CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Senna alata seeds were collected from Songkhla Province (Amphur Namom and Amphur Hat-Yai) and Narathiwas Province (Amphur Ruso) in January 2002. The Leaves and roots were collected from Songkhla Province (Amphur Namom) in March and July 2003.

3.1.2 Chemicals

Standards aloe-emodin, emodin, rhein and B5 medium were purchased from Sigma Chemical Co. Standard chrysophanol was purchased from Fluka Chemie GmbH. The plant hormones were plant tissue culture grade and the other chemicals were analytical grade or better, as available.

3.2 Methods

3.2.1 Preparation of *Senna alata* plantlets

The seeds, which were collected from various places, were cleaned with running tap water for 30 minutes and then immersed in boiling water for 5 minutes. The seeds were then subjected to a surface sterilization process by dipping into 70% ethanol for a few seconds and then soaking them in 20% Clorox® solution for 20 minutes. The sterile seeds were then washed with sterile distilled water 3 times and transferred to hormone-free B5 basal solid medium and incubated at 25 ± 2°C with a 16-hour daily light period for 2 months.

3.2.2 Medium preparation

The commercially prepared Gamborg's B5 medium in powder form was used for preparing the culture medium. The composition of the B5 medium is shown in Table 3.1. The culture medium was prepared by adding the B5 medium to distilled water (90% of the final required volume) followed by stirring. Sucrose (2% w/v) and plant hormones stock solution (as needed) were then added and stirring continued until complete dissolution. The culture medium was then adjusted to the final volume with distilled water and to pH 5.5 with 1N sodium hydroxide or 1N hydrochloric acid. In the case of solid medium, agar (0.8% w/v) was added and heated gently with continuous stirring until the solution cleared. The culture medium was sterilized by autoclaving at 121°C, 15 lb/in^2, for 15-20 minutes.

3.2.3 Selection of high yielding plants

3.2.3.1 Preparation of *Senna alata* leaf extracts

Fifty-six leaf samples were separately collected from *S. alata* plantlets (2- month old). The leaves were dried at 50°C for 24 hour and the dried powdered leaves (5 mg) were extracted with methanol (10 ml) under reflux for an hour. The extracts were filtered, evaporated the extract to dryness and adjusted to the volume of 1 ml with methanol. The contents of rhein and aloe-emodin were determined by high performance liquid chromatography (HPLC) as described below.

Table 3.1 Inorganic salt and vitamin composition of Gamborg's B5 medium.

Constituent	Concentration (mg/liter)
Macronutrients:	
KNO_3	2,500
$MgSO_4 \cdot 7H_2O$	250
$NaH_2PO_4 \cdot H_2O$	150
$CaCl_2 \cdot 2H_2O$	150
$(NH_4)_2 \cdot SO_4$	134
Micronutrients:	
H_3BO_3	3
$MnSO_4 \cdot H_2O$	10
$ZnSO_4 \cdot 7H_2O$	2
$NaMoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl2 \cdot 6H_2O$	0.025
KI	0.75
EDTA Na Ferric	40
Sucrose (g)	20
Vitamins:	
Thiamine HCl	10
Pyridoxine HCl	1
Nicotinic acid	1
myo-Inositol	100
pH	5.5

3.2.3.2 Quantitative analysis of rhein and aloe-emodin content

The samples were analyzed by HPLC. The area under the peaks of rhein and aloe-emodin were recorded and converted to concentration by using standard curves. The calibration curves were established using authentic methanolic solution of rhein and aloe-emodin in the concentration range of 4 - 64 μg/ml. Each calibration point was carried out in triplicate, and all sample analyses were also carried out in triplicate. The chromatographic conditions were as follows:

Chromatographic column	:	TSK-GEL® ODS-80TS 4.6 mm x 150 mm
Mobile phase	:	2% aq.acetic acid : methanol (35:65) (isocratic)
Flow rate	:	1 ml/minute
Run time	:	30 minutes
Detector	:	UV 254 nm
Injection volume	:	50 μl

3.2.4 Establishment of *Senna alata* tissue cultures

S. alata tissue cultures were initiated from the root of selected seedlings (top five of the low- and high-anthraquinone yielding seedlings). The root cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of liquid B5 medium supplied with 0.5 mg/l NAA and 1.0 mg/l kinetin. The flasks were incubated on a rotary shaker at 25°C, 90 rpm with a 16-hour light photoperiod. The tissue cultures were maintained under these conditions and subcultured to fresh medium every four-weeks.

3.2.5 Medium manipulation

The concentrations of NAA in liquid B5 medium supplemented with 1.0 mg/l kinetin and 2% w/v sucrose were varied to study the effect on growth of the root culture. The concentrations of NAA and kinetin in liquid B5 medium were varied again to study the effect on anthraquinone formation. To search for an optimum concentration of NAA, four concentrations of NAA, including 0.0, 0.5, 1.0 and 2.0 mg/l were used. After the optimum concentration of NAA was obtained, four concentrations of kinetin, including 0.0, 0.5, 1.0 and 2.0 mg/l were used combination with the obtained optimum concentration of NAA.

3.2.6 Determination of anthraquinone formation

3.2.6.1 Plant tissue culture harvesting

Various *Senna alata* cultures (1-month old), from different culture media, were separately harvested by vacuum filtration. The harvested tissues were dried in a hot air oven at 50°C and ground.

3.2.6.2 Sample preparation

The dried plant tissue powders of *Senna alata* (0.2 g) were extracted with 20 ml of methanol under reflux for an hour and filtered. After the filtrates were evaporated to dryness, the obtained residues were dissolved in methanol and adjusted to volume in a 10 ml-volumetric flask. These sample preparations were then subjected to analysis of anthraquinone accumulation.

3.2.6.3 Determination of anthraquinones

The anthraquinones, including rhein, aloe-emodin, emodin and chrysophanol were determined using HPLC. Peak retention times and UV absorption spectra of the corresponding peaks compared with the authentic compounds were used for identification of the anthraquinone formation. The

areas under the peaks were converted to concentration by using their calibration curves. The calibration curves of rhein, aloe-emodin and emodin were established from the authentic at the concentration range between 0.32 - 40 μg/ml, while that of chrysophanol at concentration range between 1.6 - 200 μg/ml. The conditions of HPLC were described below. The analysis of all samples was in triplicate.

HPLC conditions:

Chromatographic column : TSK-GEL® ODS-80TS 4.6 mm x 150 mm

Mobile phase : 2% aq.acetic acid : methanol (gradient from 55%methanol to 100%methanol in 60 minutes) (Figure 3.1 and Table 3.2)

Flow rate : 1 ml/minute

Run time : 60 minutes

Detector : UV 254 nm (photodiode array)

Injection volume : 20 μl

Table 3.2 Gradient profile of the mobile phase (2% aq.acetic acid : methanol)

Time (min)	%MeOH
0 - 15	55
20 - 35	60
35 - 50	70
50 - 60	100

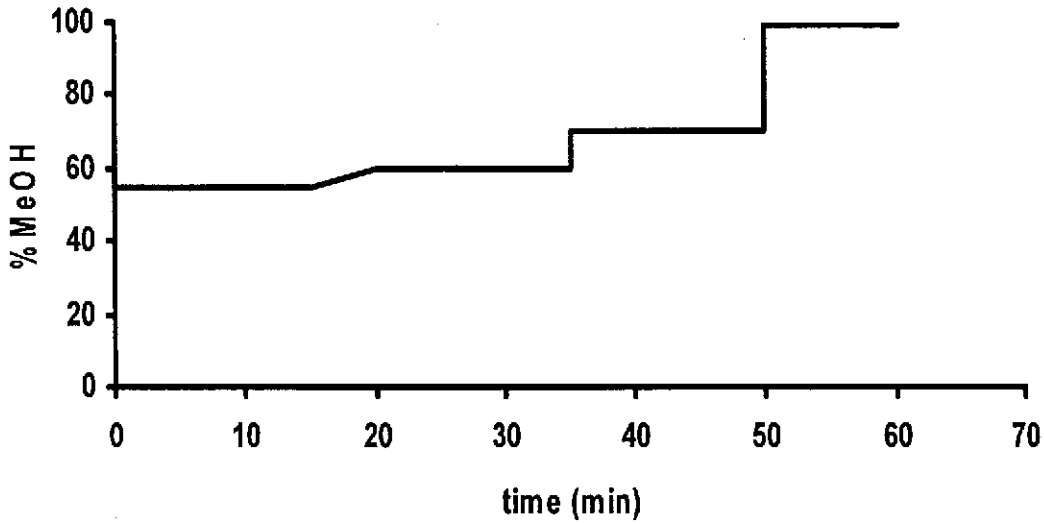

Fig 3.1 Gradient profile of the mobile phase (2% aq.acetic acid : methanol)

3.2.7 Time course of growth and anthraquinone production of *Senna alata* cell culture

One gram amounts of the cell cultures were transferred into 250-ml Erlenmeyer flasks containing 50 ml of liquid B5 medium supplemented with 0.5 mg/l kinetin and 2.0%w/v sucrose. The cell cultures (3 - 7 flasks) were harvested by filtration every 5 days for a period of 35 days. The dry weights were recorded after drying at 50°C for 24 hours. The amount of anthraquinones were determined by the HPLC method as described in the sections 3.2.6 and calculated in the units of percentages of dry weight. These data were then plotted to obtain growth and anthraquinone production curves.

3.2.8 Determination of hydroxyanthracene derivatives

Total contents of hydroxyanthracene derivatives in tissue cultures and leaves of *Senna alata* were determined by a spectroscopic method according to Thai Herbal Pharmacopoeia (1998). About 150 mg of the sample in powder was accurately weighed and placed in a 100-ml round-bottomed flask. Distilled water (30.0 ml) was added, mixed, weighed, and heated under a reflux condenser for 15 minutes. The

cooled mixture was weighed and adjusted to the original weight with water. The mixture was centrifuged and the supernatant liquid (20.0 ml) was transferred to a 150-ml separator. 2 M hydrochloric acid (0.1 ml) was added and the mixture was shaken with three 15-ml portions of chloroform. The chloroform layer was discarded. Sodium hydrogen carbonate (100 mg) was added into the aqueous part and shaken for 3 minutes. After centrifugation, the supernatant liquid (10.0 ml) was transferred to a 100-ml round-bottomed flask. A solution of iron (III) chloride 10.5 %w/v (20 ml) was added and heated for 20 minutes under a reflux condenser. Hydrochloric acid (1 ml) was added and heated for a further 20 minutes with frequent shaking. After cooling, the mixture was transferred to a separator and shaken with three 25-ml portions of ether. The ether layers were combined and washed with two 15-ml portions of water. The ether layers was then transferred to a 100-ml volumetric flask and diluted with ether to the required volume. An aliquot of the solution (25.0 ml) was carefully evaporated to dryness at low temperature and the residue was dissolved in 10.0 ml of a 0.5% w/v solution of magnesium acetate in methanol. The absorption of the resulting solution was measured at 515 nm using a Spectro UV-Vis RS Spectrophotometer, using the magnesium acetate solution as the blank. The percentage of rhein-8-glucoside was calculated from the expression: A x 0.4283/w, where A is the absorbance measured at 515 nm, and w is the weight in g of the dried leaf powder used initially. The analysis in all samples was in triplicate.