CHAPTER V

DISCUSSION

In order to select high sennosides-containing plants of Thai *Cassia angustifolia* for being used as a starting material for establishing cell cultures, a number of pod samples were first collected from various parts of Thailand. *C.angustifolia* normally grows at the middle parts of the country. Presently, there are only a few local plantation sites of this plant since the price of the product is not so attractive. In this study, the pod samples were obtained from 3 provinces including Lop-Buri, Bangkok and Nakohn Ratchasima representing the central and the north-eastern parts of Thailand.

For effectively evaluation a large number of the raw meterial of senna pods, it is necessary to have a simple, accurate and rapid method for estimation of their sennosides content. So far, several methods for quantitative analysis of sennosides have been reported (Saad, et al., 1972; Srivastana et al., 1983; Duez et al., 1984). The official methods described in the BP (British Pharmacopoeia, 1988) and USP (The United States Pharmacopoeia, 1990) required hydrolysis of the glucosides before determination of the total

anthraquinone (anthracenic content) and are, therefore, very time consuming. A number of HPLC method have been used to separate and quantitate nanogram amount of sennosides in plant extract (Gorler et al., 1979; Srivastana et al., 1983; Ohshima and Takahashi, 1983; Dence and Huizing, 1981). These methods are precise and sensitive but usually require a step of pre-purification of the extracts before quantitation and as a consequence, the overall procedure is still time consuming. For the radioimmunoassay (Atzorn, et al., 1981) although it is sensitive, specific and rapid, the method is expensive and requires a process for synthesis of sennoside protein conjugates and radioactivelly labelled tracers.

In contrast, our method developed for the determination of pod sennoside content is much simpler. The method involves solid-phase extraction followed by direct spectrophotometric determination. The advantages of this method are that it is rapid, high efficiency, high sensitivity, reliability and reproducibility. The process of solid-phase extraction allowed complete separation of the sennosides from other impurities present in the crude extract (Fig. 26). This sennoside fraction contains mainly sennoside A and sennoside B as detected by HPLC (Fig. 27). Consequently, the eluate obtained after the solid-phase extraction can be determined directly for sennoside content by simple spectrophotometric method. The whole analytical process takes about 1 hr for every sample

compared with the BP method which took at least 4 hours for each sample. Moreover, the step of solid-phase extraction can handle 24 samples simultaneously allowing large number of samples be determined at a time. Furthermore, the method is sensitive enough to detect sennosides at as low concentration as 35 μg/ml and the standard curve shows linearity up to 500 μg/ml (Fig. 38).

Based on the solid-phase extraction/spectrophotometric method, sennoside content in *C. angustifolia* pods obtained from different sources appeared to be varied, ranging from 2.38% to 5.10% dry weight (Table 13). By average, the content is 3.80 ± 0.78% dry weight. This is considerably higher than the specification of BP (2.2%) or USP (2.5%). Thai *C. angustifolia* is therefore considered superior with respect to sennoside content.

Using *C. angustifolia* plant containing highest content of total sennoside, callus culture of this plant could be established sucessfully in B5 medium. Among various nutritional and hormonal factors tested, the combination of auxins and cytokinins appeared to be essential for the formation of various types of *in vitro* cultures and their ability to produce anthraquinone.

The hormonal combination of 1.0 mg/l BA and 0.5 mg/l 2,4-D can stimulate callus formation. The callus is friable and the color of callus can be related to

anthraquinones formation. The brown freshy callus was transferred from solid medium to liquid medium and continued to grow. The suspension cultures, however do not produce detectable sennosides under various tested conditions. Instead, the anthraquinones physcion and chrysophanol were detected. The presence of both anthraquinones was confirmed by TLC (Fig. 35), UV spectrum (Fig. 32) and mass spectroscopy (Fig. 33 and Fig. 34). Considering the biosynthetic pathway of sennosides, physcion and chrysophanol are the early metabolites of the pathway. (Trease, 1866) It is, therefore, likely that *C. angustifolia* cell culture can expressed its sennoside pathway up to only the early steps. Whether the remaining steps are not wholly expressed or only a few steps are still unknown.

In order to know the relationship of culture growth and the production of physcion and chrysophanol, a growth production curve was studied (Fig. 37). It appears that this tissue cultures have a very short lag phase for only one day and then an exponential growth or log phase of 10 days, foolowed by a long linear phase of 20 days before entering stationary phase. This growth characteristic suggests that *C. angustifolia* suspension cultures spend a very short time for its adaptation to fresh medium and a high growth rate can then be induced in the cultures. The high rate of increasing in biomass goes on until presumbly the nutrient depletion or a poor

environment, the suspension cultures finally reach the stationary phase. The limiting nutrient in this system is still unknown.

For anthraquinone production, the initiation of the compound biosynthesis in *C. angustifolia* is at the exponential growth phase (Fig. 37). The maximum yield is obtained on the 8th day. At the first 2 day, the quantity of anthraquinone is very low suggesting that in this peroid of time the suspension culture is in adaptation stage. The anthraquinone producting during the exponential phase suggests that the biosynthesis is closedly associated with the growth. This is in contrast to many cell cultures in which the production phase is usually associated with the linear or exponential phased. The reason of this characteristic is still unkown.

CONCLUSION

From this research work of "Tissue Culture of *Cassia angustifolia* Vahl and Quantitative Analysis of Anthraquinones in Tissue Cultures and Senna Pods.", some conclusion can be drawn. First, the pods of *C. angustifolia* obtained from various parts of Thailand contain different level of sennosides , ranging from 2.38 to 5.10% dry weight.

Second, there are many types of *in vitro* cultures of *Cassia angustifolia*, including shoot, root, callus and suspension culture, obtained from this study. The suspension culture can produce chrysophanol and physcion which are anthraquinone also found in *C. angustifolia* plant.

Third, we found that the production of the anthraquinones (chrysophanol and physcion) occur mainly during the log phase of the culture cycle.

Finally, in this research, we have developed the solid-phase extraction and spectroscopy method for the determination of sennosides and anthraquinones in *C. angustifolia* pods and suspension culture respectively in order to study the content of sennosides in various

source of senna pods and study the anthraquinones production in the suspension culture. This developed solid-phase extraction and spectrophotometric method proved to be highly effective, accurate and reproducible in the quantitative analysis of sennosides and anthraquinones in *C. angustifolia* pods and suspension culture.

This developed Solid-Phase Extraction and spectroscopy method proved to be highly effective, accurate and reproducible in the quantitative analysis of sennosides and anthraquinones in *C. angustifolia* pods and suspension culture. Furthemore, the method is simple and rapid since there is not many stem for separate sennosides or anthraquinone. And it is very comfortable for analysis of large amount of samples.