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นางสาวจุฑามาศ จึงสุขเจริญ

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QUANTITATIVE ANALYSIS OF MAJOR ISOFLAVONOIDS IN THE LEAVES OF WHITE KWAO KRUA *Pueraria mirifica*

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จุฑามาศ จึงสุขเจริญ: การวิเคราะห์เชิงปริมาณของสารไอโซฟลาโวนอยด์หลักในใบกวาวเครือขาว *Pueraria mirifica.* (QUANTITATIVE ANALYSIS OF MAJOR ISOFLAVONOIDS IN THE LEAVES OF WHITE KWAO KRUA *Pueraria mirifica*) อ.ที่ปรึกษา: รศ.ดร.วิชัย เชิดชีวศาสตร์, อ.ที่ปรึกษาร่วม: รศ.ดร.พลกฤษณ์ แสงวณิช จำนวนหน้า 129 หน้า.

ในการศึกษานี้ได้ทำการวิเคราะห์เชิงปริมาณของสารไอโซฟลาโวนอยด์ 5 ชนิด คือ พิวรารินไดด์ซิน เจนิสตินไดด์เซอีน และ เจนิสเตอีน ในใบกวาวเครือขาว 3 สายพันธุ์ได้แก่ PM-III, PM-IV และ PM-V มาเตรียมสารสกัดหยาบ เพื่อนำไปวิเคราะห์และศึกษาความสัมพันธ์ระหว่างปริมาณสารไอโซฟลาโวนอยด์หลักเป็นเวลา 6 เดือนตั้งแต่ เดือนมีนาคมจน 2550 ถึงเดือนสิงหาคม 2550 ต่อเนื่องกัน โดยใช้เทคนิครีเวอร์สเฟสเอชพีแอลซี เทียบกับสารมาตรฐาน พบว่าในใบกวาวเครือขาวประกอบด้วยสารไอโซฟลาโวนอยด์หลัก 3 ชนิดคือ พิวรารินไดด์ซินเจนิสติน โดยค่าเฉลี่ยปริมาณสารที่สูงที่สุดคือ ไดด์ซินใน PM-V ซึ่งมีปริมาณสาร 5,059.96 ± 365.61 มิลลิกรัม/100 กรัมผงละเอียด รองลงมาคือเจนิสติน และ พิวรารินมีปริมาณสาร 3,062.35 ± 382.13 มิลลิกรัม/100 กรัมผงละเอียด และ 468.19 ± 83.55 มิลลิกรัม/100 กรัมผงละเอียด ตามลำดับ แต่ไม่พบไดด์เซอีน และ เจนิสเตอีน ปริมาณสารไอโซฟลาโวนอยด์รวมของ PM-V เดือนมิถุนายน 2550 สูงสุดโดยมีค่าเป็น 11.33 เท่าของปริมาณไอโซฟลาโวนอยด์ต่ำสุดของ PM-III ในเดือนเมษายน 2550 เมื่อเปรียบเทียบปริมาณสารไอโซฟลาโวนอยด์ทั้งหมดพบว่าเดือนมิถุนายน 2550 มีปริมาณสารสูงที่สุดคือ 5,766.34 ± 411.27 มิลลิกรัม/100 กรัมผงละเอียด โดยมีปริมาณเป็น 2.93 เท่าของปริมาณสารไอโซฟลาโวนอยด์ที่ต่ำที่สุดในเดือนเมษายน 2550 และยังพบว่า พิวรารินไดด์ซิน และเจนิสติน มีความสัมพันธ์กับสารไอโซฟลาโวนอยด์รวมในระดับนัยสำคัญ 0.01 ในการศึกษานี้แสดงให้เห็นถึงการสกัดสารไอโซฟลาโวนอยด์ คือ พิวริน ไดด์ซิน และเจนิสตินจากกวาวเครือขาว *P. mirifica* ในแปลงปลูกโดยใช้กระบวนการทางเทคโนโลยีชีวภาพทำให้ได้สารที่มีปริมาณสูง

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JUTARMAS JUNGSUKCHAROEN: QUANTITATIVE ANALYSIS OF MAJOR ISOFLAVONOIDS IN THE LEAVES OF WHITE KWAO KRUA *Pueraria mirifica*. THESIS ADVISOR: ASSOC. PROF. WICHAI CHERDSHEWASART, D.Sc., THESIS COADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 129 pp.

The RP-HPLC analysis of 5 major isoflavonoids including puerarin, diadzin, genistin, daidzein and genistein was applied to *Pueraria mirifica* leaves derived from 3 plant cultivars; PM-III, PM-IV and PM-V in every month from March, 2007 to August, 2007. The results indicated that there were only puerarin, daidzin and genistin, not daidzein and genistein present in *P. mirifica* leaves. The highest isoflavonoid contents during the 6 months were daidzin in PM-V (5,059.96 ± 365.61 mg/100g powder). The maximum amount of puerarin (468.19 ± 83.55 mg/100g powder) and genistin (3,062.35 ± 382.13 mg/100g powder) were found in PM-V, while the maximum daidzin amount was found in PM-V. The total isoflavonoid contents of PM cultivar in June 2007 were the highest (5,766.34 ± 411.27 mg/100g powder) and in April 2007 was the lowest (509.03 ± 45.06 mg/100g powder). The lowest puerarin (62.88 ± 15.02 mg/100g powder) and daidzin (194.63 ± 18.32 mg/100g powder respectively) were found in PM-III where as the minimum genistin was found in PM-V (131.59 ± 18.22 mg/100g powder). The results demonstrated that the leaf isoflavonoid contents were under the influence of plant genetics and monthly climatic change. The highest total isoflavonoid amount in PM-V was found in June 2007 which was 11.33 times higher than the lowest amount of PM-III in April 2007. The isoflavonoid contents of PM cultivar in June 2007 were the highest which was 2.93 times higher than in April 2007. The study demonstrates the highly biotechnological potential of manufacturing puerarin, daidzin and genistin from the cultivated *P. mirifica* leaves.

Field of study Biotechnology Student's signature Jutarmas Jungsukcharoen
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LIST OF ABBREVIATIONS

^{o}C	Degree Celcius
AU	Absorbance Unit
cm	Centimeter
E_2	17β-Estradiol
ER	Estrogen Receptor
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
g	Gram
h	Hour
L	Liter
M	Molarity
ml	Mililiter
nm	Nanometer
S.E.	Standard error
UV	Ultraviolet
μg	Microgram
μL	Microliter

CHAPTER I

INTRODUCTION

Isoflavonoids are major ingredient of phytoestrogens with anticancer properties (Watanabe *et al.*, 2002; Lof and Weiderpass, 2006). The important source of isoflavonoids is mainly seeds from legume plants. Soy isoflavonoids are well studied with most results derived from daidzein and genistein (Ingram *et al.*, 1997). Other plant sources for isoflavonoids are also investigated. Among the phytoestrogen-rich plant materials, the tubers of kudzu (*Pueraria lobata*) widely used in traditional Chinese, Japanese and Korean medicines, and white Kwao Krua (*Pueraria mirifica)* (Kashemsanta *et al.*, 1952) used in the Thai traditional medicines for treatment of menopausal symptoms are extensively studied. Kudzu is found to enrich with isoflavonoids, especially puerarin (Kaufman *et al.*, 1997). *P. mirifica* is found to contain active phytoestrogens including miroestrol (Jones and Pope, 1961), deoxymiroestrol (Chansakaow *et al.*, 2000[a]). The wild *P. mirifica* population exhibits 5 major isoflavonoids including puerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart et al., 2007[a]) with varied estrogenic activity in ovariectomized rats (Cherdshewasart *et al.*, 2007[b]). The cultivated plant tubers also store high amount of 5 major isoflavonoids since the first year of cultivation (Cherdshewasart and Sriwatcharakul, 2007).

Estrogenic activity of the tuberous materials was extensively evaluated in *P. mirifica*. Evaluation in female mice and monkeys revealed a dose-dependent estrogenic disruption (Jaroenporn *et al.*, 2007; and Trisomboon *et al.*, 2007). In ovariectomized rats or aging female monkeys, consumption of *P. mirifica* exhibited a dose-dependent replacement action for estrogen (Malaivijitnond *et al.*, 2006; and Trisomboon *et al.*, 2006). The plant crude extract exhibited biphasic estrogenic activity in MCF-7 cells with proliferation at low dose and antiproliferation (anti-estrogen) at high dose (Cherdshewasart *et al.*, 2004), with a possible role of metabolic activation at its cellular action (Lee *et al.*, 2002). The pretreatment with plant powder showed antiproliferation to induce mammary tumor in female rats (Cherdshewasart *et al.*, 2007). *P. mirifica* also exhibited positive response for induced osteoporosis in

orchidectomized male rats (Urasopon *et al.*, 2007). Clinical trial of the plant powder consumption exhibited successive treatment of menopausal symptoms in Thai menopausal women (Muangman and Cherdshewasart, 2001) without adverse effect in both human and animal skin tests (Cherdshewasart, 2003).

P. mirifica is a twinning vine with a lot of produced leaves during their growth and development. Leaf harvest is not complicated as tuber harvest. Leaf might be a better alternative in term of commercialized farming for phytoestrogens production, especially isoflavonoids. We therefore establish a chemical analysis of major isoflavonoids in leaves of *P. mirifica* to evaluate the potential of isoflavonoid extraction from leaves of this plant.

Aims of the study are as followed:

To analyze the major isoflavonoids from the leaves of 3 cultivars of *P. mirifica*, namely PM-III, PM-IV and PM-, by RP-HPLC analysis after chlorophyll elimination.

CHAPTER II

LITERATURE REVIEW

2.1 Phytoestrogens; chemical approach

2.1.1 Introduction and Classification

Phytoestrogens are molecules with estrogenic activity that are found in plants. Several different classes of molecules have been identified as phytoestrogens so far; these are not identical to human estrogens, but have some structural similarities which allow them to interact with estrogen receptors (a protein within certain cells which can bind to endogenous estrogens, phytoestrogens, or xenoestrogens. Once coupled with its estrogenic molecule, this protein communicates with other molecules and starts a process that ultimately leads to certain genes being switched on or off. The genes regulate many hormone-influenced systems) in human cells. 'Phytoestrogen,' then, is more of a functional classification than a chemical classification.

Following publication of the Allen Doisy bioassay for estrogens in 1923 (Allen and Doisy, 1923), plant extracts were first reported to exhibit estrogenic activity in 1926 (Loewe et al., 1927). By 1975, several hundred plants had been found to exhibit estrogenic activity on bioassay or to contain estrogenically active compounds (Farnsworth et. al., 1975). Phytoestrogens assumed biological and economic importance in the 1940s, with the outbreak of infertility in sheep grazing on pastures rich in subterranean clover in Western Australia, later known as "Clover Disease" (Bennets et al., 1946).

There were three main classes of phytoestrogens: isoflavones, coumestans, and lignans, which occur in various plants or seeds (Table 2.1). A single plant often contains more than one class of phytoestrogen for example, isoflavonoids were found predominately in soybeans (*Glycine max* L.), whereas coumestans were preliminary identified by genus of *Trifolium* such as clovers (Franke et al., 1994) and species of *Pueraria* and *Glycyrrhiza* (Dewick, 1993). Genistein and daidzein were the major

isoflavonoids also found in soybeans and their products. The available data especially in epidemiological study was examined the relationship between the consumption of natural food containing phytoestrogens and the reduced risk of cardiovascular symptoms, cancer and osteoporosis.

The chemical structure of phytoestrogen was diphenolic (better bisphenolic) compounds that comprised of 2 benzene rings (A and B) linked through a heterocyclic pyran or pyrone ring (C) in the middle (Figure 2.1). The basic structure of phytoestrogens was closely similar to natural and synthetic estrogens and antiestrogens such as resorcyclic acid lactones (e.g. zearalenone). When both structures of phytoestrogen and estradiol were superimposed, the distance between the hydroxyl groups were identical (Figure 2.2). Isoflavonoids are often present as glucoside conjugates (glycones) such as genistin, daidzin and glycitin. These glycosides can be further metabolized in gut to aglycone such as genistein, daidzein and glycitein. Based on the structural similarities, phytoestrogens could bind to estrogen receptors (ERs) (Setchell, 1998 and Hopert et al., 1998) and acted as a weak estrogen (Setchell, 1998).

Table 2.1 Common phytoestrogens and their sources (http://www.herbalchem.net/Introductory.htm).

Class	**Examples**	**Sources**	**Concentration (mg/100g powder)**
Isoflavones	Daidzein	*Soybeans and soy products:*	
		Soy flour	1.65 - 130.92
		Soybeans raw	9.89 - 124.20
		Soy protein isolate	7.70 - 68.89
		Miso	7.10 - 36.64
		Tempeh	4.67 - 27.30
		Tofu	8.00 - 25.80
		Soy cheese	0.20 - 21.10
		Soy milk	1.14 - 9.84
		Infant formulas	0.75 - 9.65
		Soy dog	3.40

		Shoyu	0.60 - 1.40
		Soy sausage	0.75
		Commercial soy sauce	0.10
		Soy oil	0
		Other sources:	
		Kudzu root	185
		Split peas, raw	0 - 7.26
		Red clover leaf *	4.2
		Mung bean sprouts	0.70
		Kudzu leaf	0.375
		Red clover seed	0.178
		Sesame seed	0.140
		Chick peas, raw	0 - 0.08
		Alfalfa (mature)	0.062
		Peanuts, raw	0.01 - 0.05
		Black-eyed peas, raw	0 - 0.03
		Pinto beans, raw	0 - 0.02
		Fenugreek seed	0.01
		Lentils, raw	0 - 0.01
		Alfalfa sprouts	0
		Black beans, raw	0**
		Kidney beans, cooked	0
		Red clover sprouts	0
	Formononetin	Red clover (mature)	1322
		Kudzu Root	7.090
		Red clover sprouts	2.28
		Red clover seed	1.270
		Alfalfa sprouts	0.34
		Chickpeas	0.094 - 0.215
		Soybeans	0.018 - 0.121
		Kudzu Leaf	0.087
		Alfalfa (mature)	trace
		Red clover tea	trace

		(flowering tops)	
	Genistein	*Soybeans and soy products*:	
		Soy flour	2.75 - 145.23
		Soybeans, raw	13.00 - 138.24
		Soy protein isolate	27.17 - 105.10
		Miso	11.70 - 52.39
		Tofu	11.10 - 42.15
		Tempeh	1.11 - 39.77
		Soy cheese	0.50 - 38.20
		Infant formulas	1.58 - 15.43
		Soy milk	1.12 - 11.28
		Soy dog	8.20
		Soy sausage	2.70
		Shoyu	0.30 - 1.54
		Commercial soy sauce	0
		Soy oil	0
		Other sources:	
		Red clover leaf*	56.0
		Kudzu root	12.60
		Kudzu leaf	2.52
		Mung bean sprouts	2.00
		Pinto beans, raw	0.52
		Peanuts, raw	0.08 - 0.39
		Red clover Sprouts	0.35
		Split peas, raw	0 - 0.10
		Chick peas, raw	0 - 0.12
		Black-eyed peas, raw	0 - 0.03
		Fenugreek seed	0.01
		Lentils, raw	0 - 0.01
		Alfalfa (mature)	trace
		Alfalfa sprouts	0
		Black beans, raw	0**
		Kidney beans, cooked	0

	Biochanin A	Red clover	833
		Chickpeas	0.838 - 3.080
		Kudzu Root	1.400
		Kudzu Leaf	1.240
		Red clover sprouts	0.44
		Red clover seed	0.381
		Peanuts	0.031
		Alfalfa (mature)	trace
		Red clover tea (flowering tops)	trace
	Glycitein	*Soybeans and soy products:*	
		Soy protein isolate	5.40 - 26.40
		Soy flours	3.95 - 28.8
		Soy beans, raw	6.72 - 20.40
		Soy cheese	2.70 - 4.10
		Soy dogs	3.40
		Miso	2.30 - 3.80
		Infant formulas	0.28 - 3.45
		Tempeh	0.90 - 3.20
		Tofu	1.70 - 2.90
		Soymilk	0.36 - 0.86
		Shoyu	0.45
		Soy sausage	0.30
		Soy oil	0
		Commercial soy sauce	0
Coumestans	Coumestrol	Red clover sprouts	28.1
		Mature alfalfa	2.5 - 6.5
		Alfalfa sprouts	4.7
		Kudzu leaf	1.57
		Soybean sprouts	0.45 - 1.21
		Mung bean sprouts	1.00
		Soybean, 'Santa Rosa' variety	0.19

		Red clover leaf	0.105
		Soybean (mature, other varieties) and soy products	~ 0
	Matairesinol	Flax seed	1.087
		Sesame seed	0.608
		Teas (brewed)	0.090 - 0.305
		Rye bran	0.167
		Oat bran	0.155
		Rye meal	0.065
		Broccoli	0.023
		Poppy seed	0.012
		Other grains, legume seeds, and nuts	Up to ~ 0.01
		Black currants	0.01
		Strawberries	0.005
	Secoisolariciresinol	Defatted flax meal	600 - 700
		Flax seed	369.9
		Pumpkin	3.870
		Teas (brewed)	1.050 – 2.887
		Lingonberry	1.510
		Strawberry	1.205
		Cranberry	1.054
		Blueberry	0.835
		Zucchini	0.817
		Sunflower seed	0.610
		Kudzu leaf	0.476
		Broccoli	0.414
		Black currant	0.388
		Garlic	0.380
		Carrot	0.370
		Peanut	0.298 – 0.333

		Soybean	0.013 – 0.273
		Cashew	0.257
		Caraway seed	0.221
		Cow pea	0.195 – 0.196
		(*Vigna unguiculata)*	
		Walnut	0.163
		Kidney bean	0.056 – 0.153
		Red cabbage	0.141
		Raspberry	0.139
		Rye bran	0.132
		Hazelnut	0.119
		Wheat bran	0.110
		Sesame seed	0.090

* Daidzein and genistein were present in their glycosidic forms, daidzin and genistin. No free aglycones were detected in red clover.

** Despite claims to the contrary, none of the scientific literature indicates that Black beans contain more than a trace of isoflavones. They are, however, rich in other flavonoids, as indicated by their dark purple-black color. See p 119 of this report [have patience downloading, it's a big PDF file] from the Institute for Environment and Health, Ministry of Agriculture, UK.

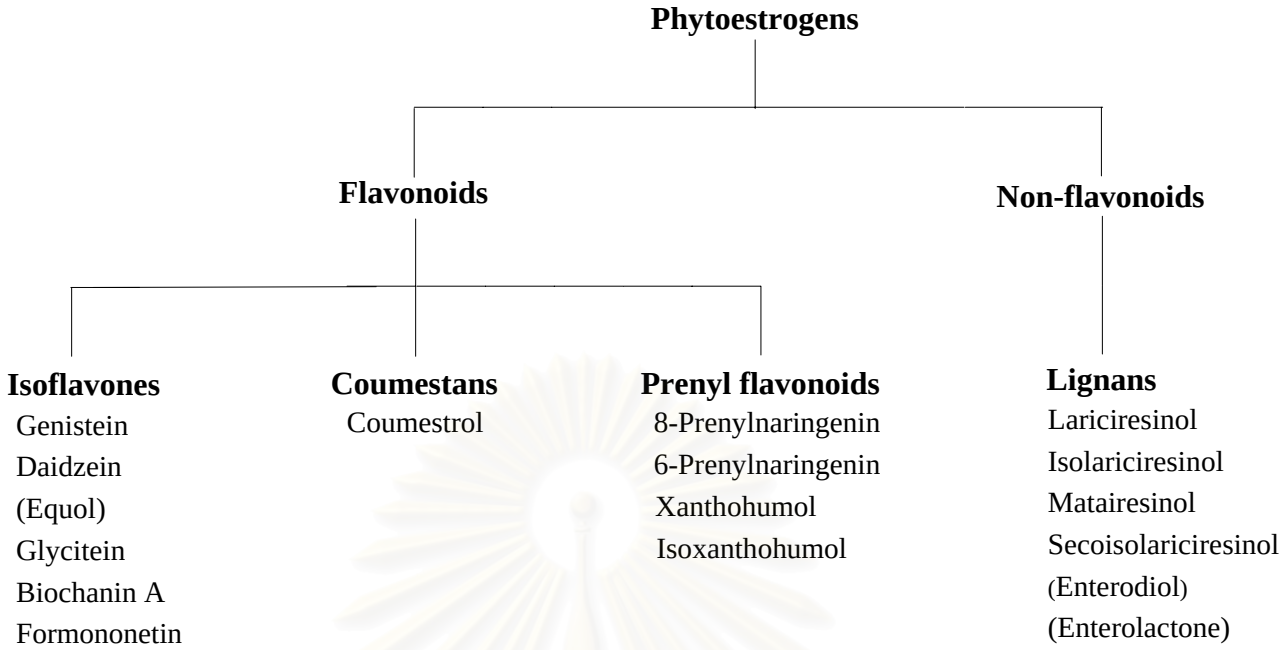


Figure 2.1. The relationship between the various groups of phytoestrogens (given in bold) and members of each group. The compounds in brackets weren't inherently presented in plants but were oestrogenic products resulting from metabolism of members of that class of phytoestrogen.

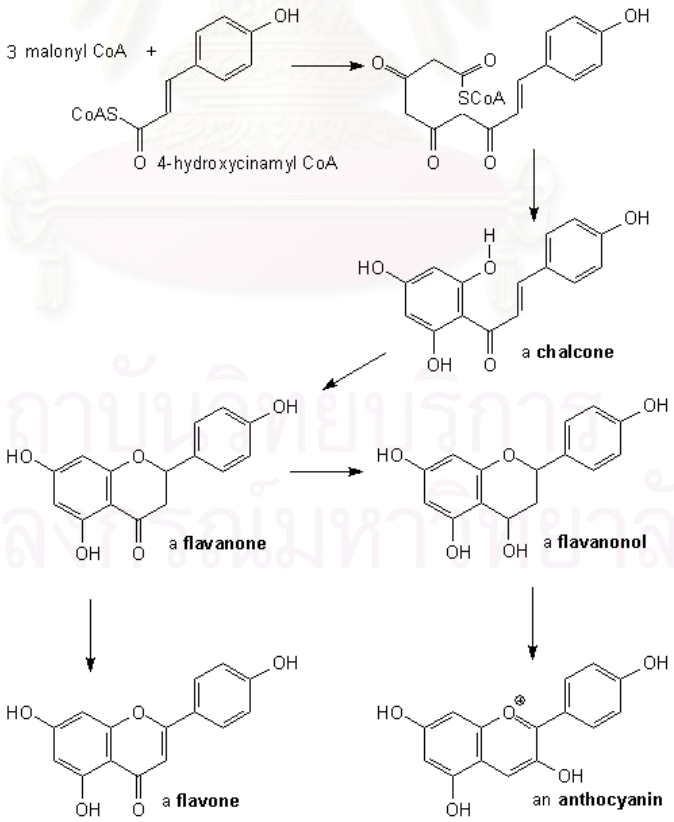


Figure 2.2. The flavone biosynthesis.

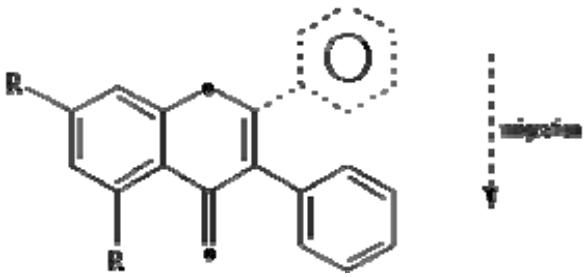


Figure 2.3. The ring migration of flavone.

Isoflavonoid had been established that acetate gives rise to ring A and that phenylalamine, cinnamate and cinnamate derivatives were incorporated into ring B and C-2, -3, and -4 of the heterocyclic ring. Since crhalcones and flavanones had been efficient precursors of isoflavonoids, the required aryl migration of ring B from the former 2 or beta position to the 3 or alpha position of the phenylpropanoid precursor must take place after formation of the basic C_{15} skeleton (Figure 2.2-2.3.).

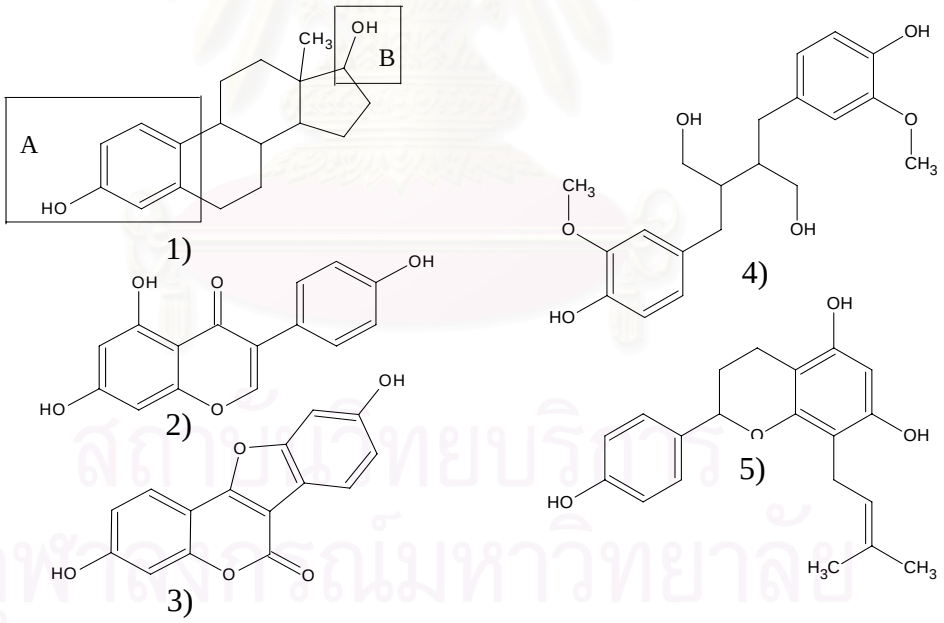


Figure 2.4. The structural similarities of phytoestrogens to oestradiol. The similarity of the structure of the human hormone 1) Oestradiol, 2) Genistein, 3) Coumestrol, 4) Secoisolariciresinol and 5) 8-Prenylnaringenin from the four classes of phytoestrogen from Figure 2.1. All the structures possess the phenolic (A) and hydroxyl (B) moieties outlined in boxes on the oestradiol structure and the distances between the two groups in each compound are similar.

2.1.2 Analysis of phytoestrogens

Generally, phytoestrogens and their metabolites are present in part per billion to part per million in plants, foodstuff as well as in biological fluids such as urine, plasma and feces. Initially, phytoestrogens were analyzed using simply techniques such as thin-layer and paper chromatography. However, the development of increasingly sensitive technologies was advanced phytoestrogen analysis considerably. The most widely used methods for quantify of phytoestrogens were high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Wang et al., 1990 and Thomas et al., 2001), gas chromatography with mass spectrometric detection (GC-MS) (Mazur et al, 1996; Tekel et al., 1999 and Nesbitt, Lam and Thompson, 1999) and liquid chromatography with mass spectrometric detection (LC-MS) (Coward et al., 1996; Cimino et al., 1999 and Doerge, Churchwell, and Delclos et al., 2000).

These developments were also useful in pharmacological and toxicological studies. Prior to analysis and their metabolites, phytoestrogens might be isolated from matrices. The extraction of phytoestrogens were required. During extraction, phytoestrogen might be lost; an appropriate internal standard might be added prior to extraction. A general schematic of the steps involved in extraction and anyalyse of phytoestrogens was shown in Figure 2.3

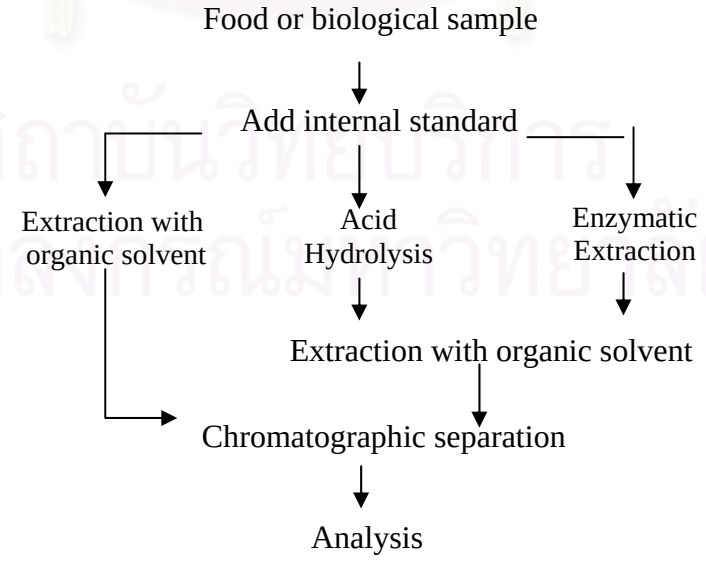


Figure 2.5. A general schematic of the steps involved in extraction and analysis of phytoestrogens (Adlercreutz et al., 1986 and Franke et al., 1994).

2.1.2.1.High performance liquid chromatography with ultraviolet detection (HPLC-UV)

The most appropriate analytical method was dependent on the type of biological matrix and compound to be analysed. The analytical methods used in phytoestrogen analysis required reference standards, which were pure, samples of the analyte and were used to calibrate the analytical method to ensure it performs in the way that is anticipated (Thompson & Wood, 1993). As methods were developed, the limits of detection and quantification were decreased so that phytoestrogens can now be measured in foodstuffs and biological samples down to concentrations of parts per billion.

HPLC-UV was a relatively rapid way of measuring phytoestrogens compared to mass spectrometric (MS) based methods (Setchell & Welsh 1987; Wang et al, 1990; Franke et al, 1994; Saloniemi et al, 1995; Obermeyer et al, 1995; Franke & Custer, 1996; Coward et al, 1998; Murphy et al, 1999; Zhang et al, 1999; Nakamura et al, 2000; Thomas et al, 2001). Following isolation of phytoestrogens from the matrix, they were directly separated and quantified. This method allows simultaneous purification and measurement of complex mixtures. UV detection was generally less sensitive than MS detection and the reported detection limits could be variable. The analytes were quantified by comparison with calibration curves derived from reference standards. However, substances presented in the sample, but not in the reference standard, which might co-elute with the analyte during chromatography, might lead to falsely high measurements of the analyte. Reference standards were not available for many phytoestrogens, such as the acetyl- and malonyl isoflavone glucosides. Therefore, measurements of these compounds were based on calibration curves of isoflavone glucosides and aglucones. Research indicated that this approach could introduce errors in measurements (Coward et al, 1993). However, when reference materials of the acetyl- and malonylglucosides were available such assumptions could be tested and results re-calculated.

2.1.2.2.Gas chromatography with mass spectrometric detection (GC-MS)

GC-MS was sufficiently sensitive to measure concentrations of phytoestrogens of less than parts per million (Adlercreutz et al, 1991a and b; 1993; 1995; 1999; Morton et al, 1994; 1997; Joannou et al, 1995; Mazur et al, 1996; Liggins et al, 1998; 2000; Tekel et al, 1999; Nesbitt et al, 1999; Lampe et al, 1999; Heinonen et al, 1999; Foster et al, 2002). Measurement was usually done with the use of internal standards (preferably isotopically labelled analogues of the analytes), but unlike HPLC-UV, the standard and the analyte was measured under exactly the same conditions. Samples might be treated to remove conjugating groups prior to analysis by GC-MS and as a result phytoestrogens couldn not be measured as they appear in the matrix. As such, the analytical results obtained using this method is expressed as quantity of 'total phytoestrogen'. Chemical and enzymatic methods were developed to remove these groups (Liggins et al, 1998; Mazur et al, 1996). Enzymatic methods were preferable for isoflavones as they could be unstable under acidic conditions (Liggins et al, 1998). However, lignan glucosides were resistanted to enzymatic hydrolysis and required strong acid to remove the sugars (Mazur et al, 1996).

2.1.2.3. Liquid chromatography with mass spectrometric detection (LC-MS)

LC-MS was also sufficiently sensitive to measure concentrations of phytoestrogens of less than parts per million (Setchell & Welsh, 1987; Barnes et al, 1994; Coward et al, 1996; Cimino et al, 1999; Doerge et al, 2000a and b; Rong et al, 2000; Chang et al, 2000). However, in contrast to GC-MS, removal of conjugating groups was not required prior to analysis. Therefore, different forms of phytoestrogens could be measured and expressed directly rather than as a value of 'total phytoestrogen'. However, measurement was dependent on the availability of internal standards for each analyte and many of these materials were unavailable at present. Laboratories using LC-MS have to use the available aglycone standards and hydrolyse samples prior to analysis. As such, the advantage of non-destructive sample preparation that LC-MS offers over GC-MS has not yet been realized. More recently, methods used LC coupled to mass spectrometers in tandem (MS/MS) were developed for the analysis of phytoestrogens (Valentin-Blasini et al, 2000; Coldham et al, 2002; Fang et al, 2002).

2.2. Phytoestrogens; physiological approach

2.2.2. Phytoestrogen action

Numerous studies were shown that phytoestrogens can exert multi-functions through genomic and non-genomic mechanisms of cellular regulation by competing to estrogen receptor binding or interfering to estrogen biosynthesis and metabolism.

2.2.1.1. Cellular and molecular mechanisms

According to the classic concept, estrogens were steroid hormones, which involved in the important functions for the sexual processes and act through protein, estrogen receptors which distributed in reproductive tissues such as ovary, mammary, uterus and vagina. Until recently, ER was identified into two subtypes, ERβ and ERα. ERβ, discovered only a few years ago, was heterodimers in DNA binding domain (over 95% amino acid identity) and splicing variants of ERα (Kuiper et al., 1996 and Mosselman et al., 1996). ERs were predominately presented in nucleus where was formed the complex with heat shock proteins when received the stimulating (Figure 2.4 and 2.5). Bound ERs were activated to the specific DNA-binding sites called estrogen receptor response elements (ERE) or AP-1 site. After binding, the target gene transcription in initiated or repressed which ultimately elicited biological responses as agonist or antagonist characters (Clark, et al., 1996; Fitzpatrick, 1999 and Diel, Smolnikar, and MIchna, 1999) depened on the phytoestrogens concentration and target organ (Setchell et al., 1998). If phytoestrogens induce biological effect as estradiol, they were considered as agonist, however, the potency was too weak that require much higher concentrations to boost the responses as well as estradiol. On the other hand, phytoestrogens could act as antagonists by block the binding of estrogen that caused an interrupted hormone response.

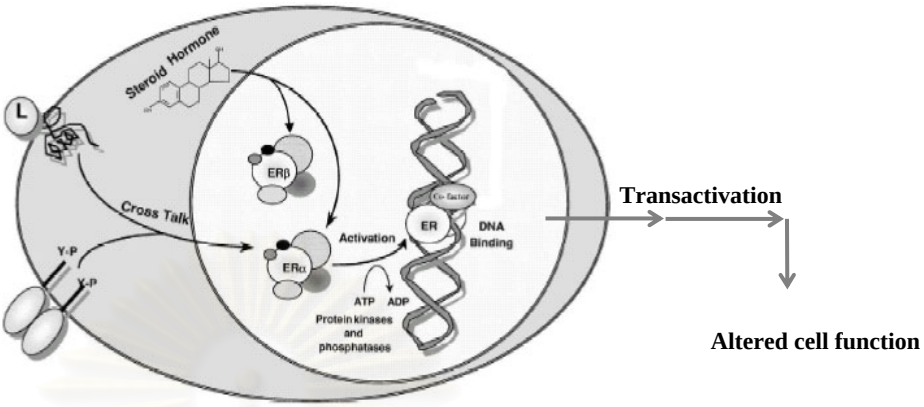


Figure 2.6. Mechanisms of estrogen receptor activation on target cells (Adapted from Diel, Schmidt, and Vollmer, 2002)

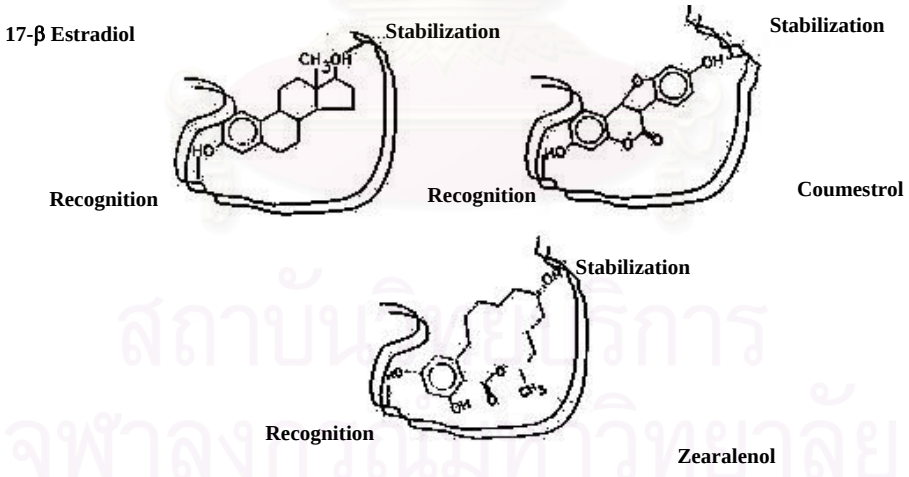


Figure 2.7. Structure-activity relationships of estrogen and some phytoestrogens (coumestrol and zearalenol) on estrogen receptor lignands (Clarke et al., 1996)

2.2.1.2. Biotransformation and metabolism

The metabolisms of isoflavonoids and lignans showed similar patterns in animal (Price and Fenwick, 1985) and human (Adlercreutz et al., 1991) whereas coumestan had not been identitied. After consumption, isoflavone and lignan glycosides were probably hydrolyzed within gastrointestinal tract by gastric acid (Xu et al., 1995) and intestinal microflora hydrolysis enzymes. The precursor of genistein and daidzein are biochanin A and formononetin, respectively (Figure 2.6). After absorption, isoflavonoids were transported to the liver, reconjugated and then excreted in urine and bile. The reconjugation of aglycone with glucuronic acid and sulfuric acid was function by hepatic phase II enzymes (Morton et al., 1994 and Adlercreutz et al., 1993). However, genistin was partly absorbed without previous cleavage (Andlauer, Kolb, and Fürst, 2000). In human, aglycones were absorbed faster and in greater amounts than their glycosides (Izumi et al., 2000). The maximum peak of isoflavonoids was range at 7- 8 hours after consuming a single soy meal (King and Bursill, 1998). Those isoflovones were detected in biological fluid including plasma (Adlercreutz et al., 1994), amniotic fluid (Adlercreutz et al., 1999), urine (Adlercreutz et al., 1991), feces (Adlercreutz, et a., 1995), milk (Franke and Custer, 1996), saliva, breast aspirate (Hargreaves et al., 1999) and prostatic fluid (Finlay et al., 1991).

Biochanin A and formononetin were metabolized by gut microflora to genistein and daidzein, respectively. Genistein could be further metabolized to 4-ethylphenol and daidzein to equol, dihydrodaidzein and *O*-desmethylangolensin (Anderson and Garner, 1997). The data suggested that equol has a greater antioxidant effect than other phytoestrogens, which often found in highest level in biological matrices and exerted significant biological effects (Hodgson et al., 1996).

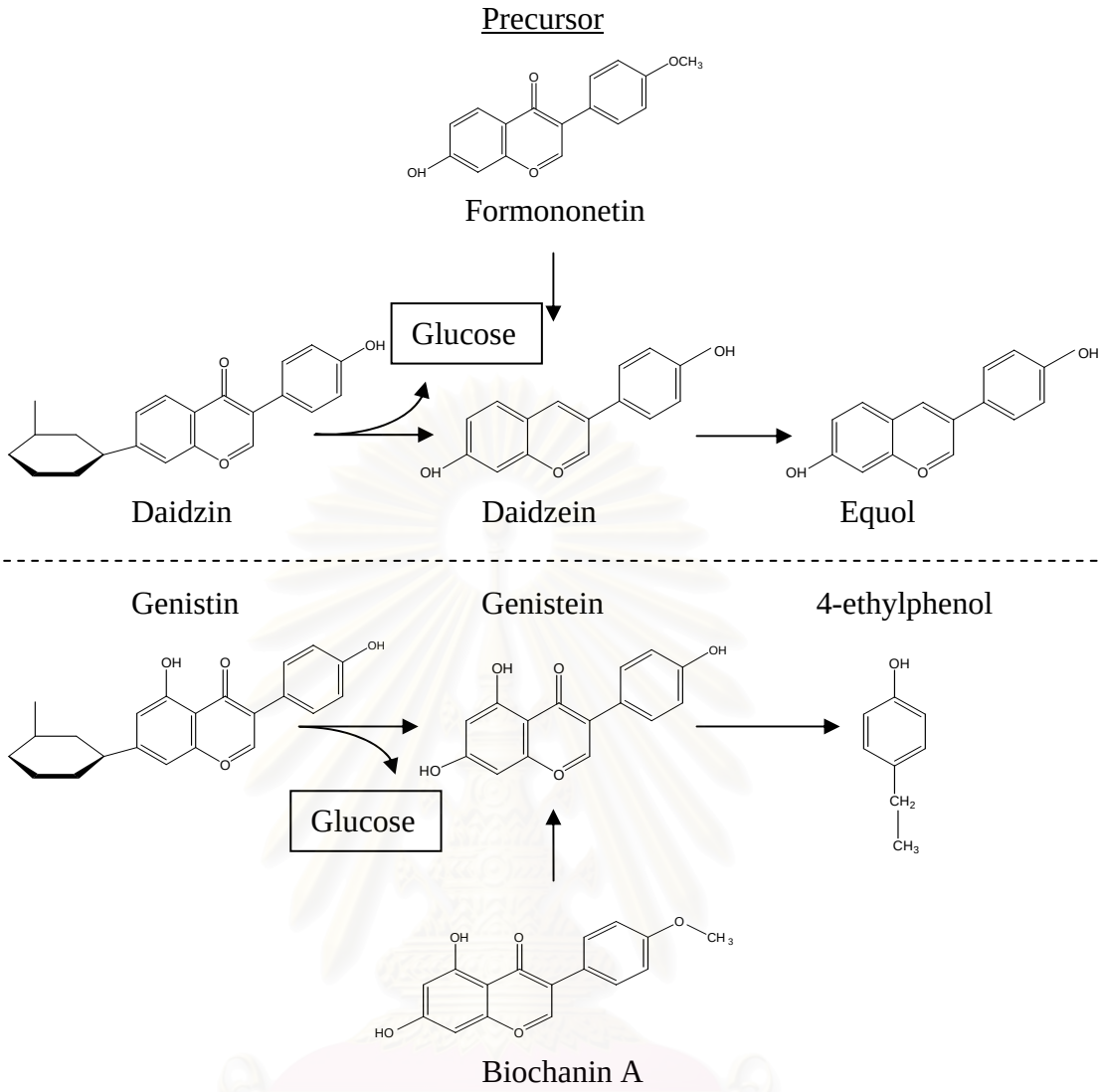


Figure 2.8. Schematic of some phytoestrogen metabolism in intestine (Anderson and Garner, 1997).

2.2.1.3. Phytoestrogens potency and active concentration

Bound compound to both type of ERs could stimulate transcription of ERE, with different tissue expression patterns. The splicing variants leaded to different relative binding affinities in different compounds. Assessment of estrogenic potency varies acrossed assays such as receptor binding affinity, transcriptional activation, cell proliferation and *in vivo* assays. It is derived from the difference in copies of EREs in used cells. Generally, a low concentration of phytoestrogen (1-100 nM) stimulates cell growth, whereas, a high concentration (5-100 μM) showed suppression. This was called a biphasic pattern.

Phytoestrogens could bind with higher affinity to ERβ on transcriptional level than to ERα (Kuiper, et al., 1997 and 1998 and Casanova et al., 1999). Phytoestrogens could affect endogenous hormonal production by inhibiting the key enzyme of hormonal biosynthesis (Evans, Griffiths and Morton, 1995; Kao et al, 1998 and Makela et al., 1998) or stimulating of sex hormone binding globulin (SHBG) (Adlercreutz et al., 1987), however, the estrogenic potency was less than estradiol. The proliferation action occured at nanomolar concentration level. *In vitro* study, the concentration of phytoestrogens to elicit a responsed on ERα and ERβ site are 0.145 and 0.0084 μM, respectively (IC_{50} values for competition of estradiol-recepter binding) (Kuiper et al., 1998). Compared with all phytoestrogens, coumestrol was the highest estrogenic potency (Kuiper et al., 1997), however, an affinity to ERα was ten times less than estradiol. It was noticed that the methoxy derivative of genistein and biochanin A did not bind ER, but they could show estrogenic activity *in vivo*. This might be the role of hydroxyl substituents at 4' and 7' positions in phytoestrogen groups (Figure 2.6). The ranking of phytoestrogens potency as compared with estradiol is estradiol > coumestrol > 8-prenylnaringenin > equol $\geq$ genistein > biochaninA > daidzein > genistin > glucoronide > daidzin > glucoronide > formononetin (Kuiper et al., 1997 and Milligan et al., 2000) (Table 2.2).

Table 2.2. Relative binding activity of estrogen and various phytoestrogens binding to ERα and ERβ

Compound	**Binding at ERα (%)**	**Binding at ERβ (%)**
17- β Estradiol[a]	100	100
Estrone[a]	60	37
Estriol[a]	14	21
Progesterone[a]	<0.001	<0.001
Testosterone[a]	<0.01	<0.01
Coumestrol[a]	94	185
Genistein[a]	5	36
Daidzein[a]	0.2	1
8-Prenylnaringenin[b]	10	10
β-Sitosterol[a]	<0.001	<0.001
Tamoxifen[a]	7	6

([a]Kuiper et al., 1997 and [b]Milligan et al., 2000)

2.2.2. Biological and pharmacological effects

2.2.2.1. Hormonal effects

The hormonal effects have been shown in both animal and human, however, the results have been inconsistency depending on the analytical methods. Phytoestrogens were quite weak according to both *in vitro* and *in vivo* assays, which possessing less than activity of estradiol between 1,000 and 10,000 (Folman and Pope, 1966 and Markiewicz et al., 1993). The phytoestrogen-rich plant, red clover demonstrated hyperestrogenize and infertility in grazing animals (Bennets, Underwood and Shier 1946 and Shutt and Braden, 1968). In uterine growth of female mice, subcutaneous injection of genistein could inhibit the stimulation of estrone (Folman and Pope, 1966). The length of the follicular phase in pre-menopausal women was increased when administered within isoflavone-rich diet (Cassidy, Bingham and Setchell, 1995 and Lu et al., 1996). On the other hand, progesterone (Lu et al., 1996 and Lu et al., 2000) and testosterone could be decreased (Strauss et al., 1998). Estradiol-17β in serum was also affected (Cassidy, Bingham, and Setchell, 1995). At high dosages of isoflavonoids, the feedback-regulating system of the hypothalamus-pituitary gland axis was interrupted and affected to hormonal status.

Genistein was shown to exert both estrogenic and antiestrogenic activity in human cell lines (MCF-7) (Wang, Sathyamoorthy, and Phang, 1996 and Sathyamoorthy, Wang, and Phang, 1994). In cell culture, the low concentration of those compounds to stimulate estrogen-dependent receptor gene activity was 10-1000 nM (Kuiper et al., 1998). Moreover, some reports were shown that soy isoflavonoids could increase nerve growth factor mRNA and brain-derived neurotropic factor mRNA in rats (Pan, Anthony and Clarkson, 1999) and also affected the cell signaling conduction on receptor expression. At high concentration of genistein, EGF receptor expression was inhibited in rat uterus and vagina (Akiyama et al., 1987 and Brown and Lamartiniere, 2000). Phytoestrogens might involve in estrogen synthesis and metabolism through interfering and/or inhibiting estrogen related enzyme such as 17-β-hydroxysteroid oxidoreductase; the conversion catalyst of estrone to estradiol (Makela et al, 1995), aromatase (Kellis and Vickery, 1984 and Pelissero et al., 1996) and steroid sulphatase (Murkies, Wilcox, and Davis, 1998 and Krazeisen et al., 2001).

Aromatase converted the androgens, dehydroepiandosterone and testrone to estrogen and estradiol, repectively. Inhibition of these enzymes would alter the balance between estradiol and the less potent of estrone. Genistein and daidzein also suppressed glucocorticoid and stimuated androgen production in human adrenal cortical cells cultures (Mesiano et al., 1999).

In HepG2 liver cancer cells culture, isoflavonoids was reported to stimulate the biosynthesis of SHBG. It was noticed that the change of SHBG concentration affect in relatively large changes in amount of free and bound hormones whereas the change of total hormone concentration affected in relatively small changes (Loukovaara et al., 1995 and Mousavi and Adlercreutz, 1993). To sum up, the action of phytoestrogen depended on the hormonal status of animal and human.

2.2.2.2. Anticarcinogenic effect

There were evidences in human studies, pointing to the potency of soy products or phytoestrogen such as genistein or daidzein to inhibit and prevent on various cancers such as endometrial (Goodman et al., 1997), prostate, (Jacobsen, Knutsen and Fraser, 1998; Herbert et al., 1998; Kolonel et al., 2000 and Strom et al., 1999), stomach (Nagata, 2000 and Nagata et al., 2002), colon (Nagata, 2000), thyroid (Horn-Ross, Hoggatt and Lee, 2002), lung (Seow et al., 2002) and mammary (Lee et al., 1991; Yuan et al., 1995; Hirose et al., 1995; Wu et al., 1996; Ingram et al., 1997; Witte et al., 1997; Key et al., 1999; Dai et al., 2001 and Shu et al., 2001). It was noticed that all these cancers are hormone-dependent.

Understandably, anticancer effects of soybean were primarily attracted attention on breast cancer. The low incidence of breast cancer in East Asia was found associated with consumption of typically Asian/oriental diet (Tham, Gardner and Haskell, 1998). Animal studies support the notion that phytoestrogen were also found to exert the inhibition of chemically induced mammary cancer (Barnes et al., 1990; Lamartiniere et al., 1995a, Murrill et al., 1996 and Gotoh et al., 1998a and 1998b). It was confirmed *in vitro* studies, isoflavonoids inhibited cancer cell growth including prostate cancer cell line (Davis et al., 1998 and Hillman et al., 2001) and MCF-7 human breast cancer cell line (Hsu, Ying, and Chen, 2000).

There was a correlation of reduced risk of breast cancer with a high plasma level of phytoestrogens such as mammalian lignan, enterolactone (Pietinen et al., 2001). Several reports were shown that genistein could inhibit the growth of both hormone-dependent and hormone-independent cancers with IC_{50} at 5 - 100 μM/L (2-25 μg/mL) (reviewed in Messina, 1999). However, genistein could stimulate those cancers at physiologically concentrations (<5-6 μM/L) (Zhang et al., 1999 and Ren et al., 2001).

The numerous mechanisms might be involved such as the inhibition of angiogenesis (Fotsis et al., 1993), protein tyrosine kinases (Akiyama et al., 1987) and related hormonal enzymes. The preventive effect of isoflavonoids might be involved the decreasing synthesis and altering metabolism form of estrogen (Xu et al., 1998 and 2000). In addition, genistein was shown to inhibit the metastatic activity of breast cancer (Scholar and Toews, 1994) and prostate cancer cells (Santibanez, Navarro and Martinez, 1997). Cell cycle progression at G_2-M was arrested by genistein that result to the differentiation and apoptosis of various cancer cell lines including human gastric cancer (Yanagihara et al., 1993), human breast carcinoma (Shao et al., 1998), leukemia (Spinozzi, et al., 1994), melanoma (Rauth, Kichina, and Green, 1997) and colon (Kuo, 1996).

In vitro and *in vivo* studies, at micro molar concentration level of phytoestrogens exert various non-hormonal related effects. Genistein was shown to inhibit DNA topoisomerase I and II (Yamashita, Kawada, and Nakono, 1990; Ji et al., 1999; Martin et al., 2000 and Salti et al., 2000) that effected to DNA damage and epidermal growth factor-induced phosphatidylinositol turnover (Imoto et al., 1988). Biochanin A was linked to increase nitric oxide level that later induction of cell apoptosis (Hsu et al., 1999). In addition, phytoestrogens might exert their effects by decreasing the activity of enzymes that activate procarcinogens, such as cytochrome P450 (CYPs) (Roberts-Kirchhoff et al., 1999).

2.2.2.3. Other effects

Epidemiological observations and laboratory animals and *in vitro* investigations were revealed a number of biological properties suggesting a prevention of western diseases such as cardiovascular, atherosclerosis,

hypercholesterolemia, menopausal symptoms and osteoporosis (review in Kurzer and Hu 1997; Bingham et al., 1998 and Tham et al., 1998).

In vivo and epidemiologic studies were demonstrated that soy protein reduced risk of coronary heart disease and atherosclerosis (Anderson et al., 1999; Anthony, 2000, and van der Schouw et al., 2000). The effects resulted from a reduction of plasma low-density lipoprotein (LDL) (Tovar-Palacio et al., 1998; Crouse et al., 1999 and Ashton and Ball, 2000) cholesterol and triglycerides (Anderson, Johnstone, and Cook-Newell, 1995 and Ho et al., 2000). Soy could reduce absorption of dietary (Greaves et al., 2000), arterial permeability, concentration and delivery of LDL (Wagner et al., 2000) and increased LDL receptor quantity and activity (Baum et al., 1998).

Many studies suggested that phytoestrogens play role in maintaining bone density in postmenopausal women (Dalais et al., 2003; Alekel et al., 2000 and Kim et al., 2002). In animal studies, isoflavonoids could prevent bone loss that occurs as a result of estrogen deficiency in ovariectomized rats (Fanti et al., 1998; Vincent and Fitzpatrick, 2000; Picherit et al., 2000 and Uesugi et al., 2001). Postmenopausal woman seemed to benefit the most from consumption of soy phytoestrogens. Bone mineral density (BMD) of the lumbar spine was increased with the treatment of 90 mg. isoflavonoids per day for 24 weeks (Potter et al., 1998). Osteoclastic bone resorption was inhibited by genistein and daidzein (Ono, Ma, and Yamaguchi, 2000) but stimuted osteoblastic bone formation (Yamaguchi, Gao, and Ma, 2000). However, data available in human about the effect of isoflavonoids on osteoporesis is limited.

Since estrogen has an important effect on the immune system. A changing of estrogen level resulted to autoimmune diseases that commonly occur in women (Enmark and Gustafsson 1998 and 1999). Isoflavonoids were also exerted an anti-inflammatory potential in various animal models. High dosages of daidzein (20 and 40 mg/kg) could enhance several immonologic function (Zhang, Li, and Wang, 1997) and was provened to increase the activation of murine lymphocytes (Wang, Higuchi, and Zhang, 1997). Moreover, isoflavone glucuronides were able to activate natural killer cells to increase the immune defenses of the body against cancer (Zhang et al., 1999).

2.3. The phytoestrogens of *Pueraria mirifica*

2.3.1. *Pueraria mirifica*

Ethnobotany and application: *P. mirifica* was long recorded as domestic consumption to promote youth in both male and female (Suntara, 1931). Traditionally, women in some area of Thailand consume a traditional Thai remedy that includes both tuberous powder of *P. mirifica* and Tripala; *Terminalia bellerica*, T*erminalis chebula* and *Phyllanthus emblica* to relieve vasomotor symptoms (hot flashes and night sweats) associated with menopause. Recently, *P. mirifica* powder was extracted and used in cosmetic industry such as breast cream, eye gel and skin moisturizer. The beneficial of skin application was used for breast firming and anti-wrinkle. Moreover, the plant powder packed in capsule was manufactured and distributed as food supplement for aging (Dweck, 2002).

Botanical characteristics and habitats: *Pueraria mirifica* Airy Shaw et. Suvatabandhu (synonym: *Pueraria candollei* Wall. ex Benth var. *mirifica* Airy Shaw et Suvatabandhu), family Leguminosae, subfamily Papilinoideae, the same as soy, bean and pea (Ridley, 1967 and Suvatti, 1978). The plant is known as “Kwao Krua” or “Kwao Krua Kao (White Kwao Krua)”. *P. mirifica* was previously referred as *B. superba* until February 1947, it was identified as a new species of *Pueraria* and was named *Pueraria mirifica* Airy Shaw et. Suvatabandhu (Lakshnakara and Suvatabandhu, 1952).

The plant is a long-living twinning wood. The leaves were pinnately three foliate stipulate; terminal leaflet. The tuberous roots were varied in sizes and shapes. The flower was bluish-purple legume shaped, blooming during late January to early April. The length of the inflorescence of certain flowers was approximately 15-40 cm. The flower contained five sepals and the petals were one standard with two keels. The pod was slender typically short or elongate, smooth or hairy, including 1-10 single seeds when fully matured and dried which turned into various color. (Smitasiri and Wungjai, 1986 and Cherdshewasart and Sriwatcharakul, 2007) (Figure 2.7).

P. mirifica was found in the deciduous forest of the northern, western and northeastern parts of Thailand at the 80-800 meters level (Panriansaen, 2000 and Lakshnakara and Suvatabandhu, 1952). Recent survey revealed that the plant is distributed in at least 28 provinces (Subtaeng and Cherdshewasart, 2003). Variation within province was also found. The co-habitated plant of *P. mirifica* was typically teak and bamboo. The plant was not found in the forest with high-density trees. The vine of *P. mirifica* elongated for climbing over the trees while spreads on the ground in an open area (Panriansaen, 2000 and Panriansaen and Cherdshewasart, 2003). Attempts had been made to establish *in vitro* multiplication and plantation of *P. mirifica*. It was found that the plant tissue was responded to plant hormones and plantlets could initiate from *in vitro* (Cherdshewasart et al., 1996). The derived plants could initiate tubers (Sompornpailin et al., 2003).

Chemical constituents: *P. mirifica* had been found to contain at least 20 chemicals in the group of phytoestrogen with similar effects to estrogen. Miroestrol was the first isolated chemical and found in the amount of approximately 1.5 mg/ 100 g dry weight (Bound and Pope, 1960) and also shown estrogenic activity in rat vaginal cornification test (Jones and Pope, 1961). The chemical structure was not classified as steroid (Benson et al., 1961). The other compounds, mainly found in *P. mirifica* were isoflavonoids, chromenes, coumarins, sterols (Table 2.3 and figure 2.8) and macromolecule such as protein, lipid and starch (Appendix C). Deoxymiroestrol was isolated and found to be the compound with higher estrogenic potency than miroestrol, approximately 10-folds. However, it was easily oxidized by the air and converted to miroestrol and isomiroestrol (Chansakaow et al., 2000[a]).

The determination of isoflavone content, puerarin, daidzin, genistin, daidzein and genistein in the extracts of *P. mirifica* root from various location of Thailand by HPLC fingerprint assay revealed a great diversity of both total and individual assayed-isoflavone (Subtang, 2002 and Subtaeng and Cherdshewasart, 2003). The five isoflavonoids and isoflavone glycosides, daidzein, genistein puerarin, daidzin, and genistin had been used as markers. Whereas miroestrol cannot be used for quantitative standardization of *P. mirifica* root extract because no available commercialized standard.

Toxicity of *P. mirifica*: Several animal toxicology studies had been completed on *P. mirifica* using both crude powders and standardized extracts. *In vitro* study, *P. mirifica* root extract was not mutagenic by Ames test. (Julsiri and Cherdshewasart, 2003). LD_{50} of *P. mirifica* root extract, a single dose of 40 g/kg BW failed to induce signs of acute or subacute toxicity in mice (Chivapat et al., 2000). In long-term feeding experiments, a chronic toxicology study in rats treated orally with *P. mirifica* root extract at daily doses of 10, 100 and 1,000 mg/kg for 90 consecutive days revealed that at doses of 10 mg/kg BW or 100 mg/kg BW exhibited no toxic effects. However, in one study, a daily dose of 1000 mg/kg BW for 90 days induced reversible anemia and pathologic changes in the kidneys and testicles (Chivapat et al., 2000). The later study was found that plant powder and extract were evaluated and no toxicity was found (Cherdshewasart et al., 2000 and Cherdshewasart, 2003).

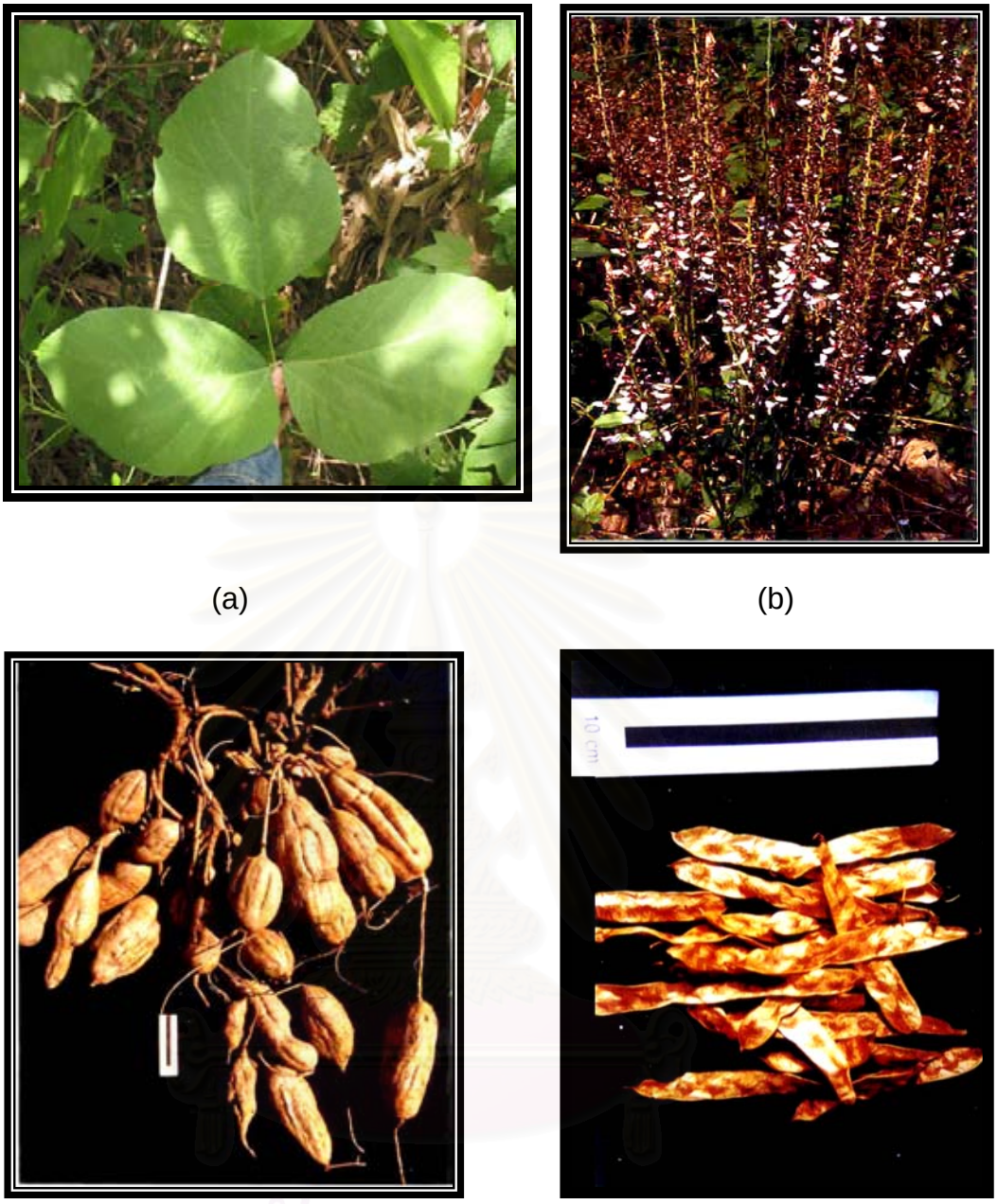
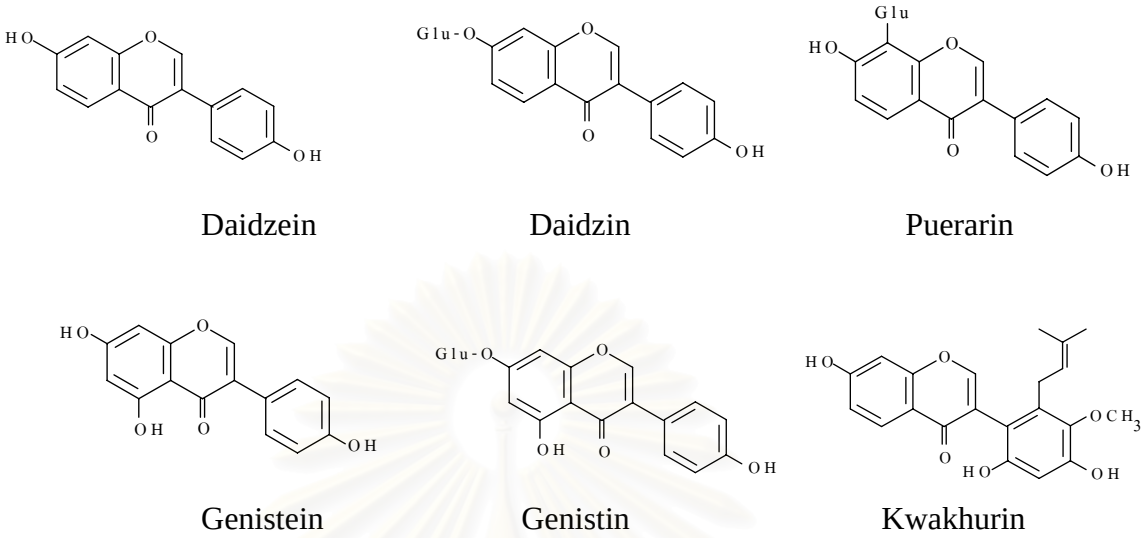


Figure 2.9. Leaves (a), flower (b), tuberous root (c) and pod (d) of *P. mirifica*

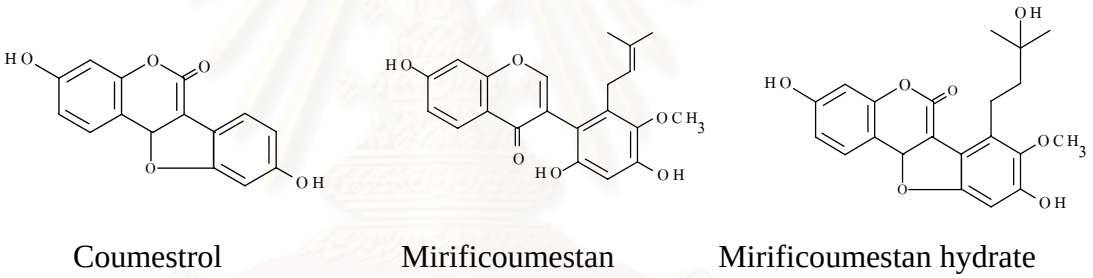
Table 2.3. Summary of the chemical constituents of *P. mirifica* (adapted from Panriansaen, 2000)

Category	Chemical	Reference
Isoflavonoids	Daidzein	Ingham et al., 1986[a]
	Genistein	Ingham et al., 1986[a]
	Kwakhurin	Ingham et al., 1986[a]
	Kwakhurin hydrate	Ingham et al., 1989
Isoflavone	Daidzin	Ingham et al., 1986[a]
glycosides	(daidzein-7-o-glucoside)	
	Genistin (genistein-7-o-glucoside)	Ingham et al., 1986[a]; Ingham et al., 1989
	Mirificin	Ingham et al., 1986[a]; Ingham et al.,
	(puerarin 6”-o-β-apiofuranoside)	1986[b]
	Puerarin (daidzein-8-glucoside)	Nilandihi et al., 1957
		Ingham et al., 1986[a]; Ingham et al.,
		1986[b]; Ingham et al., 1989
	Puerarin 6”-monoacetate	Ingham et al., 1989
Chromenes	Miroestrol	Schoeller et al., 1940
		Bound and Pope, 1960
		Jones and Pope, 1960
	Deoxymiroestrol	Chansakaow et al., 2000[a]
	Isomiroestrol	Chansakaow et al., 2000[a]
Coumestans	Coumestrol	Ingham, et al., 1986; Ingham, et al., 1988
	Mirificoumestan	Ingham et al., 1988
	Mirificoumestan glycol	Ingham et al., 1988
	Mirificoumestan hydrate	Ingham et al., 1988
Sterols	β- sitosterol	Hoyodom, 1971
	Stigmasterol	Hoyadom, 1971
Pterocapans	Pueriicapene	Chansakaow et al., 2000[b]
	Tuberosin	Chansakaow et al., 2000[b]
Acid	Tetracosanoic acid	Chansakaow et al., 2000[b]

Isoflavone and Isoflavone glycosides



Coumestans



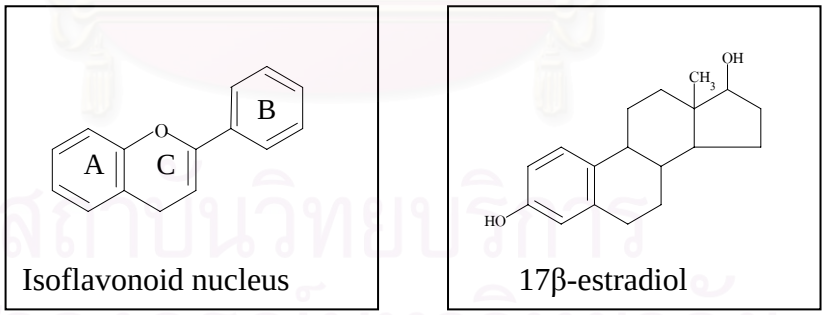
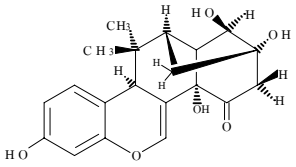
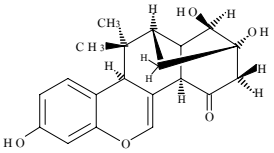


Figure 2.10. The structure of chemical compounds in *P. mirifica* and isoflavonoid nucleus compare with estrogen

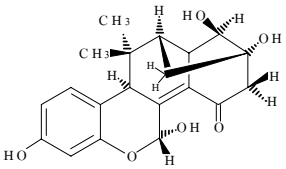
Chromenes



Miroestrol

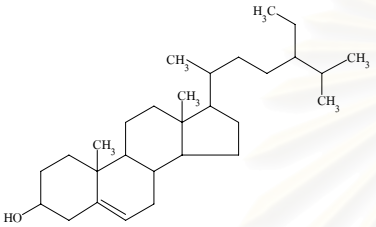


Deoxymiroestrol

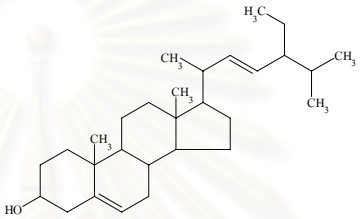


Isomiroestrol

Sterols

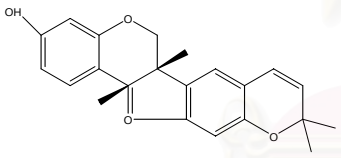


β-sitosterol

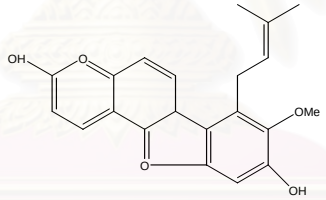


Stigmasterol

Pterocarpans



Tuberosin



Puemirificarpene

Figure 2.10. The structure of chemical compounds in *P. mirifica* and isoflavonoid nucleus compare with estrogen (continued)

Hormonal effect of *P. mirifica*: Many studies on *P. mirifica* were mostly evaluated in estrogenic activity on the reproductive system. In animal model, *P. mirifica* showed many effects depended on the method of bioassay such as dosage and period of exposure. The biological effects were identified in Table 2.4. It was revealed that miroestrol had 0.7 time of estradiol on mammary gland proliferation activity (Pope et al., 1958). In ovariectomized rat, *P. mirifica* root extraction induced proliferation of the cornified cell, increased uterus weight (Malaivijitnond, 2006) and exhibited strong estrogenic activity in uterotrophic assay (Kim et al., 2003). Uterus

and vagina weight of *P. mirifica*-treated immature rat was significantly increased (Sawatdipong, 1981). *P. mirifica* could influence the reproductive functions in both sex of rats, but the response was greater in male than female (Malaivijitnond et al., 2004; 2003[a]; 2003[b] and Kaitthaipipat, 2001). In monkey, a single dose of 1,000 mg/kg BW of *P. mirifica* disrupted ovarian function, menstrual cycle and also decreased the Parathyroid hormone and serum calcium level (Trisomboon, et al. 2004), *P. mirifica* greatly influenced menstrual cycles and may suppress ovulation by lowering serum levels of gonadotropins (Trisomboon, et al. 2005), *P. mirifica* had estrogenic action by increasing reddish sexual skin coloration in aged menopausal monkeys (Trisomboon, et al. 2006[b]). In clinical trial, miroestrol (Cain, 1960) and the crude drug showed the effectiveness in treatment of menopausal symptom (Muangman and Cherdshewasart, 2001; Lamlertkittikul and Chandeying, 2004)

Estrogenic activity of *P. mirifica* was responded in dose-dependent manner on MCF-7 cells and HepG2 cells. The mechanism of action of the plant extract was evaluated. It was found that the chemicals needed a metabolic activation to promote their actions within human cells. Recombinant yeast exhibited no estrogenic activity because it lacked metabolic enzyme (Lee et. al., 2002). Crude extract of *P. mirifica* showed biphasic effect on the growth of MCF-7 as well as 17β-estradiol with proliferative effect at low concentration and antiproliferative effect at high concentration with ED_{50} value of 642.83 μg/ml (Cheewasopit, 2001; Cheewasopit et al., 2003; Trisap et al., 2003 and Cherdshewasart et al., 2004[a]). While the crude extract of *P. mirifica* indicated no proliferative and anti-proliferative effect in HeLa cells at 100 and 1,000 μg/mL (Cherdshewasart et al., 2004[b])

To compare the estrogenic activity in each compound on MCF-7 system, it was found that those compounds had different degree of estrogenic activity (17β-estradiol (lower 10^{-12}) > deoxymiroestrol (10^{-10}-10^{-9})>miroestrol (10^{-8})>coumestrol (10^{-7}) ≈ genistein (10^{-7}) > daidzein (10^{-6}) ≈ kwakhurin (10^{-3})). Whereas daidzin, puerarin, puermicapene, tuberosin and isomiroestrol had no estrogenic activity (Chansakaow et al., 2000[a]; Chansakaow et al.,2000[b]) as shown in Table 2.5.

Table 2.4. Summary of the recent reports of the biological effects of *P. mirifica* on animal model

Effects	References
1. Reproductive system	
1.1 Reproductive organ development	
• Promoted mammary duct and breast enlargement in mice, rat, pig	• Pope et al., 1958, Smitasiri et al., 1986; Panriansaen, 2005
• Proliferated the uterus , vaginal cornification in rat	• Sukhavachana, 1940; Sawatdipong, 1981; Malaivijitnond et al., 2006
• Estrogenic effect on Gonadotrophin levels ,sexual skin coloration in monkeys	• Trisomboon et al., 2006
1.2 Fertilization and birth control	• Smitasiri, 1988
• Increased mating behavior	• Smitasiri and Pangjit, 1986, Smitasiri, 1988
• Anti-fertilization	• Sangkaew and Smitasiri, 1985, Smitasiri et al., 1986
• Induce abortion	• Langkalichan and Smitasiri, 1985
• Reduction of sperm	
2. Others	• Thaiyanun et al., 1992, Chivapat et al., 2000
• Cholesterol level	• Anuntalabhochai and Jersrichai, 1986, Bulintanthikul, 1978, Trisomboon et al., 2004
• Calcium level	

Table2.5. The growth-promoting effects of chemical compound extracts of *P. mirifica* on MCF-7 human breast cancer cells. (Chansakaow et al., 2000[a]; Chansakaow et al., 2000[b])

Compounds	Content (mg/100 g powder)	Growth-promoting effects on MCF-7 (Minimal concentrations)(M)
Isoflavone and glycoside		
Genistein	0.6	10^{-7}
Genistin	Data not shown	Data not shown
Daidzein	46.1	10^{-6}
Daidzin	8.5	No activity
Kwakhurin	0.6	$>10^{-6}$
Chromenes		
Miroestrol	3.0	10^{-8}
Deoxymiroestrol	2.0	10^{-10}-10^{-9}
Isomiroestrol	2.2	no activity
Coumestrol	0.07	10^{-7}
Pterocarpens		
Tuberosin	0.3	No activity
Puemiricarpene	1.8	No activity
Acid		
Tetracosanoic acid	15.3	-
17β-estradiol (control)	-	$<10^{-12}$

* Minimal concentration of compounds that caused 50% MCF-7 breast cancer cells growth when compared to the control

CHAPTER III

MATERIALS AND METHODS

3.1. Plant materials

The fresh mature leaves of 3 cultivars of *P. mirifica* (PM-III, PM-IV and PM-V) cultivated in a field trial at Banpong District of Ratchaburi Province (E99^{o}52^{o}/N13^{o}37^{o}) were collected in March-August 2007. The original of plant cultivar, leaf and pod morphometry of the 3 plant cultivars was previously described (Cherdshewasart and Sriwatchrakul, 2007).

3.2. Leaf harvest

The mature fresh leaves, weight at least 3 kg were randomly harvested from 3 plots of each plant cultivar since March 2007 to August 2007. The collected samples were cleaned with distilled water and dried in hot air oven at 80^{o}C for 72 hours and subsequently powdered at a size of 120 μm.

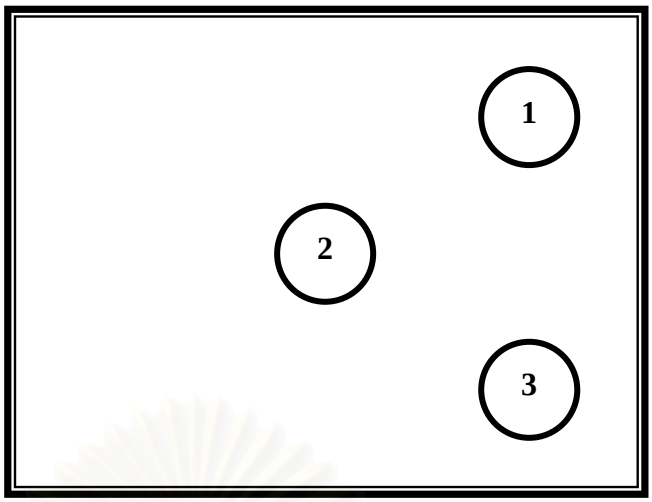
a)

Figure 3.1. The plant field trial of PM; a) PM-III, b) PM-IV and c) PM-V

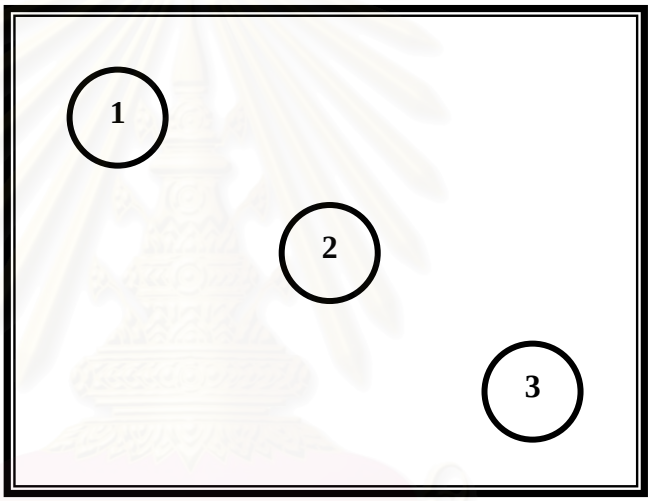
b)

c)

Figure 3.1. The plant trial field of PM; a) PM-III, b) PM-IV and c) PM-V



a)



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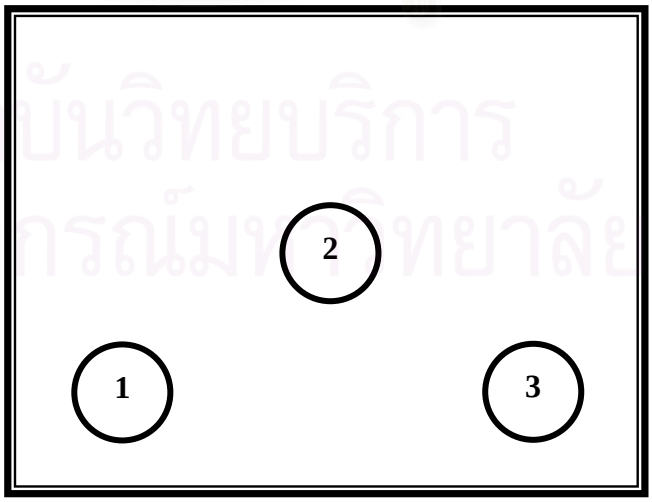

c)

Figure 3.2. The selected plot in each trial field cultivar of PM; a) PM-III, b) PM-IV and PM-V.

3.3. Plant leaf crude extraction

The 50 g of leaf powder was mixed with 500 ml methanol for 3 times by agitation in incubator-shaker overnight. The pellet was discarded and the supernatant was filtered (Whatman filter paper No.4, Whatman, USA) and evaporated in rotary evaporator *in vacuo* (Buchi, Germany) at 45^{o}C until completely dried. The plant crude extract was dissolved in distilled water and partition in chloroform to get rid of chlorophyll. The obtained crude extract was dissolved in butanol and the supernatant was subsequently evaporated at 55^{o}C until completely dried. The crude extract was weighted and stored in a tightly-capped bottle at 4^{o}C.

3.4. Analysis of isoflavonoid contents

3.4.1. Chemicals and equipments

Isoflavonoid standards, including puerarin, genistin, daidzein and genistein were purchased from Sigma (St. Louis, MO, U.S.A.), daidzin was purchased from Fluka Biochemika (Buchs, Switzerland). The organic solvents for chromatography (HPLC grade) were purchased from Merck (Germany). The water of over 16 MΩ/cm for a component of the mobile phase of HPLC was prepared by Maxima Ultrapure Water Systems (ELGA). HPLC system control and data processing were carried out by a Waters™ apparatus (Waters-717 plus Autosampler, Waters-600 Controller, Waters-2996 Photodiode Array Detector). The reversed phase C18 column (250 x 4.6 mm) was filled with 5 μm ODS2 (Waters Spherisorb, Ireland), pre-filtered with Waters Spherisorb S5 ODS2 (4.6x10 mm) guard cartridge. The filter set was Millipore membrane at 0.45 μm pore size with 13 mm diameter for the sample and 47 mm diameter for the mobile phase, of HA type for aqueous solution and HV type for organic solvent. The chromatography manager software Empower™ was operated on a personal computer.

3.4.2. HPLC sample preparation

One mg of leaf crude extract was dissolved at room temperature with 1 ml methanol (Merck). The supernatant was filtrated with a 0.45 μm pore size, 13 mm diameter membrane.

3.4.3. Quantitative HPLC

Methods for isoflavonoid analysis were modified from those previously described (Cherdshewasart *et al*., 2007[a]) by setting the linear gradient system for 50 min from 100: 0 to 55: 45 with 0.1 % acetic acid: acetonitrile, with a flow rate of 1 ml/min and analyzed at the wavelength of 254 nm. The standard isoflavonoids were serially diluted from 1:1 to 1:16 with methanol to establish the concentrations of 1/4, 1/8, 1/16, 1/32 and 1/64 mg/ml, to generate a five point calibration curve. Calibration curves were obtained for all isoflavonoids by plotting the standard concentration as a function of peak area from HPLC analysis of a 10 µl injection volume. The concentrations of standard were chosen to cover the range of isoflavonoid concentrations in the samples. The analyses of the samples were run in triplicate and identified by comparing the retention times and quantified for the amount using standard curves of peak area of the isoflavonoid standards.

3.5. Statistical analysis

The isoflavonoid contents from the samples of *P. mirifica* were analyzed for statistical significant by One-Way ANOVA, Duncan analysis, Post-Hoc Multiple Comparison test and of variance at the significance level of $P < 0.05$ with the aid of SPSS program.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1. Characteristics of the leaf powders

The 120 μm leave powders of PM-III, PM-IV and PM-V are shown in Figure 4.1.

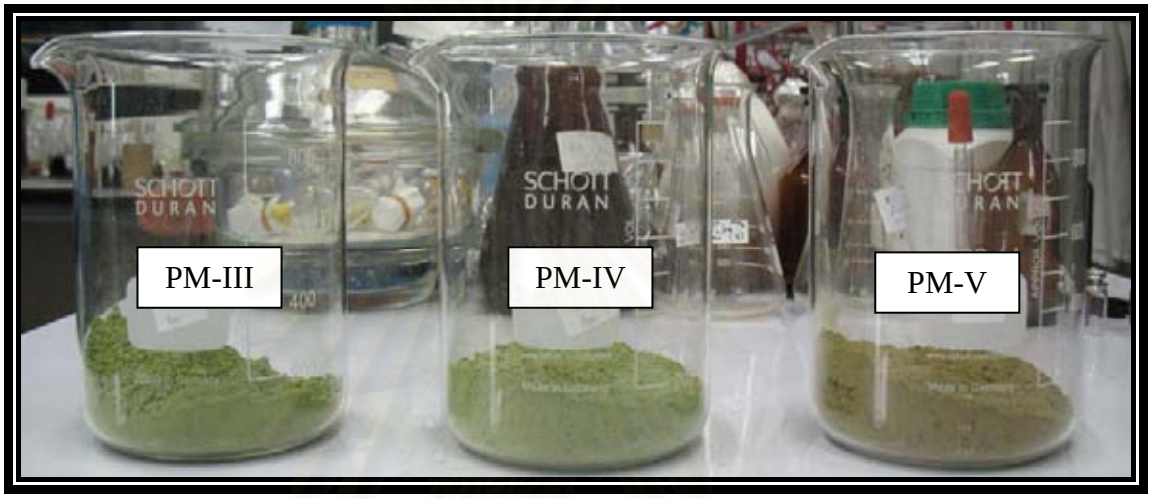

Figure 4.1. The characteristic of 3 PM leaves powder. This may notified

4.2. Plant leaf crude extraction

Plant leaf powders were extracted with solvents, 1:10 (w/v), for 3 times and evaporated in rotary evaporator *in vacuo* until completely dried and stored at 4 ^{o}C (Figure 4.2.)

Figure 4.2. The dark-brown leaf crude butanol extract of PM-III, PM-IV and PM-V.

The leaves crude butanol extract contents of 3 PM cultivars; PM-III, PM-IV and PM-V were shown in Table 4.1. Mean± S.E.M. of Area 3 in July 2007 was the highest amount that is 1.7121 ± 0.2644 g where as the maximum of cultivar was PM-III in June 2007 (2.0900 g). In the term of total area, the total area of July 2007 was the highest (4.2141 ± 0.4233). The butanol crude extract (0.2576 g) and the total content of April 2007 was the highest amount (1.3339 ± 0.3575 g). The results demonstrated the influence of both climatic changes and plant genetics on yield of plant crude extracts.

Table 4.1. The leaves crude butanol extract contents of 3 PM cultivars; PM-III, PM-IV and PM-V in each area.

Month (2007)	Cultivars	Leaf crude butanol extract contents (g)			
		Area of plant leaf collection			
		1	2	3	Total
April	PM-III	0.2994	0.2745	0.2576	0.8315
	PM-IV	0.7332	0.6694	0.6231	2.0257
	PM-V	0.3847	0.3745	0.3854	1.1446
	Mean ± S.E.M.	0.4724 ± 0.1327	0.4395 ± 0.1185	0.4220 ± 0.1071	1.3339 ± 0.3575
May	PM-III	1.3546	1.0135	0.7254	3.0935
	PM-IV	1.0312	1.0031	1.1013	3.1356
	PM-V	0.7324	0.7842	0.7856	2.3022
	Mean ± S.E.M.	1.0394 ± 0.1797	0.9336 ± 0.0748	0.8708 ± 0.1166	2.8438 ± 0.2711
June	PM-III	2.0900	1.6190	1.3458	5.0548
	PM-IV	0.7550	0.9532	1.4622	3.1704
	PM-V	1.3318	1.2618	1.4133	4.0069
	Mean ± S.E.M.	1.3923 ± 0.3866	1.2780 ± 0.1924	1.4071 ± 0.0337	4.0774 ± 0.5451
July	PM-III	1.7262	1.4963	1.8382	5.0607
	PM-IV	0.4598	1.2316	2.0938	3.7852
	PM-V	1.1624	1.4297	1.2043	3.7964
	Mean ± S.E.M.	1.1161 ± 0.3663	1.3859 ± 0.0795	1.7121 ± 0.2644	4.2141 ± 0.4233
August	PM-III	0.5241	0.2964	0.7518	1.5723
	PM-IV	1.2599	1.7224	1.144	4.1263
	PM-V	1.4079	1.3754	1.3925	4.1758
	Mean ± S.E.M.	1.0640 ± 0.2733	1.1314 ± 0.4294	1.0961 ± 0.1865	3.2915 ± 0.8597

The characteristic of the leaves crude butanol extracts is sticky wax-like material with dark brown colour.

4.3. Analysis of isoflavonoids of the leaf crude extract

4.3.1. Standard curve establishments

Standard of five major isoflavonoids; puerarin, daidzin, genistin, daidzein and genistein, were selected for the quantitative analysis of leaf crude extracts. They were detected in different retention times (Figure 4.3. - 4.8. and Table 4.2.) and their standard concentrations were properly diluted in Figure 4.9. - 4.13.

Table 4.2. The retention time of five standard isoflavonoids calculated by Empower™ program

Isoflavonoid peak	Retention time (min)	Area	% Area	Height	Amount (μg/μl)
Puerarin	14.395	256984	4.83	31995	0.0250
Daidzin	15.995	30232	0.57	2876	0.0030
Genistin	19.072	81396	1.53	5144	0.0030
Daidzein	26.682	415416	7.81	20917	0.0250
Genistein	34.843	4534289	85.26	102760	0.0250

The quantitative of each standard isoflavonoid was calculated by Empower™ program and compared with the standard chromatogram and determined for the R^2 of the standard curve (Figure 4.3-4.8) that are 0.9768, 0.9899, 0.9871, 0.9943 and 0.9985 for puerarin, daidzin, genistin, daidzein and genistein, respectively. The results demonstrated that the 5 major isoflavonoid standards were clearly distinguished from the separated retention times.

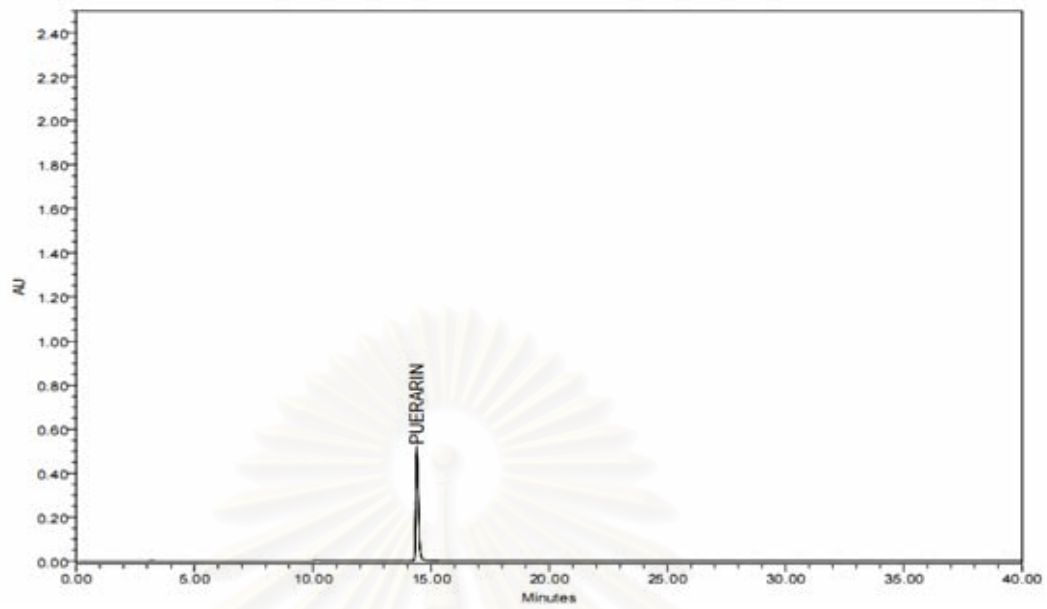

Figure 4.3. The chromatogram of standard puerarin (0.1 μg/μl) at 254 nm

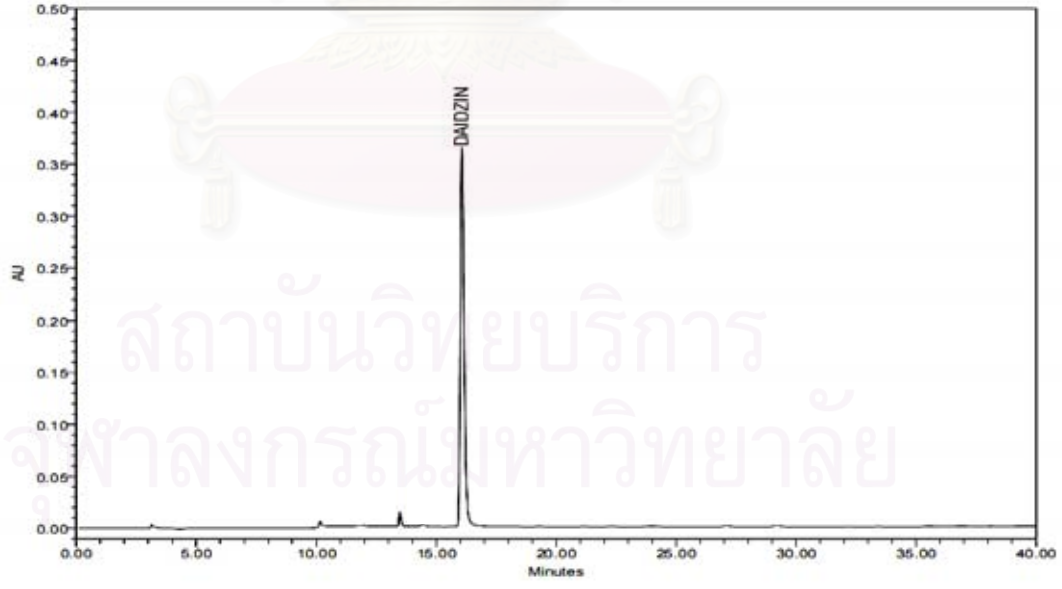

Figure 4.4. The chromatogram of standard daidzin (0.1 μg/μl) at 254 nm

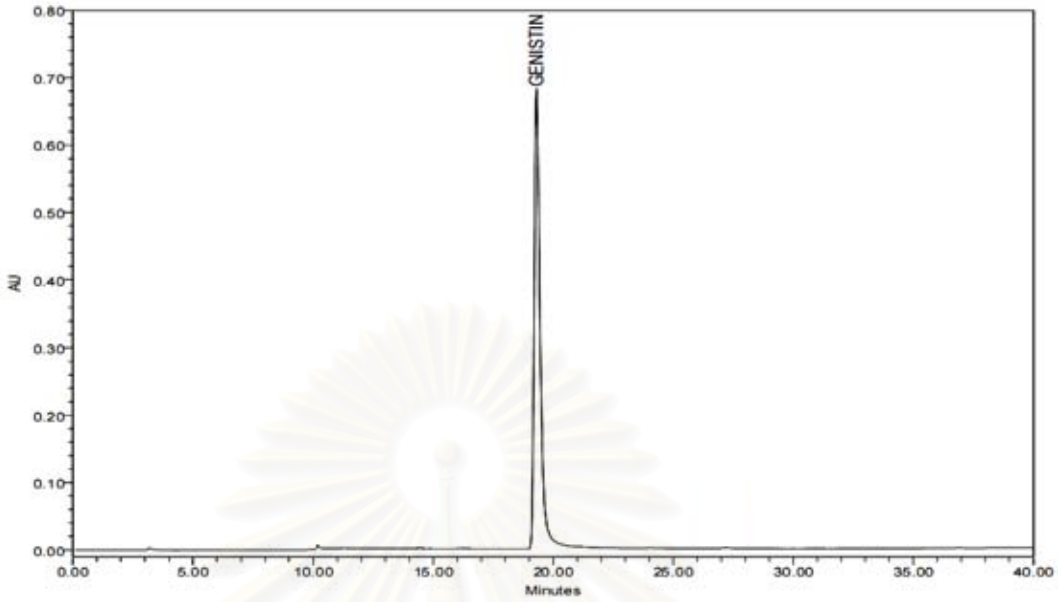

Figure 4.5. The chromatogram of standard genistin (0.1 μg/μl) at 254 nm

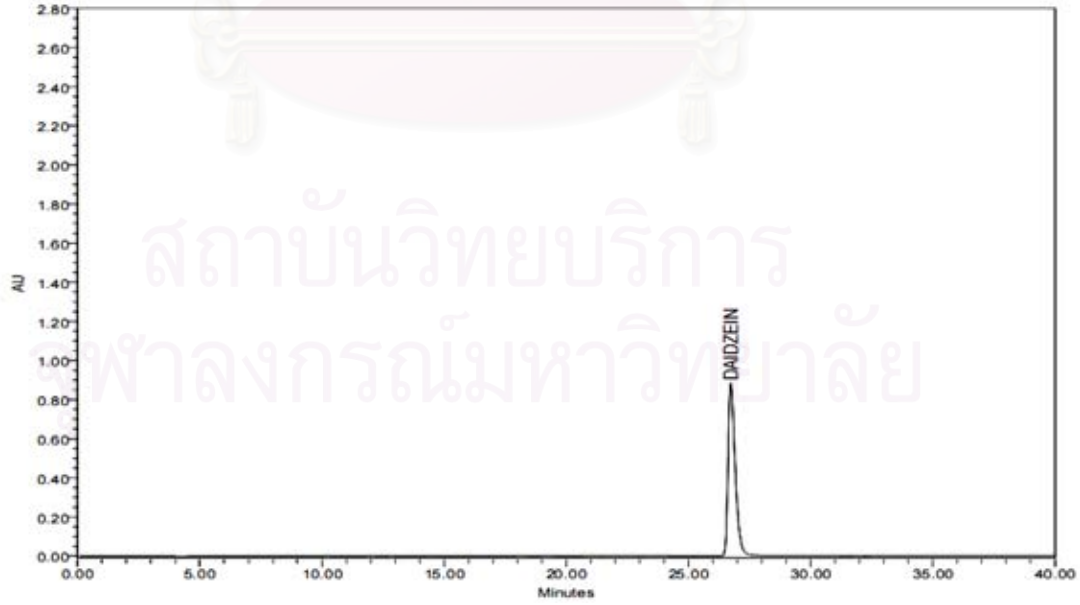

Figure 4.6. The chromatogram of standard daidzein (0.25 μg/μl) at 254 nm

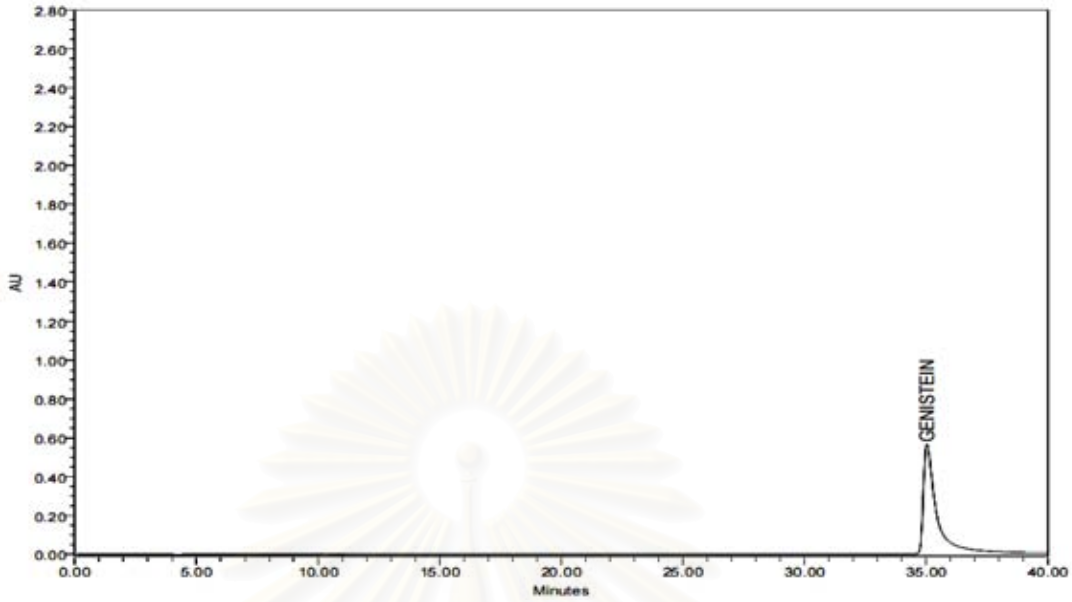

Figure 4.7. The chromatogram of standard genistein 0.25 μg/μl at 254 nm

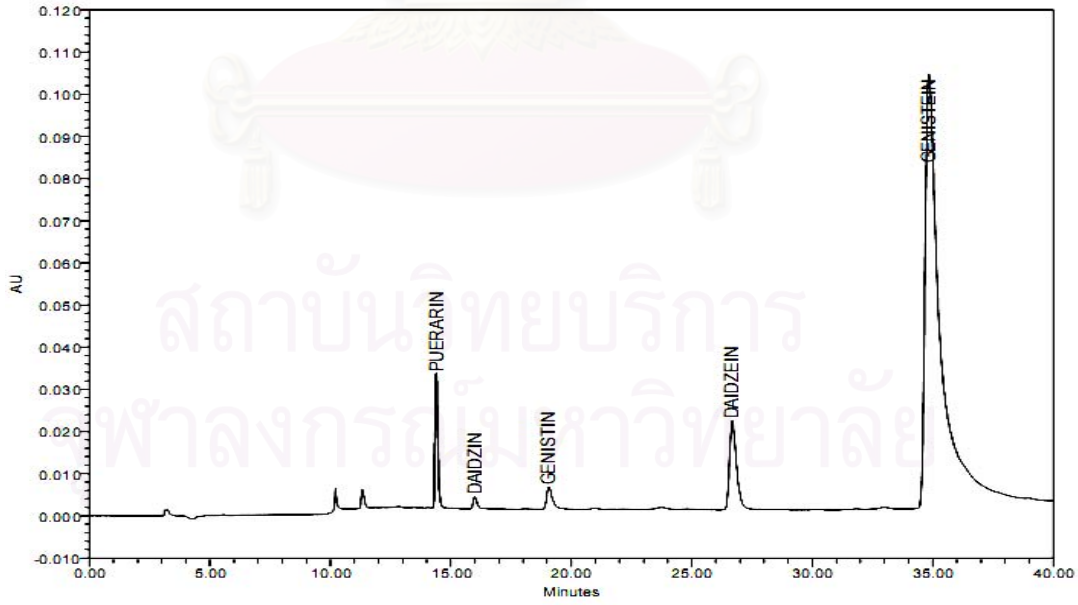

Figure 4.8. The chromatogram of 5 standard isoflavonoids; puerarin (0.025 μg/μl), daidzin (0.003 μg/μl), genistin (0.003 μg/μl), daidzein (0.025 μg/μl) and genistein (0.025 μg/μl) at 254 nm

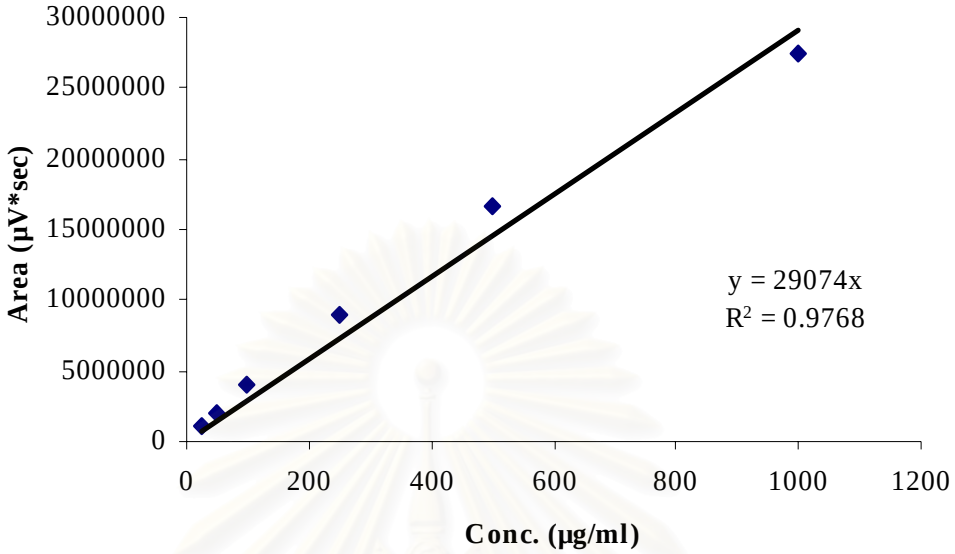

Figure 4.9. Standard curve of puerarin at 254 nm

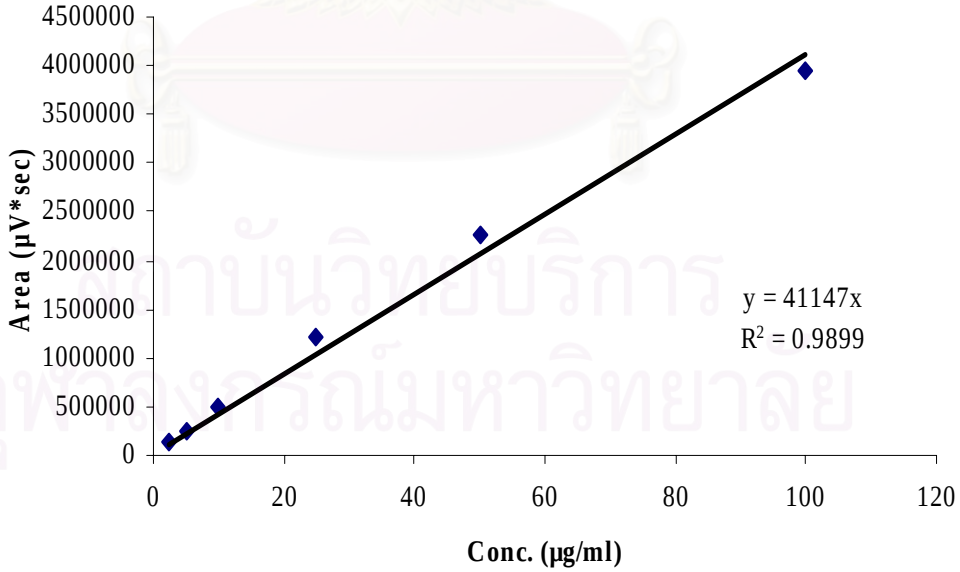

Figure 4.10. Standard curve of daidzin at 254 nm

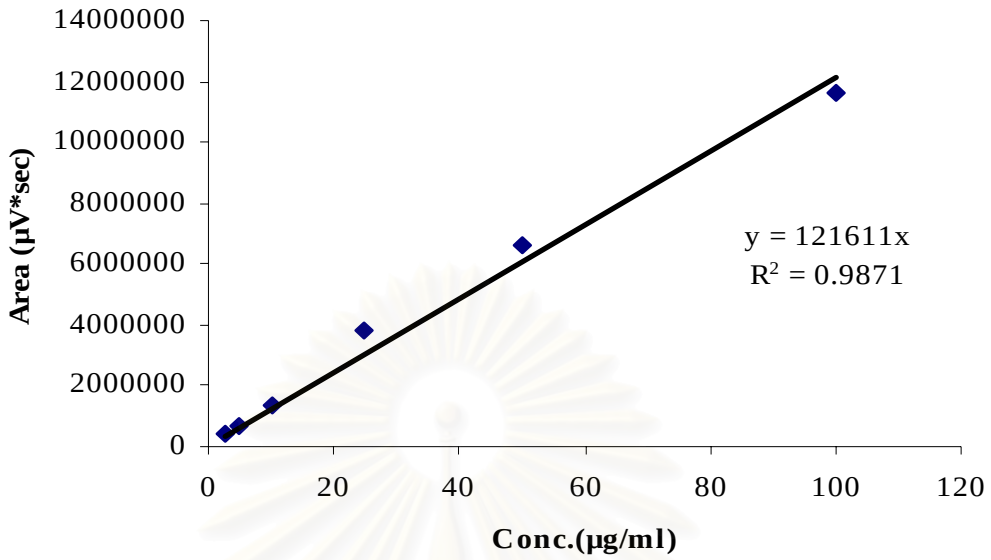

Figure 4.11. Standard curve of genistin at 254 nm

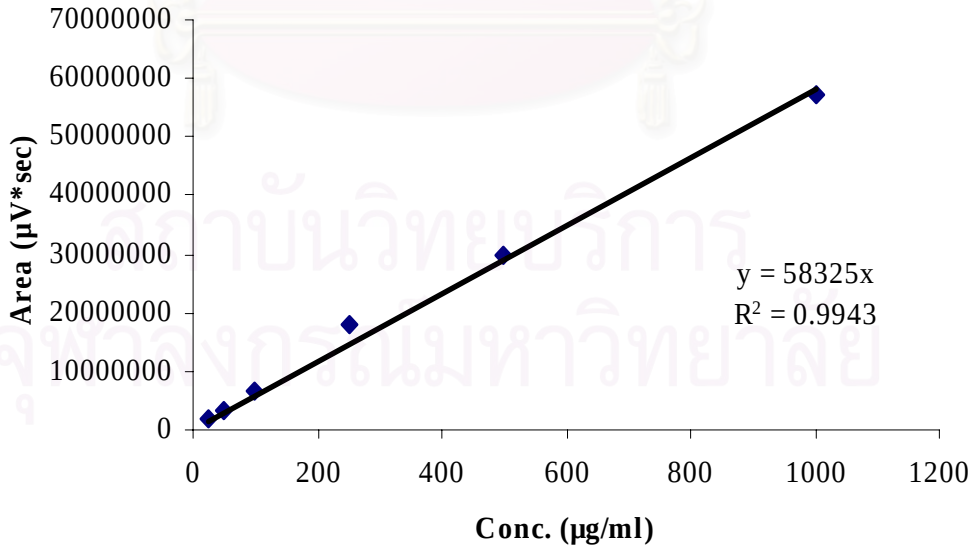

Figure 4.12. Standard curve of daidzein at 254 nm

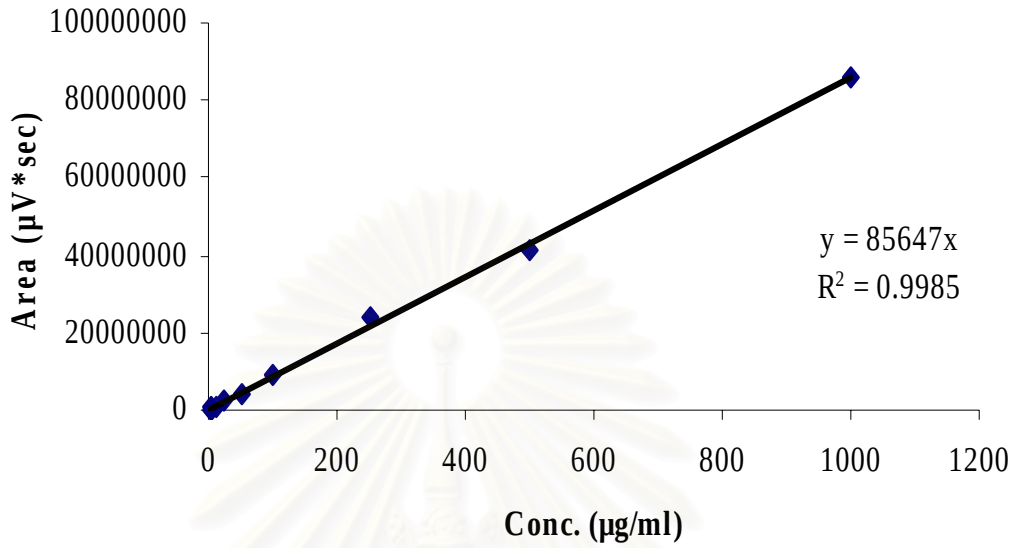

Figure 4.13. Standard curve of genistein at 254 nm

4.3.2 Quantitative analysis of isoflavonoids in PM leaf crude extracts

The leaf crude extracts were dissolved in absolute methanol and determined for 5 major isoflavonoids puerarin, daidzin, genistin, daidzein and genistein by RP-HPLC analysis at 254 nm and calculated using Empower™ program. The results were demonstrated in Figure 4.14 to 4.28

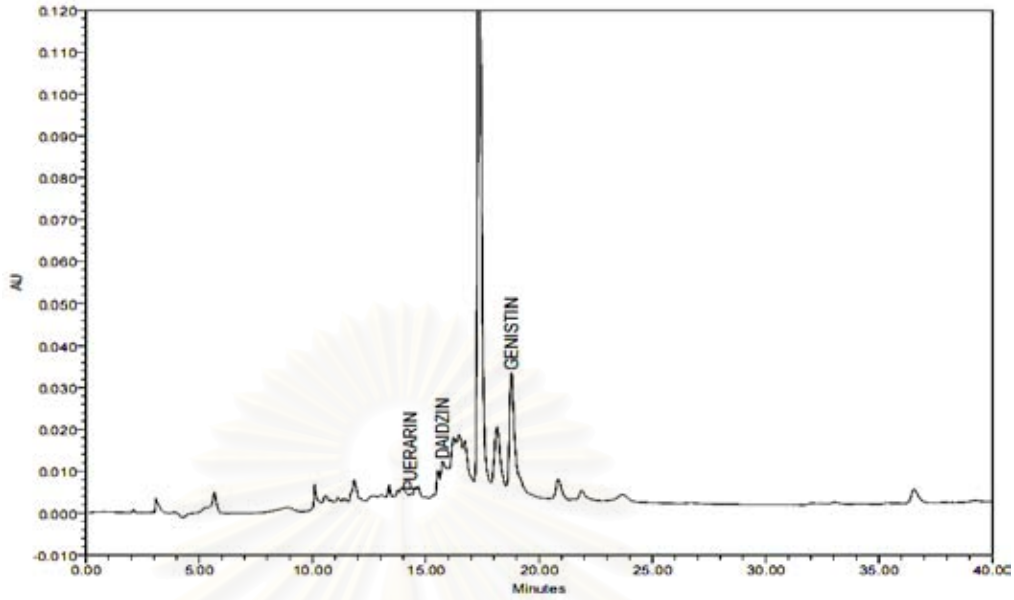

Figure 4.14. The chromatogram of PM-III leaves in April 2007

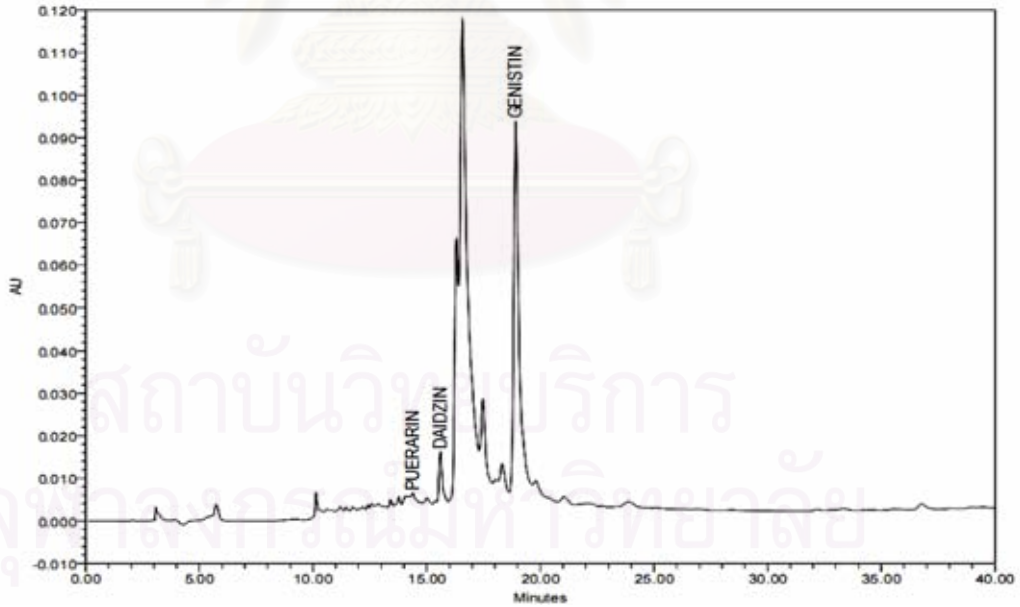

Figure 4.15. The chromatogram of PM-IV leaves in April 2007

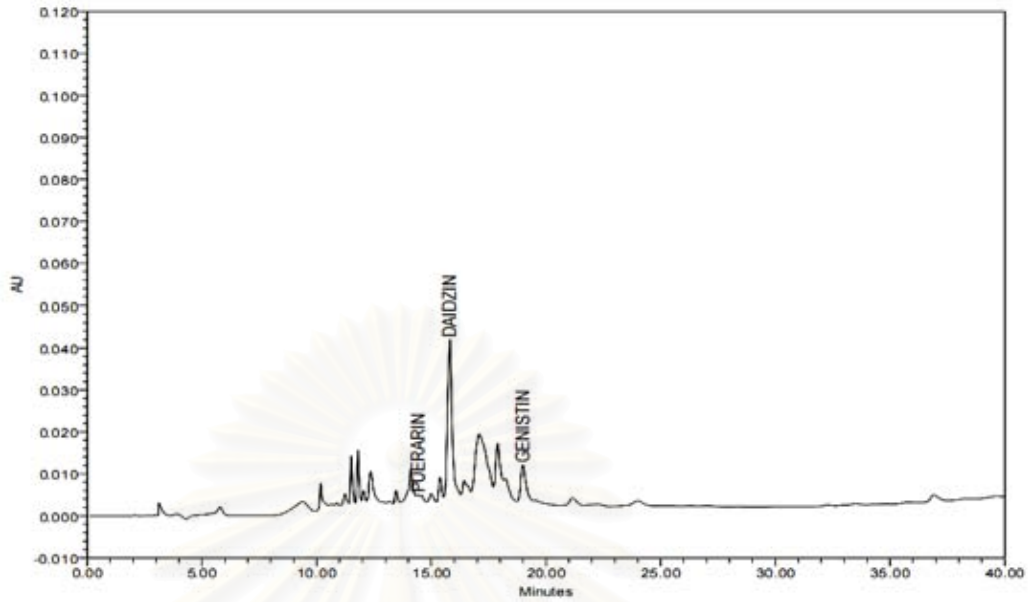

Figure 4.16. The chromatogram of PM-V leaves in April 2007

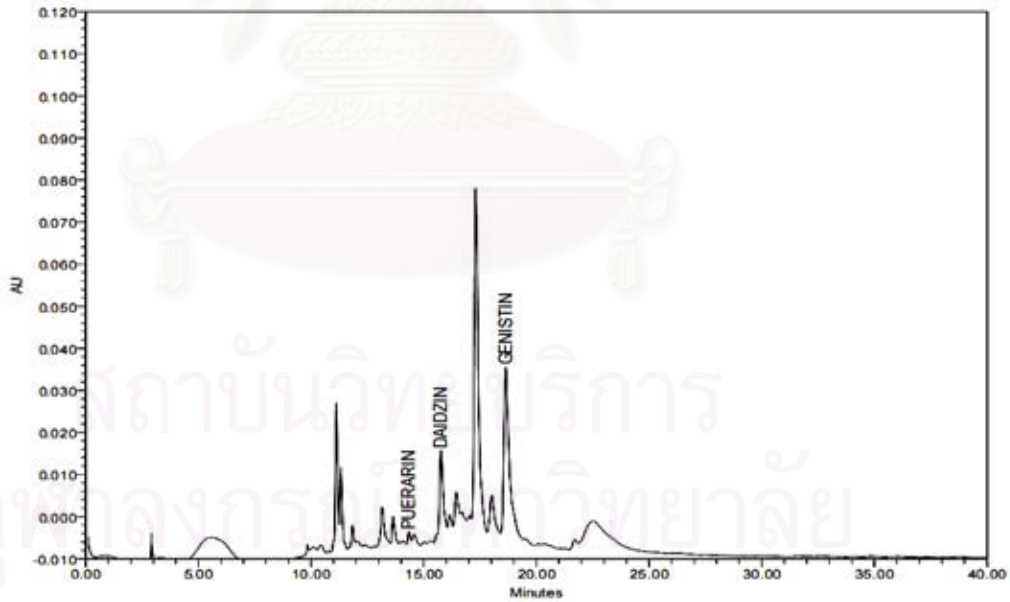

Figure 4.17. The chromatogram of PM-III leaves in May 2007

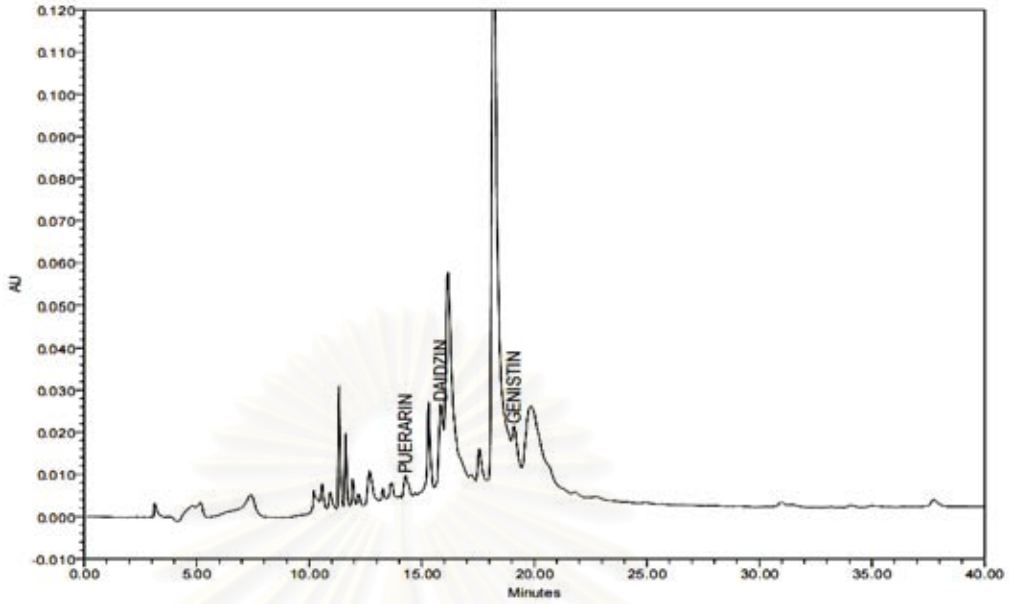

Figure 4.18. The chromatogram of PM-IV leaves in May 2007

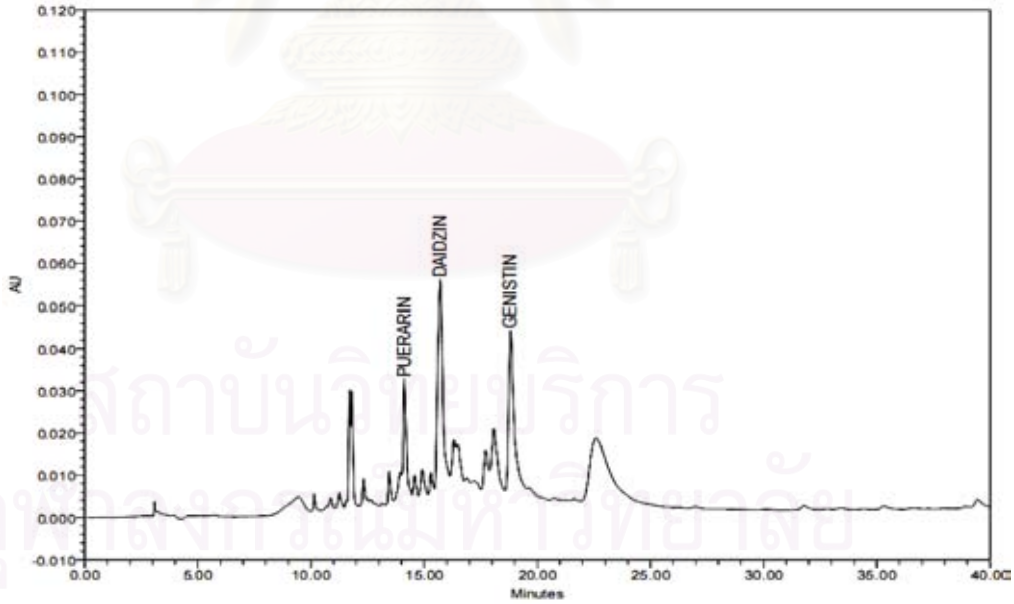

Figure 4.19. The chromatogram of PM-V leaves in May 2007

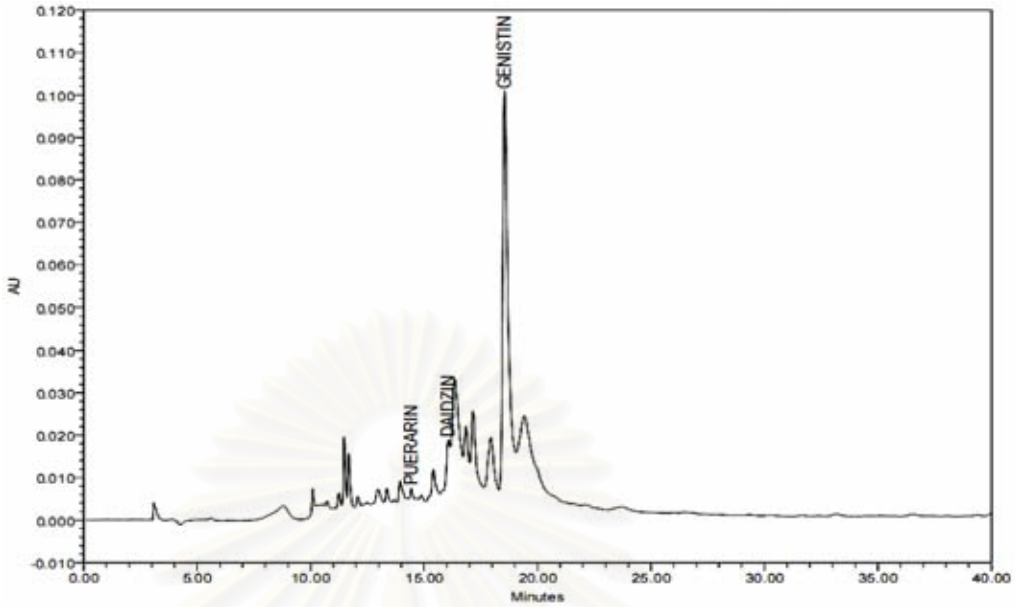

Figure 4.20. The chromatogram of PM-III leaves in June 2007

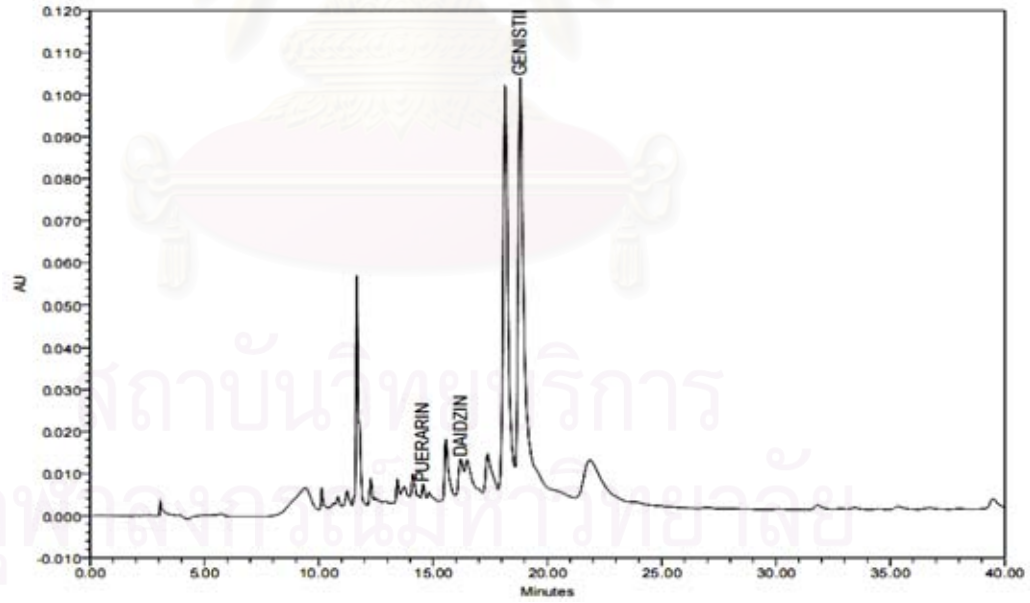

Figure 4.21. The chromatogram of PM-IV leaves in June 2007

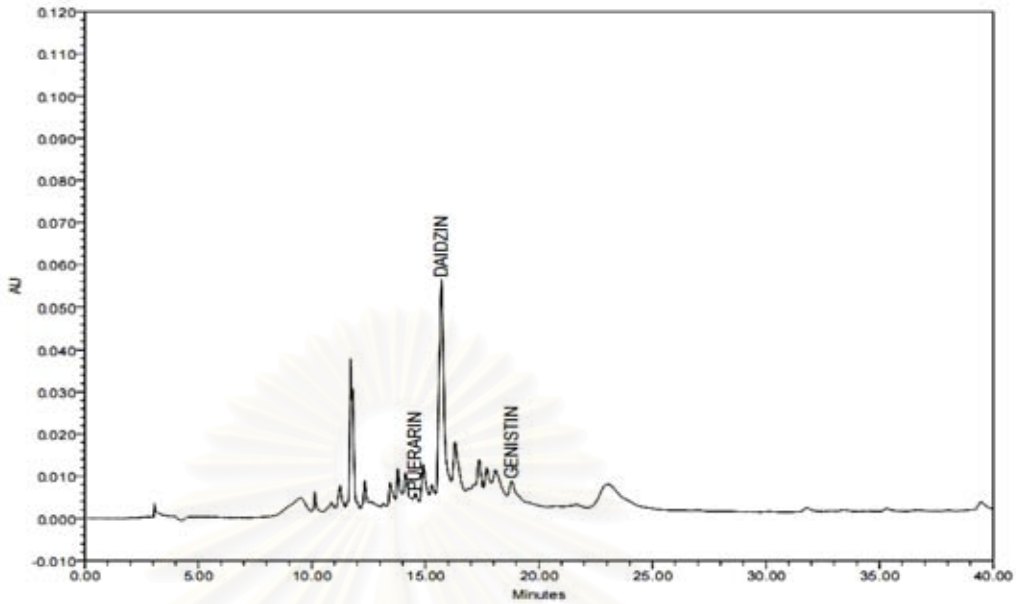

Figure 4.22. The chromatogram of PM-V leaves in June 2007

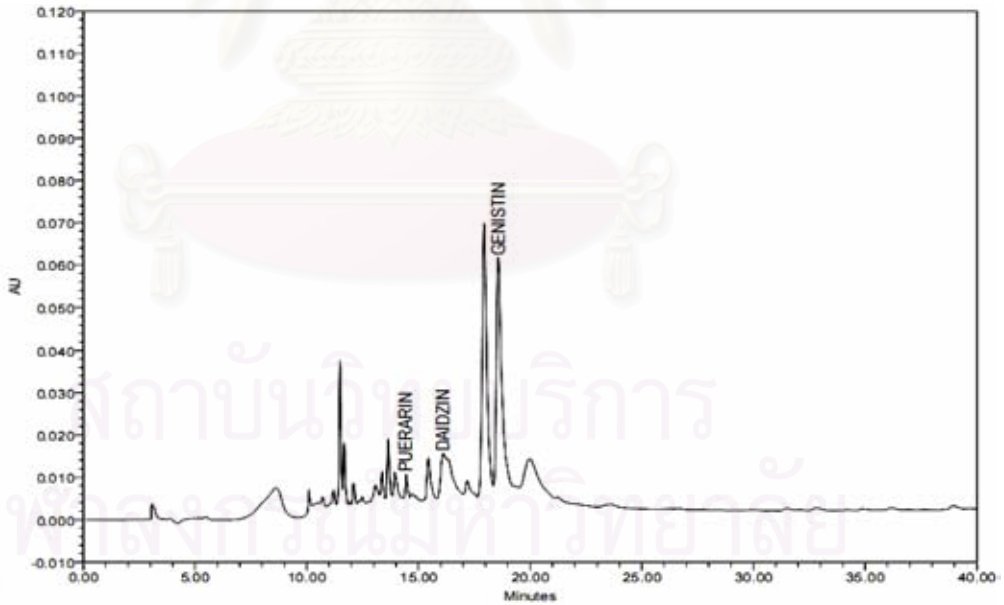

Figure 4.23. The chromatogram of PM-III leaves in July 2007

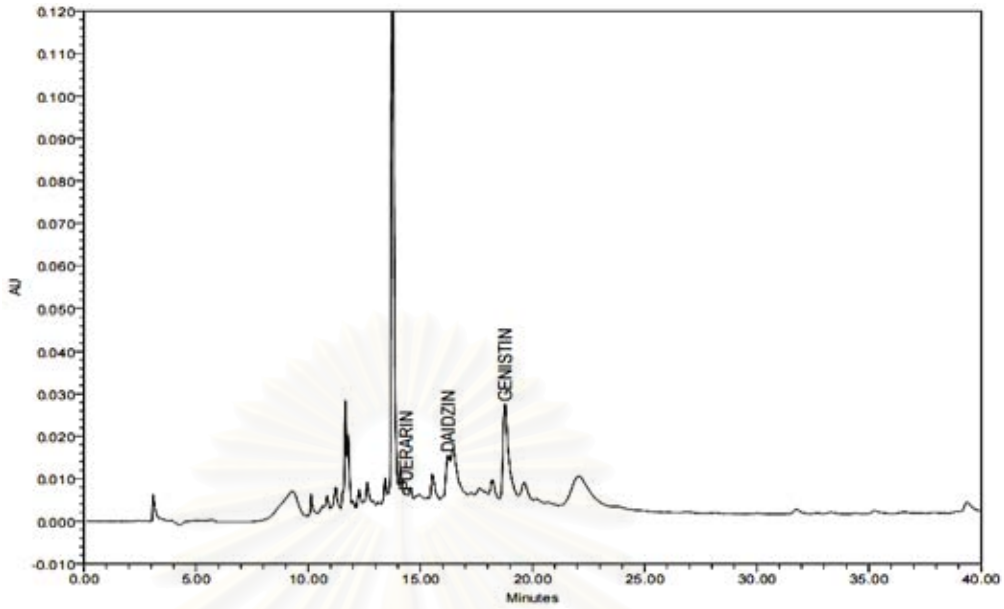

Figure 4.24. The chromatogram of PM-IV leaves in July 2007

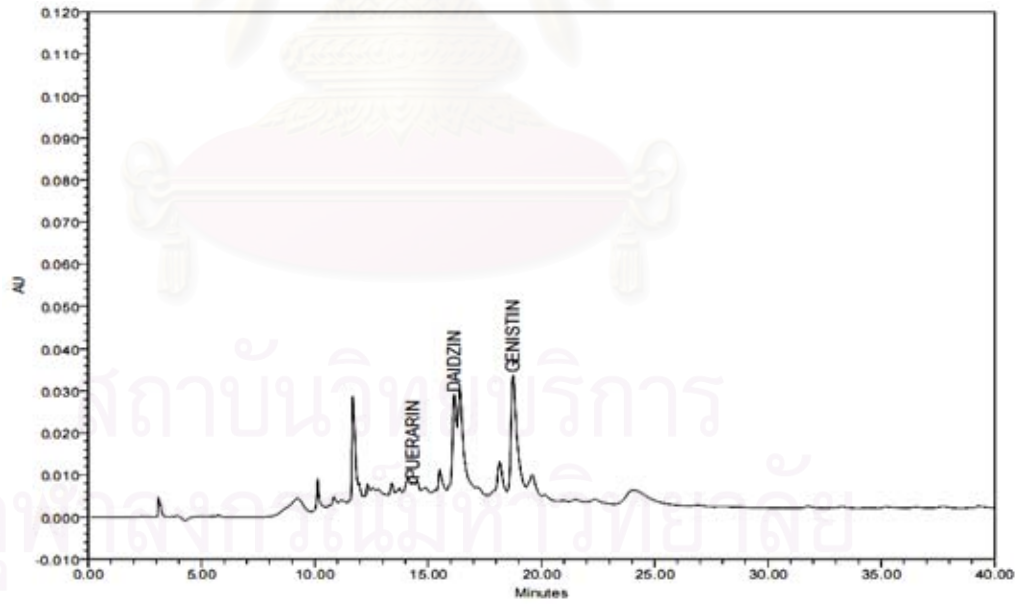

Figure 4.25. The chromatogram of PM-V leaves in July 2007

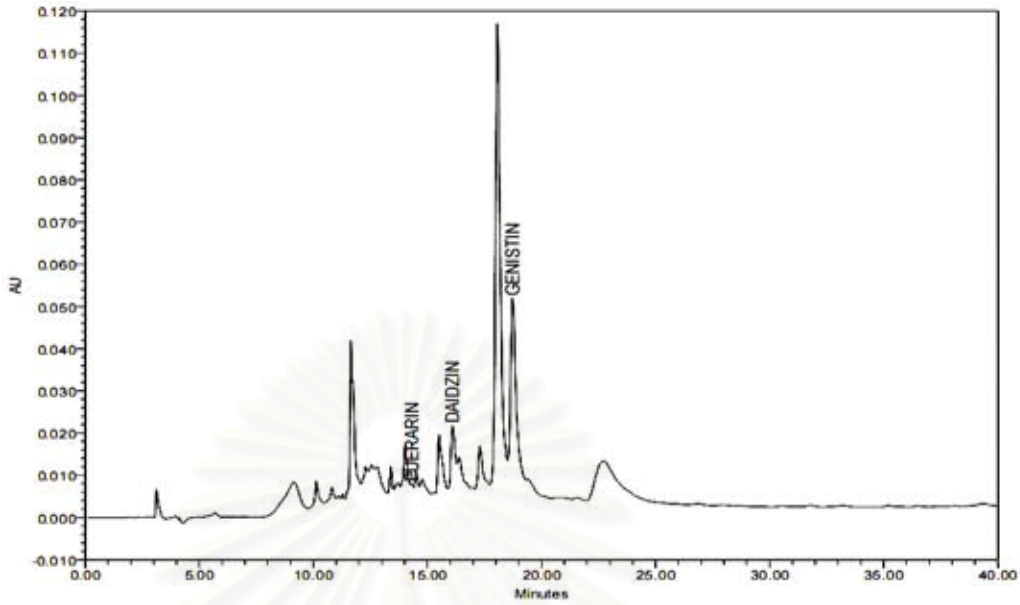

Figure 4.26. The chromatogram of PM-III leaves in August 2007

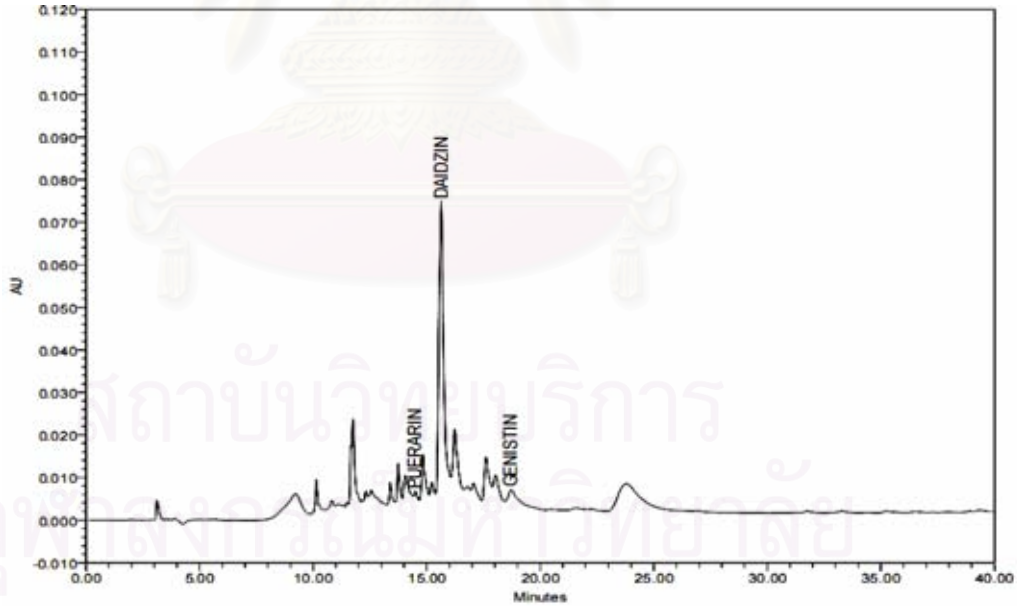

Figure 4.27. The chromatogram of PM-IV leaves in August 2007

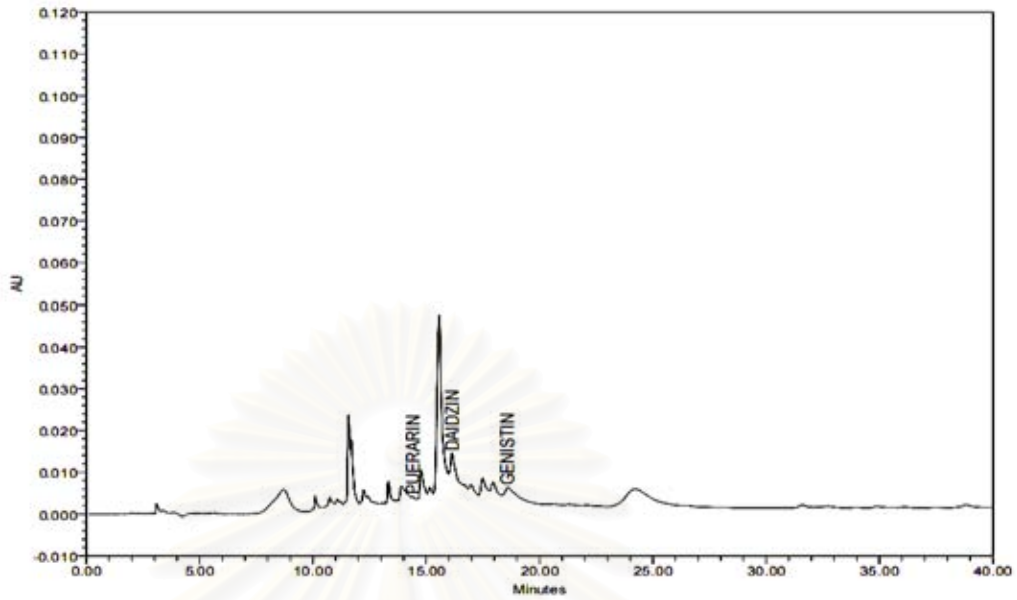

Figure 4.28. The chromatogram of PM-V leaves in August 2007

4.3.2 The analysis of isoflavonoids in PM leaf crude extracts

The leaf isoflavonoid contents of *P. mirifica* were analyzed for statistical significant by the One-Way ANOVA, Duncan analysis, Post-Hoc Multiple Comparison test and of variance at the significance level of $P < 0.05$ (Table 4.2.).

Table 4.3. The isoflavonoid contents of PM-III, PM-IV and PM-V (mg/100g powder).

Month (2007)	Cultivar	Isoflavonoid contents; Mean ± S.E. (mg/100g powder)			
		Puerarin	Daidzin	Genistin	Total
April	PM-III	62.88 ± 15.02^{a}	194.63 ± 18.32^{a}	251.52 ± 19.55^{a}	509.03 ± 45.06^{a}
	PM-IV	248.64 ± 49.67^{a,b,c}	2,269.60 ± 690.39^{d,e}	1,337.58 ± 399.8^{c,d,e}	3,855.82 ± 380.04^{d,e,f}
	PM-V	56.69 ± 11.11^{a}	568.89 ± 170.34^{a,b}	131.59 ± 18.22^{a}	757.16 ± 158.70^{a}
	Mean ± S.E.M	127.45 ± 21.68$^{a'}$	1,042.62 ± 346.95$^{a'}$	605.13 ± 59.58$^{a',b'}$	1,775.21 ± 451.92$^{a'}$
May	PM-III	182.06 ± 33.48^{a,b,c,d}	997.99 ± 167.84^{a,b}	1,277.70 ± 161.87^{b,c,d,e}	2,457.75 ± 139.70^{c}
	PM-IV	444.43 ± 75.77^{d,e}	2,541.20 ± 333.10^{e}	413.41 ± 65.79^{a}	3,399.04 ± 277.49^{c,d,e,f}
	PM-V	352.47 ± 134.80^{c,d,e}	3,082.61 ± 270.05^{e,f}	495.00 ± 161.57^{a,b}	3,930.08 ± 475.91^{d,e,f}
	Mean ± S.E.M	326.32 ± 54.97$^{b'}$	2,207.27 ± 227.49$^{b'}$	728.70 ± 107.92$^{a',b'}$	3,262.29 ± 217.68$^{b'}$
June	PM-III	286.06 ± 38.73^{b,c,d,e}	1,213.42 ± 248.81^{a,b,c}	3,062.35 ± 382.13^{f}	4,561.82 ± 454.57^{f}
	PM-IV	233.26 ± 53.72^{a,b,c,d}	861.84 ± 28.87^{a,b}	2,018.39 ± 592.42^{e}	3,113.49 ± 636.21^{c,d,e}
	PM-V	468.07 ± 80.89^{e}	5,059.96 ± 365.61^{g}	238.31 ± 20.94^{a}	5,766.34 ± 411.27^{g}
	Mean ± S.E.M	329.13 ± 38.86$^{b'}$	2,378.40 ± 399.02$^{b'}$	1,773.02 ± 321.40$^{c'}$	4,480.55 ± 353.97$^{c'}$
July	PM-III	468.19 ± 83.55^{e}	1,440.49 ± 289.30^{b,c,d}	927.92 ± 323.30^{a,b,c,d}	2,836.60 ± 295.50^{c,d}
	PM-IV	229.08 ± 42.43^{a,b,c,d}	870.90 ± 214.13^{a,b}	951.36 ± 203.69^{a,b,c,d}	2,051.34 ± 452.56^{b,c}
	PM-V	330.59 ± 34.22^{b,c,d,e}	1,321.72 ± 298.22^{b,c,d}	1,412.44 ± 80.70^{d,e}	3,064.75 ± 331.90^{c,d,e}
	Mean ± S.E.M	342.62 ± 37.28$^{b'}$	1,211.04 ± 157.24$^{a'}$	1,097.24 ± 132.5$^{b'}$	2,650.90 ± 220.24$^{a',b'}$
August	PM-III	123.29 ± 35.56^{a,b}	394.94 ± 93.59^{a,b}	563.53 ± 154.25^{a,b,c}	1,081.77 ± 280.10^{a,b}
	PM-IV	217.67 ± 11.84^{a,b,c}	3,804.92 ± 658.43^{f}	303.08 ± 30.57^{a}	4,325.68 ± 640.73^{e,f}
	PM-V	263.28 ± 43.65^{a,b,c,d,e}	2,167.33 ± 126.33^{c,d,e}	185.23 ± 22.75^{a}	2,615.84 ± 140.88^{c,d}
	Mean ± S.E.M	201.41 ± 21.68$^{a'}$	2,122.40 ± 348.68$^{b'}$	350.62 ± 59.58$^{a'}$	2,674.43 ± 345.99$^{a',b'}$

Data are mean ± SEM, $n = 3$

Means within a column followed by the same letters are not significant different at the 5% level by Duncan's test

There were differences in leaf presence and morphology among different plant cultivars. In March, PM-V was defoliated. In addition, the leaves of PM-IV were the largest and that of PM-V were the smallest.

The 5 standard major isoflavonoids were analyzed by RP-HPLC with C_{18} column to evaluate the retention time range of puerarin, daidzin, genistin, daidzein and genistein are 14.100-15.250 min, 15.900-16.100 min, 18.950-19.310 min, 26.450-26.900 min and 34.600-35.500 min respectively. The contents of leaf isoflavonoid were calculated with Empower™ program based on standard curves of major isoflavonoids. The chromatograms exhibited only 3 identified peaks of puerarin, daidzin and genistin.

The highest isoflavonoid content during the 6 months was daidzin in PM-V (5,059.96 ± 365.61 mg/100g powder). The maximum amount of puerarin (468.19 ± 83.55 mg/100g powder) and genistin (3,062.35 ± 382.13 mg/100g powder) were found in PM-V, while the maximum daidzin amount was found in PM-V. The total isoflavonoid content of *P. mirifica* in June 2007 was the highest (5,766.34 ± 411.27 mg/100g powder) and in April 2007 was the lowest (509.03 ± 45.06 mg/100g powder). The lowest puerarin (62.88 ± 15.02 mg/100g powder) and daidzin contents (194.63 ± 18.32 mg/100g powder respectively) were found in PM-III while the minimum genistin was found in PM-V (131.59 ± 18.22 mg/100g powder). The results demonstrated that the leaf isoflavonoid contents were under the influence of plant genetics and monthly climatic change. The highest total isoflavonoid amount in PM-V was found in June 2007 which was 11.33 times higher than the lowest amount of PM-III in April 2007. The isoflavonoid contents of PM in June 2007 were the highest which was 2.93 times higher than in April 2007.

In terms of statistical analysis, the isoflavonoid contents from the leaf samples of *P. mirifica* were analyzed for statistical significant by One-Way ANOVA, Duncan analysis, Post-Hoc Multiple Comparison test and of variance at the significance level of $P < 0.05$.

P. mirifica leaves showed only 3 major isoflavonoids including puerarin, daidzin and genistin. These chemicals are glycoside isoflavonoids. Unlike *P. mirifica* tubers in other independent assays and different extraction protocols, aglycoside

isoflavonoids daidzein and genistein were not present in *P. mirifica* leaves. Leaves were extracted with methanol, chloroform and butanol stepwise before dissolving the leaf crude extracts with methanol, while tuberous powders were direct extracted with methanol with the aid of sonication, prior to injection to HPLC. The leaves were most likely being the synthetic sites of isoflavonoids, and subsequently translocated to tubers which was the accumulation sites. If this is correct, *P. mirifica* may synthesis isoflavonoids only in a form of glycoside. This may be a possible protection role of such chemicals during translocation or the enzymes needed for aglycosylation are less abundant in leaf tissues. In tuberous tissues, some of the isoflavonoids could be transformed, at least the aglycosylation to form daidzein and genistein. The analysis of isoflavonoid contents in *P. mirifica* tubers in both wild plants (Cherdshewasart et al., 2007) and cultivated plants (Cherdshewasart and Sriwatcharakul 2007) had found the higher contents of puerarin over other isoflavonoids. This was contrast in leaf in which daidzin was the predominant isoflavonoids. The first possibility is that not only the aglycosylation of daidzin and genistin into daidzein and genistein but also a further transformation of daidzin and/or daidzein into puerarin was also occurred in tuberous tissues. This proposed mechanism is possible because the contents of the 3 isoflavonoids; puerarin ,daidzin and genistin in leaves were approximately 2.95, 52.45 and 52.80 times of that found in the wild plant tubers respectively (Cherdshewasart et al., 2007). The second possibility is that leaves may capable of synthesis aglycoside daidzein and genistein but the two aglycosides were eliminated by chloroform or butanol solvent during the extraction processes. This can be confirmed by using tuberous powder collected from the same plants and months, submitted to the same extraction processes as did for leaves. If there was no daidzein and genistein present in the HPLC fingerprint then the second possibility is correct.

This study demonstrated the high potential of *P. mirifica* leaves as an enrich source of plant isoflavonoids. Even the plant leaves synthesized only glycoside isoflavonoids but the human consumed isoflavonoids could be metabolized by intestinal flora (Xu et al., 1995) and metabolic activation enzymes (Lee et al., 2002; Cherdshewasart and Sriwatcharakul, 2008) into active compounds with more bioactivities.

Isoflavonoids in plants are plant secondary metabolites. The complicate isoflavonoid synthetic pathways and elicitors for these chemical syntheses were not evaluated in this study. In addition, the transformation of isoflavonoids into primary

metabolites for plant growth also not evaluated either. This study thus opens many possibilities for plant physiologists to investigate these facts. The outcome will obviously aid understanding roles of factors affecting isoflavonoid synthesis in leaf and translocation to tuber which finally results in bioactivity (estrogenic activity) of the tuberous materials for human consumption.

CHAPTER V

CONCLUSIONS

The results demonstrated that the adapted extraction methods comprised of methanol, chloroform and butanol solvents could eliminate chlorophyll from the crude extract and thus possible to determine for major isoflavonoid contents by RP-HPLC.

The results demonstrated also that only puerarin, daidzin and genistin were found in leaf crude extract derived from such extraction solvents. The data was different from the methanol extract of tuberous samples of the same plant species in which daidzein and genistein were also found. In addition, the leaf total isoflavonoid contents were found more than that of tuberous samples in another study.

In the biotechnological approach, a large scale production of puerarin, daidzin and genistin from *P. mirifica* leaves is definitely possible. *P. mirifica* is a twinning plant that produces a lot of leaves during plant growth and differentiation. Even the plants were defoliated during late winter to early summer, for example, March in this study, but cutting and watering could induce all year leaf production. Even though the tubers could accumulate 5 major isoflavonoids since the first year of age (Cherdshewasart and Sriwatcharakul, 2007), the tubers were newly differentiated with small size. Normally at least 3 year-old tubers were required for harvest while the leaves were produced since the first month of plant cultivation. Even though some of these chemicals are available from chemical synthesis but the prize is still high. Moreover, isoflavonoids from plant sources have advantage over those derived from chemical synthesis because they can be used in food and cosmetic industry without strong regulation.

This study should be completed by analysis of the annual isoflavonoid of plant leaves. This will enable us investigate the differential profile of isoflavonoids during the 12 months period. The influence of plant genetics and seasonal changes can be more precisely evaluated. This will benefit to farmers to set the right harvest period for plant leaves. Moreover, metabolomics study of the plant leaves will enable to find

more chemicals present in the leaves. Puerarin, genistin and daidzin were not only the total found chemicals in the analyzed HPLC fingerprints, but some unknown peaks were also detected. Proteomics study of plant leaves and tubers will enable the investigation into key proteins or enzymes related to isoflavonoid synthesis and transformation, including glycosylation/aglycosylation of isoflavonoids in the two plant tissues. This will open a possibility to clone some key gene(s) of these pathways and end up with construction of transgenic *P. mirifica* that produce higher amount of desired isoflavonoids in leaves or tubers.

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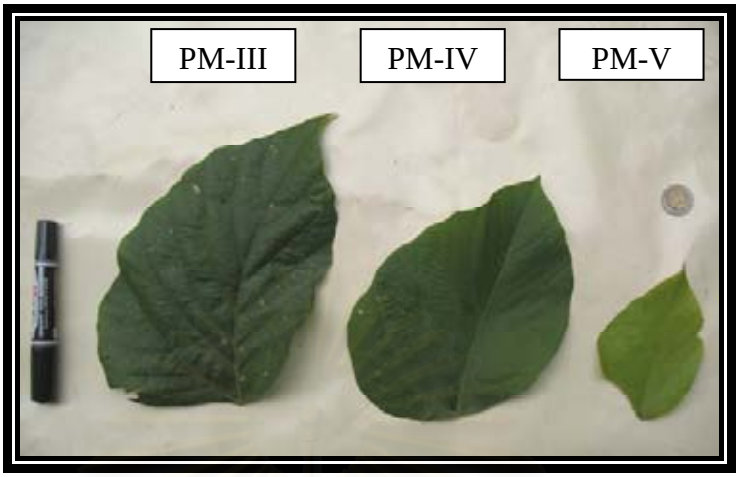
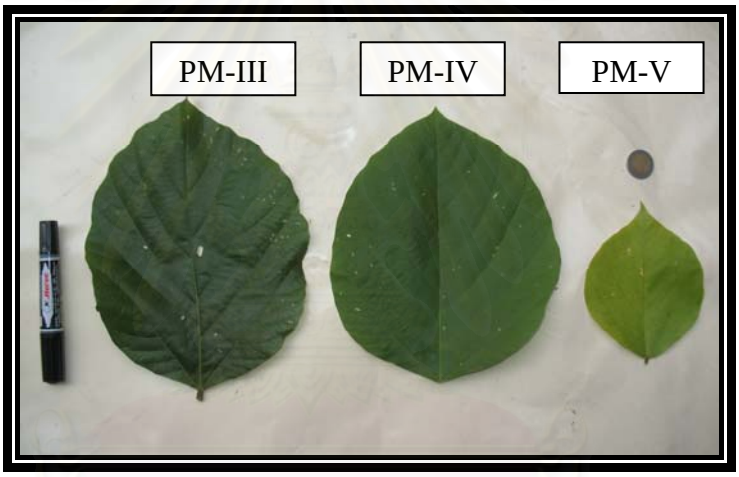
APPENDICES

Appendix A

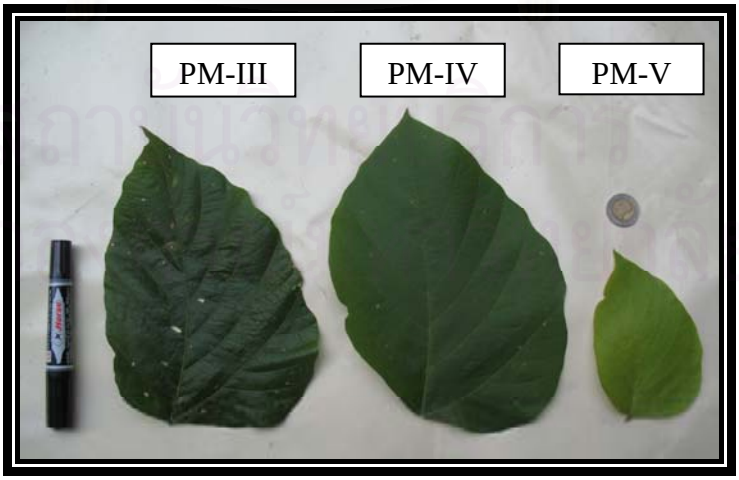
The characteristics of PM fresh leaves



a)



b)



c)

Figure 3.3. The leaves of PM; a) left-side leaf, b) center leaf and c) right-side leaf.

Appendix B

Isoflavonoid contents calculation (µg/100g powder)

The 50 g dry leave powder have a crude extract x g. Injected 10 µl of 1 mg/ml crude extract in RP-HPLC to evaluate an isoflavonoid content y mg/ml

In	10	µl evaluated isoflavonoid content		y	mg
	1000	µl evaluated isoflavonoid content		100 y	mg
	1	mg crude extract		100 y	mg
	x	mg crude extract		100 xy	g
In	50	g have a crude extract		100 xy	g
In	100	g have a crude extract	=	200 xy	g

So: The isoflavonoid contents of PM leaves are 200 xy g /100g powder

Appendix C

Correlation Graphs

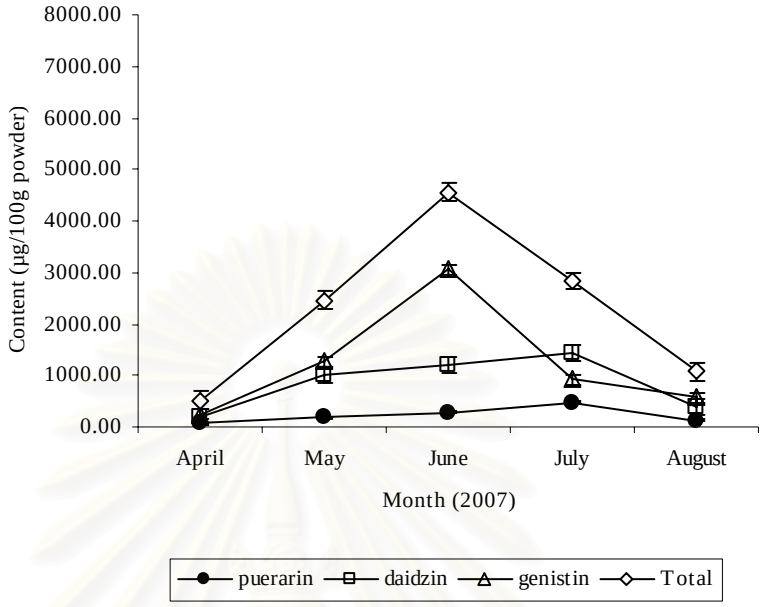


Figure 4.29. Isoflavonoid contents of PM-III (mg/100g powder). Genistin is the highest contents in June, whereas puerarin is the lowest in the same month

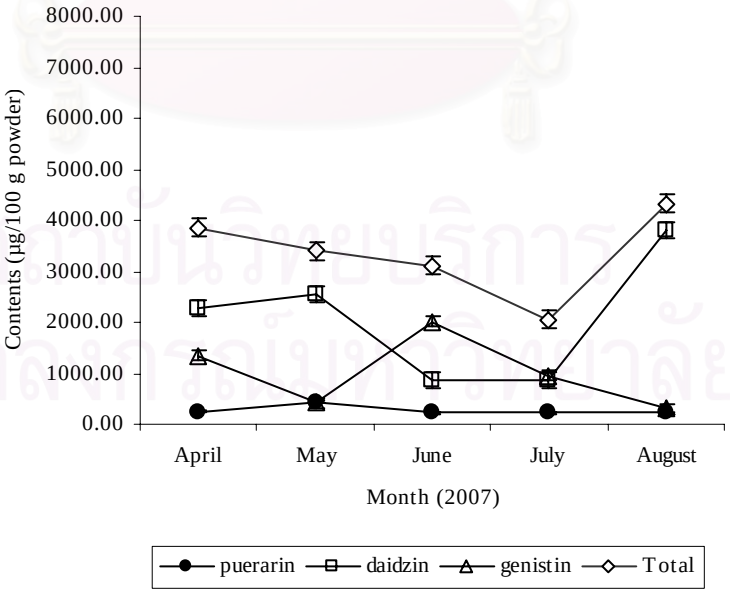


Figure 4.30. Isoflavonoid contents of PM-IV (mg/100g powder)

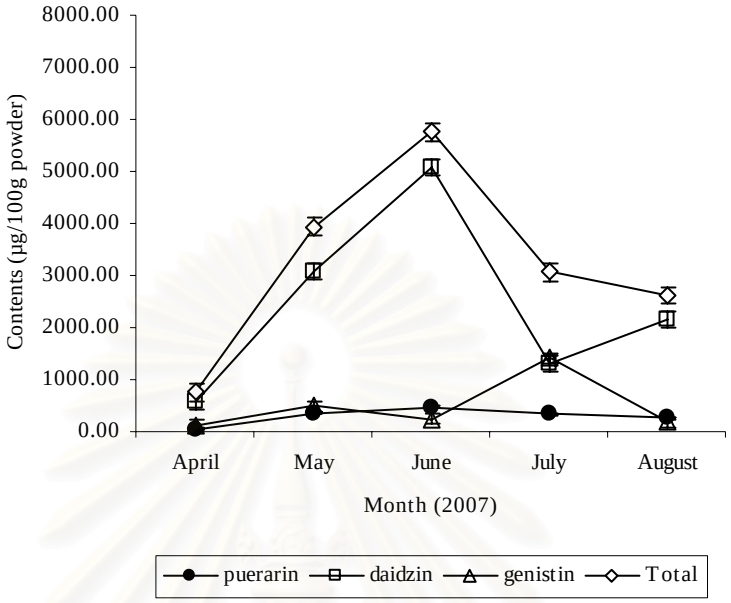


Figure 4.31. Isoflavonoid contents of PM-V (mg/100g powder). Daidzin is the highest contents in May, whereas puerarin is the lowest in August

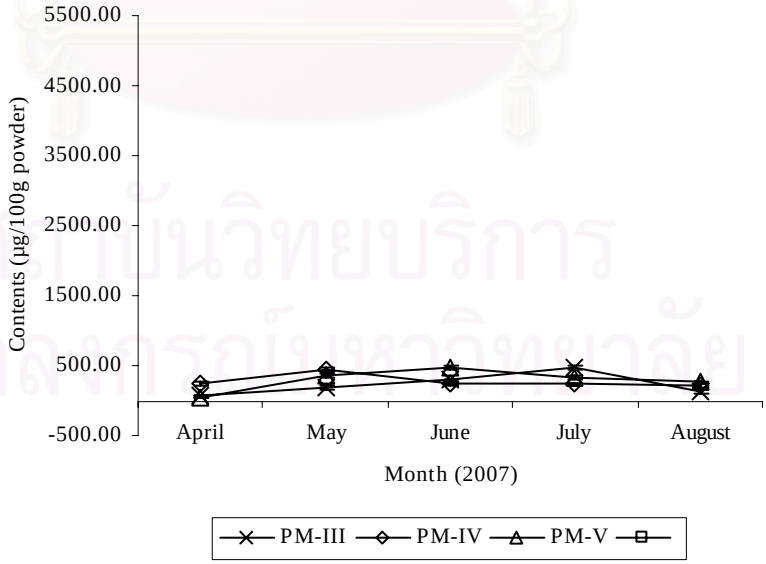


Figure 4.32. Puerarin contents of PM-III, PM-IV and PM-V in April-August 2007 (mg/100g powder)

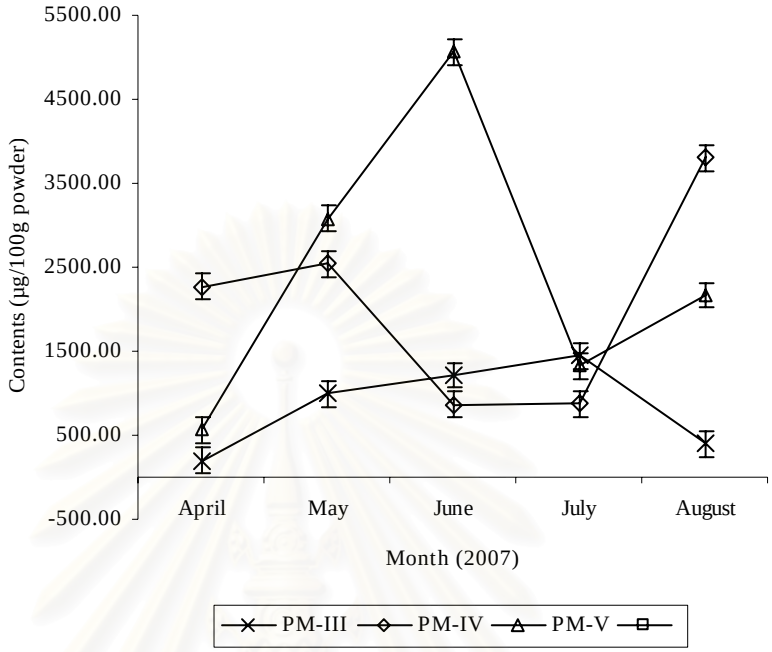


Figure 4.33. Daidzin contents of PM-III, PM-IV and PM-V in April-August 2007 (mg/100g powder)

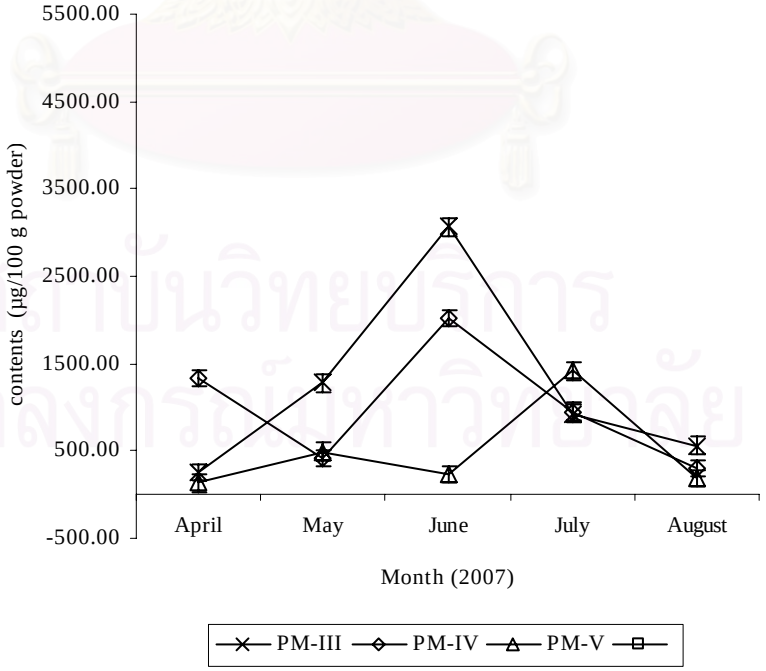


Figure 4.34. Genistin contents of PM-III, PM-IV and PM-V in April-August 2007 (mg/100g powder)

Appendix D

Statistical Analysis

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Puerarin contents (mg/100g powder)	.205	122	.000	.825	122	.000
Daidzin contents (mg/100g powder)	.148	122	.000	.873	122	.000
Genistin contents (mg/100g powder)	.204	122	.000	.752	122	.000
Total contents (mg/100g powder)	.102	122	.003	.959	122	.001

a. Lilliefors Significance Correction

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Puerarin contents (mg/100g powder)	April 2007	14	127.4529	114.23554	30.53073	61.4952	193.4105	29.94	401.64
	May 2007	27	326.3189	285.64305	54.97203	213.3223	439.3155	101.35	1414.08
	June 2007	27	329.1304	201.92454	38.86040	249.2517	409.0091	75.50	799.08
	July 2007	27	342.6200	193.72413	37.28223	265.9853	419.2547	91.96	837.52
	August 2007	27	201.4141	112.67608	21.68452	156.8409	245.9872	29.64	563.16
	Total	122	280.0852	210.83275	19.08790	242.2956	317.8747	29.64	1414.08
Daidzin contents (mg/100g powder)	April 2007	14	1042.6214	1298.17397	346.95159	293.0781	1792.1648	164.70	3480.88
	May 2007	27	2207.2674	1182.05399	227.48640	1739.6624	2674.8724	405.40	4540.88
	June 2007	27	2378.4044	2073.39053	399.02419	1558.1985	3198.6104	161.90	6392.64
	July 2007	27	1211.0356	817.02572	157.23667	887.8309	1534.2402	91.96	3107.16
	August 2007	27	2122.3970	1811.78384	348.67796	1405.6792	2839.1149	118.56	5948.80
	Total	122	1872.2338	1589.71245	143.92580	1587.2947	2157.1729	91.96	6392.64
Genistin contents (mg/100g powder)	April 2007	14	605.1329	755.14469	201.82091	169.1253	1041.1404	76.94	2639.52
	May 2007	27	728.7015	560.78582	107.92328	506.8620	950.5410	72.54	1625.52

	June 2007	27	1773.0156	1670.04454	321.40022	1112.3679	2433.6632	126.18	4533.20
	July 2007	27	1097.2415	688.52108	132.50594	824.8716	1369.6114	172.62	2216.88
	August 2007	27	350.6159	309.59773	59.58211	228.1431	473.0887	118.56	1202.88
	Total	122	943.5276	1054.22747	95.44527	754.5685	1132.4867	72.54	4533.20
Total contents (mg/100g powder)	April 2007	14	1775.2071	1687.91904	451.11534	800.6317	2749.7826	439.20	4551.92
	May 2007	27	3262.2878	1131.09961	217.68022	2814.8397	3709.7359	1722.95	6005.68
	June 2007	27	4480.5504	1839.29625	353.97273	3752.9490	5208.1517	1736.50	7458.08
	July 2007	27	2650.8970	1144.42250	220.24421	2198.1786	3103.6155	459.80	4289.10
	August 2007	27	2674.4270	1797.82131	345.99087	1963.2326	3385.6215	266.76	6406.40
	Total	122	3095.8466	1733.06293	156.90415	2785.2134	3406.4797	266.76	7458.08

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) Collecting month	(J) Collecting month	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Puerarin contents (mg/100g powder)	Tukey HSD	April 2007	May 2007	-198.86603*	65.70616	.025	-380.9233	-16.8088
			June 2007	-201.67751*	65.70616	.022	-383.7348	-19.6203
			July 2007	-215.16714*	65.70616	.012	-397.2244	-33.1099
			August 2007	-73.96122	65.70616	.793	-256.0185	108.0960
		May 2007	April 2007	198.86603*	65.70616	.025	16.8088	380.9233
			June 2007	-2.81148	54.29917	1.000	-153.2625	147.6395
			July 2007	-16.30111	54.29917	.998	-166.7521	134.1499
			August 2007	124.90481	54.29917	.152	-25.5462	275.3558
		June 2007	April 2007	201.67751*	65.70616	.022	19.6203	383.7348
			May 2007	2.81148	54.29917	1.000	-147.6395	153.2625
			July 2007	-13.48963	54.29917	.999	-163.9406	136.9614
			August 2007	127.71630	54.29917	.136	-22.7347	278.1673
		July 2007	April 2007	215.16714*	65.70616	.012	33.1099	397.2244
			May 2007	16.30111	54.29917	.998	-134.1499	166.7521

		June 2007	13.48963	54.29917	.999	-136.9614	163.9406
		August 2007	141.20593	54.29917	.077	-9.2451	291.6569
	August 2007	April 2007	73.96122	65.70616	.793	-108.0960	256.0185
		May 2007	-124.90481	54.29917	.152	-275.3558	25.5462
		June 2007	-127.71630	54.29917	.136	-278.1673	22.7347
		July 2007	-141.20593	54.29917	.077	-291.6569	9.2451
LSD	April 2007	May 2007	-198.86603*	65.70616	.003	-328.9936	-68.7384
		June 2007	-201.67751*	65.70616	.003	-331.8051	-71.5499
		July 2007	-215.16714*	65.70616	.001	-345.2948	-85.0395
		August 2007	-73.96122	65.70616	.263	-204.0888	56.1664
	May 2007	April 2007	198.86603*	65.70616	.003	68.7384	328.9936
		June 2007	-2.81148	54.29917	.959	-110.3481	104.7252
		July 2007	-16.30111	54.29917	.765	-123.8378	91.2355
		August 2007	124.90481*	54.29917	.023	17.3682	232.4415
	June 2007	April 2007	201.67751*	65.70616	.003	71.5499	331.8051
		May 2007	2.81148	54.29917	.959	-104.7252	110.3481
		July 2007	-13.48963	54.29917	.804	-121.0263	94.0470
		August 2007	127.71630*	54.29917	.020	20.1796	235.2530

		July 2007	April 2007	215.16714*	65.70616	.001	85.0395	345.2948
			May 2007	16.30111	54.29917	.765	-91.2355	123.8378
			June 2007	13.48963	54.29917	.804	-94.0470	121.0263
			August 2007	141.20593*	54.29917	.011	33.6693	248.7426
		August 2007	April 2007	73.96122	65.70616	.263	-56.1664	204.0888
			May 2007	-124.90481*	54.29917	.023	-232.4415	-17.3682
			June 2007	-127.71630*	54.29917	.020	-235.2530	-20.1796
			July 2007	-141.20593*	54.29917	.011	-248.7426	-33.6693
	Bonferroni	April 2007	May 2007	-198.86603*	65.70616	.030	-386.8674	-10.8647
			June 2007	-201.67751*	65.70616	.027	-389.6788	-13.6762
			July 2007	-215.16714*	65.70616	.014	-403.1685	-27.1658
			August 2007	-73.96122	65.70616	1.000	-261.9626	114.0401
		May 2007	April 2007	198.86603*	65.70616	.030	10.8647	386.8674
			June 2007	-2.81148	54.29917	1.000	-158.1746	152.5517
			July 2007	-16.30111	54.29917	1.000	-171.6643	139.0620
			August 2007	124.90481	54.29917	.232	-30.4583	280.2680
		June 2007	April 2007	201.67751*	65.70616	.027	13.6762	389.6788
			May 2007	2.81148	54.29917	1.000	-152.5517	158.1746

			July 2007	-13.48963	54.29917	1.000	-168.8528	141.8735
			August 2007	127.71630	54.29917	.203	-27.6469	283.0794
		July 2007	April 2007	215.16714*	65.70616	.014	27.1658	403.1685
			May 2007	16.30111	54.29917	1.000	-139.0620	171.6643
			June 2007	13.48963	54.29917	1.000	-141.8735	168.8528
			August 2007	141.20593	54.29917	.105	-14.1572	296.5691
		August 2007	April 2007	73.96122	65.70616	1.000	-114.0401	261.9626
			May 2007	-124.90481	54.29917	.232	-280.2680	30.4583
			June 2007	-127.71630	54.29917	.203	-283.0794	27.6469
			July 2007	-141.20593	54.29917	.105	-296.5691	14.1572
	Tamhane	April 2007	May 2007	-198.86603*	62.88124	.031	-385.9066	-11.8254
			June 2007	-201.67751*	49.41919	.002	-348.3924	-54.9626
			July 2007	-215.16714*	48.18807	.001	-358.3152	-72.0191
			August 2007	-73.96122	37.44788	.455	-188.4174	40.4949
		May 2007	April 2007	198.86603*	62.88124	.031	11.8254	385.9066
			June 2007	-2.81148	67.32054	1.000	-200.5870	194.9640
			July 2007	-16.30111	66.42205	1.000	-211.6578	179.0555
			August 2007	124.90481	59.09435	.349	-52.0045	301.8141

		June 2007	April 2007	201.67751*	49.41919	.002	54.9626	348.3924
			May 2007	2.81148	67.32054	1.000	-194.9640	200.5870
			July 2007	-13.48963	53.85253	1.000	-170.9353	143.9560
			August 2007	127.71630	44.50111	.063	-3.9772	259.4098
		July 2007	April 2007	215.16714*	48.18807	.001	72.0191	358.3152
			May 2007	16.30111	66.42205	1.000	-179.0555	211.6578
			June 2007	13.48963	53.85253	1.000	-143.9560	170.9353
			August 2007	141.20593*	43.12984	.021	13.7479	268.6640
		August 2007	April 2007	73.96122	37.44788	.455	-40.4949	188.4174
			May 2007	-124.90481	59.09435	.349	-301.8141	52.0045
			June 2007	-127.71630	44.50111	.063	-259.4098	3.9772
			July 2007	-141.20593*	43.12984	.021	-268.6640	-13.7479
	Dunnett T3	April 2007	May 2007	-198.86603*	62.88124	.030	-385.1836	-12.5484
			June 2007	-201.67751*	49.41919	.002	-347.8484	-55.5066
			July 2007	-215.16714*	48.18807	.001	-357.7773	-72.5570
			August 2007	-73.96122	37.44788	.423	-187.6930	39.7705
		May 2007	April 2007	198.86603*	62.88124	.030	12.5484	385.1836
			June 2007	-2.81148	67.32054	1.000	-200.0154	194.3924

			July 2007	-16.30111	66.42205	1.000	-211.0766	178.4744
			August 2007	124.90481	59.09435	.329	-51.2248	301.0344
		June 2007	April 2007	201.67751*	49.41919	.002	55.5066	347.8484
			May 2007	2.81148	67.32054	1.000	-194.3924	200.0154
			July 2007	-13.48963	53.85253	1.000	-170.5361	143.5568
			August 2007	127.71630	44.50111	.061	-3.5226	258.9552
		July 2007	April 2007	215.16714*	48.18807	.001	72.5570	357.7773
			May 2007	16.30111	66.42205	1.000	-178.4744	211.0766
			June 2007	13.48963	53.85253	1.000	-143.5568	170.5361
			August 2007	141.20593*	43.12984	.021	14.1739	268.2380
		August 2007	April 2007	73.96122	37.44788	.423	-39.7705	187.6930
			May 2007	-124.90481	59.09435	.329	-301.0344	51.2248
			June 2007	-127.71630	44.50111	.061	-258.9552	3.5226
			July 2007	-141.20593*	43.12984	.021	-268.2380	-14.1739
Daidzin contents (mg/100g powder)	Tukey HSD	April 2007	May 2007	-1164.64598	502.81144	.147	-2557.8254	228.5334
			June 2007	-1335.78302	502.81144	.067	-2728.9624	57.3964
			July 2007	-168.41413	502.81144	.997	-1561.5936	1224.7653
			August 2007	-1079.77561	502.81144	.207	-2472.9550	313.4038
		May 2007	April 2007	1164.64598	502.81144	.147	-228.5334	2557.8254

			June 2007	-171.13704	415.52029	.994	-1322.4520	980.1779
			July 2007	996.23185	415.52029	.123	-155.0831	2147.5468
			August 2007	84.87037	415.52029	1.000	-1066.4446	1236.1853
		June 2007	April 2007	1335.78302	502.81144	.067	-57.3964	2728.9624
			May 2007	171.13704	415.52029	.994	-980.1779	1322.4520
			July 2007	1167.36889*	415.52029	.045	16.0540	2318.6838
			August 2007	256.00741	415.52029	.972	-895.3075	1407.3223
		July 2007	April 2007	168.41413	502.81144	.997	-1224.7653	1561.5936
			May 2007	-996.23185	415.52029	.123	-2147.5468	155.0831
			June 2007	-1167.36889*	415.52029	.045	-2318.6838	-16.0540
			August 2007	-911.36148	415.52029	.190	-2062.6764	239.9535
		August 2007	April 2007	1079.77561	502.81144	.207	-313.4038	2472.9550
			May 2007	-84.87037	415.52029	1.000	-1236.1853	1066.4446
			June 2007	-256.00741	415.52029	.972	-1407.3223	895.3075
			July 2007	911.36148	415.52029	.190	-239.9535	2062.6764
	LSD	April 2007	May 2007	-1164.64598*	502.81144	.022	-2160.4377	-168.8543
			June 2007	-1335.78302*	502.81144	.009	-2331.5747	-339.9913
			July 2007	-168.41413	502.81144	.738	-1164.2058	827.3776
			August 2007	-1079.77561*	502.81144	.034	-2075.5673	-83.9839

		May 2007	April 2007	1164.64598*	502.81144	.022	168.8543	2160.4377
			June 2007	-171.13704	415.52029	.681	-994.0532	651.7791
			July 2007	996.23185*	415.52029	.018	173.3157	1819.1480
			August 2007	84.87037	415.52029	.839	-738.0458	907.7865
		June 2007	April 2007	1335.78302*	502.81144	.009	339.9913	2331.5747
			May 2007	171.13704	415.52029	.681	-651.7791	994.0532
			July 2007	1167.36889*	415.52029	.006	344.4527	1990.2850
			August 2007	256.00741	415.52029	.539	-566.9088	1078.9236
		July 2007	April 2007	168.41413	502.81144	.738	-827.3776	1164.2058
			May 2007	-996.23185*	415.52029	.018	-1819.1480	-173.3157
			June 2007	-1167.36889*	415.52029	.006	-1990.2850	-344.4527
			August 2007	-911.36148*	415.52029	.030	-1734.2776	-88.4453
		August 2007	April 2007	1079.77561*	502.81144	.034	83.9839	2075.5673
			May 2007	-84.87037	415.52029	.839	-907.7865	738.0458
			June 2007	-256.00741	415.52029	.539	-1078.9236	566.9088
			July 2007	911.36148*	415.52029	.030	88.4453	1734.2776
	Bonferroni	April 2007	May 2007	-1164.64598	502.81144	.223	-2603.3120	274.0200
			June 2007	-1335.78302	502.81144	.090	-2774.4490	102.8830
			July 2007	-168.41413	502.81144	1.000	-1607.0801	1270.2519

		August 2007	-1079.77561	502.81144	.338	-2518.4416	358.8904
	May 2007	April 2007	1164.64598	502.81144	.223	-274.0200	2603.3120
		June 2007	-171.13704	415.52029	1.000	-1360.0418	1017.7677
		July 2007	996.23185	415.52029	.181	-192.6729	2185.1366
		August 2007	84.87037	415.52029	1.000	-1104.0344	1273.7751
	June 2007	April 2007	1335.78302	502.81144	.090	-102.8830	2774.4490
		May 2007	171.13704	415.52029	1.000	-1017.7677	1360.0418
		July 2007	1167.36889	415.52029	.058	-21.5359	2356.2737
		August 2007	256.00741	415.52029	1.000	-932.8974	1444.9122
	July 2007	April 2007	168.41413	502.81144	1.000	-1270.2519	1607.0801
		May 2007	-996.23185	415.52029	.181	-2185.1366	192.6729
		June 2007	-1167.36889	415.52029	.058	-2356.2737	21.5359
		August 2007	-911.36148	415.52029	.303	-2100.2662	277.5433
	August 2007	April 2007	1079.77561	502.81144	.338	-358.8904	2518.4416
		May 2007	-84.87037	415.52029	1.000	-1273.7751	1104.0344
		June 2007	-256.00741	415.52029	1.000	-1444.9122	932.8974
		July 2007	911.36148	415.52029	.303	-277.5433	2100.2662
Tamhane	April 2007	May 2007	-1164.64598	414.88006	.093	-2441.1296	111.8377
		June 2007	-1335.78302	528.76811	.148	-2908.5746	237.0086

			July 2007	-168.41413	380.91833	1.000	-1377.6347	1040.8064
			August 2007	-1079.77561	491.88589	.299	-2549.7580	390.2068
		May 2007	April 2007	1164.64598	414.88006	.093	-111.8377	2441.1296
			June 2007	-171.13704	459.31511	1.000	-1529.4203	1187.1462
			July 2007	996.23185*	276.53830	.008	183.3227	1809.1410
			August 2007	84.87037	416.32485	1.000	-1140.9821	1310.7228
		June 2007	April 2007	1335.78302	528.76811	.148	-237.0086	2908.5746
			May 2007	171.13704	459.31511	1.000	-1187.1462	1529.4203
			July 2007	1167.36889	428.88656	.097	-116.6194	2451.3571
			August 2007	256.00741	529.90247	1.000	-1294.3493	1806.3641
		July 2007	April 2007	168.41413	380.91833	1.000	-1040.8064	1377.6347
			May 2007	-996.23185*	276.53830	.008	-1809.1410	-183.3227
			June 2007	-1167.36889	428.88656	.097	-2451.3571	116.6194
			August 2007	-911.36148	382.49143	.204	-2051.5179	228.7949
		August 2007	April 2007	1079.77561	491.88589	.299	-390.2068	2549.7580
			May 2007	-84.87037	416.32485	1.000	-1310.7228	1140.9821
			June 2007	-256.00741	529.90247	1.000	-1806.3641	1294.3493
			July 2007	911.36148	382.49143	.204	-228.7949	2051.5179
	Dunnett T3	April 2007	May 2007	-1164.64598	414.88006	.087	-2432.1966	102.9046

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		June 2007	-1335.78302	528.76811	.142	-2902.4976	230.9315
		July 2007	-168.41413	380.91833	1.000	-1364.9414	1028.1132
		August 2007	-1079.77561	491.88589	.283	-2543.4987	383.9475
	May 2007	April 2007	1164.64598	414.88006	.087	-102.9046	2432.1966
		June 2007	-171.13704	459.31511	1.000	-1524.8091	1182.5350
		July 2007	996.23185*	276.53830	.008	185.7088	1806.7549
		August 2007	84.87037	416.32485	1.000	-1137.2304	1306.9711
	June 2007	April 2007	1335.78302	528.76811	.142	-230.9315	2902.4976
		May 2007	171.13704	459.31511	1.000	-1182.5350	1524.8091
		July 2007	1167.36889	428.88656	.093	-110.9564	2445.6942
		August 2007	256.00741	529.90247	1.000	-1290.3383	1802.3531
	July 2007	April 2007	168.41413	380.91833	1.000	-1028.1132	1364.9414
		May 2007	-996.23185*	276.53830	.008	-1806.7549	-185.7088
		June 2007	-1167.36889	428.88656	.093	-2445.6942	110.9564
		August 2007	-911.36148	382.49143	.194	-2046.9082	224.1852
	August 2007	April 2007	1079.77561	491.88589	.283	-383.9475	2543.4987
		May 2007	-84.87037	416.32485	1.000	-1306.9711	1137.2304
		June 2007	-256.00741	529.90247	1.000	-1802.3531	1290.3383
		July 2007	911.36148	382.49143	.194	-224.1852	2046.9082

Genistin contents (mg/100g powder)	Tukey HSD	April 2007	May 2007	-123.56862	308.89336	.995	-979.4439	732.3066
			June 2007	-1167.88270*	308.89336	.002	-2023.7580	-312.0074
			July 2007	-492.10862	308.89336	.505	-1347.9839	363.7666
			August 2007	254.51693	308.89336	.923	-601.3583	1110.3922
		May 2007	April 2007	123.56862	308.89336	.995	-732.3066	979.4439
			June 2007	-1044.31407*	255.26758	.001	-1751.6041	-337.0240
			July 2007	-368.54000	255.26758	.601	-1075.8301	338.7501
			August 2007	378.08556	255.26758	.577	-329.2045	1085.3756
		June 2007	April 2007	1167.88270*	308.89336	.002	312.0074	2023.7580
			May 2007	1044.31407*	255.26758	.001	337.0240	1751.6041
			July 2007	675.77407	255.26758	.068	-31.5160	1383.0641
			August 2007	1422.39963*	255.26758	.000	715.1096	2129.6897
		July 2007	April 2007	492.10862	308.89336	.505	-363.7666	1347.9839
			May 2007	368.54000	255.26758	.601	-338.7501	1075.8301
			June 2007	-675.77407	255.26758	.068	-1383.0641	31.5160
			August 2007	746.62556*	255.26758	.033	39.3355	1453.9156
		August 2007	April 2007	-254.51693	308.89336	.923	-1110.3922	601.3583
			May 2007	-378.08556	255.26758	.577	-1085.3756	329.2045
			June 2007	-1422.39963*	255.26758	.000	-2129.6897	-715.1096

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			July 2007	-746.62556*	255.26758	.033	-1453.9156	-39.3355
	LSD	April 2007	May 2007	-123.56862	308.89336	.690	-735.3157	488.1785
			June 2007	-1167.88270*	308.89336	.000	-1779.6298	-556.1356
			July 2007	-492.10862	308.89336	.114	-1103.8557	119.6385
			August 2007	254.51693	308.89336	.412	-357.2302	866.2640
		May 2007	April 2007	123.56862	308.89336	.690	-488.1785	735.3157
			June 2007	-1044.31407*	255.26758	.000	-1549.8581	-538.7700
			July 2007	-368.54000	255.26758	.151	-874.0841	137.0041
			August 2007	378.08556	255.26758	.141	-127.4585	883.6296
		June 2007	April 2007	1167.88270*	308.89336	.000	556.1356	1779.6298
			May 2007	1044.31407*	255.26758	.000	538.7700	1549.8581
			July 2007	675.77407*	255.26758	.009	170.2300	1181.3181
			August 2007	1422.39963*	255.26758	.000	916.8556	1927.9437
		July 2007	April 2007	492.10862	308.89336	.114	-119.6385	1103.8557
			May 2007	368.54000	255.26758	.151	-137.0041	874.0841
			June 2007	-675.77407*	255.26758	.009	-1181.3181	-170.2300
			August 2007	746.62556*	255.26758	.004	241.0815	1252.1696
		August 2007	April 2007	-254.51693	308.89336	.412	-866.2640	357.2302
			May 2007	-378.08556	255.26758	.141	-883.6296	127.4585

		June 2007	-1422.39963*	255.26758	.000	-1927.9437	-916.8556
		July 2007	-746.62556*	255.26758	.004	-1252.1696	-241.0815
Bonferroni	April 2007	May 2007	-123.56862	308.89336	1.000	-1007.3878	760.2505
		June 2007	-1167.88270*	308.89336	.002	-2051.7018	-284.0636
		July 2007	-492.10862	308.89336	1.000	-1375.9278	391.7105
		August 2007	254.51693	308.89336	1.000	-629.3022	1138.3361
	May 2007	April 2007	123.56862	308.89336	1.000	-760.2505	1007.3878
		June 2007	-1044.31407*	255.26758	.001	-1774.6968	-313.9314
		July 2007	-368.54000	255.26758	1.000	-1098.9227	361.8427
		August 2007	378.08556	255.26758	1.000	-352.2972	1108.4683
	June 2007	April 2007	1167.88270*	308.89336	.002	284.0636	2051.7018
		May 2007	1044.31407*	255.26758	.001	313.9314	1774.6968
		July 2007	675.77407	255.26758	.092	-54.6086	1406.1568
		August 2007	1422.39963*	255.26758	.000	692.0169	2152.7823
	July 2007	April 2007	492.10862	308.89336	1.000	-391.7105	1375.9278
		May 2007	368.54000	255.26758	1.000	-361.8427	1098.9227
		June 2007	-675.77407	255.26758	.092	-1406.1568	54.6086
		August 2007	746.62556*	255.26758	.041	16.2428	1477.0083
	August 2007	April 2007	-254.51693	308.89336	1.000	-1138.3361	629.3022

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			May 2007	-378.08556	255.26758	1.000	-1108.4683	352.2972
			June 2007	-1422.39963*	255.26758	.000	-2152.7823	-692.0169
			July 2007	-746.62556*	255.26758	.041	-1477.0083	-16.2428
	Tamhane	April 2007	May 2007	-123.56862	228.86484	1.000	-840.2114	593.0742
			June 2007	-1167.88270*	379.51256	.038	-2294.6394	-41.1260
			July 2007	-492.10862	241.43219	.417	-1234.8516	250.6343
			August 2007	254.51693	210.43219	.940	-432.3222	941.3560
		May 2007	April 2007	123.56862	228.86484	1.000	-593.0742	840.2114
			June 2007	-1044.31407*	339.03619	.042	-2063.9380	-24.6902
			July 2007	-368.54000	170.89546	.306	-869.0427	131.9627
			August 2007	378.08556*	123.27799	.038	13.1330	743.0381
		June 2007	April 2007	1167.88270*	379.51256	.038	41.1260	2294.6394
			May 2007	1044.31407*	339.03619	.042	24.6902	2063.9380
			July 2007	675.77407	347.64339	.462	-363.5288	1715.0769
			August 2007	1422.39963*	326.87632	.002	428.8022	2415.9970
		July 2007	April 2007	492.10862	241.43219	.417	-250.6343	1234.8516
			May 2007	368.54000	170.89546	.306	-131.9627	869.0427
			June 2007	-675.77407	347.64339	.462	-1715.0769	363.5288
			August 2007	746.62556*	145.28542	.000	313.5075	1179.7436

		August 2007	April 2007	-254.51693	210.43219	.940	-941.3560	432.3222
			May 2007	-378.08556*	123.27799	.038	-743.0381	-13.1330
			June 2007	-1422.39963*	326.87632	.002	-2415.9970	-428.8022
			July 2007	-746.62556*	145.28542	.000	-1179.7436	-313.5075
	Dunnett T3	April 2007	May 2007	-123.56862	228.86484	1.000	-833.8368	586.6996
			June 2007	-1167.88270*	379.51256	.037	-2290.4561	-45.3093
			July 2007	-492.10862	241.43219	.385	-1229.6624	245.4452
			August 2007	254.51693	210.43219	.905	-422.6617	931.6956
		May 2007	April 2007	123.56862	228.86484	1.000	-586.6996	833.8368
			June 2007	-1044.31407*	339.03619	.040	-2059.0339	-29.5942
			July 2007	-368.54000	170.89546	.295	-867.7110	130.6310
			August 2007	378.08556*	123.27799	.037	14.4033	741.7678
		June 2007	April 2007	1167.88270*	379.51256	.037	45.3093	2290.4561
			May 2007	1044.31407*	339.03619	.040	29.5942	2059.0339
			July 2007	675.77407	347.64339	.437	-359.0705	1710.6187
			August 2007	1422.39963*	326.87632	.002	434.5603	2410.2390
		July 2007	April 2007	492.10862	241.43219	.385	-245.4452	1229.6624
			May 2007	368.54000	170.89546	.295	-130.6310	867.7110
			June 2007	-675.77407	347.64339	.437	-1710.6187	359.0705

			August 2007	746.62556*	145.28542	.000	315.2621	1177.9890
		August 2007	April 2007	-254.51693	210.43219	.905	-931.6956	422.6617
			May 2007	-378.08556*	123.27799	.037	-741.7678	-14.4033
			June 2007	-1422.39963*	326.87632	.002	-2410.2390	-434.5603
			July 2007	-746.62556*	145.28542	.000	-1177.9890	-315.2621
Total contents (mg/100g powder)	Tukey HSD	April 2007	May 2007	-1487.08063*	506.15301	.032	-2889.5188	-84.6425
			June 2007	-2705.34323*	506.15301	.000	-4107.7814	-1302.9051
			July 2007	-875.68989	506.15301	.420	-2278.1281	526.7483
			August 2007	-899.21989	506.15301	.392	-2301.6581	503.2183
		May 2007	April 2007	1487.08063*	506.15301	.032	84.6425	2889.5188
			June 2007	-1218.26259*	418.28174	.034	-2377.2289	-59.2963
			July 2007	611.39074	418.28174	.589	-547.5756	1770.3571
			August 2007	587.86074	418.28174	.625	-571.1056	1746.8271
		June 2007	April 2007	2705.34323*	506.15301	.000	1302.9051	4107.7814
			May 2007	1218.26259*	418.28174	.034	59.2963	2377.2289
			July 2007	1829.65333*	418.28174	.000	670.6870	2988.6196
			August 2007	1806.12333*	418.28174	.000	647.1570	2965.0896
		July 2007	April 2007	875.68989	506.15301	.420	-526.7483	2278.1281
			May 2007	-611.39074	418.28174	.589	-1770.3571	547.5756

			June 2007	-1829.65333*	418.28174	.000	-2988.6196	-670.6870
			August 2007	-23.53000	418.28174	1.000	-1182.4963	1135.4363
		August 2007	April 2007	899.21989	506.15301	.392	-503.2183	2301.6581
			May 2007	-587.86074	418.28174	.625	-1746.8271	571.1056
			June 2007	-1806.12333*	418.28174	.000	-2965.0896	-647.1570
			July 2007	23.53000	418.28174	1.000	-1135.4363	1182.4963
	LSD	April 2007	May 2007	-1487.08063*	506.15301	.004	-2489.4901	-484.6711
			June 2007	-2705.34323*	506.15301	.000	-3707.7527	-1702.9337
			July 2007	-875.68989	506.15301	.086	-1878.0994	126.7196
			August 2007	-899.21989	506.15301	.078	-1901.6294	103.1896
		May 2007	April 2007	1487.08063*	506.15301	.004	484.6711	2489.4901
			June 2007	-1218.26259*	418.28174	.004	-2046.6477	-389.8775
			July 2007	611.39074	418.28174	.147	-216.9943	1439.7758
			August 2007	587.86074	418.28174	.163	-240.5243	1416.2458
		June 2007	April 2007	2705.34323*	506.15301	.000	1702.9337	3707.7527
			May 2007	1218.26259*	418.28174	.004	389.8775	2046.6477
			July 2007	1829.65333*	418.28174	.000	1001.2683	2658.0384
			August 2007	1806.12333*	418.28174	.000	977.7383	2634.5084
		July 2007	April 2007	875.68989	506.15301	.086	-126.7196	1878.0994

			May 2007	-611.39074	418.28174	.147	-1439.7758	216.9943
			June 2007	-1829.65333*	418.28174	.000	-2658.0384	-1001.2683
			August 2007	-23.53000	418.28174	.955	-851.9151	804.8551
		August 2007	April 2007	899.21989	506.15301	.078	-103.1896	1901.6294
			May 2007	-587.86074	418.28174	.163	-1416.2458	240.5243
			June 2007	-1806.12333*	418.28174	.000	-2634.5084	-977.7383
			July 2007	23.53000	418.28174	.955	-804.8551	851.9151
	Bonferroni	April 2007	May 2007	-1487.08063*	506.15301	.040	-2935.3077	-38.8536
			June 2007	-2705.34323*	506.15301	.000	-4153.5703	-1257.1162
			July 2007	-875.68989	506.15301	.863	-2323.9169	572.5372
			August 2007	-899.21989	506.15301	.782	-2347.4469	549.0072
		May 2007	April 2007	1487.08063*	506.15301	.040	38.8536	2935.3077
			June 2007	-1218.26259*	418.28174	.043	-2415.0685	-21.4566
			July 2007	611.39074	418.28174	1.000	-585.4152	1808.1967
			August 2007	587.86074	418.28174	1.000	-608.9452	1784.6667
		June 2007	April 2007	2705.34323*	506.15301	.000	1257.1162	4153.5703
			May 2007	1218.26259*	418.28174	.043	21.4566	2415.0685
			July 2007	1829.65333*	418.28174	.000	632.8474	3026.4593
			August 2007	1806.12333*	418.28174	.000	609.3174	3002.9293

	July 2007	April 2007	875.68989	506.15301	.863	-572.5372	2323.9169
		May 2007	-611.39074	418.28174	1.000	-1808.1967	585.4152
		June 2007	-1829.65333*	418.28174	.000	-3026.4593	-632.8474
		August 2007	-23.53000	418.28174	1.000	-1220.3360	1173.2760
	August 2007	April 2007	899.21989	506.15301	.782	-549.0072	2347.4469
		May 2007	-587.86074	418.28174	1.000	-1784.6667	608.9452
		June 2007	-1806.12333*	418.28174	.000	-3002.9293	-609.3174
		July 2007	23.53000	418.28174	1.000	-1173.2760	1220.3360
Tamhane	April 2007	May 2007	-1487.08063	500.88894	.075	-3069.1216	94.9603
		June 2007	-2705.34323*	573.41237	.001	-4444.4733	-966.2131
		July 2007	-875.68989	502.00853	.639	-2459.7376	708.3578
		August 2007	-899.21989	568.51977	.737	-2626.4368	827.9970
	May 2007	April 2007	1487.08063	500.88894	.075	-94.9603	3069.1216
		June 2007	-1218.26259	415.54948	.052	-2444.0735	7.5483
		July 2007	611.39074	309.66464	.424	-293.8956	1516.6771
		August 2007	587.86074	408.77177	.820	-617.0895	1792.8109
	June 2007	April 2007	2705.34323*	573.41237	.001	966.2131	4444.4733
		May 2007	1218.26259	415.54948	.052	-7.5483	2444.0735
		July 2007	1829.65333*	416.89832	.001	600.3203	3058.9864

			August 2007	1806.12333*	494.98119	.006	359.0506	3253.1961
		July 2007	April 2007	875.68989	502.00853	.639	-708.3578	2459.7376
			May 2007	-611.39074	309.66464	.424	-1516.6771	293.8956
			June 2007	-1829.65333*	416.89832	.001	-3058.9864	-600.3203
			August 2007	-23.53000	410.14290	1.000	-1232.0830	1185.0230
		August 2007	April 2007	899.21989	568.51977	.737	-827.9970	2626.4368
			May 2007	-587.86074	408.77177	.820	-1792.8109	617.0895
			June 2007	-1806.12333*	494.98119	.006	-3253.1961	-359.0506
			July 2007	23.53000	410.14290	1.000	-1185.0230	1232.0830
	Dunnett T3	April 2007	May 2007	-1487.08063	500.88894	.070	-3053.4703	79.3091
			June 2007	-2705.34323*	573.41237	.001	-4434.7605	-975.9260
			July 2007	-875.68989	502.00853	.587	-2444.2464	692.8666
			August 2007	-899.21989	568.51977	.700	-2616.5130	818.0732
		May 2007	April 2007	1487.08063	500.88894	.070	-79.3091	3053.4703
			June 2007	-1218.26259	415.54948	.051	-2440.1498	3.6246
			July 2007	611.39074	309.66464	.408	-291.6047	1514.3862
			August 2007	587.86074	408.77177	.798	-613.2993	1789.0208
		June 2007	April 2007	2705.34323*	573.41237	.001	975.9260	4434.7605
			May 2007	1218.26259	415.54948	.051	-3.6246	2440.1498

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		July 2007	1829.65333*	416.89832	.001	604.2200	3055.0866
		August 2007	1806.12333*	494.98119	.006	362.7143	3249.5324
	July 2007	April 2007	875.68989	502.00853	.587	-692.8666	2444.2464
		May 2007	-611.39074	309.66464	.408	-1514.3862	291.6047
		June 2007	-1829.65333*	416.89832	.001	-3055.0866	-604.2200
		August 2007	-23.53000	410.14290	1.000	-1228.3153	1181.2553
	August 2007	April 2007	899.21989	568.51977	.700	-818.0732	2616.5130
		May 2007	-587.86074	408.77177	.798	-1789.0208	613.2993
		June 2007	-1806.12333*	494.98119	.006	-3249.5324	-362.7143
		July 2007	23.53000	410.14290	1.000	-1181.2553	1228.3153

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Puerarin contents (mg/100g powder)

	Collecting month	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	April 2007	14	127.4529	
	August 2007	27	201.4141	201.4141
	May 2007	27		326.3189
	June 2007	27		329.1304
	July 2007	27		342.6200
	Sig.		.722	.126
Duncan[a]	April 2007	14	127.4529	
	August 2007	27	201.4141	
	May 2007	27		326.3189
	June 2007	27		329.1304
	July 2007	27		342.6200
	Sig.		.213	.797

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

Puerarin contents (mg/100g powder)

	Collecting month	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	April 2007	14	127.4529	
	August 2007	27	201.4141	201.4141
	May 2007	27		326.3189
	June 2007	27		329.1304
	July 2007	27		342.6200
	Sig.		.722	.126
Duncan[a]	April 2007	14	127.4529	
	August 2007	27	201.4141	
	May 2007	27		326.3189
	June 2007	27		329.1304
	July 2007	27		342.6200
	Sig.		.213	.797

Means for groups in homogeneous subsets are displayed.

Daidzin contents (mg/100g powder)

	Collecting month	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	April 2007	14	1042.6214	
	July 2007	27	1211.0356	1211.0356
	August 2007	27	2122.3970	2122.3970
	May 2007	27	2207.2674	2207.2674
	June 2007	27		2378.4044
	Sig.		.082	.081

Duncan[a]	April 2007	14	1042.6214	
	July 2007	27	1211.0356	
	August 2007	27		2122.3970
	May 2007	27		2207.2674
	June 2007	27		2378.4044
	Sig.		.710	.598

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

Genistin contents (mg/100g powder)

	Collecting month	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	August 2007	27	350.6159		
	April 2007	14	605.1329		
	May 2007	27	728.7015		
	July 2007	27	1097.2415	1097.2415	
	June 2007	27		1773.0156	
	Sig.		.062	.114	

Duncan[a]	August 2007	27	350.6159		
	April 2007	14	605.1329	605.1329	
	May 2007	27	728.7015	728.7015	
	July 2007	27		1097.2415	
	June 2007	27			1773.0156
	Sig.		.203	.097	1.000

	Collecting month	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	August 2007	27	350.6159		
	April 2007	14	605.1329		
	May 2007	27	728.7015		
	July 2007	27	1097.2415	1097.2415	
	June 2007	27		1773.0156	
	Sig.		.062	.114	
Duncan[a]	August 2007	27	350.6159		
	April 2007	14	605.1329	605.1329	
	May 2007	27	728.7015	728.7015	
	July 2007	27		1097.2415	
	June 2007	27			1773.0156
	Sig.		.203	.097	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

	Collecting month	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	August 2007	27	350.6159		
	April 2007	14	605.1329		
	May 2007	27	728.7015		
	July 2007	27	1097.2415	1097.2415	
	June 2007	27		1773.0156	
	Sig.		.062	.114	
Duncan[a]	August 2007	27	350.6159		
	April 2007	14	605.1329	605.1329	
	May 2007	27	728.7015	728.7015	
	July 2007	27		1097.2415	
	June 2007	27			1773.0156
	Sig.		.203	.097	1.000

Total contents (mg/100g powder)

	Collecting month	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	April 2007	14	1775.2071		
	July 2007	27	2650.8970	2650.8970	
	August 2007	27	2674.4270	2674.4270	
	May 2007	27		3262.2878	3262.28
	June 2007	27			4480.5504
	Sig.		.285	.665	.064

Duncan[a]	April 2007	14	1775.2071		
	July 2007	27	2650.8970	2650.8970	
	August 2007	27	2674.4270	2674.4270	
	May 2007	27		3262.2878	
	June 2007	27			4480.5504
	Sig.		.064	.209	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

Homogeneous Subsets

Puerarin contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	45	4	56.6850				
	43	5	62.8800				
	83	9	123.2933	123.2933			
	53	9	182.0589	182.0589	182.0589		
	84	9	217.6733	217.6733	217.6733		

	74	9	229.0822	229.0822	229.0822		
	64	9	233.2622	233.2622	233.2622		
	44	5	248.6400	248.6400	248.6400		
	85	9	263.2756	263.2756	263.2756		
	63	9	286.0556	286.0556	286.0556		
	75	9	330.5867	330.5867	330.5867		
	55	9	352.4711	352.4711	352.4711		
	54	9		444.4267	444.4267		
	65	9			468.0733		
	73	9			468.1911		
	Sig.		.123	.061	.158		
Duncan[a]	45	4	56.6850				
	43	5	62.8800				
	83	9	123.2933	123.2933			
	53	9	182.0589	182.0589	182.0589		
	84	9	217.6733	217.6733	217.6733		
	74	9	229.0822	229.0822	229.0822	229.0822	
	64	9	233.2622	233.2622	233.2622	233.2622	
	44	5	248.6400	248.6400	248.6400	248.6400	

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	85	9	263.2756	263.2756	263.2756	263.2756	263.2756
	63	9		286.0556	286.0556	286.0556	286.0556
	75	9		330.5867	330.5867	330.5867	330.5867
	55	9			352.4711	352.4711	352.4711
	54	9				444.4267	444.4267
	65	9					468.0733
	73	9					468.1911
	Sig.		.066	.065	.132	.052	.062

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.563.

Daidzin contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Tukey HSD[a]	43	5	194.6280						
	83	9	394.9400						
	45	4	568.8900	568.8900					
	64	9	861.8400	861.8400	861.8400				
	74	9	870.8978	870.8978	870.8978				
	53	9	997.9889	997.9889	997.9889	997.9889			

	63	9	1213.4156	1213.4156	1213.4156	1213.4156			
	75	9	1321.7200	1321.7200	1321.7200	1321.7200			
	73	9	1440.4889	1440.4889	1440.4889	1440.4889			
	85	9		2167.3333	2167.3333	2167.3333	2167.3333		
	44	5			2269.6000	2269.6000	2269.6000	2269.6000	
	54	9				2541.2044	2541.2044	2541.2044	
	55	9					3082.6089	3082.6089	
	84	9						3804.9178	3804.9178
	65	9							5059.9578
	Sig.		.337	.057	.164	.079	.815	.083	.325
Duncan[a]	43	5	194.6280						
	83	9	394.9400	394.9400					
	45	4	568.8900	568.8900					
	64	9	861.8400	861.8400					
	74	9	870.8978	870.8978					
	53	9	997.9889	997.9889					
	63	9	1213.4156	1213.4156	1213.4156				
	75	9		1321.7200	1321.7200	1321.7200			
	73	9		1440.4889	1440.4889	1440.4889			

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	85	9			2167.3333	2167.3333	2167.3333		
	44	5				2269.6000	2269.6000		
	54	9					2541.2044		
	55	9					3082.6089	3082.6089	
	84	9						3804.9178	
	65	9							5059.9578
	Sig.		.061	.057	.063	.065	.075	.124	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.563.

Genistin contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Tukey HSD[a]	45	4	131.5850					
	85	9	185.2300					
	65	9	238.3089					
	43	5	251.5200					
	84	9	303.0844					
	54	9	413.4067					

	55	9	494.9978					
	83	9	563.5333					
	73	9	927.9178	927.9178				
	74	9	951.3644	951.3644				
	53	9	1277.7000	1277.7000				
	44	5	1337.5840	1337.5840				
	75	9	1412.4422	1412.4422				
	64	9		2018.3867	2018.3867			
	63	9			3062.3511			
	Sig.		.054	.198	.259			
Duncan[a]	45	4	131.5850					
	85	9	185.2300					
	65	9	238.3089					
	43	5	251.5200					
	84	9	303.0844					
	54	9	413.4067					
	55	9	494.9978	494.9978				
	83	9	563.5333	563.5333	563.5333			
	73	9	927.9178	927.9178	927.9178	927.9178		

	74	9	951.3644	951.3644	951.3644	951.3644		
	53	9		1277.7000	1277.7000	1277.7000	1277.7000	
	44	5			1337.5840	1337.5840	1337.5840	
	75	9				1412.4422	1412.4422	
	64	9					2018.3867	
	63	9						3062.3511
	Sig.		.066	.062	.065	.254	.071	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.563.

Total contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Tukey HSD[a]	43	5	509.0280						
	45	4	757.1600	757.1600					
	83	9	1081.7667	1081.7667	1081.7667				
	74	9	2051.3444	2051.3444	2051.3444	2051.3444			
	53	9	2457.7478	2457.7478	2457.7478	2457.7478	2457.7478		
	85	9		2615.8389	2615.8389	2615.8389	2615.8389	2615.8389	

	73	9		2836.5978	2836.5978	2836.5978	2836.5978	2836.5978	
	75	9			3064.7489	3064.7489	3064.7489	3064.7489	
	64	9			3113.4889	3113.4889	3113.4889	3113.4889	
	54	9				3399.0378	3399.0378	3399.0378	
	44	5				3855.8240	3855.8240	3855.8240	3855.8240
	55	9				3930.0778	3930.0778	3930.0778	3930.0778
	84	9					4325.6756	4325.6756	4325.6756
	63	9						4561.8222	4561.8222
	65	9							5766.3400
	Sig.		.100	.056	.069	.134	.140	.101	.117
Duncan[a]	43	5	509.0280						
	45	4	757.1600						
	83	9	1081.7667	1081.7667					
	74	9		2051.3444	2051.3444				
	53	9			2457.7478				
	85	9			2615.8389	2615.8389			
	73	9			2836.5978	2836.5978			
	75	9			3064.7489	3064.7489	3064.7489		
	64	9			3113.4889	3113.4889	3113.4889		

	54	9			3399.0378	3399.0378	3399.0378	3399.0378	
	44	5				3855.8240	3855.8240	3855.8240	
	55	9				3930.0778	3930.0778	3930.0778	
	84	9					4325.6756	4325.6756	
	63	9						4561.8222	
	65	9							5766.3400
	Sig.		.377	.112	.056	.062	.070	.090	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.563.

Post Hoc Tests

Puerarin contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	4	14	127.4529	
	8	27	201.4141	201.4141
	5	27		326.3189
	6	27		329.1304
	7	27		342.6200
	Sig.		.722	.126
Duncan[a]	4	14	127.4529	
	8	27	201.4141	
	5	27		326.3189
	6	27		329.1304
	7	27		342.6200
	Sig.		.213	.797

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

Daidzin contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	4	14	1042.6214	
	7	27	1211.0356	1211.0356
	8	27	2122.3970	2122.3970
	5	27	2207.2674	2207.2674
	6	27		2378.4044
	Sig.		.082	.081
Duncan[a]	4	14	1042.6214	
	7	27	1211.0356	
	8	27		2122.3970
	5	27		2207.2674
	6	27		2378.4044
	Sig.		.710	.598

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

Genistin contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	8	27	350.6159		
	4	14	605.1329		
	5	27	728.7015		
	7	27	1097.2415	1097.2415	
	6	27		1773.0156	
	Sig.		.062	.114	
Duncan[a]	8	27	350.6159		
	4	14	605.1329	605.1329	
	5	27	728.7015	728.7015	
	7	27		1097.2415	
	6	27			1773.0156
	Sig.		.203	.097	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

Total contents (mg/100g powder)

	Code	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	4	14	1775.2071		
	7	27	2650.8970	2650.8970	
	8	27	2674.4270	2674.4270	
	5	27		3262.2878	3262.2878
	6	27			4480.5504
	Sig.		.285	.665	.064
Duncan[a]	4	14	1775.2071		
	7	27	2650.8970	2650.8970	
	8	27	2674.4270	2674.4270	
	5	27		3262.2878	
	6	27			4480.5504
	Sig.		.064	.209	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.771.

BIOGRAPHY

Miss Jutarmas Jungsukcharoen was born on June 19, 1984 in Chonburi province, Thailand. She received the Bachelor Degree of Science (Biotechnology), Faculty of Engineering and Industrial Technology, Silpakorn University in 2005. She has studied for Program in Biotechnology, Faculty of Science, Chulalongkorn University since 2006.