



## วิธีเอนไซม์-ลิงค์ อิมมูโนแอสเซย์สำหรับการวิเคราะห์ปริมาณสารไมโรเอสโตรล ในผลิตภัณฑ์กาวเครือขาว

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### บทคัดย่อ

วิธีเอนไซม์-ลิงค์ อิมมูโนแอสเซย์สำหรับการวิเคราะห์สารไมโรเอสโตรลในผลิตภัณฑ์กาวเครือขาว

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**บทนำ:** ตำรับต่างๆ ของกาวเครือขาวถูกใช้อย่างแพร่หลายเพื่อบำรุงร่างกายในผู้สูงอายุ ซึ่งสอดคล้องกับฤทธิ์ทางเภสัชวิทยาทั้งในระดับสัตว์ทดลองและมนุษย์เพื่อบรรเทาอาการต่างๆ ของวัยหมดประจำเดือน ดังนั้นผลิตภัณฑ์จากกาวเครือขาวที่มีการควบคุมคุณภาพจึงเป็นที่ต้องการของผู้บริโภคและบุคลากรทางสุขภาพ สารไมโรเอสโตรลเป็นสารที่มีฤทธิ์เป็นฮอร์โมนเอสโตรเจนสูง มีศักยภาพเป็นตัวบ่งชี้ทางชีวภาพเพื่อใช้ในการควบคุมคุณภาพกาวเครือขาวและผลิตภัณฑ์ เครื่องมือและวิธีดำเนินการวิจัย: วิธีเอนไซม์-ลิงค์ อิมมูโนแอสเซย์แบบแข่งขัน หรืออิลซาถูกพัฒนาขึ้นโดยใช้โพลีโคลนอลแอนติบอดีจากกระต่าย วิธีการวิเคราะห์อิลซาถูกเปรียบเทียบความสามารถในการวัดปริมาณสารไมโรเอสโตรลกับวิธีโครมาโทกราฟีสมรรถนะสูง หรือ เอชพีแอลซี จากนั้นประยุกต์ใช้เพื่อวิเคราะห์สารในตัวอย่างผลิตภัณฑ์กาวเครือขาว **ผลการศึกษา:** วิธีอิลซามีความสามารถเทียบเท่ากับวิธีเอชพีแอลซี ( $R^2 = 0.9996$ ) ในการวิเคราะห์สารไมโรที่ความเข้มข้นต่างๆ นอกจากนั้นแล้ววิธีอิลซายังสามารถวิเคราะห์ในตัวอย่างที่ไม่สามารถวิเคราะห์ด้วยวิธีเอชพีแอลซี เนื่องจากวิธีอิลซามีความไวที่สูงกว่า **สรุปผล:** วิธีอิลซาที่พัฒนาขึ้นมีศักยภาพสูง และความน่าเชื่อถือในการวิเคราะห์ปริมาณสารไมโรเอสโตรล ซึ่งสามารถประยุกต์ใช้เพื่อการควบคุมคุณภาพกาวเครือขาวทั้งระดับการวิจัยและระดับอุตสาหกรรม

**คำสำคัญ** กาวเครือขาว สารไมโรเอสโตรล เอนไซม์-ลิงค์ อิมมูโนแอสเซย์ เอสโตรเจนจากพืช โพลีโคลนอลแอนติบอดี

### Abstract

**Enzyme-Linked Immunosorbent Assay for Determination of Miroestrol in *Pueraria candollei***

#### Associated Products

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**Introduction:** Various dosage forms of *Pueraria candollei* are widely applied in folk Thai medicine for rejuvenating purpose in aged people, which correlated with its pharmacological activities reported by pre-clinical and clinical trials for menopausal symptoms. Therefore, standardized products of this plant are needed by consumers and health care personnel. Miroestrol (ME), a potent and stable phytoestrogen in *P. candollei*, exhibited potential to be biomarker for quality control of *P. candollei* samples and its products. **Materials and methods:** Indirect competitive enzyme-linked immunosorbent assay (ELISA) for ME determination was developed and validated by using polyclonal antibody from rabbit immunization. The correlation of ME determination between our ELISA with standard HPLC method was investigated. Finally validated ELISA was applied for *P. candollei* associated products. **Results:** Developed ELISA was comparable with published HPLC method ( $R^2 = 0.9996$ ) in samples with various ME contents, moreover this ELISA could determine ME in samples undetectable by HPLC because of its



higher sensitivity. For application, the *P. candollei* involved preparations contained ME of 0.766- 12.108 µg/g dry wt.  
**Conclusion:** The developed ELISA was high performance for reliable ME determination, which could be applied for *P. candollei* quality control in research fields and industrial productions.

**Keywords:** *P. candollei*, miroestrol, enzyme-linked immunosorbant assay, phytoestrogen, polyclonal antibody

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## Introduction

*Pueraria candollei* var. *mirifica* is phytoestrogen enrich plant which its tuberous root was used widely in traditional Thai medicine system for smoothening skin, enhancing memory and rejuvenating in aged man and woman (Anusarnsoondhorn, 1931). The randomized clinical trials to evaluate efficacy and safety of *P. candollei* var. *mirifica* showed that the climacteric scores of menopause woman were declined, whereas no significant side effects were observed (Chandeyng and Lamlerkittikul, 2007 ; Lamlerkittikul and Chandeyng, 2004 ; Virojchaiwong *et al.*, 2011), which efficacy of 50 mg daily dose *P. candollei* var. *mirifica* was not significantly different from 0.625 mg conjugated equine estrogen with/without medroxyprogesterone acetate (Chandeyng and Sangthawan, 2007). Overall trials suggested that this plant benefited for menopausal related disorders.

Isoflavonoid and chromene compounds, including puerarin, daidzin, genistin, daidzein, genistein, miroestrol

(ME), deoxymiroestrol and isomiroestrol, are chemical components mediating for estrogenic effects of *P. candollei* var. *mirifica*. ME as biomarker for quality control of *P. candollei* exhibited strong estrogenic activity. Analytical method of ME using HPLC-UV was developed as described (Yusakul *et al.*, 2011) for its determination in *Pueraria candollei*. Disadvantage of HPLC method for minor compounds in plant sample is interference of other compounds which need for sample pretreatment resulting interested compound loss. Immunoassay with polyclonal antibody exhibited various advantages, including high sensitivity (Chappey *et al.*, 1992), inexpensive, short time production and low technology and skill required.

In this report, we aim to compare performance of our developed ELISA with HPLC method, and then to apply for ME determination in *P. candollei* associated products.



## Materials and Methods

### Production of polyclonal antibody

ME (>90% purity) was purified from tuberous root cortex of *P. candollei*, which obtained compound was confirmed using  $^1\text{H}$ -NMR with authentic standard. ME was conjugated to bovine serum albumin (BSA) as immunogen, whereas the compound was conjugated to ovalbumin (OVA) as non-relevant coating in ELISA test using periodate oxidation reaction. A male New Zealand White rabbit was immunized with ME-BSA emulsified with Freund's adjuvants for anti-ME antibody production, which total IgG as source of antibody was purified from rabbit serum using Protein G FF affinity column (Amersham Pharmacia Biotech, Sweden).

### Indirect competitive ELISA

Purified anti-ME antibody was characterized and used to develop competitive ELISA for ME determination. The 96-well plate was coated with 5  $\mu\text{g}/\text{mL}$  ME-OVA and then treated with 1% gelatin consecutively. In competitive step, 50  $\mu\text{L}$  serial concentrations of standard ME or sample prepared in 20% ethanol were added to each well, and then 50  $\mu\text{L}$  of 12.5  $\mu\text{g}/\text{mL}$  anti-ME antibody diluted in phosphate-buffered saline with 0.05% Tween 20 (TPBS) containing 0.25% gelatin and 0.25% BSA was allowed to react with ME in each well for 1 hour. Next, the plate was washed three times with TPBS, and a diluted solution (1:1,000) of peroxidase conjugated goat anti-rabbit IgG antibody in TPBS containing 0.5% gelatin was reacted with fixed polyclonal antibody for 1 hour. After washing the plate three times with TPBS, 100  $\mu\text{L}$  of substrate solution, 100 mM citrate buffer (pH 4.0) containing 0.003%  $\text{H}_2\text{O}_2$  and 0.3 mg/mL of ABTS, 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 carried out at 37°C.

### Method validation

*P. candollei* samples with various ME contents were used as samples for correlation experiment between developed ELISA and HPLC method (Yusakul *et al.*, 2011). The chromatography was performed using a PerkinElmer Series 200 LC pump connected to a PerkinElmer 785A

UV/VIS detector. An RP-18 column (LiChroCART®, 125 mm×4 mm, 5  $\mu\text{m}$  particle size, Merck, Germany) was used. The mobile phase consisted of 20% acetonitrile adjusted pH with 1.5% acetic acid, which flow rate of mobile phase was set at 1.0 mL/min. Finally the well characterized ELISA was applied for ME determination in *P. candollei* associated preparations.

## Results and discussions

### Correlation with HPLC method

ME contents in each sample by ELISA was correlated with value from HPLC method with high coefficient of determination ( $R^2=0.9996$ ). The high correlation indicated that developed ELISA was comparable with HPLC method in high ME containing samples. Moreover, some samples undetectable by HPLC method could be quantified by ELISA due to its 1000 times higher sensitivity approximately, when was calculated by limit of quantification (LOQ).

### Application

Characterized ELISA was applied to determine ME in *P. candollei* associated products. Because *P. candollei* products commonly were developed from part of tuber without cortex containing small amount of ME, and Thai herbal products composed of many medicinal plant, the finish product contained of too low ME content to determine using HPLC-UV method. There are several advantages of ELISA over HPLC with UV detector for ME determination in products; the firstly ELISA could detect with very low concentration requiring small sample amount, the secondly ELISA determined without any chemical interference from both *P. candollei* and other medicinal plants, the finally ELISA could be applied with many samples in short of analysis time. The *P. candollei* involved preparations contained 0.766-12.108  $\mu\text{g}/\text{g}$  dry wt. of ME, which was 0.179-3.483  $\mu\text{g}/\text{unit}$  when calculated by weight of a capsule or a tablet (table 1), which coefficient of variation (CV) for all ME determination in these products was maximum of 7.56 %. Our developed ELISA could be applied for ME quantification with various contents in different composition product with small amount of sample needed.



**Table 1** Miroestrol content in *P. candollei* associated products.

Number of product	Preparation	Weight/unit of product (mg)	Miroestrol content	
			(µg/g dry wt.)	(µg/unit)
1	Capsule	233.38	0.766±0.049	0.179±0.012
2	Capsule	512.82	1.872±0.097	0.960±0.050
3	Capsule	367.44	2.141±0.107	0.787±0.039
4	Capsule	287.64	12.108±0.285	3.483±0.082
5	Capsule	303.20	0.966±0.018	0.293±0.005
6	Capsule	516.71	1.193±0.054	0.616±0.028
7	Capsule	316.80	0.695±0.037	0.220±0.012
8	Capsule	275.21	1.138±0.019	0.313±0.005
9	Tablet	792.95	0.908±0.069	0.720±0.054
10	Tablet	153.13	1.703±0.039	0.261±0.006

## Conclusion

Our developed ELISA was the first immunoassay with high sensitivity to analyze ME, which exhibit potential as biomarker for quality control of *P. candollei* samples and its preparations. Validated ELISA was comparable with published HPLC method ( $R^2 = 0.9996$ ) in samples with various ME contents. The ELISA was applied to *P. candollei* involved preparations, which there were 0.766-12.108 µg/g dry wt. of ME content. The ELISA could be apply for quality control of *P. candollei* samples before its using in pre-clinical and clinical researches, and of *P. candollei* associated products or materials during its development and production in industrial level

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