CHAPTER V

DISCUSSIONS AND CONCLUSIONS

There is fluctuation of air temperature, with the highest temperature in April 2005 (31.3 °C) and lowest temperature in December 2005 (25.1 °C). The difference in maximum and minimum temperature is found most in April 2005 (11.5 °C) and least in October 2005 (7.2 °C). This could initiate heat shock influence to plant physiology during summer.

The annual rainfall exhibited maximum amount in October 2005 (441.5 mm) and minimum amount in January 2006 (0 mm). This could initiate water stress to the plant and its tubers during summer.

The major isoflavonoid contents of the field trial *P. mirifica* tubers including, puerarin, daidzin, genistin, daidzein and genistein were analyzed by RP-HPLC (Reverse Phase High Performance Liquid Chromatography). There is annual variation of individual and total isoflavonoid contents in the same plant clone. Puerarin, daidzin, genistin, daidzein, genistein and total isoflavonoid contents is found most in PM-III tubers collected in July 2005 (129.67±14.89 mg/100 g), February 2006 (78.01±11.25 mg/100 g), June (20.68±2.06 mg/100 g), March 2005 (24.65±0.78 mg/100 g), March 2005 (3.85±0.31mg/100 g), and July 2005 (185.88±16.74 mg/100 g), respectively. Puerarin, daidzin, genistin, daidzein, genistein and total isoflavonoid contents is found most in PM-IV tubers collected in March 2005 (187.07±1.82 mg/100 g), February 2006 (56.87±2.18 mg/100 g), March 2005 (21.27±2.75 mg/100 g), March 2005 (14.26±0.32 mg/100 g), December 2005 (2.07±0.41 mg/100 g), and March 2005 (270.79±13.14 mg/100 g), respectively.

The average annual amount of daidzin, genistin, daidzein and genistein in PM-III is higher than PM-IV. The annual of puerarin and total isoflavonoid in PM-IV is higher than PM-III.

Aglycoside, glycoside, ratio of aglycoside/glycoside, ratio of individual isoflavonoids/puerarin are analyzed. PM-III exhibited the maximum figure of aglycoside/glycoside (0.72±0.03) in March 2005 (summer) and minimum figure (0.11±0.01) in October 2005 (rainy). In PM-III, high temperature in combination of lack of water supply from rainfall is effectively promoted the accumulation of aglycoside. In the contrary, decreased in air temperature, nearly the least one (the least one is in

December 2005) and increased amount of rainfall resulted in exhibition of minimum figure of aglycoside/glycoside. PM-IV exhibited the maximum figure of aglycoside/glycoside (0.24±0.06) in March (summer) and minimum figure (0.12±0.02) in August (rainy), October (rainy) and February (winter). In PM-IV, the maximum figure of aglycoside/glycoside (0.24±0.06) is demonstrated only in summer. The minimum figure of aglycoside/glycoside (0.12±0.02) is demonstrated not only in rainy but also in winter. It means that temperature and amount of rainfall play less influence to isoflavone synthesis and storage in PM-IV than PM-III.

In comparison of aglycoside/glycoside between PM-III and PM-IV, PM-III has 3.43 times (0.72/0.21) of PM-IV in March 2005 and 0.92 times (0.11/0.12) in October 2005. Though the 2 plants exhibited varied degree of the maximum and minimum amount of aglycoside/glycoside, with much change in PM-III than PM-IV, but the difference is most present in March 2005. PM-III may exhibit a greater response to heat shock of genes in isoflavonoid synthesis and storage than PM-IV. The figures not only demonstrate the influence of temperature but also plant genetics in isoflavonoid contents.

Puerarin is the most abundant glycoside isoflavonoid in the tubers, thus this chemical can represent the accumulation trend of total isoflavonoid in tubers. Besides, not only puerarin but also glycoside daidzin and genistin, are among the most influenced isoflavonoid in the storage activity of the tubers.

The analysis of daidzin/puerarin, genistin/puerarin, daidzein/puerarin and genistein/puerarin in PM-III show the similar pattern with aglycoside/glycoside that is shown the maximum (1.29±0.23) or nearly maximum amount in March 2005. The influence of genetic is also found in this analysis where the maximum daidzin/puerarin (1.29±0.23), genistin/puerarin (0.26±0.11), daidzein/puerarin (0.68±0.13) and genistein/puerarin (0.20±0.10) in PM-IV are found in July 2005 instead of March 2005. Daiazin in both PM-III (78.01±11.25 mg/100g) and PM-IV (56.87±2.18 mg/100g) are found the maximum amount in February 2006 when the temperature was rising and was determined as early summer. Besides there is few rain since January. Taking the 2 factors into account, we may conclude that the highest tuberous daidzin accumulation should result from heat shock and low amount of accumulated water.

Isoflavonoid and total isoflavonoid contents in *P. mirifica* were not correlated with the change in air temperature (°C). The influence of amount of rainfall during the studied period in Ratchaburi Province played a great role with isoflavonoid storage in

the plant tubers. It is found that PM-III exhibited the maximum amount of puerarin in July 2005 (129.67 ± 14.89 mg/100 g, 28.9 °C, and 140.8 mm) daidzin in February 2006 (78.01 ± 11.25 mg/100 g, 28.4 °C, and 17.9 mm), genistin in December 2005 (22.12 ± 2.00 mg/100 g, 25.1 °C, and 58.7 mm), daidzein in March 2005 (24.65 ± 0.78 mg/100 g, 29.0 °C, and 9.6 mm), genistein in March 2005 (3.85 ± 0.31 mg/100 g, 29.0 °C, and 9.6 mm), and total isoflavonoid in July 2005 (185.88 ± 16.74 mg/100 g, 28.9 °C, and 140.8 mm). The plant exhibited the minimum amount of puerarin in March (31.28 ± 2.11 mg/100 g, 28.9 °C, and 9.6 mm), daidzin in January 2006 (11.62±2.25 mg/100 g, 26.4 °C, and 0 mm), genistin in March 2006 (9.34 ± 0.44 mg/100 g, 29.0 °C, and 9.6 mm), daidzein in January 2006 (3.48 ± 0.54 mg/100 g, 26.4 °C, and 0 mm), genistein in May 2005 (0.12 ± 0.04 mg/100 g, 31.0 °C, and 87.2 mm), and total isoflavonoid in January 2006 (61.75 ± 8.44 mg/100 g, 26.4 °C, and 0 mm). The plant exhibits the maximum and minimum storage of total isoflavonoid in the tubers in July 2005 (185.88 ± 16.74 mg/100 g, 28.9 °C, and 140.8 mm) and January 2006 (61.75 ± 8.44 mg/100 g, 26.4 °C, and 0 mm), respectively. It is possible to conclude that only the amount of rainfall is influenced to the accumulation of tuberous total isoflavonoid, puerarin, daidzin, genistin, daidzein and genistein in PM-III.

PM-IV exhibited the maximum amount of puerarin in March 2005 (187.07 ± 1.82 mg/100 g, 29.0 °C, and 9.6 mm), daidzin in February 2006 (56.87 ± 2.18 mg/100 g, 28.4 °C, and 17.9 mm), genistin in March 2005 (21.27 ± 2.75 mg/100 g, 29.0 °C, and 9.6 mm), daidzein in March 2005 (14.26 ± 0.32 mg/100 g, 29.0 °C, and 9.6 mm), genistein in December 2005 (2.07 ± 0.41 mg/100 g, 25.1 °C, and 58.7 mm), and total isoflavonoid in March 2005 (270.79 ± 13.14 mg/100 g, 29.0 °C, and 9.6 mm). The plant exhibits the minimum amount of puerarin in April 2005 (55.12 ± 3.45 mg/100 g, 31.3 °C, and 0.9 mm), daidzin in January 2006 (15.20 ± 2.98 mg/100 g, 26.4 °C, and 0 mm), genistin in May 2005 (4.03 ± 1.83 mg/100 g, 31.0 °C, and 87.2 mm), daidzein in August 2005 (3.69 ± 0.44 mg/100 g, 28.8 °C, and 114.8 mm), genistein in March 2005 and June 2005 (0 ± 0 mg/100 g, and 104.9 mm), and total isoflavonoid in April 2005 (55.12 ± 3.45 mg/100 g, 31.3 °C, and 0.9 mm). We can conclude that the plant exhibits the maximum and minimum storage of isoflavonoid in the tubers in March 2005 and April 2005, respectively. In addition, PM-IV exhibits the maximum ratio of aglycoside/glycoside in March 2005 and the minimum ratio in October 2005. The result is exactly correlated with PM-III. This confirms the influence of temperature and

amount of rainfall on tuberous isoflavonoid accumulation. Notice that there is the most increase (3.65 folds) of genistein from September 2005 to October 2005 in PM-IV. This may be influenced from the most increase amount of rainfall (3.03 folds) from September 2005 to October 2005. However, the best harvesting seasons for PM-III and PM-IV should be the same period that is March based on the criterion of highest aglycoside/glycoside. But, in term of antiproliferation to MCF-7 which is found only in the test with PM-III collected in April 2005, the best collected period for PM-III should be April.

The proliferation of MCF-7 is tested against the plant extracts of 2 clones of *P. mirifica,* PM-III and PM-IV, and isoflavonoid standards, in the presence and absence of S9 mixture. Proliferation effect at low dose (0.1 μg/ml *P. mirifica* extract) in the absence of S9 mixture is found from both PM-III and PM-IV in every month. The data demonstrates that low concentration (0.1 μg/ml) of plant extract is effectively acted as a physiological dose of estrogenic effect to ERα positive cells. PM-III shows more antiproliferative effect than PM-IV. PM-III show antiproliferative effect at the concentration of 1000 μg/ml in 6 collected samples including March 2005, April 2005, June 2005, September 2005, December 2005 and February 2006 while PM-IV shows the antiproliferative effect only in 3 collected samples including March 2005, May 2005 and January 2006. In the contrary, PM-IV shows more proliferative effect to MCF-7 than PM-III. PM-III shows proliferative effect only in 2 collected samples including October 2005 and January 2006 while PM-IV shows proliferative effect in 7 collected samples including April 2005 July 2005, August 2005, September 2005, October 2005, November 2005 and December 2005.

MCF-7 is also treated with plant extracts and standard isoflavonoids, in the presence of S9 mixture. This is an essential test because it represents the similar metabolism in human consumption. Proliferative effect is exhibits in every tested dose with a maximum of 8.65 folds in PM-III, and 9.18 folds in PM-IV, in comparison with the proliferative effect to MCF-7 in the absence of S9 mixture. This confirms that *P. mirifica* phytoestrogens are effectively metabolized by liver enzymes in S9 mixture and becoming potent phytoestrogens with stronger binding affinity to ERα of MCF-7.

In the absence of S9 mixture, there is correlation between 10 and 100 μg/ml PM-III with the proliferation effect of MCF-7 and with the amount of daidzin and daidzein. There is correlation between 1000 μg/ml PM-III with the antiproliferative

effect of MCF-7 and with the amount of daidzein. Our correlation analysis result of daidzein is agreed with the previous report (Sathyamoorthy, N. and Wang T.T.Y. 1997). However, there is correlation of proliferative effect of PM-III at the concentrations of 1 and 100 μg/ml with the amount of daidzin in the presence of S9 mixture.

In the absence of S9 mixture, PM-IV at the concentration of 10 μg/ml exhibits correlation between the proliferation effect of MCF-7 with the amount genistein and the antiproliferation effect at the concentration of 1 μg/ml with the amount of puerarin. In the presence of S9 mixture the results of correlation analysis in PM-IV is negative.

There are correlations between the percentage growth of MCF-7 cells at the concentrations of 100 and 1000 μg/ml of PM-III with the amount of aglycoside. There is a correlation at 10 μg/ml of PM-III with the amount of glycoside. The correlations of PM-III are found at 10 and 100 μg/ml with the amount of glycoside in the test with S9 mixture.

There are correlations between the percentage growth of MCF-7 cells with the ratio of daidzin/puerarin and daidzein/puerarin at 100 and 1000 μg/ml and the ratio of genistin/puerarin. The proliferative effect of 0.1 μg/ml PM-IV extract is correlated with the ratio of genistein/puerarin. There are correlations of PM-IV at the concentrations of 0.1, 100 and 1000 μg/ml with the ratio of daidzin/puerarin. The percentage growths of PM-IV at the concentration of 0.1, 1 and 1000 μg/ml are correlated with the ratio of genistin/puerarin. Even puerarin is the most storage isoflavonoid but the estrogenic activity is mainly influenced by daidzein.

There is correlation between the proliferation effects of 10 μg/ml PM-III with temperature. The proliferation effect to MCF-7 of PM-IV at 1 and 1000 μg/ml is found correlated with the amount of rainfall. The results demonstrated clearly that physical factors exhibit influence on tuberous isoflavonoid storage first which in consequence, influence the estrogenic effect of the chemicals derive from the plant tubers. Besides, genes in isoflavonoid synthesis and/or storage pathway might also play a great role in the presence of isoflavonoids in the two different plant clones.

The interesting result from this experiment is the antiproliferative effect at the concentrations of 10, 100 and 1000 μg/ml with IC_{50} of 5.19 μg/ml of the collected samples of PM-III in April 2005. This result demonstrates that the collected sample of PM-III in April 2005 exhibits the strongest antiproferative effect, even in the presence

of S9 mixture. The temperature and amount of rainfall is clearly influenced the antiproferative effect but the isoflavonoid contents are not. Thus the antiproliferative effect of the plant might be influences by other phytoestrogens, which are not investigated in this study.

Plant genetics should influence to not only isoflavonoid synthesis and storage in *P. mirifica* tubers because the 2 plant clones exhibit different peak of tuberous isoflavonoid storage at different month of collection of plant samples, but also the bioactivity of the plant because only PM-III exhibits anti-proliferation to MCF-7 in April 2005.

The isoflavonoid RP-HPLC is analyzed with tuberous samples collected from 2 distinct clones of *P. mirifica* in function of a monthly collected period for a total of consecutive 12 months. The work is designed to establish a common environment for all test plants. Isoflavonoid contents in PM-III showed more variation than PM-IV because the PM-III plants are derived from seeds with the same dormancy period, germinated at the same period and in the same place (greenhouse) and transferred to soil in the same field trial at the same period, and thus establish a unique age and differentiation of the plants. PM-IV derived from asexual propagation of the same period and planted in soil at the same period with PM-III. The established results definitely show the influence of both genetics and climatic (temperature and amount of rainfall) factors on isoflavonoid tuberous storage in this plant. Consider the climatic factor, the change in amount of rainfall plays more important role than the change in temperature as Thailand is in sub-tropical zone in which the temperature change is not extreme as does happen in temperate zone countries. To harvest the tuber, it has to seriously consider those 2 factors to gain plant materials with high amount of isoflavonoids.

The knowledges on influence of climate and plant genetics on tuberous isoflavonoid storage and estrogenic activity established in this study will benefit the farming of *P. mirifica* plants. The effective harvest period can be monitored via HPLC analysis of tuberous isoflavonoid storage and MCF-7 proliferation test, which are not expensive but practical methods. Besides, there is a different criterion of consuming the 2 plant cultivars. PM-III is good for antiproliferation purpose because the plant exhibited more antiproliferation than PM-IV. PM-IV is good for trophic purpose because the plant exhibited stronger proliferative effect than PM-III.