CHAPTER IV

RESULTS

1. Development of Solid Phase Extraction-Spectrophotometry for the Determination of Sennosides from Senna Pod

1.1 Solid Phase Extraction

In order to screen for *C. angustifolia* plants with high sennoside content, it is necessary to have an assay method which is simple , rapid and reliable. Among various analytical instruments, the operation of UV-VIS spectrophotometer is widely accepted to be the most convenient. However, there is a major drawback of this spectrophotometric method. That is its non-specificity and only pure solutions can be used for quantitative analysis purposes. Direct quantitation of an active constituent in a crude extract using the spectrophotometric method is impossible because there are a number of compounds that can interfere the absorption of the compounds of interest. Despite this drawback attempts were made in this study to use the spectrophotometric method for the analysis of total sennosides in senna pods. This was started by a preliminary study on the chemical constituents of crude sennoside extracts prepared from

the pods. Based on TLC system (Solvent system : Methanol : ethyl acetate : H_2O, 4:4:2, Stationary phase, silica gel plate) it was found that there are a number of compounds present in the crude extracts (Fig. 25). Among these compounds, sennoside A and sennoside B appeared to have the lowest Rf values (0.39 and 0.32, rospectively).

The technique of "solid-phase extraction" has been widely used for cleaning crude extracts before HPLC analysis (Erni, et al., 1976; 1978). The main purpose of the solid-phase extraction was to get rid of impurities which may damage the column. However, if the preparation obtained after solid-phase extraction contain only a major compound or a group of compounds of interest, it can be determined directly by using spectrophotometric method. Based on the TLC pattern of crude sennosides extract (Fig. 25), attempts were made to get rid of the compounds having the Rf values higher than 0.39 so that only sennoside A and B present in the clean extract. This is the aim of the development of the solid-phase extraction in this study which is explained in the next section.

1.2 Solvent System for Solid Phase Extraction

In the development of solid-phase extraction-spectrophotometric method for the determination of total pod sennosides, it was found that the solvent system of methanol : ethyl acetate (1:1) was suitable for allowing sennosides, particularly sennoside A and sennoside B, be

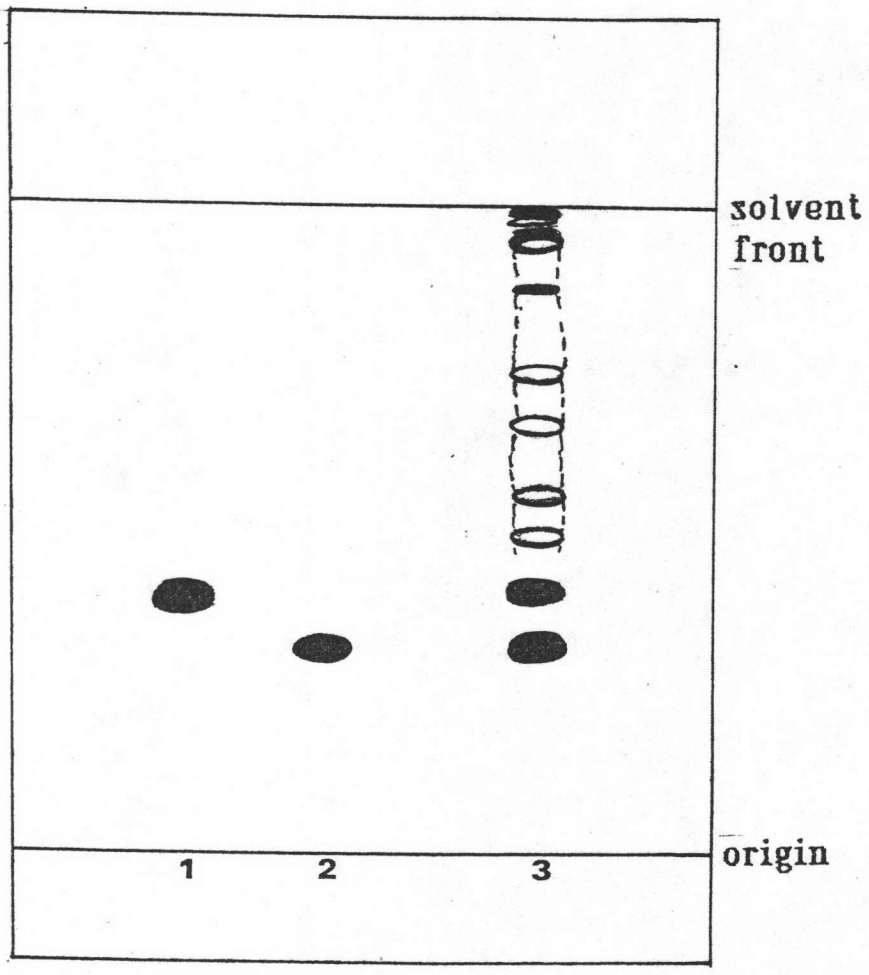


Fig. 25 TLC separation on silica gel plate of a methanolic extract of *C. angustifolia* pods (3). The plate also shows the relative mobility of sennoside A (1) and sennoside B (2). The solvent system used was methanol : ethyl acetate : H_2O, 4:4:2

bound to the column and leting other impurities be washed out of the column. To elute the sennosides out of the column, pure methanol used and complete elution was found to be within 12 ml. The results of TLC analysis of each fraction are shown in Fig. 26.

The selectivity of the solid-phase extraction was also evaluated by HPLC. As shown in the HPLC chromatograms obtained from the crude sennosides extract (Fig. 27C) and the preparation obtained after the solid-phase extraction (Fig. 27A), it can be seen that sennoside A and sennoside B are essentially, present in the solid-phase treated extract. As the results, a direct spectrophotometric method could potentially be used directly for the determination of total pod sennosides.

1.3 Percent Recovery of Sennosides

To evaluate the sennoside recovery of the developed solid-phase extraction, a solution of pure sennoside B was used. In doing this the absorbance (at 360) of sennoside solution was read before loaded onto and after eluted from the silica gel minicolumn. The results showed essentially 100% recovery of sennoside B from two separate experiments (first experiment, A_{360} before loading = 0.459 and after elution = 0.462 ; second experiment, A_{360} before loading = 0.646 and after elution = 0.653.

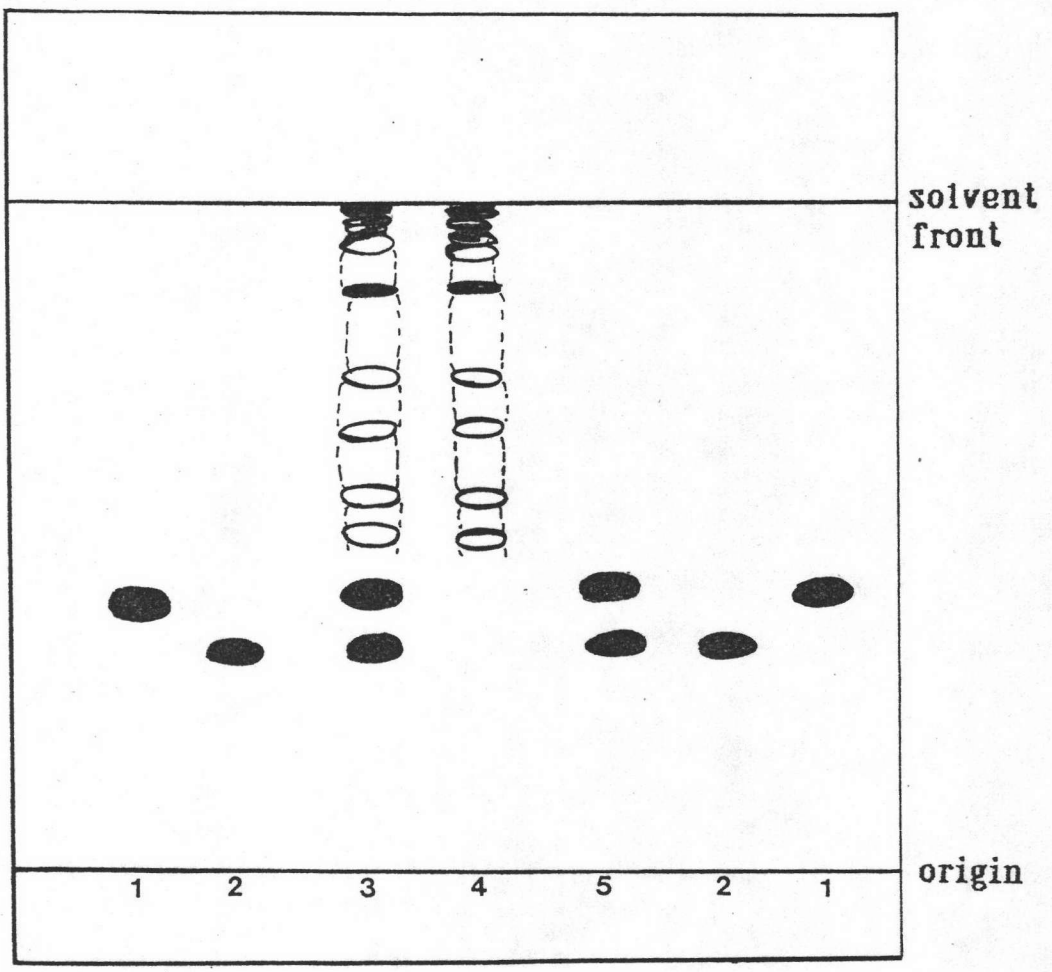
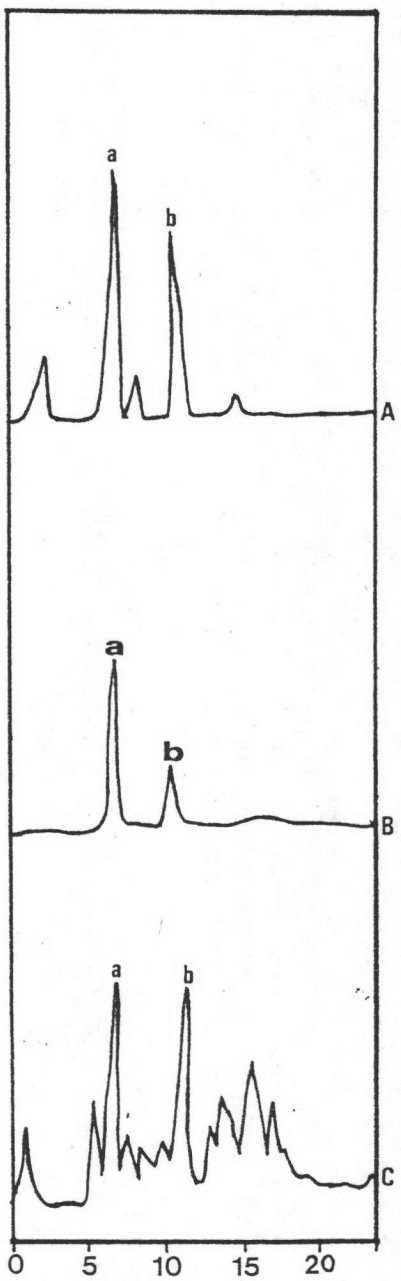


Fig. 26 Effect of the solid-phase extraction on the cleaning of crude methanolic extract of *C. angustifolia* pods. (1):standard sennoside A, (2):standard sennoside B, (3):TLC pattern of crude methanolic extract, (4): TLC pattern of the pod components washed from the column, (5):TLC pattern of the pod components eluted from the column



Solid-phase treated extract

Standard sennoside A (a) and sennoside B (b)

Crude methanolic extract

Fig. 27 HPLC Chromatograms of the crude methanolic extract of *C. angustifolia* pod (C) and the extract after treated with solid-phase extraction (A). The peak positions of sennoside A (a) and sennoside B (b) are shown in the chromatogram (B).

1.4 Accuracy, Precision and Reproducibility

In order to evaluate its accuracy and precision, the developed solid-phase extraction & spectrophotometric method was compared with the official method described in British pharmacopoeia. In doing these a number of senna pod samples from various sources were analysed for their total sennosides using the two methods and the results obtained were compared. It can be seen in Table 12 that the values of sennosides content various senna pod samples which were determine by solid-phase extraction and spectrophotometry were very closed to those obtained from the British Pharmacopoeia method. The advantages of the solid-phase extraction and spectrophotometry over the BP method are that this method is much more rapid, lower cost. Furthurmore, this simple method allowed a large number of samples to be analysed simultaneously and was therefore, useful for quality evaluation of the raw material of various sources. This method is also useful for a screening program for selecting high sennosides producing plant. In contrast, the Brithish Pharmacopoeia method is tedious and time consuming since a number of steps is involved. This can cause some mistakes and effect the precision and accuracy of the results. Also, it takes a long time and a lot of solvent for the analysis.

Table 12. Comparison of total sennosides content in *C. angustifolia* pods obtained from the method of BP and solid-phase extraction & spectrophotometry method.

Source of Sample	Total Sennosides Content % w/w		T. test
	BP	Solid-Phase Extraction	
Lop-Buri	2.90 ± 0.08	2.98 ± 0.02	1.68
G-PO Bangkok	4.36 ± 0.34	4.37 ± 0.05	0.05
G-PO Nakohn Ratchasima	3.60 ± 0.31	3.68 ± 0.01	0.44
Lop-Buri	4.20 ± 0.10	4.12 ± 0.02	1.35
Lop-Buri	5.23 ± 0.30	5.10 ± 0.10	0.71
Lop-Buri	3.72 ± 0.11	3.69 ± 0.21	0.20
G-PO Bangkok	3.33 ± 0.16	3.38 ± 0.11	0.53
Lop-Buri	3.62 ± 0.08	3.65 ± 0.13	0.34
Thai Com.Co.	2.38 ± 0.51	2.54 ± 0.14	0.54
GPO	3.07 ± 0.24	2.88 ± 0.09	1.28

Lop-Buri = samples from Lop-Buri province

GPO = samples obtained from Government Pharmaceutical Organization

Thai Com.co. = samples obtained from Thai Commonity Company.

Each method each sample was analysed in triplicate. The data showed mean value and standard variation.

In term of precision it can be said that the solid-phase extraction spectrophotometry method gave very precise results. As shown in Table 12, the three separate determination of each senna pod samples showed a very narrow standard deviation of each sennoside content value, as compared with the BP method. These results indicate that the accuracy and precision of the solid-phase extraction and spectrophotometric method in the determination of pod sennosides are reliable.

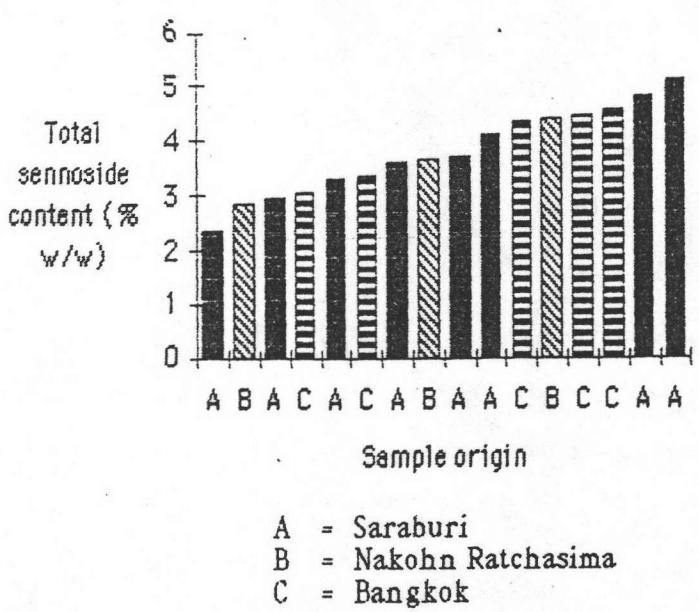
2. Sennoside Contents in the Pods of *C. angustifolia* from Different Sources

Based on the developed solid-phase extraction & spectrophotometric method, sennoside content in the *C. angustifolia* pods obtained from different sources was determined. It was found that various pod samples obtained from 4 provinces of Thailand, there were variation in the total pod sennoside content ranging from 2.38% to 5.10% of dry weight (Table 13). Among these 16 samples, sample numbers 15 and 16 from Lop-Buri gave the highest content. For average, the pod sennoside content of Thai *C. angustifolia* was found to be 3.80 $\pm$ 0.78% dry weight.

Table 13 Sennoside Contents in the Pods of *C. angustifolia* from Different Sources

No	source of sample	total sennosides content % w/w
1	Lop-Buri	2.38
2	Nakohn Ratchasima	2.88
3	Lop-Buri	2.98
4	Bangkok	3.07
5	Lop-Buri	3.32
6	Bangkok	3.38
7	Lop-Buri	3.62
8	Nakohn Ratchasima	3.68
9	Lop-Buri	3.69
10	Lop-Buri	4.12
11	Bangkok	4.37
12	Nakohn Ratchasima	4.43
13	Bangkok	4.46
14	Bangkok	4.58
15	Lop-Buri	4.84
16	Lop-Buri	5.10

Fig. 28 Variation of sennoside content in *C. angustifolia* pod obtained from different sources.



A = Saraburi
B = Nakohn Ratchasima
C = Bangkok

3. Effect of Hormonal Factors on Callus Formation of *Cassia angustifolia*

Based on the results shown in Table 13, the seeds obtained from the high sennoside producing plants were collected and used for producing seedlings. The young leaves of the seedlings were then used for induction of callus. The ability of young leaf explants of *Cassia angustifolia* (Fig. 29A) to form callus was investigated by changing the type and concentration of growth regulators in standard B5 and MS media. From 36 different recipes of the tested media (Table 11), it was found that B5 medium is generally more effective than MS media in inducing callus information from the leaf explants. Especially B5 medium supplemented with 1.0 (mg/l) BA and 0.5 mg/l 2,4-D appeared to be the best recipe to initiate callus formation (Fig. 29B). For other hormonal composition, the media showed different directions of organogenesis (Table 14). In general, NAA was found to induce the explant to initiate root (Fig. 29C), IAA to initiate shoot (Fig. 29D) and 2,4-D to initiate callus (Fig. 29B). None of the hormonal composition tested was found to initiate plantlets formation. The callus formation in the best medium appeared to be friable with pale brown color (Fig. 29B). This callus could be maintained by a regular 3 weeks subculturing.

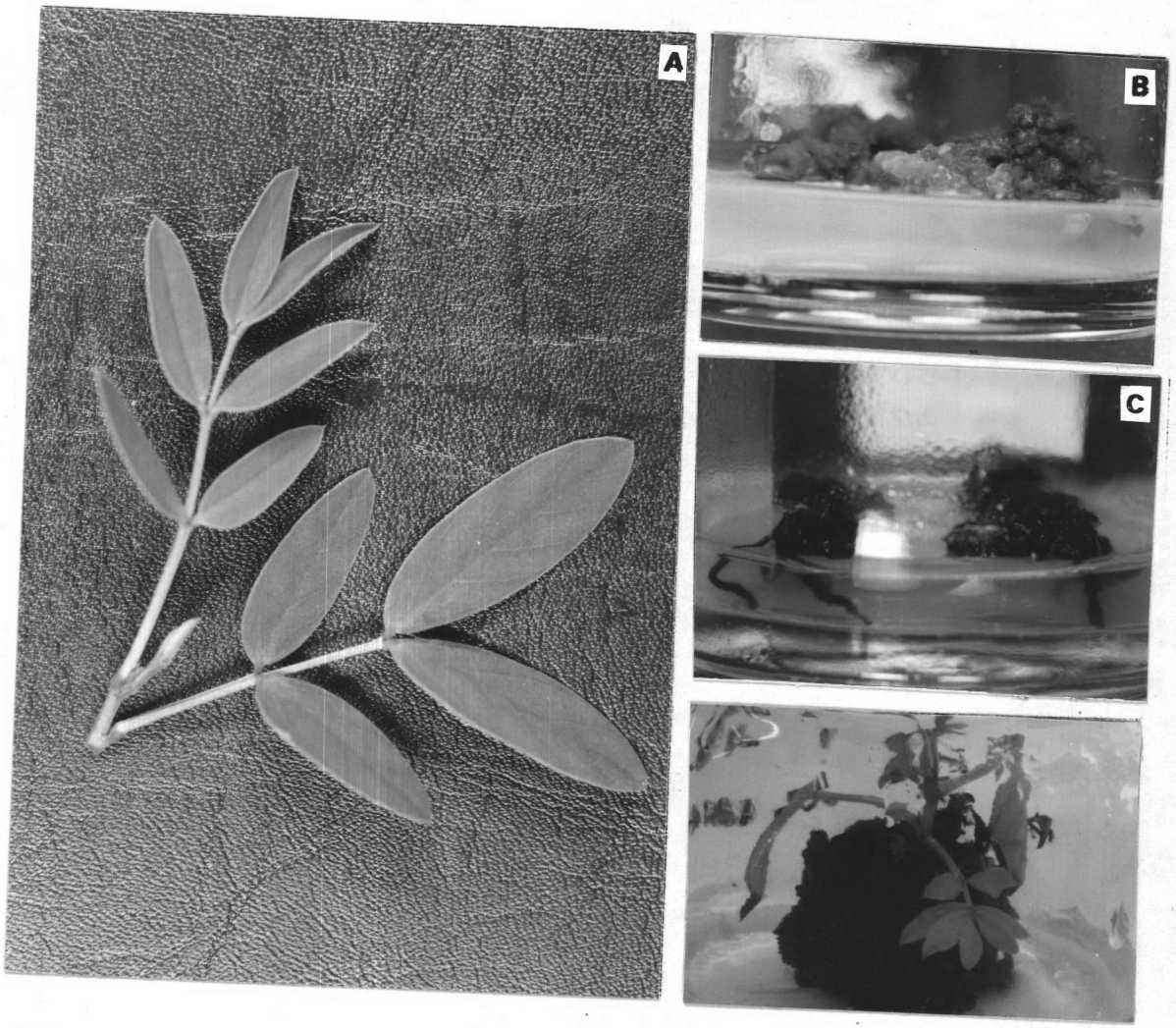


Fig. 29 A. the young leaf of *C. angustifolia* used as an explant for initiating various tissue cultures

B. Callus culture grown in B5 medium supplemented with 0.5 mg/l 2,4-D and 1.0 mg/l BA.

C. Callus culture forming root in B5 medium supplemented with 0.5 mg/l NAA and 1.0 mg/l kinetin

D. Shoot culture in MS medium supplemented with 0.5 mg/l IAA and 1 mg/l kinetin

4. Establishment of *C. angustifolia* cell suspension cultures

The pale brown callus cultured on B5 solid medium supplemented with 1.0 mg/l BA and 0.5 mg/l was used for initiating cell suspension cultures. In doing this, the young friable and soft callus was saparated into small aggregates before tranfering to B5 liquid medium containing the same hormonal composition. Under these conditions, however, the suspension culture showed a slow growth rate, and appeared to be fragile, the cell lysis caused the liquid medium being brownish. Most of the culture cells were in big aggregates and also turned to dark brown color (Fig. 30).

Attempts were then made to improve cell integrity of the suspension cultures. It was found that the higher temperature of approximately 30°C could support better culture growth than the normal 25°C. This temperature also decreased the cell damage. Subsequently, the time interval of 2-week subculturing was reduced to 7 days interval. This was found to be able to decrease the cell damage. After subculturing for a few passages, a stable suspension was obtained which showed small aggregates with healthy cells.

Table 14 Effect of hormonal factor and different media on the differentiation *Cassia angustifolia* of leave explants.

Medium cytokynin / auxin(mg/l)	Standard MS		Standard B5	
	Kinetin 1.0 mg/l	BA 1.0 mg/l	Kinetin 1.0 mg/l	BA 1.0 mg/l
NAA 1.0	R	C	R	C
NAA 0.5	S	C	R,C	C
NAA 0.1	-	-	-	C
IAA 1.0	-	-	-	C,S
IAA 0.5	S	S	-	-
IAA 0.1	-	C	R	S,C+
2,4-D 1.0	-	-	C	C+
2,4-D 0.5	-	-	C++	C+++
2,4-D 0.1	-	-	R	C+
No auxin	-	S,C+	-	S,C+

Abbreviation S = shoot
R = root
C = callus (the amount of callus is expressed by + symbol)
- = no development was observed

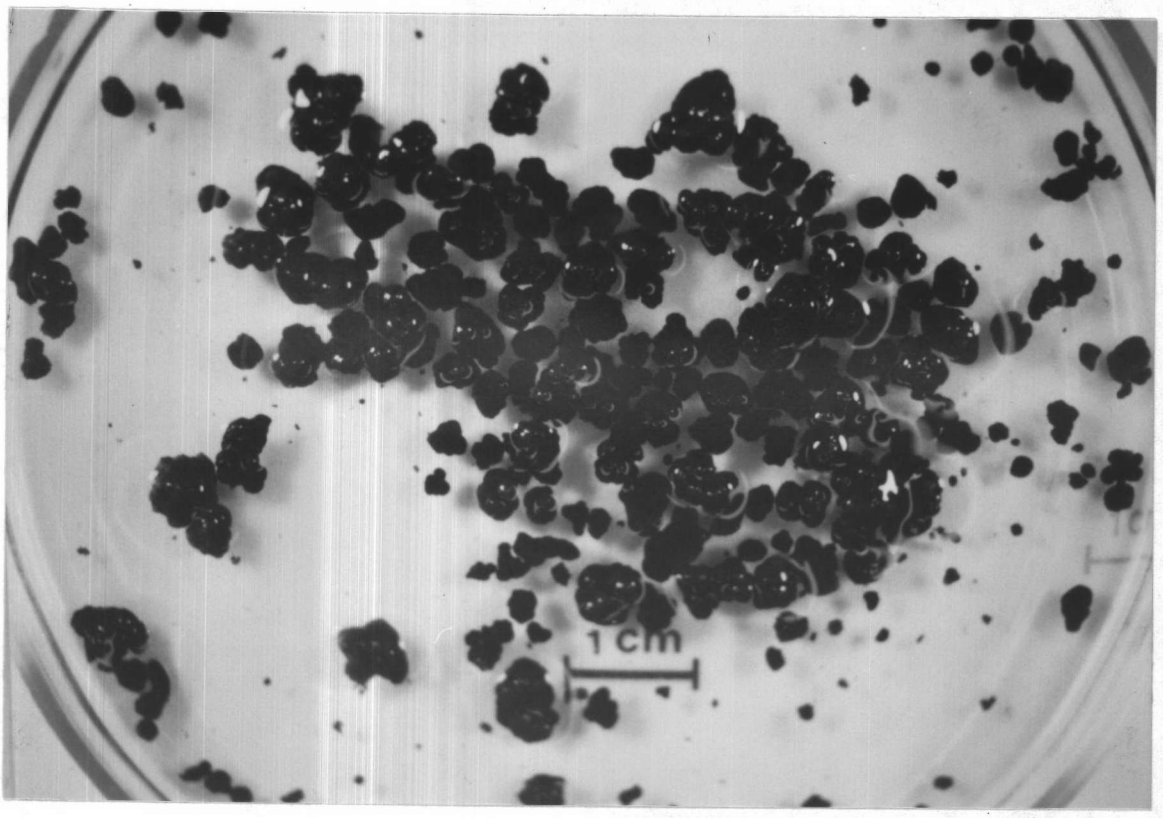


Fig. 30 Suspension cultures of *C. angustifolia* established in B5 media supplemented with 1 mg/l BA and 0.5 mg/l 2,4

5. Detection of Anthraquinones in Suspension Cultures of *Cassia angustifolia*

Simple phytochemical tests was first performed to determine compounds accumulated in *C. angustifolia* and the results were compared with those obtained from *C. angustifolia* pods. It can be seen that among chemical groups tested, only anthraquinones were detected positively in the suspension cultures (Table 15). In the pods, cyanogenic and cardiae glycosides and flavonoids were found positive in addition to the anthraqninones. Further study with thin layer chromatography showed that TLC plate of the chloroform extract, not methanolic extract, contained spots which gave positive reaction with the Borntrager test. Among various standards used in same plate (including sennoside A, sennoside B, aloe-emodin, rhein, crysophanol and physcion), the positive spot showed the same Rf values with physcion and chrysophanol (Fig. 31).

To confirm that the anthraquinones detected in the chloroform extract were physcion and chrysophanol, the UV-spectra of the two unknown anthraquinones were constructed using TLC densitometer and the resulted spectra were compared with the spectra of physcion and chrysophanol standards. The results showed that the UV-spectra of the unknown were very similar to those both standards (Figs. 32 A and B).

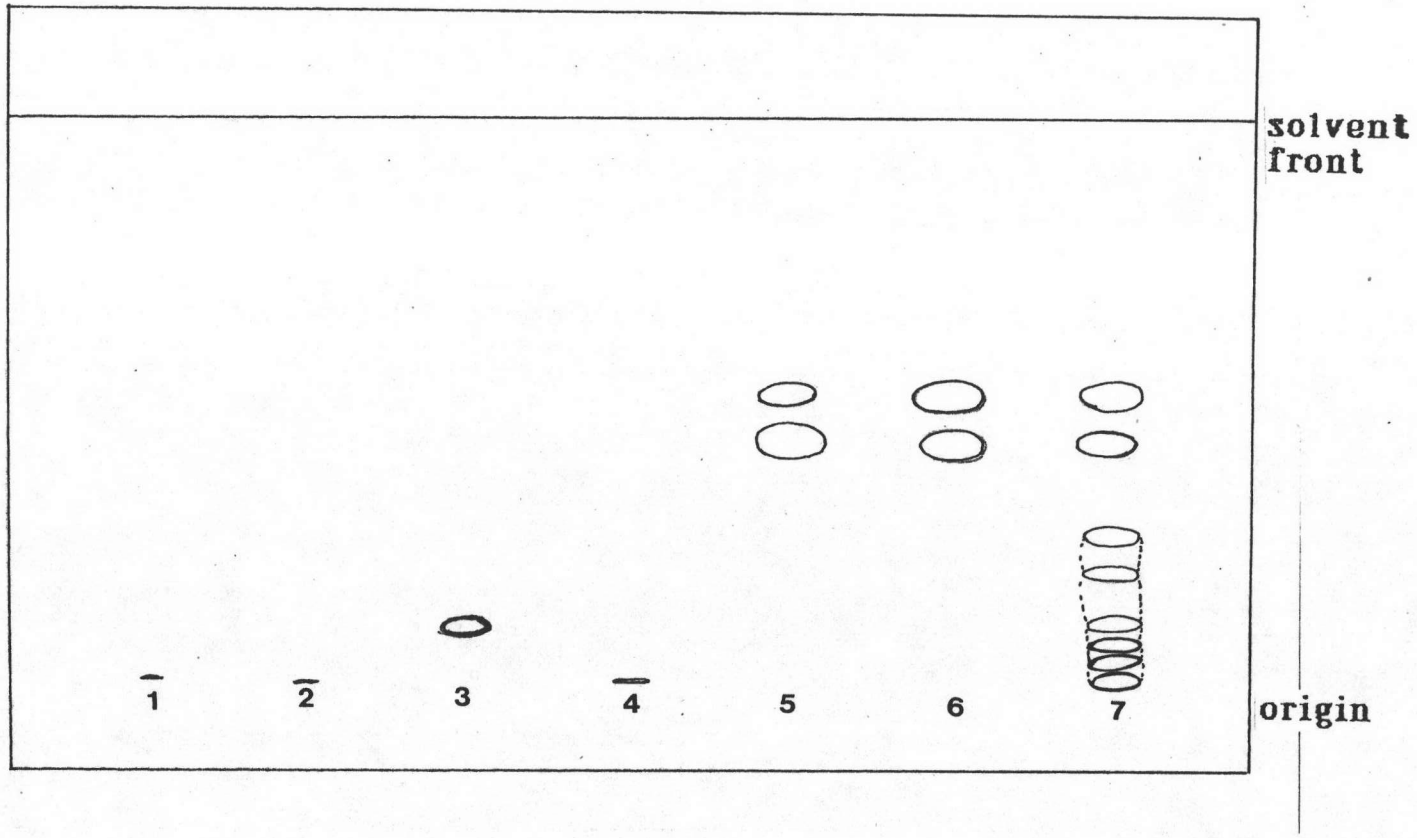


Fig. 31 TLC separation on a silica gel plate of petroleum ether extract of *C. angustifolia* suspension culture (7). The TLC plate also shows the relative mobility of sennoside A (1), sennoside B (2), aloe-emodin (3), rhein (4), physcion (5), chrysophanol (6). The solvent system used is Petroleum ether : Ethyl acetate 9:1

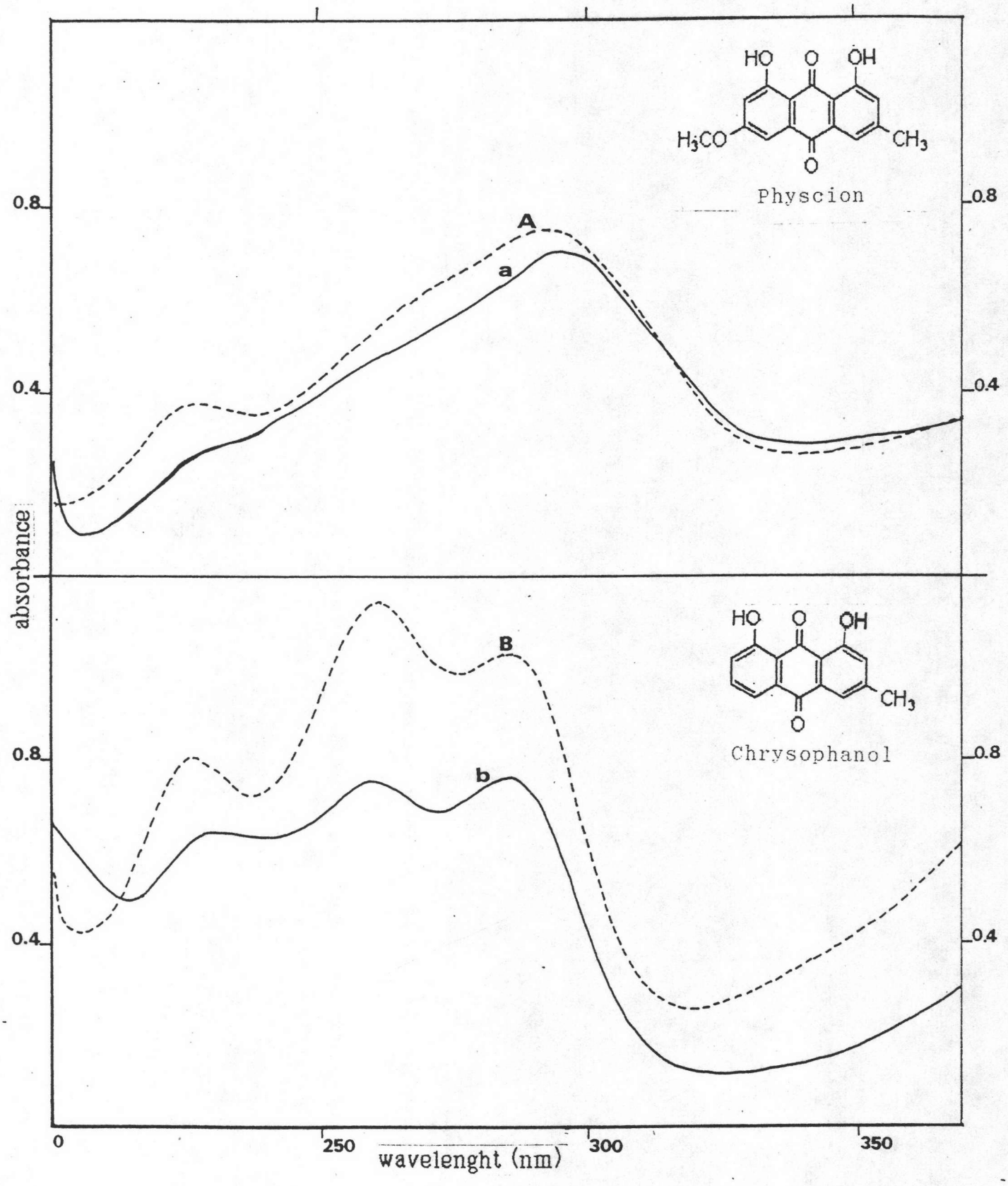


Fig. 32 UV-absorption spectra of anthraquinones produced by *C. angustifolia* suspension culture. A) the anthraquinone spectrum with similar absorption spectrum to standard physcion (-----) and B) the anthraquinone spectrum (———) with similar absorption spectrum to chrysophanol (-----)

Moreover, both anthraquinones were isolated from the chloroform extracts by preparative TLC and the compounds were subjected to mass spetroscopy. As shown in (Figs. 33 and 34), their mass spectra were corresponded to the molecular mass of physcion and chrysophanol.

Table 15 Phytochemical screening of *Cassia angustifolia* pods and suspension cultures for various chemical groups.

Phytochemical test	Pods	Cell Culture
1. Cyanogenetic glycosides	+	-
Sodium picreate paper test		
2. Cardiac glycoside		
Kedde' test (unsaturated lactone ring)	+	-
Liebermann Burchard test (steroid nucleus)	+	-
Keller-Liliani test (deoxy-sugar)	-	-
3. Alkaloid		
Dragendorff's reagent	-	-
Mayer's reagent	-	-
Valser's reagent	-	-
Wagner's reagent	-	-
Marme's reagent	-	-
Hayer's reagent	-	-

Table 15. (Continued)

Phytochemical test	Pods	Cell Culture
4. Tannin		
gelatin solution	-	-
Ferric chloride	-	-
Bromine water	-	-
5. Flavanoid		
Cyanidin test	-	-
Leucoanthocyanin	+	-
6. Anthraquinone		
Modified Borntrager test	+	+

\+ refered to positive result

\- refered to negative result

6. Determination of Anthraquinone Content in *C. angustifolia* Cell Cultures.

In order to determine the content of the anthraquinones chrysophanol and physcion produced by *C. angustifolia* suspension cultures, the techniqne, again, of solid-phase extraction and UV-spectrophotometry were used. However, the solvent system used for the solid-phase extraction of sennoside could not be used for preparing chrysophanol and physcion. A new solvent system was, therefore, developed for such a purpose, it was

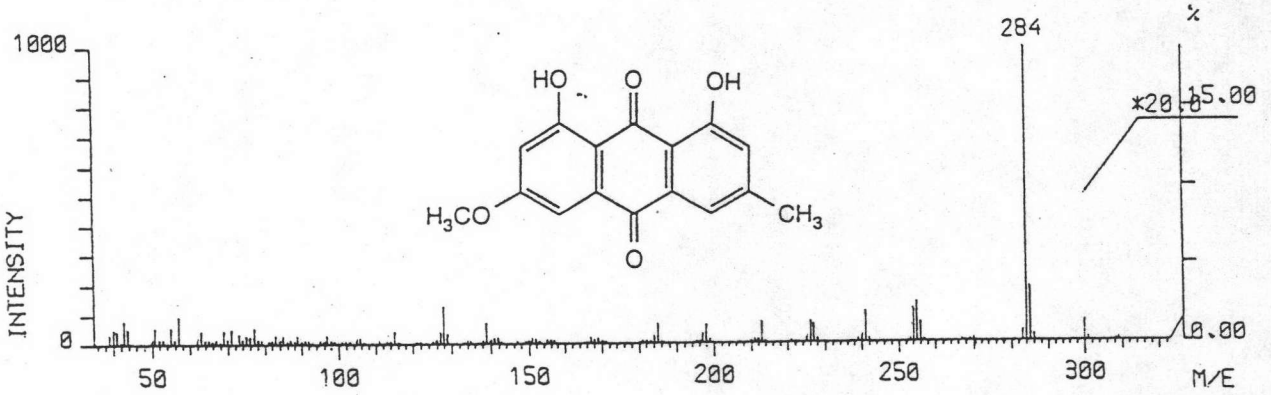


Fig. 33 Mass spectrum of physcion

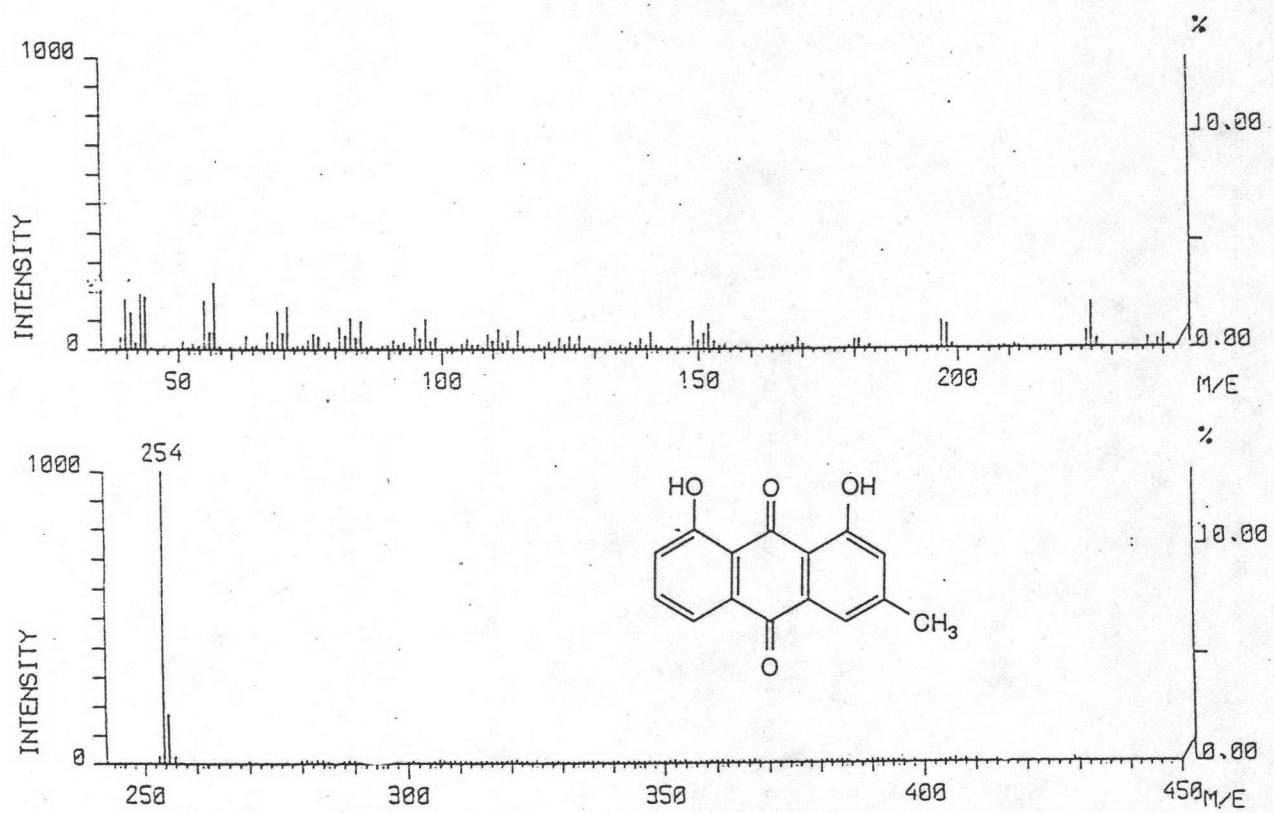


Fig. 34 Mass spectrum of chrysophanol

found that when one part of crude petroleum ether extract of *C. angustifolia* cultures was mixed with nine parts of ethyl acetate and load onto a silica gel minicolumn, chrysophanol and physcion are the extract appeared to pass through the column (Fig. 35). Other impurities, on the other hand, were trapped in the column. TLC analysis clearly support this phenomena (Fig. 25). The complete separation of the two anthraquinones from other compounds allowed the eluate be determined directly by spectrophotometer using the wavelength of 290 nm.

Using the spectrophotometric method, a standard curve of chrysophanol was constructed. It showed linearity within the concentration range of 0.1 to 6.0 µg/ml (Fig. 36). Based on this standard curve, the content of both anthraquinones in *C. angustifolia* suspension cultures during the culture growth was determined. The results will be presented in the next section.

7. Anthraquinone Production during the Culture Growth of *C. angustifolia* Cultures

The growth of *Cassia angustifolia* cell cultures during a period of 30 days were examined by using dry weight of the harvested cell mass as a growth parameter. The resulted growth cycle (Fig. 37) showed a very short lag phase. Soon after the innoculation of the fresh cell, the suspension culture appeared to increase in their dry

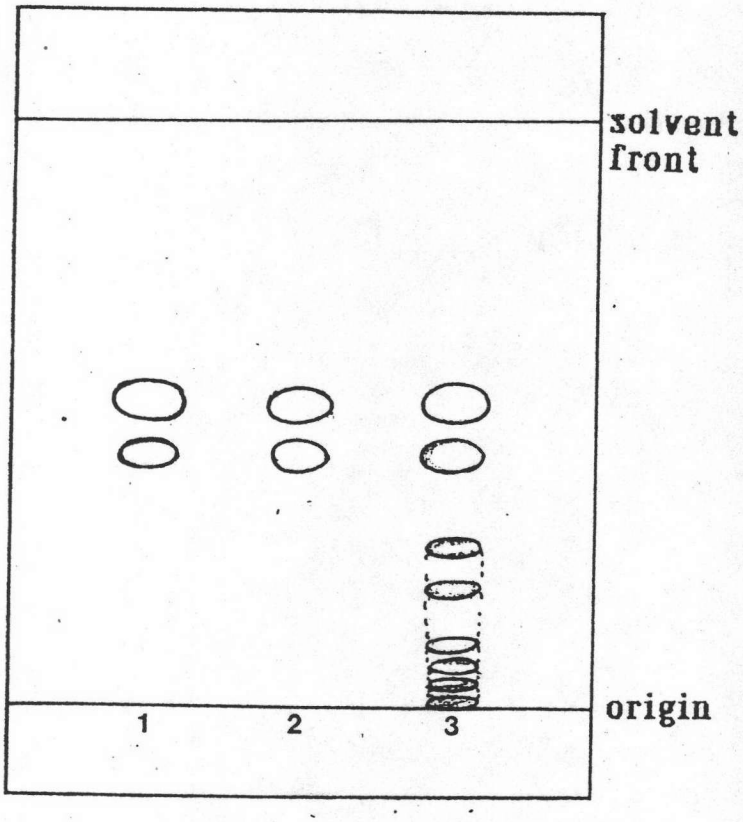


Fig. 35 TLC separtion on silica gel plate of chloroform extract of suspension culture of *C. angustifolia* solvent system : Petroleum ether : Ethyl acetate 9 : 1

1. Standard chrysophanol (1)
2. Part of Petroleum ether : Ethyl acetate 9:1 elution (2)
3. Before absorbent extraction (3)

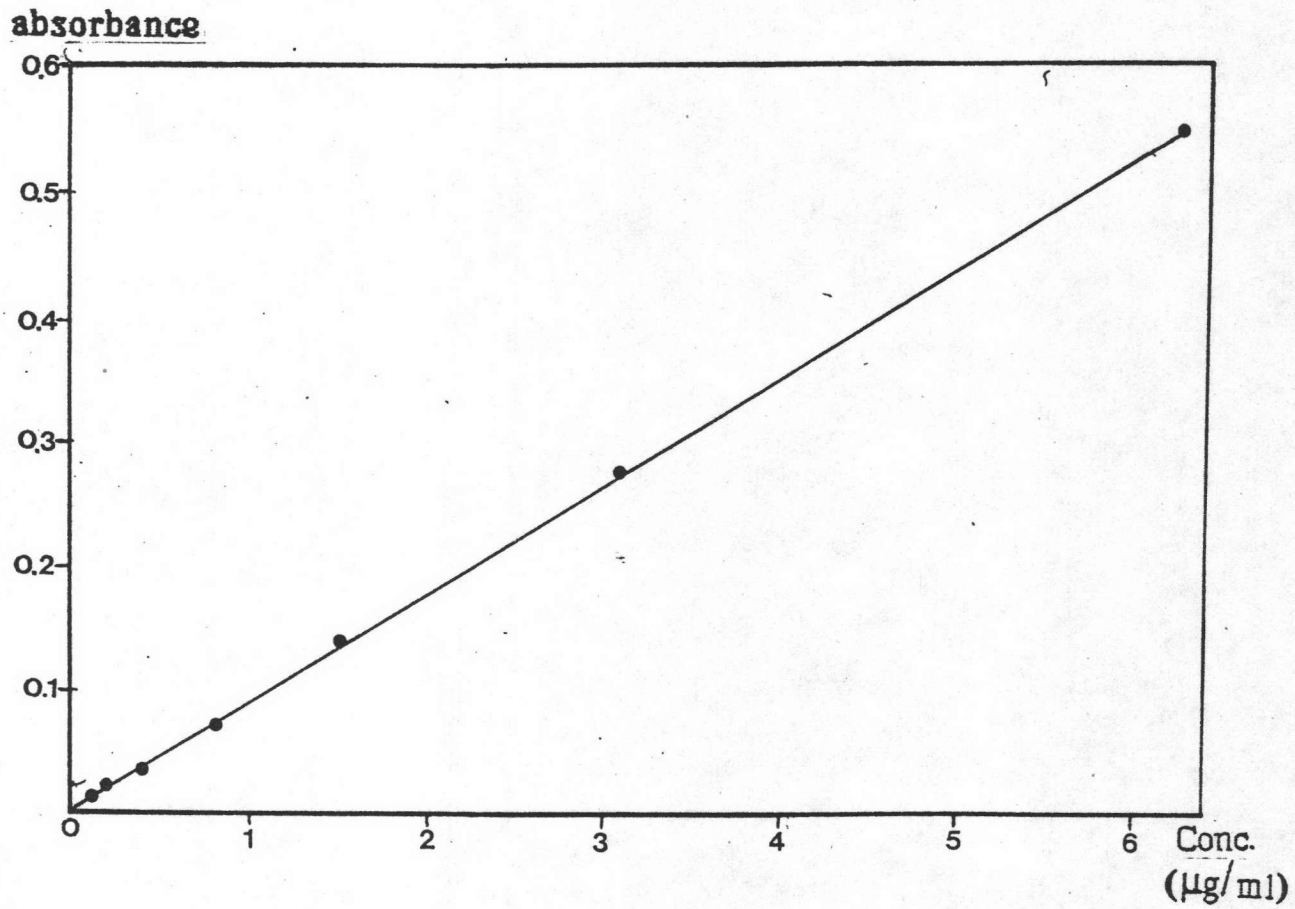


Fig. 36 Calibration curve of standard chrysophanol obtained by UV spectrophotometry

weight. The increase was continued at relatively constant rapid rate for approximately 20 days before slowing down to a zero increase rate at the day 28. There after, the culture dry weight began to decline. The highest value of biomass obtained was 0.4 g/flask (day 28-29) equivalent to approximately 8 times of the innoculated culture mass.

For anthraquinone production, it appeared that soon after innoculation the production of anthraquinone declived. After day 3 the suspension culture started to produce anthraquinones and the increase the compounds was observed for 5 days (until day 10). There after the production was declined continuously until the end of the 40 day growth cycle (Fig. 37). Based on this production curve, it can be seen that the production of anthraquinones is associated with the exponential growth phase of the culture cycle.

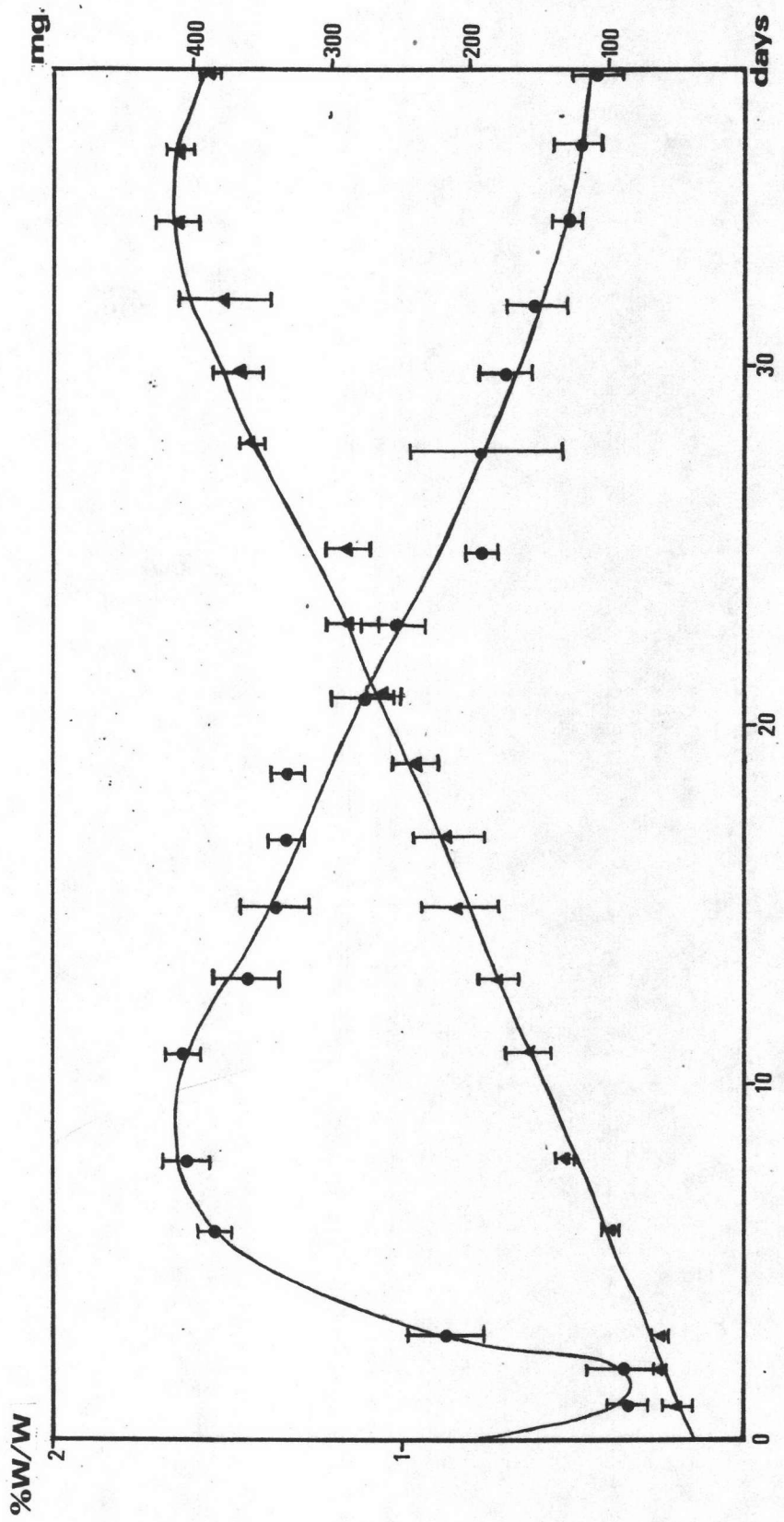


Fig. 37 Time course of dry weight (▲), and anthraquinones content in % day weight (●) for suspension culture of *C. angustifolia*

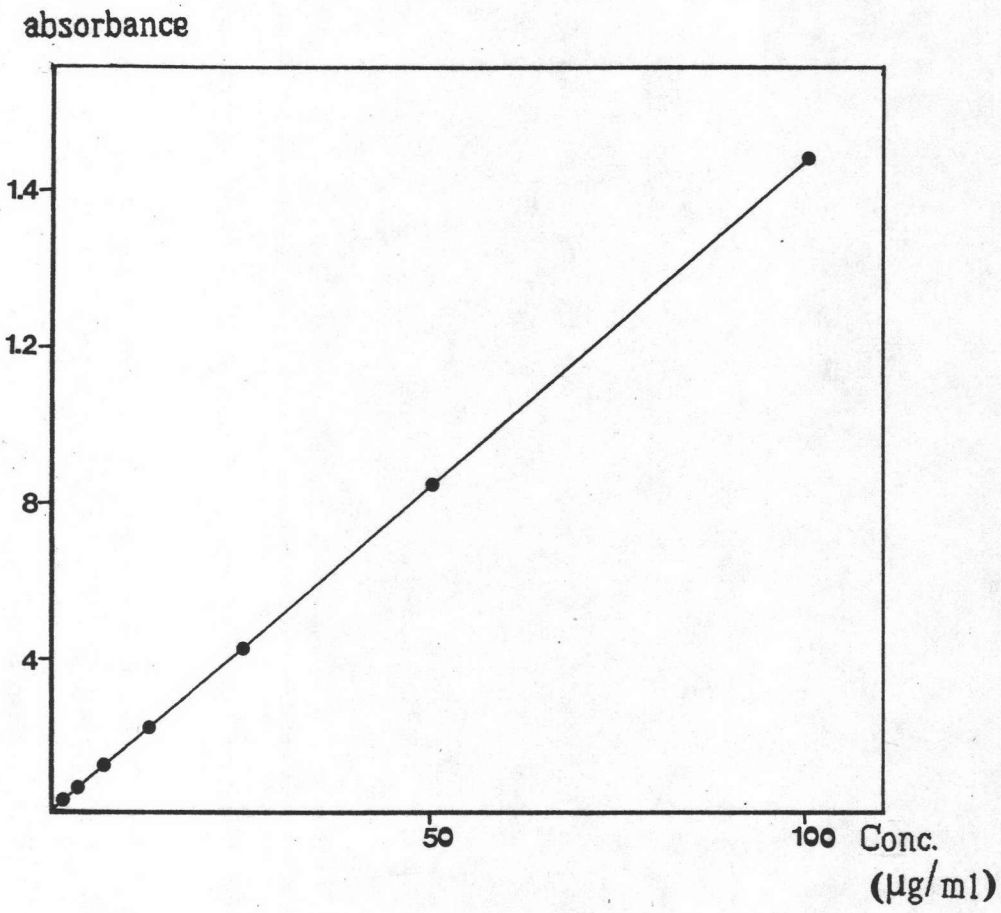


Fig. 38 Calibration curve of standard sennoside B obtained by UV spectrophotometry λ_{max} 290 nm.