CHAPTER V

DISCUSSION

The leaves of Thai *C. sublyratus* have been used since 1985 by Sankyo Co., Ltd. as raw material for the manufacturing of "Kelnac^{R}", the antipeptic ulcer drug containing plaunotol which is the active constituent isolated from the leaves. Although at least two methods of chemical synthesis of plaunotol have been reported (Corey and Yamamoto, 1970, Sato *et al.*, 1988), their cost is still too expensive as compared with the price of the natural plaunotol. As a result, plantation of *C. sublyratus* for leaf harvesting is still necessary for the drug manufacturing. Presently the Japanese pharmaceutical firm has more than one million *C. sublyratus* plants in the area more than 7000 rai at Prachuap Khiri Khan (ณรงค์ เพ็งปรีชา, 2530). Information on the plant especially the content of leaf plaunotol is usually the company's secret and therefore, very little information is available in the literatures about this Thai plant. In addition, it is widely believed that only the plants that are cultivated in Prachuap Khiri Khan can produce plaunotol. These prompted us to study in detail about the actual content of plaunotol in *C. sublyratus* and the variation of the content in the plants obtained from various areas of Thailand. This information would be very useful for the screening of high plaunotol-containing plants.

1. Development of TLC-Densitometry for the Determination of Plaunotol in *C. sublyratus* Leaves

In principle, the screening for the plants with a desired characteristic from a plant population requires an analytical technique which is simple and allows a large number of samples be analyzed. In the literatures, plaunotol content in *C. sublyratus* leaves has been reported to be determined by using gas chromatographic method (Morimoto and Murai, 1989; Ogiso *et al.*, 1981; Sununta Cajesanun, 1991). This GC method is accurate and precise but usually requires steps of complicated sample preparation and partial purification of the crude extracts before quantitation. As a consequence, the overall procedure is time consuming and unefficiency for a large number of the samples of *C. sublyratus*. To avoid these problems we established a much simpler and time-saving TLC-densitomeric method for the quantitative analysis of plaunotol in *C. sublyratus* leaves. The technique was developed carefully to maximize separation of plaunotol from other constituents in the ethanolic extracts. The resulted TLC densitometric chromatogram (Fig. 9) clearly showed the complete separation of plaunotol. Therefore, the process of prepurification of plaunotol is not necessary with this techniques and, on a regular TLC plate (20 x 20 cm^2), at least twenty samples can be analyzed by a single run. A large number of samples can therefore be analyzed simultaneously. In terms of precision and accuracy, the developed method was compared with method of GC. The results showed that the TLC-densitometry and the GC gave very similar value of plaunotol content in the same samples (Table 7 and Fig. 14) and also with narrow range in their of standard deviation (SD) (Table 7). For sensitivity, the calibration curve obtained from this method can quantitate

plaunotol even in the concentation of as low as 0.025 mg/ml and in a wide concentration range from 0.025–0.2 mg/ml (Fig. 11).

2. Screening for High Plaunotol-Producing Plants of *C. sublyratus*

In the analysis for leaf plaunotol, the leaf samples were collected from *C. sublyratus* plant growing in Rayong, Prachuap Khiri Khan, Nakhon Pathom, Bangkok and Chachoensao. The overall 68 samples were dried, powdered and subjected to the simple and accurate TLC–densitometric method. The results showed that *C. sublyratus* leaves from various sources contained highly variable plaunotol contents, ranging from 0.139 to 0.786% (w/w) (Table 9 and Fig. 15). The majority of the samples appeared to have plaunotol contents in the range 0.3–0.4% (w/w) (Table 10 and Fig. 16). In this study, there was no clear correlation between the plaunotol content and geographic conditions since both the low (less than 0.2%) and high (more than 0.6%) plaunotol containing plants were found in all areas of Prachuap Khiri Khan, Bangkok and Rayong. In addition, we found that the leaves from the young plants and mature plants had no significant differences in their plaunotol content (Table 11). It was therefore concludes that the age of the plant did not have much effect on the leaf plaunotol. As a result, it seems that, the genetic background of each individual plant is the major factor contributing to the appearent plaunotol. Apparently, this is the first report on the quantitative analysis of plaunotol in *C. sublyratus* leaves. Previously, plaunotol has been reported to be present in leaves, stems, branches and roots of this plant (ณรงค์ เพ็งปรีชา, 2530; พนิดา แสงทอง, 2528; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; วีณา วิรัจฉริยากูล และคณะ 2533).

In addition to *C. sublyratus*, screening for plaunotol–containing plants in other *Croton* species was also carried out. Among seven species

of *Croton* including *C. sublyratus*, *C. oblongifolius*, *C. crassifolius*, *C. caudatus*, *C. tiglium*, *C. cascarilloides* and *Croton* sp. (Char plao nam ngoen), only *C. sublyratus* could be detected for plaunotol. This is similar to previous reports which also found no plaunotol in other *Croton* species (ณรงค์ เพ็งปรีชา, 2530; Ogiso *et al.*, 1981; Ogiso *et al.*, 1985).

3. Preparation of Various Types of Explants and Callus Cultures from the High-Plaunotol Producing Plant

Based on the results obtained from the step of screening for high plaunotol-producing, one of *C. sublyratus* plants with the highest leaf plaunotol content (0.786%) which grows in Chulalongkorn University Campus was chosen as the parent plant for subsequent studies. Various parts of the plant potentially be used as explants were excised and studied for the optimal conditions of surface sterilization. These plant parts included : shoot tips, nodal segments with auxially buds, auxially buds, leaf segments and petioles. It was found that shoot tips, nodal segments and auxially buds needed relatively vigorous conditions to be surface sterized (see Table 6). These organ has to be treated with both 70% ethanol and 10% Clorox containing Tween 80 (a wetting agent) for 30 min. For the leaf segments and petioles, their surface sterized process is also essentially the same except the step of Clorox treatment which takes considerably shorter time (only 5 min). With these process, the explants appear to be completely free from microbial contamination, allowing us follow the development of each type of the explants.

In addition to the preparation of organ explants, we tried to establish callus cultures of *C. sublyratus*. The callus cultures was expected to be used for the plant regeneration in case of the unsuccessful results of

the micropropagation of the plant through the above-mentioned explants. With this objective, we could established *C. sublyratus* callus cultures from the leaf segments. The medium that induced callus formation was MS agar medium containing 2.0 mg/l 2,4-D and 1.0 mg/l kinetin. For subculturing, this callus culture appears to grow well on MS agar medium containing 1.0 mg/l 2,4-D and 1.0 mg/l BA and form soft, friable and yellow tissues (Fig. 24). The friable calli were subsequently used for establishing cell suspension cultures by transferring the tissues to the same medium. The cell cultures showed small aggregates with yellow color (Fig. 25). Both callus and cell suspension cultures were maintained by regular subculturing. In terms of plaunotol formation, however, both callus and cell suspension cultures failed to produce plaunotol under various conditions tested in this study (Fig. 17). This is possible that the composition of medium, type and concentration of growth regulator and other supplements still not be suitable for plaunotol formation in both cultures.

It has been reported that plaunotol accumulation is observed in callus cultures of *C. sublyratus* grown on media containing gelling agents, especially gellan gum and agarose (Morimoto and Murai, 1989). Furthermore, the increase of chlorophyll content, slow growth and light have been found to stimulate plaunotol accumulation in *C. sublyratus* callus cultures (Morimoto and Murai, 1989).

Charlwood and Rhodes (1990) explained that the dedifferentiation of plant tissue *in vitro* to produce callus or suspension cells is usually accompanied by a apparent loss of ability to accumulate secondary compound. The reasons may be :

1) the lack of expression in non-specialized cells of genes that control the essential steps in the biosynthetic pathway;
2) the diversion of substrate away from secondary product formation;
3) the non-operation of transport mechanisms by which potentially toxic end-products may be removed from the biosynthetic site;
4) the non-availability of storage sites in which secondary metabolites would normally be sequestered;
5) the unregulated catabolism of synthesized product.

4. Regeneration of *C. sublyratus* Plant through the Explants

Our preliminary study showed that various types of *C. sublyratus* explants were able to respond to MS medium supplemented with various types and concentrations of growth regulators. The explants of shoot tips, nodal segments with auxially buds and auxially buds could all form shoots while the leaf segments could not (it formed callus). The developed shoots generally showed characteristics of fast growing and leaf formation. The shoot formation from the shoot tips seemed to be faster than form the nodal segments with auxially buds and auxially buds. However, these explants did not induce multiple shoots (only one shoot appeared from each segment) under vairous tested conditions. Furthermore, the attempt to maintain the developed shoots was not successful. This may be probably due to the formation of phenolic compounds in the starting explants causing the prelonged cultures with shoots become browning and finally die.

5. Regeneration of *C. sublyratus* Plant through Callus Cultures

We were finally successful in inducing shoot regeneration from *C. sublyratus* callus cultures (Fig. 26). The callus culture was first

established from the young leaf segments on MS agar medium containing 30 g/l sucrose, 2.0 mg/l 2,4-D and 1.0 mg/l kinetin (Fig. 26A) followed by transferring onto MS medium containing 10 g/l sucrose, 2.0 mg/l NAA, 2.0 mg/l BA and 0.2% (w/v) gellan gum for friable with green tissue proliferation (Fig. 26B). Multiple shoots were regenerated from the green callus on MS medium containing 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 and 0.2 % (w/v) gellan gum (Fig. 26C and Fig. 27). These steps of shoot regeneration from the green callus of *C.sublyratus* and the medium used are similar to those studied previously by Murai *et al.* (1990). These steps are also similar to the steps of plant regeneration from the callus of rice (*Oryza sativa* L.) (สุภาพร วัฒนวีรเดช, 2531). For the shoot-induction medium, the concentrations of 1.0 mg/l BA and 0.05 mg/l GA_3 are used. BA which is the plant growth regulator of cytokinins has been reported to induce adventitions shoot formation at high concentration (1-10 mg/l) (Pierik, 1987). GA_3, on the other hand, has been found to induce elongation of internodes, the growth of meristems or buds and the plantlet regeneration (Dixon, ed., 1985; Pierik, 1987). The shoot-induction medium contains low concentration of sucrose (10 g/l). This is similar to the case of *Begonia venosa* callus cultures which form adventitions shoot in the presence of low sucrose concentration (Pierik, 1987). Using gellan gum as gelling agent, it was found that induction of green callus and shoot formation.

Attempt was also made to replace the gellan gum with normal agar. It was found that the shoot formation and greening of *C. sublyratus* callus on agar medium was not as good as those on gellan gum medium (Fig.28 and Table 13). It is possible that the agar used as gelling agent contains some inhibitory substances for the shoot formation . Ichi *et al.* (1986) have reported that the growth of tobacco callus, tobacco callus with

adventitions buds and pokeweed callus and shoot formation of cabbage are more enchanced on the gellan gum medium than the agar medium. In addition, gellan gum has the effect on plaunotol accumulation in the callus cultures of *C. sublyratus* Kurz (Morimoto and Murai, 1989; ธราธร บุญแก้ว, 2534).

When the yellowish and soft calli of *C. sublyratus* (Fig. 24) were transferred onto the shoot-induction medium directly, it formed green calli but did not regenerate shoots (Fig. 29). This may be due to the loss of regenerative capacity of the callus upon subculturing for a long period of time (more than a year) (Pierik, 1987).

Manipulation of hormonal factors by increasing the concentration of BA and GA_3 in MS gellan gum medium to 2.0 mg/l BA and 0.5 mg/l GA_3 could also induce the multiple shoot formation from the leaf-induced callus (white callus). However, the percentage of the shoot formation and the number of shoots decreased when the concentration of BA and GA_3 was increased. These results indicate that the induction of shoot formation is not necessary to start from the green callus. Instead, the white callus which is obtained directly from leaf segments can be induced directly to form shoots. Therefore, the step for establishment green callus can be omitted for the shoot regeneration via callus. However, the establishment of green callus could proliferate much tissues of *C. sublyratus* which seem to be better source for the shoot induction than the white callus obtained from the leaf segments.

Although the rooting from the regenerated shoots was not observed on the hormone-free MS medium (Fig. 30), it had been reported that rooting is observed in this medium of *Rehmannia glutinosa* (Shoyama *et al.*, 1983).

We hope that further manipulation of hormonal factors will lead to the better conditions for rooting. It is expected that the information will lead eventaully to the successful micropropagation of this plant.

CONCLUSION

From this research work of "Quantitative Analysis of Plaunotol in the Leaves and Tissue Cultures of *C. sublyratus* Kurz", the following conclusions can be drawn :

1. The developed TLC-densitometric method for plaunotol determination is effective, accurate and reproducible for the qualitative and quantitative analysis of plaunotol in the plant and culture crude extracts.

2. The leaves of *C. sublyratus* obtained from various sources of Thailand contain different level of plaunotol, ranging from 0.139 to 0.786% (w/w) dry weight. The majority of samples contain plaunotol in the range 0.3-0.4% (w/w).

3. Other *Croton* plants, including *C. oblongifolius*, *C. crassifolius*, *C. tiglium*, *C. caudatus*, *C. cascarilloides* and *Croton* sp. (Char plao nam ngoen) contain no detectable plaunotol in their plant parts.

4. Three types of the *in vitro* cultures of *C. sublyratus*, including callus, cell suspension and shoot regeneration via callus, have been obtained from this study.

5. Callus cultures of *C. sublyratus* can be established from the young leaf explants on MS medium containing 30 g/l sucrose, 2.0 mg/l 2,4-D, 1.0 mg/l kinetin and 0.8% (w/v) agar and subcultured on MS agar medium containing 1.0 mg/l 2,4-D and 1.0 mg/l BA. This callus culture does not produce plaunotol but can be used as starting material for shoot regeneration.

6. Cell suspension culture of *C. sublyratus* can be established from the callus cultures. The suspension culture is maintained on MS liquid medium containing with 30 g/l sucrose, 0.2 mg/l NAA and 0.2 mg/l 2,4-D on shaker at 120 r.p.m. Similarly, the cell suspension culture does not produce plaunotol.

7. Shoot regeneration of *C. sublyratus* via callus is possible through three kinds of media :

First, MS medium supplemented with 30 g/l sucrose, 2.0 mg/l 2,4-D, 1.0 mg/l kinetin and 0.8% (w/v) agar for inducing callus from leaf explants.

Second, MS medium supplemented with 10 g/l sucrose, 2.0 mg/l NAA, 2.0 mg/l BA and 0.2% (w/v) gellan gum for green callus formation and proliferation.

Third, MS medium supplemented with 10 g/l sucrose, 1.0 mg/l BA, 0.05 mg/l GA_3 and 0.2% (w/v) gellan gum for shoot formation from callus.