

CHAPTER III

MATERIALS AND METHODS

1. Chemical reagents

Murashige and Skoog (MS) medium was identical to appendix (Murashige and Skoog, 1962).

Standard; daidzin, genistin, daidzein and genistein were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Puerarin was obtained from Chromadex Inc. (Irvine, CA, USA). Cefotaxime sodium salt was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Miroestrol and deoxymiroestrol were isolated from the tuberous roots of *P. candollei* var. *mirifica* as described previously (Chansakaow et al., 2000a). NMR analysis was performed to compare the isolated compounds with the authentic standards of miroestrol and deoxymiroestrol and was provided by Assoc. Prof. Dr. Chaiyo Chaichantipyuth, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. All other chemicals were standard commercial products of analytical grade.

Hormone; α -Naphthaleneacetic acid (NAA) and 6-Benzyladenine (BA) were purchased from Fluka Chemical (Buchs, Switzerland). *N*-phenyl-*N'*-1, 2, 3-thiadiazol-5'-yl urea (thidiazuron, TDZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA), *N*-(2-chloro - 4- pyridyl)-*N*- phenylurea (CPPU), and abscisic acids (ABA) were purchased from Sigma.

Elicitor; Methyl jasmonate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Yeast extract was purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Chitosan was obtained from Fluka Chemical (Buchs, Switzerland).

Peroxidase-labeled anti-rabbit IgG were obtained from MP Biomedicals (OH, USA). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Wako (Osaka, Japan).

2. Plant materials

P. candollei var. *candollei* and *P. candollei* var. *mirifica* seeds were obtained from the Botanical Garden, Faculty of Pharmaceutical Sciences, Khon Kaen University and Suranaree University of Technology, Nakhon Ratchasima, Thailand, respectively. Two varieties of *P. candollei* seeds were washed with sterile distilled water and surface-sterilized with 10% sodium hypochlorite for 15 – 20 min. Then they were washed three times with sterilized water and immersed in 70% ethanol for 1 min. The sterilized seeds were moved on hormone-free Murashige and Skoog (MS) medium containing 3% sucrose (w/v), pH 5.5 and maintained at (25 ± 1) °C under continuous light for 16 h/d. The embryos were observed within 7 days after cultivation and used for hairy roots induction.

3. Preparation of elicitors

Elicitation was carried out with methyl jasmonate, yeast extract and chitosan. Methyl jasmonate 30 mM stock solution was prepared in 40% (v/v) ethanol and then filter-sterilized. Yeast extract was dissolved in de-ionized water to make 100 mg/ml stock solution and autoclaved. Chitosan was dissolved in glacial acetic acid by adding drop wise at 60 °C for a period of 15 min and adjusted to pH 5.5 before autoclaving.

4. Callus and cell suspension culture

4.1 Callus induction

According to the previous study (Udomsuk et al., 2011d) the highest level of total isoflavonoid and miroestrol contents were achieved from stem explants callus of *P. candollei* var. *mirifica* on MS medium containing of 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA. In this experiment, we induced callus from stem part of *P. candollei* var. *mirifica* by using MS medium containing of 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA as previous study.

4.2 Growth rate, chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

After subcultured callus of *P. candollei* var. *mirifica* from solid into liquid MS medium with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA hormones supplement. The cell suspension was green and friable. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). Then we observed growth rate, chromene and total isoflavonoid production of cell suspension culture in liquid MS medium with hormones supplement every 7 days for 28 days.

4.3 Elicitors treatment in *P. candollei* var. *mirifica* cell suspension culture

The 18 days of cell suspension culture were treated with various types and concentrations of elicitors. Each elicitor was added in the following final concentration: methyl jasmonate 50, 100 and 200 µM; yeast extract 0.5, 1.0 and 2.0 mg/ml; chitosan 0.5, 1 and 10 mg/l. Chromene and isoflavonoid contents were determined at 3 and 6 days after elicitation. All treatments were performed in triplicate.

5. Hairy roots culture

5.1 Hairy roots induction

The embryos (cotyledon, leaves and stems part) were infected with *Agrobacterium rhizogenes* ATCC 15834 and transferred to ½ MS medium containing 500 mg/l cefotaxime. Two weeks later, hairy roots were emerged from infection sites and observe within 14 days. Two weeks interval, the hairy roots were subculture on ½ MS medium containing 400, 300, 200, 100 mg/l cefotaxime and ½ MS medium, respectively. Transformed roots of two varieties *P. candollei* were grown in 125-ml flasks containing 30 ml of ½ MS liquid medium. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). The hairy roots were subculture every 2 weeks into fresh medium.

5.2 Growth rate and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture

After subcultured transformed root to liquid ½ MS medium, then we observed growth rate and total isoflavonoid production of hairy root culture in liquid ½ MS medium every 7 days for 35 days.

5.3 Elicitors treatment in *P. candollei* var. *mirifica* hairy root

21 days transformed hairy roots of *P. candollei* var. *mirifica* in ½ MS liquid medium were added 200 µM methyl jasmonate, 0.5 mg/ml yeast extract and 0.5 mg/l chitosan. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). Then the hairy roots were harvested every 3 and 6 days after exposing with elicitors. All treatments were performed in triplicate.

5.4 Elicitors (hormone) treatment in two varieties of *P. candollei* hairy root

21 days transformed hairy roots of two varieties *P. candollei* in ½MS liquid medium were transferred to ½MS liquid medium which each containing various concentrations of plant hormones (CPPU, TDZ at 0.01, 0.05, 0.1 and 0.5 mg/l and ABA at 0.1, 0.5, 1, 2 mg/l). Then the hairy roots were harvested at 5 and 10 days after exposing with hormone. All treatments were performed in triplicate.

5.5 Comparative analysis of hairy root and native root of both varieties of *P. candollei*

7 weeks of hairy root and native roots of both *P. candollei* var. *candollei* and *P. candollei* var. *mirifica* from seeding were collected. All roots were dried at 50°C in hot air oven to a constant weight for analysis chromene and total isoflavonoid contents.

6. Extraction of samples and chromene analysis

600 mg dried powdered samples of cell suspension or 1 g dried powdered samples of hairy root were washed with 5 ml of hexane for 1 h with sonication and then extracted four times with 5 ml of ethyl acetate–chloroform (3:1, v/v) with sonication for 1 h. The extracts were combined and evaporated at 60 °C. Then the extract

samples were dissolved in 1 ml of ethanol for HPLC analysis as described previously. The mobile phase consisted of 20% acetonitrile containing 1.5% acetic acid at 1.0 ml/min flow rate and 254 nm detection wavelengths. HPLC was performed using a PerkinElmer Series 200 LC pump connected to a PerkinElmer 785A UV/VIS detector and a PE Nelson computer. An RP-18 column (LiChroCART®, 125 mm×4 mm, 5 µm particle size, Merck, Germany) was used.

7. Extraction of samples and total isoflavonoid analysis

30 mg dried powdered samples of cell suspension or hairy root were extracted four times with 0.5 ml ethanol with sonication. The extracts were combined, evaporated and then re-dissolved in 1 ml ethanol. The total isoflavonoid content including puerarin, daidzin, genistin, daidzein and genistein of the extracted solutions were determined by an indirect competitive ELISA using anti-puerarin and anti-daidzin polyclonal antibodies (PAbs) as described previously with some modifications (Pongkitwitoon et al., 2010). Mixtures of puerarin-ovalbumin and daidzin-ovalbumin (100 µl; 0.5 µg/ml each in 50 mM carbonate buffer, pH 9.6) were adsorbed to the surfaces of the wells of a 96-well immunoplate, and the plate was incubated at 37 °C for 1 h. The plate was washed three times with 0.05% Tween 20 in phosphate-buffered saline (T-PBS). The plate was then treated with 300 µl of 1% gelatin in phosphate-buffered saline (PBS) for 1 h and washed three times with T-PBS. A 50 µl volume of various concentrations of puerarin and daidzin (ratio 1:1) or of samples dissolved in 20% ethanol was incubated with 50 µl of anti-puerarin and anti-daidzin PAbs (5 µg/ml and 2 µg/ml, respectively) for 1 h. The plate was washed three times with T-PBS, and then the PAbs were combined with 100 µl of a 1000-fold diluted solution of peroxidase-conjugated goat anti-rabbit IgG for 1 h. After washing the plate three times with T-PBS, 100 µl of a substrate solution (100 mM citrate buffer (pH 4.0) containing 0.003% H₂O₂ and 0.3 mg/ml of ABTS) was added, and the plate was incubated for 15 min. The absorbance at 405 nm was measured using a microplate reader (Model 550 Microplate, Biorad).

8. Statistical analysis

The results were expressed as the mean \pm SD. The data were analyzed using one-way analysis of variance (ANOVA). The difference between the mean of samples was analyzed by the least significant difference (LSD) at the $P < 0.01$ level.