CHAPTER V

DISCUSSION

1. HPLC Analysis of Colchicine and Colchicoside in *G. superba* Seeds

In order to evaluate the potential of Thai *G. superba* seeds as a source of colchicine, a number of seed samples were collected from various parts of Thailand. *G. superba* normally grows wild in many parts of the country, especially the areas nearby the sea such as the eastern and southern parts of Thailand. Presently, there are only a few local plantation sites of this plant since there has not been sufficient promotion and very little information is known about the uses of the plant. The seed samples collected for this study were obtained from 6 provinces representing the northern, central, eastern and southern parts of Thailand (Table 7). Most of these *G. superba* plants were originally cultivated from the tuber parts of the Thai cultivar whereas the plants from Chiang Mai were cultivated from Indian *G. superba* seeds. Among various sources described in Table 7, the seeds from Amphur Khlung, Chanthaburi is the only samples obtained from the wild-grown *G. superba*.

In this study, HPLC was chosen as a technique for the determination of the seed colchicine because the ethanolic extracts of *G. superba* seeds appear to contain only three major components under our established HPLC method (Figs.14 and 22). These components were proved to be colchicine, colchicoside and 3-demethylcolchicine by co-chromatographic methods with corresponding authentics using HPLC (Fig.14), TLC (Fig.16) and by UV-spectrum comparison of each constituent (Fig.17). The advantages of this HPLC method are its simplicity, time-saving and high sensitivity. No need for prepurification of the crude extracts before HPLC analysis and the complete separation of the three alkaloids can be accomplished within only 30 min (Fig.14). Furthermore, the method is sensitive enough to detect colchicine at as low concentrate as 5 µg/ml or 0.1 µg per injection (20 µl) and the standard curve shows linearity upto 100 µg/ml (Fig.18A).

The presence of colchicoside in the *G. superba* seeds is also interesting since it can be used as a lead compound for synthesizing thiocolchicoside. This thiocolchicoside has been reported to possess decontractant effect, anti-inflammatory, analgesic and muscle relaxant activities (Forni and Massarani, 1977 ; Merck Index, 1989). Therefore, quantitative analysis of colchicoside was also carried out simultaneously with the

analysis of colchicine. The standard curve of colchicoside shows linearity between the concentration range of 5 and 50 µg/ml (Fig.18B).

Based on the HPLC analysis , the seed samples obtained from various parts of Thailand show relatively high contents of colchicine and colchicoside, ranging from 0.83-1.46 % and 0.67-1.27 % dry weight, respectively (Table 8). The seeds from Chumphon 91 (1.46 %) and Chanthaburi 2 (1.43 %) contain the highest colchicine content. They also contain high level of colchicoside with 1.16 % and 1.05 % dry weight, respectively. These results suggested that both the cultivated (Chumphon 91) and the wild-grown (Chanthaburi 2) *G. superba* can produce seeds with similar high quality with respect to colchicine and colchicoside contents. Therefore , cultivation of this plant if economically feasible, may lead to a good source of colchicine and colchicoside.

In all seed samples examined, the colchicine content appears to be slightly higher than the colchicoside content, with the approximate ratio of 55:45. However, there is one exception for the sample from Prachin Buri which shows lower colchicine content (1.04 %) than colchicoside (1.27 %), with the ratio about 45:55 (Table 8 and Fig.19). This may be due to fungus infection of the seeds after collecting and drying of the fresh

seeds. The appearence of these Prachin Buri dried seeds was dark and different from the seeds from other sources.

It should be noted that the Chiang Mai seeds contain the lowest level of both colchicine (0.83 %) and colchicoside (0.67 %). Unlike the other sources, these *G. superba* plants were cultivated in Chiang Mai using the seeds from India (Table 7). It is possibly that the *G. superba* cultivar from India is inherently inferior to the Thai cultivar. This finding is consistent with the results reported previously that Indian *G. superba* seeds contain approximately 0.60 % dry weight of colchicine (Sarin *et al.*, 1974). Alternatively , the geographic conditions (relatively cold and highland) of Chiang Mai may be the cause of the low colchicine accumulation in the seeds. Normally, the *G. superba* plant prefers sunlight and hot weather (Bailey, 1963).

In term of total colchicine and its derivatives in *G. superba* seeds, the UV-spectrophotometry proves to be useful, convenient and reliable for determination of the total content (Table 9). The reliability of this spectrophotometric method is obvious since the crude ethanolic extract of the seeds was clearly shown to have only three components; colchicine and colchicoside are the two major constituents and 3-demethylcolchicine is the minor one (Fig.13). These three components show the same

absorption spectra (Fig.17). However, calculation of the total colchicine derivative content by this UV-spectrophotometry is based on calibration curve of colchicine (Fig.20) whereas the summation value of colchicine and colchicoside by HPLC is obtained from calculation using the calibration curve of each compound (Fig.18). Nevertheless, the values obtained by both methods seem to be closed to each other although the results obtained by the spectrophotometric method seems to be a little bit higher than those obtained by HPLC (Fig.21). This is, of course, due to the presence of the small amount of 3-demethylcolchicine in the crude extracts. Therefore, the UV-spectrophotometric method may essentially be replace the HPLC method for the determination of total colchicine and colchicoside in the seeds of *G. superba*. This method appears to be simple, rapid and allows a large number of samples to be analyzed simultaneously. Furthermore, the method is considered to be highly sensitive, it can determine colchicine in the low concentration as 0.39 µg/ml. It can also determine the content in a wide concentration range (0.39-50 µg/ml)(Fig.20). Thus, the step of sample dilution or concentration prior to the analysis may not be necessary if it gives the absorbance fallen in the wide linear range of the calibration curve. Based on its simple and rapid method, it may be useful for quality evaluation of *G. superba* seeds or also for a screening

program for selecting high colchicine-producing plants.

The results from the method of either HPLC or the UV-spectrophotometry indicate that Thai *G. superba* seeds generally contain high content of total colchicine and colchicoside with approximately two percent of dry weight (Table 9). According to the literature, this content has been the highest among various *G. superba* plants cultivated in other countries such as India, Ceylon, and Africa (Sarin *et al.*, 1974 ; Thakur *et al.*, 1975). Since both colchicine and colchicoside are pharmaceutically important especially colchicine is considered a valuable compound (up to 400,000 baht per kilogram), the *G. superba* seeds seem to be a new potential source of colchicine. This plant should, therefore, be promoted for cultivation.

2. TLC-Densitometric Analysis of Colchicine in Various Plant Parts of *G. superba*

Although colchicine has been reported to be present in the whole plant of *G. superba*, there have been no information on the quantitative distribution of this compound in this plant. In Thailand, it has long been believed that colchicine is accumulated mainly in the tuber part owing to the use of *G. superba* tuber in some traditional medicine (ชมรมธรรมชาติศึกษาไทย, 2521 ; เสงี่ยม พงษ์บุญรอด, 2522 ; ปรีดี เอกะวิภาค, 2523). This study,

therefore , aims to establish information on the distribution of colchicine in various parts of *G. superba*.

Our preliminary study suggested that the chemical constituents in ethanolic extracts of various *G. superba* parts were so complicated that the HPLC method described previously could not be used for this quantitative purpose. Prepurification of the crude extracts prior to HPLC analysis although possible, may lead to a loss of the alkaloid and affect the accuracy of the results. As a consequence, a TLC-densitometric method was developed carefully for this specific purpose of colchicine content analysis.

The developed method appears to be simple, rapid and reliable as confirmed by HPLC (Table 14). The step of sample preparation is fast and simple since colchicine in the crude ethanolic extract can be quantitated directly without prior partial purification. Moreover, the method is accurate (Table 13), reproducible (Table 15) and sensitive (Table 12). It can quantitate colchicine even the concentration is as low as 0.025 µg per spot.

In the development of this TLC-densitometry, the choosing of the solvent system for complete separation of colchicine from other interfering substances on a TLC plate is a very important step. The spot of colchicine

on the chromatographic plate must be pure so that the absorption intensity read by TLC-densitometer is not interfered by any other substances. The results from this development are satisfactory since colchicine in various plant parts is completely separated from other substances in the crude extracts (Fig.23). This allows the quantitation of colchicine be done simultaneously for all plant parts. Moreover, the application volume of each assay solution must be accurate and constant in order to obtain reproducible peak area of each spot. In this study, the micropipette apparatus was used and the spots were applied by only one person throughout the experiment in order to minimize errors due to personal parameter.

Based on the developed TLC-densitometric method, colchicine appears to be present in every part of *G. superba* plant (Figs.26 and 28). The content of colchicine in the seeds shows the highest followed by the pericarps , flowers , tubers , leaves and stems , respectively (Fig.28). Moreover, the distribution of colchicine along the stem and leaves was carried out. It seems that the colchicine content in the middle part of the stem is slightly lower than that in the bottom and the top parts (Fig.29).

It should be noted that 3-demethylcolchicine is also present in every plant part particularly in the

leaves (Fig.26). It is possible that this alkaloid is the last intermediate of colchicine biosynthesis in the plant. If this is the case, the last step in the colchicine biosynthetic pathway is the methylation of 3-demethylcolchicine to form colchicine. Alternatively, 3-demethylcolchicine may be formed from the demethylation of colchicine by an demethylase enzymes.

The significantly higher colchicine content in the young pericarps over the mature pericarps (Fig.30) prompted us to study in more detail on the effect of harvesting period or the maturity of the capsules on the colchicine content. The results show that on the maturity of the capsules, as indicated by capsules sizes, there is a continuous decline of colchicine content in the pericarps. The content decreases from 1.68 % colchicine in the pericarps of very young capsules to only 0.32 % in those of ripe and exploded capsules (Fig.32). For the seeds , on the other hand , the accumulated colchicine content appears to be highest and that of young seeds obtained from different sizes of the young capsules is still rather high and not significantly different in their quantities (Fig.31). It should be noted that the young seeds are rather small and have lower weight than the ripe seeds which may lead to high colchicine content when calculated as percent dry weight. The picture may be different if the seed colchicine content is calculated

as total content in the whole capsules of the young and the mature stages.

The mechanisms involved in a decrease of colchicine content during the maturity of the capsules are still not known. However, based on the some results of the TLC-densitometric chromatograms of young and ripe pericarps, it is clear that there is a change in their peak areas of colchicine and 3-demethylcolchicine (3-DMC). For the young pericarps, the major peak is colchicine, and the minor peak is 3-DMC. For the ripe pericarps, on the other hand, the peak of 3-DMC is bigger than that of colchicine (Fig.33). It may be that there is a demethylation enzyme that converses colchicine into 3-DMC present in the pericarps of *G. superba*. Resolution of this step must depend on further enzyme isolation.

Since the high colchicine content in the young pericarps, it seems to be interesting to use this part as colchicine sources but when considered with other results, the ripe seeds obtained from the ripe exploded capsules appear to be the best plant part for serving as high colchicine-containing sources.

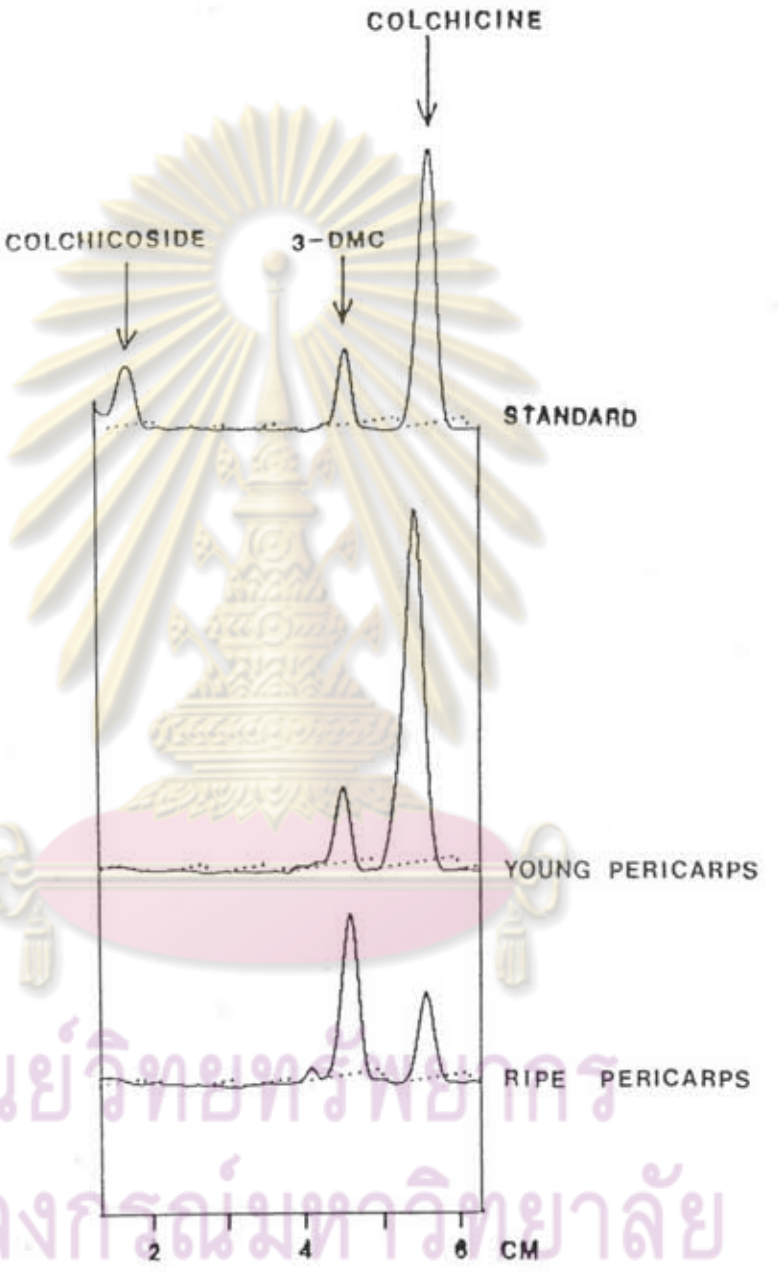

Fig.33 TLC-densitometric chromatogram of ethanolic extract of young pericarps compared with that of ripe pericarps.

CONCLUSION

From this research work of "Study on Colchicine Content in the Seeds of Thai *Gloriosa superba* L.", some conclusions can be drawn. First, the seeds of *G. superba* obtained from various parts of Thailand contain relatively high level of colchicine, ranging from 0.83 to 1.46 % dry weight. The seeds obtained from the province of Chumphon (crops of the year 1991) and Chanthaburi (wild-grown plant at Amphur Khlung) show highest colchicine content with 1.46 % and 1.43 % dry weight, respectively. On the other hand, the seeds obtained from Chiang Mai (the plant was cultivated from Indian *G. superba* seeds) show the lowest level of colchicine content (0.83 %) on dry weight basis.

Second, the *G. superba* seeds also contain colchicoside as determined by our HPLC method. Since this compound is also a major constituent and its semi-synthetic compound, thiocolchicoside, has been reported to possess many medicinal properties, we also determine the content of this compound in *G. superba* seeds simultaneously with colchicine in the same HPLC chromatogram. The results show that Thai *G. superba* seeds also contain high level of colchicoside, ranging from 0.67 to 1.27 % dry weight. The ratio of colchicine and colchicoside in all of the seed samples is essentially

about 55:45.

Third, we find that the content of colchicine and its derivatives in *G. superba* seeds which is determined by simple UV-spectrophotometric method is close to the value of total colchicine and colchicoside in the same extract as determined by the HPLC method. Therefore, in general, the spectrophotometric method can replace the HPLC method for the determination of total colchicine and colchicoside in *G. superba* seeds.

Finally, in this research, we have developed TLC-densitometric method for determination of colchicine in various parts of *G. superba* plant in order to study the distribution of colchicine in the whole plant.

This developed TLC-densitometric method proves to be highly effective, accurate and reproducible in the quantitative analysis of colchicine in various plant parts of *G. superba*. Furthermore, the method is simple and rapid since the steps of purification prior to the analysis is not necessary. As a result of TLC-densitometric analysis of colchicine in the whole *G. superba* plant, we find that colchicine is present mainly in the ripe seeds with the content of 1.35 % dry weight compared with the other parts , including pericarps, flowers, tubers, leaves and stems in which

colchicine content is found to be 0.86, 0.40, 0.26, 0.06 and 0.05 % dry weight, respectively. Besides, we have found that the period of harvesting or the maturity of the capsules has an effect on the colchicine content in the seeds and in the pericarps. For the pericarps, it seems that young pericarps contain rather high colchicine content. The more maturity of the pericarps, the less colchicine content is present. For the seeds, colchicine content in every stage of seed maturation is rather high but the seeds from the ripe-exploded capsules seem to contain the highest and are considered to be suitable plant part for using as raw materials in the colchicine extraction.