CHAPTER IV

RESULTS AND DISCUSSIONS

4.1. Metabolomic study

4.1.1. Plant harvesting

The three cultivars of *P. mirifica* produced abundant numbers and masses of leaves for harvesting every month, except when no or too few leaves were present to harvest, which was in April for the PM-III cultivar, March for the PM-IV cultivar, and February, March and April for the PM-V cultivar. During February to April, the lower of rainfall amount and temperature increase might cause water-limited environment. Plants then become water stressed and dropped their leaves (Bidwell 1964). Tubers of *P. mirifica* were harvested in seasonally term (April, August and December). Atleast 3-year old tuber was considered (Sibao et al 2007).

After Incubated in the heat oven at hot air oven at 80°C for 72 hours and subsequently powdered at a size of 120 μm, the characteristic of *P. mirifica* leaves (Figure 15) and tubers (Figure 16) powder were shown. Leaf powder has lightly green whereas tuber powder has white color.

Figure 15 The characteristic of *P. mirifica* leaf powder

Figure 16 The characteristic of *P. mirifica* tuber powder.

4.1.2. Crude leaf and tuber extract yield

The different yields of the tuber and leaf crude extract obtained for each of the three *P. mirifica* cultivars over the assayed year are summarized in Table 3 and 4 with highest mean temperature in April and rainfall amount in May (Figure 17). The crude leaf extracts derived from the three *P. mirifica* cultivars varied in their yield and relative composition of the isoflavonoids between the different cultivars and cultivation month during the 12-month period assayed. Whether this temporal monthly pattern is representative of typical years remains to be established. For the crude extract yield obtain which some of them absent in February, March and April. The absence of leaves were in paralell with the higher temperature exposured in the same months. Within each month a significant variation in the crude leaf-extract yield between the cultivars was evident although cultivar PM-III typically exhibited the highest monthly yields (in 7/12 months) and the highest average yearly yield, whilst PM-V had the lowest monthly (in 7/12 months) and yearly yield. Whereas, the total tuber crude extract in PM-V had highest yield. The tuber crude extract have a higher yield than in leaf. It may because there has no losing yield in chlorophyll removing process.

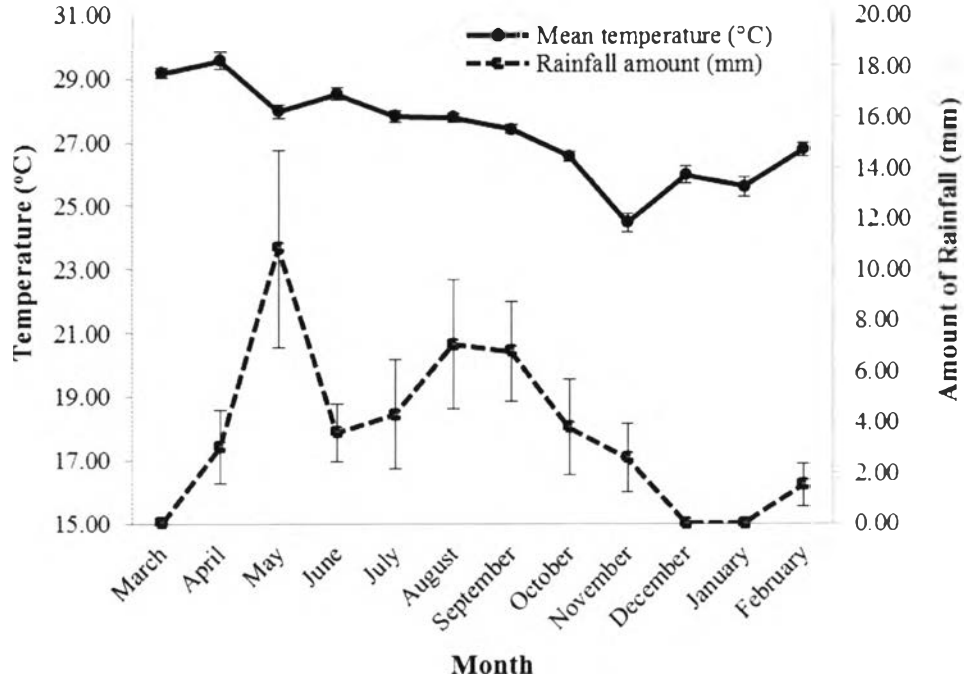


Figure 17 The mean monthly temperature (°C) and rainfall amount at Ratchaburi province, including the field trial area, during the study period (March 2007 to February 2008). Data are shown as the mean ± S.E.M.

Table 3 The tuber crude extract yields obtained (g/100 g powder) from each the three different *P. mirifica* cultivars in each season in 2007.

Month	Crude extract of *P. mirifica* (g/100g tuber powder) (Means±S.E.M.)		
	PM-III	PM-IV	PM-V
April 2007	2.50 ± 0.07[a]	2.88 ± 0.04[a]	3.37 ± 0.02[b]
August 2007	2.86 ± 0.06[a]	3.67 ± 0.48[a]	3.81 ± 0.04[c]
December 2007	2.94 ± 0.08[a]	2.55 ± 0.10[a]	2.93 ± 0.10[a]
Total mean	2.77 ± 0.03	3.03 ± 0.08	3.37 ± 0.04

Table 4 **The leaf crude extract yields obtained (g/100 g leaf powder) from each the three different *P. mirifica* cultivars in each month over the 12-month cultivation period (March 2007 to February 2008).**

Month	Crude leaf extract of *P. mirifica* (g/100g leaf powder) (Means±S.E.M.)		
	PM-III	PM-IV	PM-V
March 2007	$1.41 \pm 0.44^{a,b}$	NA	NA
April 2007	NA	$1.54 \pm 0.12^{a,b}$	NA
May 2007	$2.06 \pm 0.36^{a,b,c}$	2.41 ± 0.11^{b}	$1.96 \pm 0.08^{b,c,d}$
June 2007	$2.70 \pm 1.04^{b,c}$	2.11 ± 0.42^{b}	2.67 ± 0.09^{d}
July 2007	3.37 ± 0.20^{c}	2.52 ± 0.94^{b}	2.34 ± 0.08^{d}
August 2007	$1.26 \pm 0.13^{a,b}$	2.75 ± 0.35^{b}	2.82 ± 0.03^{d}
September 2007	$2.44 \pm 0.31^{b,c}$	1.80 ± 0.24^{b}	$2.28 \pm 0.38^{c,d}$
October 2007	3.25 ± 0.61^{c}	1.67 ± 0.43^{b}	2.44 ± 0.45^{d}
November 2007	$2.24 \pm 0.23^{a,b,c}$	$1.55 \pm 0.26^{a,b}$	$0.94 \pm 0.94^{a,b,c}$
December 2007	$2.59 \pm 1.06^{b,c}$	2.18 ± 0.10^{b}	$0.83 \pm 0.83^{a,b}$
January 2008	$2.69 \pm 0.29^{b,c}$	2.78 ± 0.27^{b}	1.27 ± 0.84^{a}
February 2008	$2.17 \pm 0.54^{a,b,c}$	2.63 ± 0.45^{b}	NA
Total mean	2.23 ± 0.18	2.02 ± 0.15	1.46 ± 0.20

NA = not applicable because the plants shed their leaves for water preservation during this month.

Data are shown as the mean ± S.E.M., derived from nine independent repeats.

^{a,b,c,d} represent groups of samples with statistically significant differences ($P<0.05$) in each column as determined by Duncan's analysis.

4.1.3. HPLC fingerprint

The five standard isoflavonoids run in triplicate were clearly separated and distinct when analyzed by RP-HPLC with the C18 column and monitoring the eluent at OD_{254}. (Figure 18). The standard puerarin, daidzin, genistin, daidzein and genistein were detected at retention time 14.349 min, 15.932 min, 18.963 min, 26.485 min and 34.642 min, respectively.

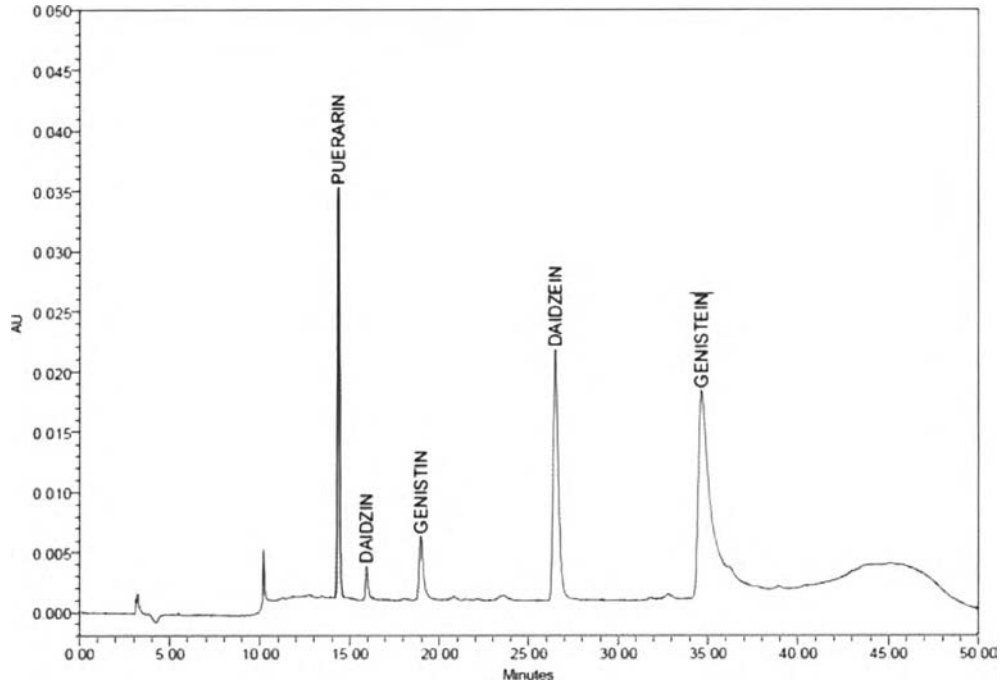


Figure 18 HPLC fingerprints of the five synthetic isoflavonoid standards of puerarin (25 mg/mL, RT = 14.35 min), daidzin (30 mg/mL, RT = 15.93 min), genistin (30 mg/mL, RT = 18.96 min), daidzein (25 mg/mL, RT = 26.49 min) and genistein (50 mg/mL, RT = 34.64 min). Profiles shown are representative of those seen from at least 27 independent trials.

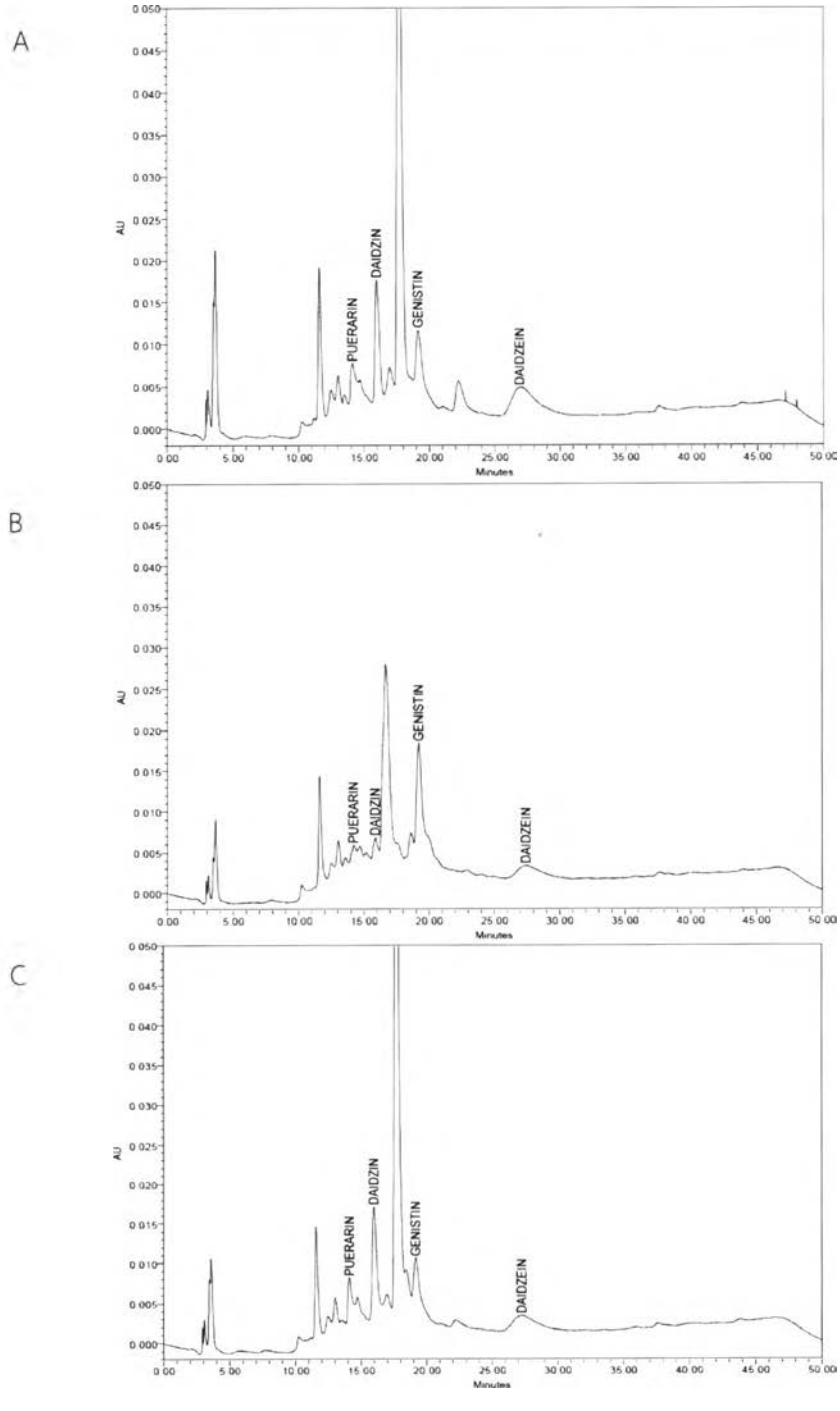


Figure 19 HPLC fingerprints of the crude leaf-extract of *P. mirifica* cultivar (A) PM-III, (B) PM-IV, and (C) PM-V, derived from samples harvested during January 2008.

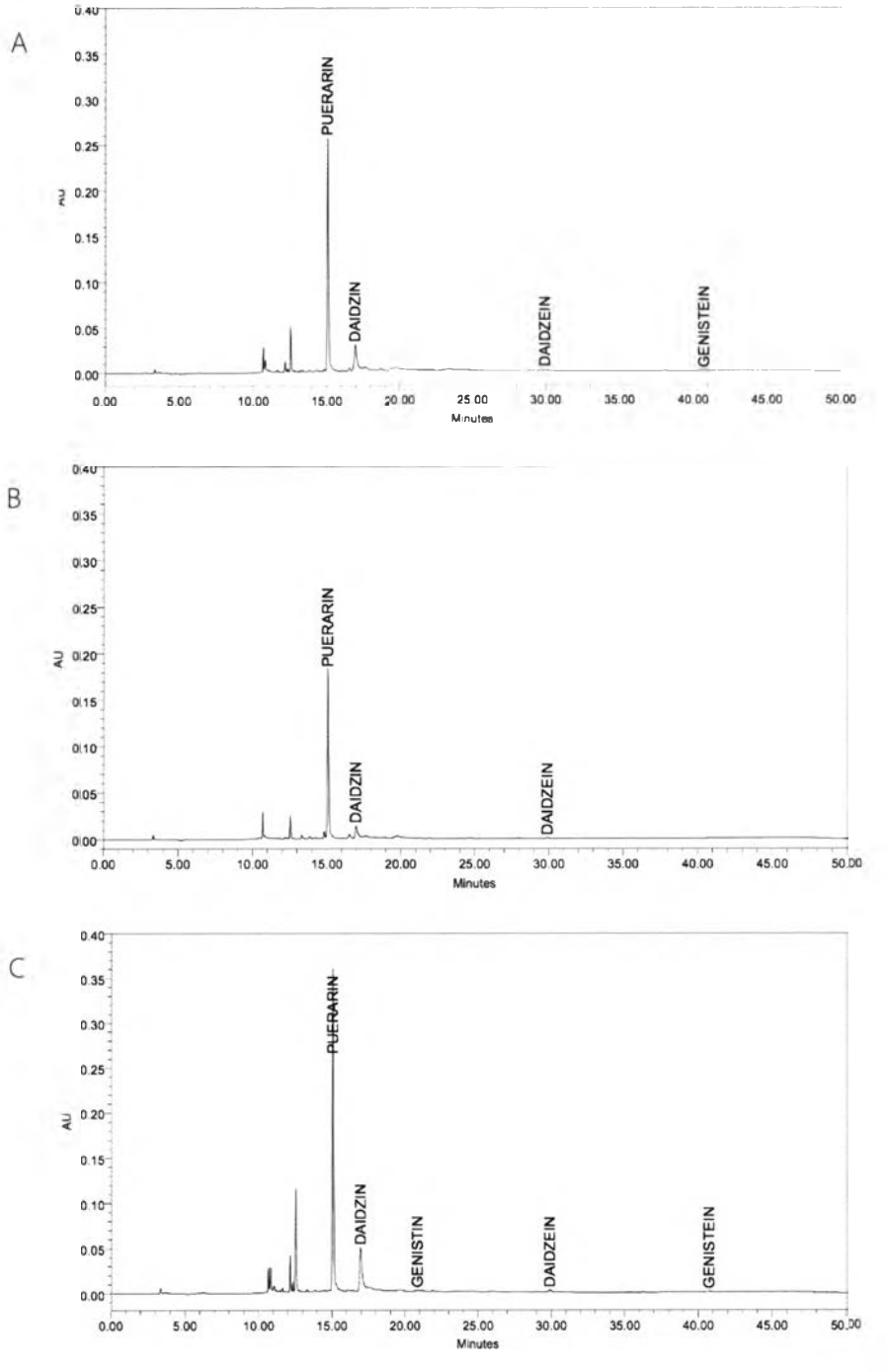


Figure 20 HPLC fingerprints of the crude tuber-extract of *P. mirifica* cultivar (A) PM-III, (B) PM-IV, and (C) PM-V, derived from samples harvested during summer season.

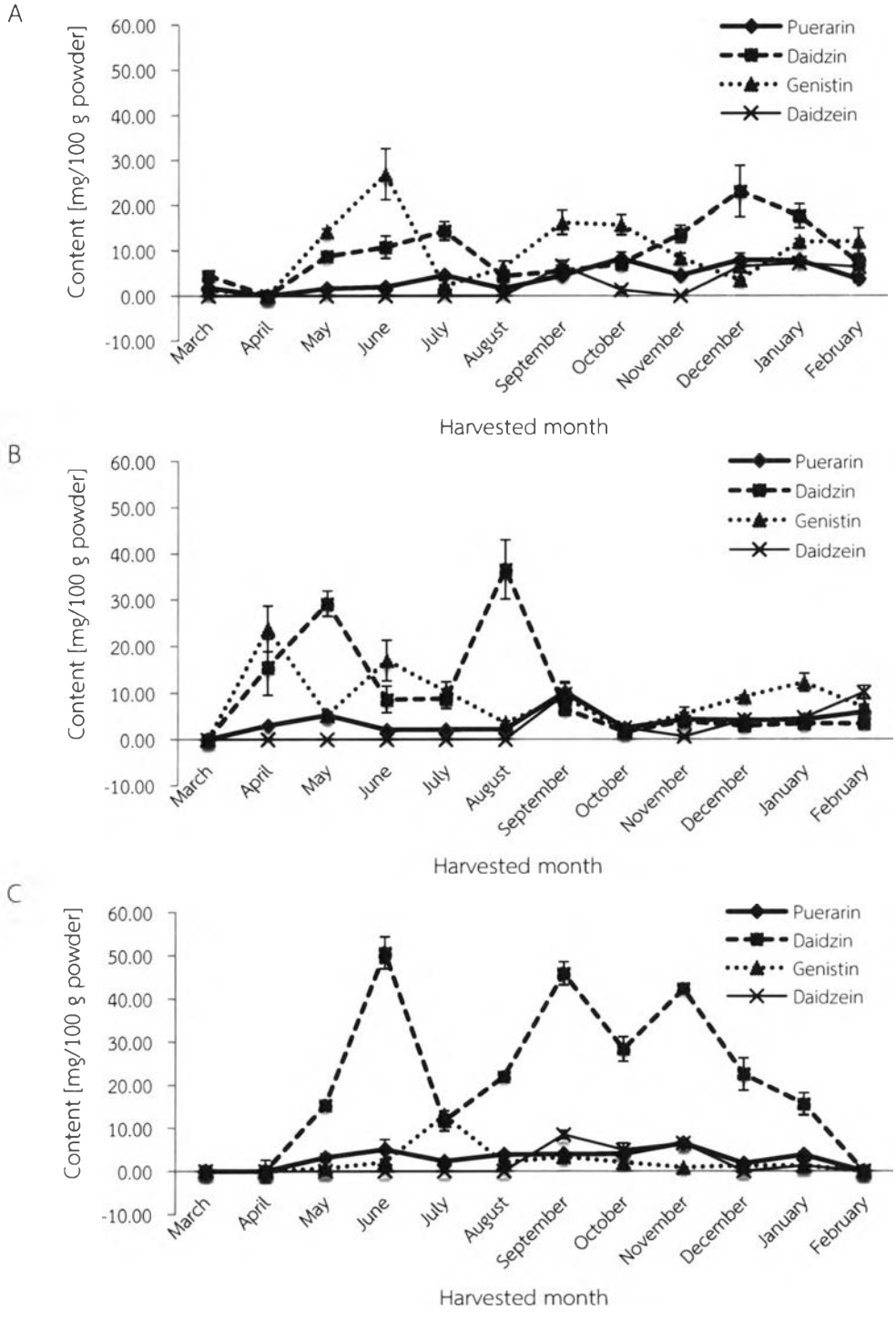


Figure 21 Major isoflavonoidprofile (mg/100 g powder) of the crude leaf-extracts from the three different cultivars of *P. mirifica* harvested in different months (A) PM-III, (B) PM-IV and (C) PM-V. Data are shown as the mean ± S.E.M

The calculated amount of each isoflavonoid found in each *P. mirifica* leaf cultivar over the 12-month cultivation period revealed the highest mean total isoflavonoid content across all three cultivars was found in June (41.7 ± 4.0 mg/100 g leaf powder) followed by September (37.9 ± 4.3 mg/100 g leaf powder) but this varied between the individual cultivars (Table 5). Cultivar PM-V had the highest total isoflavonoid contents in June (57.69 ± 4.14 mg/100 g powder) and for PM-III the optimal month was June, whilst for PM-IV it was equally optimal in April and August. Daidzin in the PM-V cultivar was the most prevalent of the isoflavonoids, where the highest daidzin concentration was found in June (50.6 ± 3.7 mg/100 g powder), however, levels of daidzin in the other two cultivars (PM-III and PM-IV) were significantly lower in this and most other months. In contrast, genistin levels were very low in the PM-V cultivar except in July (13.0 ± 0.1 mg/100 g powder), but was the isoflavonoid with the highest concentration in the PM-III and PM-IV cultivars for seven and five months, respectively, and with its levels being second to those of daidzin in several other months. In contrast, puerarin and daidzein remained at relatively low concentrations over all 12 months in all three cultivars.

The isoflavonoid content in *P. mirifica* tuber was totally difference from leaf (Table 6). The highest mean total isoflavonoid content across all three cultivars was found in summer season (178.3 ± 53.9 mg/100 g powder), while the highest isoflavonoid content was puerarin in PM-V (214.0 ± 4.4 mg/100 g powder). The highest genistin concentration was found in summer (6.0 ± 1.0 mg/100 g powder). The tuber, genistein was detected only in summer (0.45 ± 0.01 mg/100 g powder). The suitable season for PM-V tuber harvesting was summer which gave the highest yield of 5 major isoflavonoid contents.

The monthly rainfall level strongly correlated with, and so potentially had a significant impact upon the genistin content in the leaves each month ($P<0.05$), whilst the temperature likewise strongly correlated with the puerarin, daidzin, and daidzein contents ($P<0.01$) in the PM-III cultivar (in Table 12 information). In contrast, in the PM-V cultivar the rainfall strongly correlated with the puerarin content ($P<0.01$) and the temperature with the daidzin content ($P<0.01$). Note, however, that in all four of the above correlations an actual causation has yet to be established.

However, the study also confirmed the existence of a significant level of accumulated isoflavonoids in the plant leaves, including puerarin, daidzin, genistin, and daidzein. Determination of the isoflavonoid levels revealed significant cultivar-dependent difference, i.e. difference in the isoflavonoid levels among the three

cultivars grown in the same field trial and harvested at the same time, as well as between each month. Daidzin and genistin were found to have the highest average annual isoflavonoid accumulation in the leaf samples (genistin for PM-III, and daidzin for PM-IV and PM-V) with puerarin also being found in all analyzed plant samples (cultivars and harvest months), but at lower levels. In contrast, daidzein was not found in all three cultivars in March to August, plus not in cultivar PM-IV in October, PM-III in November and PM-V in November to January. Moreover, genistein was not found in any of the cultivars in any month of the year. Overall the chemovariety at the isoflavonoid level in the three *P. mirifica* cultivars, which have distinct differences in their botanical characteristics, was confirmed.

The net yield of total isoflavonoidin *P. mirifica* leaf (41.7 ± 4.0 mg/ 100 g powder) still lower than in tuber (178.3 ± 53.9 mg/ 100 g powder) about 4.3 times. However, in terms of plant cultivation, leaves harvesting is more worthwhile than tuber collection because tubers spend at least 3 years to grow maturely whereas leaves need only 3 months and can be collected quarterly. For the comparison of isoflavonoid yield between tuber and leaf harvested per year in general, the isoflavonoid yield harvested in leaf is higher than tuber 6.20 times per year.

The isoflavonoid pattern in *P. mirifica* leaves over a 12-month cultivation period remains to be ascertained if this pattern holds for other years and so is a real trend or not. However, with that caveat in mind, the puerarin, daidzin, genistin and daidzein levels in the crude leaf-extracts were found to be strongly, but differentially correlated with either the rainfall amount or the average temperature during the 12-month cultivation period assayed within the same plant cultivar in different months (seasons). In addition, whilst the plant genetics per se (as morphologically distinct cultivars in this study) also strongly correlated to the differences in the isoflavonoid types and levels found in the leaves over this 12-month assay period. The extraction method employed in this study was efficient to extract a broad range of polar isoflavones e.g. daidzin, daidzein, genistin, and genistein etc., and the isolated components showed 98-99 % purity (Yang et al 2001). Therefore, the non-detectable genistein in leave crude extract was not from this extraction method. Interestingly, genistein, the isoflavonoid not detected in this study in the crude leaf-extracts, has been found previously in the tubers of these three *P. mirifica* cultivars, although in smaller amounts compared with that of puerarin, genistin, daidzin, and daidzein (Cherdshewasart et al 2007c). The differences in the synthesis and/or accumulation of isoflavonoids between leaf and tuberous tissues is probably that genistein is an aglycosidic compound synthesized in the leaf, which is not stable enough for

transportation or storage in tuberous root, it is converted into genistin, (by glucose conjugation) to increase both its stability (through the protection of reactive nucleophilic groups) and water solubility (Gachon et al 2005b, Malaivijitnond 2012).It is possible that genistin is then cleaved to the aglycosidic form after being transported into the tubers, accounting for the detection of genistin in the leaves but genistein in the tubers. Indeed, glycoside hydrolase has been reported in roots of Medicago truncatula, another legume plant in the same family (Schenkluhn et al 2010). This enzyme can catalyze the hydrolysis of the glycosidic linkage in genistin to convert it to genistein. In contrast, a proteomic analysis of soybean leaves did not report the finding of any enzymes with glycoside hydrolase activity (Xu et al 2006) and so the actual case for *P. mirifica* remains to be resolved.

Table 5 Major isoflavonoid contents of *P. mirifica* leaves (mg/100g powder)

Month	*P. mirifica*	Isoflavonoid contents (mean± S.E.M.) (mg/100g powder)				
		Puerarin	Daidzin	Genistin	Daidzein	Total
March	PM-III	1.78 ± 0.11^{a}	4.50 ± 1.06^{a,b}	1.41 ± 0.22^{a}	0.00 ± 0.00^{a}	6.61 ± 1.37^{a}
2007	PM-IV	0.33 ± 0.17^{a}	0.33 ± 0.17^{a}	0.56 ± 0.28^{a}	0.00 ± 0.00^{a}	1.22 ± 0.61^{a}
	PM-V	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Mean ± S.E.M.	0.34 ± 0.08$^{a'}$	1.61 ± 0.53$^{a'}$	0.66 ± 0.16$^{a'}$	0.00 ± 0.00$^{a'}$	2.61 ± 0.74$^{a'}$
April	PM-III	0.80 ± 0.34^{a}	1.55 ± 0.08^{a}	2.35 ± 0.04^{a}	0.00 ± 0.00^{a}	4.69 ± 0.09^{a}
2007	PM-IV	2.99 ± 0.26^{b,c}	15.58 ± 6.04^{b}	23.75 ± 4.93^{f}	0.00 ± 0.00^{a}	42.33 ± 3.62^{e}
	PM-V	0.85 ± 0.21^{a,b}	8.30 ± 2.59^{s,b,c}	0.42 ± 0.21^{a}	0.00 ± 0.00^{a}	9.58 ± 2.95^{b,c}
	Mean ± S.E.M.	1.55 ± 0.23$^{a',b'}$	8.48 ± 2.38$^{s',b',c'}$	8.84 ± 2.61$^{b',c'}$	0.00 ± 0.00$^{a'}$	18.87 ± 3.60$^{b',c'}$
May	PM-III	1.61 ± 0.10^{a,b}	8.74 ± 0.36^{b,c,d}	14.31 ± 0.59^{c,d}	0.00 ± 0.00^{a}	24.66 ± 0.86^{c,d}
	PM-IV	5.18 ± 0.79^{c,d}	29.20 ± 2.72^{c}	5.24 ± 0.58^{a,b,c,d}	0.00 ± 0.00^{a}	39.62 ± 1.53^{e}
	PM-V	3.16 ± 0.92^{c}	15.29 ± 1.05^{c,d}	0.65 ± 0.20^{a}	0.00 ± 0.00^{a}	19.10 ± 1.81^{c,d}
	Mean ± S.E.M.	3.32 ± 0.48$^{c',d',e'}$	17.74 ± 1.92$^{d',e',f'}$	6.73 ± 1.15$^{b',c'}$	0.00 ± 0.00$^{a'}$	27.80 ± 1.88$^{c',d'}$

Data are shown as the mean ± SEM, derived from nine independent repeats.

a,b,c,d,e represent groups of isoflavonoid compounds and a',b',c',d',e' represent groups of isoflavonoid compounds (Mean ± S.E.M.) with statistically significant differences ($p < 0.05$) in each column as determined by Duncan's analysis.

Table 5 Major isoflavonoid contents of *P. mirifica* leaves (mg/100g powder) (continued)

Month	*P. mirifica*	Isoflavonoid contents (mean± S.E.M.) (mg/100g powder)				
		Puerarin	Daidzin	Genistin	Daidzein	Total
June	PM-III	$1.95 \pm 0.56^{a,b}$	$10.80 \pm 2.54^{b,c,d}$	26.94 ± 5.67^{e}	0.00 ± 0.00^{a}	39.69 ± 7.70^{e}
	PM-IV	$2.06 \pm 0.44^{a,b}$	$8.62 \pm 2.89^{a,b}$	16.97 ± 4.32^{e}	0.00 ± 0.00^{a}	27.65 ± 4.79^{d}
	PM-V	5.04 ± 2.31^{d}	50.60 ± 3.70^{f}	2.04 ± 0.32^{b}	0.00 ± 0.00^{a}	57.69 ± 4.14^{e}
	Mean ± S.E.M.	$3.02 \pm 0.44^{b',c',d'}$	$23.34 \pm 4.05^{f'}$	$15.32 \pm 3.04^{d'}$	$0.00 \pm 0.00^{a'}$	$41.68 \pm 4.01^{e'}$
July	PM-III	4.58 ± 0.47^{c}	$14.40 \pm 2.10^{d,e}$	2.07 ± 0.25^{a}	0.00 ± 0.00^{a}	$21.04 \pm 2.17^{b,c}$
	PM-IV	$2.10 \pm 0.30^{a,b}$	$8.76 \pm 2.12^{a,b}$	$10.36 \pm 2.04^{c,d,e}$	0.00 ± 0.00^{a}	$21.22 \pm 4.46^{c,d}$
	PM-V	3.12 ± 0.29^{c}	$11.67 \pm 2.37^{b,c}$	13.00 ± 0.14^{d}	0.00 ± 0.00^{a}	27.79 ± 1.96^{c}
	Mean ± S.E.M.	$3.27 \pm 0.29^{c',d',e'}$	$11.61 \pm 1.30^{c',d'}$	$8.48 \pm 1.13^{b',c'}$	$0.00 \pm 0.00^{a'}$	$23.35 \pm 1.82^{c'}$
August	PM-III	$1.58 \pm 0.37^{a,b}$	$4.38 \pm 0.80^{a,b}$	$6.25 \pm 1.47^{a,b}$	0.00 ± 0.00^{a}	$12.20 \pm 2.61^{a,b}$
	PM-IV	$2.18 \pm 0.12^{a,b}$	36.58 ± 6.42^{c}	$3.59 \pm 0.40^{a,b,c}$	0.00 ± 0.00^{a}	42.34 ± 6.04^{e}
	PM-V	$3.87 \pm 0.48^{c,d}$	$21.91 \pm 1.12^{d,e}$	1.99 ± 0.44^{b}	0.00 ± 0.00^{a}	$27.76 \pm 1.65^{c,d}$
	Mean ± S.E.M.	$2.54 \pm 0.27^{b',c'}$	$20.95 \pm 3.33^{f'}$	$3.94 \pm 0.61^{a',b'}$	$0.00 \pm 0.00^{a'}$	$27.44 \pm 3.25^{c',d'}$

Data are shown as the mean ± SEM, derived from nine independent repeats.

^{a,b,c,d,e} represent groups of isoflavonoid compounds and $^{a',b',c',d',e'}$ represent groups of isoflavonoid compounds (Mean ± S.E.M.) with statistically significant differences ($p < 0.05$) in each column as determined by Duncan's analysis.

Table 5 Major isoflavonoid contents of *P. mirifica* leaves (mg/100g powder) (continued)

Month	*P. mirifica*	Isoflavonoid contents (mean± S.E.M.) (mg/100g powder)				
		Puerarin	Daidzin	Genistin	Daidzein	Total
September	PM-III	4.51 ± 0.72^{c}	$5.50 \pm 1.15^{a,b}$	16.23 ± 2.67^{d}	6.61 ± 1.17^{b}	$26.24 \pm 4.51^{c,d}$
	PM-IV	10.19 ± 1.89^{e}	6.59 ± 1.39^{a}	$9.15 \pm 3.06^{b,c,d}$	9.72 ± 2.67^{c}	25.93 ± 6.26^{d}
	PM-V	$3.92 \pm 0.60^{c,d}$	45.84 ± 2.68^{f}	3.35 ± 0.11^{c}	0.00 ± 0.00^{d}	$53.12 \pm 3.26^{d,e}$
	Mean ± S.E.M.	$6.21 \pm 0.87^{f'}$	$19.31 \pm 3.82^{e',f'}$	$9.58 \pm 1.66^{c'}$	$5.44 \pm 1.23^{c'}$	$35.10 \pm 3.67^{d',e'}$
October	PM-III	8.20 ± 1.44^{d}	$7.03 \pm 0.42^{a,b,c}$	15.76 ± 2.28^{d}	1.29 ± 0.64^{a}	$30.98 \pm 3.30^{c,d,e}$
	PM-IV	$2.41 \pm 0.53^{a,b}$	1.67 ± 0.22^{a}	$1.94 \pm 0.09^{a,b}$	0.00 ± 0.00^{a}	$6.02 \pm 0.81^{a,b}$
	PM-V	$3.96 \pm 0.19^{c,d}$	28.33 ± 2.83^{e}	2.06 ± 0.32^{b}	0.00 ± 0.00^{c}	34.35 ± 2.96^{c}
	Mean ± S.E.M.	$4.86 \pm 0.69^{e',f'}$	$12.34 \pm 2.44^{c',d',e'}$	$6.59 \pm 1.47^{b',c'}$	$0.43 \pm 0.24^{a'}$	$23.78 \pm 2.87^{c'}$
November	PM-III	4.48 ± 0.23^{c}	$13.70 \pm 1.91^{c,d,e}$	$8.34 \pm 1.00^{a,b,c}$	0.00 ± 0.00^{a}	$26.53 \pm 1.08^{c,d}$
	PM-IV	$4.30 \pm 1.45^{b,c,d}$	3.87 ± 0.66^{a}	$5.47 \pm 1.44^{a,b,c,d}$	0.61 ± 0.31^{a}	$13.64 \pm 3.54^{b,c}$
	PM-V	$2.16 \pm 1.08^{b,c}$	$14.09 \pm 7.05^{b,c,d}$	0.28 ± 0.14^{a}	0.00 ± 0.00^{b}	$16.54 \pm 8.27^{b,c}$
	Mean ± S.E.M.	$3.65 \pm 0.62^{c',d',e'}$	$10.56 \pm 2.52^{b',c',d'}$	$4.70 \pm 0.86^{a',b',c'}$	$0.20 \pm 0.11^{a'}$	$18.90 \pm 3.10^{b',c'}$

Data are shown as the mean ± SEM, derived from nine independent repeats.

^{a,b,c,d,e} represent groups of isoflavonoid compounds and $^{a',b',c',d',e'}$ represent groups of isoflavonoid compounds (Mean ± S.E.M.) with statistically significant differences ($p < 0.05$) in each column as determined by Duncan's analysis.

Table 5 Major isoflavonoid contents of *P. mirifica* leaves (mg/100g powder) (continued)

Month	*P. mirifica*	Isoflavonoid contents (mean± S.E.M.) (mg/100g powder)				
		Puerarin	Daidzin	Genistin	Daidzein	Total
December	PM-III	7.91 ± 1.52^{d}	23.17 ± 5.67^{f}	3.52 ± 0.08^{a}	6.48 ± 1.33^{b}	34.61 ± 7.24^{b}
	PM-IV	$4.14 \pm 0.13^{b,c,d}$	2.92 ± 0.38^{a}	$9.17 \pm 0.25^{b,c,d}$	4.13 ± 0.40^{b}	$16.23 \pm 0.07^{b,c,d}$
	PM-V	$0.58 \pm 0.29^{a,b}$	$7.50 \pm 3.75^{a,b,c}$	0.42 ± 0.21^{a}	0.00 ± 0.00^{a}	$8.50 \pm 4.25^{b,c}$
	Mean ± S.E.M.	$4.21 \pm 0.77^{c',d',e'}$	$11.20 \pm 2.77^{b',c',d'}$	$4.37 \pm 0.72^{a',b'}$	$3.54 \pm 0.69^{b'}$	$19.78 \pm 3.44^{b',c'}$
January	PM-III	7.80 ± 0.49^{d}	$17.66 \pm 2.68^{e,f}$	$7.18 \pm 0.60^{a,b}$	7.18 ± 0.60^{b}	$32.65 \pm 3.07^{c,d,e}$
	PM-IV	$4.22 \pm 0.28^{b,c,d}$	3.39 ± 0.29^{a}	$12.29 \pm 1.94^{d,e}$	4.69 ± 0.69^{b}	$19.89 \pm 2.26^{c,d}$
	PM-V	$1.27 \pm 0.63^{a,b}$	$5.19 \pm 2.59^{a,b}$	0.42 ± 0.21^{a}	$0.00 \pm 0.00^{a,b}$	$6.87 \pm 3.44^{b,c}$
	Mean ± S.E.M.	$4.43 \pm 0.59^{d',e'}$	$8.75 \pm 1.73^{a',b',c'}$	$6.63 \pm 1.16^{b',c'}$	$3.96 \pm 0.65^{b',c'}$	$19.81 \pm 2.64^{b',c'}$
February	PM-III	$3.71 \pm 0.85^{b,c}$	$7.27 \pm 1.56^{a,b,c}$	$12.07 \pm 2.92^{b,c,d}$	0.00 ± 0.00^{a}	$23.05 \pm 4.88^{b,c}$
	PM-IV	5.88 ± 1.01^{d}	3.37 ± 0.77^{a}	$6.85 \pm 0.61^{a,b,c,d}$	10.01 ± 1.42^{c}	$16.09 \pm 2.37^{b,c,d}$
	PM-V	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Mean ± S.E.M.	$3.20 \pm 0.64^{b',c',d',e'}$	$3.54 \pm 0.80^{a',b'}$	$6.31 \pm 1.36^{b',c'}$	$3.37 \pm 1.04^{b'}$	$13.05 \pm 2.57^{b'}$

Data are shown as the mean ± SEM, derived from nine independent repeats.

^{a,b,c,d,e} represent groups of isoflavonoid compounds and $^{a',b',c',d',e'}$ represent groups of isoflavonoid compounds (Mean ± S.E.M.) with statistically significant differences ($p < 0.05$) in each column as determined by Duncan's analysis.

Table 6 Major isoflavonoidprofile (g/100 g powder) of the crude tuber extracts from the three different cultivars of *P. mirifica* harvested in different season

Harvested season	*P. mirifica*	Isoflavonoid contents (mean± S.E.M.) (g/100g powder)					
		Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total isoflavonoid
Summer 2007	PM-III	117.47 ± 13.29^{b}	29.63 ± 3.43^{b}	0.00 ± 0.00^{a}	1.28 ± 0.04^{b}	0.22 ± 0.02^{b}	148.59 ± 47.85^{b}
	PM-IV	93.47 ± 0.24^{a}	13.01 ± 0.17^{a}	0.00 ± 0.00^{b}	0.68 ± 0.03^{a}	0.00 ± 0.00^{a}	107.16 ± 41.14^{a}
	PM-V	213.97 ± 4.44^{c}	56.22 ± 1.39^{c}	5.96 ± 1.02^{c}	2.38 ± 0.04^{c}	0.68 ± 0.05^{c}	279.21 ± 81.72^{c}
	Mean ± S.E.M.	141.63 ± 5.44$^{b'}$	32.96 ± 1.34$^{b'}$	5.96 ± 1.02$^{a',b'}$	1.45 ± 0.05$^{a'}$	0.45 ± 0.01$^{b'}$	178.32 ± 53.90$^{b'}$
Rainy 2007	PM-III	39.77 ± 0.20^{a}	13.46 ± 0.46^{a}	0.00 ± 0.00^{a}	1.73 ± 0.10^{a}	0.00 ± 0.00	54.95 ± 15.91^{a}
	PM-IV	113.95 ± 41.55^{b}	12.86 ± 4.72^{a}	0.00 ± 0.00^{a}	2.87 ± 1.10^{a}	0.00 ± 0.00	129.68 ± 50.18^{b}
	PM-V	120.45 ± 2.82^{b}	17.31 ± 0.42^{a}	1.06 ± 0.06^{b}	6.04 ± 0.11^{b}	0.00 ± 0.00	144.85 ± 48.99^{b}
	Mean ± S.E.M.	91.39 ± 18.41$^{a'}$	14.54 ± 2.02$^{a'}$	1.06 ± 0.06$^{a'}$	3.54 ± 0.47$^{a'}$	0.00 ± 0.00$^{a'}$	109.83 ± 35.02$^{a'}$
Winter 2007	PM-III	117.75 ± 7.42^{c}	34.07 ± 2.27^{b}	1.75 ± 0.10^{a}	10.46 ± 0.63^{b}	0.00 ± 0.00	164.14 ± 45.84^{c}
	PM-IV	31.00 ± 3.30^{a}	5.74 ± 0.61^{a}	0.93 ± 0.09^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00	37.67 ± 13.19^{a}
	PM-V	46.08 ± 5.25^{b}	32.32 ± 0.42^{b}	5.14 ± 0.87^{b}	11.84 ± 1.15^{b}	0.00 ± 0.00	95.38 ± 16.28^{b}
	Mean ± S.E.M.	64.94 ± 1.69$^{a'}$	24.04 ± 1.11$^{a',b'}$	2.61 ±0.36$^{b'}$	7.94 ± 0.40$^{b'}$	0.00 ± 0.00$^{a'}$	99.06 ± 23.73$^{a'}$

Data are shown as the mean ± SEM, derived from nine independent repeats.

a,b,c,d,e represent groups of isoflavonoid compounds and a',b',c',d',e' represent groups of isoflavonoid compounds (Mean ± S.E.M.) with statistically significant differences ($p < 0.05$) in each column as determined by Duncan's analysis.

4.2. Proteomic study

4.2.1. The evaluation of protein extraction methods

The *P. mirifica* tubers were freshly harvested in seasonally term during summer, rainy season and winter. Acetone precipitation, Tris-HCl extraction, Phenol extraction method A and B were evaluated. From physical properties of the extracted protein, the pellet color of TCA-acetone and Tris-HCl was light brown because the contaminants still remain in the pellets. In contrast, the pellet in phenol extraction method is white. Similar to the previous report, the protein pellet of TCA-acetone and Tris-HCl extraction is light brown in horse gram [35] indicating that these two extraction methods might not suitable for *P. mirifica*. Some protein bands were absent in Tris-HCl and TCA-Acetone method in SDS-PAGE (Figure 22). In contrast, Phenol extraction method showed abundance proteins and clearly separated bands. The phenol extraction method has been applied in many plants; horsegram (*Macrotylomauniflorum*), rajmah (*Phaseolus vulgaris*), mah (*Vignamungo*), chickpea (*Cicerarietinum*), masoor (*Lens culinaris*) and mungbean; but also non-legume plants; rice (*Oryza sativa*), wheat (*Triticumaestivum*), bajra (*Pennisitumglaucum*) and mustard (*Brassica juncea)* (Bhardwaj & Yadav 2013). The principle of phenol extraction method according to H-bond and causes proteins becomes denatured and soluble in the organic phase. Phenol extraction was the most efficient for recalcitrant plant to remove the interfering compounds such as cell wall, storage polysaccharides, lipids and phenolic compounds and also exhibited the highest quality gels with less background and less vertical streaking (Thiellement 2007). For Phenol extraction method, this study applied phenylmethane sulfonylfluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) in method B for protecting of degradable proteins. The result showed more protein bands and sharper than method A (Figure 23). The phenol extraction method B was the most suitable extraction technique for *P. mirifica* tuber. Thus, this study was applied for protein extraction in the entire experiment prior to running two-dimensional gel electrophoresis.

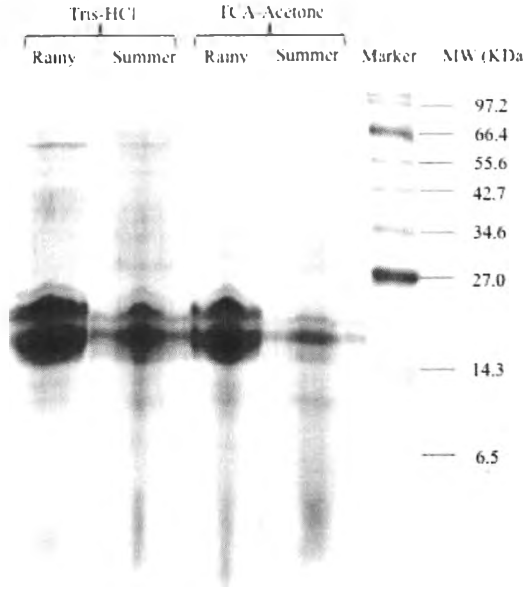


Figure 22 SDS-PAGE analysis of selected protein extraction (5 µg) method for *P. mirifica* tuber were separated on 12.5% gel comparing between two techniques ; TCA-Acetone and Tis-HCl precipitation.

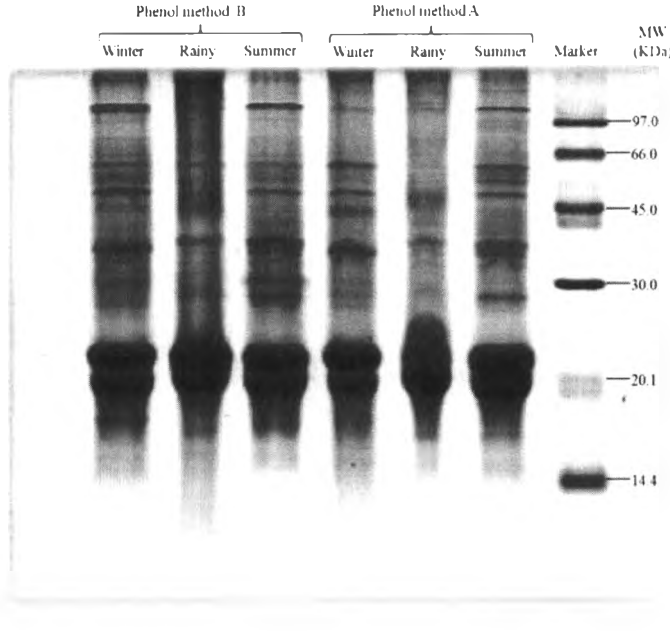


Figure 23 SDS-PAGE analysis of selected protein extraction method for *P. mirifica* tuber using phenol extraction. The 10 µg of protein extraction were separated on 12.5% gel comparing between two methods.

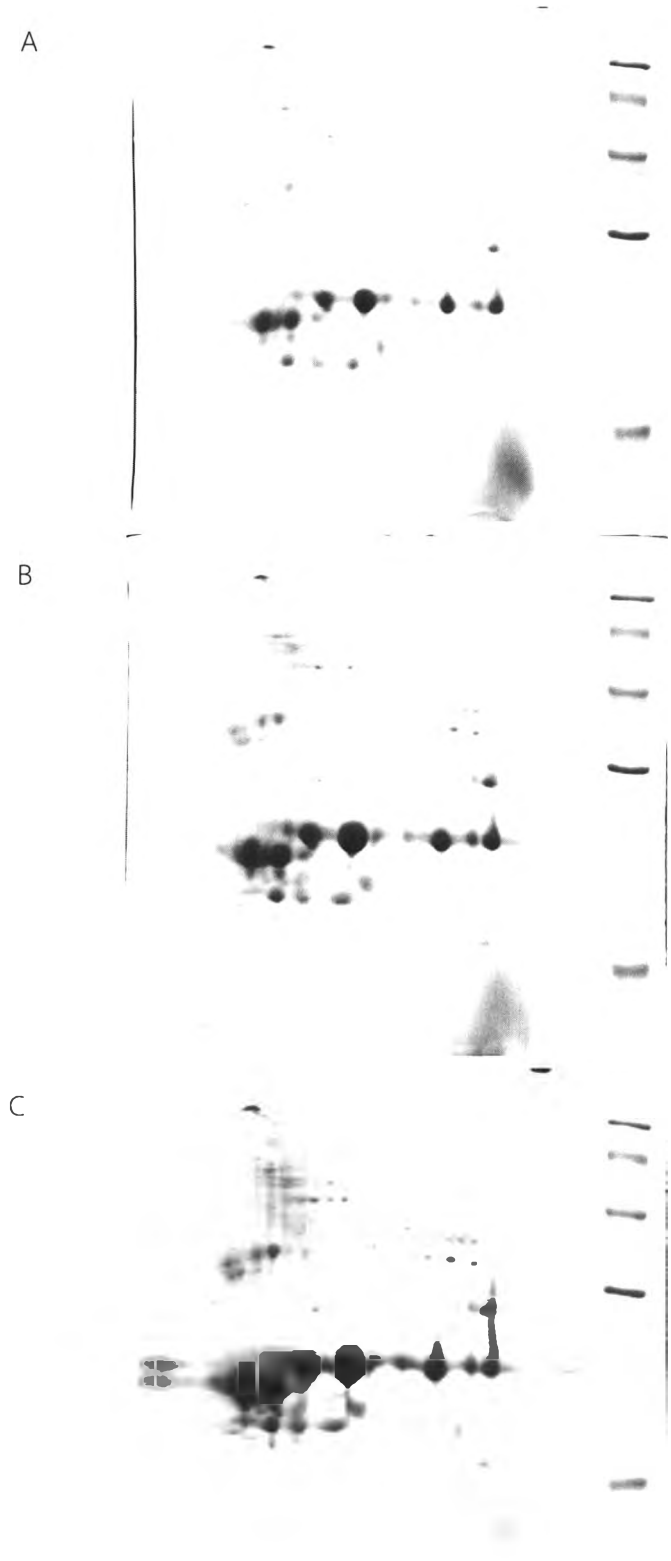


Figure 24 The verification of protein concentration in *P. mirifica* tuber (PM-III rainy season as a reference). (A) 30 μg, (B) 50 μg and (C) 100 μg

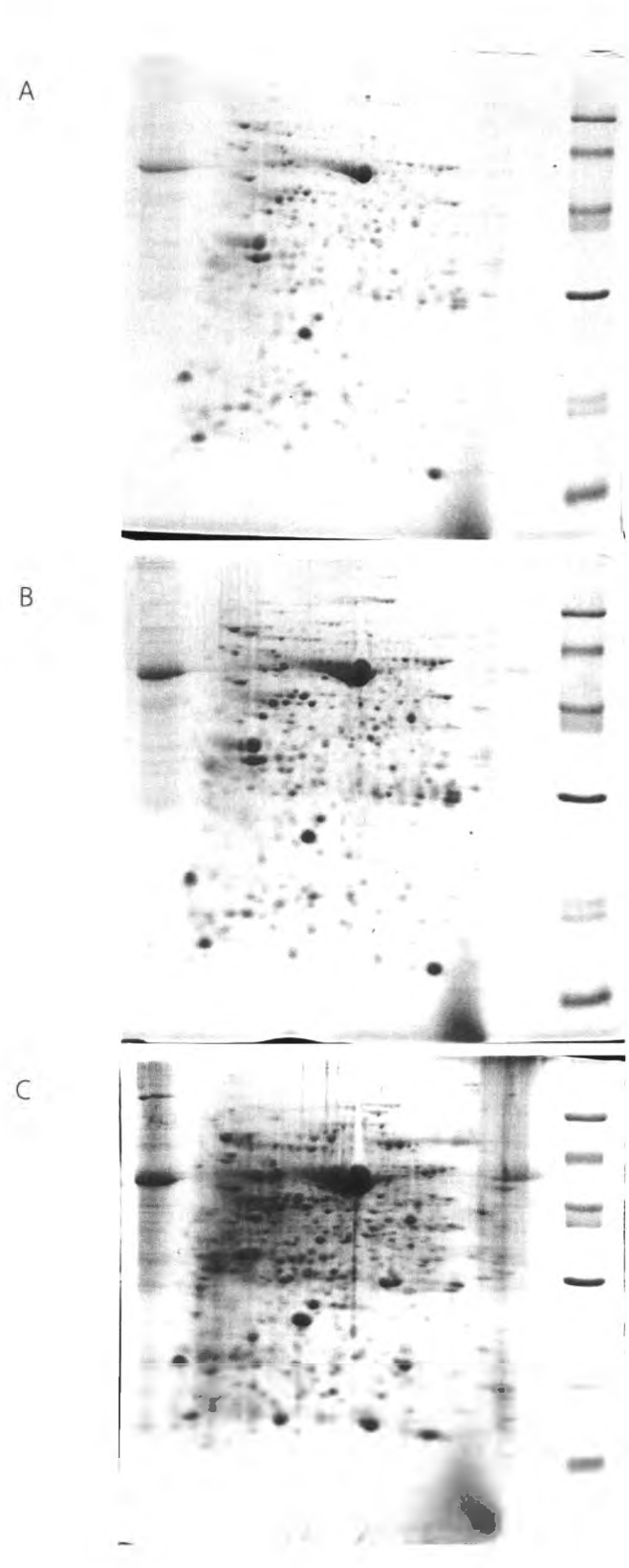


Figure 25 The verification of protein concentration in *P. mirifica* leaf (PM-IV rainy season as a reference). (A) 50 μg, (B) 80 μg and (C) 100 μg

4.2.2. The verification of protein loading concentration for 2D-PAGE pattern of *P. mirifica* tuber and leaf

The optimal protein concentration for 2D-PAGE of *P. mirifica* tuber was 50 µg (Figure 24), whereas, in leaf was 80 µg (Figure 25). These concentrations exhibited the most effective separation which yielded globular spots and good resolution compared to other concentrations. These proteins were characterized and identified by LC/MS/MS. The identified amino acid sequences were subjected to perform database searching.

4.2.3. The proteome pattern of *P. mirifica* tuber and leaf

The results of seasonal change 2D-PAGE pattern in *P. mirifica* tubers; PM-III, PM-IV and PM-V, were shown (Figure 26, Figure 27 and Figure 28, respectively). The most high intensity spots have molecular weight range 20-30 kDa. For the seasonal change in *P. mirifia* leaves; PM-III, PM-IV and PM-V, were shown (Figure 29, Figure 30 and Figure 31, respectively). The most abundant proteins were expressed in rainy season. The most high protein spots have molecular weight range 45-66 kDa. *P. mirifica* leaf proteome patterns had less expressed during winter season that totally difference from *P. mirifica* in tuber. In winter season, plant may transfer protein into storage tuberous root to preserve, so that, the proteins in leaf then become less expression.

PM-V in winter was the best protein profile pattern being employed as reference proteome pattern in tuber (Figure 32) because the abundance protein spots expression. In this study focused on PM-V for performing a proteomics pattern in *P. mirifica* tubers.

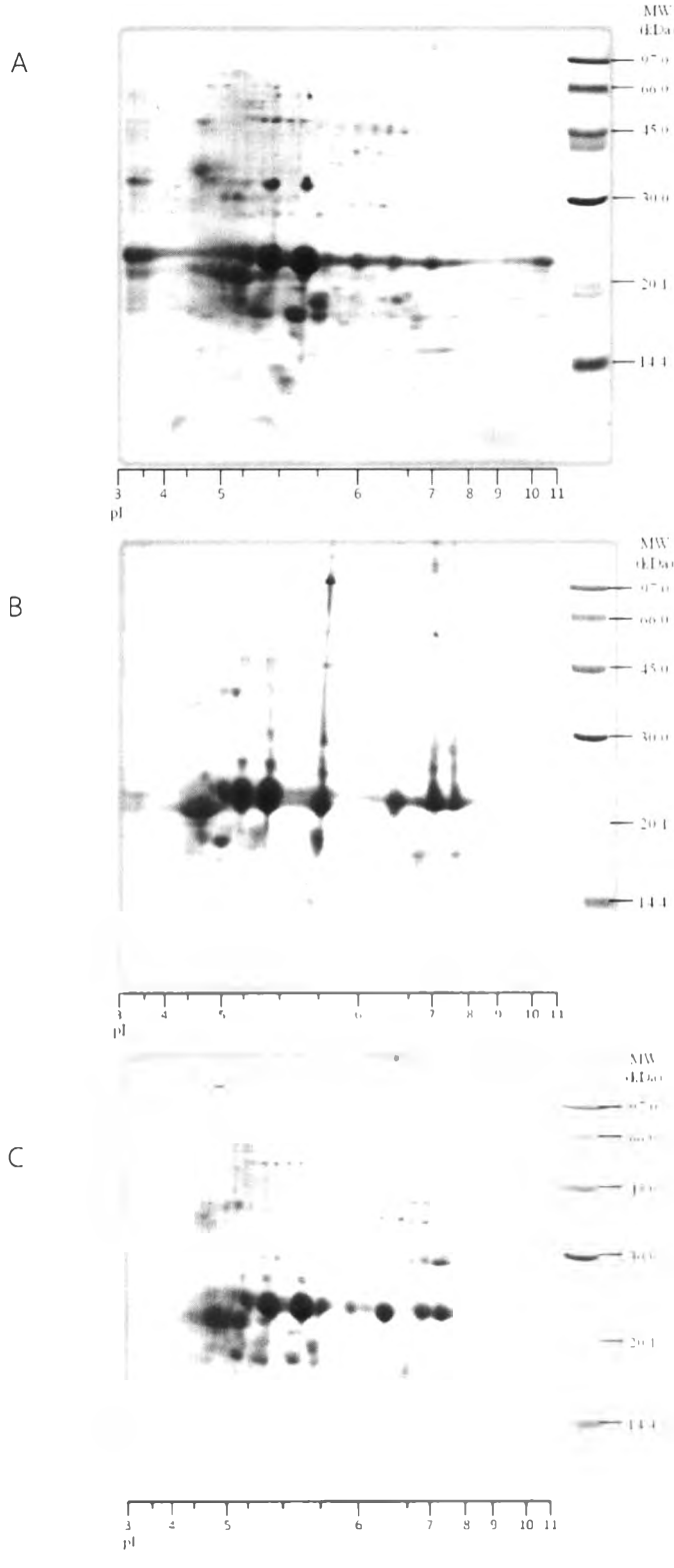


Figure 26 The proteome patterns of *P. mirifica* tubers (PM-III) in seasonal change. (A) Winter season, (B) Summer season and (C) Rainy season

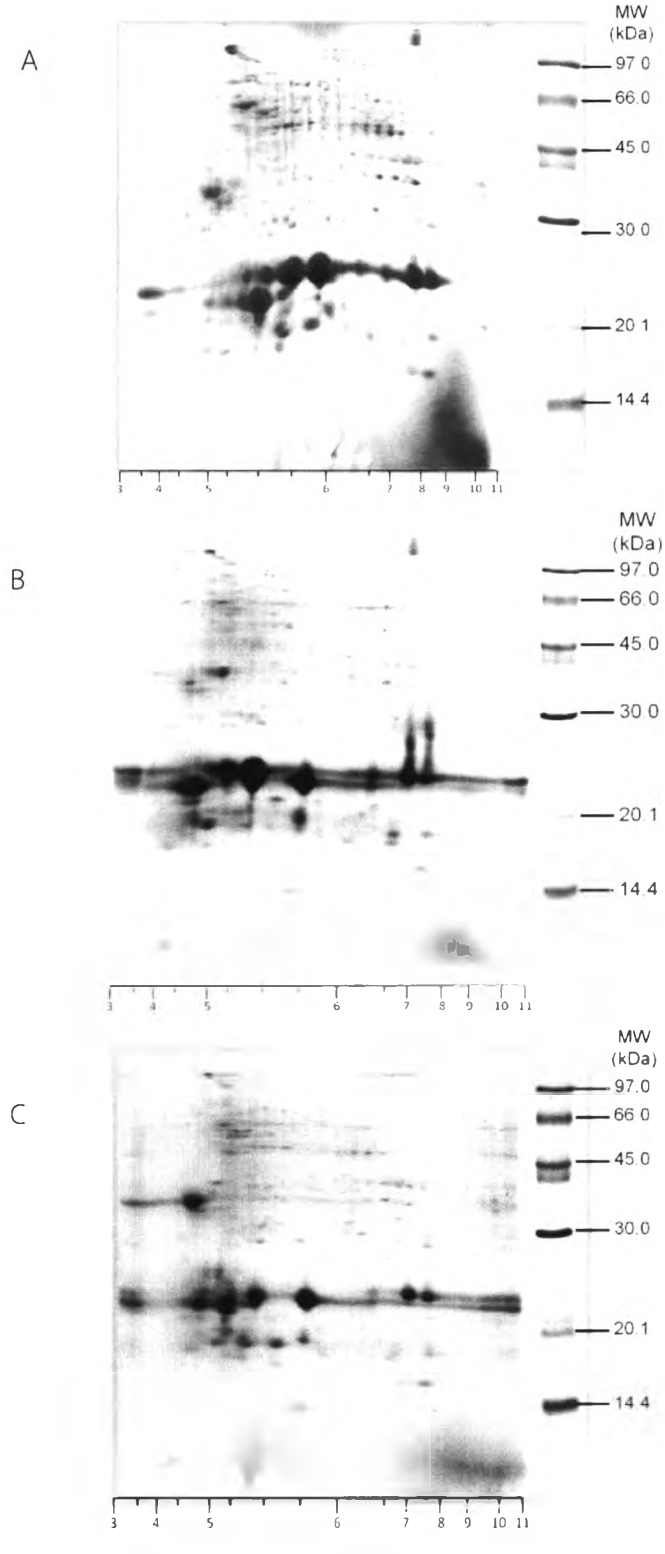


Figure 27 The proteome patterns of *P. mirifica* tubers (PM-IV) in seasonal change. (A) Winter season, (B) Summer season and (C) Rainy season.

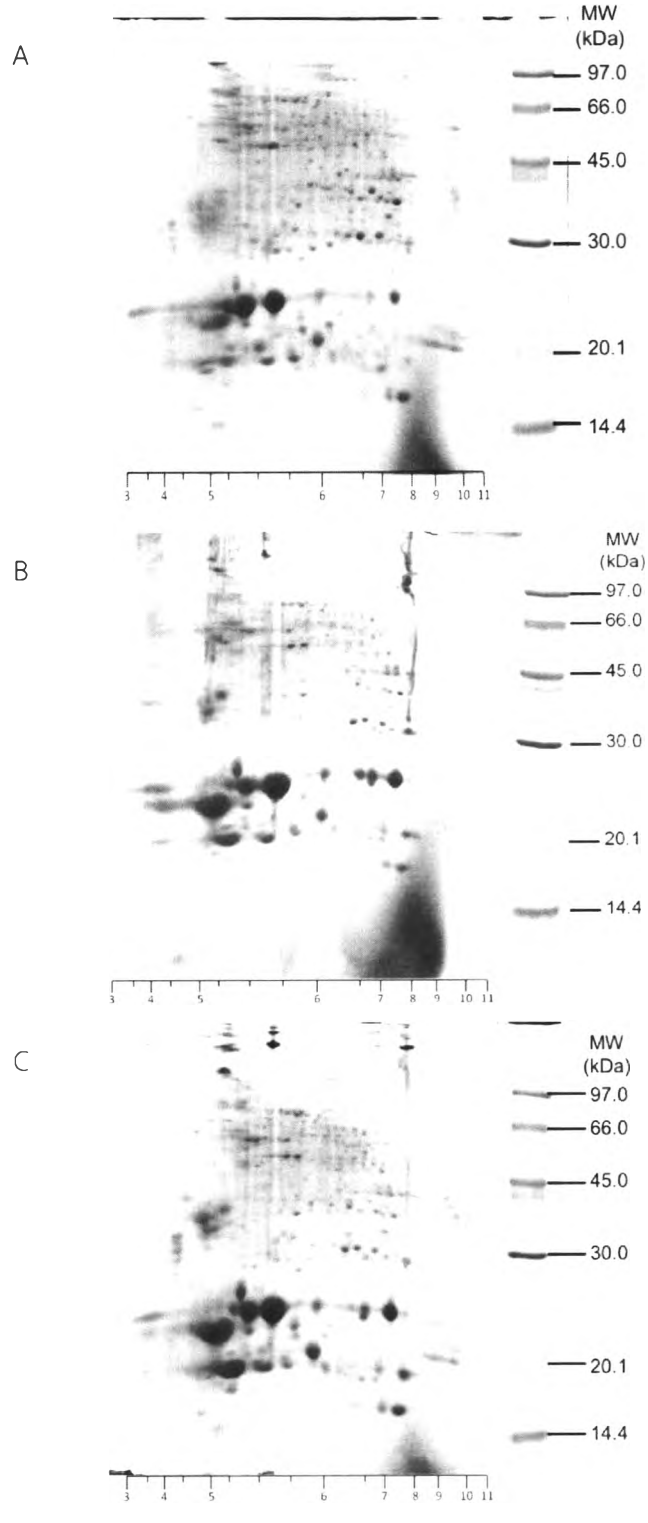


Figure 28 The proteome patterns of *P. mirifica* tubers (PM-V) in seasonal change. (A) Winter season, (B) Summer season and (C) Rainy season.

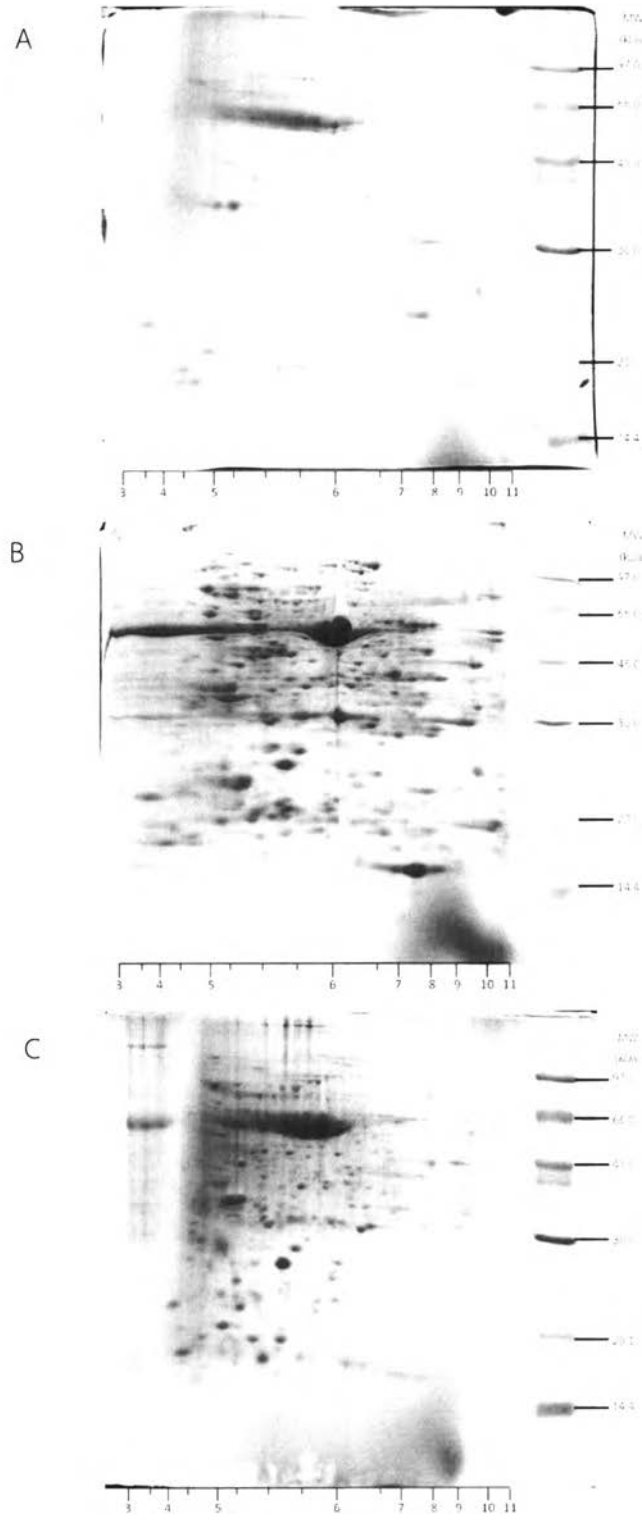


Figure 29 The proteome patterns of *P. mirifica* leaves (PM-III) in seasonal change. (A) Winter season, (B) Summer season and (C) Rainy season

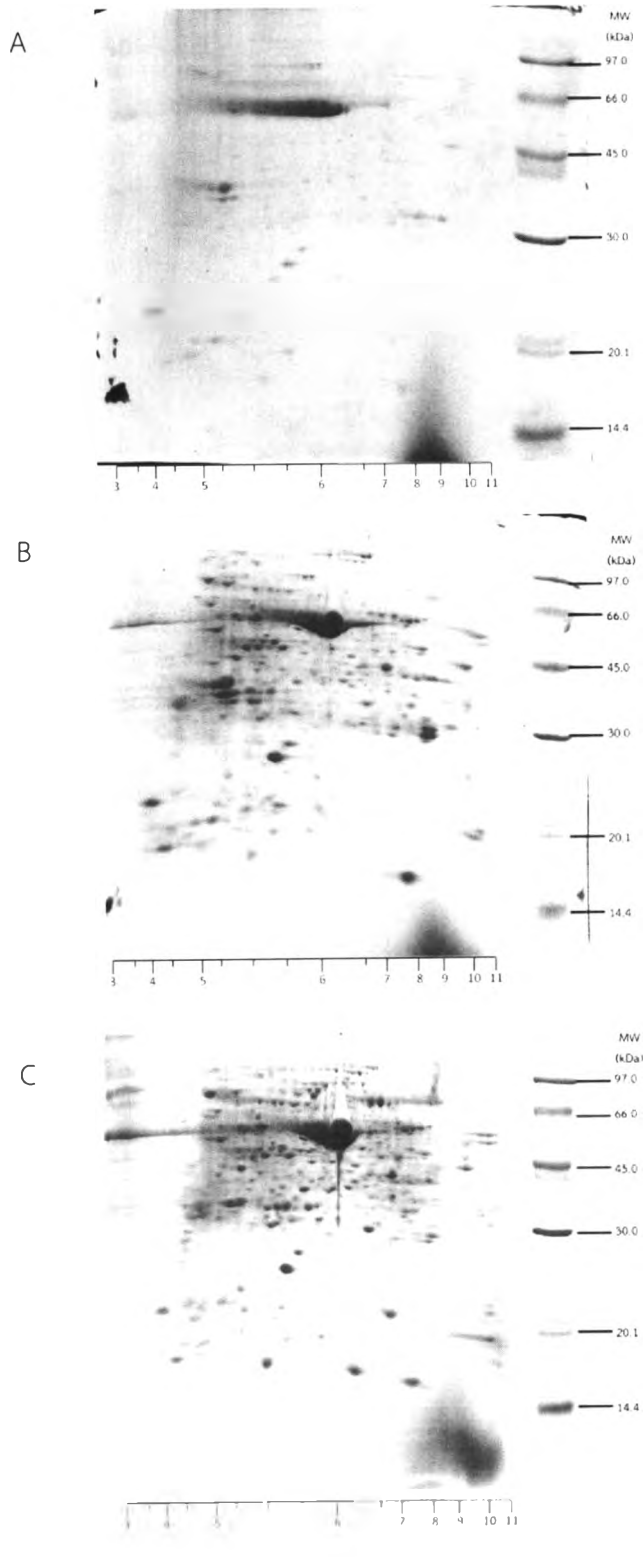


Figure 30 The proteome patterns of *P. mirifica* leaves (PM-IV) in seasonal change. (A) Winter season, (B) Summer season and (C) Rainy season

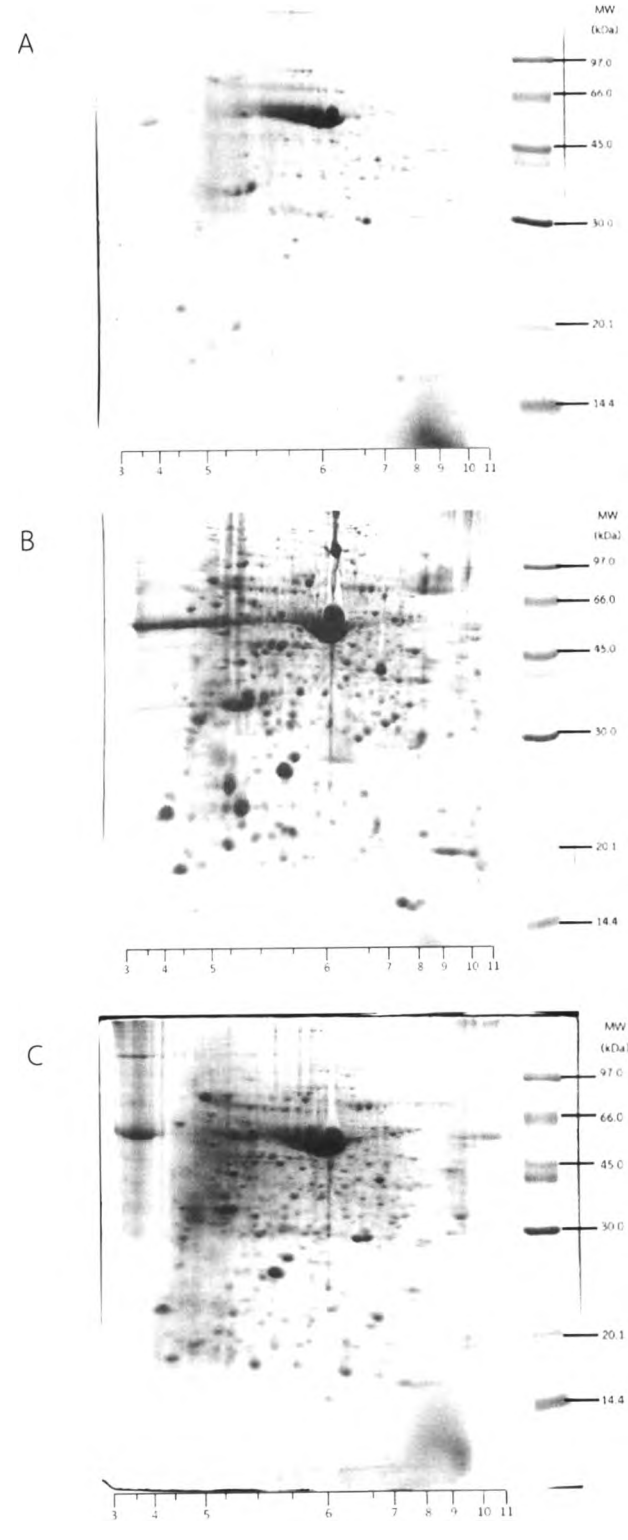


Figure 31 The proteome patterns of *P. mirifica* leaves (PM-V) in seasonal change. (A) Winter season, (B) Summer season and (C) Rainy season

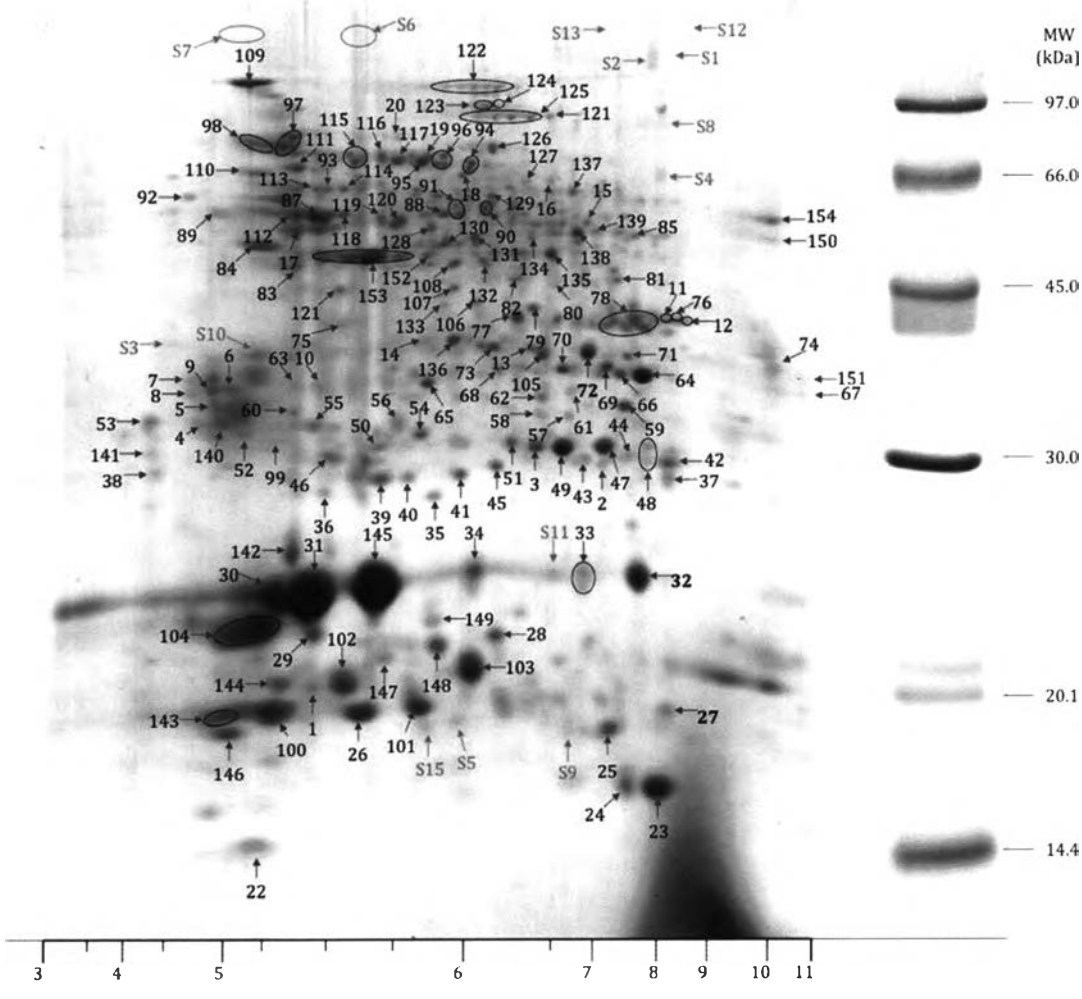


Figure 32 The representative 2D-PAGE gel of *P. mirifica* PM-V tuber (50 µg) in winter season

In tuber. from 207 protein spots, only 85.99% were identified. For example, spot no. 30, 31, 32 and 145 were characterized as Pathogenesis-related protein, Glutathione S-transferase GSTU6, UDP-glycosyltransferase (UGT) and Pathogenesis-related protein, respectively. The description of all protein spots are described in Table 13 (Appendix G)

4.2.4. Functional protein in of *P. mirifica* tuber in seasonal comparison

The 322 identified proteins were categorized according to their functions into 8 classes in different season (Figure 33). More than a half of main classified protein function was metabolic pathway. The majority of metabolism proteins were involved in carbohydrate metabolism, viz glycolysis, tricarboxylic acid (TCA) cycle and oxidative pentose phosphate pathway (PPP). The seasonal variations of functional proteins have shown in Table 7 for winter season, summer season and rainy season, respectively. The metabolism protein and protein synthesis function were up-regulated in summer, whereas, defense and transport protein function were the highest expressed in winter. Some of the identified proteins were located in the similar molecular weight but different pI, for example, spot no. 78, 104, 122, 123, 125 and 153. All of these spots were characterized as fructose-bisphosphate aldolase, fructose-bisphosphate aldolase, aconitate hydratase 1-like, 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase-like isoform 1, methionine synthase and alpha-amylase, respectively.

Because proteins may be the isoforms resulted of Post-translational Modifications (PTMs); Glycosylation, phosphorylation, etc. Proteins may change the conformation by cleaved the end of N- or C-terminal truncated with exopeptidases conduced to the pI changed (Seo & Lee 2004). The functional proteins in *P. mirifica* tuber are categorized in 8 classes (Table 8).

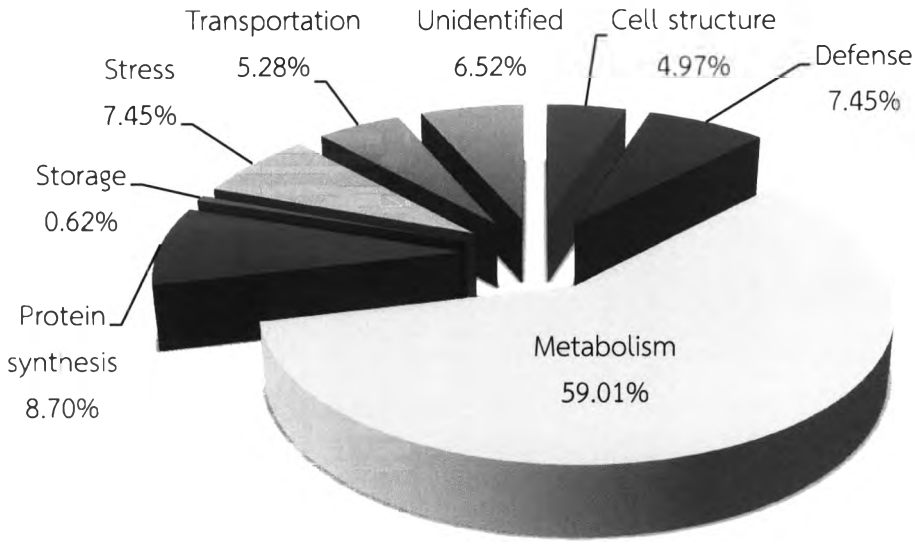


Figure 33 The functional classification of total identified proteins in *P. mirifica* tubers (Included all season of proteins expressed in this plant species (PM-III, PM-IV and PM-V).

Table 7 The functional proteins comparison of *P. mirifica* tuber during season

Function	Summer		Rainy		Winter	
	Protein (s)	Percentage (%)	Protein (s)	Percentage (%)	Protein (s)	Percentage (%)
Cell structure	17	5.52	18	5.98	18	6.06
Defense	22	7.14	20	6.64	19	6.40
Metabolism	183	59.42	180	59.80	177	59.60
Protein synthesis	27	8.77	27	8.97	26	8.75
Storage	1	0.32	1	0.33	2	0.67
Stress	21	6.82	21	6.98	23	7.74
Transportation	17	5.52	16	5.32	12	4.04
Unidentified	20	6.49	18	5.98	20	6.73
Total	308	100	301	100	297	100

Table 8 The identified protein function of *P. mirifica* tuber analyzed by LC-MS/MS.

Spot no.	Accession number	Description	MW/pI	Score	Density	Comparison between winter (fold)	
					Winter	Summer	Rainy
Cell structure							
2	gi|356535919	3-ketoacyl-CoA synthase 11-like [*Glycine max*]	58.04/9.40	21	0.10	+1.20	-1.70
83	gi|356558578	Actin-101-like [*Glycine max*]	41.60/5.31	867	0.28	-1.98	+1.94
102	gi|84028521	Actin depolymerizing factor-like protein [*Arachis hypogaea*]	16.08/6.15	152	1.95	-6.70	-3.70
Defense							
23	gi|7547630	Pathogen- and wound-inducible antifungal protein CBP20 precursor [*Nicotiana tabacum*]	22.15/8.38	49	1.36	-1.38	-1.10
27	gi|356539366	Superoxide dismutase [Cu-Zn], chloroplastic-like [*Glycine max*]	20.88/6.03	38	0.24	+5.74	+3.90
48	gi|15237409	TIR-NBS-LRR class disease resistance protein [*Arabidopsis thaliana*]	137.39/7.71	26	0.19	+1.21	-1.27
145	gi|130829	Pathogenesis-related protein 1 (PvPR1)	16.52/4.83	264	16.32	+1.32	+1.26
Metabolism							
30	gi|255088257	Fructose-1,6-bisphosphatase [*Micromonas* sp. RCC299]	39.47/5.79	64	5.13	-4.59	-3.31
32	gi|387135324	UDP-glycosyltransferase 1 [*Linum usitatissimum*] (UGT)	52.81/5.83	63	1.14	+5.44	+2.82
33	gi|131933	Ribulose bisphosphate carboxylase; RuBisCO large subunit	52.42/6.29	28	0.38	+6.19	+3.79
49	gi|77540216	Triosephosphate isomerase [*Glycine max*]	27.19/5.87	799	1.10	-2.76	-3.66
64	gi|3176098	Annexin [*Medicago truncatula*]	35.59/7.85	819	0.96	-2.90	-5.81
72	gi|356536264	Fructose-bisphosphate aldolase 3, chloroplastic-like [*Glycine max*]	41.39/7.63	291	0.53	-10.30	-8.85
127	gi|356570259	Lysosomal alpha-mannosidase-like [*Glycine max*]	115.53/5.84	639	0.26	-5.30	-3.77
136	gi|4731376	Isoflavone reductase homolog Bet v 6.0101 [*Betula pendula*]	33.13/7.82	65	0.10	-2.24	-2.50

(+) The protein density in winter is greater than that season (fold); (-) The protein density in winter is less than that season (fold)

Table 8 The identified protein function of *P. mirifica* tuber analyzed by LC-MS/MS (continued)

Spot no.	Accession number	Description	MW/pI	Score	Density	Comparison between winter (fold)	
					Winter	Summer	Rainy
Protein synthesis							
3	gi|356496249	Proteasome subunit alpha type-4-like isoform 1 [*Glycine max*]	27.29/5.96	270	0.35	+1.59	+1.13
8	gi|356516563	Elongation factor 1-delta-like [*Glycine max*]	25.02/4.42	113	0.11	-1.09	+2.77
43	gi|345450064	Ribosomal protein S4 [*Delosperma napiforme*]	20.06/10.33	30	0.14	+1.36	+1.36
Storage							
69	gi|156630208	Avenin-like protein [*Amblyopyrum muticum*]	32.58/7.83	52	0.32	-1.81	-2.33
Stress							
31	gi|357140899	Glutathione S-transferase GSTU6-like [*Brachypodium distachyon*]	25.79/6.12	50	13.53	-2.06	-1.89
91	gi|356559573	Glutathione reductase, cytosolic-like [*Glycine max*]	53.96/5.63	866	0.18	-1.18	-1.25
93	gi|356576411	Chaperonin CPN60-2, mitochondrial-like isoform 1 [*Glycine max*]	16.52/4.83	755	0.19	-2.94	-2.89
97	gi|38325811	Heat shock protein 70-1 [*Nicotiana tabacum*]	70.97/5.02	197	1.48	-2.21	-1.90
Transportation							
52	gi|384252728	E set domain-containing protein [*Coccomyxa subellipsoidea* C-169]	70.58/9.30	27	0.22	-1.04	-1.54
109	gi|356543209	Patellin-5-like [*Glycine max*]	69.52/4.87	592	1.14	-1.08	+1.09
146	gi|308803158	ABC transporter, putative (ISS) [*Ostreococcus tauri*]	71.11/8.40	45	0.58	-1.43	-2.27
Unknown							
41	gi|351734454	Uncharacterized protein LOC100306148 [*Glycine max*]	24.53/6.82	411	0.25	-2.18	-2.88
55	gi|242071029	Hypothetical protein SORBIDRAFT_05g018540 [*Sorghum bicolor*]	179.12/8.41	21	0.19	-2.09	-3.88

(+) The protein density in winter is greater than that season (fold); (-) The protein density in winter is less than that season (fold)

4.2.4.1. Cell structure

The eleven proteins were found in related with cell structure. Most are involved in actin polymerization such as Actin 101 (Spot no. 83), Actin Depolymerizing Factor (ADF) (Spot no. 101, 102) and Actin related protein (Spot no. 134). Actin was a microfilament compartment of cytoskeleton found in cytoplasm. Actin polymer was flexible and relatively strong. The amount of ADF is the most abundance in winter season (Table 1). It might require for the cytoskeletal rearrangements in low temperature exposure (Ouellet et al 2001). While, 3-ketoacyl-CoA synthase (Spot no. 2) is expressed in all season but found lowest in rainy season. This protein contributed to cuticular wax and suberin biosynthesis which protect plant tuber from water diffusion. In general, the water consumption in cell is higher than environment. Plant protects itself from water loss by waxes. The cuticular waxes form the transport-limiting barriers of plant cuticles for the diffusion of water (Post-Beittenmiller 1996, Schreiber 2001). Whereas, rainy season has high relative humidity (RH) and wax diffusion through the cuticle should be significantly reduced, since the driving force for cuticular transpiration is close to zero and thus, lower amounts of epicuticular wax should accumulate (Schreiber 2001). Therefore, we hypothesize that plant produce less wax during rainy season.

4.2.4.2. Defense

In terms of defense response, resistance proteins were expressed when plant was infected by living organism such as viruses, bacteria, fungi and nematodes (Martin et al 2003). Plant protects itself by synthesizing antimicrobial secondary compounds and by inducing defense responses (Heath 2000). Pathogen- and wound-inducible antifungal protein CBP20 precursor (spot no. 23), Pathogenesis-related protein 1 and PR10.61 (spot no. 145) played antifungal activity toward *Trichoderma viride* and *Fusarium solani* by causing cell lysis of the germ tubes and/or growth inhibition (Ponstein et al 1994). Leaf rust resistance protein Lr10 (spot no. S14) was expressed against fungal pathogen cause leaf rust diseases. There has some report that Leaf rust is normally occur a late summer or early fall but it does not occur at other times of the year (Ellison et al 2008, Feng et al 2009) that correlate with this study, the expression of Leaf rust resistance protein Lr10 in winter was stored in the tuber. Leaf Rust resistance protein (Lr10) produced for protect plant tuber against localized infection fungus that commonly occurred in winter season (Heath 2000).

NBS-LRR type disease resistance protein (Spot no. 48) was exhibited. This protein induce a series of plant defense responses, such as activation of an oxidative burst, calcium and ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis-related genes, and the hypersensitive response (McHale et al 2006). This protein was up-regulated in rainy season may because of the optimum temperature and humidity in rainy was suitable for plant diseases to grow and develop in Thailand.

Superoxide dismutase [Cu-Zn] (CuZn-SOD) (Spot no. 26, 27, 101, 144), is rapidly catalyzing the disproportionation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) within a cell that cause oxidative damage to difference cellular components (Szőllősi 2014). This enzyme also found in some plant tubers such as *Curcuma* (Boonmee et al 2011), patato (Yu et al 2012a), *Stemona tuberosa Lour.* (Niyomploy et al 2014) etc.

4.2.4.3. Metabolism

Carbohydrate metabolism was important for plant tuber during dormancy and sprout because it necessary for starch/sugar and energy production (Yu et al 2012a). Furthermore, the respiration pathway and the formation of root/shoot meristem also need energy [38]. The Fructose-biphosphate aldolase (Spot no. 72) was the one of proteins involved in carbohydrate metabolisms such as glycolysis, gluconeogenesis and Calvin cycle. This enzyme catalyzed Fructose 1,6-biphosphate to triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Fructose-biphosphate aldolase was expressed extremely in winter which greater than summer and rainy season 10.30 and 8.85 folds, respectively (Fig. 5). In the same way as Fructose-1,6-bisphosphatase (spots 30); enzyme that converted fructose-1,6-bisphosphate to fructose 6-phosphate, was expressed highest in winter as shown in Table 1. From the same reason, Triose Phosphate Isomerase (TPI) (Spot no. 49) played an important role in glycolysis metabolism by interconversion of the triose phosphate isomers between dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Alpha mannosidase (Spot no. 127) elaborated in glycan biosynthesis and degradation. It is found greatest expression in winter season. Some metabolism-related proteins, such as Fructokinase (Spot no. S10), are found only in winter season. It is functioned as phosphotransferase of fructose.

There are many reasons to support the abundant expression of metabolism-related proteins iduring winter. Firstly, it may be due to the osmotic adjustment in the cell during winter. The accumulation of carbohydrates and other solutes changes the osmotic potential of the cell and therefore decreases the difference in water potential between the ice formed in the apoplastic space and the solution within the cell (Ruelland et al 2009). Secondly, proteins might involve in the plant dormancy/sprout. Plant accumulated these enzymes for carbohydrate production in tuber during dormancy and use for sprouting by hydrolyzed carbohydrate into sugar and energy in winter as same as in the previous report in rhizomes of *Curcuma longa* (Chokchaichamnankit et al 2009). Thirdly, it's possible that plant used these enzymes to produce ATP for shedding their leaves during winter. Moreover, fructokinase is also found in plant grown at low temperature experiment (Louarn et al 2012, Shibata et al 1987). This enzyme played specific roles in the flux of carbon through starch metabolism (Gonzali et al 2001) and contributed to stem and root growth and to seed development (Odanaka et al 2002). Together, these evidences support that fructokinase may involve in carbohydrate metabolism. Some protein had less expression during winter such as Ribulose bisphosphate carboxylase; RuBisCO (Spot no. 33). Rubisco involved in Calvin cycle. It was abundance expressed in summer and rainy season when comparison in winter. Although the increasing temperature was inhibited RuBisCO activation in leaf (Feller et al 1998), (Crafts-Brandner & Law 2000). Therefore, RuBisCO may transfer from leaf to tuber for heat-stress protection.

During summer season, protein is expressed such as Deoxyuridine triphosphatase (dUTPase) (EC 3.6.1.23) (Spot S1). The dUTPase converts deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP) and pyrophosphate. This mechanism was essential for plant sprouting. dUTPase expressed 1 week before visible sprouting occurred. In tuber, dUTPase expression is used as a marker at the end of potato tuber dormancy (Senning et al 2010). The result shown Deoxyuridine triphosphatase (dUTPase) (EC 3.6.1.23) (Spot S1) was expressed in summer as same as in potato tuber, the dUTPase expression 1 week in summer before sprouting. An additional crucial role of dUTPase in is to prevent the damage that is inflicted on the DNA by the excision of misincorporated deoxyuridine (Shlomai & Kornberg 1978, Siaud et al 2010). The dUTPase (metabolism function) also showed promise as novel targets for anticancer and antimicrobial therapies by upregulation of dUTPase in human tumor cells (Vertessy & Toth 2009).

4.2.4.4. Protein synthesis

There are 30 proteins expressed in protein synthesis function. Most of them belong to Proteasome subunit alpha type. This protein has ability to cleave peptide bond with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The two of Ribosomal protein S4 (Spot no. 43) and 60s Ribosomal protein (Spot no. S12, S13) are functioning in peptide bond formation. They also prevented the hydrolysis of premature polypeptide, provided a binding site for the G-protein factors (assists initiation, elongation, and termination), and helped protein folding after synthesis. Elongation factor 1 (Spot no. 4, 7, 8) has also found in tuber. This protein was used in protein synthesis in ribosome; they facilitate translational elongation, from the formation of the first peptide bond to the formation of the last one. Serine hydroxymethyltransferase (Spot no. S6, S12, S13) is enzyme (EC 2.1.2.1) which plays an important role in cellular one-carbon pathways by catalyzing the reversible, simultaneous conversions of L-serine to glycine and tetrahydrofolate to 5,10-methylenetetrahydrofolate (hydrolysis) (Stover & Schirch 1990).

4.2.4.5. Storage

Two storage proteins; Avenin-like protein (Spot no. 69) and Glutelin type-A 2-like" (Spot no. S10) were presented. Avenin-like protein is a small family storage protein in wheat (Ma et al 2013). Glutelin type-A 2-like mainly found in rice grains (He et al 2013, Juliano & Boulter 1976), only expressed in winter. Plant stored protein during winter season and degraded in late spring (Clausen & Apel 1991).

4.2.4.6. Stress response

Plant abiotic stress (such as salinity, drought, chilling, heavily metal) is one of limiting factor for plant growth and production. When plant is under stress condition, reactive oxygen species (ROS) were overproduced to protect cell from oxidative damage (Gill et al 2013). In term of stress responding protein, 24 proteins were expressed. Glutathione S-transferase, GST was found to be the most abundance protein in *P. mirifica* tuber. GST functions in stress tolerance through a role in cell signaling (Dixon et al 2002). Gluthathione reductase, GR (Spot no. 15, 91, 139) was a second abundance exhibited in *P. mirifica* tuber. GR also play significant role in protecting cells against ROS and its reaction products-accrued potential anomalies (Gill et al 2013). Both Glutathione S-transferase and Glutathione reductase exhibited

a potential role in the detoxification of both xenobiotic and endogenous compounds (Dixon et al 1998, Gill et al 2013).

Heat stress increased membrane damage and impaired metabolic functions by enhance the risk of improper protein folding and denaturation of several intracellular protein and membrane complexes (Kosova et al 2011). A plant need to activate proper defense systems to survive under heat stress (Hashiguchi et al 2010). One of the key factors for heat tolerance is the induction of heat shock proteins. Heat shock protein and chaperonin also played stress response by preventing aggregation and stabilizing non-native proteins with directly refolded and activated of protein that has become unfolded or insoluble due to environmental stresses (Boonmee et al 2011, Chokchaichamnankit et al 2009). In this study, Heat shock protein (Spot no. 97, 103, 115) and chaperonin (Spot no. 93, 113, 114) were expressed to be highest in winter. It may because tuber is settled in underground where the temperature quite stable. In winter season, there has no HSP and chaperonin required for conserve thermo stability in leaves, plant may transfer these proteins to store in the tuber.

4.2.4.7. Transportation

The 17 proteins involved in transportation. Most of them were E-set domain containing protein (Spot no. 52, 53 and 90), Patellin-5 (Spot no. 74, 85, 109, 139 and S7), ABC transporter (Spot no. 146, S2 and S8). E-set contains protein and Patellin-5 are responsible for ion transportation, whereas ABC transporter required energy in the form of adenosine triphosphate (ATP) to translocate substrates across cell membranes (Hollenstein et al 2007).

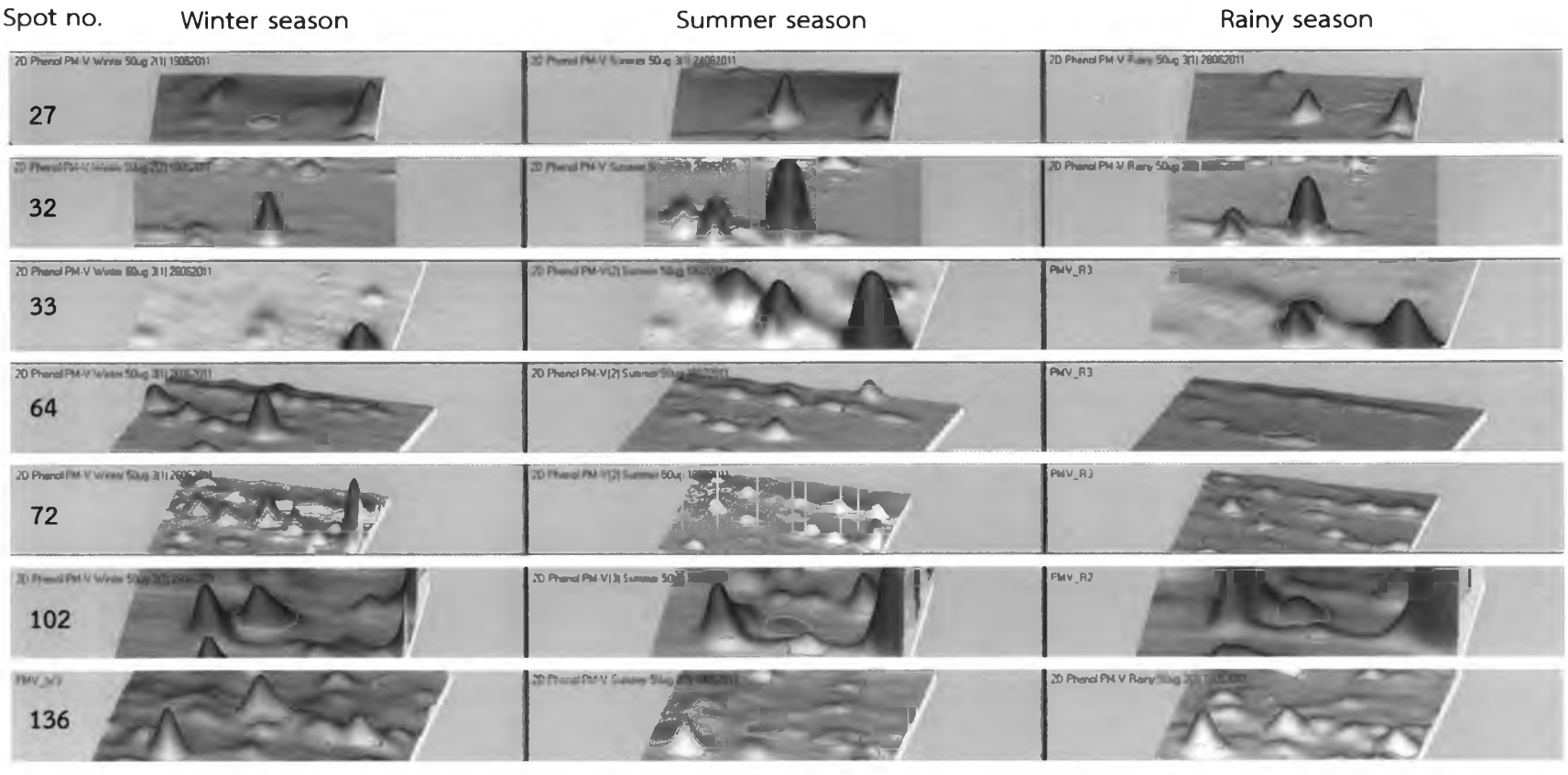
4.2.4.8. Unknown function

There were still 21 proteins resulted in unknown/hypothetical functional group such as Uncharacterized protein LOC100306148 (Spot no. 41), Hypothetical protein SORBIDRAFT_05g018540 (Spot no. 55). Proteins with unknown function have found in many living organisms. Although, the numbers of uncharacterized protein structures that have been experimentally determined and deposited in the protein data bank (PDB) remain in the thousands, trends of uncharacterized protein sequence and PDB are regression (Nadzirin & Firdaus-Raih 2012). This study performed update searching of unknown function analysis until 2014.

4.2.5. Fold comparison in *P. mirifica* tubers

In terms of fold comparison, the variation of protein spots in two-dimensional gel electrophoresis from *P. mirifica* tubers in winter, rainy and summer season were detected (Table 9). The proteins whose expression levels of *P. mirifica* tuber in summer and rainy season differed than 1.5 fold (compared with winter season) were considered significant (Table 9). The most intensity of *P. mirifica* distributed in range 14-30 kDa by using image master program (Figure 34) to calculate percent volume. The study showed the interesting spots with clearly differenced in each season. In summer season, the most intensity spot belong to Ribulosebisphosphate carboxylase; RuBisCO (Spot no. 33) (6.19 fold), both involved in metabolism activity. Superoxide dismutase (SOD) [Cu-Zn] (Spot no. 27) and UDP-glycosyltransferase (UGT) (Spot no. 32) were expressed in summer for 5.74 and 5.44 fold, respectively. For Rainy season, Rubisco, SOD [Cu-Zn] and UGT were also expressed in 3.79, 3.90 and 2.82 fold, respectively (Table 13). On the other hand, proteins which highly expressed in winter are annexin (Spot no. 64), Fructose-bisphosphate aldolase (Spot no. 72), Actin (Spot no. 102) and Isoflavone reductase (Spot no. 136). These proteins groups are higher expressed than summer and rainy season as in Table 9.

Figure 34 The 3 Dimensional protein spots of *P. mirifica* tuber in seasonal comparison by using gel image master program.



The 3-Dimensional protein spots represent the protein concentration expressed in 2D-PAGE during season

Table 9 The fold comparison of protein in summer and rainy season comparing with winter season (fold)

Spot no.	Accession number	Description	MW/pI	Fold comparison between winter	
				Summer	Rainy
27	gi|356539366	Superoxide dismutase [Cu-Zn], chloroplastic-like [*Glycine max*]	20.88/6.03	-5.73647	-3.90035
32	gi|387135324	UDP-glycosyltransferase 1 [*Linum usitatissimum*] (UGT)	52.81/5.83	+5.44276	+1.93052
33	gi|131933	Ribulosebisphosphate carboxylase; RuBisCO large subunit	52.42/6.29	-6.19078	-3.78758
64	gi|3176098	Annexin [*Medicago truncatula*]	35.59/7.85	+2.89500	+5.80895
72	gi|356536264	Fructose-bisphosphatealdolase 3 [*Glycine max*]	41.39/7.63	+10.30199	+8.84617
102	gi|84028521	Actin depolymerizing factor-like protein [*Arachis hypogaea*]	16.08/6.15	+6.69516	+3.69930
136	gi|4731376	Isoflavonereductase homolog Bet v 6.0101 [*Betula pendula*]	33.13/7.82	+2.47903	+2.23692

(+) The protein density in winter is greater than that season (fold)

(-) The protein density in winter is less than that season (fold)

4.2.6. The comparison of proteomics patterns between *P. mirifica* and *Glycine max* (soybean) leaf

The targeting isoflavonoid biosynthesis involved enzymes were studied. Their molecular weight and pI were compiled to focus on the expected area that these enzymes might be found. Surprisingly, no isoflavonoid biosynthesis involved enzymes were found. The report of proteomics in soybean leaf (Xu et al 2006) comparing with *P. mirifica* leaf are shown in Figure **35** The comparison of leaf proteomic pattern between soybean (A) (derived from (Xu et al 2006) and *P. mirifica* (B).Figure **35**. The patterns of these legume plants are quite similar. Most intensity spots are located in molecular weight range 45-60 kDa. The highest protein density was Ribulose 1,5-bisphosphate carboxylase large subunit (RuBisCO) which involved in energy/pentose phosphate pathway. There have many proteins also found in both soybean and *P. mirifica* leaf such as Photosystem II polypeptide (PSII) oxygen evolving enhancer 1, Phosphoribulokinase, Malate dehydrogenase, Glyceraldehyde-3-phosphate dehydrogenase A-subunit, Phosphoglycerate kinase, Aminomethyltransferase, etc. Glyceraldehyde-3-phosphate dehydrogenase A-subunit and Phosphoglycerate kinase are involved in Energy/glycolysis/glyoxylate cycle/gluconeogenesis pathway, whereas, photosystem II polypeptide (PSII) oxygen evolving enhancer 1 is involved in Energy/Photosynthesis. Beside, *P. mirifica* leaf has different proteins from soybean, for example, Maturase-like protein, Fructose-bisphosphate aldolase, 14-3-3-like protein, Oxygen-evolving enhancer protein 1, Haloalkane dehalogenase-like, N-glyceraldehyde-2-phosphotransferase-like, Chloroplast photosynthetic (photosynthesis) water oxidation complex, Cysteine proteinase, Maturase-like protein, Endochitinase, Proteasome subunit alpha type-7-like isoform (protein synthesis), Glycine decarboxylase complex, Thiol protease, Xyloglucan transglycosylase (XETs) (xylem cell wall biosynthesis enzyme), etc. This study is focusing on the enzyme that involved in isoflavonoid biosynthesis pathway which leads to the understanding of the correlation between metabolomics and proteomics synthesized in this plant tuber and leaf.

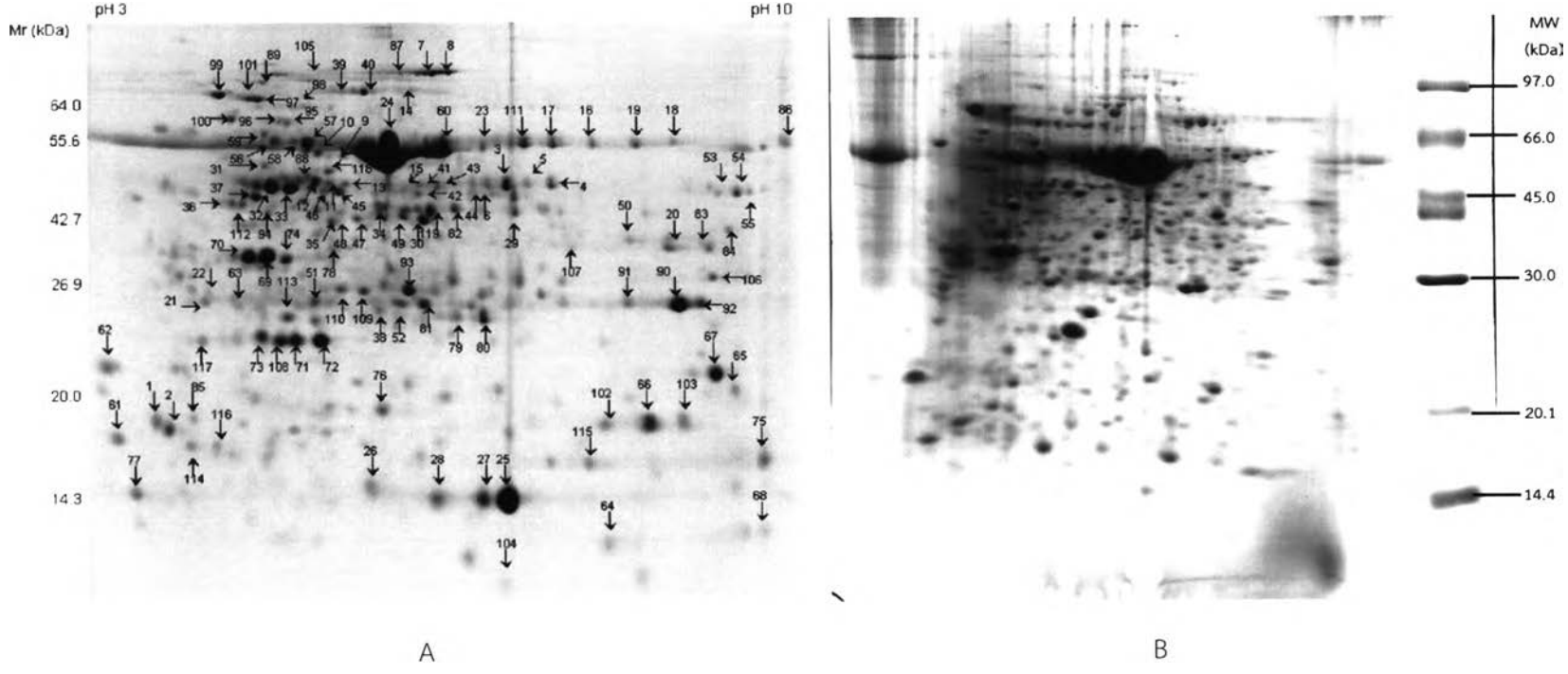


Figure 35 The comparison of leaf proteomic pattern between soybean (A) (derived from **(Xu et al 2006)** and *P. mirifica* (B).

4.2.7. Protein involved in synthesis of secondary metabolites

Plant secondary metabolites are compounds biosynthesized in specific pathway where plant content and regulation is strongly susceptible to environmental influences and to potential herbal predators. The secondary metabolism of plants, and the expressed metabolite levels, may change considerably due to the influence of several biotic (for example insects, pathogenic microorganisms and herbivores) and abiotic (temperature, light, UV, high condition of nutrients, higher latitude, drought etc.) stress signals (Pavarini et al 2012). Proteomic data revealed many proteins involved in the secondary metabolite biosynthesis pathway.

Glutathione S-transferase GSTU6 (Spot 30) belongs to flavoenzyme (contain disulfide group) that involved in flavonoid production. Glutathione S-transferase (GSTs) detoxify toxins by conjugation with Glutathione (GSH); the conjugates (toxin-SG) are then transported into the vacuole by ABC transporters prior to proteolytic processing and GSTU6 also required for transport of flavonoid pigments to the vacuole (Dixon et al 2002). Previous reported shown that these GSTs appear to be involved in the intracellular binding and stabilization of flavonoids (Cummins et al 1999), rather than in catalyzing their glutathionylation (Dixon et al 2002). Whereas, Glutathione reductase (GR) (Spot no. 15, 139) also belongs to flavoenzyme that convert oxidized glutathione (GSSG) to reduced glutathione (GSH) thus helps in maintaining high ratio of GSH/GSSG under various abiotic stresses (Szőllősi 2014). Regarding to GR catalytic mechanisms, it is to be emphasized that the availability of substrate availability largely control GR redox inter-conversions where compared to the reduced GR more stability is exhibited by the oxidized GR form which can tolerate divalent metal ions including Zn^{+2}, Cu^{+2} and Fe^{+2} (Rao & Reddy 2008).

For the synthesis of major isoflavonoids as shown in Figure 36, major key protein are Chalcone flavonone isomerase (CHI) (Spot no. 46), Isoflavone synthase (IFS), UDP-glycosyltransferase (UGT) (Spot no. 32) and Isoflavone reductase (IFR) (Spot no. 13, 73, 136), were the key enzymes for clarify how isoflavonoid biosynthesis in this plant occurred as shown in Figure 36. CHI is a key enzyme played two roles; diverted Isoliquiritigenin to liquiritigenin and *p*-coumaroyl-Co-A to naringenin (Yu et al 2003). In addition, Isoflavone synthase (IFS), another Cytochrome p450 monooxygenase (Spot no. 149), played two roles: converted liquiritigenin to daidzein and naringenin to genistein (Shimada et al 2000). UDP-glycosyltransferase (UGT) (Spot 32) played important role in defense mechanism by sugar conjugation resulted in increasing of both stability (by protecting a reactive nucleophilic group) and water

solubility (Vertessy & Toth 2009) (Gachon et al 2005a). Moreover, UGT also related to secondary metabolite glycosylation of daidzein and genistein to daidzin and genistin, respectively. Whereas, IFR catalyzed the reduction of the heterocyclic ring of isoflavone to form isoflavanone which is an intermediate of isoflavonoid biosynthesis (Shimada et al 2000).

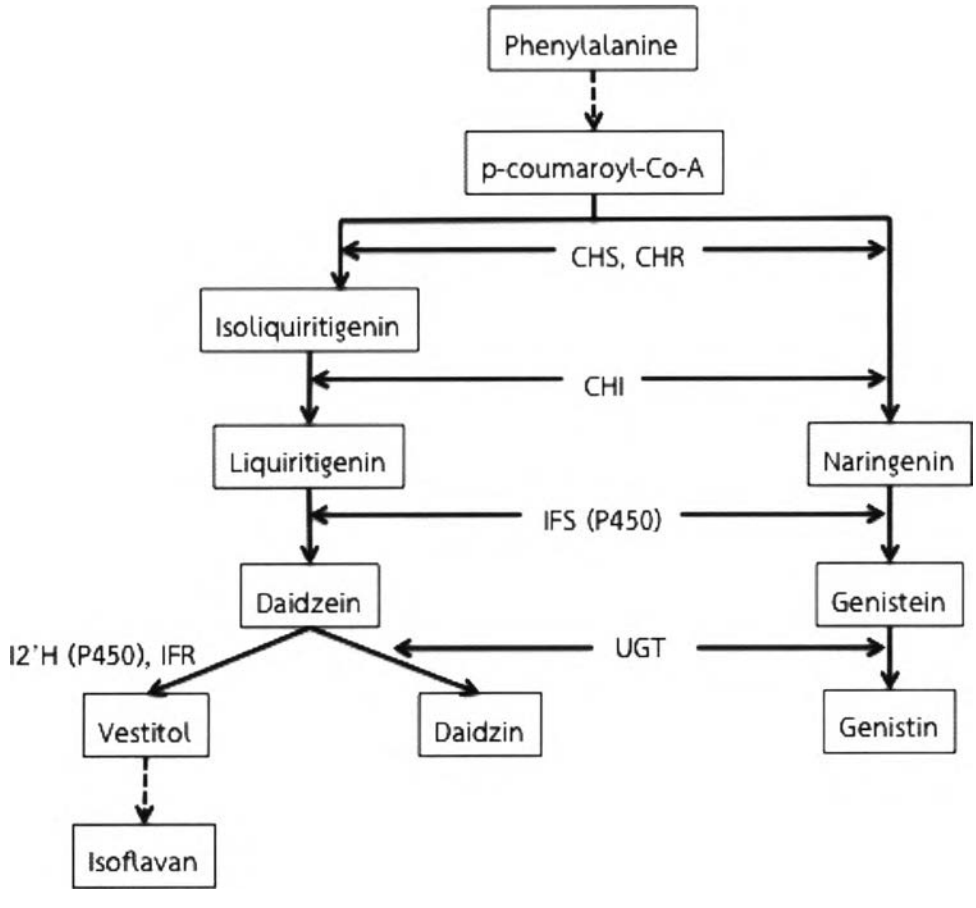


Figure 36 The isoflavonoid biosynthesis pathway (derived from [44]). This pathway stressed in the expressed enzyme in *P. mirifica* tuber that involved in the production of isoflavonoids, especially, daidzin, genistin, daidzein and genistein in previous studied [8]. CHS, Chalcone synthase; CHR, Chalcone reductase; CHI, Chalcone isomerase; IFS, Isoflavone synthase; UGT, UDP-glycosyl transferase; I2'H, Isoflavone hydroxylase; P450, cytochrome p450 monooxygenase; IFR, Isoflavone reductase.

The relationship between isoflavonoid biosynthesis related proteins and isoflavonoid compounds was shown in Table 10. The UGT involved in puerarin, daidzin and genistin synthesis whereas, IFS, P450 and CHI were involved in daidzein and genistein synthesis. From Table 10, no isoflavonoid synthesis proteins were detected in *P. mirifica* leaf but the major isoflavonoids still appeared. The result showed 2 isoflavonoid compounds, puerarin and genistein, were related with the existence and quantity of isoflavonoid synthesis proteins.

The comparison between isoflavonoid compounds and proteins was shown in

Table 11. There has been a report of the most abundance glycosyl isoflavonoids (puerarin, daidzin and genistin) were up-regulated in summer season (Cherdshewasart & Sriwatcharakul 2007) that in conformity with this study consistent, the secondary metabolites are increased in summer season. The three Dimensional spot of UGT was clearly showed the up-regulation in summer (Figure 34) by 5.44 fold comparing with winter season (Table 8). The statistical analysis results also confirmed the glycosidic addition of UGT to isoflavonoid compounds. UGT has significantly correlated with puerarin at 0.05 level and also significantly correlated with daidzin and genistin at 0.01 level. In addition, IFS (cytochrome P450) expression involved in aglycosidic isoflavonid contents, especially genistein content ($P < 0.01$). The less IFS expression is correlated to the less production of daidzein and genistein in summer (Cherdshewasart & Sriwatcharakul 2007). It is possible that daidzein and genistein were accumulated in tuber and converted to daidzin and genistin by UGT in summer season.

Table 10 The relationship between isoflavonoid biosynthesis related proteins and isoflavonoid compounds of *P. mirifica* leaf and tuber in 1 year

	Protein name	Tuber		Leaf	Related with	Tuber		Leaf	
		Quality	Quantity			Quality	Quantity	Quality	Quantity
Glycoside synthesis protein	UGT	√	High	×	Puerarin	√	High	√	Low
					Daidzin	√	Medium	×	High
					Genistin	√	Low	×	Medium
Aglycoside synthesis protein	IFS	√	Very low	×	Daidzein	√	Low	×	Low
					Genistein	√	Very low	×	-
	P450	√	Low	×					
Non-glycoside synthesis protein	CHI	√	Low	×					

Table 11 The comparison between isoflavonoids (mg/100g powder) and isoflavonoid involved proteins (density) (Isoflavonoid content data supported by [8]. Each value is the mean ± SEM.

Name	Winter	Summer	Rainy season
Isoflavonoids content (mg/100g powder)			
Puerarin††	14.63 ± 3.04^{a}	228.41 ± 87.80^{b}	14.48 ± 6.03^{a}
Daidzin†	27.91 ± 6.34^{a,b}	178.20 ± 43.55^{a,b}	13.40 ± 3.25^{a}
Genistin	19.39 ± 3.76^{a,b}	66.88 ±11.10^{a,b}	10.81 ± 2.90^{a}
Daidzein	33.92 ± 3.60^{b}	20.25 ± 7.92^{a}	47.21 ± 33.45^{a}
Genistein	25.08 ± 3.92^{a,b}	3.54 ± 1.70^{a}	13.29 ± 3.20^{a}
Total	126.13 ± 11.71	497.27 ± 125.69	99.19 ± 42.39
Total protein density			
Chalcone flavonone isomerase (CHI) ††	0.30 ± 0.01^{a}	0.09± 0.01^{a}	0.17 ± 0.00^{a}
UDP-glycosyltransferase (UGT) ††	1.13 ± 0.03^{c}	6.22 ± 0.02^{d}	3.18 ± 0.05^{c}
Cytochrome p450 monooxygenase††	0.28 ± 0.01^{a}	0.36 ± 0.01^{c}	0.34 ± 0.00^{b}
Isoflavone reductase (IFR) ††	0.61 ± 0.03^{b}	0.15 ± 0.01^{b}	0.21 ± 0.01^{a}
Total	2.32 ± 0.20	6.83 ± 1.50	3.91 ± 0.73

Each value is the mean ± SEM, n = 5.

Values with different superscripts within same column are significantly different ($P < 0.05$) by Duncan's multiple-range test.

The *P. mirifica* tuber samples expressed significant-correlation between individual and total protein density

The *P. mirifica* tuber samples expressed significant-correlation between individual isoflavonoid/protein and total isoflavonoid/total protein contents†† ($P < 0.01$), † ($P < 0.05$) as determined by Pearson correlation analysis.