PP-PS-035-KKU

ผลของสารกระตุ้นต่อการสร้างสารกลุ่มโครมีนและไอโซฟลาโวนอยด์ในการเพาะเลี้ยงรากลอยกวาวเครือขาว

อรพินทร์ อุดมศิลป์[1], ทวีศักดิ์ จึงวัฒนตระกูล[2] ,วราภรณ์ ภูตะลุน[3]*

บทคัดย่อ

ผลของสารกระตุ้นต่อการสร้างสารกลุ่มโครมีนและไอโซฟลาโวนอยด์ในการเพาะเลี้ยงรากลอยกวาวเครือขาว

อรพินทร์ อุดมศิลป์[1], ทวีศักดิ์ จึงวัฒนตระกูล[2] ,วราภรณ์ ภูตะลุน[3]*

บทนำ : กวาวเครือขาวเป็นสมุนไพรไทยที่มีการนำมาใช้และเป็นที่รู้จักมานานในแง่ของการใช้เป็นยาอายุวัฒนะ บำรุงร่างกาย กวาวเครือขาวมีชื่อวิทยาศาสตร์ว่า *Pueraria candollei* Wall. ex Benth จัดเป็นพืชในวงศ์ Leguminosae ในประเทศไทยพบกวาวเครือขาวสองสายพันธุ์ คือ *P. candollei* var. *candollei* (PC) และ *P. candollei* var. *mirifica* (PM) ซึ่งมีลักษณะทางพฤกษศาสตร์คล้ายคลึงกันแต่มีปริมาณสารสำคัญแตกต่างกัน จากรายงานการศึกษาพบว่า หัวกวาวเครือขาวมีองค์ประกอบเป็นสารกลุ่มไอโซฟลาโวนอยด์, โครมีน และอื่นๆ ซึ่งสารเหล่านี้มีฤทธิ์เป็นไฟโตเอสโตรเจน (phytoestrogen) การศึกษาวิจัยครั้งนี้จึงมุ่งเน้นถึงการศึกษาเพื่อเพิ่มขีดความสามารถในการผลิตสารกลุ่ม chromenes และ isoflavonoids ในรากลอย และศึกษาเปรียบเทียบปริมาณสารในกลุ่มดังกล่าวระหว่างการเพาะเลี้ยงกวาวเครือขาวแบบรากลอยกับรากธรรมชาติในกวาวเครือขาวทั้งสองสายพันธุ์ **วัสดุและวิธีการ :** ตรวจวัดปริมาณสารกลุ่มโครมีนโดยใช้ HPLC และใช้ competitive ELISA ในการตรวจวัดสารกลุ่มไอโซฟลาโวนอยด์ แล้ววิเคราะห์ค่าทางสถิติโดยใช้ ANOVA **ผลการศึกษา :** พบว่า 200 µM methyl jasmonate และ 0.5 mg/L yeast extract สามารถกระตุ้นการสร้างสารกลุ่ม โครมีนได้อย่างมีนัยสำคัญทางสถิติหลังการเติมสารกระตุ้น 3 วันและ 6 วัน ตามลำดับ โดยมีปริมาณโครมีน 17.03 และ 17.47 µg/g น้ำหนักแห้ง ตามลำดับ ซึ่งสูงกว่ากลุ่มควบคุมสองเท่า สารกระตุ้นทั้งสองชนิดดังกล่าวยังกระตุ้นการสร้างในสารกลุ่มไอโซฟลาโวนอยด์หลังการเติมสารกระตุ้น 3 วัน พบปริมาณไอโซฟลาโวนอยด์ 16.06 และ 14.65 mg/g น้ำหนักแห้ง ตามลำดับ ซึ่งสูงกว่ากลุ่มควบคุมสองเท่า การศึกษาเปรียบเทียบปริมาณสารทั้งสองกลุ่มดังกล่าวระหว่างการเพาะเลี้ยงกวาวเครือขาวแบบรากลอยกับรากธรรมชาติทั้งในกวาวเครือขาวทั้งสองสายพันธุ์พบปริมาณ โครมีนในรากลอยของ PM, รากลอยของ PC, ราก PM และราก PC ดังนี้ 5.51, 4.62, 18.86 และ 17.10 µg/g น้ำหนักแห้ง ตามลำดับ ส่วนปริมาณไอโซฟลาโวนอยด์พบในรากลอยของ PM, รากลอยของ PC, ราก PM และราก PC ดังนี้ 7.72, 9.10, 6.64 และ 7.05 mg/g น้ำหนักแห้ง ตามลำดับ **สรุปผล** : สารกระตุ้น methyl jasmonate และ yeast extract สามารถกระตุ้นการสร้างสารกลุ่มโครมีนและไอโซฟลาโวนอยด์ในการเพาะเลี้ยงกวาวเครือขาว (*P. candollei* var. *mirifica*) แบบรากลอยได้ ส่วนผลการศึกษาเพื่อเปรียบเทียบปริมาณสารทั้งสองกลุ่มดังกล่าวระหว่างการเพาะเลี้ยงกวาวเครือขาวแบบรากลอยกับรากธรรมชาติทั้งในกวาวเครือขาวทั้งสองสายพันธุ์พบว่า ปริมาณโครมีนในรากธรรมชาติของกวาวเครือขาวทั้งสองสายพันธุ์สูงกว่ารากธรรมชาติ ประมาณ 4 เท่า แต่พบปริมาณไอโซฟลานอยด์ในรากลอยมากกว่ารากธรรมชาติ

คำสำคัญ : กวาวเครือขาว, โครมีน และไอโซฟลาโวนอยด์

Abstract

Effect of elicitors on chromene and total isoflavonoid accumulation in *P. candollei* var. *mirifica* hairy root culture

Udomsin O[1], Juengwatanatrakul T[2], Putalun W[3]*

Introduction : *Pueraria candollei Wall.* ex Benth. (White Kwao Krua) is belong to the family Leguminosae and commonly known as a Thai herbal medicine having been used for rejuvenation. In Thailand there are two plants varieties, *P. candollei* var. *candollei* (PC) and *P. candollei* var. *mirifica* (PM) which have similar botanical characteristic but both vary in chemical

***ติดต่อผู้นิพนธ์** : โทร (66 43) 362095. โทรสาร (66 43) 202379. E-mail : waraporn@kku.ac.th

***Corresponding author :** Tel (66 43) 362095. Fax (66 43) 202379. E-mail : waraporn@kku.ac.th

component containing. Their tuberous roots contain chemical compounds, known as phytoestrogen, such as isoflavonoids, chromenes, etc. In the present study, we established a hairy root culture of *P. candollei* var. *mirifica* and optimal conditions for growth, and investigated the effects of plants elicitors (methyl jasmonate and yeast extract) on enhancing isoflavonoid and chromene accumulation. We also comparative studied on isoflavonoid and chromene production between hairy root and native root of both varieties of *P. candollei*. **Materials and methods :** Chromene analysis was performed using HPLC method (mobile phase consisted of 20% acetonitrile containing 1.5% acetic acid at 1.0 mL/min flow rate and 254 nm detection wavelengths). Total isoflavonid analysis was investigated using competitive ELISA (anti-puerarin and anti-daidzin polyclonal antibodies). The data were analyzed using one-way analysis of variance (ANOVA). **Results :** The results found 200 µM methyl jasmonate and 0.5 mg/L yeast extract significantly increased chromene production after 3 days and 6 days of elicitation, respectively (17.03 and 17.47 µg/g dry wt, 2–fold higher than control, respectively). They also increased total isoflavonoid production after 3 days of elicitation. (16.06 and 14.65 mg/g dry wt, 2–fold higher than control, respectively) For the comparative analysis of each phytoestrogen between hairy root and native root, chromene content in PM hairy root, PC hairy root, PM root and PC root were 5.51, 4.62, 18.86 and 17.10 µg/g dry wt., respectively. Total isoflavonoid content in PM hairy root, PC hairy root, PM root and PC root were 7.72, 9.10, 6.64 and 7.05 mg/g dry wt., respectively. **Conclusion :** Methyl jasmonate and yeast extract can enhance both total chromene and isoflavonoid accumulation in *P. candollei* var. *mirifica* hairy root culture. The comparative analysis of hairy root and native root of both varieties of *P. candollei* found that both native roots produced chromenes higher than hairy roots about 4-fold. In contrast total isoflavonoid content in both hairy roots were higher than native roots.

Keyword : *P. candollei* var. *candollei* (PC), *P. candollei* var. *mirifica* (PM), isoflavonoids and chromenes

Introduction

Pueraria candollei Wall. ex Benth. (White Kwao Krua) is a woody climber with tuberous root belonging to the family Leguminosae and commonly known as a Thai herbal medicine having been used for rejuvenation (Kerr, 1932). In Thailand there are two plants varieties, *P. candollei* var. *candollei* and *P. candollei* var. *mirifica* which have similar botanical characteristic but both vary in chemical component containing. Their tuberous root contain chemical compounds, known as phytoestrogen, such as major isoflavonoids and their glycosides (daidzin, daidzein, genistin, genistein and puerain), coumestans, pterocarpans and minor chromenes (miroestrol, deoxymiroestrol and isomiroestrol). Although the previously report found very low content of miroestrol and its derivatives in *P. candollei* var. *mirifica* but the estrogenic activity of miroestrol and its derivatives are much more potent than that of other isoflavones (Matsumura *et al.*, 2005). Recently, Yusakul and co-workers have been reported comparative analysis of phytoestrogen in both varieties (Yusakul *et al.*, 2011). Many studies claim the pharmacological activities of White Kwao Krua, including estrogenic activity (Chansakaow *et al.*, 2000), reduction of postmenopausal symptom (Taylor, 2003) , prevention of bone loss (Urasopan *et al.*, 2008), antioxidant activity (Cherdshewasart and Sutjit, 2008) and antihyperglycemic activity (Khitkal *et al.*, 2009).

To date, marketed various products from White Kwao Krua have been highly consumed, such as cream, serum, spay, soap, cookies, gel and capsule. The variations of active compounds in tuberous roots of *P. candollei* have been reported due to cultivation and the harvest season (Cherdshewasart et al, 2007). For enough market demand, it's necessity getting White Kwao Krua materials in short time. Then the application of plant tissue culture technique is most useful in case of scaling up and increasing secondary metabolites production in *P. candollei* cultivation. Hairy root culture is alternative method to produce isoflavonoids and chromenes because characteristic of hairy root is fast growth and highly lateral branching. Previously, Udomsuk and co-workers (Udomsuk *et al.*, 2009) reported total isoflavonoid content in *P. candollei* var. *candollei* hairy root culture higher than that

found in native root. Elicitation is one of extensively techniques to enhance secondary metabolites production in plants tissue culture. According to no appropriate determination method of chromene compound until Yusakul and co-workers (Yusakul *et al.*, 2011) have published HPLC method, therefore only studies on abiotic and biotic elicitors enhancing isoflavonoid production in both varieties of *P. candollei* culture have been reported (Khitkal *et al.*, 2009; Udomsuk *et al.*, 2009; Boonsnongcheep *et al.*, 2010; Korsangruang *et al.*, 2010; Udomsuk et al, 2011).

In the present study, we established a hairy root culture of *P. candollei* var. *mirifica* and optimal conditions for growth, and investigated the effects of plants elicitors (methyl jasmonate and yeast extract) on enhancing isoflavonoid and chromene accumulation. We also comparative studied on isoflavonoid and chromene production between hairy root and native root in both varieties of *P. candollei*.

Materials and methods

Chemicals reagent

Daidzin was purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Puerarin was obtained from ChromaDex, Inc. (CA, USA). 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). Miroestrol and deoxymiroestrol were isolated from the tuberous roots of *P. candollei* var. *mirifica* as described previously (Chansakaow *et al.*, 2000). NMR analysis was performed to compare the isolated compounds with the authentic standards of miroestrol and deoxymiroestrol and was provided by Dr. C. Chaichantipyuth, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. All other chemicals were standard commercial products of analytical grade.

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 16 h light/day. The embryos were observed within 7 days after cultivation and used for hairy roots induction.

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/ml 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 half-strength MS medium containing 400, 300, 200, 100 mg/ml 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.

Elicitors preparation and treatment

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. 21 days transformed hairy roots of *P. candollei* var. *mirifica* in ½ MS liquid medium were added 200 µM methyl jasmonate or 0.5 mg/L yeast extract. 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.

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 isoflavonoid content.

Extraction of samples and chromene analysis

1 g dried powdered samples 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 (Yusakul *et al.*, 2011). 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.

Extraction of samples and isoflavonoid analysis

30 mg dried powdered samples 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 using an indirect competitive ELISA using anti-puerarin and anti-daidzin polyclonal antibodies (PAbs) as described previously (Pongkitwitoon *et al.*, 2010) with some modifications. 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% H_2O_2 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).

Statistical analysis

The results were expressed as the mean ± 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 $P < 0.05$ level.

Results and discussion

Growth rate and total isoflavonoid accumulation from *P. candollei* var. *mirifica* hairy root culture

Fast growth and lateral branching characteristic of *P. candollei* var. *mirifica* hairy root was observed. The growth pattern and total isoflavonoid production of hairy root were shown in figure 1. The chromenes accumulation cannot be observed due to the fact that the quantity of hairy root materials was not enough for detection. The highest total isoflavonoid and biomass production was observed within 35 days and 28 days, respectively. From figure 1, 21 days hairy root ages could produce the highest total isoflavonoid and also give the highest biomass production. Adding elicitors at 21 days during hairy root culture was optimized that corresponded to the late log phase of hairy root growth.

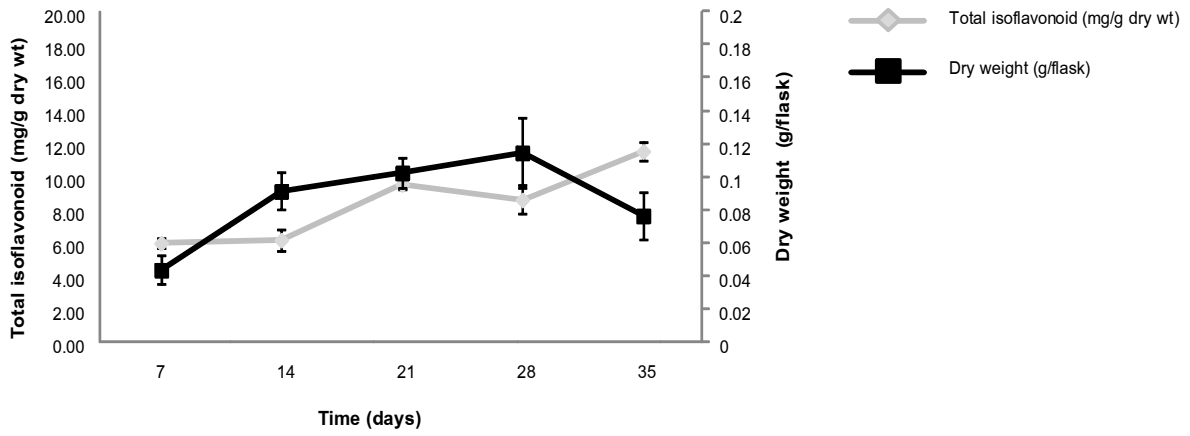

Figure 1 Growth rate and total isoflavonoid accumulation in *P. candollei* var. *mirifica* hairy root culture. Values are expressed as mean ± SD.

The effect of elicitors on growth of *P. candollei* var. *mirifica* hairy root culture

There was no significant difference in biomass production between the control and all the treatment groups indicate that elicitors were not effect to the growth of hairy root during elicitation. Base on previous data from Udomsuk and co-workers (Udomsuk et al, 2011) studying the effect of elicitors in *P. candollei* var. *candollei* hairy root culture, the best type and concentration of elicitors that elicited the highest total isoflavonoid were chosen. Then 200 µM methyl jasmonate and 0.5 mg/L yeast extract were used as elicitors in this experiment.

The effect of elicitors on chromene accumulation

Miroestrol and deoxymiroestrol are minor chromene compounds, which be found in *P. candollei* var. *mirifica* but act as highly estrogenic activity (Matsumura *et al.*, 2005). But only deoxymiroestrol, the highest estrogenic activity (Matsumura *et al.*, 2005), can be found in our experiment. Due to hypothesis of chromene biosynthesis pathway, prenyltransferases is a key enzyme in prenylation reaction converting to chromenes. According to studies reported on prenyltransferases activity which induced by methyl jasmonate and yeast extract (Sasaki *et al.*, 2008; Akashi *et al.*, 2009). The results in figure 2A found 200 µM methyl jasmonate and 0.5 mg/L yeast extract significantly increased chromene production after 3 days and 6 days of elicitation, respectively (17.03 and 17.47 µg/g dry wt., 2–fold higher than control, respectively). This data suggest that chromenes biosynthesis possible to relate with prenyltransferase enzyme.

The effect of elicitors on total isoflavonoid accumulation

Methyl jasmonate and yeast extract are widely used as elicitors, induced isoflavonoid in *P. candollei* (Korsangruang *et al.*, 2010; Udomsuk et al, 2011). The results from figure 2B demonstrate that 200 µM methyl jasmonate and 0.5 mg/L yeast extract highly significantly increased total isoflavonoid production after 3 days of elicitation (16.06 and 14.65 mg/g dry wt., 2–fold higher than control, respectively). Whereas study of Udomsuk and co-worker (Udomsuk et all, 2011) in *P. candollei* var. *candollei* hairy root culture found 200 µM methyl jasmonate and 0.5 mg/L yeast extract highly significantly increased total isoflavonoid production after 6 days and 3 days of elicitation, respectively. The variations of isoflavonoid production and treatment interval of elicitation may be due to variation of both *P. candollei* varieties (Korsangruang *et al.*, 2010).

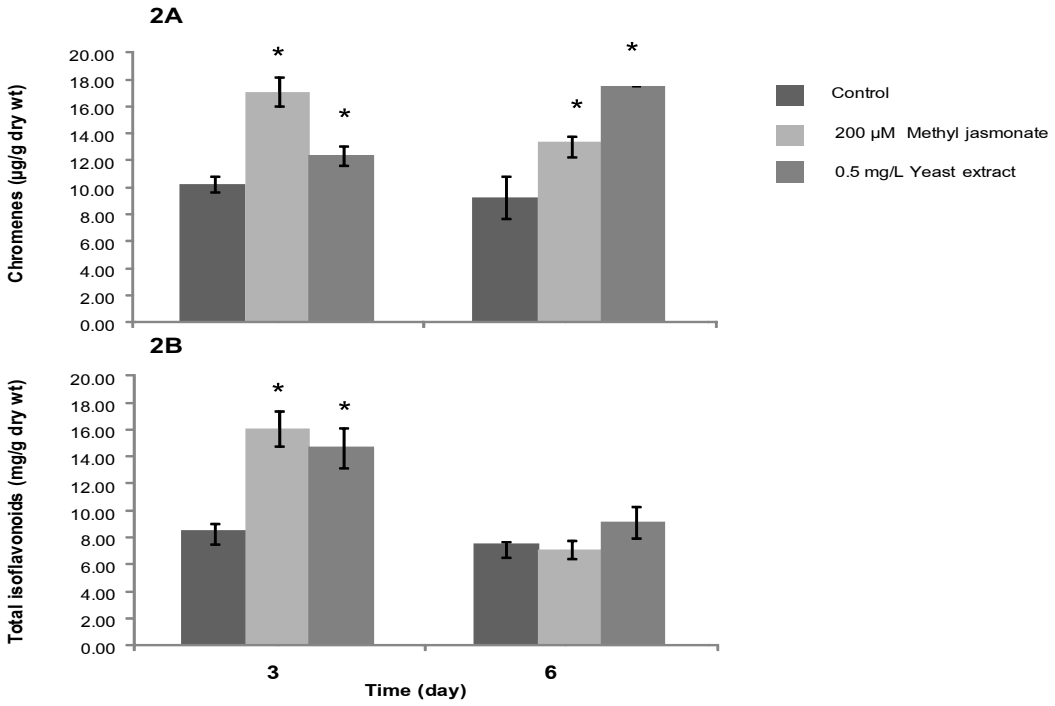

Figure 2 Effect of 200 µM methyl jasmonate and 0.5 mg/L yeast extract on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture. Values are expressed as mean ± SD.

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

Concerning the hypothesis of chromene biosynthesis pathway, it is possible that the chromene skeleton can be derived from enzymatic prenylation reaction of isoflavane and dimethylallyl pyrophosphate (DMAPP) (Yu and McGonigle, 2005; Dewick, 2009). The prenylating enzymes are generally localized to plastid, where the reaction was occurred. Therefore, chromene biosynthesis may take place in chloroplast cell. From (figure 3.) the comparative analysis of each phytoestrogen between hairy root and native root found that chromene content in PM hairy root, PC hairy root, PM root and PC root were 5.51, 4.62, 18.86 and 17.10 µg/g dry wt., respectively. Total isoflavonoid content in PM hairy root, PC hairy root, PM root and PC root were 7.72, 9.10, 6.64 and 7.05 mg/g dry wt., respectively. Because of higher chromenes accumulation in both native roots than both hairy roots about 4-fold, it is possible that its biosynthesis may take place in plastid of leaves part then being accumulated to root part.

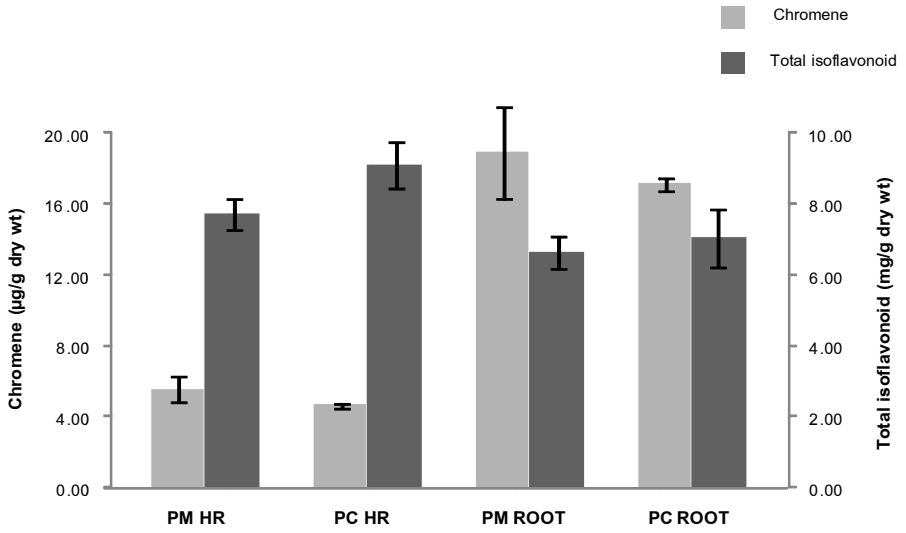

Figure 3 Comparative analysis of chromene and total isoflavonoid content in hairy root and native root PM HR; *P. candollei* var. *mirifica* hairy root, PC HR; *P. candollei* var. *candollei* hairy root, PM ROOT; *P. candollei* var. *mirifica* native root, PC ROOT; *P. candollei* var. *candollei* native root

Conclusion

From our results methyl jasmonate and yeast extract can enhance both total isoflavonoid and chromene accumulation in *P. candollei* var. *mirifica* hairy root culture. For enough White Kwao Krua materials market demand, the application of both elicitors in *P. candollei* var. *mirifica* hairy root culture may useful in case of scaling up and increasing second metabolite production during cultivation. The comparative analysis of hairy root and native root in both varieties of *P. candollei* found that both native roots produced chromenes higher than hairy roots about 4-fold, it is possible that chromene biosynthesis may take place in plastid of leaves part then being accumulated to root part. In contrast total isoflavonoid content in both hairy roots were higher than native roots.

Acknowledgments

This work was supported by a grant from Khon Kaen University (542101) and the Graduated school Khon Kaen University. We also thank Dr. Chaiyo Chaichantipyuth, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, for providing purified miroestrol and deoxymiroestrol.

References

Kerr A. A reputed rejuvenator. *J Siam Soc.* (Natural History Suppl.). 1932; 8: 336-338.

Matsumura A, Ghosh A, Pope G.S, Darbre P.D. Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *J. Steroid Biochem. & Mol. Biol.* 2005; 94: 431–443.

Yusakul G, Putalun W, Udomsin O, Juengwatanatrakul T, Chaichantipyuth C. Comparative analysis of the chemical constituents of two varieties of *Pueraria candollei*. *Fitoterapia*. 2011; 82(2): 203-207.

Chansakaow S, Ishikawa T, Sekine K, Okeda M, Higuchi Y, Kudo M and Chaichantipyuth. Isoflavonoids from *Pueraria mirifica* and their estrogenic activity. *Planta Med.* 2000; 66: 572-575.

Taylor M. Alternative to HRT: An evidence-based review. *Int J Fertil Menopausal Stud*. 2003; 48(2):64-68.

Urasopan N, Hamada Y, Cherdshewasart W, Malaivijitnond S. Preventive effects of *Pueraria mirifica* on bone loss in ovariectomized rats. *Maturitas.* 2008; 59(2): 137-148.

Cherdshewasart W and Sutjit W. Correlation of antioxidant activity and major isoflavonoid contents of the phytoestrogen-rich *Pueraria mirifica* and *Pueraria lobata* tubers. *Phytomedicine.* 2008; 15(1-2): 38-43.

Khitkal B, Kupittayanant S, Rangsriwatananon K, Manakasema Y. Antioxidant properties of puerarin and genistein from White Kwao Krua induced by elicitors and their antihyperglycemic effect on rats. *Suranaree J. Sci. Technol*. 2009; 17(1):27-37.

Cherdshewasart W, Subtang S and Dahlan W. Major isoflavonoid contents of the phytoestrogen rich-herb *Pueraria mirifica* in comparison with *Pueraria lobata*. *J Pharm Biomed Anal*. 2007; 43(2):428-434.

Udomsuk L, Jarukamjorn K, Tanaka H and Putalun W. Isoflavonoid production in hairy roots culture of *Pueraria candollei*. *Zeitschrift fur Naturforschung Section C-A Journal of Biosciences*. 2009; 64(9-10): 687-691.

Boonsnongcheep P, Korsangruang S, Soonthornchareonnon N, Chintapakorn Y, Saralamp P, Prathanturarug S. Growth and isoflavonoid accumulation of *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* cell suspension cultures. *Plant Cell Tiss Organ Cult.* 2010; 101:119–126.

Korsangruang S, Soonthornchareonnon N, Chintapakorn Y, Saralamp P, Prathanturarung S. Effects of abiotic and biotic elicitors on growth and isoflavonoid accumulation in *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* cell suspension culture. *Plant Cell Tiss Organ Cult.* 2010; 103: 333–342.

Udomsuk L, Jarukamjorn K, Tanaka H, Putalun W. Improved isoflavonoid production in *Pueraria candollei* hairy root cultures using elicitation. *Biotechnology Letters*. 2011; 33(2): 369-374.

Chansakaow S, Ishikawa T, Sekine K, Okeda M, Higuchi Y and Chaichantipyuth. Identification of deoxymiroestrol as the actual rejuvenating principle of "kwao keur", *Pueraria mirifica*. The known miroestrol may be an artifact. *J. Nat Prod*. 2000; 63(2): 173-175.

Pongkitwitoon B, Sakamoto S, Tanaka H, Tsuchihashi R, Kinjo J, Morimoto S and Putalun W. Enzyme-linked immunosorbent assay for total isoflavonoids in *Pueraria candollei* using anti-puerarin and anti-daidzin polyclonal antibodies. *Planta Medica.* 2010; 76: 831-836.

Sasaki K, Mito K, Ohara K, Yamamoto H, Yazaki K. Cloning and characterization of naringenin 8-prenyltransferase, a flavonoid-specific prenyltransferase of *Sophora flavescens*. *Plant Physiol*. 2008; 146: 1075-1084.

Akashi T, Sasaki K, Aoki T, Ayabe S, Yazaki K. Molecular cloning and characterization of a cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the key prenylation step in the biosynthesis of glyceollin, a soybean phytoalexin. *Plant Physiol*. 2009; 149: 683-693.

Yu O and McGonigle B. Metabolic engineering of isoflavone biosynthesis. *Adv Agr*. 2005; 86:147–190

Dewick PM. Medicinal natural products: a biosynthetic approach. UK: Wiley; 2009.