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Highly selective and sensitive determination of deoxymiroestrol using a polyclonal antibody-based enzyme-linked immunosorbent assay

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ABSTRACT

Pueraria candollei-associated products are of interest to worldwide consumers for their rejuvenating and cosmetic purposes. In addition, clinical trials have supported the beneficial effects of *P. candollei* on the alleviation of menopausal symptoms. Deoxymiroestrol, which was reported as the most potent phytoestrogen in the tuberous root of *P. candollei*, exhibited potential as a biomarker of *P. candollei*-derived samples and products. A polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) was developed for deoxymiroestrol determination. The raised antibody bound specifically to deoxymiroestrol with very low cross reactivities of 1.26 and 0.42% to structurally related miroestrol and isomiroestrol, respectively. The linear range was 0.73–3000.00 ng mL^{-1}, and the coefficients of variation for both the intra- and inter-plate determinations were less than 5%. In samples spiked with a known amount of deoxymiroestrol, the recoveries were 99.82–102.58% in *P. candollei* samples and 98.07–106.33% in its products samples. In comparison with other analytical methods, the validated ELISA was comparable to published HPLC-UV methods for samples with high deoxymiroestrol content (R^2=0.9993). Furthermore, ELISA can be used for samples with deoxymiroestrol concentrations that are too small to detect by HPLC and for conditions when other chemicals cause interference with chromatographic analysis. For the *P. candollei*-derived products, the preparations contained 0.154–10.998 μg g^{-1} dry wt. Our ELISA successfully measured deoxymiroestrol content with high sensitivity, selectivity, accuracy and rapidity. Therefore, this ELISA showed potential for dosage standardization of *P. candollei*-associated samples.

1. Introduction

Pueraria candollei belongs to Leguminosae and is composed of two varieties, *P. candollei* var. *mirifica* and var. *candollei*. The pharmacological activities and safety of *P. candollei* var. *mirifica* are well documented; however, both varieties could be used interchangeably due to similar chemical constituents [1,2]. This plant has been used for a long time in Thai traditional medicine for smoothing skin, enhancing memory and rejuvenation in aged people [3]. In pre-clinical investigations, *P. candollei* var. *mirifica* exhibited anti-osteoporotic [4,5], estrogenic [6–10], anti-oxidative [6,11], cardiovascular protective [12] and anti-tumor effects [13,14]. The randomized clinical trials of *P. candollei* var. *mirifica* showed that the climacteric scores of menopausal women declined after treatment, while no significant side effects were observed [15–17]. This efficacy was not significantly different from conventional hormone replacement therapy [18]. The randomized, double-blind, placebo-controlled trials indicated that *P. candollei* var. *mirifica* significantly decreased bone turnover [19] and improved vaginal health in postmenopausal women [20].

Deoxymiroestrol (DME) is the most potent phytoestrogen in *P. candollei* var. *mirifica* [7]. In vivo investigation found that DME significantly increased uterus weight and volume similar to the same dose of estradiol benzoate. Moreover, the compound significantly decreased lipid peroxidation in mouse brains [6]. A phytochemical and pharmacological study indicated that the estrogenic effect of *P. candollei* is principally caused by DME [21]; therefore, this compound should be considered as a biomarker for quality control of *P. candollei*-associated samples or products.

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From the pre-clinical through the clinical investigation, the overall results suggested that *P. candollei*-associated products showed potential as an alternative medication for menopausal symptoms. Standardization of the plant product is required to ensure the efficacy and safety for clinical applications. The DME contents in *P. candollei* samples must be standardized before using in pharmacological research and related product development because of the following reasons: (i) the two *P. candollei* varieties contained different contents of DME [2], (ii) variation of the produced secondary metabolites was found due to different culturing places and season of harvest [22,23], and (iii) DME can be oxidized by molecular oxygen under various conditions [21]. Therefore, high performance analytical methods for measuring DME are an important tool for quality control, stability testing, dosage form development and pharmacokinetic investigations.

A quantitative HPLC-UV method was developed by our research group for DME determination in *P. candollei* samples [2]. The HPLC-UV method faced several problems for minor compound analysis in plant samples. This HPLC-UV method exhibits low sensitivity compared to other modern analytical methods and requires a large sample for each analysis. The method is also non-selective, especially in plant samples or herbal products that contain multiple chemical interferences. The enzyme-linked immunosorbent assay is an analytical method with highly selective determination due to specific binding between antigen and antibody. In comparison to HPLC-UV, ELISA commonly exhibits higher sensitivity [24–26]. Additionally, ELISA requires fewer chemicals and equipment than HPLC. In the present study, we aimed to develop an ELISA for DME determination using anti-DME polyclonal antibody (PAb). This method exhibited potential as an alternative assay for the standardization of *P. candollei*-associated samples and dosage form preparations.

2. Materials and methods

2.1. Chemicals and immunological reagents

DME ($>90\%$ purity) was prepared and identified by our research group. DME was isolated from the ethyl acetate extract of the tuberous root cortex of *P. candollei*. The isolation procedure was guided by authentic standard DME from Dr. Chaiyo Chaichantipyuth, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The isolated compound was confirmed by H^1-NMR by comparison with data in the literature [21]. Bovine serum albumin (BSA), ovalbumin (OVA) and Freund's complete and incomplete adjuvants were purchased from Sigma (MO, USA). Peroxidase-labeled anti-rabbit IgG antibody was purchased from MP Biomedicals (OH, USA). 2, 2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Wako (Osaka, Japan). All other chemicals were commercial products of analytical-reagent grade.

2.2. Plant materials and preparation

Two varieties of *P. candollei* were sampled from Suranaree University of Technology, Nakhon Ratchasima province, and from Ubon Ratchathani University, Ubon Ratchathani province, Thailand. These samples were identified and collected by Dr. Thaweesak Juengwatanatrakul, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand. Reference specimens (NI-PSKKU 007–010) were deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Parts of the *P. candollei* tuberous root, including the tuber cortex, the whole tuber and the tuber without cortex, were prepared as samples for analysis by ELISA and HPLC, which was used as a comparator method in the correlation experiment. All samples were dried at 50 °C in a hot air oven and were then ground to a fine powder. Each sample powder (1 g) was washed with 5 mL hexane and extracted five times with 5 mL chloroform-ethyl acetate (1:3, v/v) using sonication for 1 h for each extraction. The pooled extract of each sample was evaporated in a water bath at 60 °C with aspiration. The solid residues of the extract were dissolved in 1 mL absolute ethanol for analysis by ELISA and HPLC. For samples of *P. candollei*-derived products, the powder was washed, extracted, evaporated and re-dissolved similar to the above procedure with some modification, e.g., ethyl acetate was used as the extraction solvent instead of chloroform-ethyl acetate (1:3, v/v) because DME was enriched in the ethyl acetate extract [21]. Each sample was performed in triplicate.

2.3. Synthesis of antigen conjugates

Because DME is a small molecule, it was essential to link the DME with bovine serum albumin (BSA) to act as an immunogen, whereas DME was linked with ovalbumin (OVA) as a non-relevant coating in the ELISA test. Briefly, a solution of sodium periodate ($NaIO_4$) (5 mg in 0.8 mL distilled water) was added dropwise to a DME solution (10 mg in 1.2 mL DMSO), and the reaction mixture was stirred at room temperature for 1 h. The mixture was gradually dropped into a BSA solution (10 mg in 2 mL 50 mM carbonate buffer, pH 9.6) and was allowed to couple for 5 h at room temperature with gentle stirring. To eliminate the excess $NaIO_4$ and uncoupled DME, the reaction mixture was dialyzed against water at 4 °C and then lyophilized to obtain DME-BSA powder. The same method was used to synthesize the DME-OVA conjugate (Fig. 1).

2.4. Determination of the hapten number for the ME-BSA conjugate

MALDI-TOF-MS was applied to determine the coupling degree between DME and BSA. Briefly, a small amount (1–10 pmol) of DME-BSA conjugate was mixed with a 1000-fold molar excess of sinapinic acid in an aqueous acetonitrile solution containing 0.10% trifluoroacetic acid. The mixture was analyzed with an Autoflex III high-performance MALDI-TOF-MS system, (Brucker Daltonics, Bremen, Germany). The number of DME molecules that were coupled to a molecule of BSA was calculated using the molecular weights of DME-BSA, DME and BSA.

2.5. Immunization

A New Zealand White rabbit, which was selected as the source for PAb production, was supplied by the National Animal Centre,

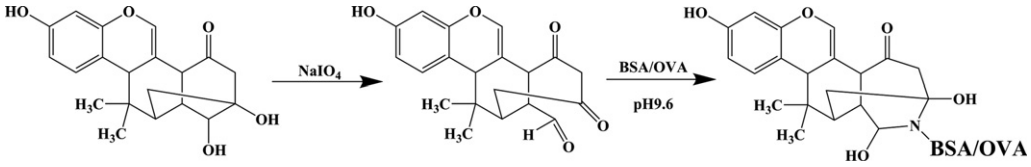

Fig. 1. Periodate oxidation method to synthesize deoxymiroestrol-BSA/OVA conjugates.