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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,

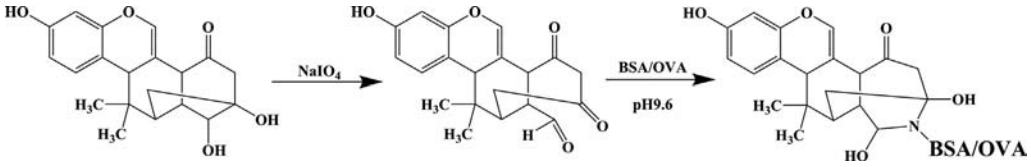

Fig. 1. Periodate oxidation method to synthesize deoxymiroestrol-BSA/OVA conjugates.

Mahidol University, Nakhon Pathom, Thailand. The rabbit was ordered, transferred and cared for by the trained staff of the Northeast Laboratory Animal Center of Khon Kaen University. The animal handing and immunization protocols were approved by the Animal Ethic Committee of Khon Kaen University (Record No. AEKKU 57/2555). The male rabbit was first immunized subcutaneously with a 1:1 emulsion mixture of Freund's complete adjuvant and DME-BSA conjugate (1.5 mg). Three weeks later, an intramuscular booster of the same conjugate (1.0 mg) emulsified with Freund's incomplete adjuvant was administered three times with a two-week interval between each booster. When the desired antibody titer was obtained, the final immunization was performed intravenously with 500 μg of the DME-BSA conjugate without any adjuvant. Finally, the rabbit was bled from the marginal ear vein. The serum was obtained by centrifuging at 7000 rpm for 10 min to remove any other blood components, and the serum was frozen at −20 °C until used for the ELISA development and validation.

2.6. Purification of polyclonal antibody

A Protein G FF affinity column (0.46 × 11 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) was used to purify total immunoglobulin G_1 from the rabbit serum. Rabbit serum (1 mL) was loaded and allowed to flow slowly through the column, which was previously equilibrated with 20 mM phosphate buffer (pH 7). The non-IgG components were washed out with 20 mM phosphate buffer (pH 7), and the bound IgG was eluted with 100 mM citrate buffer (pH 2.7) at a flow rate of 1 mL min^{-1}. The eluted IgG was neutralized with 1 M Tris–HCl buffer (pH 9.0) and then dialyzed and lyophilized to obtain IgG powder.

2.7. Indirect non-competitive ELISA

High protein-binding capacity polystyrene 96-well ELISA plates (Maxisorb Nunc, Roskilde, Denmark) were coated with 100 μL of 5.0 μg mL^{-1} DME-OVA conjugate solution prepared in 50 mM carbonate buffer (pH 9.6) and allowed to stand for 1 h. The plate was washed three times with 0.05% Tween 20 in 10 mM phosphate-buffered saline (TPBS). The unoccupied area of the 96-well plates was treated with 300 μL of phosphate buffered saline containing 1% gelatin (PBSG) for 1 h to reduce non-specific adsorption. After washing, the coated DME-OVA was reacted with 100 μL of the testing antibody prepared in TPBS containing 0.25% gelatin and 0.25% BSA (TPBS-GB). The peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 in TPBS containing 0.5% gelatin (TPBS-G) was allowed to react with the antibodies binding specifically to DME-OVA. After three washings with TPBS, 100 μL of the substrate solution, consisting of 0.003% H_2O_2 and 0.3 mg mL^{-1} of ABTS in 100 mM citrate buffer (pH 4.0), was added to each well and incubated for 15 min. The absorbance at 405 nm was measured using a microplate reader (Model 550 Microplate Reader BioRad Laboratories, CA, USA). All reactions were conducted at 37 °C.

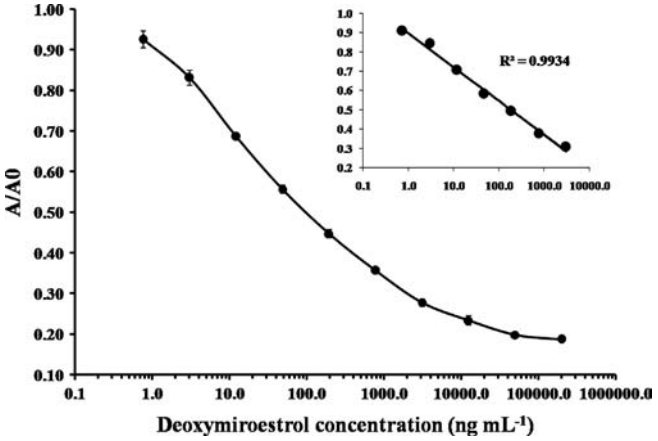

Fig. 2. The inhibitory curve of deoxymiroestrol (where A0 is the absorbance without deoxymiroestrol present and A is the absorbance with deoxymiroestrol present).

2.8. Indirect competitive ELISA

The procedure of the indirect competitive ELISA was the same as described above with a change in the competitive step. The 96-well plate was coated with DME-OVA and treated with PBSG consecutively. In the competitive step, 50 μL serial concentrations of DME or sample prepared in 20% ethanol was added to each well, and then 50 μL antibody diluted in TPBS-GB was allowed to react with DME in each well for 1 h. The plate was washed three times with TPBS, and a diluted solution (1:1000) of peroxidase-conjugated goat anti-rabbit IgG antibody in TPBS-G was reacted with fixed PAb for 1 h. After washing the plate three times with TPBS, 100 μL of substrate solution was added to each well and incubated for 15 min. The absorbance at 405 nm was measured using a microplate reader (Model 550 Microplate Reader BioRad Laboratories, CA, USA). All reactions were conducted at 37 °C.

2.9. Method validation

A quantitative ELISA was developed based on the competition between fixed DME-OVA and free DME in standard or sample solution. Indirect ELISA was selected as the model for ELISA development because signal amplification of the enzyme-labeled secondary antibody was one approach used to increase assay sensitivity. Indirect non-competitive ELISA was applied to evaluate whether PAb reacted to coated DME-OVA and to determine the appropriate concentration of PAb for indirect competitive ELISA. A quantitatively indirect competitive ELISA was developed and validated for specificity, sensitivity, precision, accuracy and

Table 1
The cross-reactivity of the antibody toward various compounds.

Compound	Cross-reactivity (%)
Chromenes	
Deoxymiroestrol	100.00
Miroestrol	1.26
Isomiroestrol	0.42
Isoflavonoids	
Daidzin	< 0.01
Daidzein	< 0.01
Genistin	< 0.01
Genistein	< 0.01
Puerarin	< 0.01
Glycitein	< 0.01
Flavonoids	
Rutin	< 0.01
Baicalin	< 0.01
Catechin	< 0.01
Quercetin	< 0.01
Anthraquinons	
Sennoside A	< 0.01
Sennoside B	< 0.01
Rhein	< 0.01
Others	
Capsaicin	< 0.01
Quinine	< 0.01
Curcumine	< 0.01
Plumbagin	< 0.01
Taxol	< 0.01

correlation with another analytical method. To determine the specificity of anti-DME PAb, several compounds with related and non-related structures were incubated with the antibody, and the percentage of cross-reactivity was calculated by comparison with DME as described previously [27]. The ELISA sensitivity and linear measurement range were calculated using the inhibitory value of different DME concentrations. Intra- and inter-plate variations in the DME determination in the measurement range concentration were used to evaluate the ELISA precision. The intra-assay variation level was evaluated using the variation of DME determination from well to well ($n=6$) within a plate, whereas the inter-plate assay variation was obtained from different plates ($n=4$). The method accuracy was investigated by a recovery experiment. Known amounts of DME were spiked into extracts of *P. candollei* and its derived products, and then DME was analyzed by the developed ELISA. *P. candollei* samples with various contents of DME were used as samples for the correlation between a published HPLC method and the developed ELISA.

2.10. High performance liquid chromatography conditions

To compare the performance of our developed ELISA with another analytical method, a high performance liquid chromatography (HPLC) method that was previously reported [2] was set as the comparator. The liquid chromatography was performed using a PerkinElmer Series 200 LC pump connected to a PerkinElmer 785 A UV/VIS detector (254 nm). An RP-18 column (LiChroCART®, 125 × 4 mm, 5 μm particle size, Merck, Germany) was used. The mobile phase consisted of 20% acetonitrile adjusted pH with 1.5% acetic acid with a flow rate of 1.0 mL min^{-1}.

Table 2
The precision of ELISAs for deoxymiroestrol determination.

Deoxymiroestrol (ng mL^{-1})	CV (%)	
	Intra-plate ($n=6$)	Inter-plate ($n=4$)
1500.00	2.12	4.68
375.00	1.98	3.96
93.75	3.65	4.73
23.44	2.92	4.31
5.68	4.99	4.10
1.46	1.76	3.84

Table 3
The recovery of deoxymiroestrol in *P. candollei* sample as determined by ELISA.

Spiked concentration (μg mL^{-1})	Measured concentration (μg mL^{-1})	CV (%)	Recovery (%)
0.00	9.27	2.98	–
0.50	9.77	5.69	99.85
1.00	10.30	2.66	102.58
5.00	14.26	2.93	99.82
10.00	19.45	1.56	101.76

Table 4
The recovery of deoxymiroestrol in sample of *P. candollei* product as determined by ELISA.

Spiked concentration (μg mL^{-1})	Measured concentration (μg mL^{-1})	CV (%)	Recovery (%)
0.000	1.22	4.24	–
0.125	1.34	4.40	98.07
0.250	1.48	8.91	105.37
0.500	1.75	4.19	106.33

3. Results and discussion

3.1. Antibody production

In the DME chemical structure, there are two hydroxyl groups on adjacent carbon atoms that can be oxidized by $NaIO_4$ to active carbonyl groups. The primary amine residues of BSA can be coupled with these reactive carbonyl groups by amination reduction in basic pH buffer. DME molecules were successfully linked with BSA and OVA with high hapten numbers. The MALDI-TOF-MS spectra showed the peak of DME-BSA at approximately 68,172.57 m z^{-1}, therefore at least 5 molecules of DME were coupled to each molecule of BSA, according to the BSA and DME molecular weights of 66,447.83 and 342.39 m z^{-1}, respectively. Rabbit serum was checked several times during immunization. Both the non-competitive and competitive ELISA suggested that an immunized rabbit produced antibody that reacted to the fixed DME-OVA and free DME after the second immunization. Overall, the results indicated that the appropriate PAb against DME was obtained for the immunoassay development. A total of 35 mg of IgG was purified from 1 mL of rabbit serum using a protein G FF column.

The indirect non-competitive ELISA using purified total IgG indicated that the PAb reacted to fixed DME-OVA in a concentration-dependent manner. A concentration of 7.8 μg mL^{-1} was set for the indirect competitive ELISA for DME determination.

Table 5
Determination of deoxymiroestrol in *P. candollei* samples using ELISA and HPLC.

Collection place/varieties	Part	Deoxymiroestrol content (μg g^{-1} dry wt.)	
		ELISA	HPLC
Ubon Ratchathani			
P. candollei var. *candollei*	Tuber cortex	162.61 ± 9.93	154.34 ± 5.70
	Whole tuber	50.06 ± 2.83	45.95 ± 1.14
	Tuber without cortex	32.28 ± 1.64	30.23 ± 2.45
P. candollei var. *mirifica*	Tuber cortex	195.04 ± 16.68	183.00 ± 8.47
	Whole tuber	88.12 ± 2.76	87.30 ± 3.75
	Tuber without cortex	10.32 ± 0.84	9.50 ± 0.44
Nakhon Ratchasima			
P. candollei var. *candollei*	Tuber cortex	16.36 ± 1.00	14.21 ± 1.14
	Whole tuber	3.06 ± 0.25	ND[a]
	Tuber without cortex	0.21 ± 0.01	ND[a]
P. candollei var. *mirifica*	Tuber cortex	17.51 ± 1.57	15.41 ± 0.71
	Whole tuber	2.29 ± 0.15	ND[a]
	Tuber without cortex	0.22 ± 0.01	ND[a]

[a] ND: not detectable.

3.2. Development and optimization of ELISA

An indirect competitive ELISA was developed and validated for sensitivity, specificity, precision, accuracy and correlation with another analytical method. Competitive binding of the antibody of free DME to fixed DME-OVA was observed in a concentration-dependent manner. The limit of detection (LOD) calculated at 10% inhibition was 0.73 ng mL^{-1}. The linear range of the ELISA measurement was 0.73–3000 ng mL^{-1} (Fig. 2). The sensitivity, was high compared to the reported HPLC-UV with an LOD of 0.78 mg mL^{-1}. This development of an anti-DME antibody was successful for DME determination with high sensitivity because the design of the DME-BSA conjugate was appropriate for antibody production with high affinity for free DME.

A cross-reactivity experiment determined whether the anti-DME antibody could react to other compounds. Several naturally occurring compounds, including estrogenic isoflavones in *Pueraria* spp., flavonoids and others, were tested. The results indicated that the PAb specifically bound to DME as listed in Table 1. The PAb could distinguish between DME and miroestrol and isomiroestrol, which have similar structures, with low cross-reactivity to miroestrol and isomiroestrol of 1.26% and 0.42%, respectively. The PAb did not recognize the estrogenic isoflavones, which indicated that the other main compounds in *Pueraria* spp. did not interfere with this PAb ELISA. Moreover, there was no cross-reactivity to other plant-originated compounds, which suggested that the ELISA could be applied to *P. candollei* products formulated with other medicinal plants without chemical interference.

The intra- and inter-plate assay precisions were determined to evaluate the ELISA reproducibility. Inhibitory variations at each concentration in the linear range were tested both within a plate ($n=6$) and between plates ($n=4$). The intra- and inter-plate coefficients of variation (CV) were both less than 5% (Table 2), which reflects the high reproducibility of the ELISA.

To investigate the ELISA accuracy, spiked *P. candollei* extracts with known concentrations of DME (0.5, 1, 5 and 10 μg mL^{-1}) were measured by the developed ELISA. The percentages of recovery were 99.82–102.58% with a maximum CV of 5.69% (Table 3), which indicated that the method is accurate in *P. candollei* plant samples. Because each *P. candollei*-associated product contained many different medicinal plants, it was necessary to evaluate ELISA accuracy in these products. The percentages of recovery in the products were 98.07–106.33% with a maximum CV of 8.91% (Table 4) for extracts spiked with 0.125–0.500 μg mL^{-1} DME. The results suggested that the developed ELISA was accurate in both *P. candollei*-associated plant and product samples. Recoveries were calculated as follows:

$$\text{Recovery}(\%) = \frac{Css - Cus}{Cs} \times 100$$

where *Css* and *Cus* are the concentrations that were measured in the spiked sample and unspiked sample, respectively, and *Cs* is the theoretical spiked concentration.

Table 6
The deoxymiroestrol contents in *P. candollei* associated products.

Number of product	Preparation	Weight/unit (mg)	Deoxymiroestrol content	
			(μg g^{-1} dry wt.)	(μg $unit^{-1}$)
1	Capsule	233.38	0.371 ± 0.029	0.087 ± 0.007
2	Capsule	512.82	1.099 ± 0.072	0.564 ± 0.037
3	Capsule	367.44	1.764 ± 0.118	0.648 ± 0.043
4	Capsule	287.64	1.769 ± 0.079	0.509 ± 0.023
5	Capsule	303.20	10.998 ± 0.727	3.335 ± 0.220
6	Capsule	516.71	0.154 ± 0.007	0.079 ± 0.004
7	Capsule	316.80	0.365 ± 0.026	0.116 ± 0.008
8	Capsule	275.21	0.894 ± 0.020	0.246 ± 0.006
9	Tablet	792.95	0.859 ± 0.027	0.681 ± 0.022
10	Tablet	153.13	1.772 ± 0.106	0.271 ± 0.016

3.3. Correlation with HPLC method

The ELISA exhibited comparable performance to the published HPLC method in high DME-containing samples with a high coefficient of determination ($R^2=0.9993$). In addition, the ELISA could measure DME at concentrations too small to be detected by the HPLC-UV method (Table 5). Because the ELISA is highly sensitive, only a small amount of sample was required for analysis, which is important for industrial applications. Subsequently, only small volumes of organic solvent were needed for sample extraction.

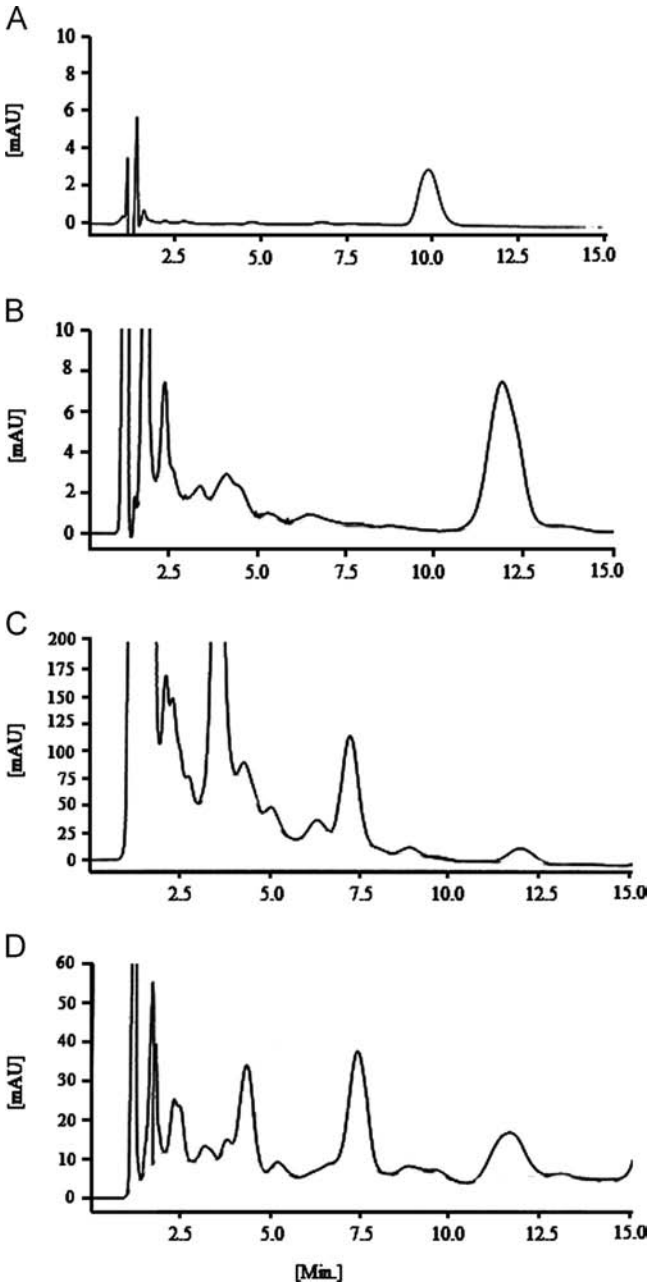

Fig. 3. The HPLC-UV chromatograms of standard deoxymiroestrol (A), product No. 1 (B), product No. 5 (C) and product No. 10 (D).

3.4. Application

The validated ELISA was applied to DME measurement in *P. candollei* products. Ten products were sampled from the markets in Khon Kaen and Ubon Ratchathani provinces. These products are commercially available as food supplements commonly prepared with other medicinal plants including *Angelica sinensis*, *Panax ginseng*, *Terminalia chebula*, *Terminalia bellirica*, *Cyperus rotundus*, *Conioselinum univitatum*, *Caesalpinia sappan* and *Carthamus tinctorius*. The *P. candollei*-derived preparations contained 0.154 ± 0.007–10.998 ± 0.727 μg g^{-1} dry wt. of DME, which was 0.079 ± 0.004–3.335 ± 0.220 μg $unit^{-1}$ when calculated using the weight of a capsule or tablet (Table 6). These products contained too little DME to be determined directly by HPLC-UV because these products were developed from the part of the tuber without cortex that contains only a small amount of DME, and because Thai herbal preparations are composed of many medicinal plants, the finished product contained a very small DME content. In addition, different products with different plants might contain chemical compounds that interfere with analysis by HPLC-UV (Fig. 3). However, these different products can be analyzed by ELISA without chemical interference as indicated in the recovery experiment of the extracted *P. candollei* products.

4. Conclusion

We have developed a PAb against DME that exhibited high potential as a biomarker of *P. candollei* samples and preparations. Our ELISA was the first immunoassay to analyze DME with high sensitivity and selectivity. The validated ELISA determined DME in the linear range of 0.73–3000 ng mL^{-1}, and the intra- and inter-plate CVs were less than 5%. The percentages of recovery were 99.82–102.58% and 98.07–106.33% in the extract of *P. candollei* and its product samples, respectively. The ELISA was comparable with the published HPLC method ($R^2 = 0.9993$) in samples with various DME contents. Overall, the validated parameters indicated that ELISA was reliable for DME determination. The *P. candollei*-derived preparations contained 0.154-10.998 μg g^{-1} dry wt. of DME. This ELISA method could be used to for the standardization of samples before use in pre-clinical and clinical research and for *P. candollei*-derived products or materials during their development and production at the industrial level. In addition, the developed ELISA had enough sensitivity and accuracy to measure small concentrations of DME in the biological fluids of experimental animals and humans for pharmacological investigations.

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