CHAPTER 4

RESULTS AND DISCUSSION

4.1 Selection of high yielding plants

To select the high anthraquinone producing, plants the seeds of *S. alata* that were collected from various places in Songkhla (Amphur Namom and Amphur Hat-Yai) and Narathiwas (Amphur Ruso) provinces were grown under aseptic conditions in solid hormine free B5 medium (Fig 4.1). Fifty-six leaf samples were separately harvested from each plantlets and the content of aloe-emodin and rhein were determined by the HPLC method as described in the section 3.2.3.2. The HPLC-chromatogram of the leaf extracts showed the peaks at retention time of 9 and 15 minutes which were identical to those of the authentic aloe-emodin and rhein, respectively (Fig 4.2). The contents of aloe-emodin and rhein were calculated using their calibration curves (Fig 4.3). The calibration curves were observed to be linear in the range from 4 to 64 μg/ml with r^2 more than 0.999 for both compounds.

Leaf extracts from the 56 samples of plantlets showed the HPLC chromatograms similar to the extracts from the intact plants. A variation of aloe-emodin and rhein content in *S. alata* leaves was assessed in fifty-six plantlet samples (Table 4.1 and Fig 4.4). The average yields of rhein and aloe-emodin in the samples were 0.09 ± 0.047 and 0.21 ± 0.151 %w/w, respectively. The content of aloe-emodin was usually higher than rhein. Although the plantlets (2 months) were younger than the intact plant (6 months). Nevertheless, some of them contained aloe-emodin and rhein content higher than the intact plants. The data show that five plantlets of numbers 5, 6, 8, 10, and 11 produced the highest amount of total anthraquinones (see Table 4.1) which were selected for the induction of tissue culture. Their roots were used as an initial explant for the establishment of *S. alata* root cultures.

Fig 4.1 *Senna alata* plantlets grown in solid B5 basal medium

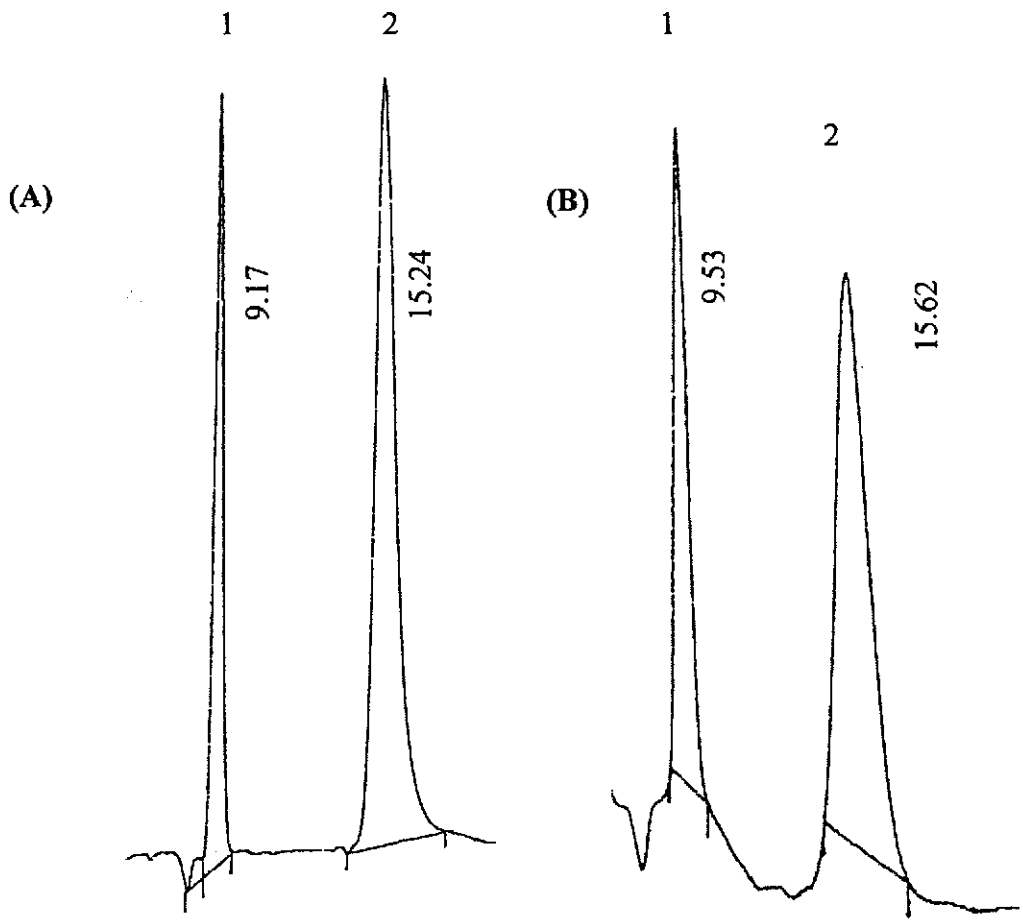
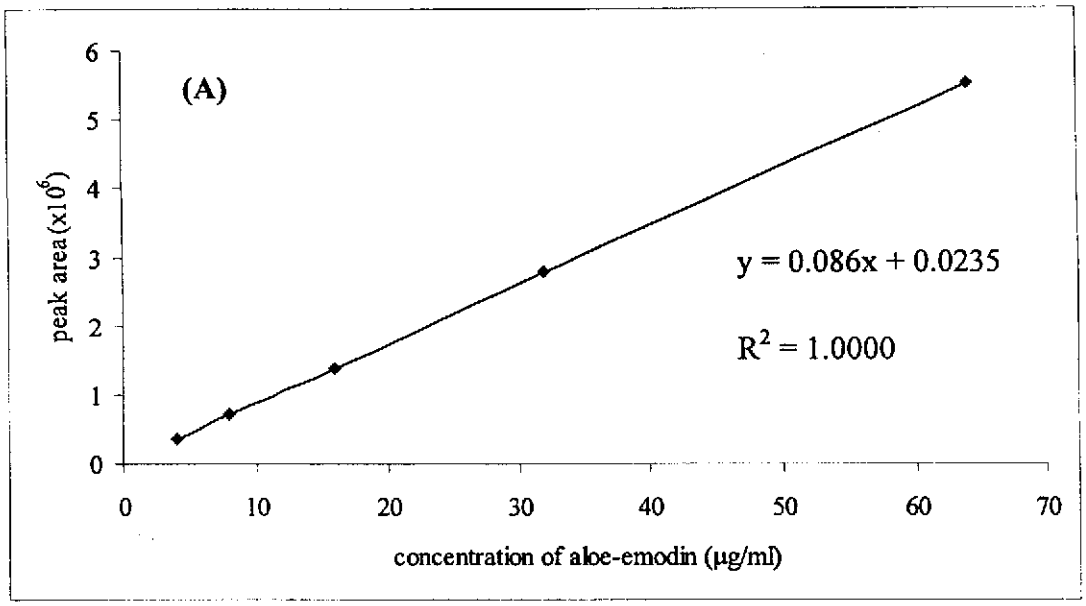


Fig 4.2 HPLC-chromatograms of; (A) the authentic aloe-emodin (RT = 9 min) (1) and rhein (RT = 15 min) (2) and (B) *Senna alata* plantlet leaf extract. The HPLC conditions are as described in section 3.2.3.2.



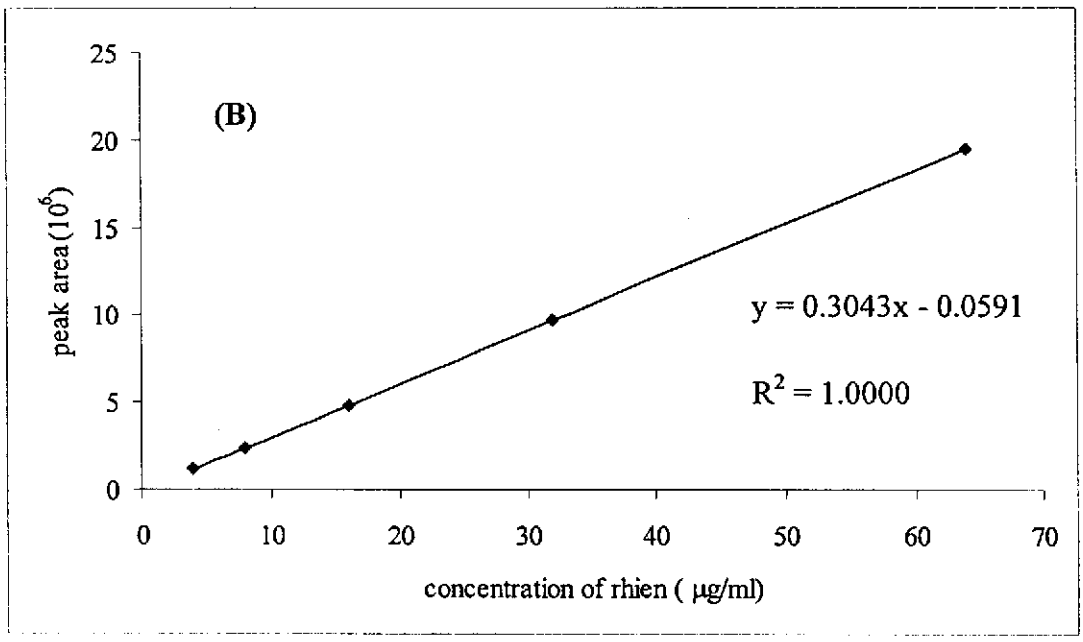


Fig 4.3 Calibration curves of aloe-emodin (A) and rhein (B). Each calibration point was a mean of three separate determination.

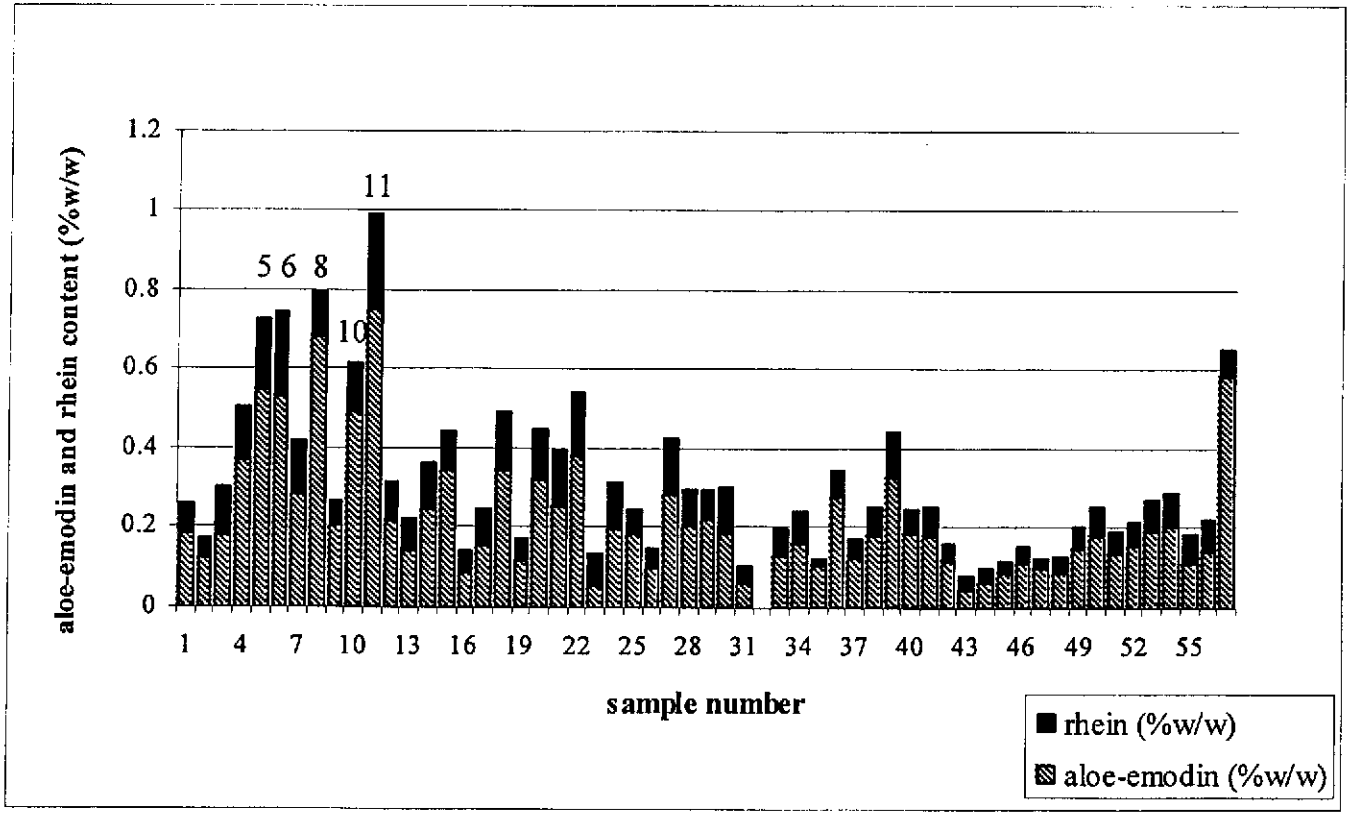


Fig 4.4 Aloe-emodin and rhein content in the leaves of *S. alata* plantlets (no. 1 – 56) and intact plant (no.57) (each data was determined of triplicate samples)

Table 4.1 Aloe-emodin and rhein content in the leaves of plantlets

Plantlet number	Aloe-emodin (%w/w ± S.D.)	Rhein (%w/w ± S.D.)
1	0.18 ± 0.001	0.08 ± 0.002
2	0.12 ± 0.005	0.05 ± 0.008
3	0.18 ± 0.000	0.13 ± 0.003
4	0.37 ± 0.001	0.13 ± 0.003
5	0.55 ± 0.004	0.18 ± 0.002
6	0.53 ± 0.004	0.21 ± 0.003
7	0.28 ± 0.002	0.14 ± 0.005
8	0.68 ± 0.009	0.11 ± 0.007
9	0.20 ± 0.005	0.06 ± 0.003
10	0.49 ± 0.009	0.13 ± 0.004
11	0.75 ± 0.008	0.24 ± 0.008
12	0.22 ± 0.006	0.10 ± 0.003
13	0.14 ± 0.002	0.08 ± 0.005
14	0.24 ± 0.005	0.12 ± 0.004
15	0.34 ± 0.002	0.10 ± 0.004
16	0.09 ± 0.007	0.06 ± 0.008
17	0.16 ± 0.005	0.09 ± 0.005
18	0.35 ± 0.006	0.15 ± 0.004
19	0.12 ± 0.008	0.06 ± 0.004
20	0.32 ± 0.007	0.13 ± 0.002
21	0.25 ± 0.008	0.14 ± 0.006

Table 4.1 (cont.)

Plantlet number	Aloe-emodin (%w/w ± S.D.)	Rhein (%w/w ± S.D.)
22	0.38 ± 0.010	0.16 ± 0.008
23	0.06 ± 0.004	0.08 ± 0.004
24	0.20 ± 0.008	0.12 ± 0.009
25	0.19 ± 0.003	0.06 ± 0.003
26	0.10 ± 0.006	0.05 ± 0.002
27	0.28 ± 0.005	0.15 ± 0.009
28	0.20 ± 0.003	0.10 ± 0.003
29	0.22 ± 0.008	0.07 ± 0.002
30	0.18 ± 0.009	0.12 ± 0.002
31	0.06 ± 0.005	0.04 ± 0.006
32	n.d.	n.d.
33	0.13 ± 0.009	0.07 ± 0.001
34	0.16 ± 0.010	0.08 ± 0.003
35	0.10 ± 0.002	0.02 ± 0.001
36	0.28 ± 0.006	0.07 ± 0.003
37	0.12 ± 0.009	0.05 ± 0.004
38	0.18 ± 0.009	0.07 ± 0.002
39	0.33 ± 0.010	0.11 ± 0.002
40	0.18 ± 0.007	0.06 ± 0.002
41	0.18 ± 0.007	0.07 ± 0.005
42	0.12 ± 0.005	0.05 ± 0.001
43	0.05 ± 0.007	0.04 ± 0.003

Table 4.1 (cont.)

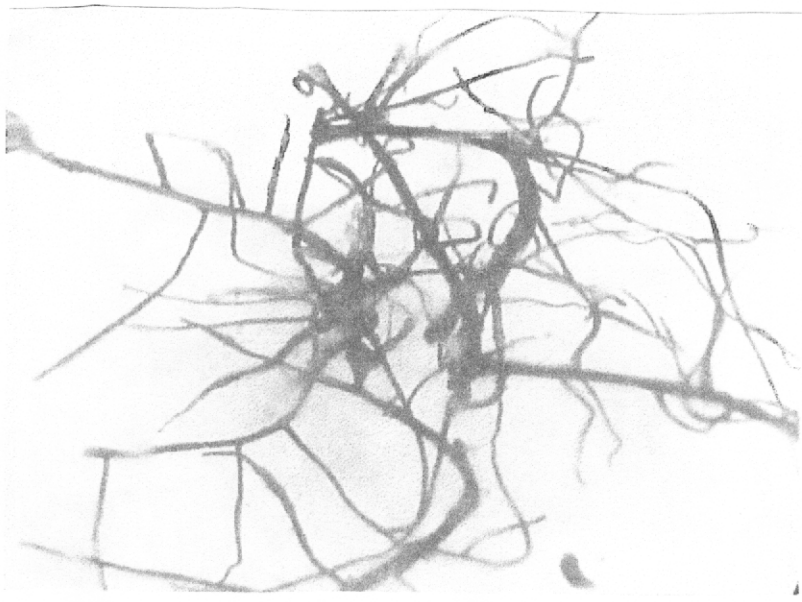
Plantlet number	Aloe-emodin (%w/w ± S.D.)	Rhein (%w/w ± S.D.)
44	0.06 ± 0.006	0.04 ± 0.001
45	0.09 ± 0.005	0.03 ± 0.001
46	0.11 ± 0.006	0.04 ± 0.002
47	0.10 ± 0.002	0.03 ± 0.003
48	0.09 ± 0.000	0.04 ± 0.003
49	0.15 ± 0.004	0.06 ± 0.001
50	0.18 ± 0.009	0.07 ± 0.002
51	0.13 ± 0.002	0.06 ± 0.003
52	0.16 ± 0.001	0.06 ± 0.003
53	0.19 ± 0.008	0.08 ± 0.002
54	0.20 ± 0.002	0.09 ± 0.002
55	0.11 ± 0.008	0.08 ± 0.003
56	0.14 ± 0.004	0.08 ± 0.002
average of plantlet no.1-56	0.21 ± 0.151	0.09 ± 0.047
Intact plant leaf	0.59 ± 0.005	0.07 ± 0.001

n.d. : can not calculated due to the area under the peak is under the lower limit of detection. Individual plantlet number data (1-56) and intact plant are a mean of triplicate determinations.

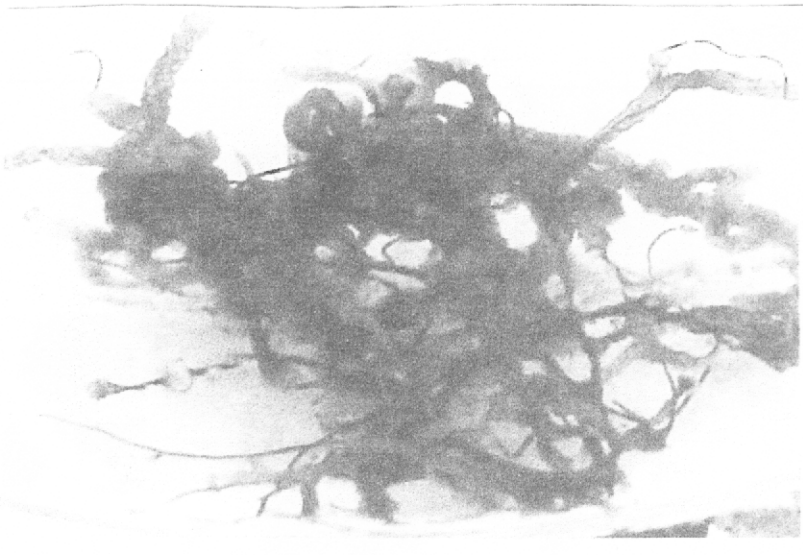
4.2 Establishment of *Senna alata* tissue cultures

The roots were cut from five plantlets containing the highest yielding of anthraquinone and used as ad explant for establishment of *S. alata* root cultures. It has been previously reported that root culture of *S. alata* was induced in the B5 medium supplemented with 1.0 mg/l NAA and 1.0 mg/l kinetin (ฐิติรัตน์ และ ณมน, 2543). However, preliminary experiment of varying the concentration of NAA in B5 medium supplemented with 1 mg/l kinetin was investigated in this study. The result show that increasing amount of NAA caused callus forming in appearance. Therefore, the B5 medium supplemented with 0.5 mg/l NAA and 1.0 mg/l kinetin was an appropriate medium for induction the root culture.

The growth of the root cultures was observed within 1 week after the initiation of the root explant in B5 liquid medium. The green cells at the root tips were observed. The old cells were changed its color to brown cell whereas the root tips were still green (Fig 4.5). The root cultures were maintained by subculturing every 4 weeks. In the 3rd subculture, the root cultures elongated with the green cells in lateral size. In the 7th subculture, the appearance of the root cultures were changed. The brown callus was formed (Fig 4.6). The callus was then scattered into small aggregate cells due to the rotation force. Suspension culture was obtained after the 7th subculture (Fig 4.7).



(A)



(B)

Fig 4.5 *Senna alata* root culture grown in liquid B5 medium supplemented with 0.5 mg/l NAA and 1.0 mg/l kinetin. (A) the 1st subculture (B) the 3rd subculture

Fig 4.6 *Senna alata* root culture (the 7th subculture) grown in liquid B5 medium supplemented with 0.5 mg/l NAA and 1.0 mg/l kinetin

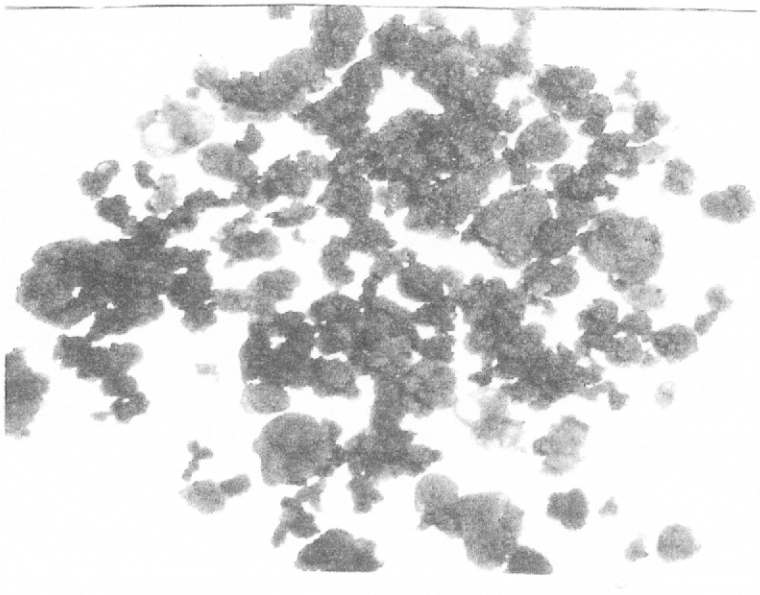


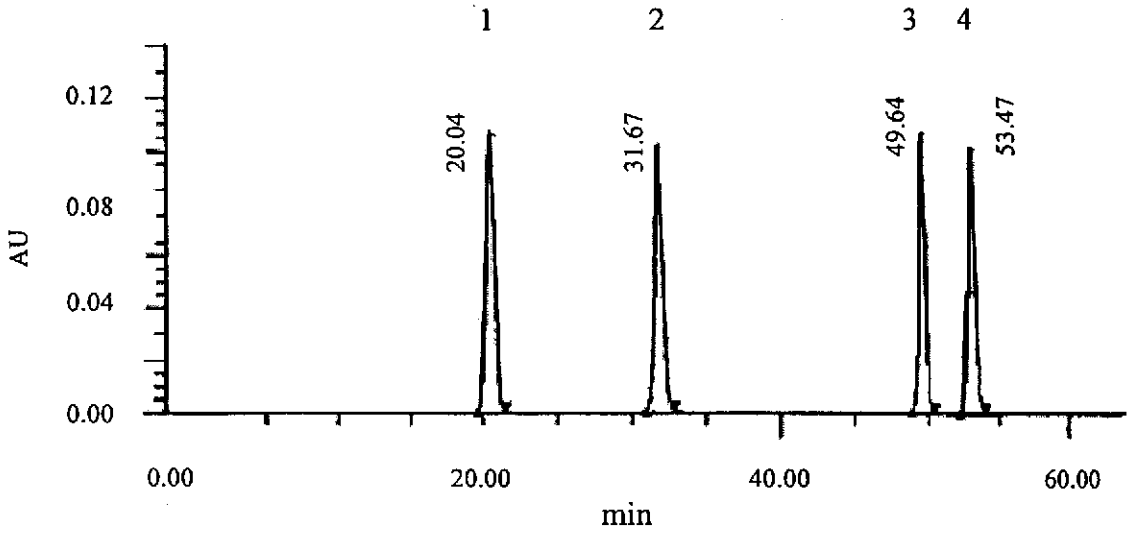
Fig 4.7 *Senna alata* cell culture (the 12th subculture)grown in liquid B5 medium supplemented with 0.5 mg/l NAA and 1.0 mg/l kinetin

4.3 Anthraquinone formation in *Senna alata* root and cell cultures

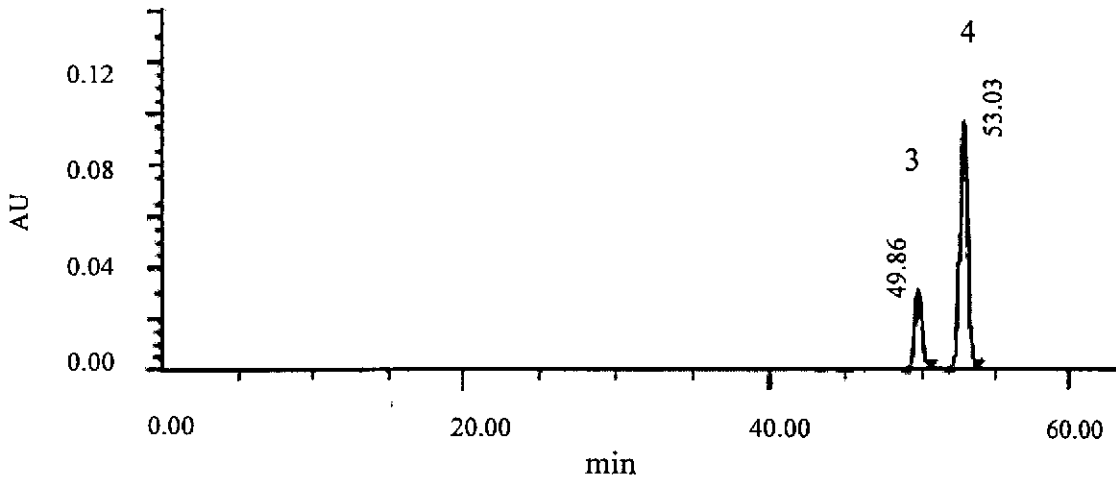
After several subcultures, the root (3^{th} subculture) and cell cultures (12^{th} subculture) of *S. alata* were examined for their potential of anthraquinones production, including aloe-emodin, rhein, emodin and chrysophanol. The extracts of the root and cell cultures were analyzed by HPLC. The HPLC-chromatograms of the extracts showed that neither rhein nor aloe-emodin were detected in the root and cell cultures of *S. alata*. However, the chromatograms showed peaks at 49 and 53 minutes, which were identical to the authentic emodin and chrysophanol, respectively (Fig 4.8). The identities of emodin and chrysophanol were also confirmed by their UV absorption spectra comparing with the authentics (Fig 4.9). The results indicated that the root and cell cultures of *S. alata*, which were grown in liquid B5 medium supplemented with 0.5 mg/l NAA and 1.0 mg/l kinetin were capable of producing emodin and chrysophanol, but not rhein and aloe-emodin. The content of emodin and chrysophanol produced in the root and cell cultures were then determined.

Table 4.2 Chrysophanol and emodin content in *S. alata* tissue culture (n = 3)

Tissue culture	**Emodin (%w/w ± S.D.)**	**Chrysophanol (%w/w ± S.D.)**
cell suspension culture (12^{th} culture)	0.01 ± 0.000	0.01 ± 0.000
root culture (3^{rd} culture)	0.27 ± 0.027	0.68 ± 0.047

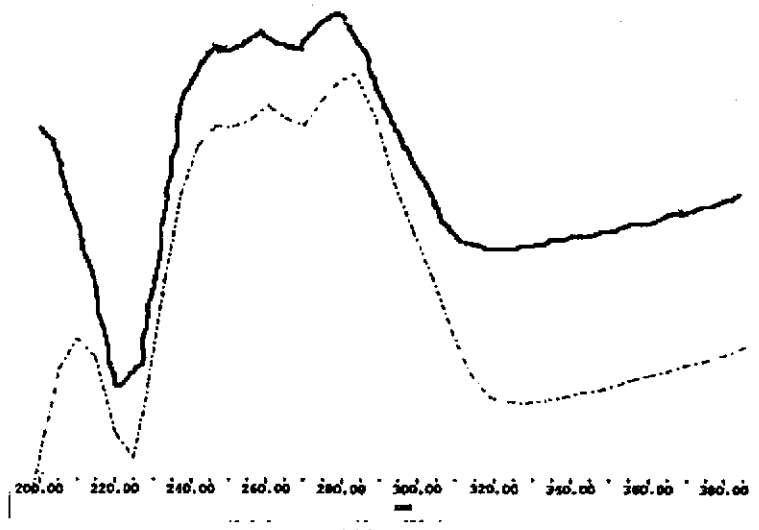


(A)

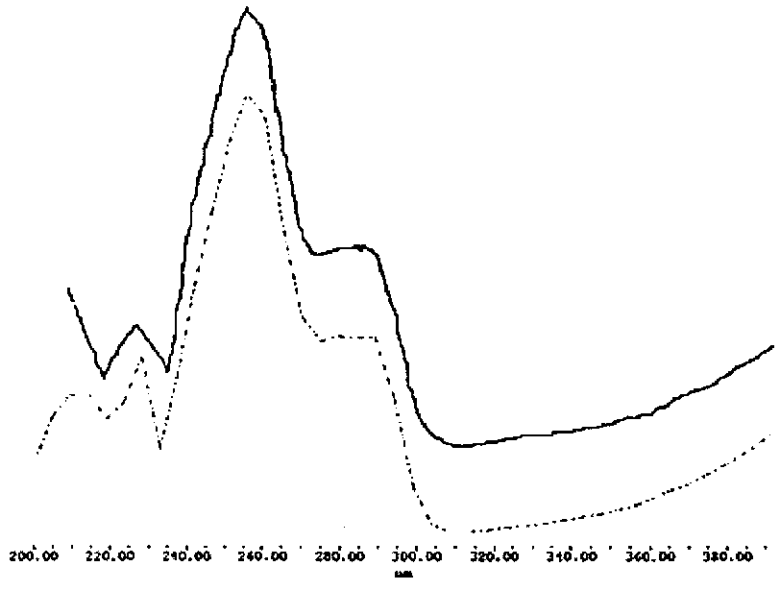


(B)

Fig 4.8 HPLC-chromatograms of; (A) the authentic aloe-emodin (RT = 20 min) (1), rhein (RT = 31 min) (2), emodin (RT = 49 min) (3) and chrysophanol (RT = 53 min) (4). (B) *Senna alata* root culture extract. HPLC conditions were described in section 3.2.6.2



(A)



(B)

Fig. 4.9 UV absorption spectra of authentic (A) emodin and (B) chrysophanol (---) and the compounds of similar RT obtained from *S. alata* root culture extract (—). UV spectra were recorded using the HPLC-UV diode array detector.

4.4 Medium manipulation on anthraquinone production in *Senna alata* cultures

Medium manipulation for increasing anthraquinone production in the cell cultures of *S. alata* was examined by variation of the concentration of two plant growth regulators, NAA and kinetin, in the basal medium. NAA concentration was varied firstly in free-hormonal liquid B5 medium. The results showed that although cell cultures in all culture media were still capable of producing emodin and chrysophanol, an increase in NAA concentration resulted in a decrease in anthraquinone production (Table 4.3). In addition, the cell cultures appeared to be large aggregated masses with a dark brown colour (Fig 4.10). Thus, NAA should be omitted in any suitable medium for optimum anthraquinone production.

The medium manipulation was further examined by variation of kinetin concentration in hormonal-free liquid B5 medium. It was found that an increase of kinetin concentration resulted in a slight decrease of anthraquinone production (Table 4.3). Whereas, the cell cultures grown in free-hormonal liquid B5 medium appeared to be large aggregated cells (Fig 4.11), those grown in medium supplemented with kinetin produced smaller cell aggregations and the cells were more homogeneous than those grown in hormone-free liquid B5 medium (Fig 4.12). Synchronous cell suspensions are necessary for culturing. They will be uniformity and have the same condition in medium. Also they are easy to subculture (Sahm, 1993; Narayanaswamy, 1994).

Anthraquinone production in *S. alata* cultures grown in B5 medium supplemented with kinetin 0.5 mg/l was not significantly different from that grown in the hormone-free medium, but gave cell cultures that were more homogeneous. Thus, B5 medium supplemented with kinetin 0.5 mg/l was selected for the maintenance of *S. alata* cell cultures.

Table 4.3 Emodin and chrysophanol content in different types and concentrations of growth regulators (n = 3)

Types and conc. of growth regulator (mg/l)	**Emodin (%w/w ± S.D.)**	**Chrysophanol (%w/w ± S.D.)**
Hormone-free medium	0.02 ± 0.001	0.03 ± 0.000
0.5 mg/l NAA	0.02 ± 0.001	0.01 ± 0.000
1 mg/l NAA	0.01 ± 0.001	0.01 ± 0.001
2 mg/l NAA	0.01 ± 0.001	0.01 ± 0.001
0.5 mg/l kinetin	0.01 ± 0.001	0.02 ± 0.002
1 mg/l kinetin	0.01 ± 0.001	0.02 ± 0.001
2 mg/l kinetin	0.01 ± 0.001	0.02 ± 0.001

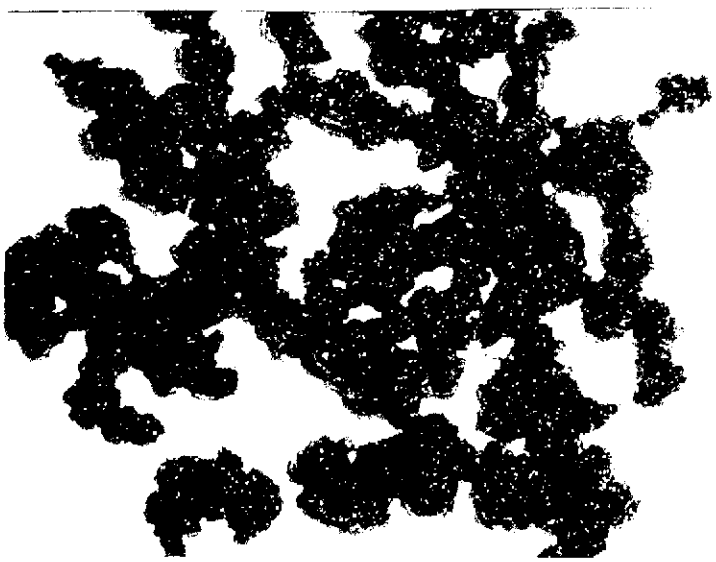


Fig 4.10 *Senna alata* cell cultures grown in liquid B5 medium supplemented with NAA 0.5 mg/l

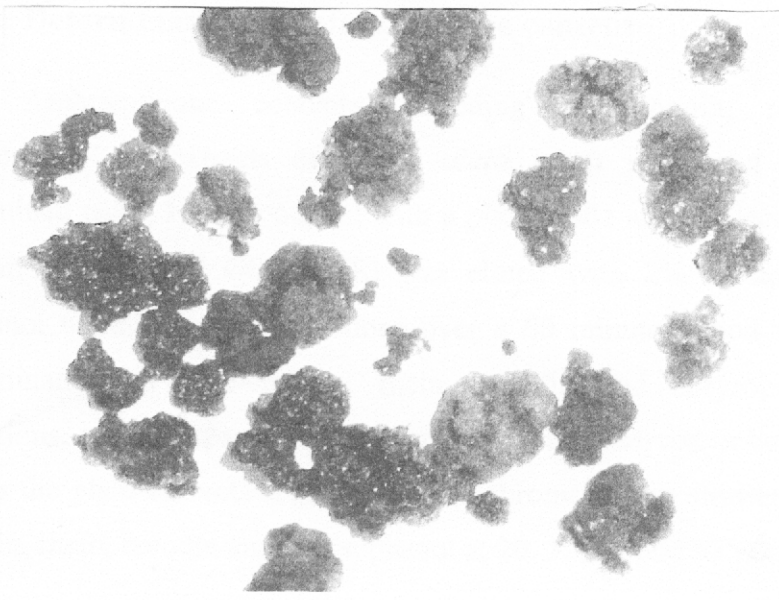


Fig 4.11 *Senna alata* cell cultures grown in hormone-free liquid B5 medium.

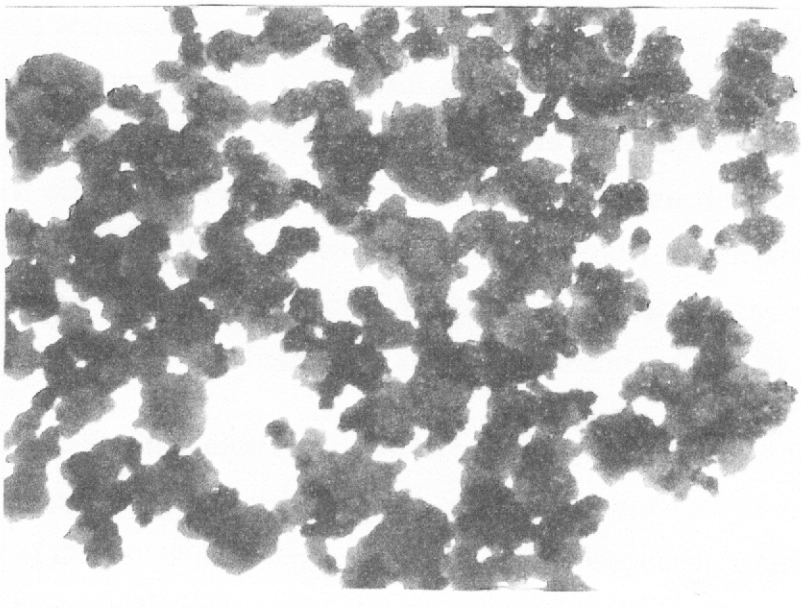


Fig 4.12 *Senna alata* cell cultures grown in liquid B5 medium supplemented with kinetin 0.5 mg/l

4.5 Determination of anthraquinone content

Anthraquinone contents, including aloe-emodin, rhein, emodin and chrysophanol in extracts of *Senna alata* intact plant and tissue culture were determined using HPLC coupled with a photodiode array detector. A reverse-phase column (TSK-GEL® ODS-80TS) was eluted with 2% acetic acid in water and methanol under gradient conditions over a 60 minute period for the simultaneous determination of aloe-emodin, rhein, emodin and chrysophanol. Under these conditions, a good separation of the analytes was observed in both standard solutions and in the plant extracts (Fig. 4.13). The chromatogram showed the peaks for aloe-emodin, rhein, emodin and chrysophanol at 20, 32, 49 and 53 minutes, respectively.

The chromatograms of the extracts obtained from root culture, cell culture, leaves and root of *S. alata* exhibited difference in the chemical patterns. The chromatogram of *S. alata* leaf extract exhibited the peaks of aloe-emodin and rhein, while those of the *in vitro* cultures showed the peaks of chrysophanol and emodin as the main anthraquinones. The root of the intact plant exhibited the peaks of rhein, chrysophanol and emodin as the main anthraquinones (Fig 4.13). Although, the peaks with the retention time about 20 and 32 minutes were detected in extracts of both the root and cell cultures, they were not identified as aloe-emodin and rhein, respectively, due to different UV absorption spectra. The identity of chrysophanol and emodin produced in the tissue cultures of *S. alata* were confirmed by comparing their UV absorption spectra with those of the authentic compound (Fig 4.9).

The results indicate that general metabolism in the root and cell cultures of *S. alata* is apparently different from that in the leaves and root of the intact plants. The ability of the root and cell cultures to produce chrysophanol, which is an intermediate in the biosynthetic pathway of aloe-emodin and rhein implies that organ cultures or cell cultures have lower potential than intact plant to produce secondary metabolites. The biosynthesis of the anthraquinone, emodin has a different intermediate from aloe-emodin and rhein (Sahm; 1993; Dewick, 2001).

For quantitative analysis of anthraquinone content in samples of *S. alata*, the area under the peaks of each anthraquinone was recorded and converted to concentration using the calibration curves (Fig. 4.14 and 4.15). The linearity of the chrysophanol calibration curves was observed in the range from 1.6 to 200 µg/ml and aloe-emodin, emodin and rhein from 0.3 to 40 µg/ml with r^2 more than 0.999 for all the compounds tested.

The results showed that the root cultures were capable of producing emodin and chrysophanol in higher amounts than that produced by the cell cultures (Table 4.2). This is a phenomenon usually found in *in vitro* cultures of plants. It has been reported that the metabolism of secondary products seems to correlate with the degree of organization of cell structures. Therefore, the root cultures are capable of accumulating a large range of secondary metabolites reflecting biosynthetic capacity (Sevón and Oksman-Caldentey, 2002).

The fundamental reasons for the difference in secondary metabolite formation in these tissue cultures are still not clear. However, based on the knowledge of morphological differentiation and the expression of secondary metabolism, it is widely accepted that the unorganized tissues such as cell cultures are usually accompanied by an apparent loss of their ability to accumulate secondary metabolites. The reasons may be a lack of gene expressions that control the essential steps of the biosynthetic pathway in non-specialized cells or the non-availability of storage site or an unregulated catabolism of secondary products (Charlwood and Rhodes, 1990).

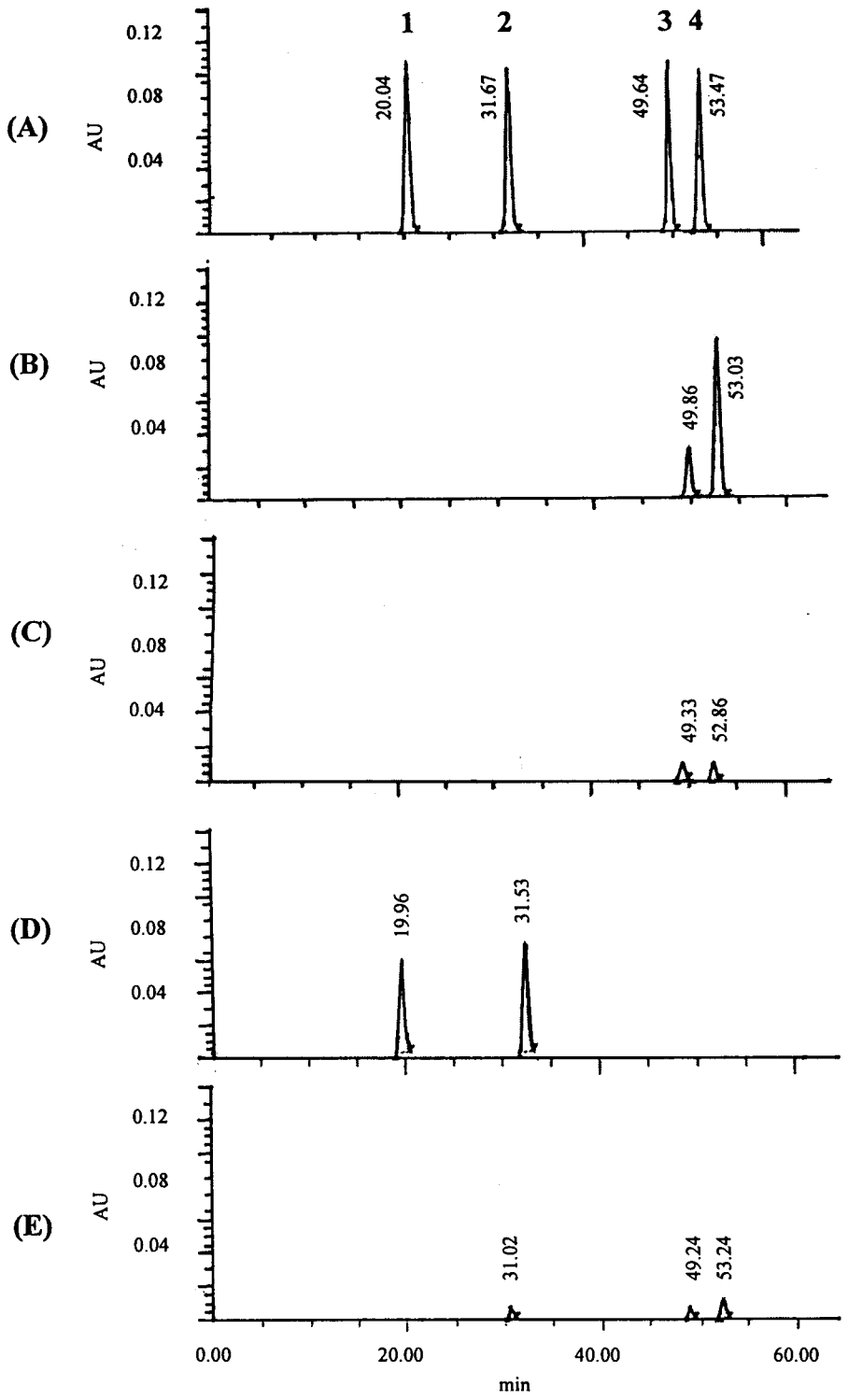


Fig 4.13 HPLC chromatograms of (A) authentics: aloe-emodin (1), rhein (2), emodin (3) and chrysophanol (4); (B) root culture extract; (C) cell culture extract; (D) leaf extract and (E) root extract.

The root cultures that were established from high and low anthraquinone producing plantlets produced emodin and chrysophanol in significantly different amounts (Table 4.4). The root culture obtained from the high anthraquinone yielding plantlets produced emodin and chrysophanol that were sixteen and twenty-six times higher, respectively, than that obtained from the low yielding plantlets. This result supports the strategy for increasing secondary metabolite production in plant tissue cultures by using high producing plantlets as initiation explants (Sahm, 1993). Although, the root culture of *S. alata* could not produce aloe-emodin and rhein, it produced higher amount of emodin and chrysophanol than the leaves and roots of the intact plant. Emodin and chrysophanol are the main bioactive components of *S. alata*. It has been reported that emodin and chrysophanol have various activity such as anticancer activity and antifungal activity. The ability of the root culture of *S. alata* to produce emodin and chrysophanol in higher amounts than the intact plant suggests that it can be the material of choice for biosynthetic studies of emodin and chrysophanol. Such ability implies that various enzymes involved in the biosynthetic pathway of emodin and chrysophanol are operating under these culture conditions.

It should also be noted that this gradient high performance liquid chromatographic method with photo diode arrays detection is highly effective for the simultaneous determination of anthraquinone content, including aloe-emodin, rhein, emodin and chrysophanol in *S. alata* and related plants. The method is fast and does not require prior purification of the sample extracts.

Table 4.4 Anthraquinone content in tissue cultures, leaves and roots of *Senna alata* (n=3)

sample	Aloe-emodin (%w/w ±S.D.)	Rhein (%w/w ±S.D.)	Emodin (%w/w ± S. D.)	Chrysophanol (%w/w ± S.D.)
High-yielding root culture	-	-	0.27 ± 0.027	0.68 ± 0.047
Low-yielding root culture	-	-	0.02 ± 0.001	0.03 ± 0.001
Cell culture	-	-	0.01 ± 0.000	0.01 ± 0.000
Natural roots	-	0.02 ± 0.013	0.01 ± 0.002	0.02 ± 0.010
Natural leaves	0.08 ± 0.001	0.09 ± 0.003	n.d.	n.d.

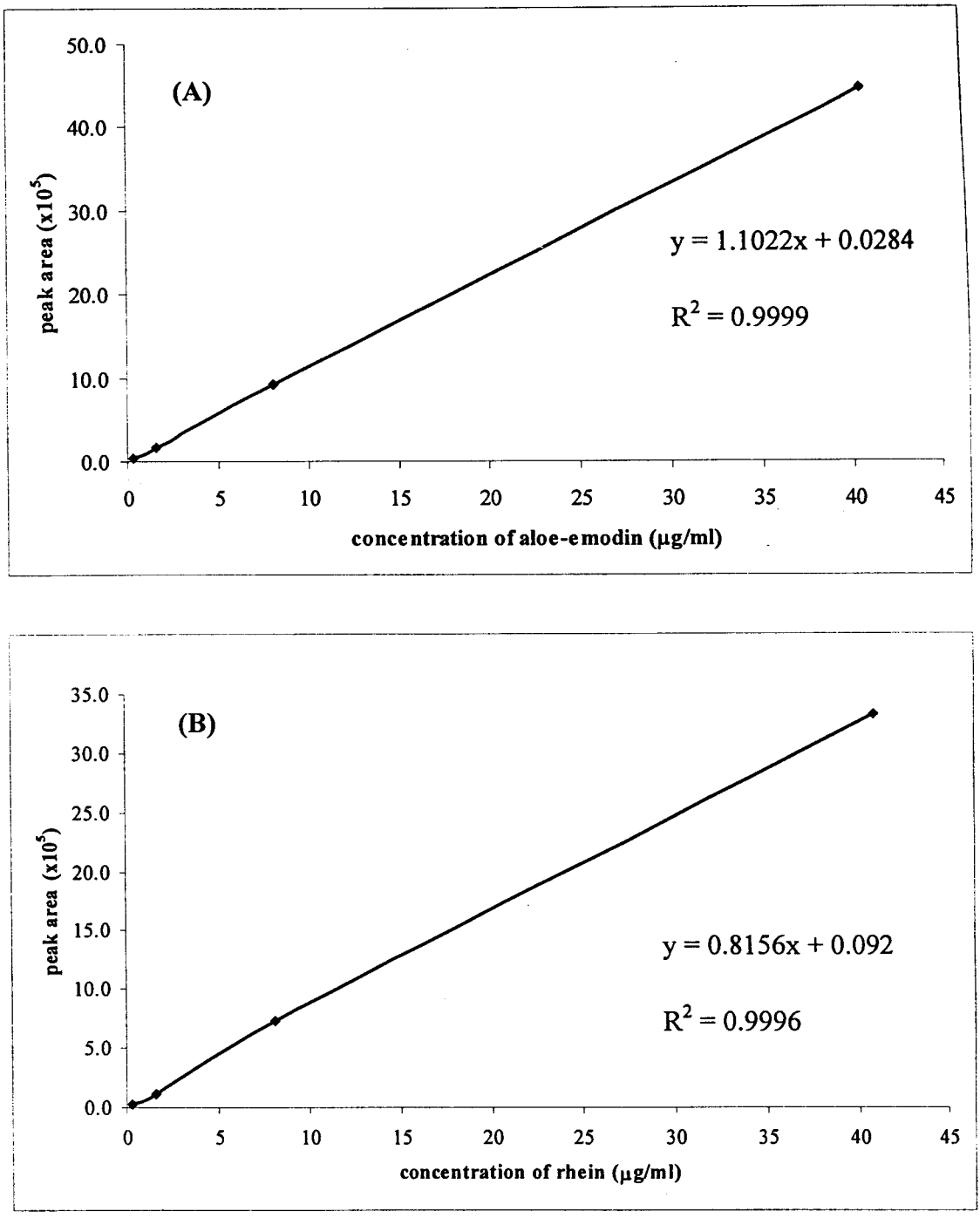
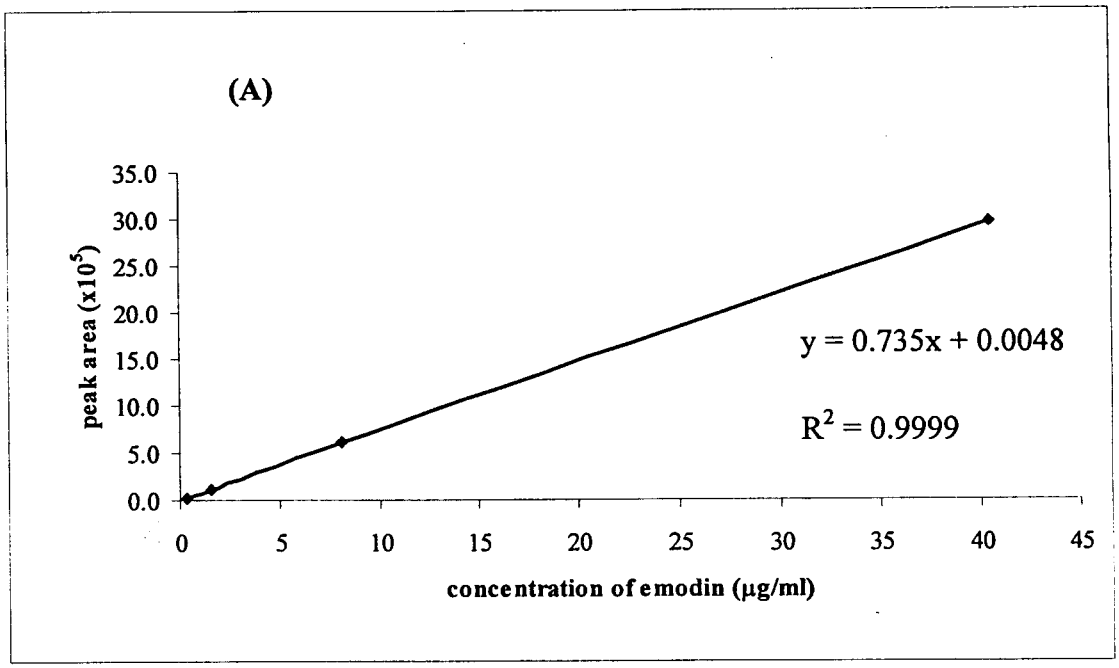


Fig 4.14 Calibration curve of aloe-emodin (A) and rhein (B). Each calibration point is a mean of three separate determination



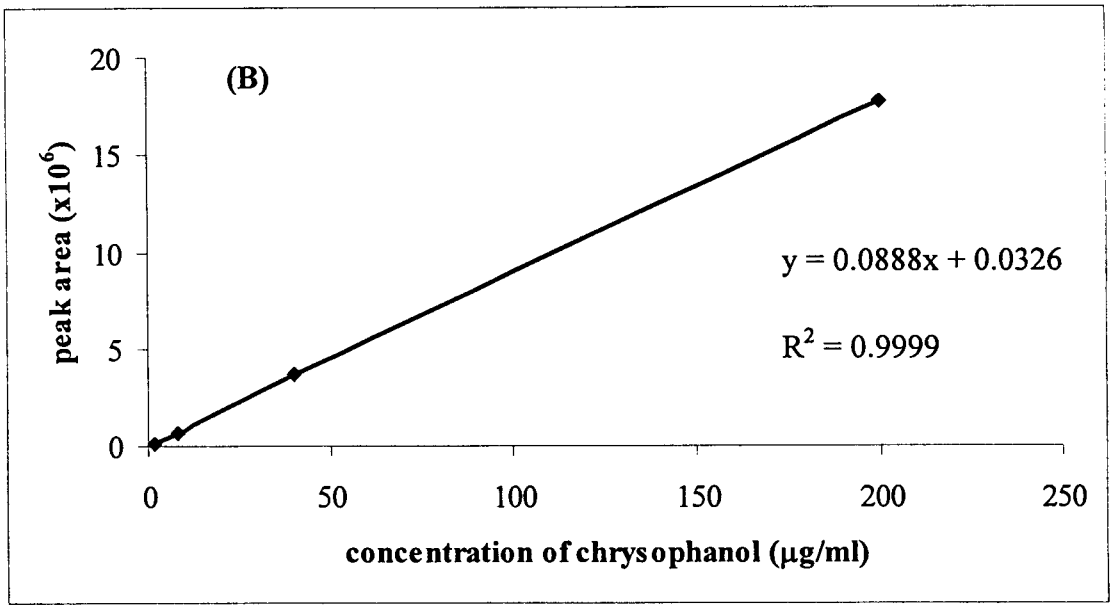


Fig 4.15 Calibration curve of emodin (A) and chrysophanol (B). Each calibration point is a mean of three separate determination

4.6 Time courses of growth and anthraquinone production

During the 35-day period of the culture growth, it was found that the fresh and dry weight of the biomass increased in the same manner. The growth pattern of *Senna alata* appears to be a normal sigmoid curve. The cell cultures spend a period of ten days for cell adaptation in a lag phase. To overcome this long period of the lag phase, increase of the initial biomass should be carried out. After the cell cultures have adapted to the fresh medium, high growth of the cells can be induced, which brings about an exponential growth phase for another ten days. The culture growth then reached a linear phase of ten days before entering a stationary phase (Fig 4.16). An increase in biomass goes on until presumably nutrient depletion, or a poor environment, when the cell cultures reach the stationary growth phase. This results in a continuous increase in the biomass throughout the period of 25 days. The cell cultures attained their highest dry biomass weight of 0.39 g, which was equivalent to about seven times of the inoculated cell culture mass.

The time course of emodin and chrysophanol production shows that emodin gradually decreases with the growth of the cell culture (Fig 4.16). This may be due to the effects of subculturing and dedifferentiation of the cells. It has been reported that several subculturings of cell cultures may lead to dedifferentiation of the cell and loss of secondary metabolite production (Narayanaswamy, 1993). For chrysophanol production, the initiation of the biosynthesis in the cell culture is at the later part of lag phase (after day 5), and chrysophanol is actively biosynthesized until the cultures reach the linear phase (30 days). The highest content of chrysophanol was observed at day 20, after which the production rate began to slow drown (Fig 4.16). This suggests that the biosynthesis of chrysophanol takes place at the same time as production of other primary metabolites used for growth promotion. This phenomenon is different from most secondary metabolite production, which usually take place when the growth rate begins to decline (Dixon, 1991).

Table 4.5 Fresh and dry weight of *Senna alata* cell biomass (n = 3 - 7)

day	Fresh weight (g)	Dry weight (g)
0	1.03	0.05
5	1.08	0.06
10	1.11	0.06
15	1.19	0.09
20	2.14	0.15
25	3.42	0.28
30	5.24	0.37
35	5.69	0.40

Table 4.6 Emodin and chrysophanol content accumulated in *Senna alata* cell biomass (n = 3)

day	Emodin (%w/w ± S.D.)	Chrysophanol (%w/w ± S.D.)
0	0.02 ± 0.001	0.02 ± 0.001
5	0.02 ± 0.001	0.01 ± 0.000
10	0.02 ± 0.000	0.01 ± 0.001
15	0.02 ± 0.001	0.01 ± 0.001
20	0.01 ± 0.000	0.02 ± 0.001
25	0.01 ± 0.001	0.02 ± 0.001
30	0.01 ± 0.000	0.01 ± 0.000
35	0.01 ± 0.001	0.01 ± 0.001

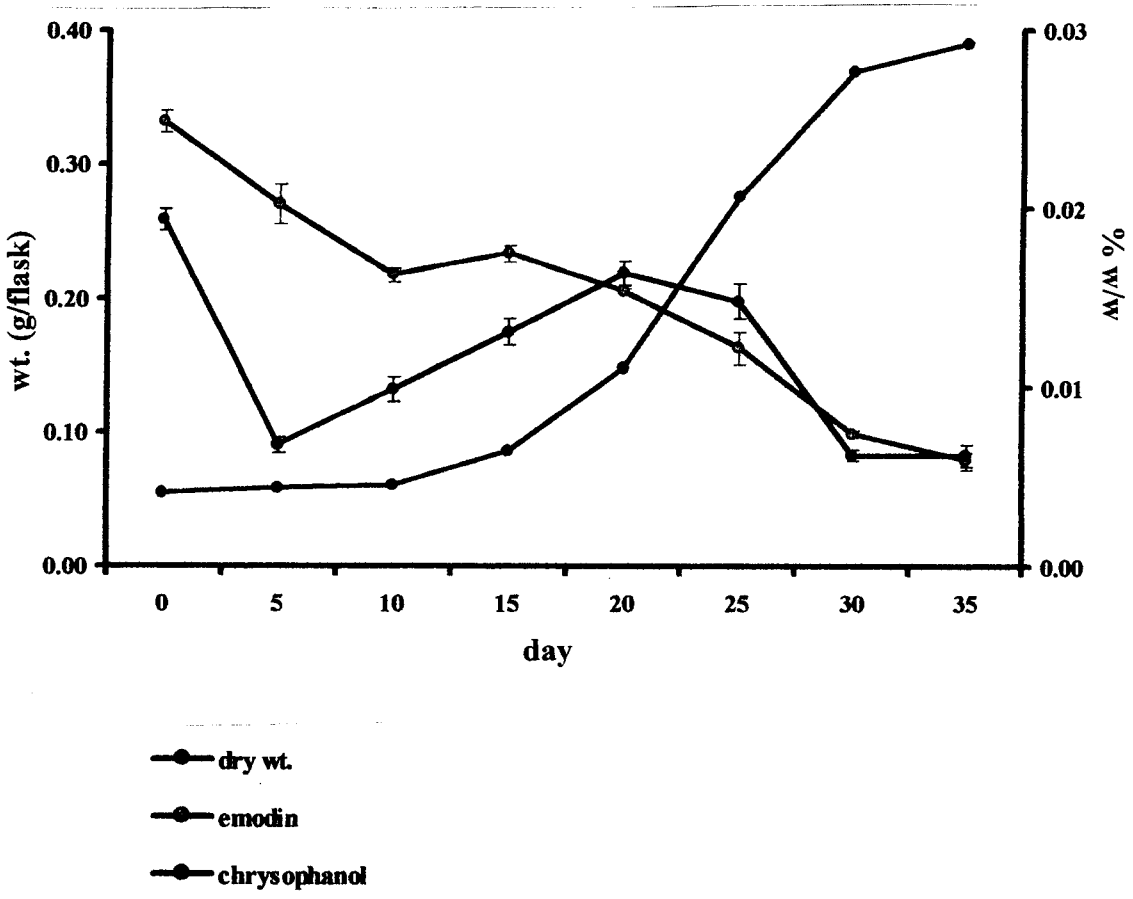


Fig 4.16 Growth curves and production curve of cell suspension cultures: fresh weight (A) and dry weight (B)

4.7 Determination of anthraquinone glycosides in *Senna alata* cell culture

Anthraquinone glycosides were demonstrated as the active constituents for laxative action (Elujoba *et al.*, 1989). Thus, we also determined the content of anthraquinone glycosides in the cell culture of *S. alata* using a spectrophotometric method described in the Thai Herbal Pharmacopoeia (1998). The method determines the total anthraquinone glycoside content as hydroxyanthracene derivatives, calculated as rhein-8-glucoside. The result showed that the hydroxyanthracene derivative content in *S. alata* cell culture was 0.5 ± 0.02 %w/w, which was less than the standard value of 1.0%w/w, indicated in the monograph of *S. alata* leaf (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998). This suggests that if we would like to use the cell culture as a source of raw material for laxative production, the cell culture should be manipulated to improve anthraquinone glycoside production. This may be done by for example, medium manipulation or establishing a transformed root culture by co-culture with *Agrobacterium sp.* (Chang *et al.*, 1998).