สารที่ออกฤทธิ์คล้ายเอสโตรเจนของกวาวเครือขาวบางสายพันธุ์ และเซลล์เพาะเลี้ยงเนื้อเยื่อ

นายบุญส่ง หวังสินทวีกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวท
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546

ISBN 974-17-5473-6

ESTROGENIC-LIKE COMPOUNDS FROM SOME *PUERARIA CANDOLLEI* VARIETIES AND CELL CULTURE

Mr.Boonsong Wungsintaweekul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy in Pharmacognosy

Department of Pharmacognosy
Faculty of Pharmaceutical Sciences
Chulalongkorn University

Academic Year 2003
ISBN 974-17-5473-6

Thesis Title	ESTROGENIC-LIKE COMPOUNDS FROM SOME *PUERARIA CANDOLLEI* VARIETIES AND CELL CULTURE
By	Mr.Boonsong Wungsintaweekul
Field of Study	Pharmacognosy
Thesis Advisor	Associate Professor Wanchai De-Eknamkul, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of the Faculty of Pharmaceutical Sciences
(Associate Professor Boonyong Tantisira, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Sumphan Wongseripipatana, Ph.D.)

……..…… Thesis Advisor
(Associate Professor Wanchai De-Eknamkul, Ph.D.)

…….. Member
(Associate Professor Ekarin Saifah, Ph.D.)

…….. Member
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

…….. Member
(Associate Professor Chaiyo Chaichantipyuth)

บุญส่ง หวังสินทวีกุล: สารที่ออกฤทธิ์คล้ายเอสโตรเจนของกวาวเครือขาวบางสาย พันธุ์และเซลล์เพาะเลี้ยงเนื้อเยื่อ. (ESTROGENIC-LIKE COMPOUNDS FROM SOME *PUERARIA CANDOLLEI* VARIETIES AND CELL CULTURE) อาจารย์ที่ปรึกษา : รศ. ดร.วันชัย ดีเอกนามกูล, 78 หน้า. ISBN : 974-17-5473-6

รากของกวาวเครือขาว 5 สายพันธุ์จากมหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน และเซลล์เพาะเลี้ยงของกวาวเครือขาวที่เตรียมจากหัวใต้ดินของกวาวเครือขาวที่เก็บมาจากจังหวัดอุดรธานี ได้ถูกนำมาทำศึกษาเกี่ยวกับสารที่ออกฤทธิ์คล้ายฮอร์โมนเอสโตรเจน โดยใช้เซลล์มะเร็งที่ไวต่อเอสโตรเจน (MCF-7 cells) การเตรียมเซลล์เพาะเลี้ยงของกวาวเครือขาว ได้ทำการเพาะเลี้ยงโดยใช้ MS medium ที่มีฮอร์โมนพืชคือ 2,4-D, BA และ NAA (1: 0.1: 0.1) ซึ่งพบว่าสารสกัดจากเซลล์เพาะเลี้ยงดังกล่าวมีฤทธิ์เอสโตรเจน จากการศึกษาทางเคมีพบว่า สารสกัดหยาบของตัวอย่างบางสายพันธุ์มีรูปแบบที่คล้ายกัน จะมีแตกต่างกันบางส่วน ทั้งชนิดของสารและปริมาณ ซึ่งเป็นประโยชน์ในการควบคุมคุณภาพต่อไป ส่วนเซลล์เพาะเลี้ยงพบว่ามีศักยภาพในการสร้างสารทุติยภูมิคือ Daidzein และ Genistein โดยเฉพาะเซลล์แขวนลอย พบว่ามีการสร้างสารทุติยภูมิมากกว่า แคลลัส Boronate Affinity Chromatography ได้ถูกนำมาประยุกต์ใช้สำหรับการแยกสารที่ออกฤทธิ์คล้ายฮอร์โมนเอสโตรเจนจากสารสกัดหยาบของกวาวเครือขาว โดยใช้ MCF-7 cells ในการติดตามฤทธิ์ที่คล้ายกับฮอร์โมนเอสโตรเจนของสารที่จับและไม่จับกับตัวดูดซับ พบว่าสารที่มี R_f value 0.4 ในการตรวจด้วยรงคเลขผิวบาง สามารถจับกับตัวดูดซับในสภาวะด่าง และถูกชะออกมาด้วยการปรับสภาวะให้เป็นกรด ซึ่งสารดังกล่าวถูกพบได้ในทุกตัวอย่างของกวาวเครือขาวที่นำมาศึกษา และในเซลล์เพาะเลี้ยง

ภาควิชา เภสัชเวท ลายมือชื่อนิสิต.....
สาขาวิชา เภสัชเวท ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2546

4476583633: MAJOR PHARMACOGNOSY
KEY WORD: *Pueraria candollei* / Cell culture / Estrogenic-like activity / Isoflavonoids / Boronate Affinity Chromatography

Boonsong Wungsintaweekul: Estrogenic-Like Compounds from Some Pueraria candollei varieties and Cell Culture. Thesis Advisor : Assoc.Prof.Wanchai De-Eknamkul, Ph.D., 78 pp. ISBN 974-17-5473-6

The tuberous roots of five varieties of *Pueraria candollei* were collected from Kasetsart University, Kampangsaen Campus and cell cultures that were established from the tuberous root tissue of *Pueraria candollei* collected from Udon Thani province were studied for their estrogenic-like activity by using of estrogen dependent cancer cells (MCF-7 cells). The cell cultures were established on MS medium with 2,4-D, BA and NAA (1: 0.1: 0.1). The crude extracts of some varieties of *Pueraria candollei* showed similar chemical patterns whereas some showed different pattern. The cell cultures appeared to produce two major isoflavonoids, daidzein and genistein. Boronate affinity chromatography was developed for separation of active ingredients from the ethyl acetate crude extracts and methanol crude extracts of some varieties and cell cultures of *Pueraria candollei*. MCF-7 cells were used for detection of estrogenic activity of unbound and bound fraction. The compound that has R_f value 0.4 on TLC plate could bind to the boronate resin (adsorbents) in alkaline solution and eluted by decreasing pH with acid. This compound can found in all samples

Department Pharmacognosy Student's signature……..
Field of study Pharmacognosy Advisor's signature……..
Academic year 2003

ACKNOWLEDGEMENTS

The author wishes to express his grateful appreciations to his thesis advisor, Associated Professor Dr.Wanchai De-Eknamkul of the Research Unit for Natural Product Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for his helpful guidance, suggestion, continue encouragement and kindness throughout the research studies.

The author would like to acknowledge his sincere thanks to Dr. Kaoru Umehara of the School of Pharmaceutical Sciences, University of Shizuoka, Japan for his helpful in estrogenic activity assay.

The author would like to acknowledge his sincere thanks to Mr. Sompoj Tubchareon of the Suwanwajogasikit Institute, Kasetsart University, Kampangsaen Campus, Nakhon Pathom for his helpful, his kindness to give specimens of *Pueraria candollei* in this study and the information about this medicinal plant.

The author would like to acknowledge his sincere thanks to Miss Nithima Sutipanta of Department of Pharmacognosy, Ubonrajathani University for her LC-MS data.

The author would like to thanks to Chulalongkorn-Chiba University Student Exchange program for supporting to visit and join the research work at Department of Medical Organic Chemistry, Chiba University, Japan. The author would also like to thank to Professor Dr. Tsutomu Ishikawa, Associate Professor Dr. Toshiko Watanabe and Dr. Takuya Kumamoto for their suggestions, encouragement throughout the research study in Chiba University, Japan

The author would like to acknowledge his sincere thanks to Graduate School of Chulalongkorn University for giving partial grant.

The author would also like to acknowledge his sincere thanks to his sister Dr. Juraithip Wungsintaweekul of Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University for her helpful suggestion and encouragement.

The author would like to thanks members of Research Unit for Natural Product Biochemistry, Department of Pharmacognosy and their friendships, kindness and love.

Finally, the author would like to sincere thanks to his father, mother, brothers and sisters for their love, encouragement and everything.

CONTENTS

Page

Page

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

ABBREVIATIONS

% = Percent (part per 100); percentage

/ = per

λ_{max} = maximum adsorption wave length

µg = microgram

2,4-D = 2,4-dichlorophenoxyacetic acid

4CL = 4-coumarate CoA ligase

ATP = adenosine triphosphate

AMP = adenosine monophosphate

BA = N_6-Benzyladenine

C-17 = carbon at 17^{th} position

C-18 = carbon at18^{th} position

C4H = cinnamate-4-hydroxylase

CHI = chalcone-flavanone isomerase

CHS = chalcone synthase

CHR = chalcone reductase

CoA = coenzyme A

cAMP = cyclic AMP (adenosine 3’’:5’’-monophosphate),

cm = centimeter(s)

DOPA = 3,4-dihydroxyphenylalanine

etc.= et cetera

et al = et alii

EtOAc = ethyl acetate

hr = hour

pH = the negative logarithm of the concentration of hydrogen ions

HCl = Hydrochloric acid

H_2O = Water

HPLC = high performance liquid chromatography

HCOOH = Acetic acid

IFS = isoflavone synthase

l = Liter (s)

kg = kilogram (s)

min = minute (s)

ml = milliter

m = meter(s)

mm = millimeter(s)

MCF = Michigan cancer foundation

M = molarity

M.W. = molecular weight

MeOH = methanol

MS = mass spectrometry/spectroscopy/spectrum

No. = Number

nm = nanometer

NAA = Naphthaleneacetic acid

NH_4OAc = Ammonium acetate

ng = nanogram

NMR = nuclear magnetic resonance

PAL = phenylalanine ammonia lyase

RT = Retention time

RP = Reverse phase

sp. = species

SD = Standard deviation

TAL = tyrosine ammonia-lyase

TLC = Thin layer chromatography

UV = Ultraviolet

w/w = weight/ weight (concentration)

CHAPTER I

INTRODUCTION

Phytoestrogens have been found in many kinds of medicinal plants, for example Red Clover (*Trifolium pratens* L.), Soybean (*Glycine max* (L.) Merr.), *etc*. These plants contain constituents that have bioactivity like estrogen in women. The physiological effect of phytoestrogen has been suggested to depend on its concentration (Hsieh *et al*., 1998). These compounds can compete with estrogen in binding with estrogenic receptor. Because of these properties, phytoestrogens are used as dietary supplement in hormone replacement therapy in menopause women and used as active ingredient in cosmetic. Miroestrol from *Pueraria candollei* var. *mirifica* is a potent phytoestrogen. It can bind with estrogenic receptor in breast and made enhancement of breast like estrone in third trimester of labor (วันชัย ดีเอกนามกูล และ ชาลี ทองเรือง, 2544). Because of this effect, miroestrol has been used in many kind of cosmetic preparation.

Pueraria candollei var.*mirifica* or Kwao Kruae Kao is a rejuvenating herb in Thai folk medicine. However, miroestrol might be artifact that occurred during separation and purification process whereas deoxymiroestrol has been found to be the true potent phytoestrogen in this medicinal plant (Chansakaow, 2000). In nature, this plant has been found many varieties. Chemical constituents of each variety can be different both qualitatively and quantitatively. In the past, miroestrol was separated from *P. candollei* var.*mirifica* or *P. mirifica*, but in only small amount. Chemical synthesis has been used for making miroestrol in laboratory (Corey and Wu, 1993). However, the chemical steps are long and complicated

Study of natural product biosynthesis can lead to yield of active compound production in plants by biotechnology (Rao *et al*., 2002). Tissue culture techniques have been used for prepared primary cells of plants as callus and cell suspension cultures. The potential of cell cultures to produce the secondary metabolites have been well established

Recently, herbal medicine has been very popular and a number of plants have been used as phytoestrogens in native medicine. Norman *et al*., 1975 suggested the increased interest in phytoestrogens was due to at least four factors:

1) The recognition that infertility in animal and humans could follow excessive ingestion of plants rich in compounds possessing estrogenic activity.
2) The known existence of “spring flush” (increased yield of improved milk) in dairy cattle ingesting certain rapidly growing grasses that contains estrogenic substance.
3) The possibility that the demonstrated improvement in carcass quality, produced by the feeding of synthetic estrogens, might also be able to be produced by the feeding of plants rich in estrogens.
4) The possibility of obtaining estrogenic substances economically form plant sources.

Now the herbal drug or native medicine was increased interest. The quality control of these herbal medicines is necessary. Because of active compound and their concentration can vary and depend on the processing conditions (Song *et al*., 1998). The analyses of active ingredients such as isoflavonoids in herbal products, soy product were used HPLC, HPLC coupling to mass spectrometer (LC-MS), GC-MS. The concentration of isoflavones also analyzed with these methods (Setchell *et al*., 2001)

Boronate affinity chromatography is one kind of affinity chromatography that used the concept of specific binding between target compound and ligand. Ligand of this technique is containing boronic acid group in the spacer arm. Boronic acid ligand can bind to *cis*-diol in the biomolecules. It has been used for *cis*-diol contains separation. Miroestrol and its derivatives are containing hydroxyl group at position C-17 and C-18. The rigidity of the structure made these hydroxyl groups look like *cis*-diol. Because of these properties, boronic acid group in alkaline condition could bind to miroestrol and its derivatives. Boronate affinity chromatography was applied to separate miroestrol derivatives.

This research works aim to

-Prepare cell cultures from the tuberous root tissue of *Pueraria candollei*

-Study of secondary metabolite production of *Pueraria candollei* cell cultures.

-Determine the estrogenic activity of crude extracts of some varieties and cell cultures of *Pueraria candollei* by MCF-7 cells

-Apply boronate affinity chromatography to separate the active ingredient of this medicinal plant.

CHAPTER II

HISTORICAL

1) *Pueraria* sp.

Pueraria is a genus of sub-family Papilionoideae, Leguminosae family. The botanical aspects of this genus are twining shrubs or herbs, with stellate 3-foliolate leaves; leaflets sometimes palmatifid. Flowers large or small, densely fascicled, in lone often compound racemes. Calyx-teeth long or short, the two upper connate. Corolla distinctly exserted; standard usually spurred at the base, equaling in length the obtuse wings and keel. Stamens more or less thoroughly monadelphous; anthers uniform. Ovary sessile or nearly so, many-ovuled; style filiform, much incurved beardless, stigma capitate. Pod linear, flattish (Hooker *et al.*, 1879)

There are seventeen species of this genus from tropical Asia to Polynesia (Niyomdham, 1992). According to Smitinand, 2001, *Pueraria* sp. that have been found in Thailand are

-*P. alopecuroides* Criab: Phit khruea (ผีดเครือ) (Chiang Mai)

-*P. candollei* Wall. ex Benth. var.*candollei*: Khruea khao pu (เครือเขาปู้), Talan khruea (ตาลานเครือ) (Lampang)

-*P. candollei* Wall. ex Benth var.*mirifica* (Airy Shaw & Suvat.) Niyomdham: Kwao khruea (กวาวเครือ) (Northern)

-*P. phaseoloides* (Roxb) Benth: Thua sian pa (ถั่วเลี้ยนป่า) (Ang Thong), Pak pit (ผักผีด) (Northeastern)

-*P. stricta* Kurz: Ka sam pik (กาสามปีก), Mapaep pa (มะแปปป่า) (Northern)

-*P. thomsonii* Benth: Khruea i thao (เครืออีเต่า) (Uthaai Thani), Phak phit (ผักผีด) (Chiang Mai)

-*P. wallichii* DC: Ma pap wo (มะแปปวอ) (Northern)

2) History of *Pueraria candollei* Wall.ex Benth. var.*mirifica* (Airy Shaw & Suvat.) Niyomdham

Pueraria candollei var.*mirifica* or *Pueraria mirifica* is well known as Kwao Khure Kao in Thailand. This medicinal plant is a member of Papilionoideae, Leguminosae. It has been use in Thai remedy in Thai folk medicine for a long time as rejuvenating herb. The compounds that have been separated from this plant are mainly activity-containing phytoestrogens. The compounds found in this plant are daidzein, genistein, puerarin, *etc*. The most estrogenically active compounds found in this plant are miroestrol, deoxymiroestrol and isomiroestrol (Chansakaow *et al*., 2000). Miroestrol was first isolated from the tuberous roots of *Pueraria candollei* var. *mirifica* (Cain, 1960) (Jones and Pope, 1961). It has been shown to be a potent estrogenic activity by systemic and oral administration, and its effects on the reproductive tract of the immature female rat (Jones *et al*., 1961). The compound has been compared with the other potent estrogens (Jones and Pope, 1960; Jones and Pope, 1961). The estrogenic activity of *P. candollei* var.*mirifica* has been studied with recombinant yeast, MCF-7 cell proliferation assay (Lee *et al*., 2002). In recombinant yeast assay, the concentration of *P. candollei* var.*mirifica* (0.025, 0.25, 2.5, 25, 2.5 X 10^2, 2.5 X 10^3, 2.5 X 10^4 ng/ ml) have shown no estrogenic activity, while 10^{-9} of 17β-estradiol (Positive control) showed high estrogenic activity. In addition, the estrogenic activities have been shown at 2.5 ng/ ml to 25 µg/ ml concentrations of *P. candollei* var.*mirifica* in a dose dependent manner on MCF-7 cell. The estrogenic effect of *P. candollei* var.*mirifica* has been shown to be blocked by tamoxifen treatment, a well known anti-estrogen (Lee *et al*., 2002). *P. candollei* var.*mirifica* may have no estrogenicity in the yeast system, but has the activity in MCF-7 cells that have human metabolic enzyme. It has, therefore, been concluded that *P. candollei* var.*mirifica* might require metabolic activation for estrogenic activity (Lee *et al*., 2002).

3) Botanical aspect of *P. candollei* Wall.ex Benth. var.*mirifica* (Airy Shaw & Suvat.) Niyomdham

P. candollei Wall.ex Benth. var.*mirifica* (Airy Shaw & Suvat.) Niyomdham is a member of sub-family Papilionoideae, Leguminosae family. This medicinal plant was first described as *P.mirifica* by Kashemsanta *et al*., 1955. The descriptions of this medicinal plant are

P. mirifica Airy shaw et Suvatabandhu, sp. Nov., *P.candollei* Grah. Affinis, foliolis subtus cinereo-tomentellis, inflorescentiis brevioribus, floribus subduplo minoribus, leguminibus minoribus pubescentibus distincta

Frutex scandens, usque 5 m. altus vel ultra; caulis glaber, striatus, 4-7 mm.diametro; radices tuberosae magnae, globosae vel ellipsoideae, usque 15 cm. Longae et 6 cm. Crassae. *Folia* trifoliolata, tota usque 60 cm. Longa. Petiolus gracilis, 25-28 cm. Longus, 1-2 mm. Crassus, supra canaliculatus, minute adpresse puberulus, pulvina basali 8-15 mm. Longa usque 4 mm. Crassa rugulosa dense puberula. *Rhachis* (interfoliola lateralia et foliolum terminale) 3.5-5.5 cm. Longa, 1 mm. Crassa. *Stipellae* subulatae, 4-5 mm. Longae, vix 1 mm. Latae, acutissimae, striatae, adpresse fulvo-pubescentes. *Petioluli* 6-7 mm. Longi, 1.5-2 mm. crassi, adpresse fulvo-pubescentes. *Foliola lateralia* oblique, latere inferiore (postico) late ovata, late ovata, latere superiore (antico) oblongo-elliptica, 15-18 (-20-25) cm. longa, 12-15 cm. lata, basi altero latere late truncato-rotundata altero latere late cuneato-rotundata, apice brevissime late acuminata acuta; *foliolum terminale* rhomboïdeo-suborbiculare vel latissime obovatum vel interdum fere subhexagonum, 14-15 cm.diametro, basi late cuneata, apice latissime rotundato et brevissime late acuminato subacuto ; foliola omnia siccitate tenuiter chartacea et viridia, margine subsinuosa, supra minute dissite adpresse pubescentia, subtus glauca et insuper dense adpresse cinereo-tomentella ; costa et nervi gracillimi, utrinque prominuli ; nervi primarii 6-8 jugi, patuli, subrecti vel leviter procurvi, usque ad ipsum marginem attingentes. *Inflorescentiae* praecoces, racemosae vel paniculatae, fasciculatae, 20-30 cm. longae, angustissimae, multiflorae, rhachibus inferne subteretibus superne angulatis minute subvulvo-tomentellis (pilis deflexis). *Flores* parvi, 7-8 mm. longi, pedicello 1 mm. longo pubescente, bracteolis minutis ovatis adpressis vix 1 mm. longis. *Calyx* campanulatus, 4 mm. longus, 2-2.5 mm. latus, purpureo-brunneus, adpresse fulvo-pubescens, dente superiore late ovato rotunato, dentibus 3 inferioribus parvis deltoïdeis acutis. Corolla purpureo-caerulea : vexillum (explanatum) orbiculare, 7-8 mm. diametro, brevissime unguiculatum, dente parvo prope basin utroque latere auctum ; alae oblongae, levissime falcate, circiter 9 mm. longae et 2 mm. latae, obtusiusculae, circiter 1.5 mm. unguiculatae, lobulo acuto prope basin calcaratae ; carinae petala apice tantum cohaerentia, oblique apatulata, 8-9 mm. longa, 2.5-3 mm. lata, circiter 3 mm. unguiculata et dente brevissimo acuto aucta, apice oblique rotundato-truncata. *Stamina* 9 mm.longa. *Ovarium* cum stylo 9 mm. longum. *Legumen* (an omnino maturum?) parvum, oblongum, valde applanatum,

usque 3 cm. longum et 7 mm. latum, 1-4-spermum, ubisemina aborta varie constrictum, basi et apice breviter attenuatum, sutures anguste incrassates, undique breviter adpresse pubescens. *Semen* suborbiculare, applanatum, basi intrusum, 2.5 mm. diametro, leave, brunneo-coccineum

P. mirifica Airy Shaw et Suvatabandhu is closely related to *P. candollei* Wall.ex Benth. of Burma and Thailand, but differs clearly in the cinereous, grey-green, closely tomentellous lower surface of the leaflets, in the shorter inflorescences (though this may not be constant), and in the much smaller flowers and fruits, the latter being distinctly pubescent (Kashemsanta *et al*., 1955). *P. candollei* and *P. mirifica* are deciduous plants. Herbarium specimens of both species are very similar. They have many intermediate forms among them. Because of this data, *P. mirifica* was ranked into *P. candollei* var.*mirifica* (Niyomdham, 1992). Now, *P. mirifica* is one variety of *P. candollei* as *P. candollei* var.*mirifica* (Smitinand, 2001).

P. candollei var.*mirifica* can be found in Thailand and Burma. They grow on the deciduous forest and hill slopes. The flower of this medicinal plant has bloomed on February and March. We can find the fruits of on April (Lakshnakara *et al*., 1955).

(A)

(B)

(C)

Figures 1: The pictures of *P. candollei*; **(A)**: flowers and leaves, **(B)**: pods and **(C)**: tuberous roots. **Place**: Kasetsart University, Kampangsaen campus.

4) Phytochemical studies of *P. candollei* var.*mirifica*

Isoflavonoids have been isolated and characterized from the tuberous root extract of *P. candollei* var.*mirifica* and miroestrol has been reported as potent estrogenic principle (Cain, 1960). The pharmacological and chemical study of miroestrol have been determined and compared with natural estrogen (Bounds and Pope, 1960). The method of isolation had been simplified and recoveries of miroestrol have been obtained at 15 micrograms per kilograms of powder drug. Periodate is a reagent which very sensitive and specific color test with miroestrol. Miroestrol has been found to crystallize in two forms, a stable hydrated form (stout needles), which after desiccation quickly regains its water of crystallization when exposed to the moist air, and a non-hygroscopic, anhydrous form (thin rectangular plates). Its ultra-violet and infrared adsorption curves have also been a useful criteria of the purity of the various specimens isolated (Cain, 1960).

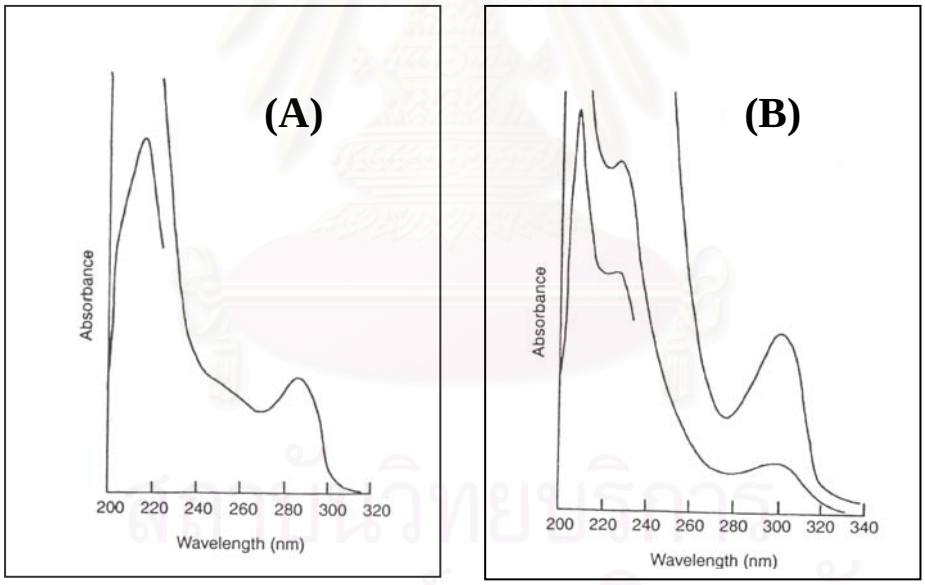


Figure 2: UV spectrum of miroestrol determined in methanol **(A)** and methanol plus aqueous sodium hydroxide **(B)**

5) Synthesis of miroestrol

Miroestrol is the most potent estrogenic compound in *P. candollei* var.*mirifica*. Corey and Wu, 1993 eventually succeeded in the chemical preparation of miroestrol as shown in Fig.3.

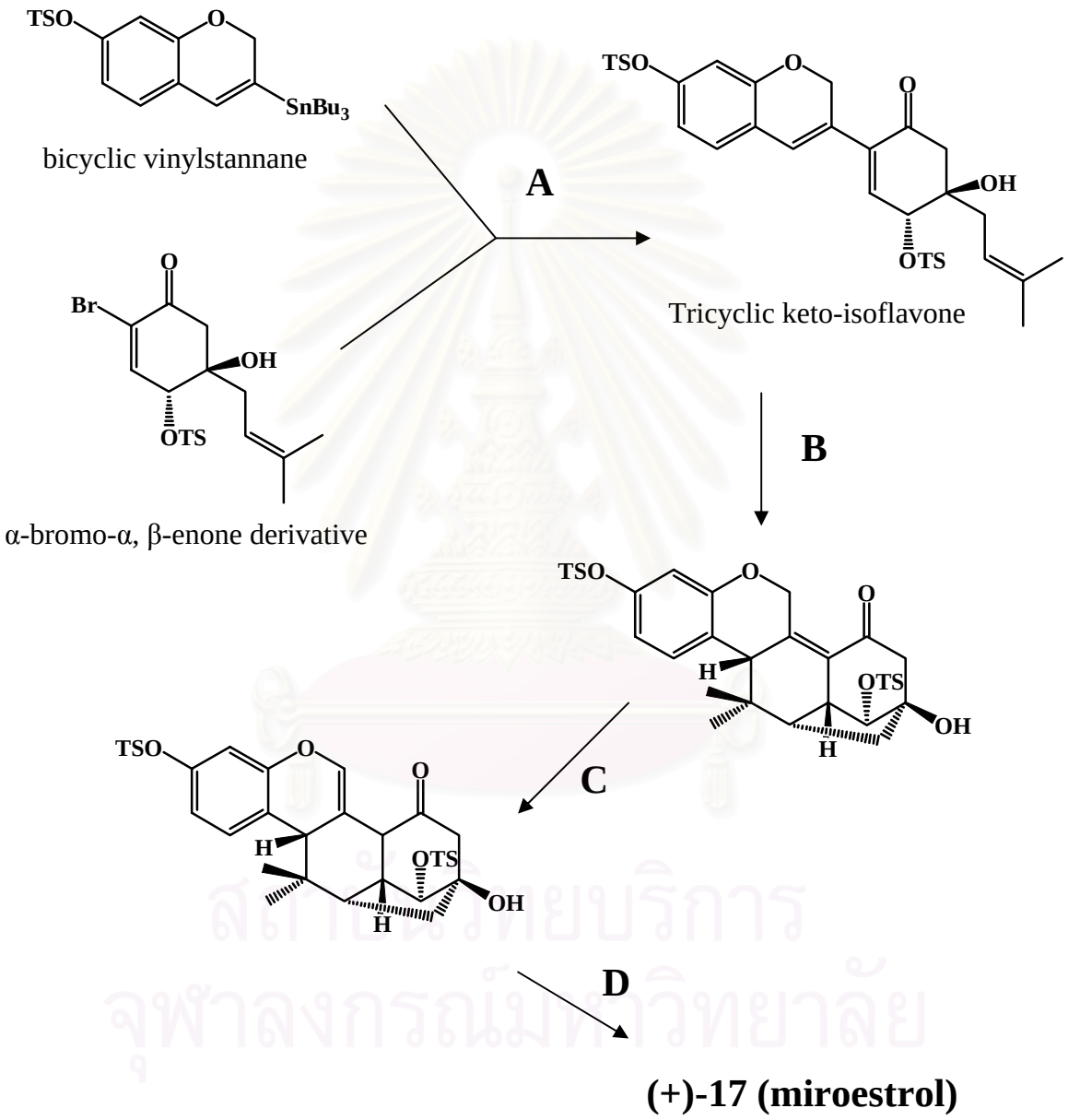


Figure 3: Synthetic route to miroestrol (Corey and Wu, 1993). TS = tri-isopropylsilyl protecting group; SnBu3 = tri-n-butylstannyl residue; A = coupling reaction, B = cyclo-isomerization, C = isomerization, D = oxidation/ desilylation

6) Constituents of *P. candollei* var. *mirifica*

The Isoflavonoids that found in *P. candollei* var. *mirifica* can be classified to 5 main groups: chromenes, Isoflavones, Isoflavone glycosides, coumestans and pterocarpans as summarized in Table 1 and Table 2.

Table 1: Natural isoflavonoids found in *P. candollei* var.*mirifica*

Group	Compounds
Chromenes	-Miroestrol -Deoxymiroestrol -Isomiroestrol
Isoflavones	-Daidzein -Genistein -Kwakhurin -Kwakhurin hydrate
Isoflavone glycosides	-Daidzin -Genistin -Mirificin -Puerarin -Puerarin-6”-monoacetate
Coumestans	-Coumestrol -Mirificoumestan -Mirificoumestan glycol -Mirificoumestan hydrate
Pterocarpans	-Tuberosin -Puemiricarpene

The other compounds that have been found in this plant are β-sitosterol, stigmasterol, alkane alcohol, fat and sugar (วันชัย ดีเอกนามกูล และ ชาลี ทองเรือง, 2544).

Table 2: Structures of isoflavonoids in *P. candollei* var.*mirifica*

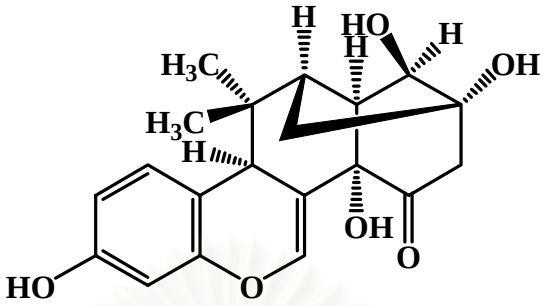
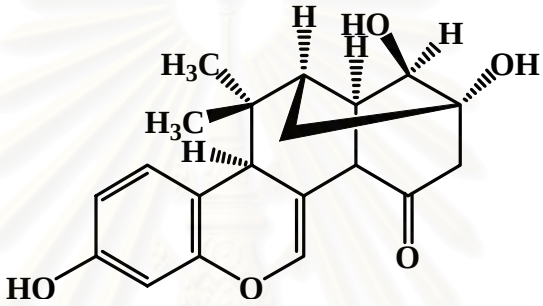
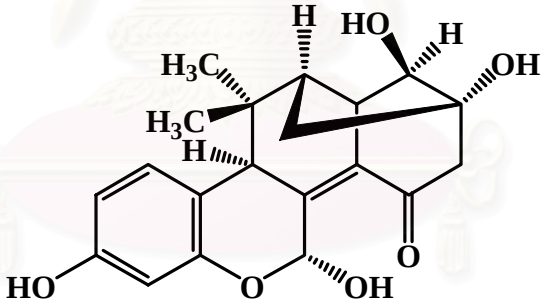
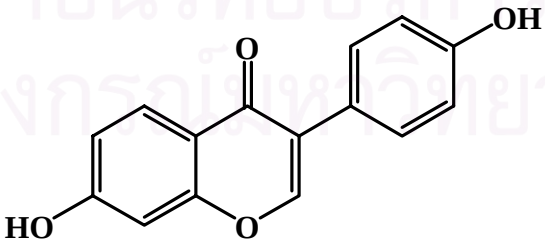
Compound	Structure	References
Miroestrol -$C_{20}H_{22}O_6$ -MW= 358.39		Cain, 1960
Deoxymiroestrol -$C_{20}H_{22}O_5$ -MW= 342.39		Chansakaow *et al*., 2000
Isomiroestrol -$C_{20}H_{22}O_6$ -MW=358.39		Chansakaow *et al*., 2000
Daidzein -$C_{15}H_{10}O_4$ -MW= 254.24		Ingram *et al*., 1986 Chansakaow *et al*., 2000

Table 2: Structures of isoflavonoids in *P. candollei* var.*mirifica* (continued)

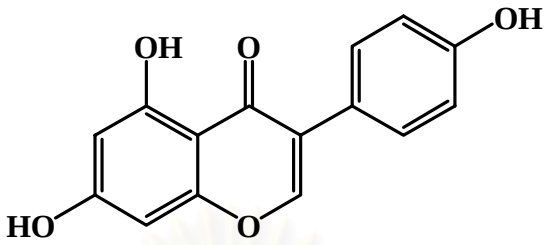
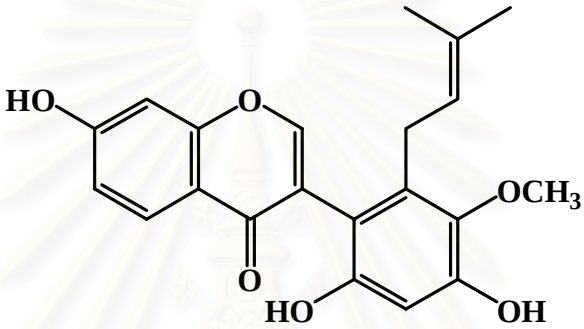
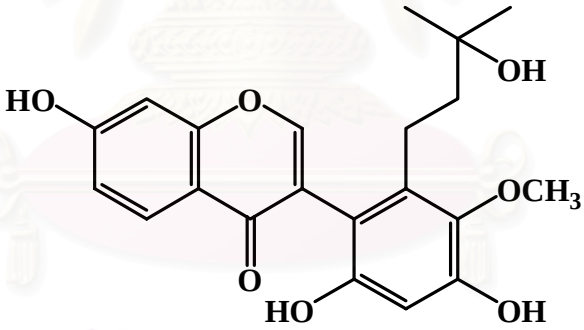
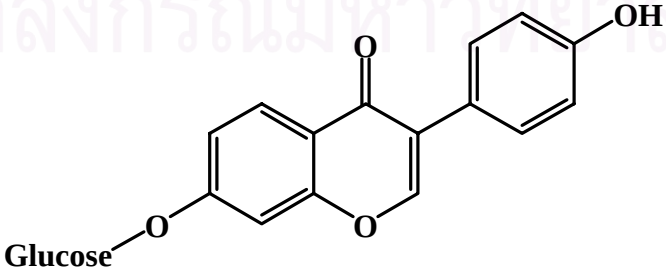
Compound	Structure	References
Genistein -$C_{15}H_{10}O_5$ -MW= 270.24		Ingram *et al*., 1986 Chansakaow *et al*., 2000
Kwakhurin -$C_{21}H_{20}O_6$ -MW= 368.38		Ingram *et al*., 1986 Tahara *et al*., 1987 Chansakaow *et al*., 2000
Kwakhurin hydrate -$C_{21}H_{22}O_7$ -MW= 386.40		Ingram *et al*., 1989
Daidzin -$C_{21}H_{20}O_8$ -MW= 400.38		Ingram *et al*., 1986 Chansakaow *et al*., 2000

Table 2: Structures of isoflavonoids in *P. candollei* var.*mirifica* (continued)

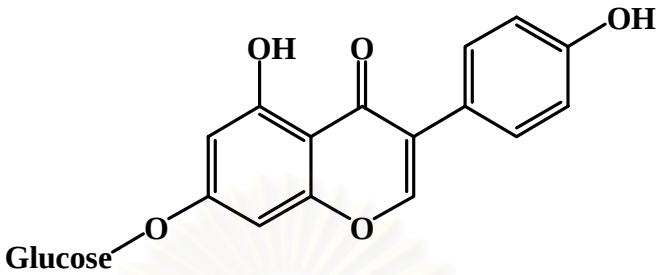
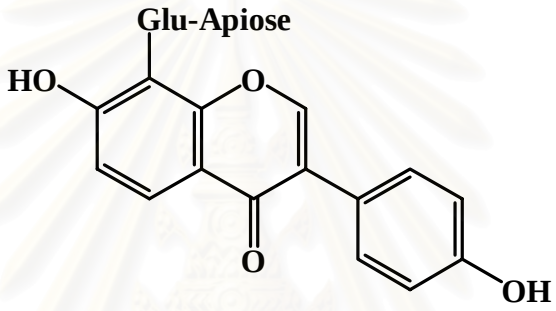
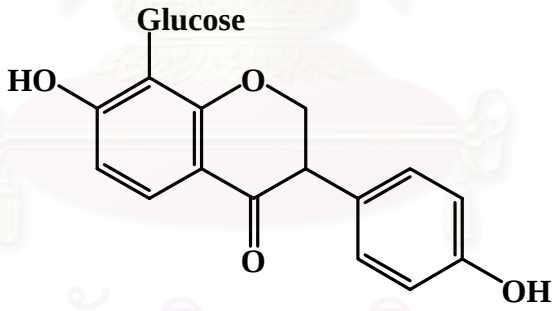
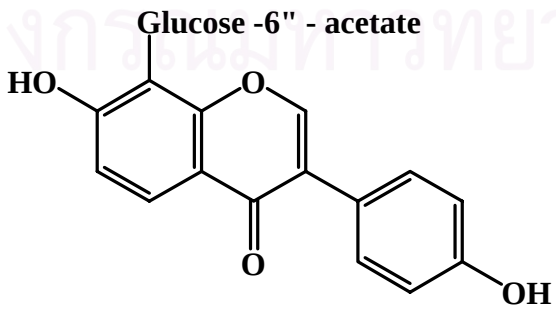
Compound	Structure	References
Genistin -$C_{21}H_{20}O_9$ -MW= 416.38		Ingram *et al*., 1989
Mirificin -$C_{26}H_{28}O_{13}$ -MW= 548.49		Ingram *et al*., 1986 Chansakaow *et al*., 2000
Puerarin -$C_{21}H_{20}O_9$ -MW= 416.38		Ingram *et al*., 1986
Puerarin-6”-monoacetate -$C_{22}H_{22}O_{10}$ -MW= 446.40		Ingram *et al*., 1989

Table 2: Structures of isoflavonoids in *P. candollei* var.*mirifica* (continued)

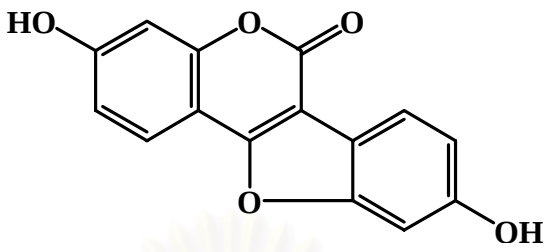
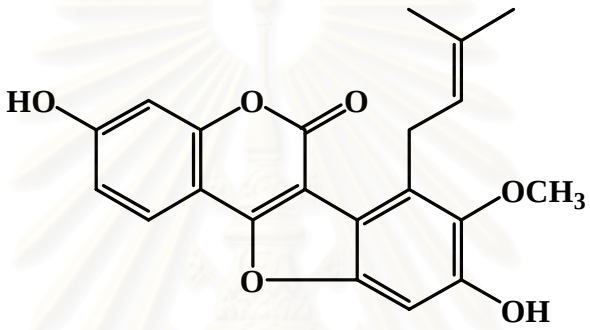
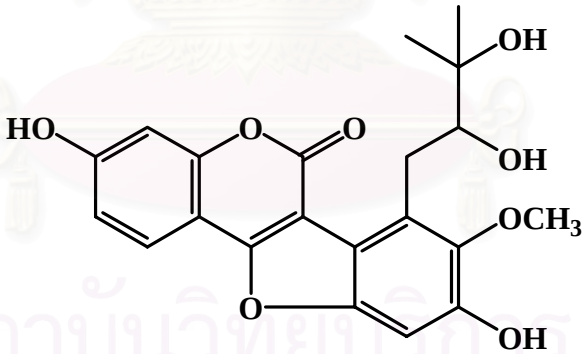
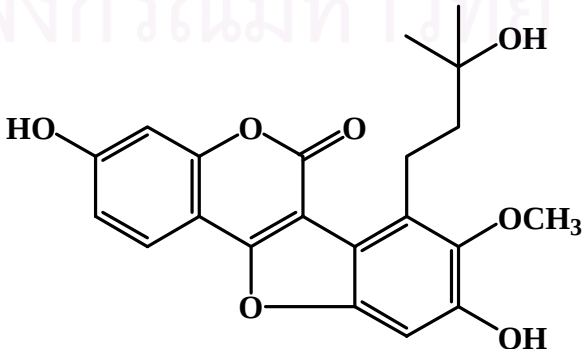
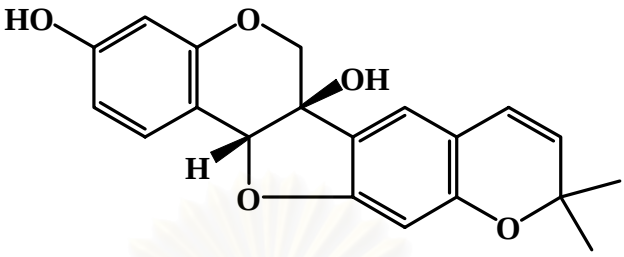
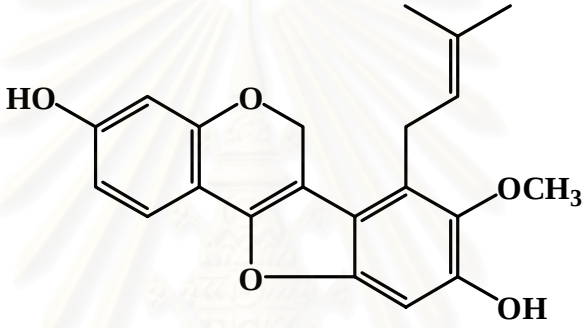
Compound	Structure	References
Coumestrol -$C_{15}H_8O_5$ -MW= 268.22		Ingram *et al*., 1988 Chansakaow *et al*., 2000
Mirificoumestan -$C_{21}H_{18}O_6$ -MW=366.36		Tahara *et al*., 1987
Mirificoumestan glycol -$C_{21}H_{22}O_8$ -MW= 402.39		Tahara *et al*., 1987
Mirificoumestan hydrate -$C_{21}H_{20}O_7$ -MW= 384.38		Tahara *et al*., 1987

Table 2: Structures of isoflavonoids in *Pueraria candollei* var.*mirifica* (continued)

Compound	Structure	References
Tuberosin -$C_{21}H_{22}O_7$ -MW= 338.35		Joshi & Kamat, 1973 Chansakaow *et al*., 2000
Puemiricarpene -$C_{21}H_{20}O_5$ -MW= 352.38		Chansakaow *et al*., 2000

7) Affinity chromatography (Scouten, 1981)

Affinity chromatography is a type of liquid chromatography that makes use of biological-like interactions for the separation and specific analysis of sample components. Some applications of this approach include the use of Boronate, lectin, protein A or protein G, and immunoaffinity support for the direct quantification of solutes. The newer techniques are methods that use affinity chromatography in combination with other analytical methods, such as reverse-phase liquid chromatography, gas chromatography, and capillary electrophoresis. (Hage, 1999) Boronate affinity chromatography that use boronic acid or boronates as ligands are one group of chromatographic techniques that have been used in clinical samples. At a pH above 8, most boronate derivatives form covalent bonds with compounds that contain cis-diol groups in their structure. After washing step, a decreasing in mobile phase pH could be used for elution. (Mallia et *al*., 1981)

Boronate affinity chromatography was used for separation the low to moderate molecular weight compounds such as mistletoe lectins (Li et *al*., 2001), glycohemoglobin of human (Frantzen et *al*., 1997), 3,4-Dihydroxyphenylalanine-containing proteins (Hawkins et *al*., 1986), 2-Hydroxycarboxylic acids (Higa et *al*., 1986). Boronate derivatives on various solid supports have been investigated and applied.

In alkaline condition, the planar boronic acid has been changed to tetrahedral boronate anion and they can be interact with polyhydroxyl compounds as shown in Fig.4

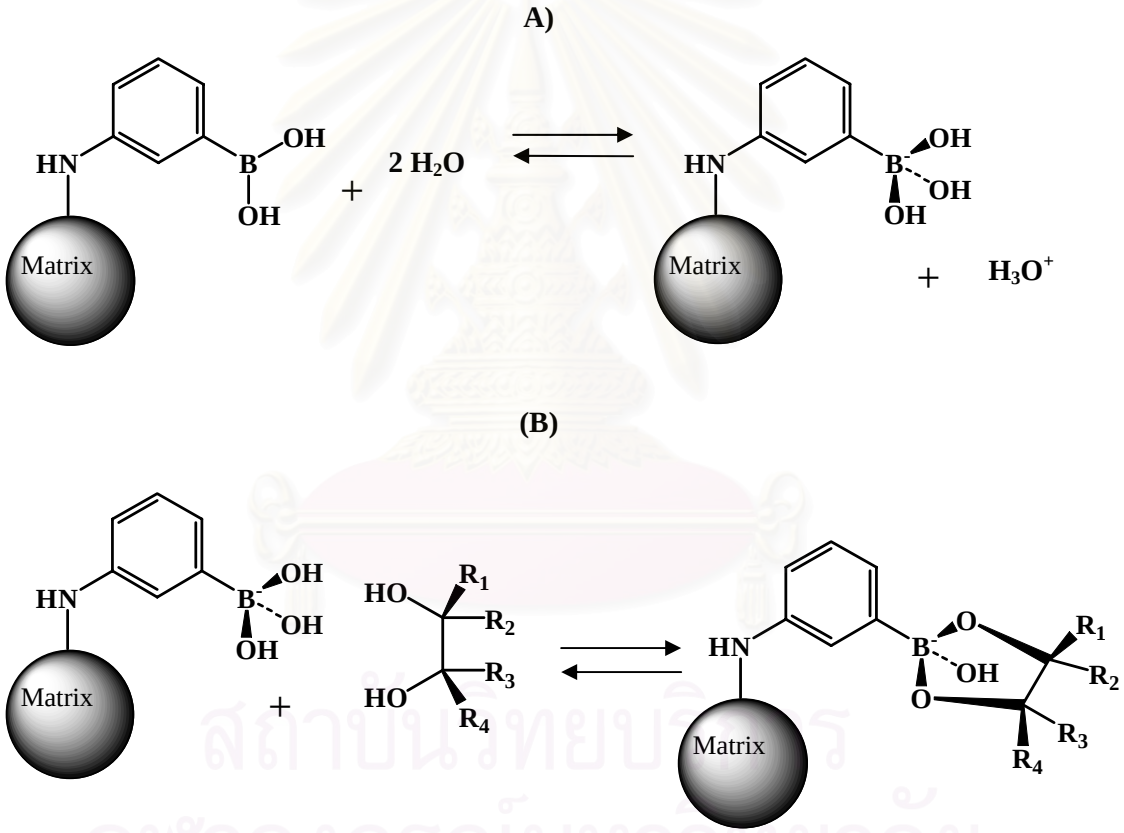


Figure 4: Schematic presentation of: **(A)** the transition of planar boronic acid to a tetrahedral boronate anion under alkaline conditions, and **(B)** the interaction between a boronate anion with a tetrahedral conformation and polyhydroxyl compounds

Boronate affinity chromatography has been used for separate the target compounds from the sample. They have many applications as shown in the Table 3.

Table 3: Applications of boronate affinity supports (Bio-Rad laboratories, 1986)

Application	**Application buffer**	**V_0 buffer**	**Retained fraction(s)**	**Elution buffer**	**Support used**
Adenylate cyclase assay	HEPES pH 7.5 + $MgCl_2$	CAMP	ATP, AMP and Adenosine	0.05 M NaOAc	Bio-Gel P-150 boronate
Isolation of catecholamines from urine	0.1 M phosphate, pH 7.0 + EDTA	Other urine components and DOPA	Norepinephrine, epinephrine	0.025 N HCl	Boric acid gel (Aldrich)
Modified nucleosides in urine	0.25 M NH_4OAc, pH 8.8	Thymidine, adenine	Pseudo uridine	0.1 M HCOOH	Bio-Gel P-2, 200-400 boronate
Separation of mononucleotides, oligonucleotides	Triethyl ammonium	Deoxyribo-nucleosides	Ribonucleosides	H_2O and other	Dihydroxyboryl methacrylate
Sugars	0.05 M N-methyl morpholinium-Cl, pH 7.5 + 1 M NaOAc	Erythritol, adonitol, sucrose and D-glucose	L-Arabinitol, Xylitol, D-mannitol, Dulcitol, Sorbitol and D-Fructose	Same as V_0 buffer	Cellulose dihydroxyboryl
Assay of ribonu-cleotide reductase	Tris, $MgCl_2$	dUMP, dCMP	CDP	Citrate	Cellulose di-Hydroxyboryl Dowex 1 (AG)

These derivatives have been variously term dihydroxy boryl, boric cid gel, and boronic acid gel, all referring to m-aminophenylboronic acid immobilized to solid supports. Supports have included aminoethyl cellulose, porous glass, methacrylate gel, polyacrylamide gel and acrylic beads.

Boronate affinity chromatography and *P. candollei* var.*mirifica*

Miroestrol and its derivatives from *P. candollei* var.*mirifica* have high potency of estrogenic activity when compared with estradiol, but it is very small amount in this plant. Miroestrol has hydroxyl group at C-17 and C-18 of ring D and the ethylene bridge made 2 hydroxyl groups look like *cis- d*iol group as shown in Fig.5 and Fig.6. Boronate affinity chromatography has been used for separation of the compounds that contain *cis-diol* group in molecule.

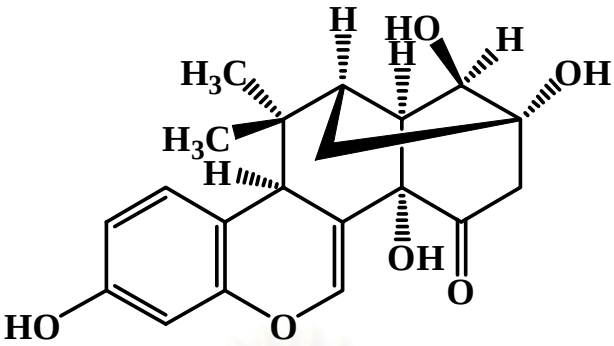


Figure 5: Structure of miroestrol

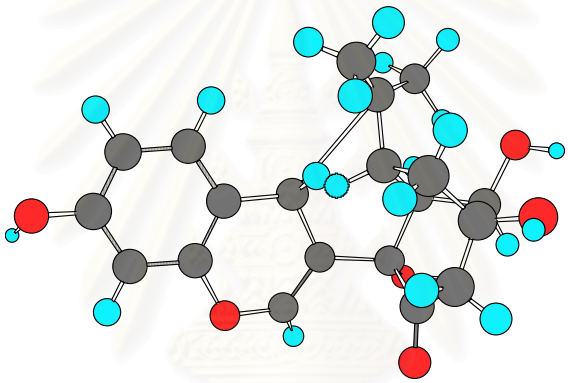


Figure 6: Ball and stick model of miroestrol of miroestrol

The adsorbent of this study is m-aminophenylboronic acid on acrylic beads. Buffer A is 0.25 M ammonium acetate in methanol, pH 8.8, Buffer B is 0.1 M formic acid in methanol, 0.1 acetic acid in methanol can be used for clean up the adsorbent before re-equilibration.

8) MCF-7 cell proliferation assay

The MCF-7 cell assay has been purposed as a primary screen for the detection of xenobiotic estrogen (Hsieh *et al*., 1998) and (Odum *et al*., 1998). The assay is based on the ability of human breast cancer cell (MCF-7) to proliferate in response to estrogen. The use of in vitro screens such as MCF-7 cells offers a potentially useful primary screen for environmental estrogen.

Proliferation of estrogen dependent cancer cells has been used to detect estrogenicity of the compounds in the environment. Sato *et al.,* 1995 have introduced a cell proliferation assay termed the E-Screen test. For this bioassay, MCF-7 breast cancer cells were chosen. These cells are genuine human estrogen-sensitive cells; they remain quiescent when inoculated into ovariectomized hosts. They require the presence of estrogen to grow as tumors in hosts. The MCF-7 cell assay is based on the ability of this polyclonal human breast cancer cell line to proliferate in response to estrogen. This cell line has been used for many years in the search for estrogen agonists or estrogen antagonists for breast cancer therapies (Odum *et al.,* 1998).

When MCF-7 cells are grown in culture medium supplemented with nonestrogen charcoal-stripped human serum, proliferation is prevented. When estrogen is added, the cells proliferate. The E-screen assay compares the cell yield achieved after 4 to 6 days of culture in medium supplemented with 5 to 10 % charcoal-dextran stripped human serum in the presence (positive control) or absence (negative control) of estradiol and with diverse concentration of xenobiotics suspected of being estrogenic. When cell yield is examined over a wide range of test-compound concentrations, it is possible to distinguish agonists, partial agonists, and inactive compounds from one another (Korach *et al.,* 1995).

9) Biosynthetic pathway of isoflavonoids

Flavonoids represent a large class of secondary plant metabolites. They are important secondary metabolites with several functions, including as pigments, signaling molecules, protectants against biotic and abiotic stresses and in fertility (Wade *et al*., 2003) Isoflavonoids belong to a class of Flavonoids with C_6-C_3-C_6 skeleton derived from *p*-coumaryl CoA and three molecules of malonyl CoA. They share the early steps of their biosynthesis with the general flavonoid biosynthetic pathway until the branching point at formation of the flavanone. Isoflavone biosynthesis consists of three enzymatic reactions, namely chalcone synthase (CHS), chalcone-flavanone isomerase (CHI) and isoflavone synthase (IFS). CHI is a very stable enzyme with catalyzed the conversion of chalcone into flavanones. The established cell culture of *Pueraria lobata* produced isoflavones, only CHI activity was detected in cell free preparations under the various assay conditions used.

However, the activities of CHS and IFS could be detected when the cell cultures are challenged with elicitors (Hakamatsuka *et al*., 1998).

The phenylpropanoid pathway in plants converts phenylalanine into naringenin chalcone. As the first step, Phenylalanine is deaminated to yield cinnamic acid by the action of phenylalanine ammonia lyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to 4-coumaric acid, which is then activated to 4-coumaroyl-coenzyme A (CoA) by the action of 4-coumarate CoA ligase (4CL). Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to yield naringenin chalcone, the precursor for a large number of flavonoids. Naringenin chalcone is converted to naringenin by chalcone isomerase or nonenzymatically in vitro. Because some of the PALs show tyrosine ammonia lyase activity, tyrosine is also used as the precursor. The steps of isoflavonoid production have been shown in the Fig.7 and Fig.8.

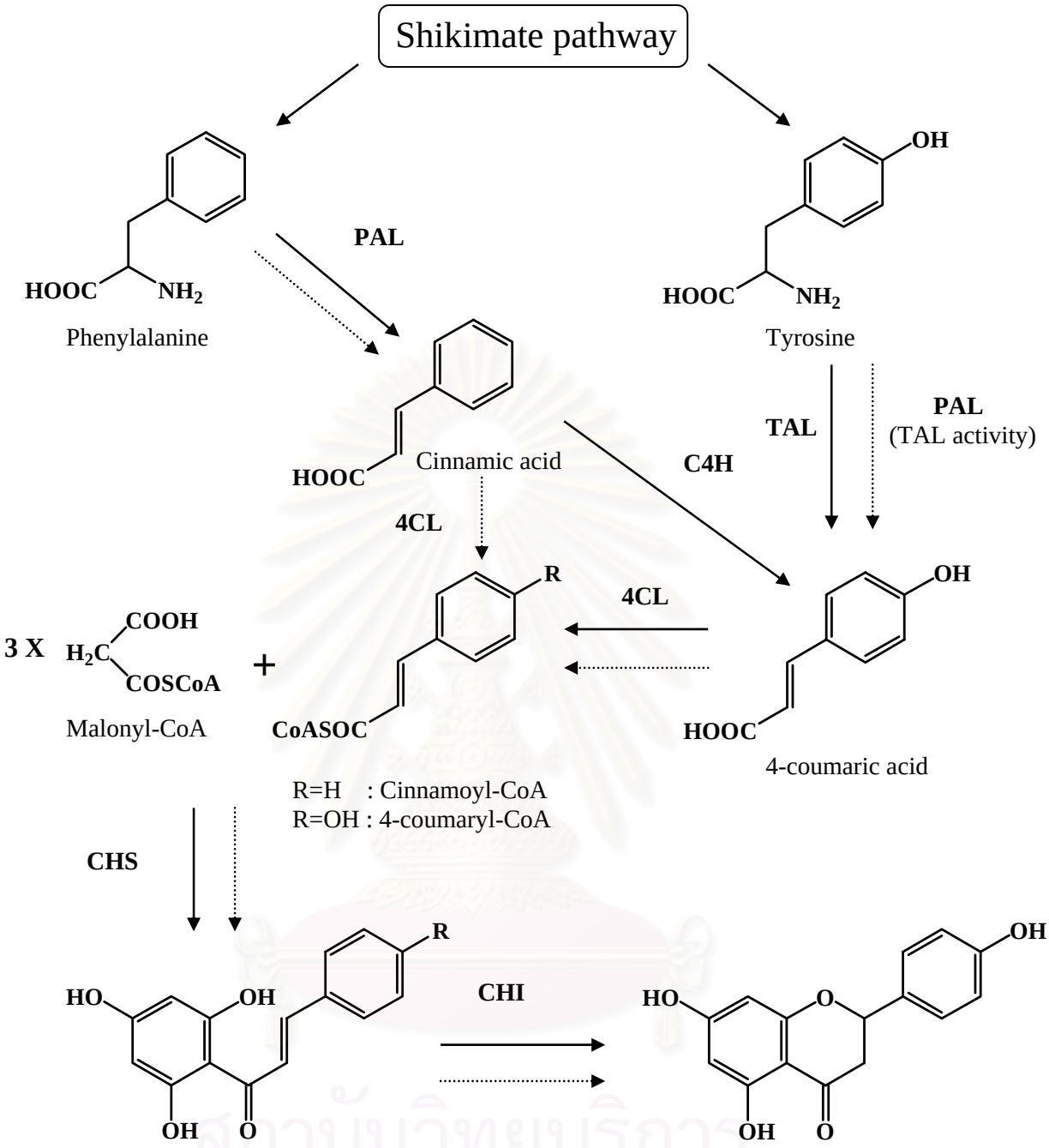


Figure 7: Flavanone biosynthetic pathway in plants. The dashed arrows represent the expected flavanone biosynthetic pathway in *E. coli* containing the artificial gene cluster including *PAL*, *4CL*, and *CHS*. TAL, tyrosine ammonia-lyase; CHI, chalcone isomerase (Hwang *et al*., 2003)

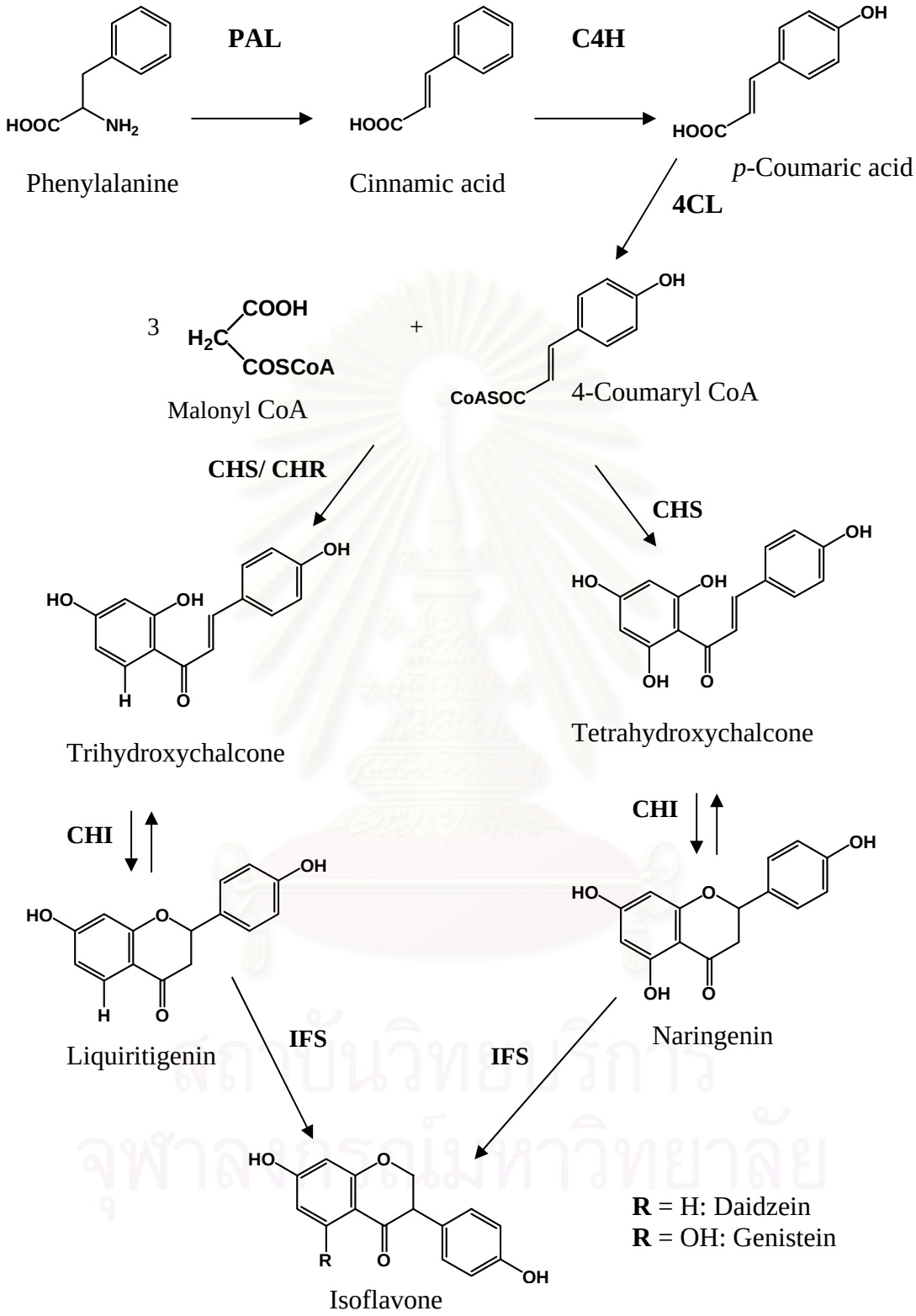


Figure 8: Isoflavone biosynthesis partway

10) Plant cell culture as a source of secondary metabolites

Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites as shown in Table 4. Plant cells are biosyntically totipotent, which means that each cell in culture retains complete genetic information and hence is able to produce the range of chemicals found in the parent plant. The advancetages of this technology over the conventional agricultural production are as follows.

-It is independent of geographical and seasonal variations and various environmental factors.

-It offers a defined production system, which ensures the continuous supply of products, uniform quality and yield.

-It is possible to produce novel compounds that are not normally found in parent plant.

Table 4: Natural products that have been isolated from tissue and cell suspension culture of higher plants (Ramachanda *et al*., 2002)

Phenylrpopanoids	**Alkaloids**	**Terpenoids**	**Quinones**	**Steroids**
1)Anthocyanins 2)Coumarins 3)Flavonoids 4)Hydroxycinnamoyl derivatives 5)Isoflavonoids 6)Lignans 7)Phenolenones 8)Proanthocyanidins 9)Stilbenes 10)Tanins	1)Acridines 2)Betalains 3)Quinolizidines 4)Furonoquinones 5)Harringtonines 6)Isoquinolines 7)Indoles 8)Purines 9)Pyridines 10)Tropane alkaloid	1)Carotenes 2)Monoterpenes 3)Sesquiterpenes 4)Diterpenes 5)Triterpenes	1)Anthroquinones 2)Benzoquinones 3)Naphthoqui-nones	1)Cardiac glycosides 2)Pregnenolone derivatives

CHAPTER III

MATERIALS AND METHODS

1) Chemicals

Adsorbent: m-aminophenylboronic acid was purchased from Sigma-Aldrich Company. Chemicals for culture media are tissue culture grade. They were purchased from Sigma-Aldrich Company. Plant growth regulators were purchased from Gibco laboratories. Agar was purchased from Merk Company. Organic solvents for phytochemical study were analytical grade (Labscan Company) and distilled organic solvents were used for extraction. TLC plates of silica gel 60 F254 0.2 mm. thick on aluminium sheet were obtained from Merk Company. Organic solvents for HPLC analysis were HPLC grade that purchased from Labscan Company. Water for HPLC was ultrapure water and filtrated with 0.45 μm. pore size nylon filter before use.

2) Plant material

The tuberous roots of five varieties of *P. candollei* were collected from Kasetsart University, Kampangsaen campus. They were used for studying estrogenic activity and for the source of active compound separation by boronate affinity chromatography.

The five varieties were labeled KW001, KW002, KW003, KW004 and KW005. Among these, KW005 is the most popular variety used in farming since it gives good yield for cultivation. For the establishment of cell cultures from the tuberous root tissue, the tuberous root samples collected from Udon Thani Province was used.

3) Plant tissue culture techniques

3.1 Preparation of explants

The collected tuberous root was cleaned with tap water for remove soil and other contaminations. The clean tuber was cut into small pieces. These were then sterilized with 15% Clorox solution for 15 minutes and cleaned with sterilized water for 3-4 times.

3.2 Preparation of Medium

Murashige & Skoog Medium (MS Medium) was used in this experiment. This medium was supplemented with plant regulators or plant hormones. They were 2,4 D (2,4 dichloroacetic acid), BA and NAA in the concentrations of 0.1mg: 0.01mg: 0.01mg in 1 Liter. 0.8% Agar was used for preparation of semi-solid MS medium. 30% sugar was used as carbon source. For cell suspension culture 50 ml of liquid MS medium were transferred into 250 ml flask and covered with aluminum foil.

3.3 Callus culture

The sterilized tuberous root tissue was cut into small pieces of explants. Each explant was transferred to solidified MS medium (plus 0.8% agar) by aseptic techniques. They were incubating under 16 hours light shelf. Temperature was controlled at 25 ºC. The callus was observed to be formed in three weeks.

3.4 Cell suspension culture

After callus culture was established and sub-cultured for 3-4 cycles, the friable callus was obtained and it was used for cell suspension culture preparation. The callus tissue was transferred to liquid MS medium with the same supplement of plant regulators. They were incubated on a shaker with 120 rounds per minute. The cell suspension cultures were sub-cultured every 14 days by transferred 10 ml inoculum to 50 ml fresh medium.

4) Phytochemical techniques

4.1 Preparation of *Pueraria candollei* powder from 5 varieties, callus and cell suspension culture

4.1.1 *Pueraria candollei*: the tuberous roots of 5 varieties were cleaned with tap water to remove soil. The tuberous roots were sliced into small piece and dried with hot air oven at 45- 50 ºC and freeze drying. Both samples were ground into powder.

4.1.2 Callus: 21 days the callus was harvested and dried by freeze drying. The dried callus was ground into powder.

4.1.3 Cell suspension: 14 days the cell suspension was harvested and cleaned with distilled water for media removal. It was dried by freeze drying. The dried cell suspension was ground into powder.

4.2 Extraction

Each dried powder obtained from either the tuberous root, callus or cell suspension were weighted for 150 gm and macerated sequentially with 500 ml of hexane three times, 500 ml of ethyl acetate three times and 500 ml of methanol three times respectively. The extract of hexane, ethyl acetate and methanol were then evaporated using rota-evaporator.

4.3 TLC (thin layer chromatography) study

Technique	: one dimension, ascending, simple development
Stationary phase	: aluminium sheet silica gel 60 F254 (precoated, Merck)
Layer thickness	: 0.2 mm
Solvent System	: chloroform: methanol (9.2: 0.8)
Temperature	: 25- 30 ºC
Detection	: ultraviolet light at 254 and 365 nm, TLC densitometer at 280 nm

4.4 Boronate affinity chromatography

Boronate affinity chromatography is one kind of affinity chromatography. This technique has been used for separate the *cis-diol* containing molecules by specific binding. The adsorbent in this experiment is m-aminophenylboronic acid on acrylic beads. For this technique, the *cis-diol* molecules are bound to the column under basic conditions and they are eluted under the acidic condition.

4.4.1 Buffer:

(1) Buffer A: 0.25 M Ammonium acetate in methanol, adjusted pH to 8.8. It was used for equilibrate column and wash the column to remove the unbound compounds from the adsorbent.

(2) Buffer B: 0.1 M Formic acid in methanol. It was used as eluent for eluting the bound compounds from the adsorbent.

(3) 0.1 M acetic acid in methanol. It was used for cleaning up the adsorbent before column re-equilibration.

4.4.2 Adsorbent: m-aminophenylboronic acid on acrylic beads.

One gram of the dry adsorbent was used in this experiment. It was added into 15 ml buffer A. The adsorbent was swelled to 4 ml adsorbent volume. The wet adsorbent was transferred into the glass column as packing material. Equilibrate the column to pH 8.8 with buffer A before being used.

4.4.3 Sample preparation:

Samples of ethyl acetate and methanolic crude extract of some varieties of *P. candollei* and cell cultures was each weighted for 50 mg and dissolved with 5 ml of Buffer A. Sonicator was used for helping dissolvation. After that, it was centrifuged for residue removal. The clear solution was used for loading into the column.

4.4.4 Boronate affinity chromatography procedure

1) Equilibrate the column with Buffer A, pH 8.8
2) Load the solution of sample into the column with flow rate 1 ml/ min.
3) Wash the adsorbent with 50 ml Buffer A for removal the unbound compounds from the adsorbent. Flow rate 1 ml/ min. This fraction was collected as unbound fraction or wash fraction.
4) The bound compounds were eluted from the adsorbent with Buffer B. Flow rate 1 ml/ min. This fraction was collected as bound fraction or elute fraction.
5) Before re-equilibration of the column, 0.1 M acetic acid was used for cleaning up the adsorbent in the column, flow rate 1 ml/ min. After that, the adsorbent in the column was re-equilibrated with Buffer A.
6) Unbound fraction and bound fractions were evaporated under vacuum by rota-evaporator.

4.5 HPLC analysis

The ethyl acetate or methanol crude extract of each varieties of *P. candollei* was separated by boronate affinity chromatography. The unbound and bound compounds were analyzed by HPLC analysis. The column of TSKgel Super-ODS (100 X 4.6 mm) C_{18} reverse phase was used in this experiment. Mobile phase of gradient of methanol and water was used 0 min: 30% methanol, 60 min: 60% methanol, 65 min: 95% methanol and 70 min: 30% methanol. HPLC instrument of SHIMADZU HPLC with diode array detector was used. The wavelengths for monitoring were 254, 365 and 280 nm.

5) Bioassay for estrogenic activity using MCF-7 cells (estrogen dependent cancer cells)

5.1 MCF-7 cell culture

Human breast cancer cells, MCF-7 was purchased from the American Type Culture Collection (Manassas, VA). The MCF-7 cells were grown in MEM supplemented with 6 ng/ml insulin, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM glutamine, 10 % FBS, and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) under a 5 % CO_2 humidified atmosphere at 37 ºC.

5.2 Cell proliferation assay

Cells were seeded into 96-well tissue culture plates in 5 % DCC treated- FBS supplemented RPMI phenol red-free medium at a density of 1 x 10 exp 4 cells/well. Test compounds were added in 70 % EtOH solution (control contained 0.7 % ethanol) and incubated at 37 ºC with 5 % CO_2 for 96 hr. In all experiments, serial dilutions of estradiol were added as positive control. To evaluate relative cell concentrations, Alamar Blue reagent was used. After 3h, fluorescence was measured at 590 nm with excitation at 530 nm using a FL500 spectrophotometer (BIO-TEK Instruments Inc, USA).

Samples in paste condition were weighed by using balance and serial dilutions of the samples to be tested were made in a 70 % aqueous ethanol solution at

concentrations 100-fold higher than the desired final concentrations in unit of microgram/ml. Samples in liquid condition were diluted serially with 70 % aqueous ethanol at 1, 10, and 100 times diluted solutions. Then all sample solutions were diluted for 10 times with culture medium, and 10 micro liter of each sample solutions were added to 90 micro liter of the cell suspension seeded in 96 well plates.

6) The quantitative analysis of secondary metabolite production of *Pueraria candollei* cell cultures

The cell cultures were established from tuberous root tissues of *Pueraria candollei* var.*mirifica*. MS media (Murashige and Skoog media) was used in this experiment. The plant regulators are 2,4-D (2,4-dichlorophenoxyacetic acid), BA (N_6-Benzyladenine) and NAA (Naphthaleneacetic acid) (1: 0.1: 0.1). Callus and cell suspension were harvested and dried. They were extracted with ethyl acetate by maceration method. The ethyl acetate crude of each sample was spotted on TLC plate, separated with solvent system and compared with standard daidzein, standard genistein. HPLC technique was also used to confirm the result and measured the amount of secondary metabolites.

6.1 TLC (thin layer chromatography) analysis

Technique : one dimension, ascending, simple development
Stationary phase : aluminium sheet silica gel 60 F254 (precoated, Merck)
Layer thickness : 0.2 mm
Solvent System : chloroform: methanol (9.2: 0.8)
Temperature : 25- 30 ºC
Detection : ultraviolet light at 254 and 365 nm, TLC densitometer at 280 nm

6.2 HPLC (high performance liquid chromatography) analysis

The TSKgel Super-ODS (100 X 4.6 mm) C_{18} reverse phase HPLC column was used in this experiment. Mobile phase: methanol and water; 0 min: 30% methanol, 60 min: 60% methanol, 65 min: 95% methanol and 70 min: 30% methanol.

HPLC instrument is SHIMADZU HPLC with diode array detector. The wavelengths for monitoring are 254, 365 and 280 nm.

The ethyl acetate crude extract of callus and cell suspension culture of *Pueraria candollei* var.*mirifica* were analyzed by HPLC method. The calibration curve of standard daidzein and standard genistein were prepared. 1 mg of each standard was dissolved in 10 ml methanol. This stock solution of each standard was diluted with methanol to the concentration range 0.0001-0.1000 mg/ ml for constructing their calibration curve of each standard by HPLC analysis.

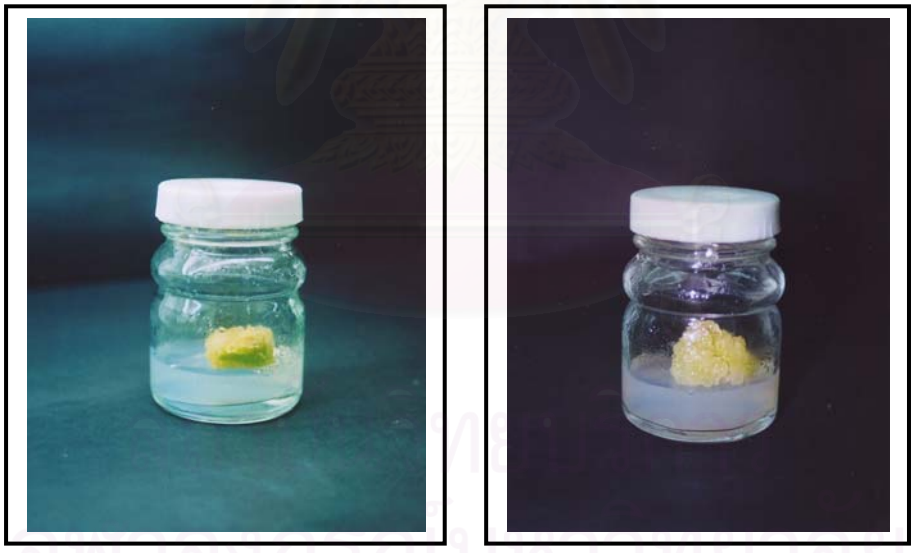
CHAPTER IV

RESULTS

1) Plant tissue culture of *Pueraria candollei*

1.1 Establishment of callus cultures

Callus culture was induced successfully on MS medium supplemented with 2,4-D, BA, NAA (1: 0.1: 0.1) and 0.8% (w/v) agar. The tuberous root tissue of *Pueraria candollei* is look like sheets. The callus was initiated at the area between sheets of tuberous root tissue. The color of initiated callus is pale green. The first callus culture is compact callus. These callus cultures could be maintained by sub-culturing with the same media for every 3 – 4 weeks. After sub-culturing for several months, the callus culture formed friable, soft callus and the color is pale green to yellow as shown in Fig.10 A and Fig.10 B



A **B**

Figure 9: A) the induction of callus from the tuberous root tissue of *Pueraria candollei* var. *mirifica* on MS media supplemented with 2,4-D, BA, NAA (1: 0.1: 0.1)

B) the friable callus of *Pueraria candollei* var.*mirifica* on MS media supplemented with 2,4-D, BA, NAA (1: 0.1: 0.1)

1.2 Establishment of cell suspension cultures

Cell suspension culture was induced form callus culture of *Pueraria candollei*. The friable callus was transferred into the liquid media of MS medium supplemented with the same plant regulators without agar. The flask contains callus and liquid MS media was shake on the shaker at 120 r.p.m. In this condition, the cell suspension was appeared a fast growth rate. After stable cell suspension cultures were obtained, it was maintained in the same media by sub-culturing every 14 days

Figure 10: The cell suspension culture of *Pueraria candollei* in MS medium supplemented with 2,4-D, BA, NAA (1: 0.1: 0.1)

2) Phytochemical study of some varieties and cell cultures of *Pueraria candollei*

The biodiversity of the nature and geographical variation can cause the variation in plant. *Pueraria candollei* is a sample of this event. The specimens that collected from difference place have been studied the chemical pattern and determined the estrogenic activity with MCF-7 cell. 5 varieties of *Pueraria candollei* were collected from Kasetsart University, Kampangsaen campus. They were collected from another places in Thailand and cultivated in the area of Kasetsart University, Kampangsaen campus for 2 years. After dry and ground into powder, they were extract with hexane, ethyl acetate and methanol. The ethyl acetate and methanol crude extracts were determined the estrogenic activity by MCF-7 cell. Chansakaow *et al*., 2000 found the most potent isoflavonoids such as miroestrol and deoxymiroestrol in ethyl acetate crude extract.

2.1 Some varieties of *Pueraria candollei*

Some varieties of *Pueraria candollei* were collected from Kasetsart University, Kampangsaen campus. They are KW001, KW002, KW003, KW004 and KW005. The samples collected from different area and grow in Kasetsart University, Kampangsaen campus for 2 years, KW005 for 1 year.

Table 5: Some varieties of *Pueraria candollei* and information

Cultivar	Places	Remarks
KW001	Tabkwang, Saraburi	Pod has hair
KW002	Lampang 1	Green, big pod, No hair
KW003	Lampang 2	Green, small pod, No hair
KW004	Lampang 3	Purple, small pod, No hair
KW005	Lampoon	พันธุ์ ศูนย์สุกร 1 (ศก1)

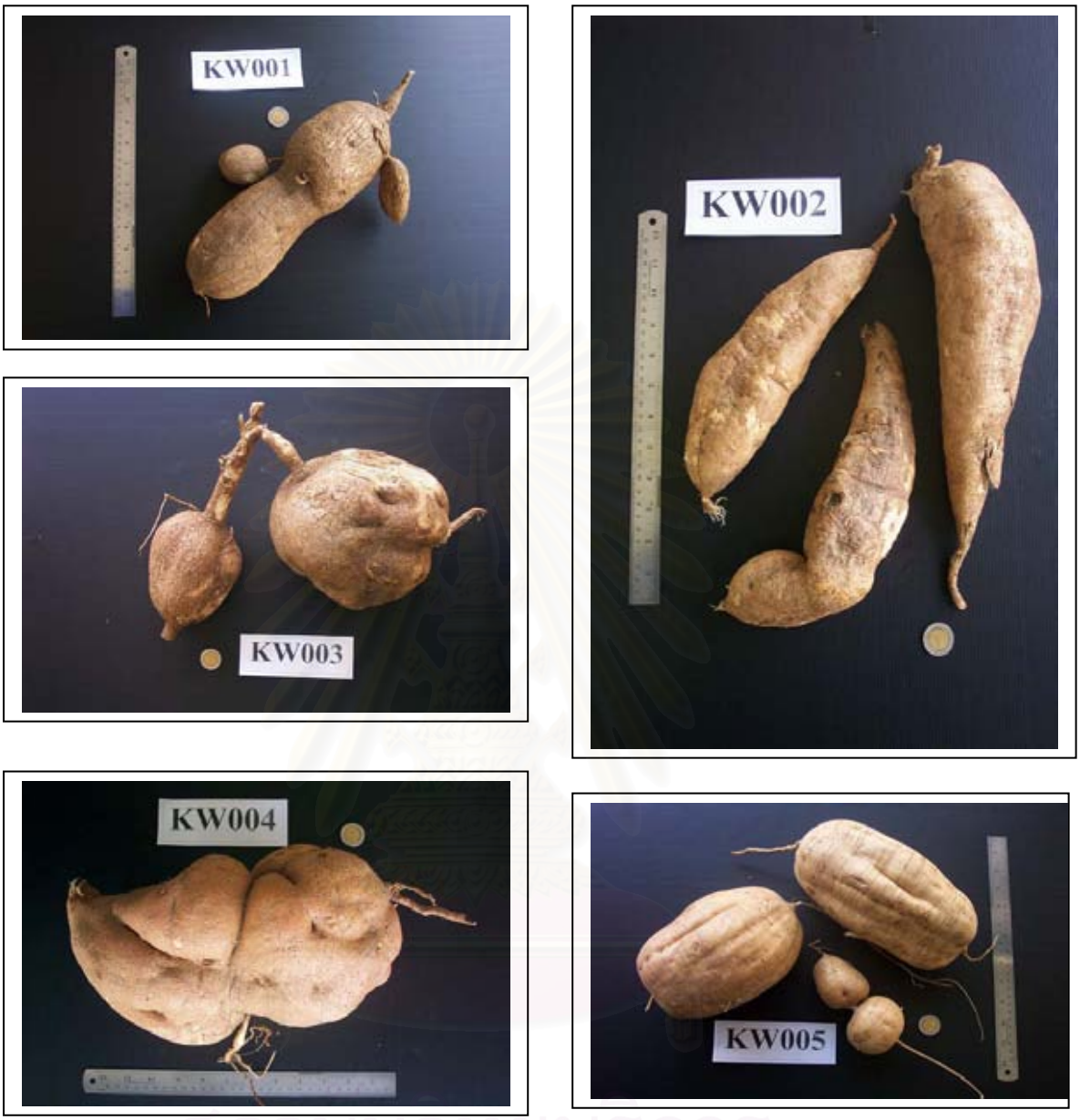


Figure 11: The tuberous roots of *Pueraria candollei* (KW001, KW002, KW003, KW004 and KW005).

Table 6: The samples were dried with hot air oven and freeze dry method.

2.1A) Hot air oven method

Specimens	Wet weight (kg)	Dry weight (kg)	%
KW001	7.00	0.97	13.86
KW002	6.70	0.91	13.58
KW003	7.00	0.78	11.14
KW004	7.40	0.73	9.86
KW005	10.00	1.14	11.4

2.1B) Freeze dry method

Specimens	Wet weight (kg)	Dry weight (kg)	%
KW001	1.15	0.1385	12.04
KW002	1.00	0.1389	13.89
KW003	0.85	0.0731	8.60
KW004	1.00	0.1079	10.79
KW005	1.00	0.1134	11.34

The effect of heat to chemical constituents of sample had observed. Both samples had provided into 2 groups, first group had dried with hot air oven and second group had dried with freeze dry method. The crude extracts of both samples had compared between hot air oven method and freeze dry method. The chemical patterns on TLC plate under the UV-light were similar.

2.2 Chemical pattern of ethyl acetate crude extract of some varieties of *Pueraria candollei*

The ethyl acetate crude extracts of some varieties of *Pueraria candollei* were studied for the chemical pattern on the TLC plate. They were spotted on the TLC plate and developed with the solvent system; Chloroform and methanol (9.2: 0.8). Daidzein and genistein were standard authentic that used compare to those samples. After that, TLC plate was observed under the UV light at 254 and 365 nm.

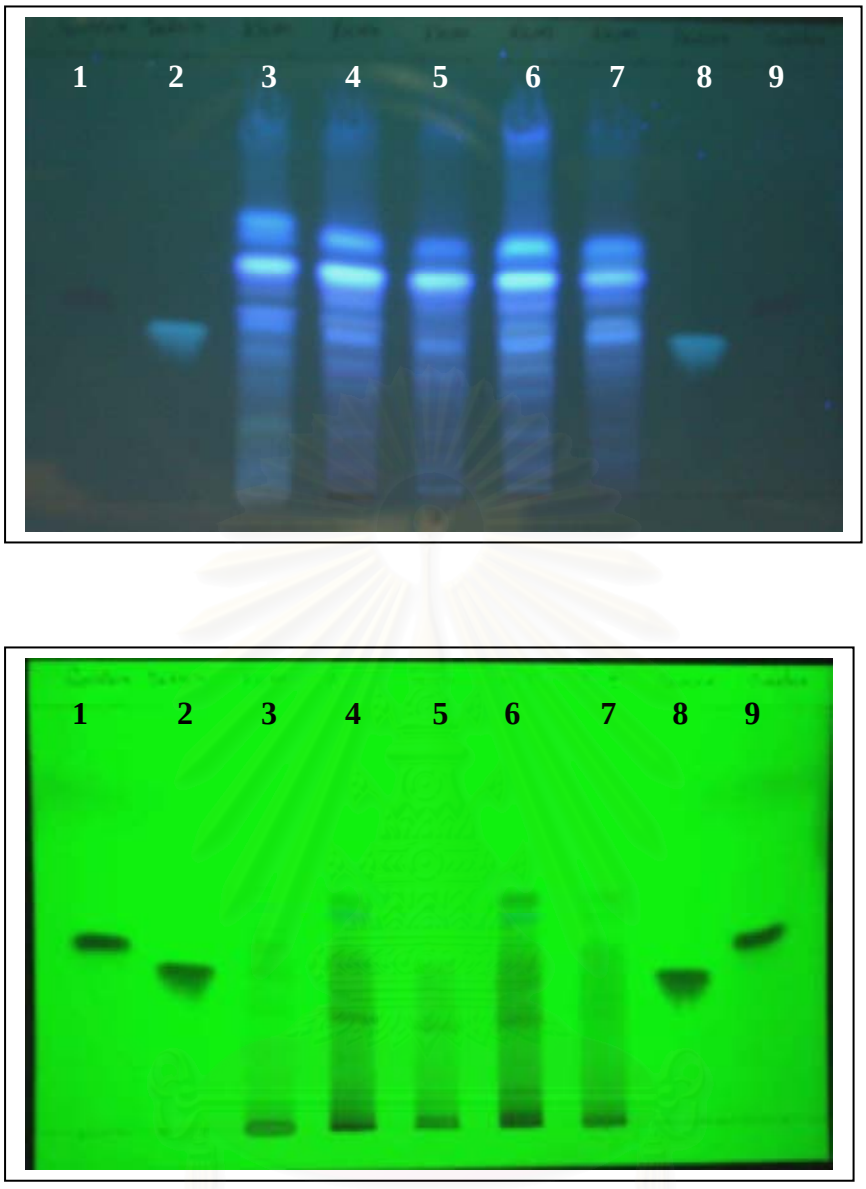
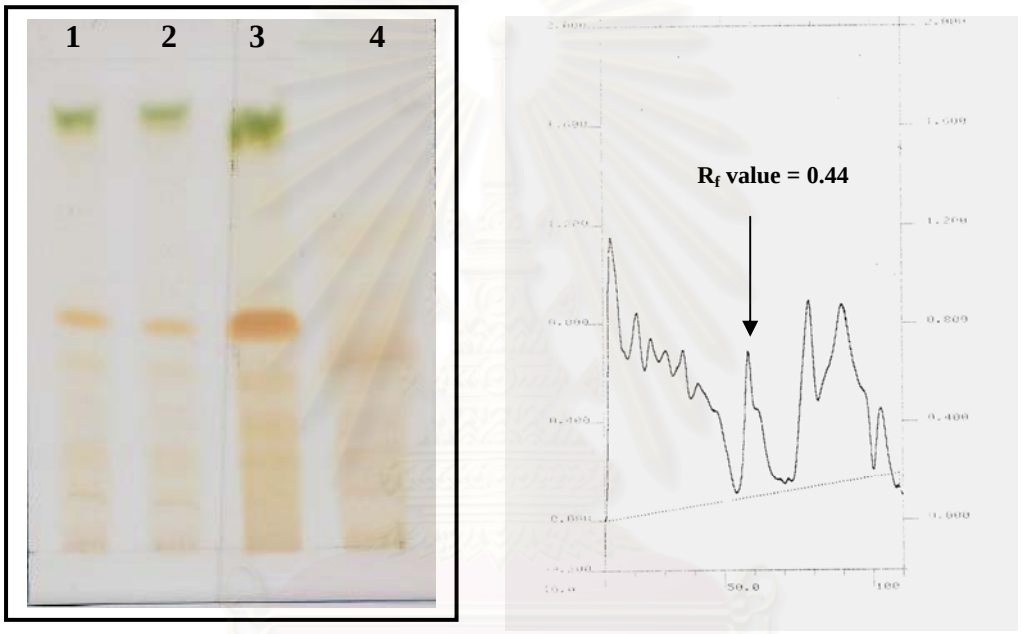


Figure 12: The chemical patterns of the ethyl acetate crude extracts of *Pueraria candollei*: (1) and (9): genistein, (2) and (8): daidzein, (3): KW001, (4): KW002, (5): KW003, (6): KW004 and (7): KW005. (A): at 365 nm, (B): at 254 nm.

2.3 Chemical pattern of ethyl acetate crude extract of cell cultures of *Pueraria candollei*

The tuberous root of *Pueraria candollei* that collected from Udon Thani province has been used for prepare cell cultures. The callus culture and cell suspension culture were harvested and dried with freeze dry method. They were ground into powder with mortar and pestle. The powder drug of both had been macerated with ethyl acetate and methanol respectively.



(A) (B)

Figure 13: (A) The chemical patterns on a TLC plate of ethyl acetate crude extracts of callus (Udon Thani) and powder drug of *Pueraria candollei* (whole plant). **1-2**; callus 33 days old, **3**; callus 58 days old and **4**; ethyl acetate crude extract of *Pueraria candollei* (whole plant). The solvent system: chloroform and methanol (9.2:0.8)

(B) The TLC densitometric chromatogram of lane 3

The ethyl acetate crude extract of callus culture of *Pueraria candollei* was compared with standard daidzein and standard genistein on TLC plate. It was found that the callus culture of this plant could produce the daidzein and genistein. They were confirmed the result with HPLC analysis with photo diode array detector.

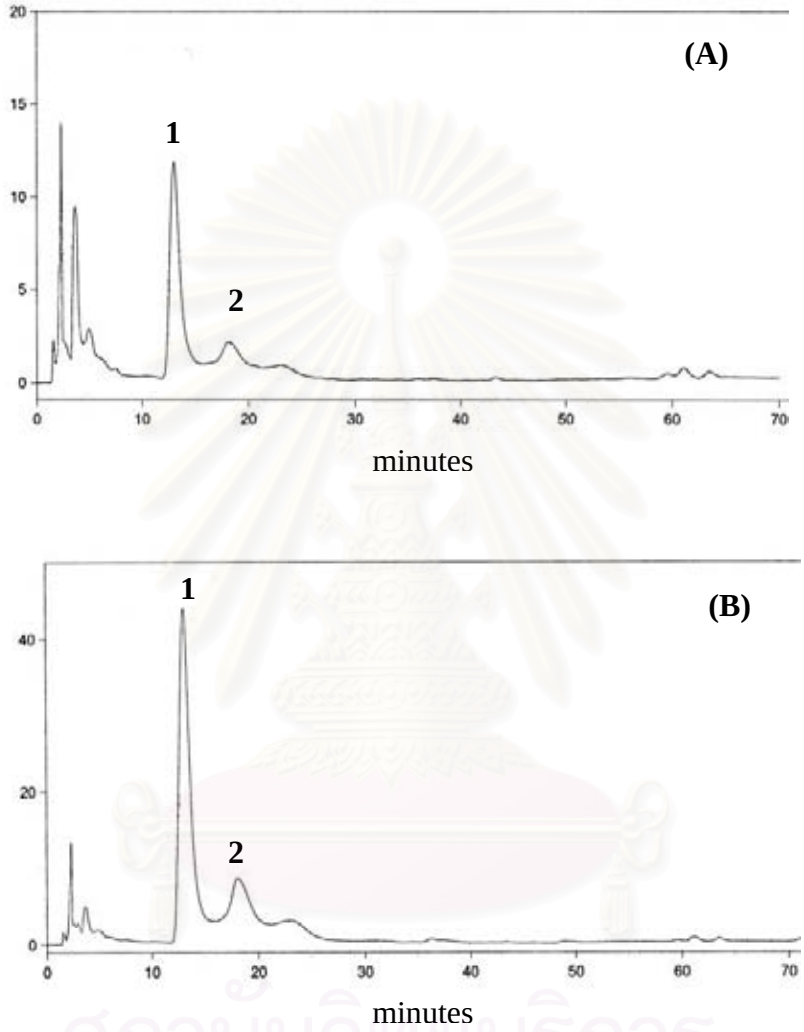


Figure 14: HPLC chromatograms of ethyl acetate crude extract of callus culture **(A)** and cell suspension culture **(B)** of *Pueraria candollei* (Udon Thani). These chromatograms were compared with standard daidzein and standard genistein. (1= daidzein and 2= genistein)

2.4 HPLC analysis of ethyl acetate crude extracts of some varieties and cell cultures of *Pueraria candollei*

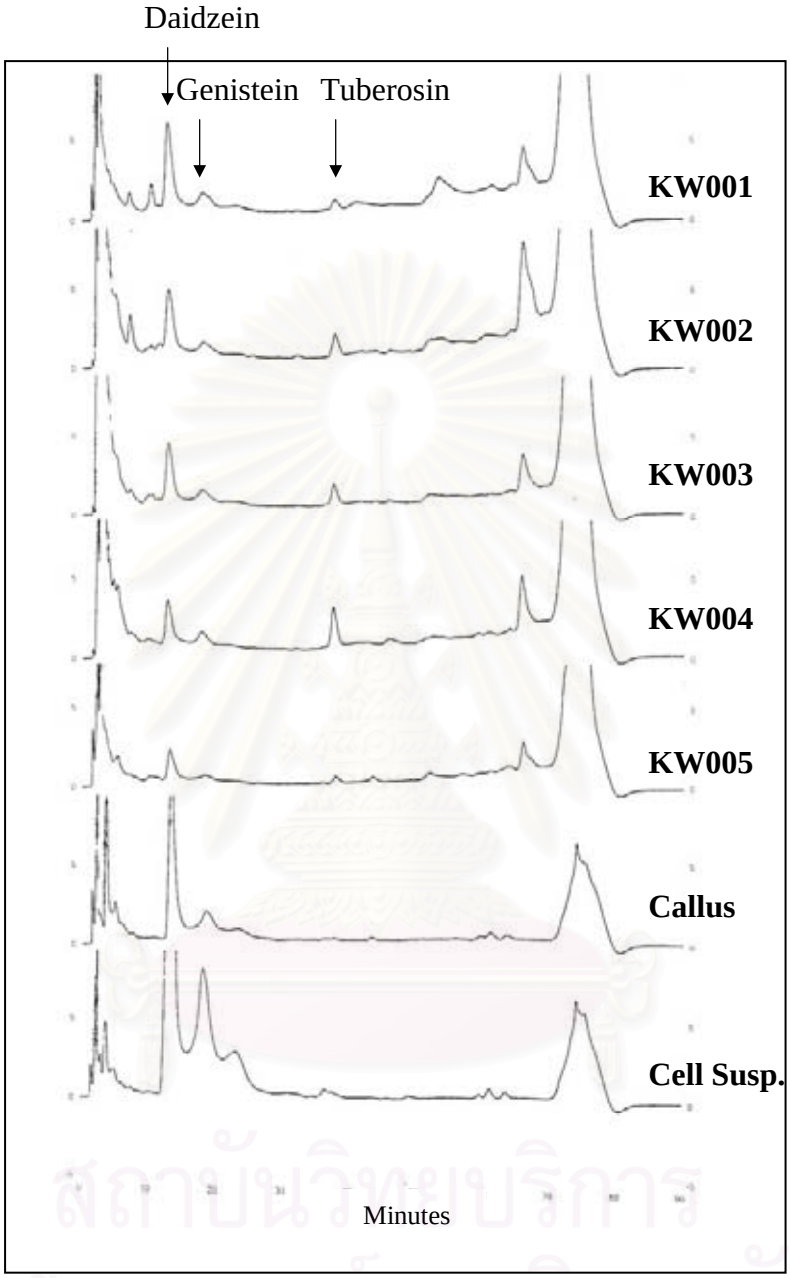


Figure 15: The chromatograms from HPLC analysis of ethyl acetate crude extracts from some varieties and cell cultures of *Pueraria candollei*

2.5 HPLC analysis of methanol crude extracts of some varieties and cell cultures of *Pueraria candollei*

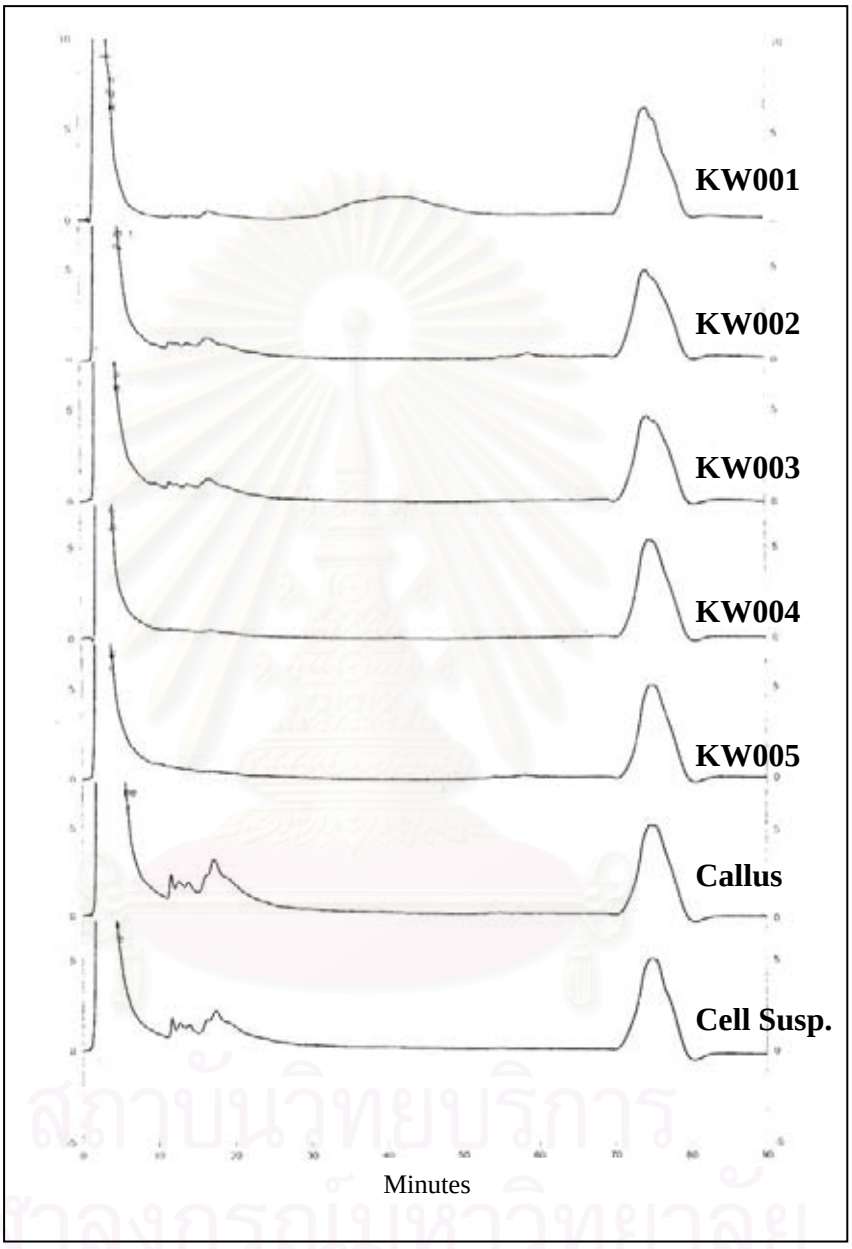


Figure 16: The chromatograms from HPLC analysis of methanol crude extracts from some varieties and cell cultures of *Pueraria candollei*.

3) The quantitative study of secondary metabolite in *Pueraria candollei*

The ethyl acetate and methanol crude extracts of *Pueraria candollei* cell cultures were determined the estrogenic activity with MCF-7 cell. It was found that they had estrogenic activity at low concentration and had cytotoxic activity at high concentration. The ethyl acetate crude extracts of *Pueraria candollei* (Udon Thani) were spotted on TLC plate compared with standard compounds, daidzein and genistein. They were developed in solvent system; chloroform and methanol (9.2: 0.8). The TLC pattern under UV light shown cell cultures produced daidzein and genistein. This result was confirmed with HPLC analysis. The quantitative analysis of daidzein and genistein production was measured by standard curve of each standard.

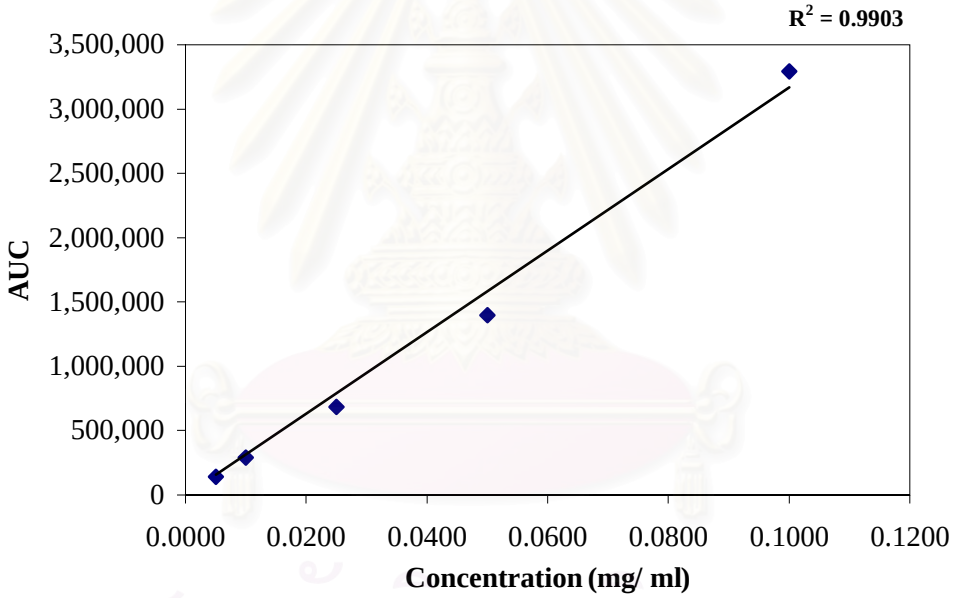


Figure 17: Standard curve of daidzein, AUC at 280 nm

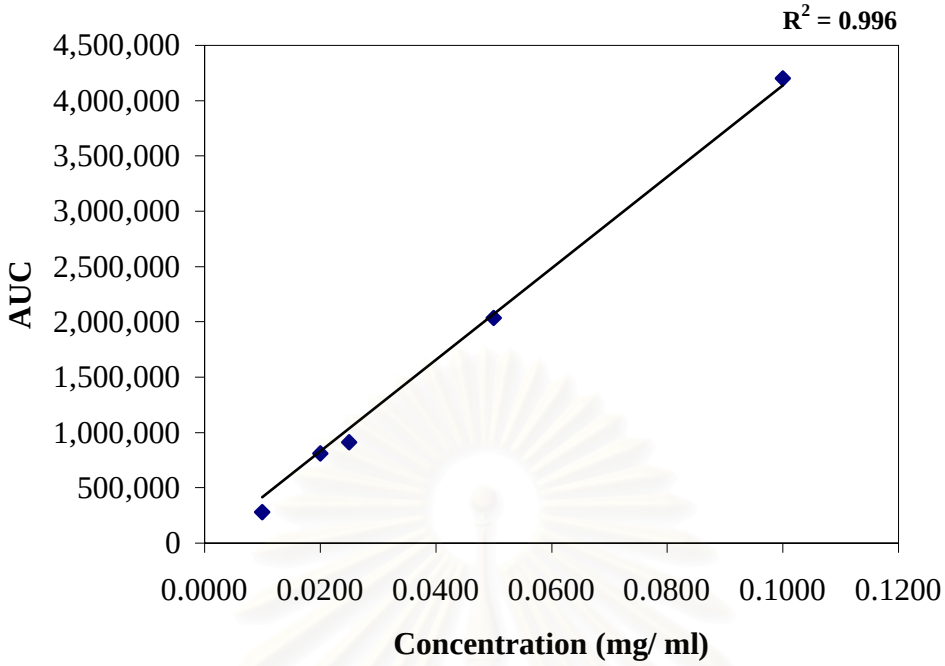


Figure 18: Standard curve of genistein, AUC at 280 nm

The quantitative analyses of secondary metabolite production in *Pueraria candollei* cell cultures were determined with HPLC analysis. The area under the curve of each sample was compared with the standard curve of daidzein and standard genistein. The ethyl acetate crude extract of callus and cell suspension culture of *Pueraria candollei* were measured. Daidzein and genistein were found in both cell cultures. The ethyl acetate crude extract of some cultivars of *Pueraria candollei* were also measured with this method.

3.1 Quantification of daidzein

Table 7: The result of quantification analysis of daidzein in cell cultures and some varieties of *Pueraria candollei*

Sample	Daidzein (mg)	%
Callus of *P. candollei*	14.9095 ± 7.5229	0.76
Cell suspension of *P. candollei*	75.6373 ± 42.3444	3.07
P. candollei (KW001)	2.0836 ± 0.9005	0.40
P. candollei (KW002)	2.6220 ± 0.9966	0.29
P. candollei (KW003)	2.3074 ± 0.8399	0.27
P. candollei (KW004)	0.9196 ± 0.9107	0.10
P. candollei (KW005)	1.0321 ± 0.1555	0.13

3.2 Quantification of genistein

Table 8: The result of quantification analysis of genistein in cell cultures and some varieties of *Pueraria candollei*

Sample	Genistein (mg)	%
Callus of *P. candollei*	0.9120 ± 0.6067	0.05
Cell suspension of *P. candollei*	11.9698 ± 5.5051	0.49
P. candollei (KW001)	0.0387 ± 0.0014	0.01
P. candollei (KW002)	0.0904 ± 0.0042	0.01
P. candollei (KW003)	0.1548 ± 0.0245	0.02
P. candollei (KW004)	0.2302 ± 0.4101	0.03
P. candollei (KW005)	0.0009 ± 0.0000	< 0.01

The yields of daidzein are 0.76% and 3.07% in callus and cell suspension culture, respectively. The yields of genistein are 0.05% and 0.49% in callus and cell suspension culture, respectively. After that, the daidzein in ethyl acetate crude extracts of some varieties of *Pueraria candollei* were measured with HPLC analysis. The ethyl acetate crude extract of five varieties were analyzed with HPLC method and compared the area under the curve of each sample with standard curve. The yields of

daidzein of five cultivars were shown in the Table 7. The qualitative analysis of ethyl acetate crude extract found genistein in all cultivars of *Pueraria candollei*. The yields of genistein in these cultivars ethyl acetate crude extract were shown in Table 8.

In process of cell suspension culture, we found the color of MS medium changed to orange. After harvest the cells, MS medium was partition with ethyl acetate. The ethyl acetate layer was collected, removed the water with sodium sulfate and evaporated under the vacuum. It was spotted on TLC plate compared with ethyl acetate crude extract of cell suspension and ethyl acetate crude extract of *Pueraria candollei*

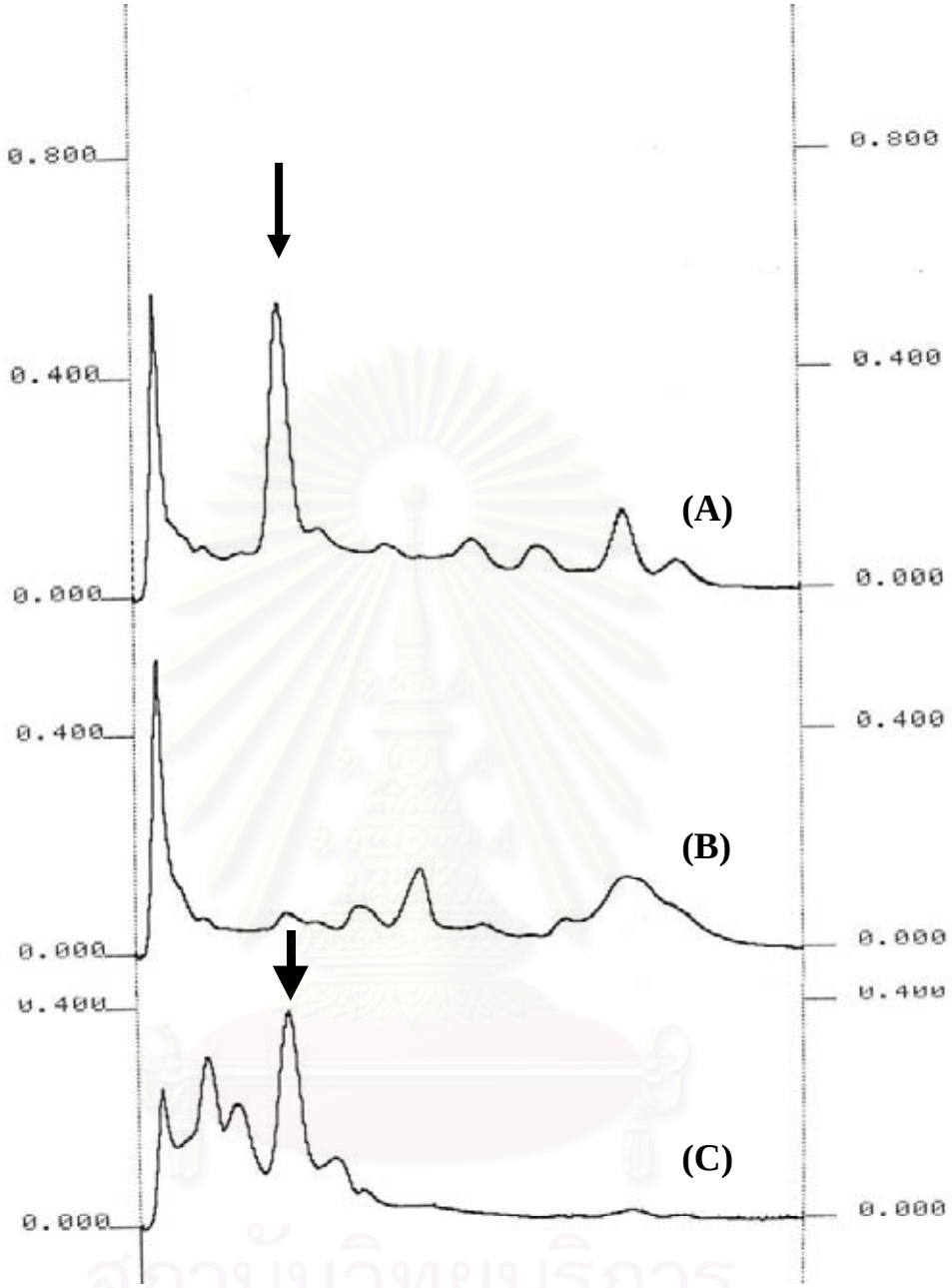


Figure 19: These are chromatograms of ethyl acetate crude extract. They were scanned with TLC densitometer at 280 nm. **(A)** Ethyl acetate crude extract of *Pueraria candollei* cell suspension culture **(B)** Ethyl acetate crude extract of *Pueraria candollei* and **(C)** Ethyl acetate extract of used MS medium from cell suspension culture of *Pueraria candollei*.

4) Estrogenic activity of various fraction of *P. candollei*

The proliferation of estrogen-dependent cancer cells has been used for estrogenic activity test. MCF-7 cells are estrogen-dependent cancer cells used in this study. The positive control is estradiol (Fig.20). All samples were determined for their estrogenic activity. First, the crude methanolic crude extracts of some varieties of *P. candollei* and cell cultures were tested with MCF-7 cells. Subsequently, the ethyl acetate crude extracts were tested. The determination of estrogenic activity with MCF-7 cells used the procedure of Sato *et al*., 1995. It was found that all the methanolic extracts of these samples had estrogenic activity (Fig.21). The most of the ethyl acetate extracts of these varieties had cytotoxic activity to MCF-7 cells (Fig.22).

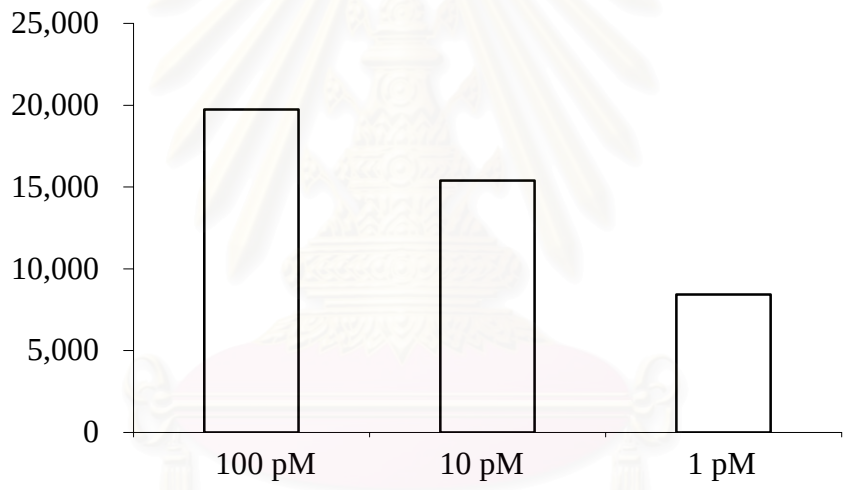


Figure 20: The estrogenic activity of positive control

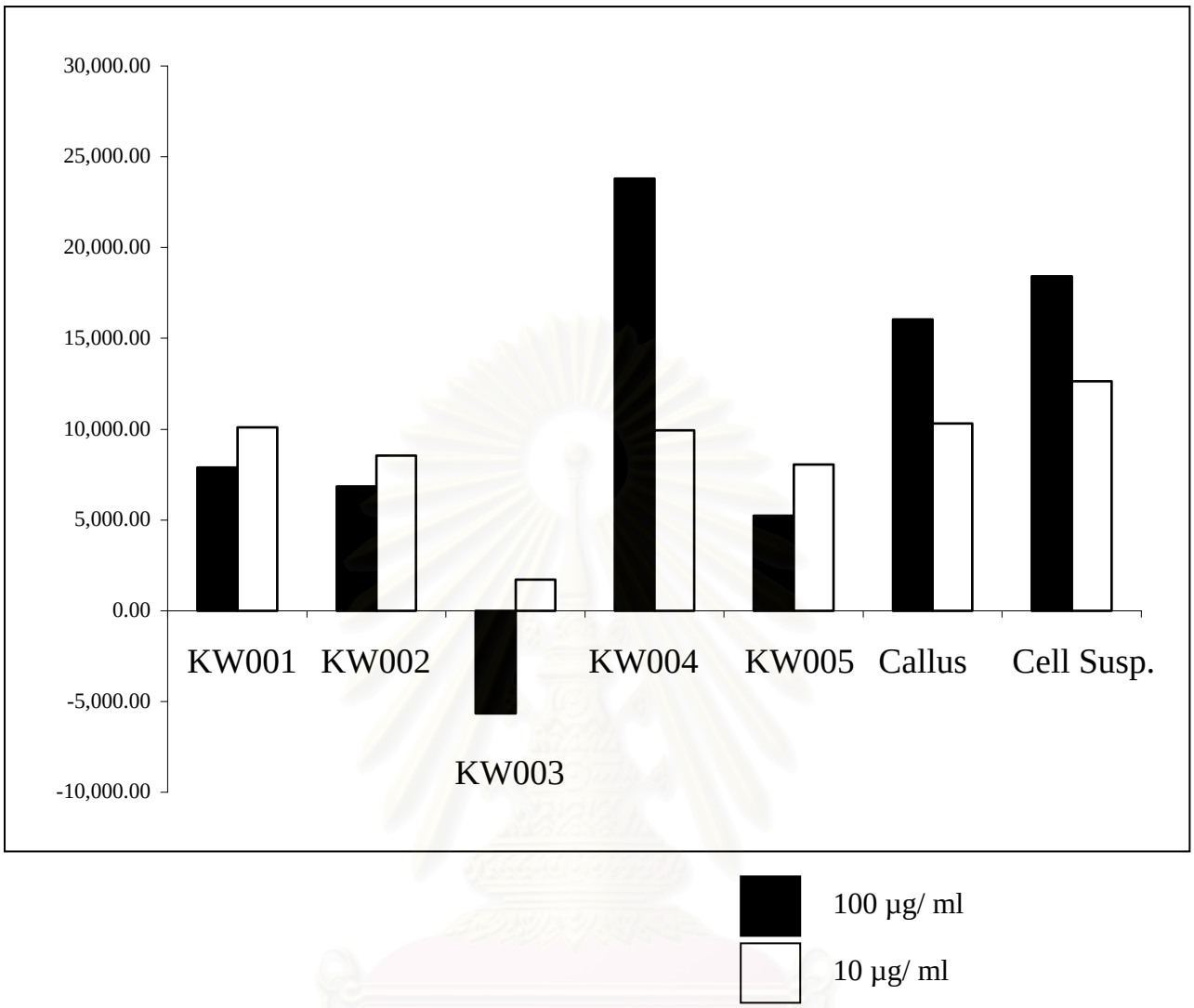


Figure 21: Estrogenic activity results of methanolic crude extracts of some *Pueraria candollei* varieties and cell cultures

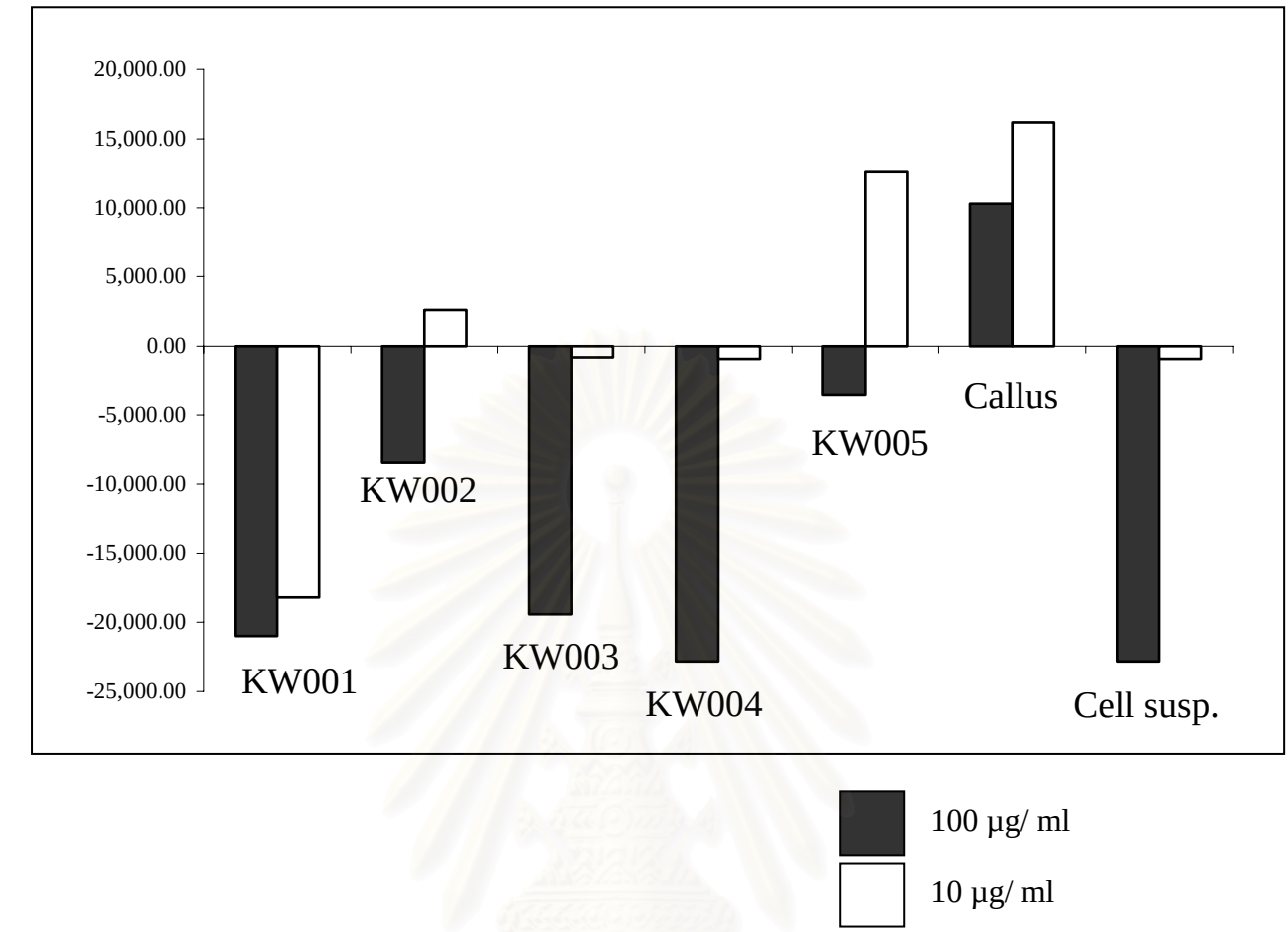


Figure 22: Estrogenic activity results of ethyl acetate crude extracts of some *Pueraria candollei* varieties and cell cultures

5) Boronate affinity chromatography

In our study, boronate affinity chromatography was applied for the separation of prospective active compounds from the crude extracts of *Pueraria candollei*. The adsorbents used was *m*-aminophenyl boronic acid on acrylic beads. In alkaline solution (pH 8.8), they could bind to the *cis-diol* containing compounds. From the study of structure modeling with stick and ball model, miroestrol and its derivative might be able to bind to the adsorbents in alkaline solution. The hydroxyl groups at C-18 and C-19 of these structures look like *cis-diol* when the 5-membered ring was form.

In practice, the crude extract of each sample was weighted and dissolved in 5 ml of 0.25 M ammonium acetate in methanol, pH 8.8. The solution was loaded into the glass column containing *m*-aminophenyl boronic acid on acrylic beads in alkaline solution. The stopcock was opened to let the solution pass through the adsorbents slowly, approximately 1 ml/ min. For the next step, 50 ml of 0.25 M ammonium acetate in methanol, pH 8.8 was used for wash the unbound compounds from the column followed by the changing the pH of the column by passing 50 ml of 0.1 M formic acid in methanol to elute the bound compounds from the adsorbents. The bound fraction of each sample was the spotted on to a TLC plate and the plate was developed with the solvent system: chloroform and methanol (9.2: 0.8). After the development, the TLC plate was scanned with the TLC densitometer at 280 nm. The signal was expanded to 8 fold.

5.1 Boronate affinity chromatography of *Pueraria candollei* (KW004) methanolic and ethyl acetate extracts

Pueraria candollei (KW004) crude extracts were chosen to try with boronate affinity chromatography. All fractions of this sample were evaporated under vacuum. They were analyzed by TLC method and HPLC (with photo diode array detector).

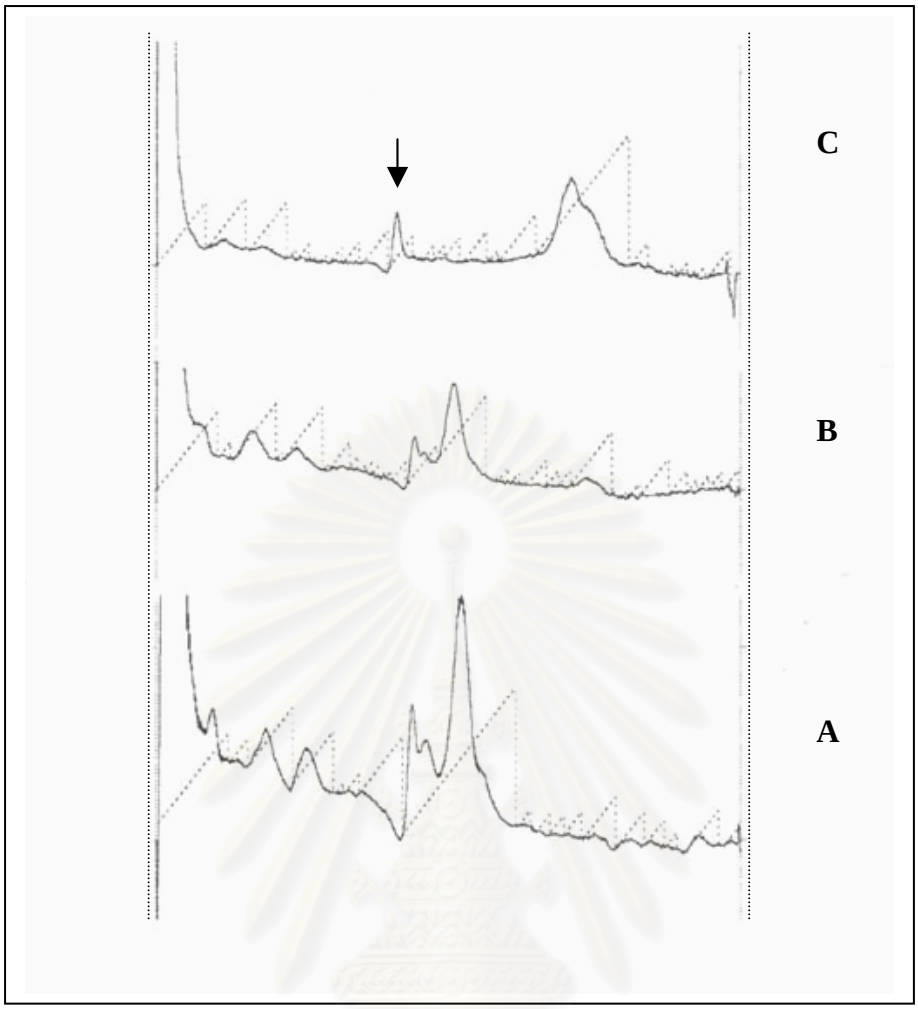


Figure 23: TLC densitometric chromatograms of methanolic extracts of *Pueraria candollei* (KW004) by scanned at 280 nm, X 8. **A**; the chromatogram of load fraction (unbound fraction) **B**; the chromatogram of wash fraction (unbound fraction) and **C**; the chromatogram of elute fraction (bound fraction)

Figure 23 shows the TLC densitometric chromatogram of 3 fractions obtained from the boronate column namely load, wash and elute fractions. It can be seen that some compounds in methanolic extract could bind to the functional group of *m*-aminophenylboronic acid and could be eluted from the column by decreasing the pH. Some compounds can bind loosely or non specific to the adsorbents. These compounds could be removed from the adsorbent in washing process. Both bound and unbound fractions obtained from the column were tested for their estrogenic activity with MCF-7 cell.

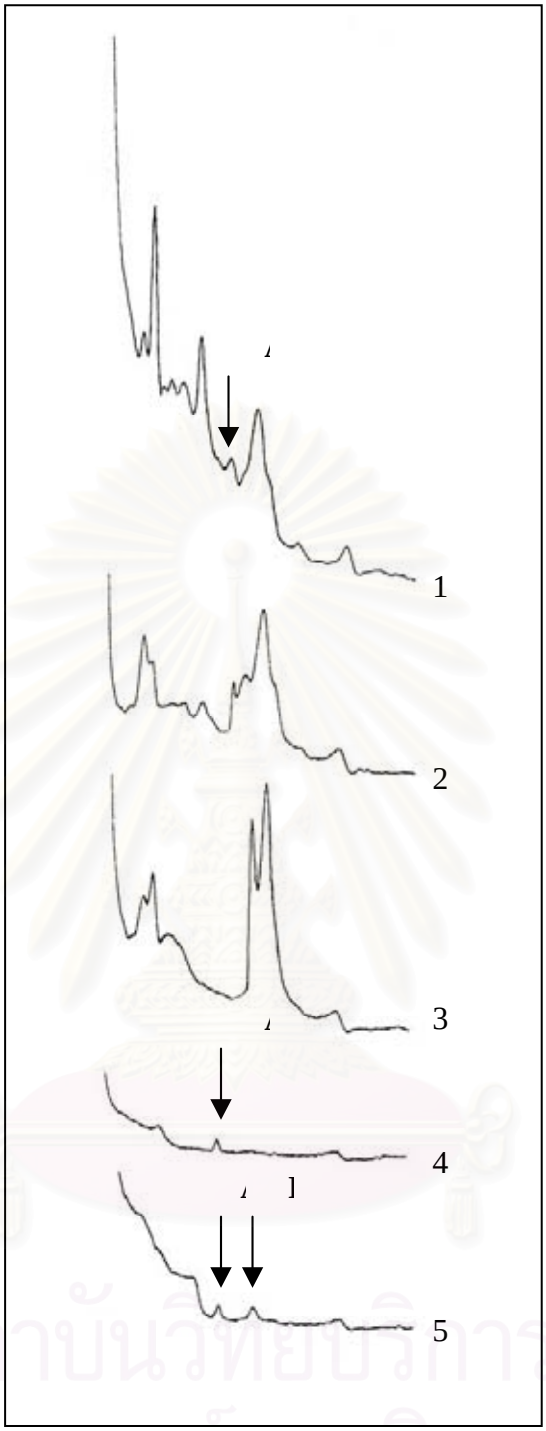


Figure 24: TLC densitometric chromatograms by scanned at 280 nm of *P. candollei* (KW004); 1: crude ethyl acetate extract, 2: load, 3: wash, 4: elute and 5: clean with 0.1 M acetic acid.

From the TLC densitometric chromatograms (Fig.24), the crude ethyl acetate extract of *P. candollei* (KW004) was tried for the partial separation by boronate affinity chromatography. After that, all the fractions include the crude ethyl acetate

extract were all spotted on the TLC plate. The solvent system was chloroform and methanol (9.2: 0.8). TLC densitometer used for the scanning was at 280 nm. The signal was expanded to 8 fold. From the result of TLC densitometry, it was found that some compounds from the ethyl acetate crude extract could be bound to the boronate adsorbents (A: R_f value = 0.4). Some amount of this compound could be eluted with 0.1 M formic acid and another part of this compound could elute from the adsorbents with 0.1 M acetic acid. In the process of cleaning of the adsorbents before re-equilibration, we could find some compounds eluted from the adsorbents (B: R_f value = 0.5). The binding of **B** to the adsorbents may be stronger than **A** and it was eluted from the adsorbents when the stronger acid was used

.

The HPLC analysis with higher sensitivity and equipped with photo diode array detector was subsequently used. All fractions of *P. candollei* (KW004) from boronate affinity chromatography were analyzed with this method. The comparison with standard and previous data, we can identify the peak of daidzein, genistein, kwakhurin, tuberosin, puemiricarpene and mirificoumestan (Fig.25).

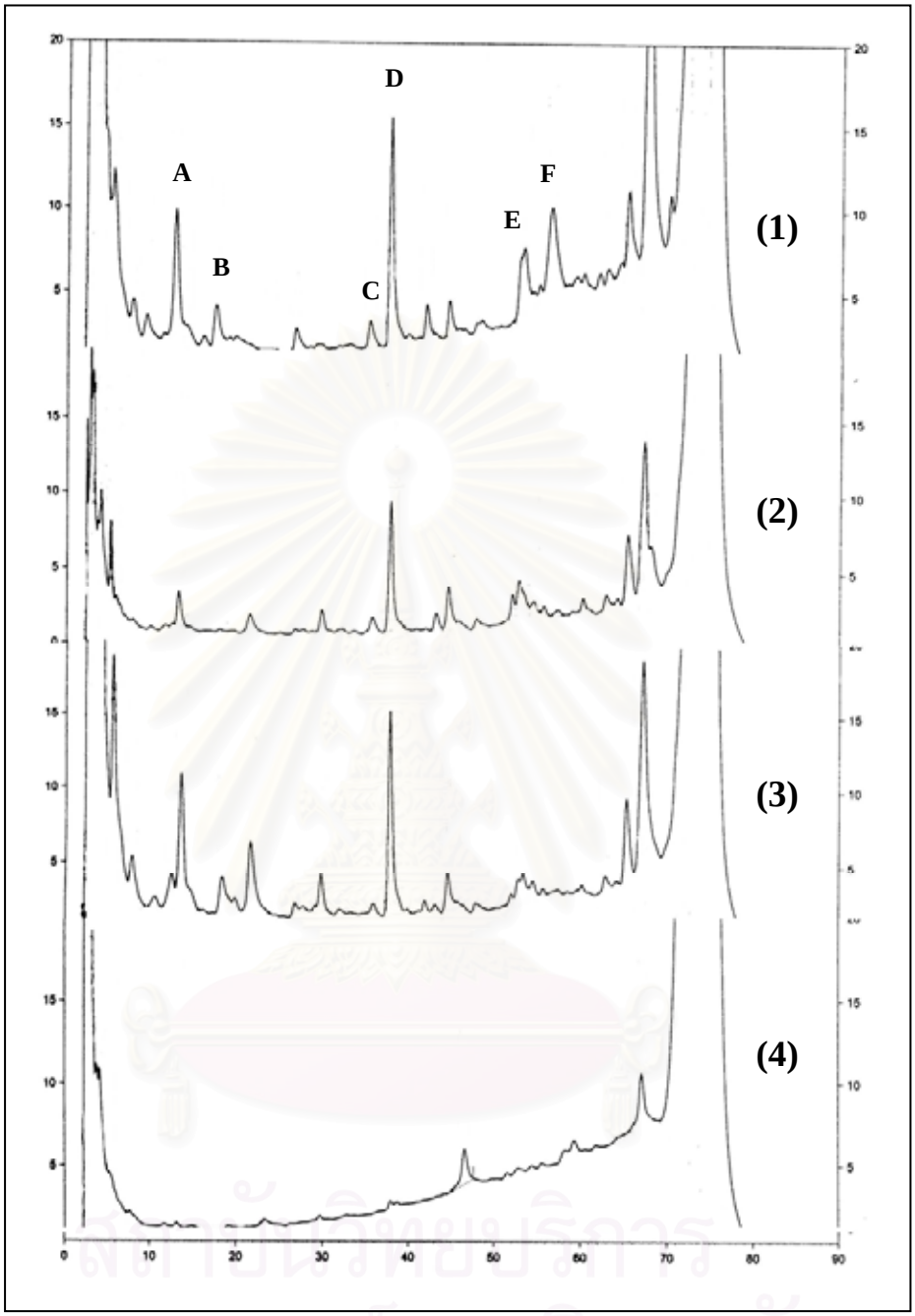


Figure 25: The HPLC chromatograms of *P. candollei* (KW004) crude ethyl acetate extract **(1)**, load fraction **(2)**, wash fraction **(3)**, elute fraction **(4)**. Some peaks of the chromatogram were identified by comparing with standard; **A**: daidzein, **B**: genistein, **C**: kwakhurin, **D**: tuberosin, **E**: puemiricarpene and **F**: mirificoumestan

The crude methanolic extract of *P. candollei* (KW004) was also studied with boronate affinity chromatography. The bound fractions of the crude ethyl acetate extract and crude methanolic extract were compared and spotted on the TLC plate and developed with the same solvent system of chloroform and methanol (9.2: 0.8). After developed, the TLC plate was scanned with the TLC densitometer at 280 nm.

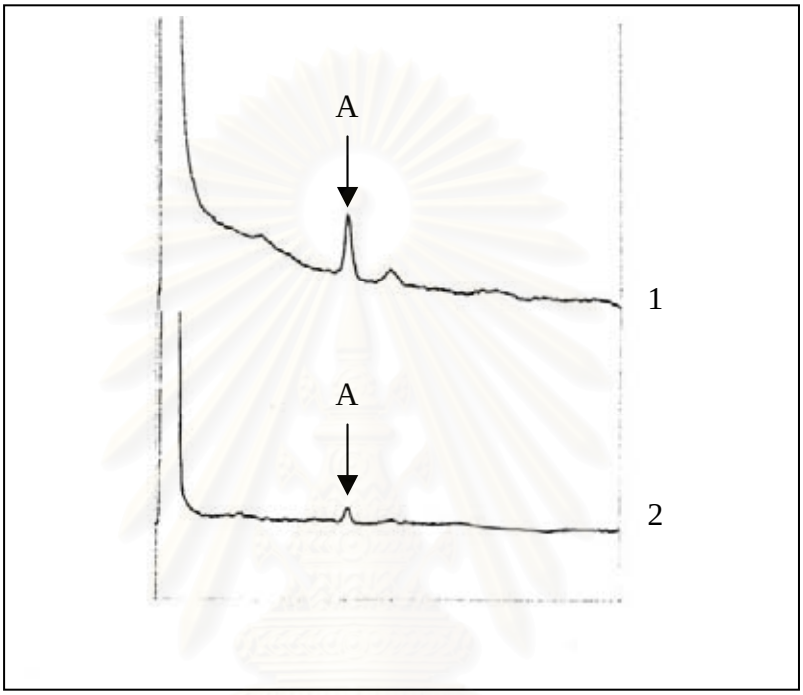


Figure 26: TLC densitometric chromatograms of *P. candollei* (KW004). **A**: bound compound R_f value = 0.4, 1: bound fraction of ethyl acetate crude extract and 2: bound fraction of methanol crude extract.

From TLC densitometric chromatograms in Fig.26, a small amount of an unknown **A** can be found in the methanolic extract. The extraction by maceration method with ethyl acetate could extract an unknown A from the powder drug, but not completely since it could subsequently be extracted with methanol. However, most of unknown A was extracted by ethyl acetate.

5.2 Boronate affinity chromatography of some varieties and cell cultures of *Pueraria candollei* methanolic and ethyl acetate extracts

The ethyl acetate crude and methanolic extracts of the other varieties: KW001, KW002, KW003, KW005 and the callus and cell suspension culture also studied by the partial separation with boronate affinity chromatography. All bound fractions of these samples were also showed the similar TLC densitometric patterns (Fig.27 and Fig.28) although with different size of peak an unknown A.

However the methanolic fraction of *P. candollei* (KW002) was found to have another big peak of high R_f value. The KW004 also showed the same peak with smaller peak size (Fig.28)

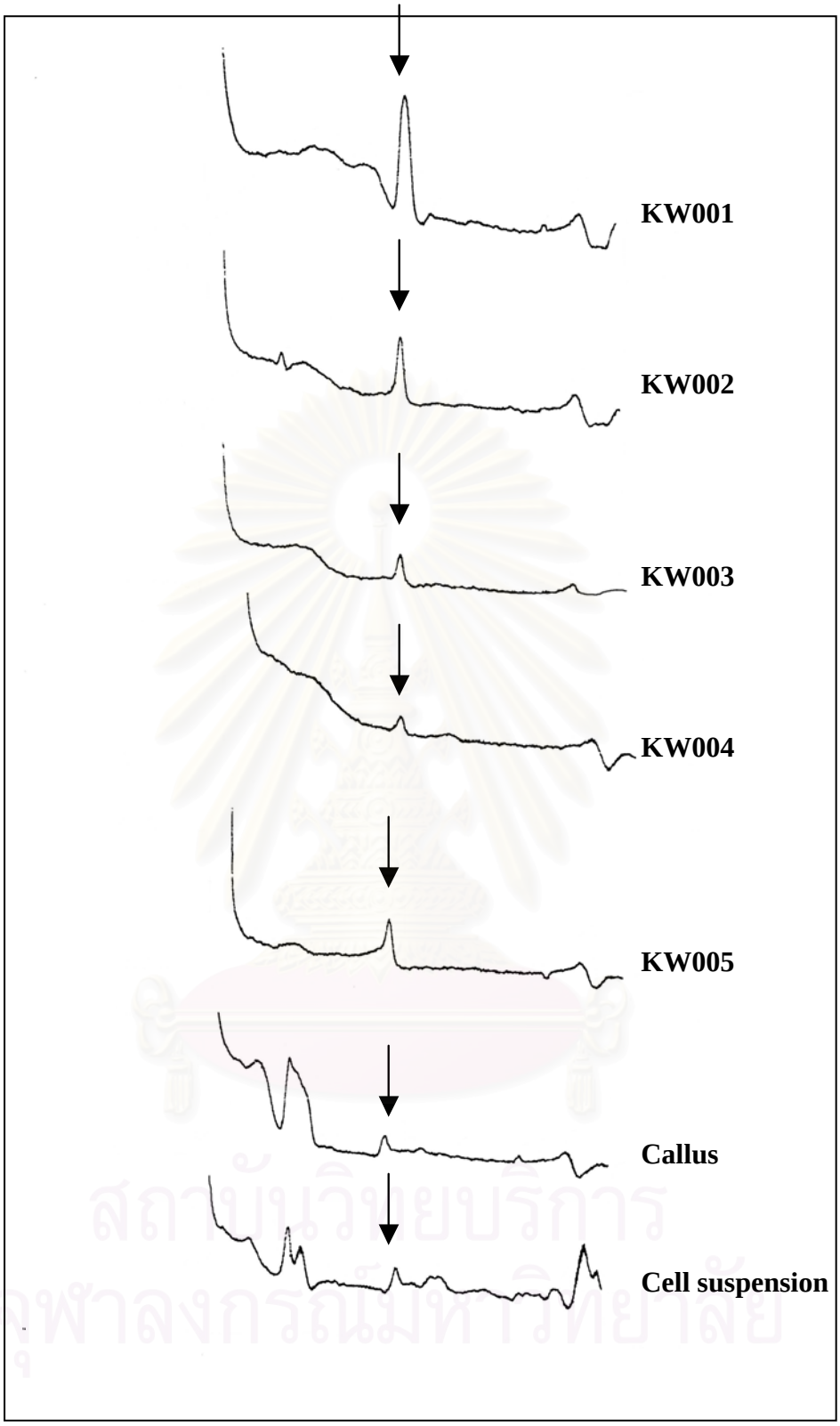


Figure 27: TLC densitometric chromatograms of the ethyl acetate crude extracts of *Pueraria candollei* KW001, KW002, KW003, KW004, KW005, Callus and Cell suspension

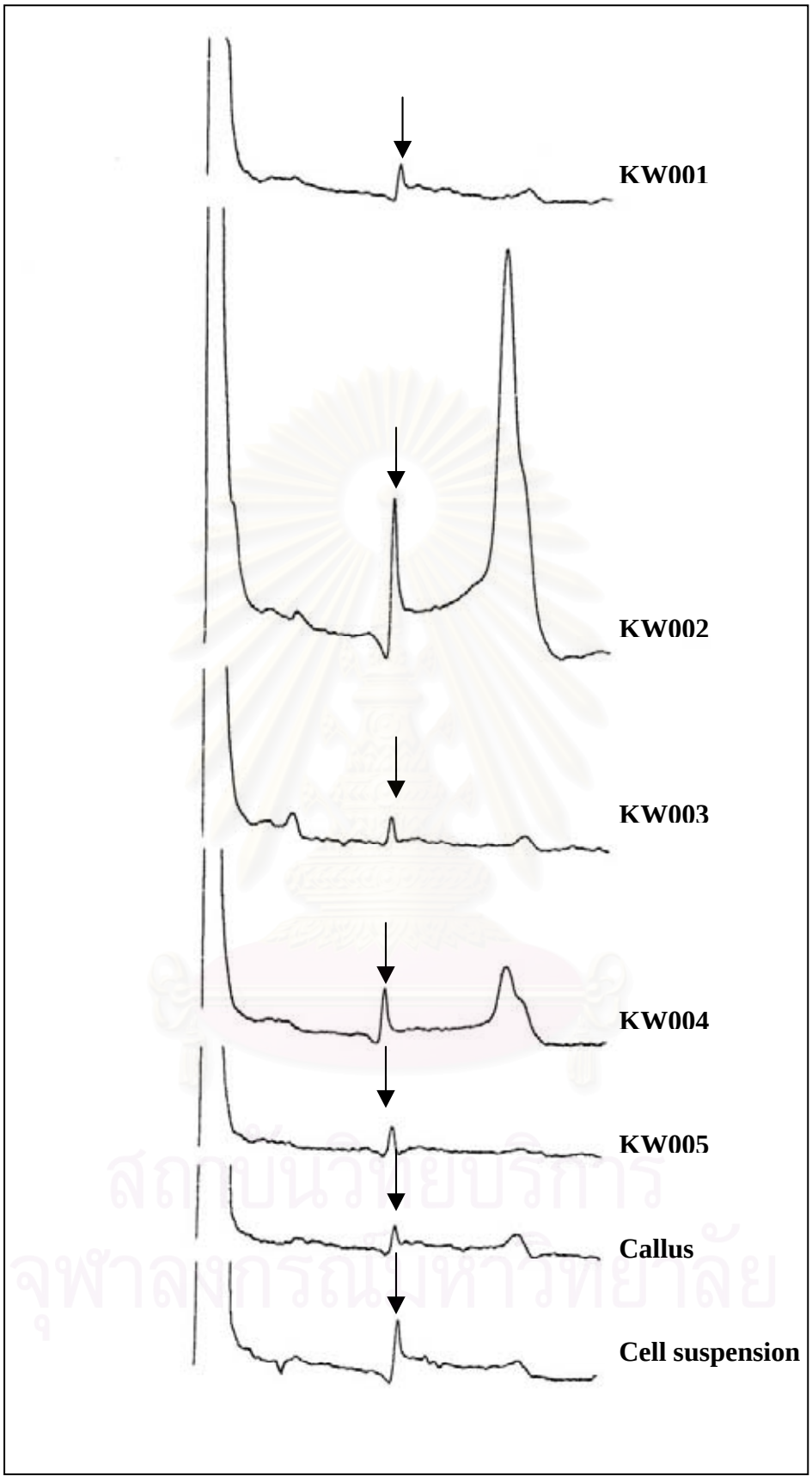


Figure 28: TLC densitometric chromatograms methanol crude extract of *Pueraria candollei* KW001, KW002, KW003, KW004, KW005, Callus and Cell suspension

The crude methanolic extract of each sample was again separated with boronate affinity chromatography. Load fraction, wash fraction and elute fraction were all tested with MCF-7 cells. The activities of all fractions were compared with the activity of methanol crude extract of each sample. The results are shown in Fig.29

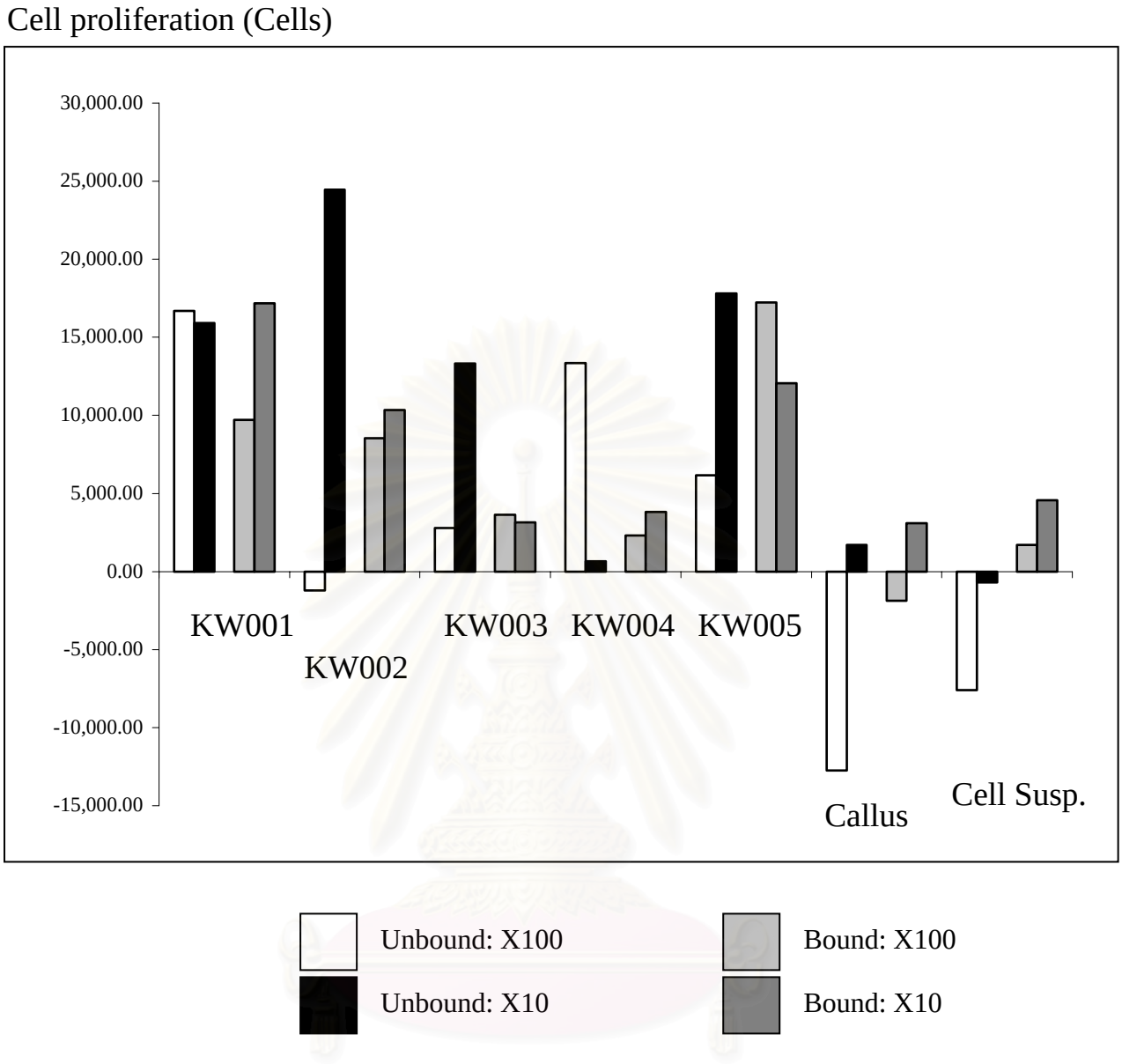


Figure 29: Estrogenic activity results of bound and unbound fraction of methanolic crude extracts of some *Pueraria candollei* varieties and cell cultures

The ethyl acetate crude extract of each sample was separated with boronate affinity chromatography. Unbound fraction, and bound fraction were tested with MCF-7 cells. The results are shown in Fig 30.

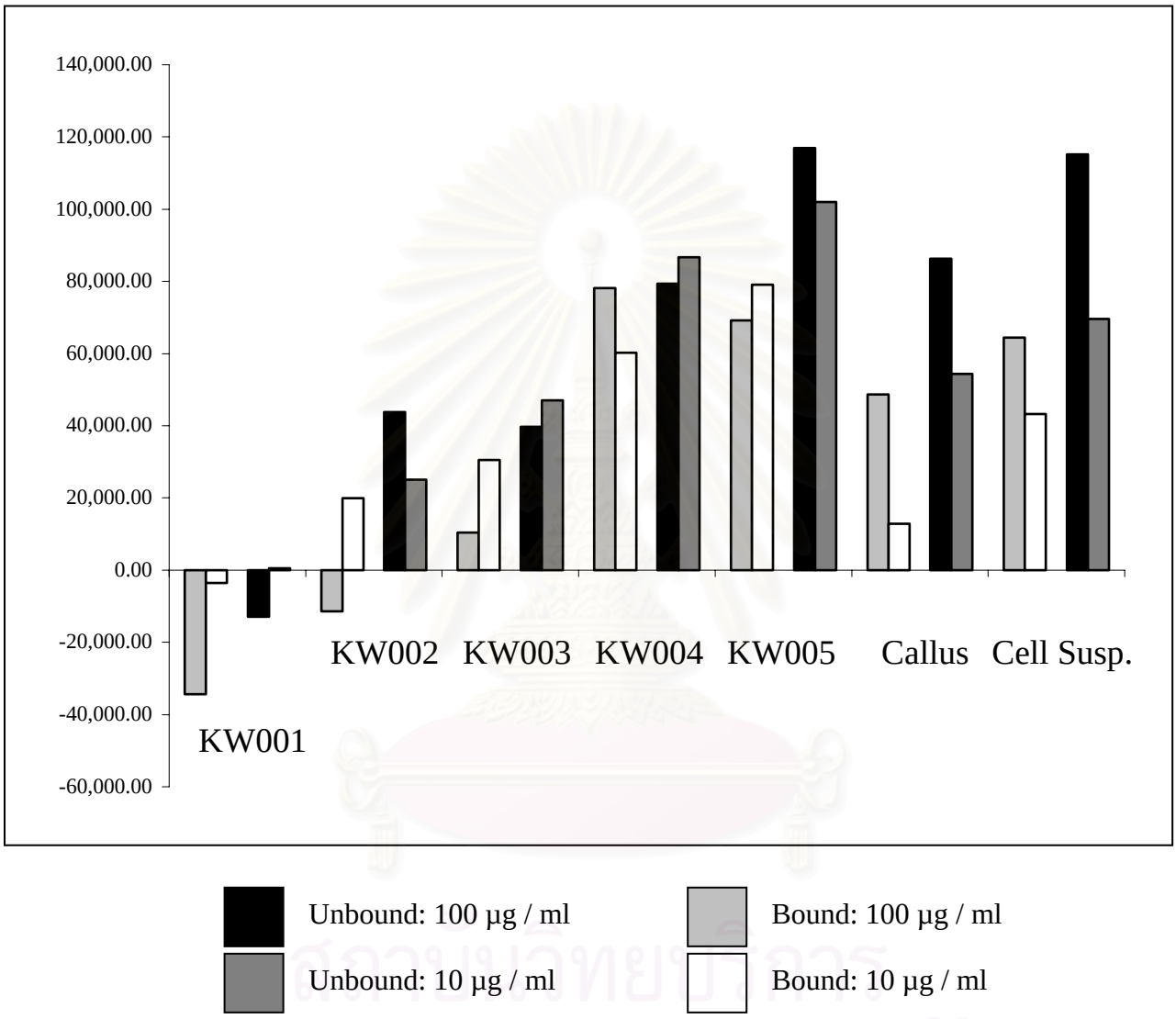


Figure 30: Estrogenic activity results of bound and unbound fraction of ethyl acetate crude extracts of some *Pueraria candollei* varieties and cell cultures

CHAPTER V

DISCUSSION

The biological activities of estrogen were reproduction and physiological processes in human and animals. The excess of estrogen can cause the estrogen dependent cancer cell such as breast cancer, ovarian cancer, endometrial cancer, etc. The deficiency of estrogen can result in menopausal symptoms, cardiovascular disease and osteoporosis. The major causes of estrogen deficiency are postmenopausal and surgical operation. The hormone replacement therapy has been required. The phytoestrogens were attention as alternative hormone replacement therapy (Oh *et al*., 2004).

Phytoestrogen is the secondary metabolite from plant that has estrogenic activity in living creatures. The methods for estrogenic activity assay were developed. MCF-7 cell is the tool for determines the estrogenic activity as E-screen. These are the estrogen dependent cancer cells. The proliferation of MCF-7 cells was observed with test compounds. Other methods are gene activation in recombinant yeast, vitellogenin or lactoferrin as biomarker etc (Korach *et al*., 1995). Some techniques had limitation in activity assay for example the crude extract of *Pueraria candollei* var.*mirifica* did not show any activity in recombinant yeast assay but show activity dose dependent on MCF-7 cell. This result indicated some compounds must have metabolic activation for estrogenic activity (Lee *et al*., 2002).

The Phytoestrogens are biological interest because they exhibit both *in vitro* and *in vivo* weak estrogenic and antiestrogenic action. The consumption of phytoestrogen-rich diet protects against breast and prostate cancers, cardiovascular disease and alleviates estrogen deficiency symptom in postmenopausal women. Phytoestrogen were unable to antagonized E2-stimulated growth, suggesting that the mechanism where by phytoestrogen may function as an antiestrogen is different from the mechanism by which phytoestrogen stimulate cell proliferation (Ramanathan *et al*., 2003). Genistein has been the phytoestrogen of greatest interest, *in vitro*, has been shown to exert both proliferation (estrogenic) and antiproliferative (antiestrogen). In the human estrogen receptor (ER)-positive MCF-7 breast cancer cell line, these

effects are biphasic and concentration dependent, with stimulation of cell proliferation at low concentration of genistein (10^{-5} – 10^{-8} M) and inhibition at higher concentration (10^{-4} – 10^{-5}M). The antiproliferative effects of genistein occurred in both ER-positive and ER-negative cell lines. It has been proposed that genistein and other phytoestrogens inhibit tumor cell growth by interfering with the tyrosine kinase activity of activated growth factor receptors and cytoplasmic tyrosine kinase, which are essential for the transduction of mitogenic signals (Murkies *et al*., 1998). The molecular mechanism of action of the genistein was identified. The concentration at 0.15 mM of genistein caused MCF-7 apoptotic cell death. Genistein's dual roles of protein tyrosine kinase inhibitor and topoisomerase II inhibitor are essential for the initiation of apoptosis (Constantinou *et al*., 1998). The novel mechanism of genistein is inhibiting the proteasome activity. The proteasome is a potential target of genistein in human tumors cells and that inhibition of the proteasome activity by genistein might contribute to its cancer-preventive properties (Kazi *et al*., 2003). The up-regulated genes in MCF-7 cells in response to high concentration of genistein treatment are genes associated with stress response (HSP 105), transcription (protein kinase, Y linked, PRKY) and salvage-pathway enzyme for dTTP formation (TK 1). A recent study, the over expression of the HSP 105 can enhance the stress-induced apoptosis. The up-regulation of HSP by genistein in MCF-7 cells provides direct evidence for the activation of stress pathways possibly an enhancement of the process of apoptosis (Chen *et al*., 2003).

Isoflavonoids were found in tuberous root of *Pueraria candollei* var.*mirifica*. They have estrogenic activity (Chansakaow *et al*., 2000). In Leguminosae members, many kind of plant have isoflavonoids and expressed the estrogenic activity in biological assay. *Pueraria lobata* is a member of this family that has been study. A methanol extract from this plant showed a suppressive effect on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen furylfuramide (Miyazawa *et al*., 2001). The other activity of isoflavonoids from the *Pueraria* sp. was determined as free radical scavenging activity. The free radical scavenging activity of isoflavonoid compounds were attributable to the hydroxyl groups on the backbone structure (Wenli *et al*., 2003)

Pueraria candollei is belonging to the Leguminosae family. It has been used as rejuvenating herb in Thai folk medicine and Burmese folk medicine. The nutritional values of this plant were determined. It was found high concentration of calcium (7.56% of powder drug) (วันชัย ดีเอกนามกูล และชาลี ทองเรือง, 2544). The histological study of *Pueraria candollei* tuberous root tissue with microscopic method was shown the crystal of calcium oxalate on the vessels. This character can cause the high level of calcium source. The shape of starch grain was similar to maize starch grain.

The biodiversity of the nature and variation such as geographical variation can cause the intermediate and variety in plant. *Pueraria candollei* is a good sample of this event. The cultivars that collected from difference places have been studied the chemical pattern and determined the estrogenic activity with MCF-7 cell. 5 varieties of *Pueraria candollei* were collected from Kasetsart University, Kampangsaen campus. They were collected from difference places in Thailand and cultivated in the area of Kasetsart University, Kampangsaen campus for 2 years (KW005; 1 year). The tuberous roots of them were prepared into powder and extract with hexane, ethyl acetate and methanol. The ethyl acetate and methanol crude extracts were determined the estrogenic activity by MCF-7 cell. Chansakaow *et al*., 2000 found the most potent isoflavonoids such as miroestrol and deoxymiroestrol are in ethyl acetate crude extract. The estrogenic activity of ethyl acetate crude extract were most cytotoxic effect, but methanol crude extracts found proliferation of MCF-7 cell.

From the results of estrogenic activity test, the methanol crude extracts of varieties KW001, KW002, KW003, KW004, KW005, callus and cell suspension of *Pueraria candollei* had estrogenic activity, The ability of MCF-7 cells in proliferation is up to kind of phytoestrogen and concentration. Previous data reported that the potent phytoestrogen of this medicinal plant are in ethyl acetate crude extract and we can found the glycoside or glucoside in methanol crude extract. MCF-7 cells proliferation assay results found that methanol crude extracts had estrogenic activity while most ethyl acetate crude extracts had cytotoxic effect to MCF-7 cells. Because of these data, the methanol crude extract and ethyl acetate crude extract of each sample were analyzed by HPLC method with photo diode array detector. The HPLC

chromatograms were compared with authentic sample and previous data, ethyl acetate crude extracts found daidzein, genistein and tuberosin and other compounds while HPLC chromatograms of methanol crude extracts shown the chemical pattern in each samples difference from the ethyl acetate crude extracts. The polar compounds in methanol crude extracts such as glycoside, glucoside and sucrose were appeared in first 10 minutes of the cycle time. Although in methanol crude extracts had polar compounds, they could produce the proliferation of MCF-7 cells. In case of ethyl acetate crude extracts, the results of estrogenic activity assay with MCF-7 cells had shown the cytotoxic effect in high concentration of samples. Because of high concentration of phytoestrogen can express the antiestrogen activity, especially genistein. From the HPLC chromatograms of ethyl acetate crude extract, we can found daidzein, genistein and tuberosin. The amount of these compounds was higher than methanol crude extracts, so MCF-7 cells assay shown cytotoxic effect. The chemical pattern in each sample had main compounds similar, but difference in concentration. They had some differences in kind of some compounds and concentration. Because of these results, the estrogenic activity test results were shown the proliferation of MCF-7 cells up to kind and concentration of phytoestrogen. The concentrations of ethyl acetate crude extracts that used for estrogenic activity test were 1, 10 and 100µg/ ml. The lower concentration such as 0.1, 0.01µg/ ml of each sample may bring a good result. The cytotoxic and false negative results were express in high concentration of phytoestrogen.

Boronate affinity chromatography was used for separation the low to moderate molecular weight compounds. Affinity chromatography or bioselective adsorption is the technique for purification of the protein as enzyme. It is a simple method and contains a few steps in procedure with higher yield of purification and save the time in work (Scouten, 1981). Miroestrol and its derivatives had a hydroxyl group at C-17 and C-18 of ring D and the ethylene bridge made 2 hydroxyl groups look like *cis*- diol group. Boronate affinity chromatography has been used for separation of the compound that contain *cis*-diol group in molecule. This technique was applied for separate miroestrol and its derivatives from this plant. The *cis-diol* hydroxyl groups of these derivatives were monitor on computer modeling. It is look like *cis-diol* but not clear. We can call them pseudo-*cis*-diol group. Both crude extract from these samples were used to separate with boronate affinity chromatography. Load, wash and elute

fractions were determined the estrogenic activity with MCF-7 cell and they were analyzed chemical pattern with TLC densitometry and HPLC analysis.

First, the methanol crude extract of *Pueraria candollei* (KW004) that has potent estrogenic activity separated with boronate affinity chromatography. Load, wash and elute fraction were spotted on TLC plate and developed with chloroform and methanol (9.2: 0.8). The TLC plate was scanned with TLC densitometer for chromatogram. It was found that some compounds in methanol crude extract could bind to boronic acid group and eluted with decreasing pH. Second, the ethyl acetate crude extract of *Pueraria candollei* (KW004) was separated with boronate affinity chromatography. The elute fraction was spotted on TLC plate and developed with same solvent system and scanned with TLC densitometer. It was found some peak between R_f values 0.6-0.7. After that all fractions from boronate affinity chromatography of *Pueraria candollei* (KW004) were analyzed by HPLC with photo diode array detector. At the retention time 46.645 min, we could found one peak that has UV spectrum pattern similar to miroestrol. The collection of this peak for determined the real structure with spectroscopy method (NMR, MS, IR, etc) is necessary. In ethyl acetate crude extract of *Pueraria candollei* (KW004), six isoflavonoids were identified with comparison of chromatogram. They were found daidzein, genistein, kwakhurin, tuberosin, puemiricarpene and mirificoumestan. Both six isoflavonoids were found in load and wash fraction. It was indicated both six isoflavonoids could not bind to boronic acid group.

The potential of plant cell cultures for secondary metabolite production is interest. The tuberous root tissue of *Pueraria candollei* was used for established the cell cultures. Callus culture and cell suspension culture of this plant were harvested, dried and extract with organic solvents. The ethyl acetate crude extract of both samples were determined the secondary metabolites production with TLC analysis and HPLC analysis. It was found that both type of cell culture could produce daidzein and genistein that are the general isoflavonoids in leguminous plant. The quantitative analysis of both compounds was measured by compare with standard curve of daidzein and genistein. The amount of daidzein and genistein in cell suspension culture higher than callus culture and mother plan. This event indicated that some enzymes in phenylpropanoid biosynthesis pathway are express and some enzymes not

express in this condition. In case of *Pueraria lobata* cell cultures, some enzymes in biosynthesis partway were express when elicited with elicitors (Hakamatsuka *et al*., 1994).

The ethyl acetate crude extract and methanol crude extract of *Pueraria candollei* callus culture has estrogenic activity with MCF-7 cells. In case of ethyl acetate crude extract, low concentration of ethyl acetate crude extract can cause the MCF-7 cells proliferate more than higher concentration. This result is similar to genistein, the proliferation of MCF-7 cell depend on the concentration of phytoestrogen. In case of methanol crude extract, it contains a polar part of this sample. It was expressed the estrogenic activity in MCF-7 cells, higher concentration of this crude made more proliferation of MCF-7 cells. These results suggest that phytoestrogen in ethyl acetate crude extract has more estrogenic activity than phytoestrogen in methanol crude extract. The ethyl acetate crude extract and methanol crude extract of *Pueraria candollei* cell suspension culture also has estrogenic activity similar to *Pueraria candollei* callus culture, but the concentration of phytoestrogen in *Pueraria candollei* cell suspension ethyl acetate crude extract may higher than *Pueraria candollei* callus ethyl acetate crude extract. The cell suspension culture of this plant is good material for isoflavonoids and prenylated isoflavonoids biosynthesis partway study.

Cell suspension of *Pueraria candollei* has been produced isoflavonoids. In 10^{th} day, cell suspension culture media had changed the color. It was become orange. Cell suspension was collected and media from culturing process was collected. The medium from culturing process was partition with ethyl acetate. The ethyl acetate layer was collected and evaporated under the vacuum. This extract was spotted on TLC plate compared with ethyl acetate crude extract of cell suspension. They were developed with chloroform and methanol (9.2: 0.8). TLC plate was scanned with TLC densitometer at 280 nm. Chromatograms were show some compound excreted from cell suspension to the media. Because of small vacuole of cell, the secondary metabolites that produced by cell were excreted out of cell. These compounds may be toxic to the cell. This result indicated that sub-culturing of cell suspension was necessary in 10^{th}-14^{th} day.

CHAPTER VI

CONCLUSION

Following the results and discussions of this research work, the conclusions are:

1) *Pueraria candollei* has many varieties. The geographical variation made them change in the morphology and chemical pattern.
2) Cell cultures of *Pueraria candollei* has been established with MS medium supplemented with plant hormones: 2, 4-D, BA, NAA (1: 0.1: 0.1) and 3 % sucrose as carbon source.
3) Cell cultured of *Pueraria candollei* has potential to produce the secondary metabolites. Callus and cell suspension of this medicinal plant has produce daidzein and genistein. These are common isoflavones that can found in the leguminous plants. The yield of isoflavones production in cell suspension is higher than callus culture.
4) MCF-7 cells are tool for estrogenic activity scanning. Both samples have estrogenic activity. The methanol crude extracts of *Pueraria candollei* has high estrogenic activity. The proliferation of MCF-7 cells was increased in high concentration of crude extract and at higher concentration of crude extract, they has cytotoxic to MCF-7 cells.
5) High concentration of isoflavones can cause the cytotoxic to the MCF-7 cells, but low concentration of isoflavones can cause the proliferation of the MCF-7 cells.
6) The ethyl acetate crude extract of *Pueraria candollei* has higher concentration of isoflavones than methanol crude extract.
7) Boronate affinity chromatography is partial separation of the active ingredient. Bound fraction of the methanol crude extract has higher estrogenic activity than other fraction.
8) Both samples of *Pueraria candollei* ethyl acetate crude extract and the ethyl acetate crude extract of callus and cell suspension culture of this plant has compound that bound to adsorbent in the alkali condition of boronate affinity chromatography. This compound has R_f value between 0.5-0.6 on the TLC plate that developed in the solvent system of chloroform and methanol (9.2: 0.8).

REFERENCES

ภาษาไทย

วันชัย ดีเอกนามกูล และชาลี ทองเรือง สถานภาพการวิจัยและพัฒนากวาวเครือขาวในประเทศไทย และสิ่งที่ควรดำเนินการวิจัย รายงานการศึกษาเสนอต่อศูนย์พันธุวิศวกรรมและเทคโนโลยีแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ, 2544

English

Bio-Rad laboratories Affi-Gel 601 Boronate Affinity Gel to Separate Ribonucleotides, Ribonucleosides, Sugars, Catecholamines, and Coenzymes (1986)

Bounds D. G. and Pope G. S. Light absorption and chemical properties of miroestrol, the estrogenic substance of *Pueraria mirifica* Journal of the Chemical Society, October-November, (1960): 3,696-3,705

Cain J. C. Miroestrol: an estrogen from the plant *Pueraria mirifica* Nature, December 3(1960): 774-777

Chansakaow S., Ishikawa T., Sekine K., Okada M., Higuchi Y. , Kudo M., and Chaichantipyuth C. Isoflavonoids from *Pueraria mirifica* and their Estrogenic Activity Planta Med, 66(2000): 572-575

Chansakaow S., Ishikawa T., Seki H., Sekine K., Okada M., and Chaichantipyuth C. Identification of Deoxymiroestrol as the actual Rejuvinating Principle of Kwao Keur: Pueraria mirifica, the known miroestrol may be an artifact Journal of Natural Products, 63, (2000): 173-175

Chen W. F., Huang M. H., Chi-Hung, Yang M., and Wong M. S. Inhibitory actions of genistein in human breast cancer (MCF-7) cells Biochemica et Biophysica Acta, 1638, (2003): 187-196

Constantinou A. I., Kamath N., and Murley J. S. Genistein Inactivate bcl-2, Delays the G2/M Phase of the cell cycle, and Induces Apoptosis of Human Breast Adrenocarcinoma MCF-7 cells European Journal of Cancer, 34, 12(1998): 1,927-1,934

Corey E. J. and Wu L. I. Enantioselective total synthesis of miroestrol J. Am. Chem. Soc., 115, (1993): 9,327-9,328

Farnsworth N. R., Bingel A. S., Cordell G. A., Crane F. A., and Fong H. H. S. Potential value of plants as sources of new antifertility agents II Journal of Pharmaceutical Sciences, 64, 5(1975): 717-753

Frantzen F., Grimsrud K., Heggli D. E., Faaren A. L., Løvli T. , and Sundrehagen E. Glycohemoglobin filter assay for doctor's offices based on boronic acid affinity principle Clinical Chemistry, 43, 12(1997): 2,390-2,396

Hage D. S. Affinity Chromatography: A Review of Clinical Applications Clinical Chemistry, 45, 5(1999): 593-615

Hakamatsuka T., Ebizuka Y., and Sankawa U. *Pueraria lobata* (Kudzu vine): In Vitro Culture and the Production of Isoflavonoids Biotechnology in Agriculture and Forestry, Medicinal and Aromatic plants VII (ed. by Y.P.S. Bajaj), 28(1994): 386-400

Hakamatsuka T., Mori K., Ishida S., Ebizuka Y., and Sankawa U. Purification of 2-Hydroxyisoflavone dehydratase from the cell cultures of *Pueraria lobata* Phytochemistry, 49, 2(1998): 497-505

Hawkins C. J. Isolation of 3,4-Dihydroxyphenylalanine-containing proteins Using Boronate Affinity Chromatography Analytical Biochemistry, 159, (1986): 187-190

Higa S. and Kishimoto S. Isolation of 2-Hydroxycarboxylic acids with a boronate affinity gel Analytical Biochemistry, 154, (1986): 71-74

Hooker J. D. Vol. II (Sabiaceae-Cornaceae) Flora of British India, (1879)

Hsieh C.Y., Santell R. C., Haslam S. Z., and Helferich W. G. Estrogenic Effects of Genistein on the Growth of Estrogen Receptor-positive Human Breast Cancer (MCF-7) Cells *in Vitro* and *in Vivo* Cancer Research, 58, (1998): 3,833-3,838

Hwang E. I., Kaneko M., Ohnishi Y., and Horinouchi S. Production of Plant-Specific Flavanones by *Escherichia coli* Containing an Artificial Gene Cluster Applied and Environmental Microbiology, (2003): 2,699-2,706

Ingram J. L., Tahara S., and Dziedzic S. Z. Minor Isoflavones from the root of *Pueraria mirifica* Zeitschrift fuer Naturforschchung section C Biosciences, 44 (9-10), (1989): 724-726

Ingram J. L., Markham K. R., Dziedzic S. Z., and Pope G. S. Puerarin 6”-O-β-Apiofuranoside, A *C*-Glycosylisoflavone *O*-Glycoside from *Pueraria mirifica* Phytochemistry, 25, 7(1986): 1,772-1,775

Jones H. E. H. and Pope G. S. A Study of the Action of Miroestrol and other Oestrogens on the Reproductive tract of the Immature Female Mouse J. Endrocrin, 20, (1960): 229-235

Jones H. E. H., Waynforth H. B. and Pope G. S. The effect of Miroestrol on Vaginal cornification, Pituitary function and Pregnancy in the rat J. Endrocrin, 22, (1961): 293-302

Jones H. E. H. and Pope G. S. A Method for the Isolation of Miroestrol from *Pueraria mirifica* J. Endrocrin, 22, (1961): 303-312

Joshi B. S. and Kamat V. N. Tuberosin, a New Pterocarpan from *Pueraria tuberosa* DC J. Chem. Soc. Perkin 1, (1973): 907-911

Kazi A., Daniel K. G., Smith D. M., Kumar N. B., and Dou Q. Ping Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell apoptosis-inducing ability of genistein Biochemical Pharmacology, 66, (2003): 965-976

Korach K. S. and McLachlan J. A. Techniques for Detection of Estrogenicity Environmental Health Perspectives (Supplement 7), 130, (1995): 5-8

Kashemsanta L. M. C. and Suvatabandhu K., and Airy Shaw H. K. A New species of Pueraria (Leguminosae) from Thailand, yielding an oestrogenic principle Kew Bulletin, Royal Botanic Gardens, (1955): 549-551

Lee Y. S., Park J. S., Cho S. D., Son J. K., Cherdshewasart W., and Kang K. S. Requirement of metabolic activation of estrogenic activity of *Pueraria mirifica* J. Vet. Sci., 3(4), (2002): 273-277

Li Y. C., Pfüller U., Larsson E. L., Jungvid H., Galaev I. Y., and Mattiasson B. Separation of Mistletoe lectins based on the degree of glycosylation using boronate affinity chromatography Journal of Chromatography A, 925, (2001): 115-121

Mallia A.K., Hermanson G.T., Krohn R. I., Fujimoto E. K., Smith P. K. Preparation and use of the boronic affinity support for the separation and quantitation of glycosylated hemoglobins Anal. Lett., 14, (1981): 649-661

Miyazawa M., Sakano K., Nakamura S., and Kosaka H. Antimutagenic activity of Isoflavone from *Pueraria lobata* J.Agric. Food Chem., 49, (2001): 336-341

Murkies A. L., Wilcox G., and Susan D. R. Phytoestrogens: Clinical Review 92 Journal of Clinical Endocrinology and Metabolism, (1998): 297-303

Niyomdham C. Note on Thai and Indo-Chinese Phaseoleae (Leguminosae-Papilionoideae) Nord. J. Bot, section of tropical taxonomy, 12(3), (1992): 345

Odum J., Tittensor S., and Ashby J. Limitations of the MCF-7 Cell Proliferation Assay for Detecting Xenobiotic Oestrogen Toxicology in vitro, 12, (1998): 273-278

Oh S. M., Chung K.H. Estrogenic activities of *Ginko biloba* extracts Life Sciences, 74, (2004): 1,325-1,335

Ramanathan L. and Gray W. G. Identification and Characterization of Phytoestrogen-specific gene from the MCF-7 human breast cancer cell Toxicology and Applied Pharmacology, 191, (2003): 107-117

Rao S. R. and Ravishankar G. A. Plant cell cultures: Chemical factories of secondary metabolites Biotechnology Advances, 20, (2002): 101-153

Setchell K. D. R., Brown N.M., Desai P., Zimmer-Nechemias L., Wolfe B.E., Brashear W. T., Kirschner A.S., Cassidy A. and Heubi J.E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavones supplements American Society for Nutritional Sciences, (2001): 1,362S-1,375S

Soto A.M., Sonnenschein C., Chung K.L., Fernandez M.F., Olea N., Serrano F.O.The E-SCREEN assay as a tool to identify estrogens: an update on Estrogenic environmental pollutants Environ Health Perspect, (1995): 103-113

Scouten W. H Affinity chromatography: Bioselective Adsorption on inert Matrices Chemistry Department, Bucknell University, Lewisburg, Pennsylvania, (1981)

Song T., Barua K., Buseman G., and Murphy P. A. (1998) Soy isoflavone analysis: quality control and a new internal standard Am. J. Clin. Nutr., 68(suppl.), (1998): 1,474S-1,479S

Tahara S., Ingram J. L., and Dziedzic S. Z. Structure elucidation of Kwakhurin, a New Prenylated isoflavone from *Pueraria mirifica* root Zeitschrift fuer Naturforschchung, section C Biosciences, 44, (1987): 510-518

Smitinand T. Thai plant names revised edition 2001, the Forest Herbarium, Royal Forest Department, (2001)

Wade H. K., Sohal A. K., and Jenkins G. I. Arabidopsis *ICX1* Is a Negative Regulator of Several Pathways Regulating Flavonoid Biosynthesis genes Plant Physiology, Febuary, 131, (2003): 707-715

Wenli Y., Yaping Z., and Shu B. The radical scavenging activities of *radix puerariae* isoflavonoids: A chemiluminescence study Food Chemistry, (2003)

Appendix

(1) Murashige and Skoog Media (MS Media): Stock solution

Stock 1 (Macronutrients) NH_4NO_3 KNO_3 $MgSO_4.7H_2O$ KH_2PO_4	**gm/ 1,000 ml** 33.00 38.00 7.40 3.40	Store in refrigerator
Stock 2 (Micronutrients) H_3BO_3 $MnSO_4.H_2O$ $ZnSO_4.7H_2O$ $Na_2MoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	**mg/ 100 ml** 620.00 1,690.00 860.00 25.00 2.50 2.50	Store in refrigerator
Stock 3 (Calcium Stock) $CaCl_2.2H_2O$	**gm/ 100 ml** 3.00	Store in refrigerator
Stock 4 (KI Stock) KI	**mg/ 100 ml** 75.00	Store in amber bottle in refrigerator
Stock 5 (Vitamins) Thiamine.HCl i-inositol	**mg/ 100 ml** 8.00 10,000.00	Store in freezer (25 ml fraction)
Stock 6 (Fe-EDTA Stock) Na_2EDTA $FeSO_4.7H_2O$	**gm/ 500 ml** 3.73 2.78	Store in refrigerator
2,4-D Stock solution 2,4-D	**mg/ 100 ml** 10.00	Dissolve 2,4-D in 5 ml Ethanol, heat slightly and gradually dilute to 100 ml with water
NAA Stock solution NAA	**mg/ 100 ml** 10.00	Dissolve NAA in 5 ml Ethanol, heat slightly and gradually dilute to 100 ml with water. Add little alkaline solution for good dissolve
BA Stock solution BA	**mg/ 100 ml** 10.00	Dissolve with water

(2) Preparation of Murashige & Skoog Media (MS Media)

Ingredients	Amount
Distilled Water	1,000 ml
Stock 1 (Macronutrients)	50 ml
Stock 2 (Micronutrients)	1 ml
Stock 3 (Calcium Stock)	5 ml
Stock 4 (KI Stock)	1 ml
Stock 5 (Vitamins)	10 ml
Stock 6 (Fe-EDTA Stock)	5 ml
Sucrose	30 gm
Agar (Solid medium)	8 gm
2,4-D Stock solution	10 ml
NAA Stock solution	1 ml
BA Stock solution	1 ml
Final pH adjust to	5.8

Methods:

1) Mix the stock solution 1 - 6 together in the beaker, stirred with magnetic bar

2) Add sucrose 30 gm.

3) Adjust the volume with distilled water to 1,000 ml in the cylinder

4) Add plant regulators

5) Adjust pH of the solution with 1 M Sodium hydroxide to 5.8

6) For callus culture, add 8 gm of agar into the solution and boil on the hot plate. Pour 20 ml of media in each glass bottle.

7) For cell suspension culture, pour 50 ml of media without agar in flask 250 ml and covered with aluminum foil.

8) Sterile with Autoclave; Temperature: 121 ºC, Pressure: 15 pound per inch^2 and time: 15 minutes.

VITA

Mr.Boonsong Wungsintaweekul was born on August 26, 1973 in Surat Thani, Thailand. He received his Bachelor of Science in Pharmacy in 1996 from the Faculty of Pharmaceutical Sciences, Prince of Sonkla University, Thailand. He received the grant as student exchange to visit the laboratory at Department of medical organic chemistry, Chiba University, Japan. At present, he is a pharmacist in the Department of Consumer Protection, Surat Thani provincial health office, Ministry of Public health, Thailand.