CHAPTER V

DISCUSSION

Study on the geographic distribution of *P. mirifica* plants

P. mirifica plant was explored from difference places in different regions of Thailand. Even though each site was far from each other, except for the northern and some part of central such as Kanchanaburi province which was in the same mountainous line, the plant could be found in all sites which was foothill, hill, slope and slant **(Table 3)**. *P. mirifica* could be well grown in the loamy, the mixing of sandy and gravel but not in the clay.

In natural sources, many twinning plants could be found in the same habitat, some of them were similar to *P. mirifica*. For identify of the plant, it was necessary to consider many botanical character such as leaf, flower and tuberous root. The flower was the clear marker for classifying the plant (**Figure 6 - 7**). In some places, the plant exhibited no flower in some year due to the serious change in the climate and the fall of flower before seed set, so the seed could not be collected. Therefore, the seed could not be collected from all of the studied sites (**Table 4** and **Figure 9**). Thus, this study focused only two sites, Chiangmai and Kanchanaburi province, where the flowering and seed set have been annually recorded.

The first problem of this study was the long distance of each study site that should be studied at the same time as flowering and seed set. The second problem was the limit of the budget of trips for collecting the plants, so this study was carried out only the site where the existing of the plant had been informed. It should be noted that this study was not a full-scale survey. However, such topography characteristic in each study site could be used as a guide in the next exploration.

It was also found that the risk problems of *P. mirifica* faced in the study period wwas the fire and deforestation. The fire occurred in every year during the summer were the important factor to the interruption of the seed setting. The plant may die also before maturation. So the pods and seed were not produced.

Since all of seeds were collected from the nature, it might be implied that were the varieties in nature. Therefore, seed character was found to be highly variable (**Table 4** and **Figure 9)** which might reflect the differences in the formation of tuberous and chemical contents. The future study could be done in this aspect, since the seed was the important reproductive organ and was the key tool for the plant breeder.

The shape of tuberous root was also varied (**Figure 4**). It can not defined the type of that shape due to the formation was not resulted from genetic but also geographic such as the long shape which caused that of tuber laid under the rock Some tuberous roots were as long shaping as that found in the *B. superba.*

The qualitative analysis of methanolic crude extracts, TLC method can be used for seperating some of chemical concents. Either numbers of TLC band or intensity appeared their own character of each various place. These results might be caused the different of age and source of the collected samples. TLC patterns could be use as a guidance to compare the different of each varities for the futhur study.

In TLC study, it was shown that puerarin could be quantified by TLC-densitometry, the simple and rapid method as the analyzed result could be examined directly from the crude extracts without prior purification. The analysis of puerarin comprised of two main steps; the extraction and quantitation.

The quantitative analysis using amount of puerarin as a comparison the difference between places. It was found that the puerarin contents varied among all tested plants. Puerarin extracted from *P. mirifica* tuberous root powder was seperated on TLC plate by applying appropriate solvent system. The best solvent system was found to be chloroform: methanol: H_2O in the ratio of 40: 40: 2. The R_f value of puerarin was approximately 0.70 (**Figure 33**). To ensure the accuracy of the extracted puerarin, the standard puerarin had to be applied as standard marker. Furthermore, other factors had to be aware such as an overloaded sample since the solvent system could not elute all puerarin and the TLC densitometer could not detect the actual amount accurately.

The results from the collected samples from ten various places during the summer of 1999 revealed difference in total and intensity of TLC. The Chiangmai, Kanchanaburi, Sakonnakorn and Lumpang collected samples showed the similar

pattern while the others were totally different (**Table 5. - 8, Figure 10 and 14**). It implied that the active ingredients in the Thai *P. mirifica* may be nearly totally different from each other which may resulted from the differences in physical factors such as soil content, climatic difference or even latitude difference as well as the genetics.

Study on the genetic differences of *P. mirifica* plants

Comparison of the tuberous roots of field grown *P. mirifica* population in Chiangmai and Kanchanaburi varieties was found from the field grown population that the both of varieties were able to flower in the first year of plantation (**Table 9 and Figure 15**). It might be induced by the heavy rain during the year 1999-2000 which supported the growth of the plant and made the plants became mature within only 1 year period. The flowering occurred from February to May, but only 2 out of 7 plants could establish seed set.

It was found that there was no difference in the growth rate between Chiangmai and Kanchanburi varieties in the field trial as analyzed from the length of the peristem and that parameter was not related to the yield of tuberous root (**Table 10** and **Figure 16 - 17**).

In the determination of some characters of the tuberous roots such as the number, fresh and dry weights, no difference was found between the two varieties. It was found that three of twenty plants (15%) in both varieties had no tuberous root formation (**Figure 18(A)**) which may resulted from their genetics. The tuberous roots could be found in four different shapes (**Figure 18(D)**) in both varieties even though the tuberous roots were reproduced in the same place with no difference in the soil and other physical factors. The tendency of tuberous root shapes appeared in the long that might be in the first of growth. It is interested that how the shape changing in other year later. The plant with the longest peristem length could not exhibit the

tuberous root (**Table 10)** In term of tuberous root formation, it was found only the difference of the mean value of main root length of Chaingmai and Kanachanaburi varieties was found to be 67.40 and 47.50, respectively. It could be concluded that there was no relationship between the growth of the stem and the production of the tuber. The yields of tuberous root obtained from the fresh and dry weight in both varieties was not difference (**Table.13-14**). It was also found that the water content in the tuberous roots of the two varieties that was approximately 90% (**Table 15**). The formation of the difference shape may also depend upon the difference in genetics.

The macromolecules ranged from starch, protein and fat (**Figure 16**) which reflect a lot of starch accumulation that was commonly found among tuberous plant. The results in **Table 16** revealed that Kanchanaburi variety contained higher starch content of 24.86 $\pm$ 8.48 as compared to 16.57 $\pm$ 1.80 of Chaingmai variety. It implied that at least the starch content could be adapted to differentiate the different varieties. Macromolecules and other contents may reflect the differences of the chemical structures and cellular contents of *P. mirifica*, which may in turn reflect directly to the quality of the powder or extract, derived from this plant. Proximate analysis can help to discriminate the difference between the two varieties, once the contents of starch, fiber, ash, protein and fat are analyzed.

Quantitative analysis of puerarin contents in the field grown *P. mirifica* tuberous root by TLC densitometry was also found also difference in chemical contents as the same results were observed for the collected tubeous roots from

various places (**Table 8** and **Figure 14**). The results confirmed that chemical contents varied among *P. mirifica* in different location.

To proof that genetics is the most important factor that resulted in diversity of the active ingredients, the two varieties of plants from Chiangmai and Kanchanaburi were seed collected to establish subclones which were germinated at the same period and planted at the same day in the same small sized field plot.

The results from the 18 month old field grown plants, Kanchanaburi variety showed significantly higher content of puerarin than that of Chiangmai variety (**Table 17** and **Figure 34**). As two varieties were grown in the same field which were also submitted to the same climatic and soil content conditions, such significantly different puerarin contents should be deduced to the genetic factor which implied that the two varieties were inherited with different genotypes concerning puerarin synthesis and /or storage. This remarkable puerarin content difference may reflect to the difference in other active ingredients as well.

Eventhough, miroestrol is believed to be the most important active ingredient and recently was found to be isomiroestol (Chansakaew et al, 2000) but no authentic miroestrol was commercially available. Puerarin was selected to be marker, because from the preliminary analysis found that puerarin was present in highest amount among all isoflavone found in *P. mirifica*. Eventhough, there are also commercial supply for the other isoflavones such as daidzein, daidzin, genistein and genistin. Besides, puerarin is a lower price product as compare with other isoflavones. Thus, puerarin was chosen to be a preliminary standard marker to establish for the

primary isoflavone screening system for *P. mirifica* from various varieties. At least puerarin could also be used as primary marker to compare the content among the selected clones and could used to search for the "highest puerarin content clone" among the investigated plants.

Due to the fact that there is no definite ratio among the five isoflavones present in *P. mirifica*; puerarin, daidzin, daidzein, genistin and genistein, thus the puerarin content will not reflect exactly to such ratio. But it is well defined to be an exellent marker model to sort out *P. mirifica* sublone with high particular chemical content such as puerarin and adapt the analysis to sort for the clone with different chemical content criteria. For example, the plant breeder could select for the highest genistein content of *P. mirifica* subclone by replace the marker chemical from puerarin to genistein and also adapt the analysis condition. By this low cost laboratory analysis, it is possible for *P. mirifica* breeder to establish a commercialized mother plant as well.