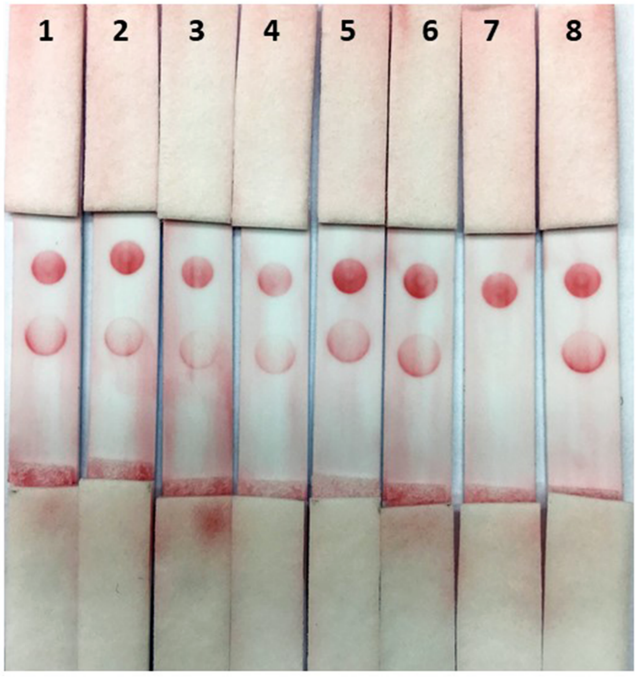

FIGURE 3 Cross-reactivity the immunochromatographic assay using daidzin, daidzein, genistin, genistein, and puerarin at a concentration of 50 μg/mL, as shown in lanes 1–5, respectively, and coumestrol (10 μg/mL) in lane 6. The positive control was 160 ng/mL kwakhurin (Kwa) (lane 7), and the negative control was 5% (*v*/*v*) ethanol (lane 8)

plants. The CR of the ICA toward chemical constituents of many other herbs was also evaluated, including those in *B. rotunda*, *B. superba*, *K. parviflora*, *Z. cassumunar*, *A. sinensis*, *Z. officinale*, and *D. scandens*, which were extracted and used as a mixture of compounds for evaluation of the possible CR of the ICA toward their phytochemicals. There have been no previous reports of the presence of Kwa in these plants. As expected, the ICA showed negative results when these samples were tested, which implied that other phytochemicals did not interfere with the ICA analysis. The experiment was performed to evaluate the possibility of false-positive, which the 160 ng/mL of Kwa at final concentration was spiked into the sample with negative results [whole roots of *P. phaseoloides* (Nakhon Si Thammarat, Thailand), whole roots of *P. lobata* (Tenkaido Co., Osaka, Japan), seeds of *G. max* (Thanya Farm Co., Ltd., Nonthaburi, Thailand), ground tissue of PM roots (Nakhon Ratchasima, Thailand), and ground tissue of PM stems (Ratchaburi, Thailand)]. The results of the ICA analysis showed clear positive results of Kwa detection (Figure 4). It was confirmed that there were no false-negative results caused by interactions of other components in the sample extracts.

3.2 | ICA and HPLC analysis of the sample

The ICA was applied to detect Kwa in various types of samples, including various parts of PM, *P. phaseoloides*, *P. lobata*, *Butea superba*, seeds of *Glycine max*, and others. The results are summarized and presented in Table 1. The ICA was able to differentiate between *white Kwao Krua* (PM) and *red Kwao Krua* (*B. superba*). The developed method is simple and valuable for the identification of PM. In addition, we found that Kwa mainly accumulated in the periderm and bark

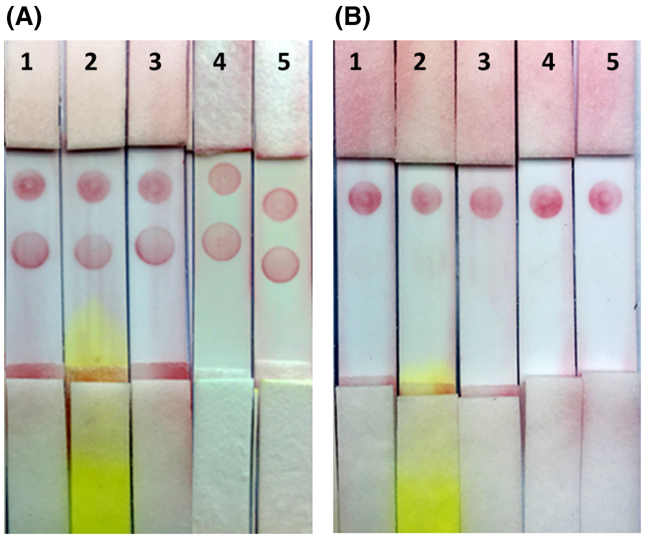

FIGURE 4 The accuracy of the immunochromatographic assay (ICA) was tested for the non-false-negative results. Lanes 1–5 show the results of samples, including the whole root of *Pueraria phaseoloides* (Nakhon Si Thammarat, Thailand), the whole root of *Pueraria lobata* (Tenkaido co., Osaka, Japan), seeds of *Glycine max* (Thanya Farm Co., Ltd., Nonthaburi, Thailand), ground tissue of *Pueraria mirifica* roots (Nakhon Ratchasima, Thailand), and ground tissue of *Pueraria mirifica* stems (Ratchaburi, Thailand), respectively. All samples show negative results (A) in the initial tests, and then they produce positive results when kwakhurin (Kwa) was spiked in with 160 ng/mL (B)

TABLE 1 Kwa analysis of samples using the immunochromatographic assay (ICA) and high-performance liquid chromatography (HPLC)

Samples (trade name or source of the sample)	Results of Kwa analysis	
	ICA strip test	HPLC (μg/mL)
Pueraria candollei var. *mirifica* root		
Whole root, lot no. 01/62 (Nakhon Pathom, Thailand)	–	ND
Whole root, lot no. 02/62 (Nakhon Pathom, Thailand)	–	ND
Whole root (Suphanburi, Thailand)	+	10.5 ± 0.0
Cortex-1 (Nakhon Ratchasima, Thailand)	+	10.1 ± 0.2
Cortex-2 (Nakhon Ratchasima, Thailand)	+	39.8 ± 0.4
Cortex (Ubon Ratchathani, Thailand)	+	8.91 ± 0.59
Periderm (Nakhon Pathom, Thailand)	+	132 ± 3
Ground tissue of roots (Nakhon Ratchasima, Thailand)	–	ND
Root hairs, lot no. 04/62 (Nakhon Pathom, Thailand)	–	0.620 ± 0.083
Root hairs, lot no. 03/62 (Nakhon Pathom, Thailand)	–	ND
Pueraria candollei var. *mirifica* stem		
Whole stem (Chiang Mai, Thailand)	+	35.5 ± 0.2
Stem cortex (Ratchaburi, Thailand)	+	16.5 ± 0.4
Ground tissue of stems (Ratchaburi, Thailand)	–	ND
Other *Pueraria* species		
Whole root of *P. phaseoloides* (Nakhon Si Thammarat, Thailand)	–	ND
Whole root of *P. lobata* (Tenkaido co., Osaka, Japan)	–	ND
Butea superba		
Stem (Thaprachan herb co., ltd., Thailand)	–	ND
Stem (Nakhon Si Thammarat, Thailand)	–	ND
Others		
Seeds of *Glycine max* (Thanya farm co., ltd., Nonthaburi, Thailand)	–	ND
Rhizome of *Boesenbergia rotunda* (Thaprachan herb co., ltd., Thailand)	–	ND
Rhizome of *Kaempferia parviflora* (Thaprachan herb co., ltd., Thailand)	–	ND
Rhizome of *Zingiber cassumunar* (Thaprachan herb co., ltd., Thailand)	–	ND
Root of *Angelica sinensis* (Thaprachan herb co., ltd., Thailand)	–	ND
Rhizome of *Zingiber officinale* (Thaprachan herb co., ltd., Thailand)	–	ND
Stem of *Derris scandens* (Thaprachan herb co., ltd., Thailand)	–	ND

Abbreviations: Kwa, kwakhurin; ND, not detected.

of PM roots. Kwa was not detected in the ground tissue of PM. Therefore, some whole roots of PM may contain a low amount of Kwa because large PM root tubers are composed of a small portion of the cortex compared with that in the ground tissue. Interestingly, Kwa accumulates in high amounts in the stem of PM. The phytochemicals present in PM stems should be further identified and characterized to

evaluate their usefulness. The ICA produced positive results when the extract contained Kwa concentrations greater than 3.2 μg/mL, corresponding to a Kwa standard solution concentration of 160 ng/mL. Analysis of the root hair of PM lot No. 04/62 (Nakhon Pathom, Thailand) yielded a negative result for Kwa detection. HPLC determination indicated that the root hairs contain Kwa at 0.620 μg/mL. Therefore, the developed ICA exhibited a negative result. The results from this study showed the ICA and HPLC were generally in agreement. Kwa and other estrogenic compounds contained in PM that were determined by HPLC are presented in Table S1 (supporting information).

The ICA did not detect Kwa in the extracts of *P. phaseoloides* and *P. lobata*. Therefore, the developed ICA is useful for distinguishing PM from other *Pueraria* species. Both of the abovementioned plants have been reported not to contain Kwa. The seeds of *G. max* are composed of isoflavones and their glycosides, including daidzin, genistin, and glycitin,[18] and these soy isoflavones exhibit weak to moderate estrogenic effects. Therefore, the ICA did not detect Kwa in these sample extracts. These tests confirmed that soybeans do not contain Kwa and that these isoflavone phytochemicals did not produce false-positive Kwa detection with the developed ICA. Corresponding HPLC analyses were conducted, and authentic isoflavone standards were used to establish calibration curves. The linearity of their determination was in the range of 1.56–25.0 μg/mL (R^2 = 0.9953), and the coefficients of variation were less than 7%.

The chromatogram of the standard isoflavonoids contained in PM and other samples, including the whole root of PM (Suphanburi, Thailand), the whole root of *P. lobata* (Tenkaido Co., Osaka, Japan), seeds of *G. max* (Thanya Farm Co., Ltd., Nonthaburi, Thailand), and the rhizome of *B. rotunda* (Thaprachan Herb Co., Ltd., Thailand) are presented in Figure S2 (supporting information). The retention times of puerarin, daidzin, genistin, daidzein, Kwa, and genistein were 5.74, 812, 11.94, 15.03, 18.53, and 19.33 min, respectively.

In terms of Kwa detection with these two analytical procedures, the duration of the ICA analysis was shorter than that of HPLC. For HPLC, time is needed to obtain a suitable resolution of Kwa separation from other components with similar polarities, such as genistein. The retention time of Kwa in the HPLC method was 18.53 min; therefore, at least 25–30 min of analysis time was required for each analysis. The major disadvantage of HPLC is that a large volume of mobile phase (acetonitrile) is often consumed, which leads to waste management costs. In addition, organic solvents are often toxic and hazardous to the working personnel. Therefore, Kwa detection via ICA has the advantage of being a rapid and simple analytical procedure that does not require expensive equipment or toxic organic solvents. In view of the specificity of the analysis, the ICA also exhibited superiority over the HPLC method. Traditionally, PM is composed of many bioactive compounds, leading to analytical difficulties when implementing HPLC analysis. The specificity of the HPLC analysis may require a relatively long time for separation and a complicated mobile phase system to sufficiently separate the constituents of such an extract.

The detection of Kwa using the ICA developed in this method is simple, rapid, and convenient for the identification of PM. The cutoff limit of Kwa detection was 160 ng/mL. The validation of analytical performance indicated the reliability of the developed ICA. The ICA was able to analyze Kwa in the extracts of PM materials or formulations of PM mixed with other medicinal plants. The specificity of the antigen–antibody binding resulted in no false-positive or false-negative detections due to other PM compounds and those contained in other plants. Therefore, the developed ICA for Kwa detection is able to differentiate PM from *red Kwao Krua* and other *Pueraria* species. This method can be applied in the agricultural and industrial sectors for quality control and consumer safety.

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COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST/COMPETING INTERESTS

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with animals and human participants performed by any of the authors.

CONSENT TO PARTICIPATE

Not applicable.

INFORMED CONSENT

All authors have read the manuscript and agreed upon its submission to the journal.

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SUPPORTING INFORMATION

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